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Reconstruction of a *Streptomyces* linear replicon from separately cloned DNA fragments: Existence of a cryptic origin of circular replication within the linear plasmid

(circular plasmid/DNA replication/pSCL)

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ABSTRACT **We report here the reconstruction of a functional linear replicon, the 12-kilobase *Streptomyces clavuligerus* plasmid pSCL, from separate DNA fragments cloned in *Escherichia coli* on the pUC19 plasmid. Protein-free DNA molecules containing the full-length pSCL sequence, an internally inserted thiostrepton-resistance gene, and adventitious nucleotides external to the pSCL termini were introduced into *Streptomyces lividans*, where the synthesis and functional attachment of replication proteins occurred and pSCL was established as an extrachromosomal linear replicon. Transformation of *S. lividans* with uncut supercoiled pUC19/pSCL DNA from *E. coli* or with a circularized 8-kilobase internal fragment of pSCL yielded circular replicons, indicating the existence of a cryptic origin of circular replication within the linear plasmid. Insertion mutations at sites that prevented the replication of pSCL linear plasmids also interfered with its replication in the circular mode.**

Linear plasmids ranging in size from 12 to several hundred kilobases (kb) are common in *Streptomyces* species (e.g., see refs. 1–7). While at least one of these plasmids encodes antibiotic synthesis pathways (4), little else is known about their biological functions. The few *Streptomyces* linear plasmids that have thus far been characterized contain terminal inverted repeat sequences and have protein bound covalently to the 5′ termini of their DNA strands (1, 3). These properties are reminiscent of linear DNA viruses such as *Bacillus* phage ϕ29 and adenovirus (see ref. 8 for a recent review), which replicate by a protein-primed strand-displacement mechanism (see refs. 9 and 10 for reviews).

We have initiated a study of the smallest known linear plasmid, pSCL, a 12-kb replicon identified originally in *Streptomyces clavuligerus* (5). In this paper, we describe the reconstruction of pSCL as a functional *Streptomyces* linear replicon using separate DNA fragments cloned in *Escherichia coli*. We report also the surprising finding that the pSCL plasmid has dual replication capabilities in *Streptomyces lividans* and can replicate extrachromosomally in this host as either circular or linear DNA.

MATERIALS AND METHODS

Bacteria. *S. lividans* TK64 (11) and *S. lividans* C14 (12) were used for *Streptomyces* hosts. *S. clavuligerus* ATCC27064 was a kind gift from Y. Aharonowitz (Tel Aviv) and was used as the source of pSCL DNA. *E. coli* strain DH10 was purchased from Life Technologies (Grand Island, NY) and was used as the recipient in *E. coli* transformations.

Culture Conditions and Transformation. Standard procedures were used for the growth and transformation of strains of *E. coli* (13) and *Streptomyces* (11). Thiostrepton was added to 50 μg/ml and hygromycin was added to 200 μg/ml where appropriate. *S. clavuligerus* was grown in tryptic soy broth (Becton Dickinson).

DNA Isolation. Linear plasmid DNA was isolated from 50-ml liquid cultures according to Chardon-Loriaux *et al.* (3) with the following modifications: DNA was precipitated from cleared lysate by the addition of 2.5 vol of absolute ethanol and suspended in 1–2 ml of TE (10 mM Tris·HCl, pH 8.0/1 mM EDTA). The solution was extracted with phenol/chloroform/isoamyl alcohol (25:24:1), ethanol precipitated a second time, resuspended in 1–2 ml of TE, and treated with λ exonuclease (Life Technologies) according to the manufacturer's recommendations. Total bacterial DNA was isolated the same way except that λ exonuclease treatment was omitted.

DNA Manipulations. The pSCL termini were cloned as described for pSLA2 of *Streptomyces rochei* by Hirochika *et al.* (14). Briefly, preparations of linear plasmid DNA were cleaved with *Kpn* I for left-end cloning and with *Sph* I for right-end cloning. The endonuclease-treated DNA was then treated with 0.1 M NaOH at 37°C for 90 min to remove remnants of terminal protein(s) and was then neutralized by the addition of an equimolar amount of HCl. Tris·HCl (pH 8.0) was added to 50 mM and the denatured DNA strands were reannealed by incubation at 65°C for 2 hr. The pSCL DNA was precipitated and ligated to pUC19 DNA that had been cut with *Hin*cII and *Kpn* I, or by *Hin*cII and *Sph* I. A 1.8-kb *Bam*HI fragment containing the thiostrepton-resistance (*tsr*^{r}) gene and a 1.2-kb *Bam*HI fragment containing the hygromycin-resistance (*hyg*^{r}) gene, both cloned in pUC19, were kindly supplied by M. O. Vogtli (this laboratory). Hybridizations were carried out according to Church and Gilbert (15). Other DNA manipulations were carried out according to standard procedures (13). Restriction endonucleases and other DNA modifying enzymes were purchased from commercial sources and were used as recommended by the manufacturers.

RESULTS

Cloning and Reconstruction of pSCL Plasmids in *E. coli*. As no phenotype is known to be associated with pSCL, selection of *Streptomyces* cells containing the native plasmid is not practical. To facilitate the manipulation and study of pSCL, we undertook to clone and insert a selectable genetic marker within the plasmid genome.

Linear pSCL DNA was purified from total cellular DNA preparations of *S. clavuligerus* by treatment of total DNA with λ exonuclease (see *Materials and Methods*). Findings by Keen *et al.* (5) have suggested that terminal proteins are present on pSCL DNA, as is the case with other linear

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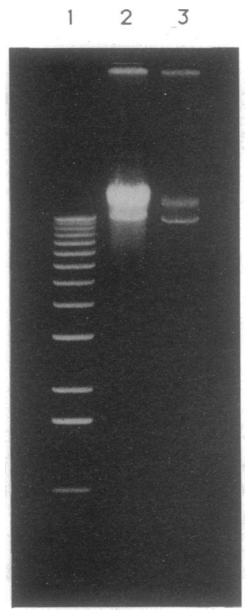

FIG. 1. Partial purification of linear pSCL DNA by λ exonuclease treatment. Ethidium bromide-stained agarose gel of 1-kb ladder (Life Technologies) (lane 1), total DNA from *S. clavuligerus* (lane 2), and total DNA from *S. clavuligerus* treated with λ exonuclease (lane 3).

plasmids (8); consequently, we expected that linear pSCL DNA would be protected from λ exonuclease digestion, which requires exposed 5′ termini, whereas linear fragments of chromosomal DNA would be digested, yielding DNA preparations enriched for the linear plasmid. As shown in Fig. 1, λ exonuclease treatment proved to be an efficient method of purifying pSCL DNA from *S. clavuligerus* cells.

As a prelude to cloning pSCL DNA in *E. coli*, we treated partially purified pSCL DNA with 0.1 M NaOH to remove the terminal-bound protein. As this treatment denatures the plasmid DNA, we attempted to re-form duplex pSCL molecules by reannealing the strands (see *Materials and Methods*). However, we were unable to directly reconstitute full-length pSCL DNA molecules that could be ligated to an *E. coli* plasmid vector. Therefore, only the terminal DNA fragments of pSCL were subjected to NaOH treatment; these subsequently were reannealed and separately cloned. Full-length linear plasmid DNA was reconstituted by sequential cloning in *E. coli* of separate component fragments of the plasmid, as diagrammed in Fig. 2. The resulting construct, pLPE2, contains a pSCL DNA segment indistinguishable by restriction endonuclease mapping from wild-type linear pSCL molecules isolated from *S. clavuligerus* (5). However, unlike native linear pSCL DNA, the pSCL segment of pLPE2 contains no terminal protein(s).

Replication of Linear pSCL-Derived Plasmids in *S. lividans*. To facilitate genetic manipulation of pSCL in *Streptomyces*, the location of *Hin*cII sites was mapped on pLPE2 DNA isolated from *E. coli*, the plasmid DNA was linearized by partial digestion with *Hin*cII, and *Bgl* II linkers were inserted at 5 of its 10 *Hin*cII sites; Fig. 2 shows their locations. A 1.8-kb *Bam*HI fragment containing a *tsr*^{r} determinant derived from *Streptomyces azureus* (16) was then introduced into each of the linker insertions. The pSCL segment of each of the five *tsr*^{r}-containing pLPE2 derivatives was separated from pUC19 by cutting the plasmid with both *Hin*dIII and *Bam*HI, each of which cleaves pLPE2 at a single site flanking the pSCL segment (see Fig. 2); these cleavage sites within pUC19 vector sequences are located 14 and 16 nucleotides external to the *Hin*cII site of insertion of pSCL.

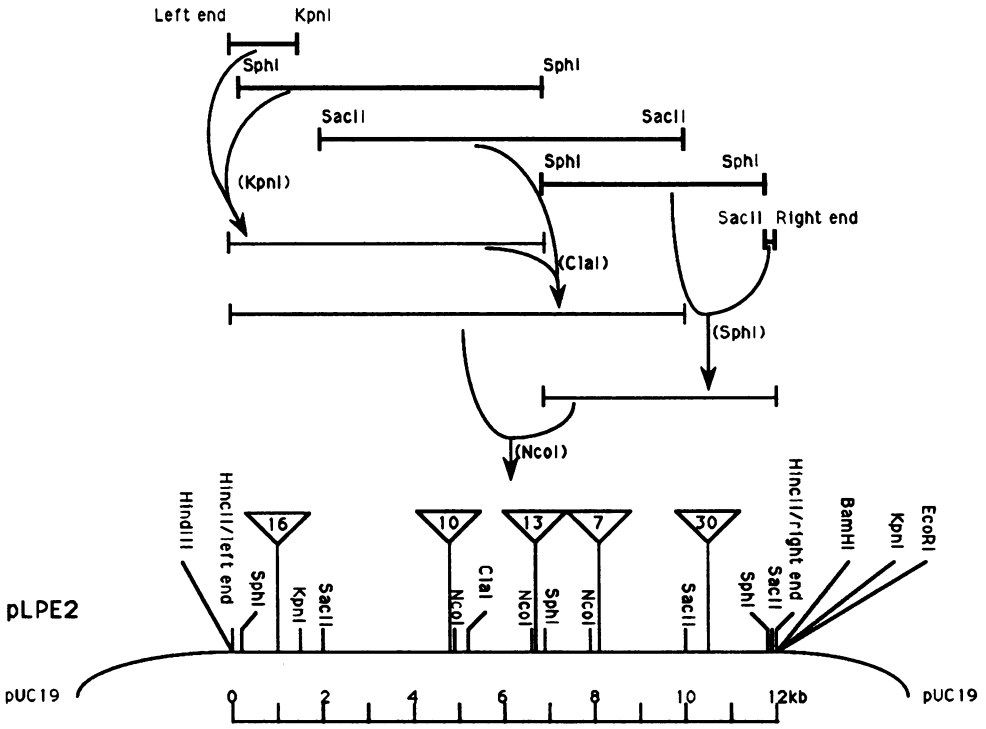

FIG. 2. Reconstruction of the pSCL plasmid and restriction map of pLPE2. Fragments of pSCL and the cloning procedure used for reconstruction are diagrammed. pSCL DNA was cleaved with *Kpn* I or *Sph* I, generating a ≈2-kb left-end DNA fragment and a 0.2-kb right-end DNA fragment, respectively. These terminal fragments of pSCL were then treated with alkali and their DNA strands were reannealed as described, yielding protein-free DNA fragments containing one end that can be ligated to blunt-ended DNA and one end that can be ligated to a *Kpn* I or *Sph* I site. Two *Sph* I fragments, 4.6 and 6.6 kb, were each cloned in pUC19. An 8-kb *Sac* II fragment was cloned in a pUC19 plasmid that had a *Sac* II linker inserted at its *Hin*cII site. Arrows indicate pairs of fragments, cloned on separate pUC19 plasmids, used to reconstruct pSCL; each pair was fused at the restriction site indicated in parentheses. The pUC19 segment is not drawn to scale. Triangles indicate sites of *Bgl* II linker insertions into pLPE2. Number in each triangle refers to the construct carrying the specific linker insertion.

Linear DNA fragments containing pSCL that had been cleaved from pLPE2 derivatives pLPE2-7, pLPE2-16, and pLPE2-30 generated *tsr*^{r} *S. lividans* transformants at low frequency (≈10 transformants per μg of DNA), whereas two insertion derivatives, pLPE2-10 and pLPE2-13, yielded no *tsr*^{r} transformants at all in three separate experiments. Attempts to transform *S. clavuligerus* with pLPE2 derivatives gave no transformants with any of the constructs, possibly because of the much lower transformability of *S. clavuligerus* in our hands. As shown in Fig. 3, pLPE2-hybridizable DNA was observed in transformant clones of *S. lividans* by Southern blot analysis; however, plasmid DNA molecules were not detected in *tsr*^{r} *S. lividans* transformants by ethidium bromide staining of agarose gels, which revealed the presence of wild-type pSCL linear replicons in *S. clavuligerus*.

To determine the molecular nature of the pLPE2 hybridizable sequences, total DNA from *S. lividans* transformants was cleaved with *Kpn* I, which cuts at a single site in linear pSCL DNA; DNA fragments measuring 1.5 and 12.3 kb are predicted from cleavage of pLPE2-7 and pLPE2-30 linear DNA molecules by *Kpn* I, while 3.3- and 10.5-kb fragments

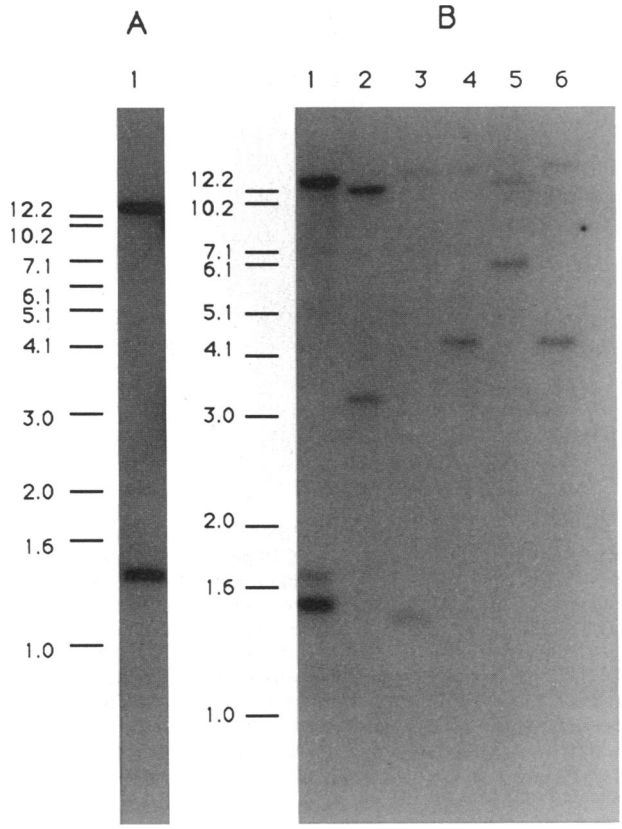

FIG. 3. Southern blot analysis of total DNA from *S. lividans* TK64 transformed with linear DNA segments of pLPE2 derivatives generated by *Hin*dIII and *Bam*HI digests (*A*, lane 1; *B*, lanes 1–3), or circular pLPE2 constructs (*B*, lanes 4–6). DNA in all lanes was digested with *Kpn* I and probed with pLPE2. (*A*) Linear pLPE2-7. (*B*) Lanes: 1, variant clone of linear pLPE2-7; 2, linear pLPE2-16; 3, linear pLPE2-30; 4, circular pLPE2-7; 5, circular pLPE2-16; 6, circular pLPE2-30. Molecular size markers (kb) are indicated on the left. Expected fragment sizes for linear replicons containing a 1.8-kb *tsr*^{r} gene insert at the sites shown in Fig. 2 are as follows: pLPE2-7 and pLPE2-30; 1.5 kb (from the *Kpn* I site to the pSCL left end) and 12.3 kb (from the *Kpn* I site to the pSCL right end); pLPE2-16, 3.3 kb (from the *Kpn* I site to the left end) and 10.5 kb (from the *Kpn* I site to the right end). Expected fragment sizes for the corresponding circular replicons, which include the 2.7-kb pUC19 segment are as follows: pLPE2-7 and pLPE2-30, 4.2 and 12.3 kb; pLPE2-16, 6 and 10.5 kb.

are expected from the cleavage of pLPE2-16. As shown in Fig. 3, *S. lividans* transformants contained pLPE2-hybridizable *Kpn* I-generated DNA fragments of the predicted size (Fig. 3*A*, lane 1; Fig. 3*B*, lanes 2 and 3), indicating the presence of pSCL DNA molecules that are both extrachromosomal and linear. However, other *S. lividans* clones receiving a linear pSCL-containing DNA segment of pLPE2-7 DNA showed three bands, corresponding to DNA fragments 12.3, 1.55, and 1.65 kb (Fig. 3*B*, lane 1), while some clones transformed with the linear pSCL DNA segment of pLPE2-16 showed two bands corresponding to fragments of 10.5 and 3.6 kb (data not shown). The results of further analysis have suggested that these unexpected fragment sizes result from sequence duplications within these constructs. While the linear plasmid DNA molecules replicated in *S. lividans* and were partitioned into spores, they were not stably inherited by all of the progeny of transformed clones. After one cycle of growth on nonselective medium, only 0.01% of spores from transformants gave rise to clones containing the plasmid, as measured by their ability to yield thiostrepton-resistant colonies. Under selective growth conditions, ≈1% of *S. lividans* spores contained the plasmid. The linear plasmid could be propagated under selection in *S. lividans* cells grown in serially diluted liquid cultures.

Replication of Circular pSCL-Derived Plasmids in *S. lividans*. The same constructs that transformed *S. lividans* as linear molecules—specifically pLPE2-7, pLPE2-16, and pLPE2-30—were also able to transform *S. lividans* as uncleaved circular DNA. Those constructs that did not transform *S. lividans* as linear DNA—namely, pLPE2-10 and pLPE2-13—similarly failed to yield *tsr*^{r} *S. lividans* transformants when introduced as circular DNA molecules. The transformation efficiency for circular DNA constructs was at least several hundred times higher than for the analogous linear constructs and was comparable to, albeit lower than, the frequency we observed for circular *Streptomyces* shuttle vectors made in *E. coli* (2×10^3 transformants per μg of DNA). However, the thiostrepton-resistance phenotype showed the same unstable inheritance in these transformants as in clones receiving linear pSCL-derived DNA segments (see above).

Southern blotting of DNA from *S. lividans* clones receiving the circular pLPE2 plasmid was carried out after digestion of total cellular DNA with *Kpn* I. Rather than showing cleavage fragments of the sizes found for the linear extrachromosomal pSCL replicon, the DNA isolated from these transformants yielded fragments of 4.2 and 12.3 kb for pLPE2-7 and pLPE2-30 and fragments measuring 6 and 10.5 kb for pLPE2-16 (Fig. 3*B*, lanes 4–6). As indicated in Fig. 3, these are the sizes expected for fragments generated by cleavage of *tsr*^{r}-containing circular pLPE2 DNA molecules by *Kpn* I. As shown in Fig. 4, DNA species characteristic of both types of pSCL-derived replicons were observed following introduction of differentially marked circular and linear pSCL-derived plasmids, indicating that the two forms of pSCL can coexist in *S. lividans*.

The dual replication capabilities of pSCL were confirmed by the ability of an internal fragment of pSCL to replicate as a circular plasmid in *S. lividans*. The internal pSCL segment was separated from the pUC19 portion of pLPB3-T by cleavage with both *Bam*HI and *Bgl* II, generating a linear DNA fragment having compatible cohesive ends. Total DNA from a transformant clone obtained by introducing self-ligated pLPB3-T DNA was analyzed by Southern blotting (Fig. 5). A single hybridizable fragment of ≈9.8 kb was detected after digestion with *Eco*RV, as expected for an extrachromosomal circular replicon consisting of the 8-kb pSCL DNA fragment bracketed by the *Bam*HI and *Bgl* II sites, plus the *tsr*^{r} DNA insert (lane 3). DNA species of expected sizes were also detected after digestion with *Cla* I

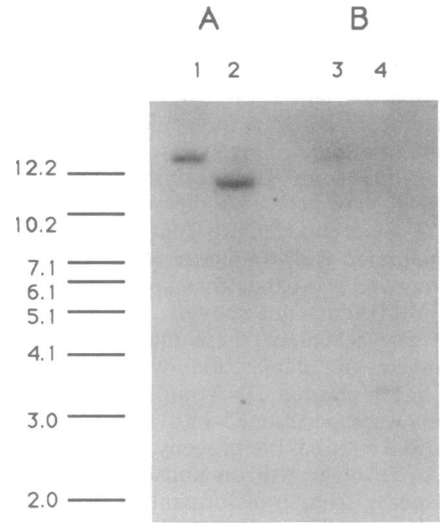

FIG. 4. Southern blot analysis of total DNA from *S. lividans* TK64 containing both linear and circular derivatives of pSCL. A clone carrying the linear pLPE2-16 *tsr*^{r} plasmid was transformed with a circular derivative of pLPE2-30 in which a 1.2-kb fragment containing the *hyg*^{r} gene of *Streptomyces hygroscopicus* (17) was inserted. Transformants were selected on medium containing both antibiotics. Total DNA from a transformant clone was cut with *Kpn* I and then probed with the *hyg*^{r} gene for detection of incoming circular plasmid. Lanes: 1 and 3, uncut DNA; 2 and 4, DNA cut with *Kpn* I. (*A*) Blot probed with *hyg*^{r}-containing DNA fragment showing a single band of the size expected (11.7 kb) for circular pSCL DNA. (*B*) Blot probed with *tsr*^{r}-containing DNA fragment showing a single 3.3-kb band, as expected for the linear extrachromosomal replicon. Molecular size markers (kb) are indicated on the left.

and *Sac* II (lanes 2 and 4). Unlike full-length pSCL-containing terminal sequences, the internal segment cleaved from pSCL was not propagated as a linear replicon when introduced into *S. lividans* as a linear DNA molecule.

DISCUSSION

We set out to establish a model system to study the biology of linear plasmids in *Streptomyces*. We initially cloned full-length pSCL DNA molecules in *E. coli* to facilitate the manipulation of plasmid DNA; this in turn permitted the insertion of genetic markers that allowed both the selection of transformants in *S. lividans* and the identification of loci essential for plasmid replication and maintenance. As insertions can be made readily into the pSCL segment of circular plasmids isolated from *E. coli*, the three-component ligations necessary for the introduction of selectable markers into linear plasmid molecules were avoided.

Functional linear *Streptomyces* replicons were reconstituted from pSCL plasmid DNA that had been cloned in *E. coli*, indicating that naked linear pSCL DNA introduced into *S. lividans* by transformation can synthesize, and serve as a substrate for, proteins required for plasmid DNA replication. It previously has been shown that protease treatment of other linear extrachromosomal replicons prevents replication by the protein-primed strand-displacement mechanism common to such molecules (refs. 9, 10, and 18 and references therein), possibly because attachment of a small peptide remnant at the DNA ends interferes with the binding of functional replicase. However, complete removal of terminal proteins by treatment of linear bacteriophage ϕ29 DNA with piperidine allows its replication *in vitro* in reaction mixtures containing these proteins (19), implying that the required replicase can reattach to naked DNA termini. Our results show that functional attachment of the replication protein(s) of pSCL to naked plasmid DNA can occur *in vivo*. Because

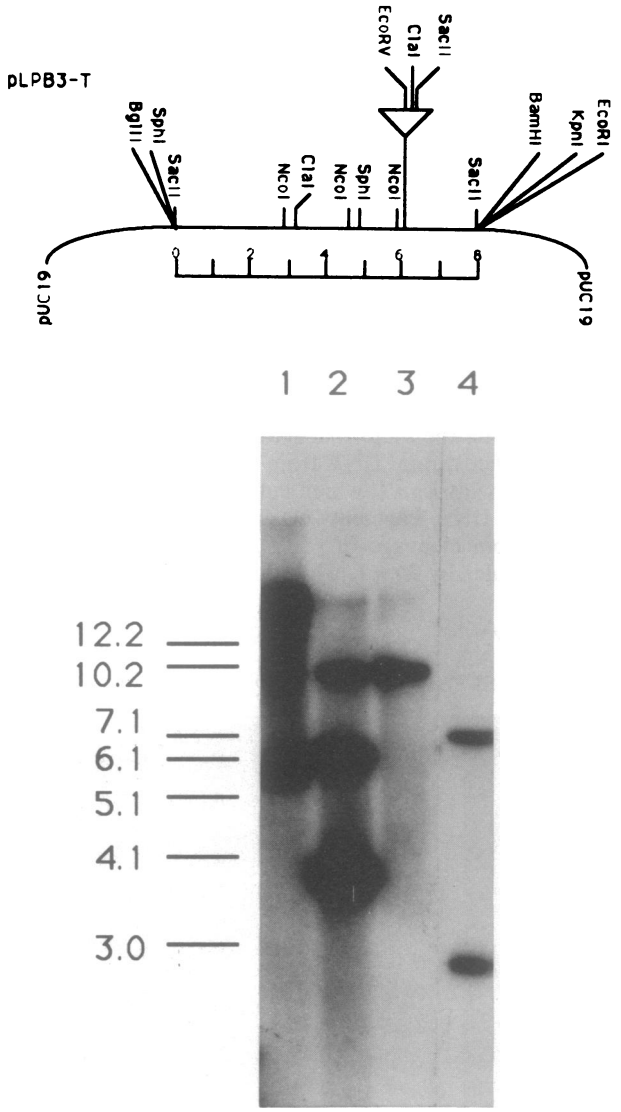

FIG. 5. Restriction map of pLPB3-T. An 8-kb internal *Sac* II fragment of pSCL was cloned in pUC19 plasmid containing a *Sac* II linker in its *Hin*cII site. A *Bgl* II linker and subsequently a 1.8-kb fragment containing the *tsr*^{r} gene were inserted at a *Hin*cII site on this fragment that corresponds to the insertion site of pLPE2-7. A second *Bgl* II linker was inserted at the flanking *Hin*dIII site resulting in pLPB3-T. Southern blot analysis was done using pLPB3-T as a probe. Total DNA isolated from a clone of *S. lividans* TK64 that had been transformed with a self-ligated *Bam*HI/*Bgl* II-digested pLPB3-T DNA was run undigested (lane 1) and digested with *Cla* I, *Eco*RV, and *Sac* II (lanes 2–4). Molecular size markers (kb) are indicated on the left.

the replication of other linear extrachromosomal DNA elements is known to require only the terminal sequences and associated replicases, the identification of two separate sites internal to pSCL that are essential for replication suggests that these regions may encode replication protein(s).

As the terminal DNA fragments of adenovirus genomes can replicate transiently *in vivo* when introduced with 20 adventitious nucleotides attached to them externally (20), we reasoned that if (*i*) naked linear pSCL DNA having nucleotides external to the termini can similarly serve as a substrate for DNA replication *in vivo* by protein-primed strand displacement, (*ii*) linear pSCL DNA molecules devoid of protein at their termini can synthesize any required replicases they encode, and (*iii*) proteins encoded by the *S. clavuligerus* host chromosome, but lacking in *S. lividans*, are not necessary for pSCL replication, then linear extrachromosomal replicons

that could be selected by using the *tsr*^{r} gene inserted into pSCL would be established in transformants.

Sufficient naked linear pSCL DNA fragments containing adventitious external DNA sequences survived the action of intracellular exonucleases long enough to enable the synthesis and attachment of replication proteins and, consequently, the establishment of linear replicons. The maximum number of nucleotides that can be added externally to pSCL without preventing attachment of replicase and whether nucleotides external to the pSCL termini may actually facilitate the establishment of functional linear replicons by delaying exonucleolytic digestion of essential sequences at the pSCL termini are unknown. In addition, we have not determined whether the pSCL replicons generated from fragments containing adventitious external nucleotides have exactly the same ends as native pSCL DNA in *S. clavuligerus*.

There are no reports of circular extrachromosomal replicons in *S. clavuligerus*, and we found no evidence of circular plasmids in this organism. However, when circular DNA containing pSCL sequences was introduced into *S. lividans*, the DNA was maintained as a circular plasmid replicon. We did not detect linear replicons following the introduction of the circular pUC19/pSCL composite plasmid pLPE2 by transformation, possibly because the ends of pSCL are embedded in a long stretch of pUC19 DNA in pLPE2. The terminal segments of ϕ29 similarly failed to replicate *in vitro* when tested on circular DNA molecules (19). Alternatively, if linear replicons are produced from pUC19/pSCL circular DNA molecules, our inability to detect them may result from our inability to specifically select for unstable linear plasmid in the presence of circular plasmids carrying the same genetic marker.

pSCL molecules replicating in either the linear or circular mode were maintained at a low copy number in *S. lividans*. In addition, neither linear nor circular pSCL replicons were stably inherited in *S. lividans* in the absence of selection, and certain of the linear constructs contained a duplication of a short sequence near their left end (unpublished data). These effects were independent of the site of insertion of the *tsr*^{r} gene. While the stability of pLPE2-7, -16, or -30 was not increased in *S. lividans* C14, a strain that contains another linear plasmid (SLP2; C. Chen, personal communication), the stable inheritance of pSCL and other linear plasmids we have detected in *S. clavuligerus* raises the possibility that either the *S. clavuligerus* host or the large linear plasmids that accompany pSCL in this organism (our unpublished results) may provide a function that affects the inheritance or copy number of pSCL.

The evolutionary basis for the unexpected occurrence of an origin of circular DNA replication in an extrachromosomal element that exists in its native state as a linear plasmid is unclear. However, the observation that insertions eliminating the ability of pSCL to replicate in the linear mode also inactivate its circular mode of DNA replication raises the possibility that the two replication modes may require the same plasmid-encoded gene product.

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1. Hirochika, H. & Sakaguchi, K. (1982) *Plasmid* **7**, 59–65.
2. Ogata, S., Koyama, Y., Sakaki, Y. & Hayashida, S. (1983) *Agric. Biol. Chem.* **47**, 2127–2129.
3. Chardon-Loriaux, I., Charpentier, M. & Pecheron, F. (1986) *FEMS Microbiol. Lett.* **35**, 151–155.
4. Kinashi, H., Shimaji, M. & Sakai, M. (1987) *Nature (London)* **328**, 454–456.
5. Keen, C. L., Mendelovitz, S., Cohen, G., Aharonowitz, Y. & Roy, K. L. (1988) *Mol. Gen. Genet.* **212**, 172–176.
6. Leblond, P., Francou, F. X., Simonet, J. M. & Decaris, B. (1990) *FEMS Microbiol. Lett.* **60**, 79–88.
7. Solenberg, P. J. & Baltz, R. H. (1991) *J. Bacteriol.* **173**, 1096–1104.
8. Sakaguchi, K. (1990) *Microbiol. Rev.* **54**, 66–74.
9. Stillman, B. W. (1983) *Cell* **35**, 7–9.
10. Salas, M. (1988) *Curr. Top. Microbiol. Immunol.* **136**, 71–88.
11. Hopwood, D. A., Bibb, M. J., Chater, K. F., Kieser, T., Bruton, C. J., Kieser, H. M., Lydiate, D. J., Smith, C. P., Ward, J. M. & Schrempf, H. (1985) *Genetic Manipulation of Streptomyces: A Laboratory Manual* (John Innes Found., Norwich, U.K.).
12. Omer, C. A. & Cohen, S. N. (1984) *Mol. Gen. Genet.* **196**, 429–438.
13. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
14. Hirochika, H., Nakamura, K. & Sakaguchi, K. (1984) *EMBO J.* **3**, 761–766.
15. Church, M. G. & Gilbert, W. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 1991–1995.
16. Thompson, C. J., Kieser, T., Ward, J. M. & Hopwood, D. A. (1982) *Gene* **20**, 51–62.
17. Zalacain, M., Gonzalez, A., Guerrero, M. C., Mattalian, R. J., Malpartida, F. & Jimenez, A. (1986) *Nucleic Acids Res.* **14**, 1565–1581.
18. Lyra, C., Savilhati, H. & Bamford, D. H. (1991) *Mol. Gen. Genet.* **228**, 65–69.
19. Gutierrez, J., Garcia, J. A., Blanco, L. & Salas, M. (1986) *Gene* **43**, 1–11.
20. Hay, R. T., Stow, N. D. & McDougall, I. M. (1984) *J. Mol. Biol.* **175**, 493–510.