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Isolation and characterization of plasmids isolated from *Streptomyces* spp. and construction of recombinant plasmids

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Streptomyces 속으로 부터 분리한 플라스미드의 특성 및 재조합 유도체의 제조

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ABSTRACT: Five independent Actinomycetes harboring plasmids were isolated from soil. Molecular weight of these plasmids was 55 kb, 6.2 kb, 4.4 kb, 55 kb and 7.0 kb, respectively. Among them, small and apprent high copy number plasmids, pJY501 of 4.4 kb and pJY711 of 7.0 kb, were selected. The plasmids purified by CsCl-EtBr density gradient centrifugation preserved the conformation of super-coiled covalently closed circular molecule, and an apparent copy number was estimated about 150 and about 35 per chromosome. The isolates carrying plasmids were assigned to the genus *Streptomyces*. For the purpose of introducing selection markers into the isolated plasmids, the *tsr* fragment of pIJ702 was inserted into the *Bcl*I site of pJY501 and pJY711. And the recombinant plasmids constructed designated as pJY502 and pJY712 respectively.

KEY WORDS□*Streptomyces*, high copy number, recombinant plasmid.

Streptomyces are Gram-positive multi-cellular microorganism of biological, medical, as well as industrial importance and produce over 70 % of all antibiotics (Hopwood and Merrick, 1977). Evidence of involvements of plasmids in antibiotic biosynthesis has been reviewed (Hopwood, 1978; Okanish, 1978). Transfer of plasmid DNA by protoplast transformation and methods for preparation and regeneration of protoplasts have been developed (Bibb *et al.*, 1977; Thompson *et al.*, 1980; Baltz, 1978; Hopwood and Wright, 1979; Baltz and Matsushima, 1981). Recent developments in *Streptomyces* genetics should facillitate the research of antibiotic biosynthesis at the molecular level and also be used to generate novel structure by cloning antibiotics production genes from one species into another. A branch of recent developments in *Streptomyces* is the isolation and analysis of *Streptomyces* plasmids, and several *Streptomyces* antibiotic resistance genes have been cloned and used as vectors (Thompson *et al.*, 1980; Thompson *et al.*, 1982; Kieser *et al.*, 1982; Lydiate *et al.*, 1985).

The purpose of this paper is to report the isolation and characterization of small and multi-copy plasmids, pJY501 and pJY711, isolated from soil. We also describe the construction of derivatives carrying the thiostrepton resistance gene (*tsr*) (Thompson *et al.*, 1982b).

MATERIALS AND METHODS

Bacterial strains and plasmids

Bacterial strains and plasmids used in this

Table 1. *List of bacterial strains and plasmids*

Strains/Plasmids	Genetic marker	Source
Strain No. 9(pJY091)		This study
Strain No. 47(pJY471)		"
Streptomyces sp. No. 50 (pJY501)		"
Strain No. 56(pJY561)		"
Streptomyces sp. No. 71(pJY711)		"
S. lividans TK24	*str*-6	Hopwood
S. lividans TK150 (pIJ702)	Thio^r, Mel$^+$	Katz *et al.*
E. coli V517	CCC plasmids marker	Macrina *et al.*

study are shown in Table 1.

Culture conditions and transformation procedures

Actinomycetes strains were isolated on glucose asparagine agar (10 g glucose, 0.5 g K_2HPO_4, 0.5 g asparagine, 15 g agar). Culture media for *Streptomyces* strains were YEME medium (Bibb *et al.*, 1977), containing 34 % sucrose, 0.2 % $MgCl_2$ $6H_2O$ and 0.5 % glycine, and Tryptic soy broth (Difco) and R2YE (Thompson *et al.*, 1980). Protoplast formation and transformation of *S. lividans* were as in Thompson *et al.* (1982a). Direct selection for thiostrepton resistant transformants was made by overlaying regeneration medium (R2YE) with 2.5 m*l* soft nutrient agar (Difco) containing 500 μg thiostrepton per m*l* after incubation for 20 hrs (Kieser, 1982). Thiostrepton (kindly donated by Mr. S.J. Lucania of E.R. Squibb and Son, New Brunswick, USA) was used in liquid at 5 μg per m*l* and solid media at 50 μg per m*l*.

Isolation of Actinomycetes from soil and Screening of plasmids

Soil samples dried at 37°C for 7 days were diluted with distilled water and spread on glucose asparagine agar. After incubation at 28°C for 5 days, the colonies regarded as Actinomycetes were selected and preserved in slants. Actinomycetes strains isolated from soil were incubated at 28°C in YEME medium or Tryptic soy broth. Plasmid DNAs were isolated using the alkaline extraction procedure described by Birnboim and Doly (1979) and methods of Kieser (1984) and Hopwood *et al.* (1985).

Identification of the isolates carrying plasmids

Morphological test, physiological tests, diaminopimelic acid (DAP) and monosaccharide analysis of cell wall were performed following procedures described by Berd (1973), Lechevalier (1970) and Bergey's manual of determinative bacteriology (1974).

DNA isolation and manipulation

Plasmids were isolated from *Streptomyces* by the procedure of Birnboim and Doly (1979) and methods of Kieser (1984) and Hopwood *et al.* (1985). For restriction enzyme endonuclease digestion, plasmid DNAs were purified further by CsCl-EtBr gradient centrifugation. All restriction enzymes, ligase and bacteral alkaline phosphatase were used under the condition recommended by the manufacturer. Reactions were terminated by heating to 65°C for 5 min, except for *BclI* where ethanol precipitation was used to stop the reaction. The recovery of DNA fragments from agarose gel was performed using Unidirectional electroeluter (International Biotechnologies, Inc. Model UEA).

Plasmid copy number determination

Plasmid copy number was determinated using method described by Kieser *et al.* (1982). For the determination of plasmid copy number mycelium was grown for plasmid preparation. Total DNA was prepared using procedure of Hopwood *et al.* (1985). Samples for each dilution were loaded on to agarose gels for electrophoresis. The gels were stained for 30 min in ethidium bromide (0.5 μg per m*l*). The gels were destained in several changes of water and photographed. The negatives were scanned with a LKB densitometer to determine the peak areas of chromosomal and plasmid DNA. Then plasmid copy number was determined by the peak areas that gave the ratio of plasmid to chromosomal DNA.

RESULT AND DISCUSSION

Isolation of Actinomycetes strains and screening

of plasmids

A total of 81 Actinomycetes isolated from soil were investigated for the presence of plasmids. Plasmids were detected in 5 strains and designated as pJY091, pJY471, pJY501, pJY561 and pJY711 (Fig. 1). 6.2 % plasmid-containing strains was lower than 15-25 % that had been reported (Hayakawa *et al.*, 1979; Okanish *et al.*, 1980; Hopwood, 1981) but was similiar percentage with Ohunuki (1983). All plasmids were easily isolated with procedures to be used. The molecular weight of plasmids was estimated about 55 kb, 6.2 kb, 4.4 kb, 55 kb and 7.0 kb, respectively by using CCC plasmids of *E. coli* V517 as standard size markers. Among them, small and apparent high copy number plasmids, pJY501 and pJY711, were selected for further experiments.

Identification of the isolates carrying plasmids

Morphology was observed after 2 to 3 weeks on yeast-malt extract agar. The chains of spore in both strain No. 50 and strain No. 71 formed spirals. The surface of spores was smooth in case of strain No. 50 and warty in case of strain No. 71. Fig. 2 showed the electronmicroscopic photograph of strain No. 50 and strain No. 71. The cultural characteristics of the isolates are listed on Table 2 and physiological characteristics are on Table 3. Two strains showed good growth on yeast extract-malt extract agar and glucose-peptone agar and produced soluble pigments. Two isolates decomposed starch, casein, hypoxanthine and tyrosine. And Two strains were sensitive to lysozyme and utilized all carbohydrates except salicin. The hydrolysate of two isolates was found to contain LL-diaminopimelic acid by thin layer chromatography. And the monosaccharide analysis demonstrated that cell walls of the isolates did not contain any monosaccharides (Fig. 3). Two isolates were cell wall type I according to the classification by Lechevalier *et al.* (1970). In comparison with the description of Bergey's manual of determinative bacteriology, the strain No. 50 and the strain No. 71 were identified as the genus *Streptomyces* and designated *Streptomyces* sp. No. 50 and *Streptomyces* sp. No. 71, respectively.

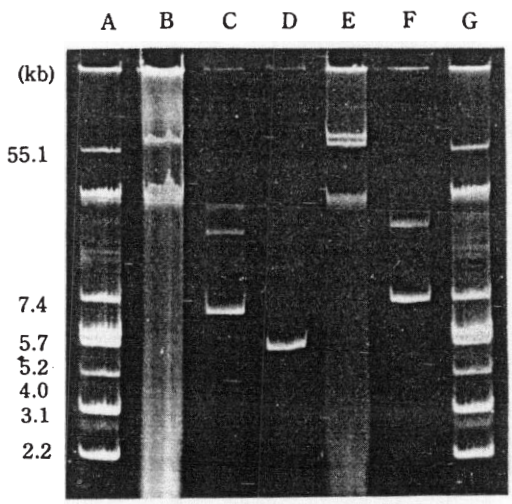

Fig. 1. *Agarose gel electrophoresis pattern of plasmids of the isolates.*
A, G; *E. coli* V517 B; pJY091 C; pJY471 D; pJY501 E; pJY561 F; pJY711

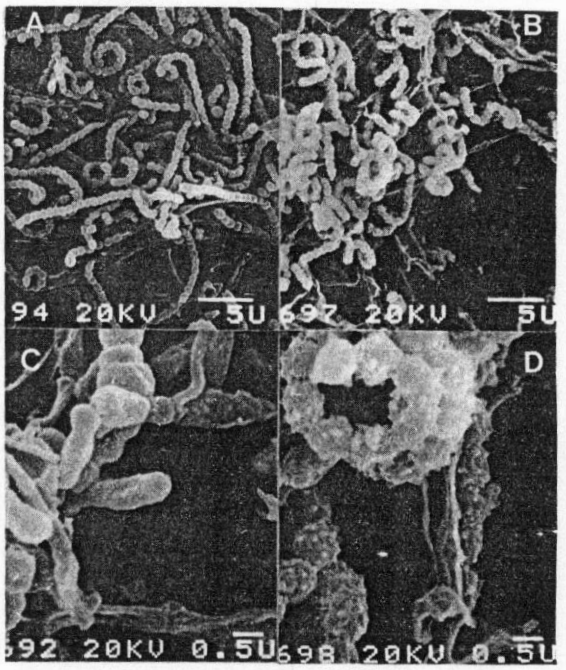

Fig. 2. *Electronmicroscopic photographs of the isolates.*
A; Spore chains of strain No. 50 B; Spore chains of strain No. 71 C; Spore surface of strain No. 50 D; Sore surface of strain No. 71

Characterization of pJY501 and pJY711

The plasmids purified by CsCl-EtBr density

Table 2. *Culture characteristics of the isolates*

Characteristics / Strain number	Growth		Aerial Mycelium		Reverse side		Soluble pigment	
Media	50	71	50	71	50	71	50	71
Yeast extract-malt extract agar	a	a	g	g	y	b	y	b
Oat meal agar	p	p	b	w	y	b	y	-
Inorganic salts-starch agar	p	p	b	w	y	b	y	-
Glycerol-asparagine agar	p	p	w	b	y	b	y	-
Tyrosine agar	p	p	y	w	y	b	-	b
Glucose asparagine agar	p	p	y	w	y	b	y	-
Glucose-nitrate agar	p	p	g	w	y	b	y	b
Glucose-peptone agar	a	a	b	w	b	b	-	-
Nutrient agar	p	p	-	-	y	y	y	-

a; abundant m; moderate p; poor y; yellow b; brown g; gray w; white

Table 3. *Physiological properties of te isolates*

Tests	Strain No. 50	No. 71
Melanin production	-	+
Nitrate reduction	-	-
Indol test	-	-
Catalase test	+	+
Starch hydrolysis	+	+
Casein hydrolysis	+	+
Gelatin liquifection	+	-
Litmus milk test	-	-
Urease test	-	-
Lysozyme resistance	sensitive	sensitive
CMC hydrolysis	-	-
Decomposition of xanthine	-	+
Decomposition of hypoxanthine	+	+
Decomposition of tyrosine	+	+
Carbohydrate utilization		
mannitol	+	+
salicin	-	-
galactose	+	+
arabinose	+	+
sucrose	+	+
raffinose	+	-
glucose	+	+
inositol	+	+
fructose	+	+
rhamnose	+	+
xylose	+	+

+ ; positive - ; negative

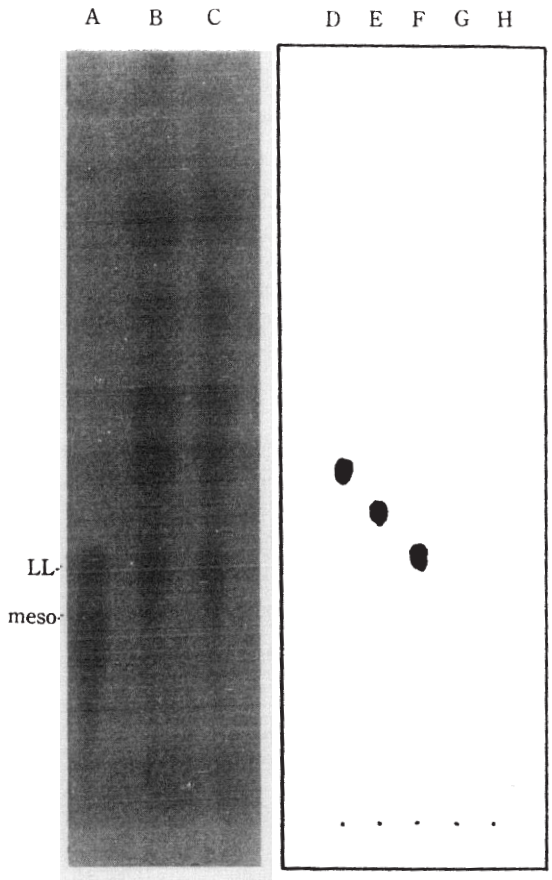

Fig. 3. *This layer chromatography of DAP isomers and monosaccharide of cell wall.*
A; Mixture of LL-, DD- and meso-DAP B; Strain No. 50 C; Strain No. 71 D; Xylose E; Arabinose F; Galactose G; Strain No. 50 H; Strain No. 71

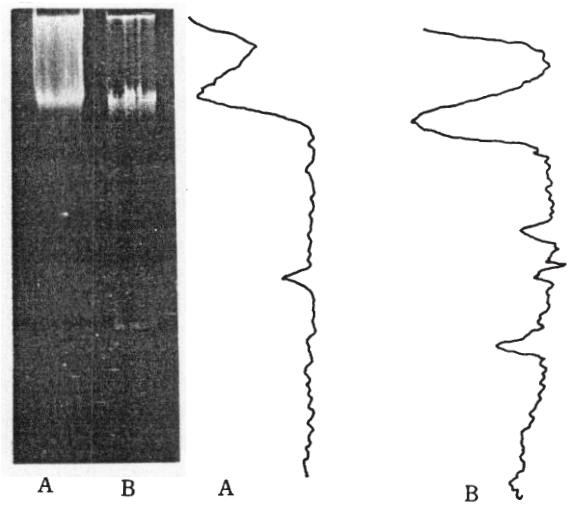

Fig. 4. *Patterns of micro-densitometric analysis.*
A; pJY711 B; pJY501

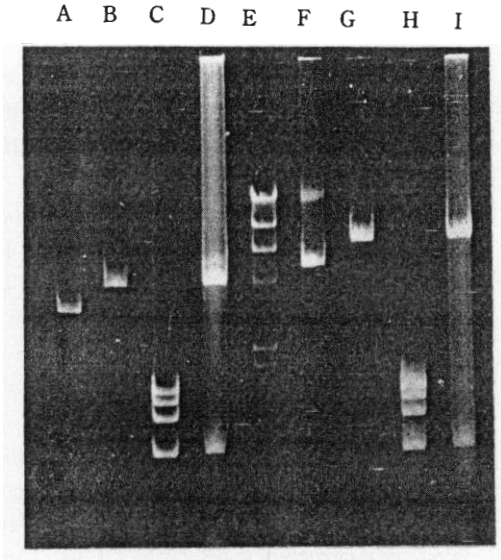

Fig. 5. *Agarose gel electrophoresis pattern of recombinant plasmids pJY502 and pJY712.*
A; pJY501 B; pJY501 digested with *Bcl*I C, H; pIJ702 digested with *Bcl*I D; pJY502 digested with *Bcl*I E; λ DNA digested with *Hin*dIII (23.13, 9.42, 6.68, 4.36, 2.32, 2.03, 0.56 kb) F; pJY711 G; pJY711 digested with *Bcl*I I; pJY712 digested with *Bcl*I

gradient centrifugation preserved the conformation of supercoiled covalently closed circular molecular. And the digestion of pJY501 and pJY711 with *Bgl*II, *Bcl*I generated single linear fragments. A apparent copy number of pJY501 and pJY711 was estimated 150-200 and 30-40 per chromosome by microdensitometric analysis (Fig. 4).

Construction of pJY501 and pJY711 derivatives carrying the *tsr* gene

Plasmids pJY501 and pJY711, were digested with *Bcl*I and treated with bacterial alkaline phosphatase. As a source of thiostrepton resistance gene (*tsr;* 1.09 kb), pIJ702 (Katz *et al.*, 1983) was digested at its four *Bcl*I sites and eluted using electroeluter. Each two DNA preparations were ligated and the ligated DNA was used to transform protoplasts of *S. lividans*. 5 thiostrepton-resistant colonies in case of pJY501 and 12 colonies in case of pJY711 were obtained. Two recombinant plasmids (designated pJY502 and pJY712) were detected by BclI digestion and agarose gel electrophoresis (Fig. 5). Both pJY502 and pJY712 readily transformed protoplasts of *S. lividans* to thiostrepton resistance.

Plasmids, pJY501 and pJY711, isolated from *Streptomyces* sp. No. 50 and *Streptomyces* sp. No. 71 were small (4.4 kb and 7.0 kb) and high copy number plasmids (about 150 per chromosome for pJY501 and about 35 for pJY711). Because of their originally small size and high copy number, the derivatives of pJY501 and pJY711 were attractive for development of potential vectors in *Streptomyces*.

적 요

토양으로부터 plasmid를 갖고 있는 5주의 방선균을 분리하였다. 이들 plasmid의 분자량은 각각 55kb, 6.2kb, 4.4kb, 55 kb와 7.0kb였다. 이들중 크기가 작고 높은 copy수를 가진 4.4kb의 pJY501과 7.0kb의 pJY711를 선택하였다. CsCl 밀도 구배 초원심분리를 행한결과 두 plasmid는 covalently closed circular 형태로 존재하였으며 chromosome당 copy수는 각각 150과 35 정도였다. Plasmid를 갖고 있는 두 분리균은 *Streptomyces* 속으로 동정되었다. 분리된 두 plasmid에 selection marker를 삽입하기 위하여 pJY501의 *Bcl*I 인식부위에 pIJ702의 *tsr* 단편을 삽입하고 pJY711의 *Bcl*I 인식부위에 *tsr* 단편을 삽입하여 재조합 plasmid pJY502와 pJY712을 제조하였다.

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