ISOLATION AND CHARACTERIZATION OF LINEAR PLASMIDS FROM LANKACIDIN-PRODUCING *Streptomyces* SPECIES

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Streptomyces rochei 7434AN4, a producer of lankacidin and lankamycin contains three large linear plasmids, pSLA2-L (200 kb), M (100 kb), and S (17 kb). Studies on the mutants of 7434AN4 having a different plasmid profile showed a parallel relationship between the presence of pSLA2-L and the production of both lankacidin and lankamycin. When pSLA2-L was transferred by protoplast fusion to *S. rochei* 2-39, a non-antibiotic-producing mutant of 7434AN4 which contained no detectable plasmid, the fusants gained the capacity to produce both antibiotics. From the physical maps of pSLA2-L and pSLA2-L1, a deletion plasmid (160 kb) of pSLA2-L, the latter plasmid was determined to contain a symmetrical linear repeat composed of the right 80-kb part of pSLA2-L. Four other lankacidin-producing *Streptomyces* strains were also found to have distinctive large linear plasmids which hybridized with the pSLA2-L probe. These results support the involvement of pSLA2-L in the production of lankacidin and lankamycin in *S. rochei* 7434AN4.

The plasmid SCP1, which carries the methylenomycin biosynthetic gene cluster in *Streptomyces coelicolor* A3 (2), was shown to be a giant linear plasmid of 350 kb by pulsed field gel electrophoresis (PFGE)[1,2]. In an effort to investigate the involvement of linear plasmids in antibiotic biosynthesis following SCP1, large linear plasmids were detected in five *Streptomyces* strains[3]. Among them, *Streptomyces rochei* 7434AN4 had three linear plasmids of 200, 100 and 17 kb. This strain produces two structurally unrelated antibiotics, lankacidin[4,5], a 17-membered macrolide antibiotic and lankamycin[6], a 16-membered macrolide antibiotic. The same strain was reported to possess plasmid pSLA2, which was the first linear plasmid isolated from bacteria[7~9]. The smallest of the threes plasmids was found to be identical to pSLA2. We named the three linear plasmids pSLA2-S, M, and L, respectively. Curing experiments of pSLA2-S by HAYAKAWA *et al.*[7] suggested that pSLA2-S was involved in the lankacidin production.

Based on these data, we selected *S. rochei* 7434AN4 and other lankacidin-producing strains, to study the involvement of plasmid in antibiotic production. In this paper, we report the detection of large linear plasmids in all the lankacidin-producers tested and their homology by Southern hybridization. In particular, we describe the characterization of the three linear plasmids from *S. rochei* 7434AN4 and the involvement of the largest plasmid, pSLA2-L, in the production of lankacidin and lankamycin.

Materials and Methods

Bacterial Strains and Media

Lankacidin-producers and nonproducing mutants used in this study are listed in Table 1. *S. rochei* 7434AN4, 491, and 6642GC1 were described previously by HAYAKAWA *et al.*[7]. Other lankacidin-producers were obtained from ATCC (American Type Culture Collections, Rockville, U.S.A), JCM (Japan Collection of Microorganisms, Wako, Saitama, Japan), and IFO (Institute for Fermentation, Osaka, Japan). Mutants

Table 1. Lankacidin-producing *Streptomyces* strains and mutants of *Streptomyces rochei* 7434AN4.

Strain	Linear plasmids (kb)	Origin/Reference
S. griseofuscus JCM4276	30, 50, 200	SAKAMOTO *et al.*[10]
S. violaceoniger IFO14166	410	GAUMANN *et al.*[11]
S. rochei subsp. *volubilis* ATCC 21250	410	HIGASHIDE *et al.*[12]
S. rochei 6642GC1	17, 100, 200	URAMOTO *et al.*[5]
S. rochei 7434AN4	17, 100, 200	HAYAKAWA *et al.*[7]
S. rochei 491	—	HAYAKAWA *et al.*[7]
S. rochei 7434AN4R	17, 100, 200	This work
41227	100, 200	This work
51252	200	This work
1-2	17, 100	This work
1-3-1	17, 100	This work
F-7	17, 200	This work
K3A-12	100	This work
KE32	160	This work
2-39	—	This work
3-44	—	This work

of *S. rochei* 7434AN4 were isolated as part of this study. *Streptomyces lividans* TK64 and *Micrococcus luteus* IAM386 were obtained from John Innes Institute and Institute of Applied Microbiology, University of Tokyo, respectively. TSB medium contained 30 g of Tryptic Soy Broth, Difco, per liter. YM medium contained 4 g of yeast extract, 10 g of malt extract, and 4 g of glucose per liter (pH 7.3).

Mutagenesis of *S. rochei* 7434AN4

UV irradiation, NTG treatment, and protoplast regeneration of *S. rochei* 7434AN4 were performed by the methods of KINASHI *et al.*[13]. High temperature mutants were obtained by spreading the spores of *S. rochei* 7434AN4 on YM agar plates and incubated at 42°C. Colonies grown at this temperature were picked and analyzed further by PFGE and bioautography.

Protoplast Fusion

Protoplasts of the lankacidin producers, *S. rochei* 7434AN4R and 51252, and the nonproducers, *S. rochei* 2-39 and *S. lividans* TK64 were made and fused as previously described[13]. Colonies grown on R2YE[14] plates were transferred to YM master plates and their properties were further studied.

Bioassay of the Antibiotic Activity and the Lankacidin Resistance

Colonies to be tested were inoculated onto agar plugs containing YM medium and incubated at 28°C for 5 days. The agar plugs were put on a bioassay plate and incubated at 37°C overnight. The bioassay plate was composed of two layers. The bottom layer contained TSB medium-1% agar, while the top layer contained TSB medium-0.7% agar supplemented with 2% of the overnight culture in TSB medium of the indicator organism, *M. luteus* IAM386. Minimal inhibitory concentration of lankacidin against various mutants of *S. rochei* 7434AN4 was determined by inoculating mycelial suspension of the mutants onto YM plates containing a series of concentrations of lankacidin C followed by incubating at 28°C for 3 days.

TLC Bioautography

The lankacidin-producers and their mutants were reciprocally cultured in 100 ml of YM medium in a 500 ml Sakaguchi flask at 28°C for 3 days. The broth filtrates were extracted with an equal volume of ethyl acetate and concentrated *in vacuo*. The concentrates were applied to two silica gel TLC plates (Kieselgel 60 F254, Merck) and developed with a mixture of chloroform and methanol (10 : 1). Spots on the first TLC plate were detected under a UV light and then by spraying 10% sulfuric acid followed by heating. The second TLC plate was placed in contact with a bioautography plate for 20 minutes. After exposure to the TLC plate the bioautography plate was incubated at 37°C overnight. The inhibitory zones

corresponding to lankacidin C and lankamycin appeared at Rf 0.50 and 0.70, respectively. A third inhibitory zone sometimes appeared at Rf 0.35. The UV spectrum of this substance (278 nm) suggested that it belongs the lankacidin group of antibiotics, but we did not characterize it further.

Pulsed Field Gel Electrophoresis

The DNA samples of the lankacidin-producers for PFGE analysis were prepared by the protoplast method as described previously (2) or by the mycelium method. 2 ml of mycelial suspension in TE25Suc[15] and 2 ml of molten 2.0% low-melting agarose in TE25Suc kept at 40°C were poured into a Falcon 3002 plate and mixed well. After solidifying the agarose at 4°C for 10 minutes, 4 ml of lysozyme (2 mg/ml) in TE25Suc was added and incubated at 37°C for 1~4hours. The agarose gel was detached from the bottom of the plate and floated in the buffer during protoplasting. The lysozyme solution was replaced by 1ml of 0.5 M EDTA, 2 ml of pronase (5 mg/ml) in TE25Suc and 1ml of 10% SDS, and incubated at 50°C overnight. The buffer was replaced by 0.5 M EDTA and the plate could be stored at 4°C for several months. We used contour-clamped homogeneous electric fields (CHEF)[16] gel electrophoresis for PFGE analysis. It was carried out at 15°C with a switching interval of 16 to 24 seconds at 170 V in 0.5 × TBE using 1.0% agarose gel. SDS-CHEF was performed by adding 0.2 % sodium dodecyl sulfate (SDS) to the buffer and agarose gel. After PFGE the gel was stained in an ethidium bromide solution (5 μg/ml) for 30 minutes followed by washing in water for more than 1 hour.

Enzyme Digestion and Southern Hybridization

Linear plasmids were separated by CHEF gel electrophoresis using low-melting agarose, excised out from the gel and digested with a restriction enzyme as described (2). The digested fragments were separated again by the second CHEF and transferred to a nylon membrane filter for hybridization. To make probes, plasmids and DNA fragments were extracted from the gel by electroelution or by the glass powder method (Geneclean II, Bio101, Inc., La Jolla, CA) and labeled with digoxigenin-labeled dUTP (Beohringer, Mannheim, Germany). Hybridization and detection were carried out as recommended by supplier's protocol (Beohringer).

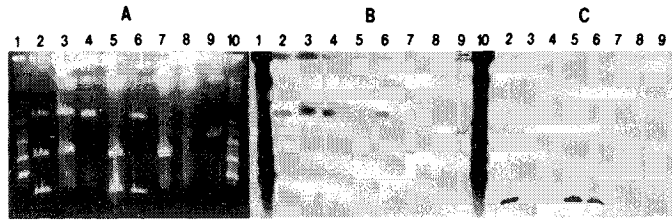
Results

Various Mutants of *S. rochei* 7434AN4 with a Different Plasmid Profile

Although HAYAKAWA *et al.*[7] suggested the involvement of pSLA2-S in the lankacidin production in *S. rochei* 7434AN4 based on curing experiments, we detected two additional plasmids, pSLA2-L and M in the same strain. We tested *S. rochei* 491, the cured strain of HAYAKAWA *et al.* used for their analysis, and found that this strain lost all three linear plasmids.

In order to know the relation between the presence of the pSLA2 plasmids and the production of and resistance to the two antibiotics, we first made mutants having a different plasmid profile. The 7434AN4 strain was subjected to UV irradiation, NTG treatment, protoplast regeneration, and growing at a high temperature. Colonies with no or weak antibiotic activity were selected for further studies. Some mutants were further mutagenized by an additional treatment in order to obtain all possible different combination of the three plasmids except the one containing only pSLA2-S (Fig. 1A and Table 2). *S. rochei* 51252, a mutant with only pSLA2-L obtained by UV irradiation of the 7434AN4 strain, was further subjected to protoplast regeneration to make plasmid-negative mutants, *S. rochei* 2-39 and 3-44. *S. rochei* 1-2, a UV-irradiated mutant with pSLA2-M and S, was further incubated at a high temperature in order to obtain strain K3A-12 with only the pSLA2-M plasmid. High temperature culture of the 51252 strain gave a mutant KE32 which contained a 160-kb linear plasmid named pSLA2-L1. Protoplast fusion of *S. rochei* 7434AN4R, a spontaneous rifampicin-resistant mutant of 7434AN4, and 2-39 gave a fusant F-7 containing pSLA2-L and S.

Fig. 1. CHEF and Southern blot analyses of various mutants of *S. rochei* 7434AN4.



(A) CHEF electrophoresis. CHEF was carried out at 15°C with a switching interval of 18 seconds at 130 V for 19 hours in 0.5 × TBE using 1.0% agarose gel. (B) and (C) Southern hybridization. Hybridization was carried out using digoxigenin-labeled pSLA2-L and lambda DNA (B), and digoxigenin-labeled pSLA2-S (C), respectively, as probes. Lanes: 1, 10, lambda DNA ladder; 2, *S. rochei* 7434AN4; 3, 41227; 4, 51252; 5, 1-2; 6, F-7; 7, K3A-12; 8, 2-39; 9, KE32.

Table 2. Plasmid profiles and functional properties of various mutants of *Streptomyces rochei* 7434AN4.

Strain	pSLA2 plasmids			Productivity		MIC of LC	Origin/Treatment
	L	M	S	LC	LM		
7434AN4	+	+	+	+	+	>100	Wild type
491	−	−	−	−	−	3.13	7434AN4/EtBr
7434AN4R	+	+	+	+	+	>100	7434AN4/SP
41227	+	+	−	+	+	>100	7434AN4/SP
51252	+	−	−	+	+	>100	7434AN4/UV
1-2	−	+	+	−	−	6.25	7434AN4/UV
1-3-1	−	+	+	−	−	3.13	7434AN4/UV
F-7	+	−	+	+	+	>100	7434AN4R, 2-39/PF
K3A-12	−	+	−	−	−	3.13	1-2/HT
KE32	*	−	−	−	−	3.13	51252/HT
2-39	−	−	−	−	−	3.13	51252/PR
3-44	−	−	−	−	−	3.13	51252/PR

* pSLA2-L1, a deletion plasmid of pSLA2-L; EtBr, ethidium bromide; SP, spontaneous mutant; UV, ultraviolet irradiation; NTG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; PF, protoplast fusion; HT, incubation at a high temperature; PR, protoplast regeneration. MIC, minimal inhibitory concentration of lankacidin C (μg/ml).

As summarized in Table 2, all the mutants were analyzed by CHEF for the presence of linear plasmids, by bioautography for the production of lankacidin and/or lankamycin, and by bioassay for the lankacidin resistance. The results clearly indicate a relationship between the presence of pSLA2-L and the production of the two antibiotics, lankacidin and lankamycin. All the non-producers were sensitive to lankacidin (the lankamycin sensitivity was not tested). *S. rochei* KE32 containing pSLA2-L1 did not produce lankacidin nor lankamycin and was sensitive to lankacidin. In all the strains studied, production of the two antibiotics, lankacidin and lankamycin was always linked.

Physical Mapping of pSLA2-L and pSLA2-L1

Restriction endonucleases, *Afl*II, *Ase*I, *Spe*I, *Ssp*I and *Xba*I did not cut pSLA2-L, while *Dra*I, *Eco*RV, *Hin*dIII and *Hpa*I gave several fragments suitable for physical mapping. Numbers of the fragments with each restriction endonuclease and their sizes are summarized in Table 3.

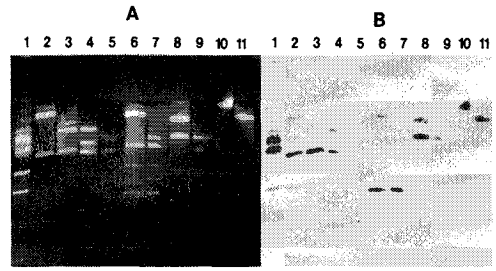
As shown in Fig. 2A, *Dra*I and *Hpa*I cut pSLA2-L into two fragments of 135 (A), 65 (B) kb and 175 (A), 25 (B) kb, respectively; fragments with each enzyme were named in alphabetical order starting with the largest one. Digestion of pSLA2-L with *Hin*dIII gave five fragments of 155 (A), 25 (B), 10.0 (C), 5.8

Table 3. Fragments of pSLA2-L and pSLA2-L1 with various restriction endonucleases.

Plasmid	Enzyme	Fragments (kb)
pSLA2-L	*Dra*I	A (135), B (65)
	*Hpa*I	A (175), B (25)
	*Hin*dIII	A (155), B (25), C (10.0), D (5.8), E (2.5)
	*Eco*RV	A (105), B (50), C (19), D (19), E (4.0)
pSLA2-L1	*Dra*I	A (two 65), B (25)
	*Hpa*I	A (110), B (two 25)
	*Hin*dIII	A (75), B (two 25), C (two 10.0), D (two 5.8), E (two 2.5)
	*Eco*RV	A (75), B (two 19), C (two 19), D (two 4.0)

(D), and 2.5 (E) kb. In order to construct the physical map of pSLA2-L, the following experiments were carried out (data not shown). Double digestion with *Dra*I and *Hin*dIII afforded the *Dra*I-A fragment and the *Hin*dIII-B, C, D, and E fragments, which could localize HindIII-A on the left end. Hybridization using the *Hpa*I-A and B fragments determined the sequence *Hin*dIII-A-B. The order of the *Hin*dIII-C, D, and E fragments were determined by hybridization as follows: pSLA2-L was digested with *Cla*I and *Eco*RI, respectively, and probed by the *Hin*dIII-C, D and E fragments. *Hin*dIII-D and E hybridized to a 5.3-kb *Eco*RI band, and *Hin*dIII-C and E hybridized to a 2.7-kb *Eco*RI band, indicating the order of *Hin*dIII-C-E-D. Connection of *Hin*dIII-B and C was confirmed by hybridization of these fragments to a same 5.2-kb *Cla*I band. Therefore, the order of *Hin*dIII-A-B-C-E-D was determined as shown in Fig. 3.

Fig. 2. Analysis of the restriction endonuclease fragments of pSLA2-L and pSLA2-L1 by CHEF electrophoresis (A) and Southern hybridization (B).

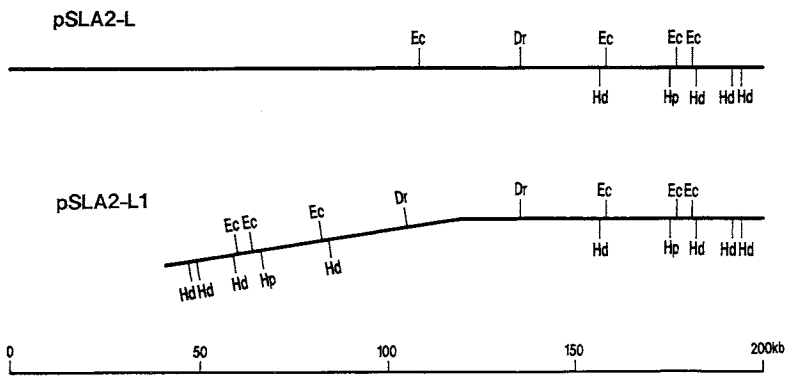


CHEF electrophoresis was carried out at 170 V for 10 hours with 16-s pulse times. The HindIII-D fragment of pSLA2-L and lambda DNA were labeled with digoxigenin-dUTP and used as probes for hybridization. Lanes: 1, lambda DNA ladder and lambda DNA digested with *Hin*dIII; 2, 4, 6, 8, the pSLA2-L digest; 3, 5, 7, 9, the pSLA2-L1 digest; 10, pSLA2-L; 11, pSLA2-L1. The following enzymes were used for digestion; lanes 2, 3, *Hpa*I; 4, 5, *Eco*RV; 6, 7, *Hin*dIII; 8, 9, *Dra*I.

Digestion of pSLA2-L with *Eco*RV gave five fragments of 105 (A), 50 (B), 19 × 2 (C and D), and 4.0 (E) kb (Fig. 2, lane 4). Double digestion with *Dra*I and *Eco*RV afforded all the *Eco*RV fragments except *Eco*RV-B, determining the sequence *Eco*RV-A-B from the left end. The *Hpa*I-A probe hybridized to the *Eco*RV-A and B fragments and one 19-kb fragment, while the *Hpa*I-B probe hybridized to the *Eco*RV-E fragment and another 19-kb fragment. These results extended the sequence to *Eco*RV-A-B-C; one of the 19-kb fragments present here was named *Eco*RV-C. The order of the remained *Eco*RV-D and E fragments was determined by hybridization of *Eco*RV-E to the inner *Hin*dIII-B fragment to give the sequence *Eco*RV-A-B-C-E-D (Fig. 3).

pSLA2-L1 is a 160-kb plasmid isolated from *S. rochei* KE32, a lankacidin-nonproducing and lankacidin-sensitive strain. Digestion of pSLA2-L1 with *Hin*dIII gave the same fragments as those of pSLA2-L (*Hin*dIII-B, C, D and E) except *Hin*dIII-A whose size changed to 75 kb (A') (Fig. 2A, lane 7). The total size (118 kb) of these fragments was much smaller than the size of pSLA2-L1 itself. This discrepancy was solved by considering duplication of the B, C, D and E fragments. In accordance with

Fig. 3. Physical maps of pSLA2-L and pSLA2-L1.



The length of the fragments is given in Table 3. SDS-CHEF and Southern blot analyses suggested that pSLA2-L might have a short TIR and a protein binding on both ends. pSLA2-L1 is a symmetrical linear plasmid containing the duplicated right 80-kb part of pSLA2-L in an inverted orientation. Ec, *Eco*RV; Dr, *Dra*I; Hd, *Hind*III; Hp, *Hpa*I.

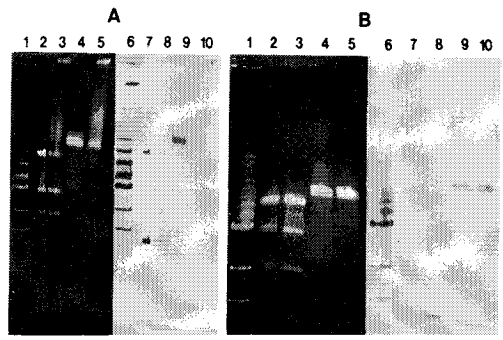
this interpretation, the intensity of the *Hind*III-B fragment is stronger than that of the *Hind*III-A′ fragment instead of its small size. Therefore, pSLA2-L1 was deduced to be a symmetrical plasmid containing the duplicated right 80-kb part of pSLA2-L as shown in Fig. 3.

Linear plasmids isolated from *Streptomyces* species had a terminal inverted repeat (TIR) on both ends[9, 17~19]. In order to test the presence of TIR on pSLA2-L, a hybridization experiment was carried out. As shown in Fig. 2B, the *Hind*III-D probe hybridized strongly to *Hind*III-D itself and slightly to *Hind*III-A, suggesting that a relatively short TIR is present on both ends. The *Hind*III-D of pSLA2-L hybridized to the corresponding band of pSLA2-L1 but not to the A′ band. This result suggested the presence of TIR only on the *Hind*III-D fragment, supporting the symmetrical structure of pSLA2-L1.

SDS-agarose Gel Electrophoresis

The pSLA2-L plasmid was characterized by SDS-agarose gel electrophoresis. The gel samples of *S. rochei* 51252 were prepared with and without pronase treatment. The two samples were subjected to normal CHEF and SDS-CHEF to compare their mobility. As shown in Fig. 4, non-pronase-treated pSLA2-L remained at the origin in normal CHEF (A, lane 5), but moved with the same speed as the pronase-treated counterpart in SDS-CHEF (B, lane 5),

Fig. 4. Normal CHEF (A) and SDS-CHEF (B) analyses of pronase-treated and non-pronase-treated pSLA2-L.



(A) Normal CHEF analysis (lanes 1 to 5) and Southern blot analysis (lanes 6 to 10). Pronase-treated and non-pronase-treated pSLA2-L and their *Hind*III digests were separated by CHEF gel electrophoresis at 170 V for 10 hours with 16-s pulse times. Hybridization was carried out with digoxigenin-labeled pSLA2-L and lambda DNA as probes. Lanes; 1, 6, lambda DNA ladder and lambda DNA digested with *Hind*III; 2, 7, *Hind*III digest of pronase-treated pSLA2-L; 3, 8, *Hind*III digest of non-pronase-treated pSLA2-L; 4, 9, pronase-treated pSLA2-L; 5, 10, non-pronase-treated pSLA2-L. (B) SDS-CHEF analysis (lanes 1 to 5) and Southern blot analysis (lanes 6 to 10). The same samples as those used in panel A were subjected to CHEF analysis with 1% agarose containing 0.2% SDS in 0.5 × TBE buffer containing 0.2% SDS at 150 V for 12 hours with 48-s pulse times.

indicating that a protein was bound to pSLA2-L. The same analysis of the *Hin*dIII digest showed that the terminal HindIII-A and D fragments of non-pronase-treated pSLA2-L remained at the origin in normal CHEF (A, lane 3), indicating a protein binding to these fragments. The *Hin*dIII-D probe hybridized to the origins of the non-pronase-treated samples (A, lanes 8 and 10). These results suggest that a protein is bound to the termini of pSLA2-L as in the cases of other linear plasmids[2,8,18]. SDS-CHEF analysis further indicated that a protein was bound to the *Hin*dIII-D fragment of pSLA2-L1 but not to the *Hin*dIII-A′ fragment, supporting its symmetrical structure (data not shown).

Homology of the Linear Plasmids in *S. rochei*

pSLA2-L, M, S, and L1 from various *S. rochei* mutants were separated by CHEF gel electrophoresis and subjected to Southern hybridization. As shown in Fig. 1B and 1C, the pSLA2-L probe hybridized to pSLA2-L itself and pSLA2-L1 strongly, and to pSLA2-M weakly, but not to pSLA2-S. On the other hand, the pSLA2-S probe hybridized only to pSLA2-S itself. Therefore, pSLA2-L has strong homology to pSLA2-L1, some homology to pSLA2-M, but no homology to pSLA2-S. These results together with the phenotypic correlation observed in the mutants suggest that pSLA2-S is a different plasmid from pSLA2-L.

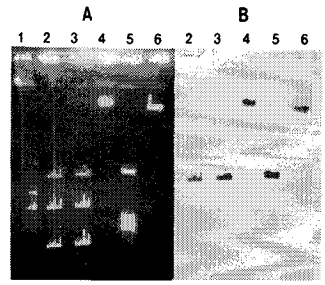
Transfer of pSLA2-L by Protoplast Fusion

The pSLA2-L plasmid was transferred by protoplast fusion from the rifampicin-resistant *S. rochei* 7434AN4R to the rifampicin-sensitive *S. rochei* 2-39. Among 150 colonies tested, 23 colonies were judged to originate from the 2-39 strain based on their rifampicin sensitivity. The plasmid profiles and antibiotic-producing properties were also studied in these 23 strains. Seventeen (74%) of the colonies had plasmids. All the colonies except for the F-7 strain which contained pSLA2-L and S, had three plasmids. No rifampicin-sensitive revertant was detected from 481 regenerated colonies in a control experiment using only 7434AN4R. Bioautography was done for the 23 rifampicin-sensitive colonies and a complete correlation was seen again between the presence of pSLA2-L and antibiotic production. It should be stressed that the production ability of the two antibiotics was never separated in any fusant. We also tried to transfer pSLA2-L from 7434AN4R to *S. lividans* TK64 by protoplast fusion. *S. lividans* TK64 is a streptomycin-resistant and proline-requiring mutant of *S. lividans* 66, and does not produce lankacidin nor lankamycin. The fusants originated from the TK64 strain were selected based on streptomycin resistance. All of the 221 streptomycin-resistant colonies were found to be proline-auxotrophs and did not have the antibiotic-producing ability. CHEF analysis showed that none of the pSLA2 plasmids was transferred to 20 randomly selected colonies.

Large Linear Plasmids from Various Lankacidin-producers

All the results described in the previous sections suggested that pSLA2-L rather than pSLA2-S is involved in the production of lankacidin and

Fig. 5. CHEF (A) and Southern blot (B) analyses of various lankacidin-producers of *Streptomyces* species.



CHEF electrophoresis was carried out at 170 V for 19 hours with 24-s pulse times. Digoxigenin-labeled pSLA2-L was used as a probe. Lanes; 1, lambda DNA ladder; 2, *S. rochei* 7434AN4; 3, *S. rochei* 6642GC1; 4, *S. rochei* subsp. *volubilis* ATCC 21250; 5, *S. griseofuscus* JCM4276; 6, *S. violaceoniger* IFO 14166.

lankamycin. If this is the case, we speculated that homologous linear plasmids would be present in the following lankacidin producing strains; *S. griseofuscus* JCM4276, *S. violaceoniger* IFO14166, *S. rochei* subsp. *volubilis* ATCC21250, *S. rochei* 6642GC1. All the strains were found to have large linear plasmids (Fig. 5A and Table 1). *S. rochei* 6642GC1 has three plasmids of the same sizes with pSLA2-L, M, and S, respectively. HIROCHIKA and SAKAGUCHI[8] reported that this strain contained pSLA1, a linear plasmid almost identical to pSLA2-S except that its *Sma*I-E fragment is 40 bp smaller than that of pSLA2-S. To study the homology of these plasmids to pSLA2-L, we did a hybridization experiment. As shown in Fig. 5B, the pSLA2-L probe hybridized to the linear plasmids from all four lankacidin-producers.

Discussion

Streptomyces rochei 7434AN4 has three large linear plasmids, pSLA2-L, M and S. We made various mutants from this strain and characterized their plasmid profile and antibiotic production capacity. The results indicated a parallel relationship between the presence of pSLA2-L and the production of both lankacidin and lankamycin. pSLA2-L was transferred by protoplast fusion from the rifampicin-resistant 7434AN4R strain to the rifampicin-sensitive nonproducer (2-39). Since protoplast fusion causes frequent recombinations between chromosomes, its analysis should be done carefully. But the 100 % correlation observed in the rifampicin-sensitive fusants suggest that pSLA2-L is involved in antibiotic production.

The inability to isolate a mutant of *S. rochei* 7434AN4 containing only pSLA2-S could have happened by chance or may suggest that the pSLA2-L or M plasmids are indispensable for the maintenance of pSLA2-S. In the yeast *Kluyveromyces lactis*, a linear plasmid pGKL2 was reported to be necessary for the maintenance of another linear plasmid pGKL1[20]. We successfully transferred the pSLA2 plasmids from *S. rochei* 7434AN4R to 2-39 but not to *S. lividans* TK64. This result could also suggest that the *S. rochei* chromosome contains a region necessary to maintain the pSLA2 plasmids which is missing in the *S. lividans* chromosome.

From the physical map, pSLA2-L1 was deduced to be a symmetrical linear plasmid containing the right 80-kb part of pSLA2-L. KIKUCHI *et al.*[21] also reported the structure of a similar symmetrical linear plasmid, F1 formed during transformation of *Saccharomyces cerevisiae* by pGKL1. Comparison of the maps of pSLA2-L and pSLA2-L1 suggested that the antibiotic biosynthetic and resistant genes might be located on the deleted left part of pSLA2-L. The weak hybridization of the *Hin*dIII-D fragment to the *Hin*dIII-A fragment suggested the presence of a short TIR on the ends of pSLA2-L. Further, SDS-CHEF indicated that a protein is bound to the termini of pSLA2-L. Linear plasmids so far isolated from *Streptomyces* species have TIR on both ends and a terminal protein on the 5′ ends. Therefore, these two properties seem to be common in linear plasmids in *Streptomyces* species.

We studied four other lankacidin-producers and found large linear plasmids in all the strains tested. The probe pSLA2-L hybridized to the plasmids from all the producers. SOLENBERG *et al.*[22] studied the transposition of Tn5096 in *S. griseofuscus* C581 which was derived from strain ATCC23916 (JCM4276) used in this work. This transposon was randomly inserted into both the chromosome and two linear plasmids of 200 and 65 kb in this strain. In contrast we detected three plasmids in the JCM4276 strain. Insertion of Tn5096 into the larger plasmid eliminated the capacity of the strain to produce lankacidin, which supports the involvement of this plasmid in lankacidin production. GRAVIUS *et al.*[23] reported that the *otr*B oxytracycline resistance gene was hybridized to a giant linear plasmid in *Streptomyces rimosus*. Therefore, large linear plasmids might be widely involved in antibiotic production in *Streptomyces* species in addition to the case of SCP1 in methylenomycin production.

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