A Conjugative Linear Plasmid in *Streptomyces laurentii* ATCC31255

Chizuru Kinoshita-Iramina, Maki Kitahara, Katsumi Doi, and Seiya Ogata

*Microbial Genetics Division, Institute of Genetic Resources, Faculty of Agriculture, Kyushu University, Hakozaki, Higashi-ku, Fukuoka 812-81, Japan*

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Plasmid pSLL of *Streptomyces laurentii* ATCC31255 (wild-type strain P0) is a 93-kilobase linear DNA plasmid that carries a protein bound to each 5' end of the DNA. It was self-transmitted to the pSLL-cured strain by conjugation in solid culture. The pSLL-cured strain carried a circular plasmid, pSLS, and showed a marked decrease in spore formation and thioestrepton productivity, owing to the pSLS. However, by retransmission of pSLL, these things reverted to levels seen in strain P0. Thus, plasmid pSLL suppressed the injurious effects of pSLS on the host mycelia.

**Key words:** *Streptomyces*, linear plasmid; conjugative plasmid

Thioestrepton-producing *Streptomyces laurentii* ATCC 31255, wild-type strain P0, forms spontaneously developing pocks, as do *Streptomyces aureus* and some other strains. Two plasmids are related to the pock formation in strain P0. A covalently closed circular plasmid, pSLS (16 kbp), was excised from an integrated form in the chromosome, after which the free pSLS plasmid participated in pock formation. Another plasmid, pSLL (about 90 kbp), was also isolated from strain P0, and seemed to have a linear DNA structure. Plasmid pSLL simultaneously suppressed excision of the integrated pSLS (pSLS$_{in}$) from the chromosome, and pock formation.

Since Hayakawa et al. discovered pSL2 in *Streptomyces rochei*, many linear plasmids of broad size ranges (12–1700 kbp) have been isolated from actinomycetes. All the *Streptomyces* linear plasmids characterized so far have protein covalently attached to the 5' DNA termini, and some such as SCP1 (350 kbp), SL2 (50 kbp), and pBL1 (43 kbp) are transmissible. Most of the genes directing the synthesis of antibiotics in streptomycetes appear to be chromosomal, however, the plasmid SCP1 of *S. coelicolor* carries the methylenomycin biosynthetic (mmr) gene cluster, pKSL (520 kbp) of *S. lasalliensis* may possibly be required for production of lasalocid and echinomycin and pSLA2-L (200 kbp) of *S. rochei* may have a role in the production of the lankacidin group of antibiotics. The plasmid SCP1 of *S. coelicolor* codes for several spore-associated proteins (Sap) and pSRM (43 kbp) of *S. rimosus* might be involved in aerial mycelium formation. In other linear plasmids in *Streptomyces*, there is little evidence to support their relation to morphological and metabolic differentiation.

We report here that pSLL is a conjugative linear DNA molecule with protein attached. The influence of pSLL on host organisms is discussed.

**Materials and Methods**

*Bacterial strains and plasmid.* *Streptomyces laurentii* ATCC31255 (wild-type strain P0) and its derivatives, P1 and P1a, were used throughout this work. Strain P0 harbors a large plasmid, pSLL, and a chromosomal integrated pSLS (pSLS$_{in}$). Strain P1 was spontaneously isolated from the wild-type strain P0. While strain P1 carried no pSLL, it was composed of two cell populations, free pSLS-carrying cells and pSLS$_{in}$ cells. Free pSLS was generated from pSLS$_{in}$ during development of host mycelia in solid culture. Therefore, it had one copy or less of free plasmid pSLS per host genome equivalent. Strain P1a was isolated for this work from the strain P1, after UV irradiation. This strain was isoleucine and valine auxotroph (Iv~ mutant) and carried approximately 60 copies of pSLS and no pSLL. Plasmid pUC119 DNA was used as an internal control for the exonuclease treatment.

*Media.* Bennett broth and agar were used for the growth of *S. laurentii*. MG-1 broth was used for extraction of plasmids and total DNA of *S. laurentii*. Minimal essential medium was used for selection of auxotrophic mutants.

*Preparation of total DNA for detection of linear plasmid.* Cells of *S. laurentii* were embedded in agarose and lysed according to Kinashi and Murayama, using lysozyme and Pronase, as described previously. DNA inserts, agarose plugs containing total DNA, were cut out of the agarose gel and used for PFGE analysis.

*Electrophoresis.* Conventional agarose gel electrophoresis was done as described by Sambrook et al. Pulsed-field gel electrophoresis (PFGE) was done using the Atto AE-6800 Cross Field electrophoresis unit for detection of plasmid pSLL and CHEF-DR II pulsed field electrophoresis systems (Bio Rad) for analysis of DNA fragments of restriction digestion. For all separations, 1% agarose gels in 0.5 × TBE buffer were used. SDS-PFGE was done by adding 0.2% SDS to the buffer and agarose gel. The pulse times were used: 30 s at 150 V for the detection of plasmid pSLL; 50 s at 150 V for SDS–PFGE; and 0.2–13 s at 200 V for analysis of DNA fragments of restriction digestion.

*Exonuclease digestion of pSLL.* Exonuclease treatments were based on those of Cong et al. After preparation of agarose plugs containing pSLL DNA and EcoRI-linearized pUC119 DNA, the gel pieces were washed twice for 2 h each time in dH$_2$O, were equilibrated with the digestion buffer for 2 h, and then were treated with 100 U Exonuclease III (New England Biolabs) or 10 U A exonuclease (Gene) at 37°C for 2 h.

For analysis of the terminal fragment of BamHI-digested pSLL, the pSLL in the agarose gel was treated with exonuclease III for 10–20 min before BamHI digestion.

*Restriction digestion of pSLL.* The gel pieces of pSLL were equilibrated with digestion buffer and treated with restriction endonucleases. The concentration of enzymes and the time of incubation were chosen according to the manufacturer's recommendations (New England BioLabs).

Non-Pronase-treated pSLL was obtained by omitting the Pronase treatment of the DNA inserts. After separation by SDS–PFGE, the untreated pSLL band was cut out of the gel and washed in dH$_2$O to
remove SDS.

Construction of transconjugants. A mixture of donor and recipient strains at a 1:50 ratio was incubated for 96 h at 28 °C on Bennett agar or Bennett media. Cells on the agar plate were suspended in Triton X-100 glycerol solution (Triton X-100, 0.1%; glycerol, 20%), and appropriate dilutions were plated onto selective media. The mycelia in liquid culture were diluted and plated on Bennett agar to obtain single colonies, then the colonies were transferred to the selective media described above.

Isolation of circular plasmid. Spores or mycelia were cultivated for 24 h in MG-1 medium at 28 °C on a rotary shaker (250 rpm), and the mycelia were used for the plasmid DNA isolation. Plasmid DNA was isolated by the alkaline lysis methods.(20)

Southern blot analysis. DNA fragments separated by PFGE or conventional agarose gel electrophoresis were transferred onto nitro cellulose filters (BA85; Schleicher & Schuell, Federal Republic of Germany), as described by Hopwood et al.21) Probe DNA was prepared using a DIG DNA labeling and detection kit (Boehringer). DNA fragments for probes were recovered from the agarose gel by a glass powder method, using a Genclean II kit (Bio 101, Inc., U.S.A.).

Thiostrepton productivity. Thiostrepton productivity was measured as described by Ogata et al.20) The mycelia grown for 24 h in Bennett media were harvested by centrifugation, followed by DMSO-extraction. The concentration of thiostrepton in the extract was measured by the paper disk method with Bacillus subtilis ATCC6633 as the test organism.

Measurement of number of spores. Spores were cultivated on Bennett agar plate (Nunc multidish; diameter of the dish was 1.8cm). The spores were scraped from an 8-day plate and suspended in Triton X-100-saline (Triton X-100, 0.1%; NaCl, 0.9%). The number of spores was estimated by microscopic counts of ten random haemocytometer fields, as described by Ogata et al.20)

Results

Evidence for the existence of terminal proteins

If there are proteins linked to a DNA molecule, the electrophoretic mobility of non-Pronase-treated DNA should be lower than that of Pronase-treated DNA.7) To search for the presence of protein bound to pSLL, the genome DNA inserts, with or without Pronase treatment, were analyzed by PFGE and SDS-PFGE. Non-Pronase-treated pSLL remained at the origin in the agarose gel of normal PFGE (Fig. 1A, lane 1), but moved at the same speed as the Pronase-treated pSLL in the agarose gel of SDS-PFGE (Fig. 1B, lane 1). Thus, proteins are bound to pSLL DNA.

To explore the structural composition of the ends of pSLL, the degradability of the plasmid by S' and S" end-specific DNA exonuclease was examined. The total DNA inserts with Pronase treatment were put on a PFGE gel. The pSLL band was cut out of the gel and incubated with either exonuclease III or λ exonuclease. As shown in Fig. 2, the DNA of pSLL was degraded only by the action of exonuclease III, not by the λ exonuclease (lane 2). The linearized pUC119 DNA (an internal control) was degraded by both enzymes. These results suggest that each S" end of pSLL DNA is covalently linked with a protein as are other Streptomyces linear plasmids.3)

Restriction mapping of the plasmid

To examine the terminal fragment of BamHI-digested pSLL, two examinations were done: Total DNA inserts, with or without Pronase treatment, were put on a SDS-PFGE gel. The bands were cut out of the gel and washed in dH2O. These Pronase-treated and non-Pronase-treated pSLL were digested with BamHI and electrophoresed on a conventional agarose gel. The 4-kbp fragment of untreated pSLL did not migrate (Fig. 3). To identify which was the first fragment to be degraded with exonuclease III, Pronase-treated pSLL was digested with exonuclease III, before BamHI digestion. As shown in Fig. 4, the 4-kbp and 1-kbp fragments were degraded by exposure to exonuclease III for 20 min and the other fragments remained. These results suggest that BamHI fragments of 4-kbp and 1-kbp were present on the right and left termini of pSLL, respectively.

We then did single or double digestion of Pronase-treated pSLL with EcoRI, EcoRV, AseI, and XbaI and separated the preparation by PFGE (Fig. 5). The 4-kbp BamHI fragment eluted from a gel was labeled with digoxigenin
and served as a hybridization probe against Southern transfer of a gel digested with the four enzymes. It hybridized to the 5-kbp fragment of EcoRI, 25-kbp fragment of EcoRV, 51-kbp fragment of Asel, and 83-kbp fragment of XbaI.

When ramping pulse times of 0.1–2.5 s were used for 8 h, EcoRV gave four fragments (data not shown). Double digests with EcoRV and Asel (data not shown) located the Asel sites within the EcoRV-B2 fragment. Double digests with EcoRI and XbaI (Fig. 5) located the XbaI sites within the EcoRI-A fragment. Double digests with EcoRV and EcoRI gave a 6-kbp fragment. Therefore, the order of the EcoRI-B and -C fragments was B–C. These data make up the map for EcoRI, EcoRV, Asel, and XbaI shown in Fig. 6.

**Conjugative transfer of pSLL**

The wild-type strain P0 and the pSLL-cured strain P1a (Iv− mutant) were used as donor and recipient strains, respectively, and their mixture was incubated for 96 h in Bennett agar. pSLL-carrying Iv− colonies were obtained at frequencies of $10^{-3}$–$10^{-2}$ transconjugant per donor (Fig. 7, A). The transconjugants obtained also carried about 60 copies of the circular plasmid pSLS, a feature of recipient strain P1a (Fig. 7, B). These results indicate that pSLL is a conjugative plasmid, based on its infectious transfer in mixed culture from a pSLL+ strain P0 to a pSLL− derivative P1a. Transconjugants were never detected from the mixture in liquid culture.

**Influence of pSLS and pSLL on host cells**

The potential of spore formation and thiostrepton production of a transconjugant, named P1aT, was compared with strains P0, P1, and P1a. The wild-type strain P0 formed Indian pink spores. However, P1 and P1a, formed non-colored spores and the number of spores decreased markedly (Fig. 8, Table). Thiostrepton productivity was diminished in these strains, while the productivity in
There were unique _AaeI_ and _XbaI_ sites on the _pSLL_. _EcoR1_ and _EcoRV_ gave four fragments. In the linear map the 8 restriction sites are indicated. In the block diagram the restriction fragments are denoted by letters and the sizes are given in kbp. _Cep45I, BamH1, Kpn1, BglII_ and _BsuIII_ cut _pSLL_ into more than six fragments, and _DraI, AluI, HpaI, SpiI, HindIII_, and _MunI_ did not cut _pSLL_.

**Fig. 6. Restriction Enzyme Cleavage Map of pSLL DNA.**

**Fig. 7. Southern Hybridization Analysis of pSLL and pSLS DNAs from S. laurentii (P0) and Its Derivatives.** DIG-labeled _pSLL_ DNA and _pSLS_ DNA were used as a probe in panels _A_ and _B_, respectively. _A_, PFGE of total DNAs; _B_, conventional agarose gel electrophoresis of plasmid DNAs. Plasmid DNAs were extracted by the alkaline lysis methods described in materials and methods. Lane 1, strain P0; Lane 2, strain P1; Lane 3, strain P1a; Lane 4, strain PlaT.

**Table** Properties of _S. laurentii_ ATCC31255 and Its Derivatives

<table>
<thead>
<tr>
<th>Strains</th>
<th>pSLL</th>
<th>Pock formation</th>
<th>Number of spores/plate</th>
<th>Thioestrepton production (µg/g of mycelia)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P0</td>
<td>+</td>
<td>–</td>
<td>1.5 ± 10^7</td>
<td>21.6 ± 3.2(^a)</td>
</tr>
<tr>
<td>P1</td>
<td>–</td>
<td>+</td>
<td>8.4 ± 10^7</td>
<td>10.3 ± 2.2(^a)</td>
</tr>
<tr>
<td>P1a</td>
<td>–</td>
<td>+</td>
<td>5.0 ± 10^7</td>
<td>7.0 ± 1.3(^a)</td>
</tr>
<tr>
<td>PlaT</td>
<td>+</td>
<td>–</td>
<td>1.8 ± 10^7</td>
<td>24.2 ± 2.0(^a)</td>
</tr>
</tbody>
</table>

\(^a\) Spontaneously developing pocks.

\(^b\) Values are means ± SD.

Transconjugant PlaT reverted to the level seen in strain P0 by transmission of _pSLL_ (Table). It also formed colored spores (Indian pink) as seen in strain P0 (Fig. 8). These results suggested that in _S. laurentii_, sporulation and thioestrepton productivity were diminished by the action of the free plasmid _pSLS_ and were stimulated by the function _pSLL_.

**Discussion**

We obtained evidence that _pSLL_ is a conjugative linear plasmid with protein attached. On conjugative transfer of _pSLL_ to _pSLL_-cured strains, stimulation or restoration of spore formation and thioestrepton production occurred.

The diminished sporulation and thioestrepton productivity of _pSLL_-cured strains, P1 and P1a, is attributed to the existence of the free plasmid _pSLS_. Some pock-forming plasmids of actinomycetes are known to lead to retardation of growth and development of host mycelia. Sometimes growth inhibition is accompanied by production of antibiotics. These inhibitions may relate to activity of _kil_ genes such as _tra_ of _pIJ101, 23^\(^a\) _traB_ of _pSN22, 24^\(^a\) _traSA_ of _pSAM2, 25^\(^a\) _and _) of _pSA1.1_: the _spi_ gene affects sporulation and thioestrepton production, and is essential for _pSA1.1_-transfer. 14, 26, 28 Based on these observations, we propose that plasmid _pSLS_ of _S. laurentii_ has an injurious effect on its host mycelia, as do the _tra, traB, traSA_, and _spi_ genes.

Another gene, _kor_ (kil-override), 23^\(^a\) regulates transcription of the _kil_ genes. The gene products of _korA, traR_, and...
**Fig. 8.** Lawn of *S. laurentii* (P0) and Its Derivatives.
A, strain P0; B, strain P1; C, strain P1a; D, strain P1aT.

**korSA** control the expression of the *tra*, *traB*, and *traSA*, respectively. pSA1.1 also has a *kor*-like gene (in preparation). A linear plasmid pSCL1 (12 kbp) of *S. clavuligerus* encodes a putative protein with a high sequence similarity to the deduced *KorA* protein of pLI101, but a *kil* gene has not been identified on this plasmid.29

pSLL-cured strains, P1 and P1a, were not completely deficient in sporulation and thiostrepton production, which indicates that genes directing the sporulation and thiostrepton synthesis are not present on plasmid pSLL. It may be that pSLL has a *kor*-like gene and its product suppresses the expression of *kil* genes of pSLS.

In conclusion, the linear plasmid pSLL seems to diminish the injurious effect of free pSLS on host mycelia, in addition to suppressing the excision of pSLS. Further investigations are in progress to search for a *kor* gene on the linear plasmid pSLL and for a *kil* gene on the circular plasmid pSLS.

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