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Isolation and Characterizaton of Plasmids from *Streptomyces*

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Streptomyces spp. purchased from American Type Culture Collection and Institute for Fermentation in Osaka, and donated from Northern Regional Research Laboratory, and those isolated from soil samples were assayed to isolate many plasmids harboring streptomycetes. Among these organisms, 5 small size-plasmid carrying organisms SNUS 8810-597A, 8810-600, 8810-754, 8811-344, and 8811-347 were characterized and their plasmids pSJ597, pSJ600, pSJ754, pSJ344, and pSJ347 were isolated in a large scale. The plasmid harboring organisms were sensitive to neomycin, kanamycin, gentamicin, and thiostreptone, but some of them showed weak or strong resistance against streptomycin, chloramphenicol, ampicillin, and tetracycline. It was confirmed that pSJ597 and pSJ600 do not carry antibiotic biosynthetic genes. pSJ600 showed a pock-forming character.

Approximately 70% of all naturally occurring antibiotics are isolated from *Streptomyces* spp. and it is in the commercial production of antibiotics that this genus has been exploited (6). Still the genetics and molecular biology of many *Streptomyces* are poorly understood. Recent development of many vectors and improvement in protoplast preparation, regeneration, and transformation has facilitated cloning of many genes or gene clusters which are responsible for biosynthesis of antibiotics (11, 18 20). Thus, a lot of studies are carried out on genetic organization, gene expression, and gene regulation in *Streptomyces* and now we are about to understand the genetics of *Streptomyces* more clearly (25).

Development of broad host range plasmid mediated gene cloning systems has aided molecular studies of *Streptomyces*. Although currently available plasmid vectors appear to have utility in many *Streptomyces* species (7), restriction barriers between species and the possible instability of some plasmids in a certain species could result in need for different plasmid replicons.

We have been interested in development of new vectors for *Streptomyces*. We screened many Streptomyces species obtained from American Type Culture Collection (ATCC), National Regional Research Laboratory (NRRL) and Institute for Fermentation in Osaka (IFO), and those isolated from soil samples collected from the campus of Seoul National University. In this paper we wish to report the isolation and partial characterization of 5 new plasmid-harboring streptomycetes.

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Key words: plasmids, *Streptomyces*, screening, Pock formation, biological activities

MATERIALS AND METHODS

Strain and Culture Condition

NRRL strains were obtained from the culture collection of the Nothern Regional Research Laboratory, Agricultural Research Service, Peoria, Illinois, USA. IFO and ATCC strains were purchased from Institute for Fermentation in Osaka and American Type Culture Collection, Rockville, MD, USA. They were cultured by spreading or by streaking spores on V-8 agar (5) plates or on ISP No. 4 agar (purchased from Difco) plates. The freeze-dried spores were used to prepare V-8 agar (5) slants. The spores grown on agar slants were used to inoculate broth cultures.

Preparation of Spore Suspensions of *Streptomyces*

Spore suspensions were prepared by the reported method (8). After they were cultured for 14 days or more spores were harvested by scratching the agar surfaces with 24% sucrose solution-tryptic soy broth (TSB, purchased from Sigma) or nutrient broth (1 : 1). The spore suspensions were freeze-dried and stored at 4°C.

Preparation of Spore Suspension of *B. subtilis*

Sterilized 20% glycerol (1~2 ml) was added to the nutrient agar plates in Petri dish grown with *B. subtilis* 6633 for 7 days at 29°C and scratched spores were transferred to a tube and stored under nitrogen gas at 4°C.

Sterilization

Sterilization was carried out either with autoclave at

121°C for 15 minutes or by filtering the solution on membrane (2 μm pore size, Gelman Co.).

Isolation of Soil Streptomycetes

Soil microbes were cultured on actinomycetes selection media and streptomycetes were isolated by the agar disk method (5) The selected streptomycetes were pure-cultured on V-8 juice or on ISP No. 4 agar plates at 28°C for 7~14 days. Agar slants of the same media were prepared and the organisms were cultured and stored at 4°C.

Susceptibility to Antibiotics of the Isolated Streptomycetes

The solutions of antibiotics which were prepared by dissolving streptomycin sulfate, neomycin sulfate, gentamicin sulfate, tetracycline hydrogen chloride, kanamycin sulfate and ampicillin sodium salt in distilled water (20 mg/ml) and by diluting with an equal volume of distilled water, or by dissolving thiostreptone in EtOH (20 mg/100 μl) or chloramphenicol in EtOH (10 mg/ml) and by diluting these solutions with an equal volume of DMSO (thiostreptone) or of ethanol (chloramphenicol). The first solution was filtered through membrane (0.22 μM in pore size) and the dilution was repeated and the diluted solutions were added to ISP No 4 agar solutions stored at 50°C to give the final antibiotic concentrations of 1000, 500, 250, 125, 62.5, 31.2, 15.6, 7.8, 3.9 and 1.9 μg/ml. The agar solution was poured into petri dishes and hardened. The plates were placed on a desk overnight and streaked with streptomycetes. The plates were incubated at 28°C for 4 or 7 days and growth of organisms was observed.

Antibiotic Production Assay and Antimicrobial Activity Assay

Bacillus subtilis ATCC 6633, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* IFO 13738, *Proteus vulgaris* IFO 3167 were cultured in nutrient broth either at 28°C or at 37°C (*E. coli* only) for 16~18 hours on a reciprocal shaker (180 rpm). The culture was added to a liquid nutrient agar medium stored at 47°C to give 2% (v/v) inocula. The liquid media (20 ml) were mixed and dispensed in Petri dishes and hardened. Agar disks (8 mm in diameter) grown with *Streptomyces* for 4 days or paper disks (12 mm in diameter) wetted with 50 or 100 μl of TSB cultured with *Streptomyces* also for 4 days were placed on the agar plates seeded with test organisms. The agar plates were incubated at 28°C or 37°C for 18 hours. The inhibition zones around agar disks or paper disks were measured.

Alternaria longipes ATCC 1502 was grown on V-8 agar plates at 28°C for one week. Triton X-100 solution (5 ml), which was made by mixing 50 μl of Triton X-100 with 200 ml of distilled water was added to each Petri dish. Spores and mycelia were scratched off from the agar surface and the liquid suspended with organisms was transferred to 200 ml of Trypotic Soy agar liquid medium stored at 45°C. The liquid agar medium (20 ml) was dispensed on Petri dishes and hardened at room temperature. The plate was kept at 4°C and used for assay of antifungal compounds by the same method as that for unicellular eubacteria.

Culture of Organisms for Plasmid Isolation

The organisms on V-8 agar slants were used to inoculate TSB (50 ml). Culture was carried out on a reciprocal shaker (200 rpm) at 28°C for 48 hours. Mycelium was harvested from the liquid culture by centrifugation and washed with 0.3 M sucrose solution. The mycelium was stored at −20°C.

Isolation and Detection of Plasmids

Isolation of plasmids from *Streptomyces* mycelium was carried out by following the reported procedure (14) with mycelium (150~200 mg wet weight) stored at −20°C.

Large Scale Culture for Large Scale Isolation of Plasmid DNAs

The seed cultures were obtained by incubation of 50 ml of TSB or 34%-sucrose-TSB which were inoculated with mycelium from V-8 agar slants for 24 hours at 28°C. The seed culture (10 ml) was used to inoculate 500 ml of the medium in 2-liters Erlenmyer flasks with the same composition as the seed culture. The medium in 2-liter flasks was incubated for 48 hours at 28°C on a reciprocal shaker. The mycelium was harvested by centrifugation at 6000 rpm for 10 min. and washed with 0.3 M sucrose solution for 2 times. The mycelium was then stored at −20°C.

Large Scale Isolation of Plasmid DNAs

The same procedure was employed as described for detection of plasmids in *Streptomyces* with frozen mycelium (2.5~3.5 g). The crude solution was further purified by treatment of spermin to precipitate plasmids and by cesium chloride gradient centrifugation.

Isolation of total DNAs

Total DNAs were isolated from *Streptomyces* by following the reported method (15).

Transformation of *S. lividans* 1326 or TK 24 with Plasmids and Isolation of Pock Forming Colonies

The protoplasts solution (4×10^8) prepared by the reported method (23) of *S. lividans* 1326 or TK24 was centrifuged for 10 min with a desk-top-centrifuger and the supernatant was removed. The protoplasts were suspended in the liquid left over. To this solution, 0.2 μg (*S. lividans* TK 24) or 1.0 μg (*S. lividans* 1326) of plasmids pSJ597, pSJ600, pSJ754, pSJ344, or pSJ347 were added. The solution was diluted with 500 μl of T buffer, mixed by pippetting and cooled in ice water for 1 min. (*S. lividans* TK 24) or 2 min (*S. lividans* 1326).

The solution was then further diluted with 2~3 ml of P buffer and centrifuged for 10 min. with a desktop-centrifuger. After removing the supernatant, the protoplasts were suspended in the residual liquid and spreaded over R2YE mdium plates. After the plates were cultured at 28°C for 4~7 days, pock-forming colonies were observed.

Confirmation of Pock-forming Characters

The transformant of *S. lividans* 1326 by plasmid pSJ 600 was cultured on ISP No. 4 plates for 14 days at 28°C. Spore solution obtained from the plates was diluted 10 times with distilled water and 100 μl of the solution was spread over an ISP no 4 agar plate spreaded already with 100 μl of the spore solution of *S. lividans* 1326. The plates were cultured at 28°C for 4~7 days and pock-forming colonies were confirmed.

RESULTS AND DISCUSSION

Screening of Plasmids from *Streptomyces*

Many diverse plasmids have been isolated from *Streptomyces* species and some of them have specific biological functions (9, 10). When we examined the extracts of 43 *Streptomyces* species listed in Table 1 for extrachromosomal DNA elements by the method reported by Kieser (14), we could confirm plasmids in some of the species. For plasmid isolation, they were cultured in 34%-sucrose-tryptic soy broth (34% sucrose-TSB) and harvested at the logarithmic phase. *Streptomyces bifurcus* NRRL 3539, *Streptomyces forma paramomycinus* NRRL 2455, *Streptomyces aureofaciens* NRRL B-1288, *Streptomyces viridofaciens* NRRL B-1679, *Streptomyces cacaoi var. asoensis* ATCC 19093, *Streptomyces griseus* ATCC 23345, *Streptomyces galbus* ATCC 14077, *Streptomyces kanamyceticus* NRRL 2535 did not grow in 34 %-sucrose-TSB well and they are cultured in TSB without addition of sucrose. The mycelia were lysed by treatment of lysozyme and alkaline SDS. After treatment of acidic phenol-chloroform, the aqueous layer was separated and analyzed for plasmids on agarose gel by electrophoresis.

As reported by Manis and Highlander (19), *Streptomyces espinosus* NRRL 11439 harbored a plasmid with a low copy number. The organism grew well on ISP No. 4 agar plate but did not grow well in 34%-sucrose-TSB. While Wang *et al.* (26) reported isolation of 4 plasmids (pSE1: 11.04 kb, pSE2: 12.34 kb, pSE3: 17.46 kb, and pSE6: 140.3 kb) in *Streptomyces erythreus* NRRL 2338, we could not confirm any plasmids in this organism. This organism is also reported to harbor a plasmid (pSG1: 16.6 kb) existing as a free form or as one that is integrated in chromosome (3). In *Streptomyces griseus* no plasmid has been confirmed yet. The extracts of

Table 1. *Streptomyces* species assayed for plasmids

Strains		Antibiotic production	Culture in 34%-sucrose-TSB	days in TSB
S. fradiae	NRRL 3718	Neomycin	1~2	
	NRRL B-1195	"	3	
S. griseus	IFO 3357	Streptomycin	2~3	
	NRRL B-2682	"	2	
	ATCC e10137	"	2	
	ATCC 12475	"	1~2	
	ATCC 23345	"		2
	ATCC 15395	"	2	
	ATCC 27001	"	2	
	ATCC 31087	"	2	
	NRRL 3851	Cephamycin	2	
S. kanamyceticus	*IFO 13414*	*Kanamycin*	2	
	NRRL 2535	"		2
S. hygroscopicus	IFO 12703		3	
S. griseus subsp. *rubidofaciens*	NRRL 3383		3	
S. ribodidificus	NRRL B-11466	Ribostamycin		4
S. coeruleo-rubidus	NRRL 3045		2~3	
S. bifurcus	NRRL 3539		2	
S. f. paramo-mycinus	NRRL 2455	Paramomycin	2	
S. rimosus	NRRL 2234		1~2	
S. erythreus	NRRL 2338	Erythromycin	2	
S. espinosus	NRRL 11439			2~3
S. kitosatoensis	NRRL 2486		2	
S. lividans	3131 JI		3	
	1326 JI		3	
S. coelicolor	A3(2)	Methyleno-mycin	3	
S. aureofaciens	*NRRL 2209*		*1~2*	
	NRRL B-1287		2	
	NRRL B-1288			8
S. chrysomallis	NRRL 2250		2	
S. venezuelae	NRRL B-902		3	
	NRRL B-2277		3	
S. parvulus	NRRL B-1628		3	
S. viridofaciens	*NRRL B-1679*			2
S. galbus	ATCC 14077	Streptomycin		2
S. murinus	*NRRL 2286*		*3*	
S. cacaoi var. asoensis	ATCC 19093			?
	ATCC 19094		3	
S. lincolensis	NRRL 2936		1~2	
S. moderatus	NRRL 3150		2	
S. clavuligerus	NRRL 3585		3	
S. albogriseolus	NRRL B-1305		1~2	

Streptomyces fradiae NRRL B-1195, *Streptomyces ribosidificus* NRRL 11466, and *Streptomyces moderatus* NRRL 3150 showed faint bands which are moving slower than chromosomal bands. Actually many groups (4, 17, 24, 27) reported the existence of a large plasmid in *S. fradiae* ATCC 10745 (NRRL B1195). Okanishi *et al.* (24) and Nojiri *et al.* (22) confirmed a large plasmid in *S. ribosidifi-*

Table 2. The soil streptomycetes found to harbor plasmids

SNUS-8810-:	597A, 668B, 619, 683A, 331, 751, 754, 758, 758-1, 331A1, 277A, 600, 688, 171, 336, 2, 608, 334, 597B, 321A, 277A, 277B, 124, 667, 856, 725, 684, 635, 272.
SNUS-8811-:	433, 371, 347, 451, 329, 344, 303, 225, 82, 525, 193, 495, 451A1

cus. In the present isolation work, we could confirm a large plasmid in *Streptomyces moderatus* NRRL 3150 for the first time. It is known that *Streptomyces clavuligerus* contains pSCL (12 kb) which is a linear plasmid that has a protein at the 5'-OH terminal (12).

Screening of Plasmids in Soil Streptomycetes

In 1988, we isolated about 1500 streptomycetes from soil samples collected from campus of Seoul National University (5). Among these organisms, we examined 470 organisms for plasmids and we confirmed 34 plasmid-harboring streptomycetes at the first screening. Several other species were also suspected to have plasmids. Reexamination of the 34 organisms revealed that 29 organisms were plasmid-harboring. These organisms and others suspected to harbor plasmids were streaked on V-8 agar plates and some of the organisms were further pure-cultured. All the organisms grew with formation of hyphae on agar plates and showed characteristics of streptomycetes. Reexamination of the pure-cultured organisms for the presence of plasmids revealed that 41 organisms were plasmid-harboring (Table 2). Among these organisms, organisms SNUS 8810-2, 8810-331A1, 8810-597A, 8810-600, 8810-608, 8810-754, 88 11-344, and 8811-347 harbored plasmids which showed faster mobility than the chromosomal DNA bands, but organisms SNUS 8810-272, 8810-495, 8810-619, 8810-635, 8810-683A, 8810-684, 8811-82, 8811-225, 8811-371, and 8811-525 harbored large plasmids showing slower mobility than the chromosomal band. The organisms SNUS 8810-684, 8810-683 and 8810-619 formed colonies with grey aerial mycelia and with the same backside color on ISP No. 4 agar plates. They also showed same antimicrobial spectra and harbored plasmids showing same mobilities under electric field. Thus, we concluded that they were identical organisms. After reconfirmation of the presence of plasmids several times, we selected 5 small-plasmid harboring organisms, SNUS 8810-597A, 8810-600, 8810-754, 8811-344, and 8811-347 and 5 large plasmid harboring organisms SNUS 8811-82, 8810-684, 8810-683, 8810-619 and 8811-525. The picture of Fig. 1 shows plasmid bands on an agarose gel. Table 3 describes some characteristics of the small size-plasmid-haboring organisms when they were grown on V-8 agar plates. The plasmids isolated were named pSJ.

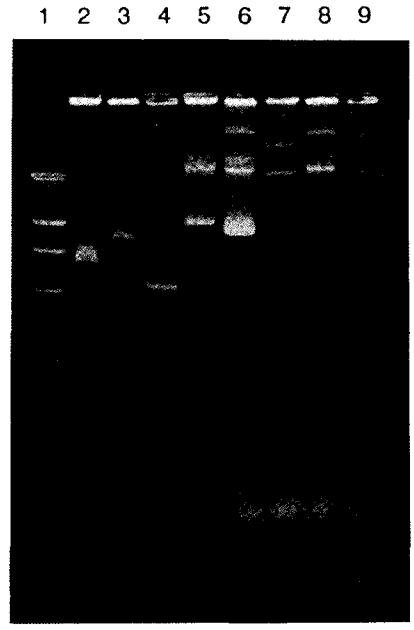

Fig. 1. Confirmation of plasmid DNAs isolated from soil bacteria by agarose gel electrophoresis.
The picture shows phage lamda DNA digested with *Hin*dIII (1), small size plasmids isolated from SNUS-8810-597A (2), 8810-600 (3), 8810-754 (4), 8811-344 (5) and 8811-347 (6) and large size plasmids isolated from SNUS 8810-684 (7), 8811-82 (8) and 8811-525 (9).

Large Scale Isolation of Plasmids

Small size plasmids might be better for developing new cloning vectors. In order to characterize more precisely these plasmids, large scale isolation was carried out for the small-plasmid haboring organisms, SNUS 8810-597A, 8810-600, 8810-754, 8811-344, and 8811-347 by employing the same method as used for the screening of plasmids. The mycelia harvested from 2 liters broth (TSB) cultures (50~60 g of wet weight; 100 g of wet weight for the organism SNUS 8810-754) were treated with lysozyme and lysed with alkaline SDS solution. The lysed mixture was treated with acidic phenol-chloroform and the supernatant was treated with RNase followed by treatment of neutral phenol-chloroform. The aqueous supernatant layer was separated and treated with spermine to precipitate DNA. The precipitated DNA was redissolved and applied on CsCl-EtBr solution. The solution was ultracentrifuged and the plasmid bands were collected and dialyzed. The isolated amounts of plasmids estimated by UV absorption value are given in Table 4. Their restriction maps will be published elsewhere.

Estimation of Copy Numbers of Plasmids

The fluorescence intensities of the chromosomal and the plasmid DNA bands obtained by electrophoresis of the total DNA extracts isolated from these organisms

Table 3. Growth characteristics on V-8 agar plates of the 5 plasmid-harboring soil streptomycetes and the maximum concentrations (μg/ml) of streptomycin (SM), neomycin (NM), kanamycin (KM), ampicillin (Amp), gentamicin (GM), chloramphenicol (CM), thiostreptone (TS), and tetracycline (TC) that allows growth of streptomycetes on ISP No. 4 agar plates after 4 or 7 days' culture

Strain No.	Color aerial mycelium	Color reverse colony	Antibiotic concentration							
			SM	NM	KM	Amp	GM	CM	TS	TC
SNUS 8810-597A	pale	brownish	–	–	–	63	–	1.9	–	15
	grey	brown	3.8	–	–	125	–	3.8	–	31
8810-600	grey	brown	–	–	–	31	–	3.8	–	15
			1.9	–	–	63	–	7.5	–	31
8810-754	brownish	orange	500	3.8	–	1000	7.5	15	–	15
	yellow	yellow	500	7.5	1.9	1000	15	15	–	31
8811-344	brownish	brick	500	15	7.5	500	63	1.9	1.9	63
	yellow		1000	15	15	500	31	63	7.5	125
8811-347	dark	yellowish	7.5	1.9	3.8	1000	3.8	15	–	65
	grey	brown	7.5	1.9	7.5	500	7.5	31	–	125
S. lividans 1326			15	1.9	3.8	1000	3.8	15	–	65
			15	1.9	7.5	500	7.5	31	–	125
S. lividans 3131			ND	1.9	3.8	1000	15	63	1000	65
			ND	3.8	7.5	1000	15	63	1000	125

Table 4. The amounts of plasmid DNAs isolated from soil streptomycetes

Strain	*Plasmid*	Amounts of plasmid isolated
SNUS-8810-597A	pSJ597A	307 μg
8810-600	pSJ600	50 μg
8810-754	pSJ754	67 μg
8811-344	pSJ344	360 μg
8811-347	pSJ347	327 μg

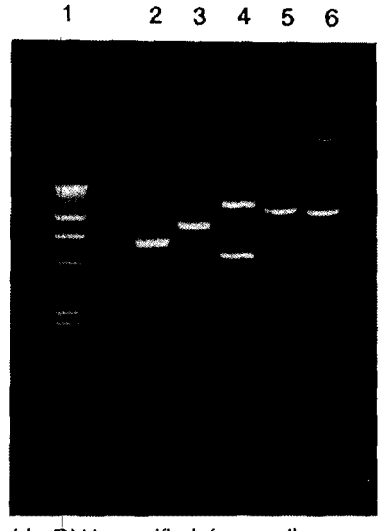

Fig. 2. Plasmids DNAs purified from soil streptomycetes in a large scale by CsCl-EtBr centrifugation.
The pictures shows DNA bands of phage lamda DNA digested with *HindIII* (1), pSJ597 plasmid (2), pSJ600 plasmid (3), pSJ754 plasmid (4), pSJ344 plasmid (5) and pSJ347 plasmid(6)

by the literature method (1) were compared to estimate copy numbers of plasmids. The result is shown in Fig. 2. When we compare the intensities of the plasmid and the chromosomal DNA bands of *Streptomyces lividans* 3131, which are known to produce pIJ702 at the copy number of 4-200 (the copy numbers of plasmids in this organisms are dependent on its growth phase (1)) with those of newly isolated from soil streptomycetes, it was indicated that all the plasmids are high copy number ones as pIJ702.

Taxanomical Studies of Isolated Streptomycetes

Examination of culture characteristics on ISP media, physiological characteristics, the spore surface of the organisms by the scanning electron microscopy and the photomicrographs of the mycelium of the ogranisms suggested that all the five organisms belonged to streptomycetes (10).

Biological Characteristics of Plasmids or Plasmid-haboring Organisms

pSV1 plasmid possesses biosynthetic and resistance genes for antibiotic methylenomycin (2). SCP2 or SCP2* are gender factors (21). Some plasmids are found to have pock-forming characters (1, 8, 16). We examined the plasmid-harboring *Streptomyces* for the antibiotics they produce and their resistance characteristics against various antibiotics. Examination of these characteristics is important since the vectors developed from plasmids need specific markers to use as cloning vehicles. Usually antibiotic producing organisms show resistance against

Table 5. Antimicrobial activities (mm)* of antibiotics produced by plasmid carrying streptomycetes

Strain	*B. subtilis*	*E. coli*	*Pr. vulgaris*	*Ps. aeruginosa*	*Py. pruzae*
SNUS 8810-597A	30	–	–	–	23
8810-600	28	–	–	–	20
8810-754	18	–	–	–	–
8811-344	17	–	–	–	–
8811-347	17	–	–	–	–

* Antimicrobial activities were obtained by the agar disk diffusion method. Agardisks (8 μm in diameter) grown with the streptomycetes for 4 days were placed on agar plates seeded with the test organisms and the diameter of th growth inhibitionzones were measured after 18 hours' incubation.

the antibiotics they produce. Thus, the resistance characteristics against certain antibiotics may imply that the organisms may produce antibiotics in the same class.

Antibiotic resistance characteristics of the plasmid-harboring organisms are summarized in Table 3. The results suggest that the plasmid-harboring soil streptomycetes do not possess antibiotic resistance genes against neomycin, kanamycin, gentamicin, chloramphenicol, thiostreptone and spectinomycin. Organisms SNUS 8810-754 and 8811-344 showed strong resistance against streptomycin. *S. lividans* 1326 and its plasmid pIJ702 haboring transformant 3131 strain was employed as controls. *S. lividans* 3131 contains pIJ702 which has a resistance gene against thiostreptone and a melanine formation gene. Many streptomycetes including *S. lividans* showed resistance against ampicillin. The organisms SNUS 8810-597A and 8810-600 showed very weak resistance against some antibiotics tested. Antibiotic resistance data were obtained by culturing the organisms on agar disks in which antibiotics are added in series by 2-fold dilution. Growth of organisms was checked after 4 or 7 days' incubation.

Examination of antibiotic-production was carried out by culturing the plasmid harboring organisms on TSB agar or V-8 juice agar disks. These agar disks grown with the organisms for 4 days were laid over agar plates seeded with antimicrobial activity assay organisms. As shown in Table 5, some of the plasmid-haboring organisms showed production of antibiotics which are active against *Bacillus subtilis* and *Pyricularia oryzae*. When the organisms were cultured in TSB, none of them showed antimicrobial activity against these organisms. We do not know yet why they do not produce antibiotics in liquid culture while they produce antibiotics on surface culture on agar plates.

Formation of Pock

When pock-forming characteristics were examined, pSJ600 which were isolated from organisms SNUS 8810-600 showed pock-forming characteristics. The pock-forming characteristics of pSJ600 will be published elsewhere.

Examination of Plasmids for Encoding Antibiotic Resistance or Antibiotic Biosynthesis Genes

S. lividans 1326 which was transformed by pSJ600 were obtained by cross of the organisms with the pock-forming plasmid harboring organism, SNUS 8810-600 did not show resistance against streptomycin. During cloning of a streptomycin resistance gene from *S. kanamyceticus*, with pSJ597 as a vector, pSJ597 harboring *S. lividans* 1326 were obtained. Examination of the pSJ 597 harboring transformant showed no resistance against streptomycin. Thus, the resistance gene against streptomycin in SNUS 8810-597A is existing in chromosome rather than the plasmid. When both the transformant was grown on V-8 agar disks and tested for the production of antibiotics, they did not show any antimicrobial activity. Thus, we concluded that in these two plasmids neither antibiotic production genes nor antibiotic (those examined in this paper) resistance genes are present.

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