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Formation of Spontaneously Developing Pocks Regulated by Two Unhomologous Plasmids in *Streptomyces Zaurentii*

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Streptomyces laurentii ATCC 31255, wild-type strain PO, formed spontaneously developing pocks with the production of phage taillike particles. Electron micrographs showed that much hyphae in the pocks were broken or lysed. Around the broken hyphae, there were many hexagonal ringlike particles and some kinds of tubelikc particles. A large (SO-90 kbp) plasmid designated as pSLL was isolated from strain PO. Another covalently closed circular plasmid, pSLS(16 kbp), was found in pSLL-cured PO cells (named P1), which formed spontaneously pocks on a solid medium. The frequency of the generation of pSLL-loss P1 cells coordinated positively with the frequency of pock appearance. Thiostrepton productivity of strain P1 showed a decrease of about 50%, compared with that of wild-type strain PO. The pSLS was also found as an integrated form in the chromosome of strains PO and P1. The results indicated that. strain PO harbored two plasmids, pSLL and integrated form pSLS, and pSLS seemed to be generated as a free-form plasmid with the loss of pSLL. It was also suggested that, pSLL may suppress the excision of the integrated pSLS from the chromosome and that the free form of pSLS may participate in pock formation.

INTRODUCTION

Streptomyces *azureus* and some others form unique colonies or lawn with spontaneously developing pocks in solid medium (Ogata, 1991, 1995). These pocks appear spontaneously during the growth of the organisms, and increase in number during subculturing. In the pocks, lysis of the aerial and sporulating hyphae results from the production of a large number of defective phage particles. However, neither the lysis nor the particle production occur in the substrate or submerged hyphae. The eroded colonies or lawns with the pocks temporarily disappeared by the subculturing through five or six cycles in the liquid medium. The spontaneously developing pocks were first reported in thiostrepton-producing Streptomyces axureus ATCC 14921 (Ogata et *al.*,1981), and their naming was first recognized in S. *endus* (Ogata et *al., 1982).* Similar observations have been reported in several species of *Streptomyces* (Suenaga *et al.*, 1983, 1984) and *Streptoverticilium* (Ogata *et al.*, 1983a). There are some distinct differences between the formation of conjugative pocks (Bibb *et al.*, 1978) and that of spontaneously developing pocks: the former requires for two types of strains, a donor and a recipient for pock-forming plasmid transfer; whereas the latter occurrs in homotype strain originating from a single spore, as described elsewhere (Ogata *et al., 1982;* Ogata, 1991)

A low-copy number plasmid pSA1, which elicited the pocks, was isolated from S. axureus (Ogata *et al.*, 1983b; Miyoshi *et* **al.,** 1986). In this report, we describe an

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additional example of the formation of spontaneously developing pocks with the production of phage taillike particles, and the isolation of the plasmids relating to the formation of spontaneously developing pocks in another thiostrepton-producing *Streptomyces laurentii.*

MATERIALS AND METHODS

Bacterial strains and plasmid. *Streptomyces laurentii* ATCC 31255, wild-type strain PO, was used throughout this work. A pSLL-cured mutant, P1 was isolated from the wild-type strain PO, as described in Results. *Escherichia coli* JM109 (Yanisch *et al.*, 1985) and plasmid pUC 119 (Yanisch *et al.*, 1985) were used for the cloning of pSLS fragments. *Bacillus subtilis* ATCC 6633 was used for the determination of thiostrepton productivity.

Media. Rye flakes agar (Ogata *et al.*, 1992) was used for sporulation and Bennett medium and agar (Ogata *et al.*, 1985a) were used for the growth of S. *laurentii.* MG-1 medium (Ogata *et al.*, 1985a) was used for extraction of plasmids and total DNA of *S. Zaurentii.* LB broth and LB agar were used for the growth of *E. coli.* (Sambrook *et al.*, 1989)

Electron microscopy. Single or overlapping pocks were directly picked up with a platinum loop, suspended directly in potassium phosphotungstate solution (pH 6.0) and placed on grids with collodion-carbon matrix (Ogata *et al.*, 1992). Electron micrographs were taken with a JEM 2000EX.

Thiostrepton productivity. Thiostrepton productivity of strains PO and P1 were determined as previously described by Ogata *et al.* (198513). Thiostrepton production in the liquid medium was determined using the Bennett broth. The mycelia grown for 22, 26, and 36 hrs were gatherd by the centrifugation at 3,000 X g for 10 min and then applied to DMSO-extraction. Thiostrepton activity in the extract was determined by the paper disk method with *Bacillus subtilis* as test organism.

Isolation of plasmid and bacterial DNAs, and DNA manipulation. Spores or mycelia were cultivated in MG-1 medium at 28°C on a rotary shaker (250 rpm) for 24 hr or 48 hr, and the wet mycelia were used for the plasmid and total DNA isolation. Plasmid DNA was isolated by the alkaline lysis methods (Hopwood *et al.*, 1985), and purified by CsCl-ethidium bromide density gradient centrifugation. Isolation of total DNA was done by the method of Hopwood *et al.* (1985). Restriction endonuclease digestions, ligation with T4 DNA ligase, transformation, and conventional agarose gel electrophoresis were done as described by Sambrook *et al.* (1989)

PFGE analysis. DNA inserts for Pulsed-field gel electrophoresis (PFGE) were prepared from *Streptomyces* mycelia as described by Kinashi *et al.* (1991) with modifications as follows. S. *laurentii* was cultivated for 24 hr at 28°C in MG-1 medium,

and washed twice with TE25suc buffer composed of 25 mM Tris-hydrochloride (pH 8.0), 25 mM EDTA, and 10.3% sucrose. One gram of the wet mycelia was suspended in 5 ml TE25suc. A sample (2 ml) of the suspension was transferred to a Falcon 1007 plate, and 2 ml of 1% low melting point agarose(type VII Sigma) in TE25suc was added and mixed gently. After the agarose solidified at 4°C, 5 ml lysozyme solution (2 mg per ml in TE25suc) was overlaid and incubated at 37°C for 2 hr. After the lysozyme solution was removed, it was overlaid with a 5 ml Pronase solution composed of 10 mM Tris-hydrochloride (pH 8.6), 1% lauroyl-sarcosine, 0.5% EDTA, and 2 mg/ml Pronase (Actinase E, Kaken Pherm., Tokyo, Japan), and incubated at 37°C for 24 hr. The liquid layer was replaced by TE buffer, and the plate was stored at 4°C. DNA inserts (1 × 3 × 8 mm) were cut out of the agarose gel and used for PFGE analysis. PFGE was done in the Atto AE-6800 Cross Field electrophoresis unit in 0.5 × TBE buffer at 160V. A TBE and a TE were prepared as described by Sambrook et *al.*(1989). Pulse time for PFGE analysis was usually 30 sec. The relative mobility of pSLL was estimated with different pulse times (25-50sec).

Southern blot analysis. The DNA fragments separated by PFGE or conventional agarose gel electrophoresis were transferred to nitrocellulose filters (BA85; Schleicher & Schuell, Dassel, Federal Republic of Germany) as described by Hopwood *et al.* (1985). Probe DNA was labeled using the DIG labeling and detection kit (Boehringer). DNA fragments for probes were recovered from the gel by a glass powder method using a Geneclean II kit (Bio 101, Inc., La Jolla, Calif.).

RESULTS

Morphologies of confluent lawns and colonies

S. laurentii PO generated spontaneously developing pocks after several successive cultivations on a solid medium. (Fig. 1 B, C). Inhibition of spore formation and aerial hyphal growth was seen in the pocks. These pocks increase in number during subculturing.

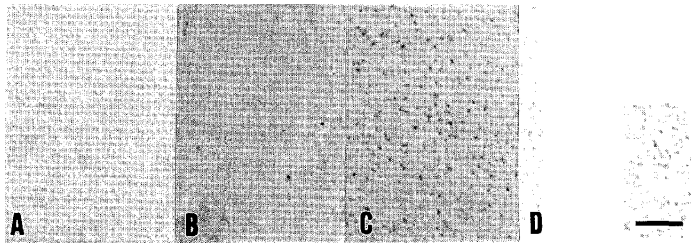

Fig. 1. Confluent lawns of strains PO and P1. A, Strain PO, which was grown from stock spores; B and C, strain PO, which were subcultured on solid medium for two and four cycles, respectively; D, strain P1. No or few pocks are seen in Figs. A and B. Fig. C shows scores of pocks. Fig. D shows abundant or confluent pocks. Bar, 3mm.

Detection of defective phage particles by electron microscopy

Electron micrographs showed that much hyphae in the pocks were broken or lysed, as observed with S. *azureus* and others (Ogata *et al.*, 1981, 1982, 1983a; Suenaga *et al.*, 1983, 1984). Around the broken hyphae, there were many hexagonal ringlike particles, as shown in Fig. 2. These particles morphologically resembled the distal view of an end structure of phage tail, which was termed as base plate or tail tip. Small tubelike particles were also seen as shown by small arrows. These particles looked like a part of tail core. The tail corelike structure was also observed in the center of the ringlike patricles which seemed to attach to the small tubelike particles.

Small numbers of different type particles with hollow tubelike structure were also observed (large arrow in Fig. 2). Their width was 14nm but length was not constant. Morphologically they were similar with the phage tail sheaths. No other morphology was found. In the pocks of S. *azureus*, a variety of phage parts were observed (Ogata *et al.*,1981, 1982). Some of them were similar to the particles of S. *laurentii*. Production of defective phage particles with two different types of ringlike and tubelike structures was also reported in S. *chrysomallus* (Krugel *et al.*, 1987). Among the particles of S. *laurentii* and other species reported, there are some similarity in the morphology but not in the size.

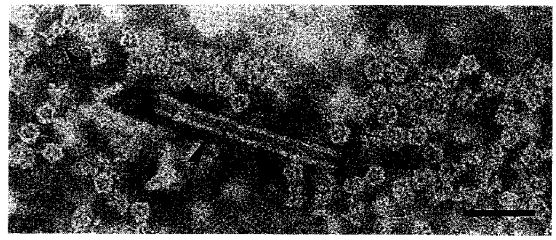

Fig. 2. Electron micrographs of phage taillike particles. Hexagonal ringlike particles, small tubelike particles (small arrows) and hollow tubelike particles (large arrow) are seen. Bar, 50 nm.

Isolation of a plasmid pSLL

Since pock formation in streptomycetes is generally related to plasmid function, we tried to isolate plasmids from strain PO. No circular plasmid was detected from the wild-type strain PO by an alkaline lysis method, but a large extrachromosomal molecule was detected as well as chromosomal DNA by PFGE analysis as shown in Fig. 3, lane 2. The extrachromosomal DNA was expected to have a linear DNA structure by referring the results described by Kinashi and shimaji (1987); circular DNA molecules move very slowly compared to linear DNAs on the PFGE gel, and the DNA molecules migrated on the gel at the similar position to that of concatemers of λ DNA even though different pulse times were applied (data are not shown). The DNA molecule was named pSLL; it was estimated to be 80-90 kbp on the basis of the migration distance of concatemeric λ DNA. The copy number of pSLL was also estimated at 20 to 40 according to Kinashi and Murayama (1991).

Isolation of pSLL-cured strain *P1*

To examine the nature of spontaneously developing pocks, spores and mycelia were harvested from cells showing pocks on the lawn and single colonies were isolated of a fresh agar medium. The resultant strain, P1, generated a large number of pocks on the lawn (Fig. 1D). Strain P1, however, had lost pSLL. Southern hybridization analysis using pSLL DNA as a probe showed also no possibility that pSLL had integrated into the chromosomal DNA, as shown in Fig. 3.

As shown in Table I, the population of pSLL-loss cells (P1 type cells) increased through serial transplantations on the solid medium along with the increase of pocks. However, the segregation of P1 type cells from strain PO was suppressed in liquid culture;

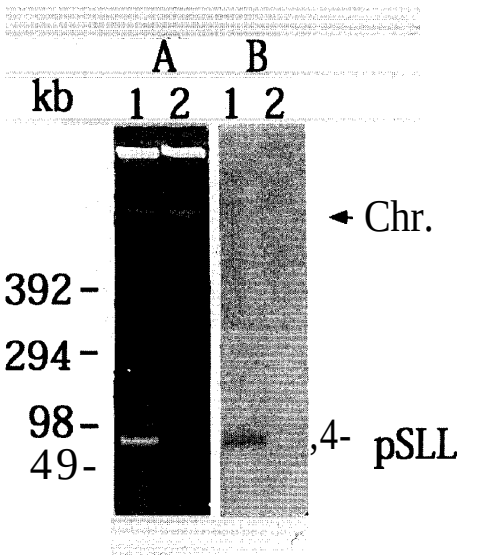

Fig. 3. PFGE of total DNA from strains PO and P1 of *S.laurentii* and hybridization with DIG-labeled pSLL DNA.
A, PFGE; B, Hybridization.
1, DNA from strain PO; 2, DNA from strain P1.

Table 1. Increase of pock-carrying colonies and decrease of plasmid pSLL-carrying colonies during serial subculturings.

Subcultured cycles	Ratio pock-carrying colonies,	pSLL-carrying colonies/Total colonies
0	0120	20/20
4	2/20	10/20
10	9/20	2/20

the population of pSLL-carrying cells increased greatly in successive cultures in the liquid medium (data not shown).

This suggests that pSLL may suppress the formation of spontaneously developing pocks.

Thiostrepton productivity of Strains PO and P1

As shown in Fig. 4, the thiostrepton production of strain P1 decreased to about 50%, compared with that of wild-type strain PO. However, there was no significant defference in the submerged mycelial growth of each strain (data not shown). Such a decreasing tendency of thiostrepton productivity was seen on strain the PK100 of S. *azureus* that harbored a pock plasmid, pSA1.1 (Miyoshi et al., 1986). Pock plasmid might supress the spore formation and thiostrepton productivity. So, we attempted to detect pock plasmids from strain P1.

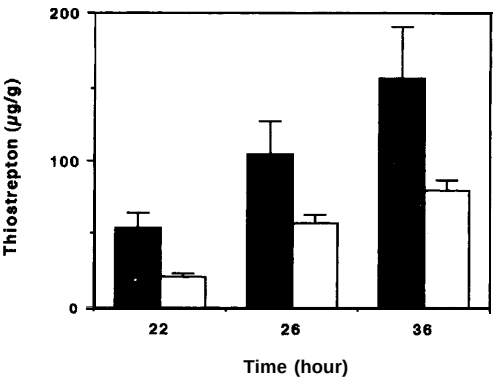

Fig. 4. Thiostrepton productivity of strains PO and P1. Closed column, Strain PO; Open column, Strain P1.

Isolation of circular plasmid pSLS

Though strain PO had no circular plasmid, a circular plasmid was isolated from strain P1 and named pSLS (Fig. 5). Cleavage sites of restriction endonucleases in the pSLS are shown in Fig. 6. The size of the plasmid was estimated to be 16 kbp by restriction analysis.

Similarities among pSLS, pSLL, and plasmid pSA1 of S. *azureus* (Miyoshi *et* al., 1986) were examined by Southern hybridization using a DIG-labeled probe of pSLS, but no hybridization was found (data not shown), indicating an absence of homology among these three plasmids.

Chromosomal integrated sequence of plasmid pSLS

The total DNAs of strains PO and P1 were digested by *Pst*I, *Bcl*I, or *Bam*HI, electrophoresed on an agarose gel, and after blotting, hybridized with DIG-labeled pSLS DNA.

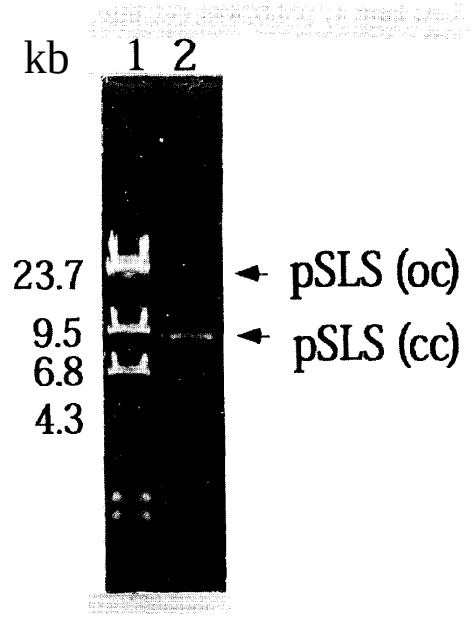

Fig. 5. Agarose gel electrophoresis of pSLS isolated from strain P1 of *S. laurentii*.
1, *Hin*dIII-digested λ DNA; 2, pSLS DNA.

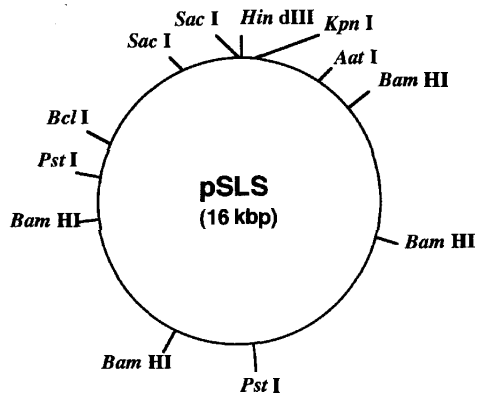

Fig. 6. Restriction enzyme cleavage map of pSLS DNA
There were the unique *Hin*dIII, *Kpn*I, *Aat*I, and *Bcl*I sites on the pSLS. *Pst*I, *Bam*HI, and *Sac*I gave two, four, and two fragments, respectively. *Sma*I, *Sal*I, and *Ban*II cut pSLS into more than seven fragments, and *Eco*RI, *Eco*RV, and *Sph*I did not cut pSLS.

As shown in Fig. 7-A, lane 1, pSLS was cut into two fragments (12 and 4 kbp respectively) by *Pst*I. The hybridization signals with EM-digested total DNA were detected at the position of the 4 kbp fragment of pSLS. No signal was, however observed at the position of the 12 kbp fragment of pSLS. In exchange for the 12 kbp These bands must be originated from the integrated sequence of pSLS. They would contain the junction site between pSLS DNA and the host chromosomal DNA. Only one hybridized fragment in PO and P1 (Fig. 7-A-lane 5 and lane 6) showed that the junction sites might locate adjacent to the *Bcl*I site on pSLS. To confirm this observation, *Pst*I-*Hin*dIII fragment which would be containing junction sites was cloned in pUC119, and this fragment was used as a probe of hybridization analysis. As shown in Fig. 7-B, two hybridization signals were observed at the different position from that of free plasmid (Fig. 7-B).

These results indicated that strains PO and P1 have chromosomal integrated sequence of pSLS. However, the free form of pSLS could not detect in this method in strains PO and P1. That was due to the low concentration of the free pSLS compared with chromosomal DNAs.

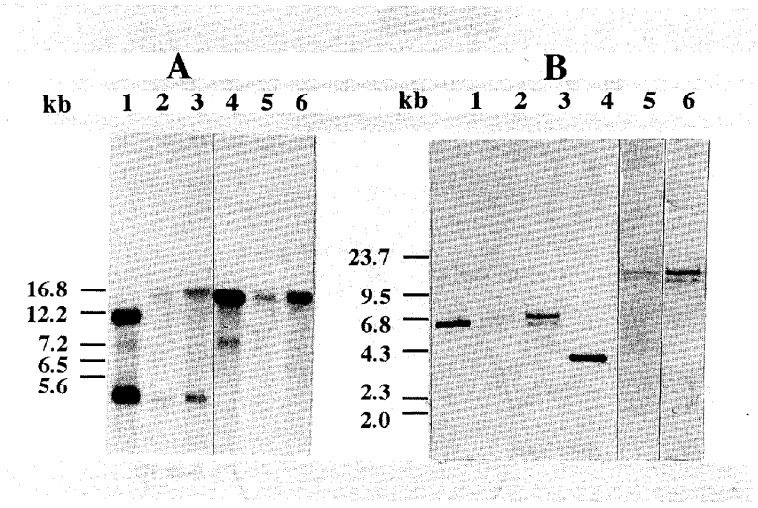

Fig. 7. Southern hybridization analysis of total DNAs from strain PO and strain P1 with DIG-labeled pSLS DNA.

pSLS and its *Pst*I-*Hind*III fragment were used as a probe in panels A and B, respectively. Endnuclease-digested DNAs were used for 0.6% agarose gel electrophoresis.

Panel A; Lanes 1 and 4, pSLS DNA; Lanes 2 and 5, total DNAs from strain PO; Lanes 3 and 6, total DNAs from strain P1; Lanes 1, 2 and 3, *Pst*I-digested DNA; Lanes 4, 5 and 6, &&digested DNA. *Bam*HI-digested λ DNA was used as size marker.

Panel B; Lanes 1 and 4, pSLS DNA; Lanes 2 and 5, total DNAs from strain PO; Lanes 3 and 6, total DNAs from strain P1; Lanes 1, 2 and 3, *Bam*HI-digested DNA; Lanes 4, 5 and 6, *Pst*I, *Hin*dIII-digested DNA. *Hin*dIII-digested λ DNA was used as size marker.

Relationship between the pock formation and freeform of pSLS

To clarify the effect of the cultural conditions of strains PO and P1 or of the state of pSLS replicon on the pock formation, their mycelia, which were different in number of pocks, were prepared for the isolation of DNAs as described in Materials and Methods. For these experiments, the submerged mycelium were cultivated for 24hr. The DNAs were isolated by alkaline lysis method, because free form of pSLS was not detected by SDS lysis method.

The DNAs obtained were subjected to agarose gel electrophoresis and after blotting, hybridized with DIG-labeled pSLS DNA. As shown in Fig. 8-lane 2, the free form of pSLS replicon was not detected. However, the hybridized band was seen in the chromosome DNAs of strain PO from the stocked culture, which had not formed any pocks. This band was the integrated sequence of pSLS replicon. On the other hands, both of free and integrated forms of pSLS were detected in the PO that had been subcultured through four cycles on agar media and formed pocks (Fig. 8-lane 3). In the case of strain P1, the pocks disappeared after the transplantations in liquid culture, and simultaneously, the free form of pSLS remarkably decreased (Fig. 8-lane 5). These results suggest that the excision of pSLS replicon would occur on the solid culture, but not in the liquid culture, and that the free form of pSLS participates in the formation of spontaneously developing pocks.

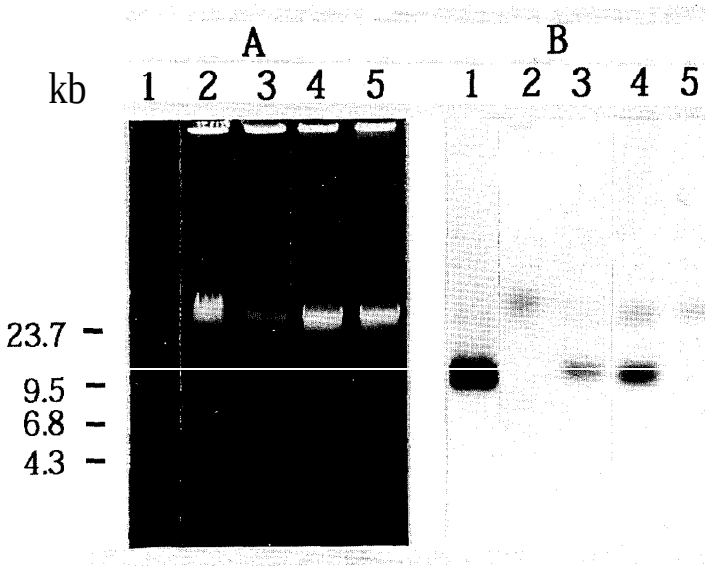

Fig. 8. Agarose gel electrophoresis (left) of DNA isolated from strains PO and P1 followed by hybridization with DIG-labeled pSLS DNA (right).

1, pSLS DNA; 2, DNAs of strain PO cultured from stock spores; 3, DNAs of strain PO subcultured in solid medium (four cycles); 4, DNAs of strain P1 cultured from stock spores; 5, DNAs of strain P1 subcultured in liquid medium (four cycles).

DISCUSSION

A plasmid pSLL was isolated from the wild-type strain PO using the PFGE analysis. Another plasmid, pSLS, was isolated from the pSLL-cured derivative P1. Plasmid pSLL was maintained stably in the substrate or submerged hyphae, and the curing of pSLL might occur in the aerial and sporulating hyphae during the process of morphological differentiation. The free form of pSLS was excised from its chromosomal integrated sequence in absence of pSLL.

The excision events of plasmid in streptomycetes have been described on pSAM2, SLP1 and pSA1 (Pernodet *et al.*, 1984; Bibb *et al.*, 1981; Ogata *et* al., 1989). The excision of pSAM2 and SLP1 were induced by UV-irradiation and interspecific matings, respectively. In the case of pSA1 in S. axureus, the excision occurred in the aerial and sporulating hyphae and was stimulated by transplantations on the solid medium and UV-irradiation. In particular, the excision events of plasmids in S. *laurentii* and S. *azureus* were very similar to each other. However, there was a clear difference. The excision of pSLS only occurred in large plasmid pSLL-cured strain or cells. These results suggest that plasmid pSLL relates to the regulation of the excision of pSLS replicon. There was no DNA homology among these three plasmids, pSLS, pSLL and pSA1. The role of pSLL in the regulation of the excision event of pSLS is a subject of considerable interest. The physical and functional characterization of pSLL is worthy for further study.

Spontaneously developing pocks appeared in the lawn of solid culture of S. *laurentii*, when the free form of pSLS existed in the cells. This suggests that the free form would be directly concerned with pock formation. The pock formation was always accompanied by the production of defective phage particles. The induction of defective phage replicon may be caused by the cooperation of pSLS replicon. The same event was observed on S. *axureus*, but the defective phage morphology differed in each species (Ogata *et al.*, 1992). We suggested that the free form may promote the excision of integrated defective

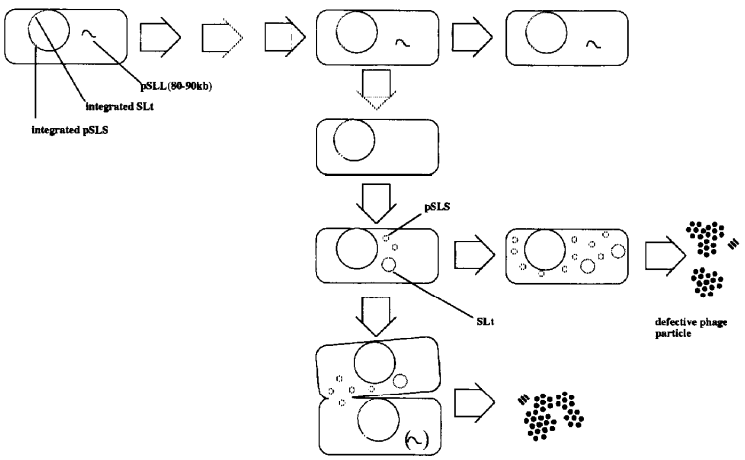

Fig. 9. A proposed scheme for the formation of spontaneously developing pock in S. *laurentii*.

phage replicon. The lysis of hyphae would be due to the endolysin, which acts at the last stage of phage multiplication. These observations led to a proposed scheme for the appearance of spontaneously pocks, as illustrated in Fig. 9.

The event of spontaneously developing pocks also may be limited to the aerial and sporulating hyphae. We will report in the near future the role of defective phage replicon on the pock formation.

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