Characterization of the Genetic Components of *Streptomyces lividans* Linear Plasmid SLP2 for Replication in Circular and Linear Modes

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The nucleotide sequence of *Streptomyces lividans* linear plasmid SLP2 consists of 50,410 bp (C. H. Huang, C. Y. Chen, H. H. Tsai, C. Chen, Y. S. Lin, and C. W. Chen, Mol. Microbiol. 47:1563–1576, 2003). Here we report that the basic SLP2 locus for plasmid replication in circular mode resembles that of *Streptomyces* linear plasmids pSLA2 and SCP1 and comprises iterons_{SLP2} and the adjacent rep_{SLP2} gene. More efficient replication additionally required the 47-bp sequence between bp 581 and 628 upstream of the iterons. Replacement of either the iterons or the rep gene of SLP2 by the corresponding genes of pSLA2 or SCP1 still allows propagation in *Streptomyces*, although the transformation frequencies were 3 orders of magnitude lower than the original plasmids, suggesting that these plasmids share similar replication mechanisms. To replicate SLP2 in linear mode, additional SLP2 loci—either mtap_{SLP2}/tap_{SLP2} or ilrA_{SLP2}/ilrA_{SLP2}—were required. IlrA_{SLP2} protein binds specifically to the iterons_{SLP2} in vitro. Interactions were detected between these SLP2-borne replication proteins (Mtap_{SLP2}, Tap_{SLP2}, and IlrA_{SLP2}) and the telomeric replication proteins (TpgL, TapL, and TpgL) of the *S. lividans* chromosome, respectively, but the SLP2 proteins failed to interact. These results suggest that SLP2 recruits chromosomally encoded replication proteins for its telomere replication.

Unlike most eubacteria, *Streptomyces* species usually contain linear chromosomes and plasmids (9, 16, 17). The linear plasmids are 12 to 1,700 kb long (16, 27). Their telomeres contain inverted repeat sequences from 44 bp (7) to 180 kb (19), and their 5’ telomeric ends are linked covalently to terminal proteins (1, 28). Unlike linear replicons of adenoviruses and bacteriophage λ29, which also contain terminal proteins linking covalently to 5’ telomeric DNA ends and undergo replication by a mechanism of strand displacement (23), replication of *Streptomyces* linear plasmids starts at centrally located loci (5, 25) and continues bidirectionally towards the telomeres—leaving an ~280-nucleotide 3’ single-strand overhang as an inter-locus (25). This is converted to a double strand by a postulated “folding back” of multiply short palindromes on the telomere extension (20). The chromosomal telomere-associated protein (TapL, encoded by *tapL*) binds to the palindromes II/III then to recruit telomere terminal protein (TpgL, encoded by *tpgL*) (1, 2). Neither *tapL* nor *tpgL* homologous genes are carried by the linear plasmids pSLA2 and pSCL1 (1, 2), and the mechanism of recruitment or activation of these chromosomal telomere proteins for plasmid telomere replication is unknown.

The centrally located loci of *Streptomyces* linear plasmids can also maintain propagation in circular mode when the telomeres are deleted (5, 8, 22, 25). The centrally located locus for replication of linear plasmid pSLA2 is composed of the iterons located within the essential genes rep1 (encoding DNA-binding protein) and rep2 (DNA helicase) (6). Experimental evidence shows that the replication origin of linear SCP1 plasmids contains a rep2_{SLP2}-like gene and its adjacent regions contain different iteron sequences (22). Similar loci are also indicated in linear plasmids pSCL1 and SLP2 (11, 27). The extent of functional similarity of these individual replicating components, and consequently the extent to which mechanisms of replication are similar among linear plasmids, is not known.

The minimal locus required for maintaining the replication of pSLA2 in circular mode cannot allow its propagation in linear mode unless it also contains a new plasmid locus, rlrApSLA2 (required for linear replication) (21). Plasmids containing rlrA_{SLP2} are detrimental for propagation in circular mode, the effect of which can be reversed by an adjacent and divergently transcribed locus, rorA_{SLP2} (rorA override), which resembles korA (kilA override) of *Streptomyces* circular plasmid pIJ101 (14, 26). rorA_{SLP2} and rlrA_{SLP2} increase inheritance and copy number of pSLA2 circular plasmids, suggesting that they may affect the origin locus (e.g., iterons) (21). Although rorA_{SLP2}-homologous genes are found in linear plasmids SCP1, pSLA2, and SLP2, no rlrA_{SLP2}-homologous loci of the plasmids are found (3, 11, 18), suggesting that they carry a distinct locus that enables replication in linear mode.

SLP2 is a large (50,410-bp) linear plasmid of *Streptomyces lividans* (7, 10, 11). Here we report that the basic locus of SLP2 for replication in circular mode consists of the iterons_{SLP2} and rep_{SLP2}, while efficient replication requires an additional sequence upstream of the iterons_{SLP2}. Combination of the iterons and rep genes of linear plasmids SLP2, pSLA2, and SCP1 enables propagation in *Streptomyces*. Replication of SLP2 in linear mode required both the SLP2-borne genes mtap_{SLP2} and

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either \textit{tpgSLP2} or \textit{ilrASLP2}, while interactions between these SLP2 proteins and the \textit{S. lividans} chromosomal telomeric proteins were also detected. These results suggest that structurally distinct linear plasmids of different \textit{Streptomycyes} species have highly conserved replication modes and functions.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and general methods.** \textit{Escherichia coli} strain DH5\textalpha\ (Life Technologies, Inc.) and plasmid pSP72 (Promega) or pBluescript KS (Strategene) were used as cloning host and vector. \textit{E. coli} plasmid isolation, transformation, and PCR amplification followed the methods of Sambrook et al. (24). \textit{Streptomycyes} strains ZX7 and TK20 were the native hosts for linear plasmid SLP2 (15). \textit{Streptomycyes} culture, plasmid isolation, preparation of protoplasts, and transformation followed the methods of Kieser et al. (15). To compare the transformation frequencies of plasmids at different times in the experiments, we used 0.1 ng plasmid DNA of pIJ702 as a control each time.

**Cloning, sequencing, and analysis of the DNA fragment of the SLP2 portion of pQC542.** The 0.8-kb SLP2 telomeres from pLUS450 (containing the 2.6-kb chromosomal telomeres; kindly provided by Carton Chen) were cloned into pSP72 to yield pQC154. pQC177 was obtained by ligating three DNA fragments: a 3.3-kb fragment of Xba/IHpaI-cleaved pQC154; a 1.2-kb fragment of Xba/IspI-cleaved pQC154; and a 2.6-kb fragment of XbaI-cleaved pQC98 (containing the \textit{melC} gene). The restriction endonuclease BstEII-linearized pQC177 DNA was ligated with the isoschizomer Sau3AI-digested partially genomic DNA of \textit{S. lividans} 1326 (harboring plasmids SLP2 and SLP3 [10]) and transformed into a plasmid-free host, \textit{S. lividans} ZX7. An ~30-kb plasmid DNA band from one transformant was detected, and restriction endonuclease analysis indicated that it contained an ~23-kb portion of SLP2 (15). The SLP2 portion of plasmid pQC542 DNA was sequenced via a "shotgun" strategy by the Chinese Human Genome Center in Shanghai. \textit{Strep-}
tomyces open reading frames (ORFs) were predicted (4, 13; see also the website http://watsen.niho.jp/~jun/cgbin/frameplot-3.0.pl).

**RT-PCR assays of the expressed RNAs in \textit{Streptomycyes}.** Total RNA of \textit{Streptomycyes} sp. strain TK20 (harboring plasmid pSP72) was prepared by the standard procedure (15). Approximately 1 \mu g of RNA was reversely transcribed into cDNA by using the RevertAid first-strand cDNA synthesis kit (MBI Fermentas). Then, 2 \mu l of product was subjected to PCR amplification. The pair of primers for reverse transcription-PCR (RT-PCR) amplification of the predicted mtp2 was 5'-CTGACTCATGTAGCTAGCTTGTACG-3' and 5'-CTGAC TGAAATTGCTACTCATTCTGG-3'. PCR conditions were 94°C for 3 min and then 94°C for 35 s, 63°C for 50 s, and 72°C for 1 min for 30 cycles.

**Construction of plasmids containing various pQC542 fragments for linear DNA replication.** Plasmids pQC654, pQC656, and pQC658 were obtained by deletion of the 7.5-kb MosI, 7.5-kb MnlI, and 4.9-kb Sau3AI fragments of pQC542, respectively. Plasmids pQC656 and pQC653 were obtained by ligation of the 15.9-kb Ascl and 7.7-kb MnlI fragments with pQC177. Deletions of the 7.5-kb MnlI fragment of pQC656 and the 0.36-kb Nhel fragment of pQC653 were performed to obtain pQC702 and pQC736, respectively. Further deletions of the 1.6-kb StuI/HindIII fragments of pQC653 and pQC730 were made to obtain pQC549 and pQC742, respectively. Plasmids pQC546 and pQC742 were obtained by ligation of the 3.7-kb Sau3AI fragment and the 3.0-kb PCR-amplified fragment (containing ORFs pQC542.19 and pQC542.20) of pQC542 with pQC717 treated with BclI, respectively. Plasmids pQC709, pQC734, and pQC743 were obtained by ligation of the 1.1-kb Nhel fragment of pQC542 with pQC546 treated with XbaI, with pQCX23 treated with XbaI, and deletion of a 0.36-kb Nhel fragment of pQC709, respectively. These plasmid DNAs were isolated from \textit{E. coli}, linearized by DraI, and introduced by transformation into \textit{Streptomycyes} sp. strain ZX7.

**Yeast two-hybrid assays for detecting interactions between SLP2 proteins and chromosomal telomeric proteins.** The four SLP2-borne replication genes (\textit{ilrASLP2}, \textit{tapotSLP2}, \textit{tpgSLP2}, and \textit{repSLP2}) and two chromosomal telomeric genes (\textit{tapL} and \textit{tpgL}) were PCR amplified individually and cloned into both the DNA-binding domain of "bait" vector pGBK7 and into the GAL4 activation domain of "target" vector pADT7. The pairs of bait and target plasmids were coexpressed by transformation into the recipient yeast strain AH109. Yeast cells were streaked on SD medium lacking leucine and tryptophan (SD-Leu/Trp) and SD medium lacking leucine, tryptophan, and histidine but containing 3 mM 3-amino-1,2,4-triazole (SD-Leu/Trp/His/+3AT). Colonies grown on SD-Leu/-Trp/-His/+3AT were inoculated into the corresponding liquid broth to deter-

**RESULTS**

**Identification of SLP2 loci for basic and efficient replication in circular mode.** The complete nucleotide sequence of SLP2 reveals a locus containing the “three pairs of long repeats” (iterons\textit{SLP2}) from bp 7896 to 8080 and an adjacent gene, SLP2.8 (designated \textit{repSLP2}), encoding DNA helicase (11). To investigate the capacity for replication of this locus in \textit{Streptomycyes}, we employed pQC516 (21), a derivative of \textit{E. coli} plasmid pSP72 bearing the \textit{Streptomycyes} \textit{melC} and \textit{Itrg} markers, to clone various lengths of SLP2 fragments containing the locus. The resulting plasmid DNAs were introduced by transformation into \textit{Streptomycyes} \textit{lividans} ZX7 (using 0.1 ng plasmid pIJ702 DNA as a control each time). As shown in Fig. 1, pZUR245 containing the iterons\textit{SLP2} and rep\textit{SLP2} was able to propagate in ZX7, although the transformation frequency was very low. When plasmids (e.g., pPXQ20, pPXQ57, and pPXQ55) contained the 47-bp sequence between bp 581 and 628 upstream of the iterons\textit{SLP2}, the transformation frequency increased ~1,000 times compared to that of pPXQ8, indicating that this sequence affected the efficiency of replication. Like plasmid pSL2A2 (21), plasmid (e.g., pPXQ20) containing the basic locus of replication propagated in strain ZX7 at a low copy number (data not shown) and was inherited unstably (~1%).

**Combinations of the \textit{rep} genes and iterons of the origins of \textit{Streptomycyes} linear plasmids SLP2, pSL2A, and SCP1 still propagate.** As seen in Fig. 2, reversing the native orientation of the iterons\textit{SLP2} and rep\textit{SLP2} (e.g., pPXQ153) did not affect transformation, indicating that the two components could be separated. To investigate whether combinations of these similar components from \textit{Streptomycyes} linear plasmids pSL2A, SCP1, and SLP2 were functional, the rep\textit{I} (containing ~0.3-kb iterons) and rep\textit{II} genes of pSL2A, the iterons (plus the 491-bp upstream region) and rep of SCP1, and the iterons (plus the 628-bp upstream region) and rep of SLP2 were amplified individually by PCR and then paired and cloned into pQC516. The resulting plasmids were introduced by transformation into strain ZX7, as shown in Fig. 2; they were able to propagate in ZX7, although the transformation frequencies were ~1,000 times lower than that of pPXQ20 (containing the native SLP2 origin).
Construction and characterization of SLP2-derived linear plasmid pQC542. The *Streptomyces* large linear plasmid SLP2 is only detected by using pulsed-field gel electrophoresis (7). To investigate its essential function of replication in linear mode, we employed plasmid pQC177 (Fig. 3A), which contained SLP2 telomeres and the *melC/tsr* markers, to clone a 23-kb SLP2 in *Streptomyces* to yield plasmid pQC542 (Fig. 3A) (see Materials and Methods). DraI-linearized (removing an 0.7-kb *E. coli* fragment) pQC542 DNA was introduced into strain ZX7, and a 27-kb band from ZX7 transformants was detected on the gel. It was sensitive to treatment with *E. coli* exonuclease III but resistant to *H. pylori* exonuclease (Fig. 3B), suggesting that pQC542 can propagate in linear mode in strain ZX7.

The SLP2 portion of pQC542 was sequenced independently and consisted of 22,816 bp—highly resembling the corresponding sequence from nucleotides 2573 to 25674 in the 50,410-bp complete nucleotide sequence of plasmid SLP2 published by Huang et al. (11), except there were five single-nucleotide polymorphisms and SLP2 had an additional 287-bp sequence (from nucleotides 3680 to 3967) which was missing in pQC542. An unpredicted mini-ORF from nucleotides 8794 to 9057 in SLP2 (Fig. 3F) encoded a small protein of 88 amino acids, resembling (48% identity; expectation value of \(10^{-11}\)) the N-terminal fragment of the *S. lividans* chromosomal telomere-associated protein (TapL, 739 amino acids) (2). This ORF, verified to be transcribed in vivo by RT-PCR examination (Fig. 3C) (see Materials and Methods), was designated *mtapSLP2* (mini-tap).

In addition to the origin, the SLP2-borne genes *mtapSLP2* and either *tpgSLP2* or *ilrASLP2* are required for plasmid replication in linear mode. To define the loci for linear plasmid replication, various lengths of the pQC542 fragment were cloned into pQC177, and the resulting plasmids (see Materials and Methods) were DraI linearized and introduced into ZX7. As shown in Fig. 3D, the linearized plasmids pQC709, containing the additional loci *mtapSLP2* and SLP2.13 (designated *ilrASLP2*, for involved in linear SLP2 replication), and pQC699, containing the other additional loci (*mtapSLP2* and *tpgSLP2*), were able to propagate in ZX7. The linear conformations of pQC709 (Fig. 3E) and pQC699 (data not shown) in ZX7 were verified. These results indicated that two alternative sets of loci, *mtapSLP2*/*ilrASLP2* and *mtapSLP2*/*tpgSLP2*, were required for SLP2 replication in linear mode.

*IlrASLP2* binds specifically to the iterons in *vitro*. Like the organization of *rlrA–rorA* of plasmid pSLA2, the *rorA* region, adjacent and divergently transcribed gene was *ilrASLP2*. It was suggested that the action of the RlrA protein of plasmid pSLA2 might be at the plasmid iterons’ structure (21). To investigate if there was an interaction between the *IlrA* protein...
### Transformation frequency of Dral linearized plasmids in *Streptomyces* (transformants/µg DNA)

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<thead>
<tr>
<th>Plasmids</th>
<th>DNA fragments cloned into pQC177</th>
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<tr>
<td>pQC542 ORFs</td>
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<tr>
<td>pQC542</td>
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<tr>
<td>pQC654</td>
<td></td>
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<td>pQC734</td>
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<tr>
<td>pXQ25</td>
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**Legend:**
- **Ms**: MspI
- **As**: AsclI
- **Mi**: MluI
- **Nh**: NheI
- **St**: StuI
- **Hi**: HindIII

**Plasmids:**
- pQC542 (30 kb)
- pQC542 (TE)
- pQC542 (exo)
- pQC542 (Kod)
- 1 kb ladder

**DNA fragments:**
- Chr 27 kb L
- 27.5 kb
- 23.1 kb
- 9.4 kb
- 6.6 kb
- 500 bp
- 250 bp
- 276 bp

**PCR:**
- DNA marker
- TK20 RNAs
- TK20 DNA
- TK20 cDNA

**Transformation frequency:**
- 1X10³
- 2X10³
- 1X10³
- 1X10³
- 0
- 5X10³
- 0
- 1X10³
- 0
- 0
- 2X10¹
- 0
- 0
- 0
of SLP2 and the iterons\textsuperscript{SLP2} sequence, electrophoretic mobility shift assays for DNA-protein complex formation were employed. Purified Ilr\textsuperscript{A}\textsubscript{SLP2} protein was incubated with \[^{32}\text{P}]/\text{H}_9251\text{dCTP}-labeled 249-bp DNA of the core sequence of iterons SLP2 and then electrophoresed and autoradiographed. As shown in Fig. 4, the Ilr\textsuperscript{A}\textsubscript{SLP2} protein could bind to the DNA probe to form a DNA-protein complex. Formation of this complex was competed by adding 10\textsuperscript{3} or 25\textsuperscript{3} unlabeled probe DNA but was unaffected by the addition of 100\textsuperscript{3} nonspecific DNA, suggesting that the binding reaction of the Ilr\textsuperscript{A}\textsubscript{SLP2} protein and iterons\textsuperscript{SLP2} DNA was specific.

**DISCUSSION**

Previous experiments showed that the centrally located loci for replication of *Streptomyces* linear plasmids pSLA2, SCP1, and pSLA2-L in circular mode consisted of the different iterons and the *rep* genes (6, 8, 22). Huang et al. (11) proposed that the chromosomal TpgL and TapL may be involved in replication of plasmid SLP2 telomeres.
the replication loci of SLP2 containing tpgSLP2, repSLP2 (DNA helicase), and the complex repeat sequences (iterons SLP2) are at kb 7.8 to 8.1 of SLP2. Here we show that the basic SLP2 locus for circular plasmid replication consisted of iterons SLP2 and the adjacent repSLP2 gene. We found that plasmids containing combinations of the iterons and rep genes of SLP2, pSLA2, and SCP1 still propagate in Streptomyces, although the transformation frequencies were 3 orders of magnitude lower were initiated, for support and suggestions, David Hopwood for Streptomyces lividans strains, and Carton Chen for the plasmid pLUS450 containing a chromosomal telomere. We are also grateful to David Hopwood, Stanley Cohen, and Christine Miller for critical reading and useful suggestions on the manuscript.

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REFERENCES


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<th>Protein combination</th>
<th>Growth on*: SD/-Leu/Trypt SD/-Leu/Trypt/His/3AT</th>
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<td>pGBKT7-53 + pGADT7-RecT</td>
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<tr>
<td>TpgL + TapL</td>
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<tr>
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<tr>
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<tr>
<td>TpgL + RepSLP2</td>
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<tr>
<td>TpgL + TmpSLP2</td>
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</tr>
<tr>
<td>TpgL + TmpSLP2/repSLP2</td>
<td>+</td>
<td>-</td>
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* Abbreviations: SD medium, synthetic complete medium; SD/-Leu/Trypt, SD lacking leucine and tryptophan; SD/-Leu/Trypt/His/3AT, SD lacking leucine, tryptophan, and histidine and with 3 mM 3-AT added.

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We thank Stanley N. Cohen, in whose laboratory these experiments were initiated, for support and suggestions, David Hopwood for Streptomyces lividans strains, and Carton Chen for the plasmid pLUS450 containing a chromosomal telomere. We are also grateful to David Hopwood, Stanley Cohen, and Christine Miller for critical reading and useful suggestions on the manuscript.