POCK FORMING PLASMIDS FROM ANTIBIOTIC-PRODUCING STREPTOMYCES

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Pock forming ability of fifteen plasmids isolated from antibiotic-producing Streptomyces was examined with polyethylene glycol mediated protoplast transformation. Four plasmids (pSF765, pSF689, pSF674 and pSF601) were found to have pock forming ability on *Streptomyces lividans* 66. Each putative transformants isolated from the center of pock regions harbored plasmids with same restriction enzyme cleavage sites as original plasmids. The plasmids from the transformants produced pock, which were morphologically identical to the original plasmids.

Transformation frequency of *S. lividans* 66 with pSF689 from *S. lividans* 66 was at least 500-fold higher (5×10^5 transformants per 1 μg DNA) than that with the plasmid isolated from the original strain (10^3 transformants per 1 μg DNA). In case of pSF765 the transformation frequency was not changed (about 10^5 transformants per 1 μg DNA) by replications in *S. lividans* 66. Expanded restriction enzyme maps for the four plasmids are reported.

The development of Streptomyces host-vector systems has numerous applications in fermentation based industries and expands the genetic knowledge of these unique microbes.

Efficient transformation and in turn, cloning systems have been established with *Streptomyces coelicolor*[1,2], *S. lividans*[3,4], *S. fradiae*[5] and *S. espinosus*[6]. The plasmids used in these studies were SCP2, SLP1.2, pIJ101, pUC1 and pUC6. The phenotype of lethal zygosis[7], "pock" formation, was used in these studies for the detection of transformants.

Antibiotic-resistance genes of antibiotic-producers have recently been cloned[3,8,9] and new vectors developed such as pIJ41[10] and pIJ365[4] for use in Streptomyces.

In an effort to establish new host-vector systems for the antibiotic-producing Streptomyces, we examined the pock forming ability of the plasmids from antibiotic-producing strains.

Materials and Methods

Bacterial Strains

Antibiotic-producing Streptomyces culture which harbors pSF plasmids[11] were obtained from Meiji Seika Kaisha Collection. The recipient organism used for transformation experiments was *S. lividans* 66 which was kindly supplied by Dr. N. D. LOMOVSKAYA and Dr. K. F. CHATER.

Growth Conditions

Streptomyces SF strains were grown in MYG medium (malt extract 1%, yeast extract 0.4%, glucose 0.8%) containing 0.5~2.0% glycine at 28°C for 48 hours.

S. lividans 66 was grown in YEME medium (yeast extract 0.3%, Bacto peptone 0.5%, malt extract 0.3%, glucose 1%) containing 34% sucrose and 5 mM $MgCl_2$[3,7,10] at 32°C for 48 hours for plasmid preparation. Protoplasts were prepared from cells grown in YEME medium containing 34% sucrose, 5 mM $MgCl_2$ and 0.5% glycine at 32°C for 32~36 hours.

Isolation of Plasmid DNA

Methods for pSF plasmid preparation were previously described by NOJIRI *et al.*[12] or OKANISHI *et al.*[13]. The plasmid isolation method for *S. lividans* 66 was as described by HANSEN *et al.*[14]. Mycelium (about 3 g wet weight) harvested from a 80-ml culture in YEME medium in 500-ml flasks was suspended in 30 ml of TES buffer (tris-HCl 0.1 M, EDTA 20 mM, sucrose 25%, pH 8.0), and 0.6 ml of a lysozyme solution (30 mg/ml) and 0.6 ml of a ribonuclease A solution (5 mg/ml) were added. After digestion for 30 minutes at 37°C, 12 ml of 20% sodium dodecyl sulfate solution was added and mixed. Then 2.4 ml of 3 N NaOH was added and shaked for 60 minutes at room temperature.

After adding 9 ml of 2 M tris-HCl buffer, 15 ml of 5 M NaCl solution was added, then the mixture allowed to stand over night at 0°C. A cleared lysate was obtained by centrifugation at $10,000 \times g$ for 15 minutes. Polyethylene glycol (PEG) #1000 was added to a final concentration 10% and then the solution was kept at 0°C for 3 hours to complete the DNA precipitation.

After centrifugation at $5,000 \times g$ for 15 minutes, the DNA was dissolved in TESH buffer (tris-HCl 0.2 M, EDTA 20 mM, NaCl 50 mM, pH 8.0). The plasmid DNA was purified by ethidium bromide-CsCl dye-buoyant density gradient centrifugation[15].

Preparation and Transformation of Protoplasts

The original procedure for the preparation of protoplast was described by OKANISHI *et al.*[16] and the procedure for the transformation method was as described by THOMPSON *et al.*[3] or CHATER *et al.*[10]. Mycelium grown in 80 ml of YEME medium (500-ml flasks) was washed twice in 10.3% sucrose and the pellet suspended in P medium[16]. Protoplasts were generated by digesting the cell wall with lysozyme (1 mg/ml) in P medium for 1 hour at 32°C. The intact mycelium was removed by cotton filtration. Protoplasts were then collected by centrifugation ($1,500 \times g$ 10 minutes) and washed once with P medium. The pellet was finally suspended in 2 ml of P medium. The transformation mixture contained 50 μl of plasmid DNA (about 1 μg DNA is dissolved in 1/20 concentration TESH buffer), 100 μl of 3/2 concentration of T medium[3,10] and 100 μl of protoplasts in P medium. T medium differs from P medium in the concentrations of sucrose (2.5%) and $CaCl_2$ (0.1 M), it has 50 mM tris-maleic acid (pH 8.0) instead of TES buffer. Transformation was initiated by adding 375 μl of 33.3% PEG #1000 dissolved in T medium. After 60 seconds transformation was terminated by addition of 5 ml of P medium. Protoplasts were pelleted by centrifugation ($1,000 \times g$ 10 minutes) suspended in 0.5 ml of P medium, and spread on plates of R2YE[3,10] agar medium. The R2YE plates were dried to remove about 20% of the weight of the medium prior to inoculation. Pocks appeared on regeneration medium after 4 days incubation at 32°C.

In the instances when pocks did not appear clearly, spore suspensions from R2YE medium were diluted properly and plated again on fresh R2YE medium, seeded with a large excess of the parental plasmid negative strain to give a confluent lawn. The spores from the center of pock were isolated using a fine wire under a low-power microscope.

Restriction Digestion and Agarose Gel Electrophoresis

Restriction endonucleases were obtained from either Takara Shuzo Kaisha, Ltd. or Bethesda Research Laboratories, Inc. and used as directed. Electrophoresis was carried out on a horizontal slab gel of 1% agarose in E buffer (tris-HCl 40 mM, EDTA 1 mM, CH_3COONa 5 mM, pH 8.0). After electrophoresis for 2 hours at a constant voltage of 100 volts, the bands were visualized by fluorescence under 302 nm UV light.

Results

"Pock" formation was observed on regeneration plates after transformation of *S. lividans* 66 with fifteen pSF plasmids[11]. Only four of the plasmids tested, pSF765, pSF689, pSF674 and pSF601, formed pocks (Table 1). Two days after transformation the pocks began to appear and after four days they became clearer. The photographs (Fig. 1) show the pocks on the regeneration plates with the four plasmids. The size and clearness of pocks were different with each plasmid. The largest size pocks

Table 1. Pock formation with pSF plasmids on *S. lividans* 66.

Plasmid	Pock formation	Molecular weight ($\times 10^6$)	Host	Product
pSF425	−	ND*	*S.* sp. SF425	Nojirimycin
pSF506	−	20	*S.* sp. SF506	Azomycin
pSF588	−	3.0	*S. albofaciens* SF588	Oxytetracycline
pSF601	+	21	*S.* sp. SF601	Tubercidin
pSF609-1	−	5.5	*S. lavendulae* SF609	Cycloserine
pSF619	−	6.2	*S. hygroscopicus* SF619	Paromomycin
pSF674	+	15	*S.* sp. SF674	Rhizomycin
pSF689	+	9.1	*S. platensis* SF689	Oxytetracycline
pSF701-1	−	4.7	*S. griseochromogenes* SF701	SF701 substance
pSF760	−	ND	*S.* sp. SF760	Oleandomycin
pSF733	−	52	*S. ribosidificus* SF733	Ribostamycin
pSF765	+	4.8	*S. fradiae* SF765	Neomycin
pSF930	−	ND	*S.* sp. SF930	β-Glucuronidase
pSF1223	−	ND	*S.* sp. SF1223	SF1223 substance
pSF1306	−	ND	*S.* sp. SF1306	SF1306 substance

* ND: Not determined.

Fig. 1. Pocks produced by the growth of individual plasmid-containing *S. lividans* 66 in back grounds of plasmid-free *S. lividans* 66.
A: pSF601, B: pSF765, C: pSF674, D: pSF689.

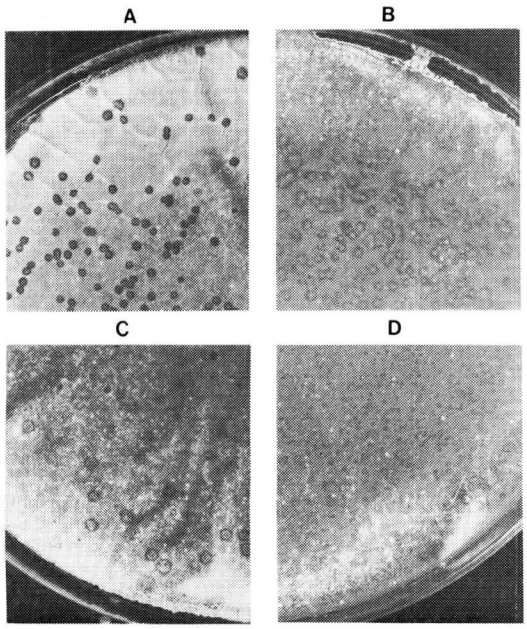
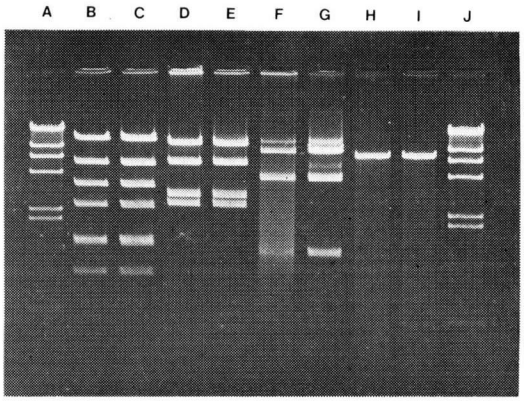


Fig. 2. The digestion patterns of pSF plasmids isolated from each original strain and *S. lividans* 66. *Hind* III digested phage λcI DNA (A and J). *Bam* HI digested DNAs: (B) pSF601 from original strain, (C) pSF601 from *S. lividans* 66. *Pst* I digested DNAs: (D) pSF674 from original strain, (E) pSF674 from *S. lividans* 66. *Kpn* I digested DNAs: (F) pSF689 from original strain, (G) pSF689 from *S. lividans* 66. *Bcl* I digested DNAs: (H) pSF765 from original strain, (I) pSF765 from *S. lividans* 66.



were observed with pSF765 plasmid, and the smallest with pSF689. Plasmid pSF601 produced clear pocks.

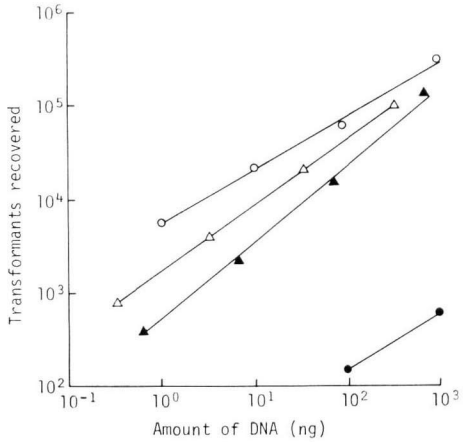
The putative transformants from the center of each pock region was isolated and the plasmids isolated from the resulting cultures.

The plasmids obtained from the original strain was compared with the plasmid DNA from *S. lividans* 66 transformant. The pock morphology (size and clearity) and the restriction cleavage pattern of plasmid DNA from the parent and transformant were the same (Fig. 2).

The transformation frequency with plasmid isolated from the original strain and the respective *S. lividans* 66 transformant was compared (Fig. 3). The transformation frequency was almost equal (10^5 transformants per 1 μg DNA) with the pSF765 plasmid. In contrast, the transformation frequency with the pSF689 plasmid from the original strain and the *S. lividans* 66 transformant was 10^3 transformants per 1 μg DNA and higher than 10^5 transformants per 1 μg DNA, respectively.

Fig. 3. Frequency of transformation with pSF plasmids isolated from original strains and *S. lividans* 66.

The pSF765 from *S. fradiae* SF765 (▲), pSF765 from *S. lividans* 66 (△), pSF689 from *S. platensis* SF689 (●), and pSF689 from *S. lividans* 66 (○).



The number of cleavage sites of pSF601, pSF674, pSF689 and pSF765 are shown in Table 2. The cleavage maps of pSF601 and pSF674 were constructed (Fig. 4), by single and multiple enzyme digestion.

Fig. 4. Cleavage maps of pock forming plasmids.

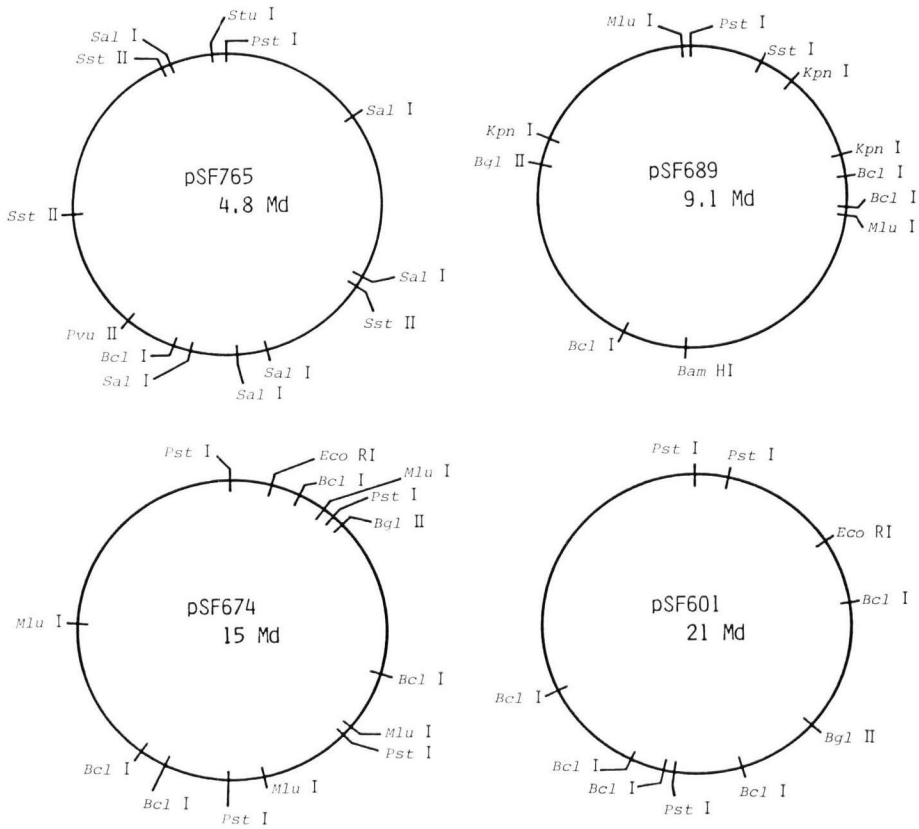


Table 2. Number of cleavage sites with restriction enzymes.

Restriction enzyme	Number of cleavage sites			
	pSF765	pSF689	pSF674	pSF601
Bam HI	0	1	0	7
Bcl I	1	3	4	5
Bgl II	0	1	1	1
Eco RI	0	0	1	1
Kpn I	0	3	4	ND*
Mlu I	7	2	4	6
Pst I	1	1	4	3
Pvu II	1	6	0	7
Sal I	6	5	>10	>4
Sst I	0	1	5	ND
Sst II	3	>6	ND	ND
Stu I	1	0	0	ND

* ND: Not determined.

Discussion

BIBB[17] reported that lethal zygosis appears to be a common phenotype of Streptomyces plasmids. HOPWOOD *et al.*[18] reported that the plasmids of *S. violaceoruber* species group were transferred to *S. coelicolor* A3 (2) and *S. lividans* 66, and the transconjugants or transformants were recognized by the lethal zygosis phenotype. The plasmids of pIJ101 series isolated from *S. lividans* ISP 5434 were transferred to both *S. lividans* 66 and other Streptomyces species[4].

Fifteen pSF plasmids were isolated in our laboratory from various antibiotic-producing Streptomyces which were taxonomically distinct from *S. violaceoruber* group. Four (pSF601, pSF674, pSF689, pSF765) of these plasmids formed pocks on *S. lividans* 66. In addition, the plasmids formed pocks on other species such as *S. chartreusis* (unpublished data). The pock phenotype appears to be widely distributed amongst plasmids from various species of Streptomyces. These plasmids studied in this paper were readily transferred to other Streptomyces species.

CHUNG[5] reported that pock formation was caused by an actinophage which exists in a Streptomyces host as a plasmid. OGATA *et al.*[19] also reported that the pock phenomena in various Streptomyces was caused by phage tail-like particles. Actinophage was not involved in the pock phenomena described in this paper.

The mechanism of pock formation with the small plasmids used in this studies is presently under investigation.

Although a restriction-modification system has not been previously described in *S. lividans* 66[9], the results shown in Fig. 3 suggest that pSF689 isolated from its original strain was altered when grown in *S. lividans* 66. Futher work will be necessary to identify the bases for this apparent change.

pSF765 and pSF689 are relatively small, molecular weight of 4.8 and 9.1 megadaltons respectively. Both plasmids have single recognition sites for *Bam* HI, *Bgl*II, *Pst* I, *Sst* I, *Bcl* I, *Pvu* II and *Stu* I, and retain the pock phenotype when transformed into *S. lividans* 66. It may be possible to obtain smaller plasmids from either pSF765 or pSF689, which could be used as vectors for Streptomyces.

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