Linear *Streptomyces* plasmids form superhelical circles through interactions between their terminal proteins

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ABSTRACT

Linear chromosomes and linear plasmids of *Streptomyces* possess covalently bound terminal proteins (TPs) at the 5′ ends of their telomeres. These TPs are proposed to act as primers for DNA synthesis that patches the single-stranded gaps at the 3′ ends during replication. Most (‘archetypal’) *Streptomyces* TPs (designated Tpg) are highly conserved in size and sequence. In addition, there are a number of atypical TPs with heterologous sequences and sizes, one of which is Tpc that caps SCP1 plasmid of *Streptomyces coelicolor*. Interactions between the TPs on the linear *Streptomyces* replicons have been suggested by electrophoretic behaviors of TP-capped DNA and circular genetic maps of *Streptomyces* chromosomes. Using chemical cross-linking, we demonstrated intramolecular and intermolecular interactions between the TPs in vivo between Tpgs, between Tpcs and between Tpg and Tpc. Interactions between the chromosomal and plasmid telomeres were also detected in vivo. The intramolecular telomere interactions produced negative superhelicity in the linear DNA, which was relaxed by topoisomerase I. Such intramolecular association between the TPs poses a post-replicational complication in the formation of a pseudo-dimeric structure that requires resolution by exchanging TPs or DNA.

INTRODUCTION

Linear chromosomes (1) and linear plasmids (2) of *Streptomyces* are capped at the 5′ ends of their telomeres by covalently bound terminal proteins (TPs). These linear replicons are replicated bidirectionally from an internal origin, which leaves single-stranded gaps of 250–300 nt at the 3′ ends (3, C.-H. Huang, unpublished data). These gaps are proposed to be patched by TP-primed DNA synthesis (reviewed in 4).

Most *Streptomyces* telomeres are highly conserved in their terminal DNA sequences as well as the capping TPs. They are referred to as ‘archetypal telomeres’ and ‘archetypal TPs’, respectively (5,6). A number of *Streptomyces* telomeres, however, are dissimilar to the archetypal telomeres and to one another. These atypical telomeres are capped by TPs that share no homology with the archetypal TPs (5,6).

The archetypal TPs are of similar sizes (about 185 amino acid), and contain a DNA-binding domain of HIV reverse transcriptase type and an amphiphilic domain that may be involved in protein–protein interactions or membrane binding (7,8). They are encoded by the *tpg* gene, which forms an operon with an upstream gene *tap* (7,8). The product of *tap* is also essential for end patching by binding to the secondary structures formed by the 3′ overhangs, and supposedly recruiting Tpg to the site of action (9).

Atypical telomeres are present mostly on linear plasmids, such as SCP1 of *Streptomyces coelicolor* (10), pRL1 of *Streptomyces* strain 44030 and pRL2 of *Streptomyces* strain 44424 (11). They are also found in the chromosomes of *Streptomyces griseus* IFO 13350 (12) and *S. griseus* 2247 (13). The telomeric sequence of SCP1 begins with a string of four-six G’s instead of the three C’s in the archetypal telomeres (10,14). This atypical telomere is capped by a 259 amino acid atypical TP encoded by the *tpc* gene on SCP1 (5). Tpc differs totally from Tpgs in sequences and size.

The availability of matching TPs is absolutely necessary for the replication of linear *Streptomyces* replicons. All *Streptomyces* chromosomes have been shown to encode their own TP (mostly Tpg), and deletion of *tpg* resulted in circularization of the chromosomes (8). Many linear
**Materials and Methods**

**Bacterial strains and plasmids**

Bacterial strains and plasmids used in this study are listed in Table 1. *Streptomyces* cultures were grown in liquid YEME medium at 30°C. Linear plasmid pLUS891L (5) contained an autonomously replicating sequence (ARS) of pSLA2 (26) and a thiostrepton-resistance gene (*tsr*) flanked by a pair of 320-bp telomere sequence of SCP1 (Figure 1A). pLUS892L (5) contained, in addition, the 3.2-kb sequence of SCP1 spanning the *tac* and *tpc* genes (Figure 1B). *Streptomyces lividans* TK64 and 3200 were used for propagation of linear plasmids.

**General bacteriological and molecular biological procedures**

Genetic manipulations of *Streptomyces* were performed according to the methods of Kieser et al. (2000). The buffer of agarose gel electrophoresis contained 0.1% sodium dodecyl sulphate (SDS), without which TP–DNA complex cannot enter the gel (27).

**In vivo cross-linking**

*Streptomyces* cultures containing linear plasmids were grown in YEME medium to exponential phase, harvested by centrifugation, washed with water twice and suspended in phosphate-buffered saline (PBS). Disuccinimidyl suberate (DSS; Pierce) or disuccinimidyl glutarate (DSG; Pierce) was added to a final concentration of 25 mM, and the mixture was incubated at 4°C for 2 h and quenched with 10 mM Tris–HCl at room temperature for 10 min. Genomic DNA was isolated according to Kieser et al. (2000) without pronase E treatment for analysis of TP–TP cross-linking. To remove TP, proteinase K (AMRESCO) was added to the loading buffer containing the DNA sample to a final concentration of 20 U/ml before electrophoresis.

**Table 1. Bacterial strains and plasmids used in this study**

| Strains/plasmids | Description | References |
|------------------|-------------|------------|
| *S. coelicolor* M146 | hisA1 uraA1 strA1 SCP1+ SCP2− | (37) |
| *S. lividans* TK64 | pro-2 str-6 SLP2− SLP3− | (37) |
| *S. lividans* 3200 | pro-2 str-6 SLP2− SLP3− SCP1+ | (37) |
| pLUS971 | Linear plasmid containing the ARS of pSLA2, and a pair of *S. lividans* telomeres | (35) |
| pLUS890L | Linear plasmid containing the ARS of pSLA2, an end of SCP1 telomere and an end of *S. lividans* telomere | (5) |
| pLUS891L | Linear plasmid containing the ARS of pSLA2, and a pair of SCP1 telomeres | (5) |
| pLUS892L | Linear plasmid containing the *tac-tpc* operon, ARS of pSLA2, and a pair of SCP1 telomeres | (5) |
In vitro cross-linking

Streptomyces cultures containing linear plasmids were grown in YEME medium to exponential phase. DNA isolated according to Kieser et al. (2000) without pronase E treatment was dissolved in PBS. Subsequent treatment with DSS or DSG was identical to that for in vivo cross-linking.

Chromosome conformation capture (3C) procedure (28)

After in vivo cross-linking, the mycelium was harvested by centrifugation, treated with lysozyme (1 mg/ml) at 37°C for 30 min and osmotically lysed by dilution into 10 volumes of TE buffer. Five microliters of the lysate was digested with MluI and ligated with T4 DNA ligase (TaKaRa Bio Inc.) in a final volume of 20 µl. Another 5 µl of the lysate was digested with MluI and ligated in a final volume of 1 ml. PCR was performed on the ligated DNA products using primer sets listed in Table 2. The ligated DNA (200 ng) and the primers (50 pmol) were added to PCR tubes containing 0.2 mM dNTPs, 2.5 U of Expand High Fidelity DNA polymerase (Roche Applied Science) in 50 µl of 1 x PCR reaction buffer. The program used for the PCR consisted of 2 min of initial denaturation at 95°C, followed by 20 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 1 min/kb. The final extension step was done at 72°C for 7 min.

GnHCl-CsCl density gradient centrifugation

Protoplasts of Streptomyces were lysed by osmotic shock, and the debris was removed by centrifugation. The supernatant was subjected to three rounds of density gradient centrifugation in 10 mM Tris–HCl, 1 mM ethylenediaminetetraacetic acid (EDTA) (pH 8.0) buffer containing 0.24 mg/ml ethidium bromide and (i) first run: CsCl (mean density 1.5); (ii) second run: CsCl (mean density 1.3) and 4 M GnHCl; and (iii) third run: CsCl (mean density 1.2) and 6 M GnHCl. The final DNA fraction collected was extracted with 1-butanol and precipitated with isopropanol.

Atomic force microscopy

Atomic force microscopy (AFM) generally followed the procedure described by Wang et al. (29). Plasmid DNA was isolated from S. lividans 3200/plus891L mycelium...
and fractionated by gel electrophoresis. For imaging, the DNA sample was diluted in HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer (20 mM HEPES, pH 8.0, 50 mM NaCl, 5 mM MgCl2) and deposited onto freshly cleaved mica at room temperature. After 1 min incubation, the mica surface was rinsed with HPLC-grade water, blotted dry and then dried under a stream of nitrogen. To remove TP, proteinase K was added to the HEPES buffer containing the DNA sample to a final concentration of 20 U/ml before application to the mica surface. The images were captured in air using a NanoScope IIIa Atomic Force Microscope (Digital Instruments) in tapping mode at a scan rate of 3 Hz with pixel resolution of 512 × 512. Silicon probes (Molecular Imaging) with spring constants of 50 Nm⁻¹ and resonance frequencies of 170 kHz were used in all imaging.

RESULTS

The telomeres of the SCP1 plasmid can be cross-linked in vivo

Possible TP–TP interaction was first tested on two high copy linear plasmids that possessed SCP1 telomeres, 8.8-kb pLUS891L and 12.1-kb pLUS892L (5). These plasmids possessed the autonomous replication sequence of pSLA2 plasmid of Streptomyces rochei, and exist in high copy numbers (about 130–180 per chromosome) in Streptomyces (30). pLUS892L differs from pLUS891L in containing two additional genes, tac and tpc (encoding the terminal protein of SCP1) of SCP1, which allow it to replicate independently of SCP1 (5) and to be maintained more stably. On the other hand, replication of pLUS891L requires the presence of SCP1 in the host, and the plasmid was less stably maintained.

Both pLUS891L and pLUS892L were propagated in S. lividans 3200, which harbored SCP1. Mycelium harvested from liquid cultures was treated with DSS or DSG, two membrane permeable compounds commonly used for cross-linking interacting proteins (but not DNA and protein or DNA and DNA) in vivo (31,32). Total DNA was isolated from the mycelium without proteolytic treatment to preserve TP, and digested with BclI or BglII. The restriction fragments were separated electrophoretically and hybridized to labeled pLUS891L and pLUS892L probes, respectively. If the TPs at the telomeres were cross-linked, the terminal restriction fragments would be linked together, whereas the internal fragments would not. The cross-linking result for pLUS891L DNA (Figure 1A, left) shows that the terminal 2.9- and 1.4-kb BclI fragments and the 4.5-kb BglII fragment (0.3-kb BglII fragment ran off the gel) disappeared in the DSS- (lanes 5 and 6) and DSG-treated (lanes 8 and 9) mycelium, respectively. Instead, hybridizing DNA of larger and heterogeneous sizes appeared (marked by the asterisks). On treatment of the sample with proteinase K, some proteins in vivo. The interactions were unlikely to be exclusively between two TPs, which would give rise to complexes of discrete molecular weights. The heterogeneous size of the cross-linked product suggested a more complex pattern of interactions, perhaps involving one or more other proteins that were not covalently bound to DNA (See below).

The telomeres of the SCP1 plasmid can be cross-linked in vitro

Cross-linking of TPs was also investigated in vitro. Total DNA of S. lividans 3200/pLUS891L was isolated without proteolytic treatment and subjected to the same cross-linking, restriction and Southern hybridization analyses. The results (Figure 2A) were essentially identical to those of in vivo cross-linking (Figure 1A); i.e. the terminal fragments were cross-linked (lanes 4–9). Likewise, treatment with proteinase K eliminated the cross-linking (data not shown). These results indicated that the telomere interaction was relatively stable, withstanding the isolation procedure.

To eliminate possible involvement of non-covalently bound proteins, a lysate of S. lividans 3200/pLUS892L was first purified in a GnHCl–CsCl gradient (5) to remove non-covalently bound proteins. The isolated DNA fraction was then subjected to cross-linking, digested with BclI or MluI, fractionated by gel electrophoresis and hybridized to the SCP1 terminal DNA probe.

The results (Figure 2B) showed that the terminal 2.4- and 1.4-kb BclI fragments were cross-linked to produce a 3.8-kb fragment (lanes 2 and 3), and the terminal 4.7- and 1.8-kb MluI fragments were cross-linked to produce a 6.5-kb fragment (lanes 5 and 6). These cross-linked products were expected from intramolecular interactions between the TPs on opposite telomeres on the same plasmid. No products corresponding to cross-linking between two left terminal fragments (4.8 kb in the BclI digest and 9.4 kb in the MluI digest) or between two right terminal fragments (2.8 kb in the BclI digest and 3.6 kb in the MluI digest) were detected, indicating the absence of intermolecular cross-linking in this in vitro procedure.

These results were consistent with the notion that at least some of the heterogeneous cross-linked products generated from plasmid DNA that had not been subjected to GnHCl–CsCl gradient centrifugation purification (Figure 1) involved non-covalently bound proteins. They also indicated that direct interactions between TPs without the involvement of the non-covalently bound proteins were possible at least in vitro.
Telomere proteins interact intermolecurally

To further confirm telomere–telomere interactions in vivo, the chromosome conformation capture (3C) procedure (28) was adopted for analysis. In vivo cross-linked pLUS892L DNA was first digested with MluI, which would separate the internal 5.8-kb fragment from the cross-linked terminal fragments. The digested DNA was then ligated at relatively low concentrations to minimize intermolecular ligation, followed by PCR against the site of ligation using a set of ‘left’ (L) and ‘right’ (R) primers to detect the joined terminal fragments (Figure 3A). There were three possible types of cross-linking: L+R, L+L and R+R. The L+R type could be the products of either intramolecular or intermolecular interaction between a left and a right telomere, and might be detected by PCR using a mixture of L and R primers. On the other hand, the L+L and R+R types could be produced only by intermolecular cross-linking between two left and two right telomeres, respectively. These products might be detected by the L primer and R primer singly, respectively.

Initially, only the mixture of L and R primers produced a PCR product (of the expected size of 1.6 kb) under the ‘standard’ conditions (Figure 3A, lane 4), and no PCR product was found using the L or R primers singly. It was reasoned that ligation of two identical terminal fragments would create long inverted repeats with the center of symmetry at the ligation site, which on denaturation would form extensive secondary structures, thus hindering the polymerization reaction during PCR. To overcome such an obstacle, 10% DMSO (dimethyl sulfoxide) was added to the PCR reaction (33,34). Under these conditions, the L primer generated a 2.4-kb product (lane 12), and the R primer a 0.8-kb product (lane 8), which were expected from intermolecular cross-linking. Under this condition, the mixture of L and R primer also generated these two products, but not the 1.6-kb product (lane 10). A 50-fold dilution of the ligation reaction produced about the same quantities of the PCR products, confirming that the ligation products were produced intramolecularly from two cross-linked terminal fragments. The identities of all these PCR products were confirmed by sequencing (data not shown).

These results indicated that the telomeres of Streptomyces linear plasmids are closely associated both intramolecularly and intermolecularly in vivo. It further
implies that the telomeres of linear plasmids and linear chromosomes are clustered in vivo.

Plasmid and chromosomal telomeres interact

The same 3C procedure was extended to the linear plasmid pLUS971 (35), which contained the telomeres of the S. lividans chromosome capped by Tpg, encoded by the S. lividans chromosome. For this plasmid, the expected 0.8-kb (lanes 8 and 10) and 1.2-kb (lanes 10 and 12) products were detected in both 1x and 50x dilutions (Figure 4A). The results indicated that Tpgs capping a linear replicon also interact in vivo.

The next question is whether an archetypal and an atypical telomere on the same linear plasmid may interact in vivo. To answer this question, we used linear plasmid pLUS890L with a Tpc-capped SCP1 telomere and a Tpg-capped S. lividans chromosomal telomere (5). The 3C procedure (Figure 4B) revealed interactions in vivo between the heterologous telomeres (L+R) as well as between homologous telomeres (L+L and R+R). This result was intriguing, because it implies that, despite their distinct telomere structures, the telomeres of SCP1 plasmid may interact with those of the host chromosome in vivo.

To test this possibility, cross-linking was performed on S. coelicolor M146/SCP1, and the 3C procedure was used to detect cross-linking between the telomeres. The results (Figure 5) revealed all three types of telomere interactions: chromosome–chromosome (0.6-kb; lanes 8 and 10), plasmid–plasmid (0.8-kb; lanes 10 and 12) and chromosome–plasmid (0.7-kb; lane 4). The identities of all these PCR products were confirmed by sequencing (data not shown). These results indicated that the telomeres of the S. coelicolor chromosomes and SCP1 plasmids are clustered in the mycelium.

Linear plasmids form superhelical circles in vivo

The observed telomere interactions suggested that we might be able to isolate linear plasmid DNA in a circular configuration formed by the intramolecular telomere–telomere interactions. To preserve such circular configuration during isolation, the mycelium of S. lividans 3200/pLUS892L was treated with lysozyme, and lysed by osmotic shock and osmotic shock. The clear lysate was loaded directly onto an agarose gel for electrophoretic separation. Hybridization using the plasmid DNA probe showed a substantial portion of hybridizing DNA migrating ahead of the linear plasmid DNA (Figure 6A, lane 1). This fast-migrating plasmid DNA disappeared after proteinase K digestion (lane 2). These results suggested that the fast-migrating DNA (Figure 6A) consists of superhelical circles formed by telomere–telomere interactions. This supposition was tested by treating the DNA with Escherichia coli topoisomerase I, which reduced the mobility of the fast-migrating DNA to a position slightly behind the linear plasmid DNA (lane 3), as expected for the relaxed circular form. Treatment of the putative relaxed circular form with proteinase K restored its mobility to that of the
DISCUSSION

Here, we have demonstrated in vivo telomere–telomere associations, which cause the linear plasmids to assume a circular configuration in Streptomyces mycelia (Figures 1 and 6A). The circular form of the linear plasmids is negatively supercoiled, and may be relaxed by topoisomerase I in vitro. In this regard, it is noteworthy that Bao and Cohen (16), using Tap as a scaffold, have identified topoisomerase I as a component of the Streptomyces telomere complex.

Telomere–telomere interactions have also been demonstrated for the linear chromosomes of Streptomyces. Our results could not tell whether the interactions were intermolecular or intramolecular due to the multinucleoid nature of the substrate mycelia. Intermolecular interactions would result in end-to-end joining of two adjacent chromosomes, whereas intramolecular interactions would result in a circular configuration as the linear plasmid. We hypothesize that the latter is the norm in vivo.

The observed telomere–telomere interactions in vivo appear to involve one or more non-covalently bound proteins, the identities of which are not clear. One possible candidate is Tap, which has been shown to interact with Tpg in vitro (9). Tap binds specifically to the secondary structure formed by the 3' overhang, and was proposed to recruit Tpg to the terminal location to complete the end patching process. In vitro, Tap also binds DNA polymerase I and topoisomerase I (16). Whether these proteins may be involved in the observed cross-linking in vivo remains to be seen.

The telomere–telomere interactions may be visualized by AFM imaging analysis (Figure 6B and more images and quantitative analysis in Supplementary Figure S1). In the AFM images, most of the DNA molecules in the cross-linked sample (left and middle) were in a compact and often supercoiled state, and some appear to be linear form (lane 5). These results indicate that the linear plasmids were negatively supercoiled in vivo.
involved in intermolecular interactions. Proteolytic
digestion of the DSS-cross-linked DNA by proteinase K
transformed all the compact DNA molecules into single,
relaxed, linear structures (Figure 6B right).

The fact that TP–TP interactions were demonstrated in
linear plasmids capped by Tpc (Figures 2 and 3), Tpg
(Figure 4A) and both (Figures 4B and 5) suggests that
such telomere–telomere interactions are universal among
the linear plasmids and chromosomes of Streptomyces.
The intermolecular interactions, particularly those
between a chromosomal TP (Tpg) and a plasmid TP
(Tpc), indicate that the telomeres of linear plasmids
interact with those of the chromosome in vivo.

Such clustering of the telomeres of the linear plasmids
and chromosomes may facilitate coordination of replica-
tion and post-replication segregation of these replicons.
For instance, C.-C. Hsu and C. W. Chen (unpublished
data) recently discovered that the partitioning defect
(ΔparAB) of the linear plasmid SLP2 was partially
compensated by the partitioning system of the host
chromosome. While it is possible that the compensation
was be due to cross-talk between the chromosomal and
SLP2 partitioning systems (despite the difference in their
parS sequences), another possible explanation is for SLP2
as-dimer to hitchhike’ on the chromosome through telomere
interactions during post-replication partitioning.

The observed superhelicity of the circular configuration
of the linear plasmid indicates that the phosphodiester
linkage between the TP and the 5′-end of the DNA
cannot freely rotate. This further implies the existence of
one or more non-covalent interactions between the TP or
one or more non-covalently bound proteins and the
telomere DNA that prevent free rotation.

Such superhelical circular configuration by linear
replicons has been previously observed in adenoviruses
(21,22), ϕ29 phage (23) and GA-1 phage (24). In the
case of adenoviruses, the superhelicity was suggested to
facilitate unwinding of DNA during transcription (25).
Similarly, the negative superhelicity of the linear replicons
in Streptomyces may also be important for transcription,
or alternatively replication, packaging or other DNA
transaction activities.

The telomere–telomere association poses at least two
potential post-replication complications. Firstly, at the
completion of replication, the newly synthesized DNA
strands are capped by new TPs. If the ‘old’ TPs on the
parental strains of the linear plasmid or chromosome
remain associated with each other through replication,
and the new TPs capping the daughter DNA become
associated with each other, a pseudo-dimeric structure
would result that resemble the long strip produced when
a classical Möbius strip is cut along the center line
(Figure 7A–D) (6). We designated this structure, a
‘pseudo-dimer’.

A second pathway that would produce pseudo-dimers
in Streptomyces is homologous recombination between
two linear replicons with associated telomeres, just as
circular dimers are generated by recombination between
two circular replicons.

In either case, the pseudo-dimer needs to be resolved for
proper segregation and partitioning. There are two
possible solutions. Firstly, the pseudo-dimers might be
resolved by exchanging the interacting TPs, i.e. the ‘old’
TPs switching to associate with the ‘new’ ones at the
opposing telomere (Figure 7E). An immediate question
on this model would be how the ‘old’ and ‘new’ TPs
assort among themselves to find the correct partners.
Alternatively, the Möbius strip-like structure may be
resolved by DNA recombination (Figure 7F). The
system employed would not be homologous recom-
bination, because the chromosomes remain linear in the
recA mutants of Streptomyces (36). It would be more
likely a site-specific recombination system such as the
XerCD-dif system for circular chromosomes in
E. coli and many other bacteria. In these bacteria, dimeric chromosomes generated by homologous recombination between sister chromosomes are resolved by resolvase XerCD at the dif site in the replication termination region. However, there is no XerCD homolog in Streptomyces species, although Streptomyces chromosomes are circularized at relatively high frequencies, and the mutants with circular chromosomes generally grow normally and exhibit no defect in chromosome partitioning during sporulation (our unpublished data). Therefore, it is possible that Streptomyces possesses a novel site-specific recombination to resolve dimers and pseudo-dimers. It is noteworthy that the TPs are segregated semi-conservatively (‘old’ with ‘new’) in the TP exchange pathway, and conservatively (‘old’ with ‘old’ and ‘new’ with ‘new’) in the DNA exchange pathway. This may be experimentally tested.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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