Published online 24 November 2010 *Nucleic Acids Research, 2011, Vol. 39, No. 6* **2165–2174**
doi:10.1093/nar/gkq1204

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

Hsiu-Hui Tsai[1], Chih-Hung Huang[2], Ingrid Tessmer[3], Dorothy A. Erie[3] and Carton W. Chen[1,*]

[1]Department of Life Sciences and Institute of Genome Sciences, National Yang-Ming University, Shih-Pai, Taipei 112, [2]Institute of Biotechnology, National Taipei University of Technology, Taipei 106, Taiwan and [3]Department of Chemistry, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA

Received June 16, 2010; Revised and Accepted November 8, 2010

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 *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from an internal origin, which leaves single-stranded gaps of 250–300 nt at the 3′ ends (3, C.-H. Huang, unpublished data).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 an 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

*To whom correspondence should be addressed. Tel: +886 2 2872 8800; Fax: +886 2 2826 4930; Email: cwchen@ym.edu.tw

Streptomyces plasmids also encode their own TPs (as in SCP1). However, some of them rely on another linear (usually larger) plasmid or the chromosome to supply the necessary TPs (8).

Interestingly, both Tpg and Tpc contain a nuclear localization signal (NLS), which have been shown to be functional in targeting into the nuclei of human cells (15) and plant cells (our unpublished data). The NLS may be destroyed without affecting the ability of the TP to support replication of the linear replicons. The significance of the NLS in the TPs can only be speculated on.

Two other telomere-associated proteins, DNA polymerase I and topoisomerase I, were identified using Tap as a scaffold (16). Intriguingly, these two enzymes display an additional reverse transcriptase activity, the significance of which is not clear. Despite the identification of PolI as a component of the telomere complex, it does not appear to be essential for end patching, because deletion mutation of *polA* may be generated without affecting the linearity of *Streptomyces* chromosomes (17).

Despite the linearity of the chromosomes, genetic maps constructed by conjugation and protoplast fusion are circular for different *Streptomyces* species (reviewed by 18). Addressing this paradox, Wang *et al*. (19) showed that recombination between linear chromosomes during conjugation is strongly biased toward even-numbered cross-overs, which would automatically give rise to circular genetic maps (20). It was proposed that the bias toward even-numbered cross-overs reflects an association of the chromosomal telomeres presumably mediated by interactions between the capping TPs. Moreover, TP–TP interactions are also suggested by immobilization of the TP–DNA complex during electrophoresis (1).

Intramolecular TP–TP interactions have previously been observed in the viral particles of adenoviruses (21,22), ϕ29 phage (23) and GA-1 phage (24). The TP–TP interactions allow these linear replicons to assume more compact superhelical structures. The superhelicity increases transcription of adenoviral genes in a promoter-specific manner (25).

In this study, *in vivo* and *in vitro* telomere–telomere interactions at the telomeres of linear *Streptomyces* plasmids and chromosomes were demonstrated by cross-linking studies. While direct TP–TP interactions could be demonstrated *in vitro*, other non-covalently bound proteins also appeared to be involved in telomere–telomere interactions *in vivo*. The telomere–telomere interactions occur both intramolecularly and intermolecularly. Intramolecular interaction causes linear plasmids (and presumably linear chromosomes) to assume a circular configuration with negative superhelicity, which presents a complication in post-replication segregation. Both telomere–telomere interactions and superhelicity are probably important for replication and/or gene expression at the terminal regions. Moreover, interactions between the chromosomal and plasmid telomeres were observed *in vivo*, indicating that the telomeres of the chromosome and plasmid are clustered in the mycelium.

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)

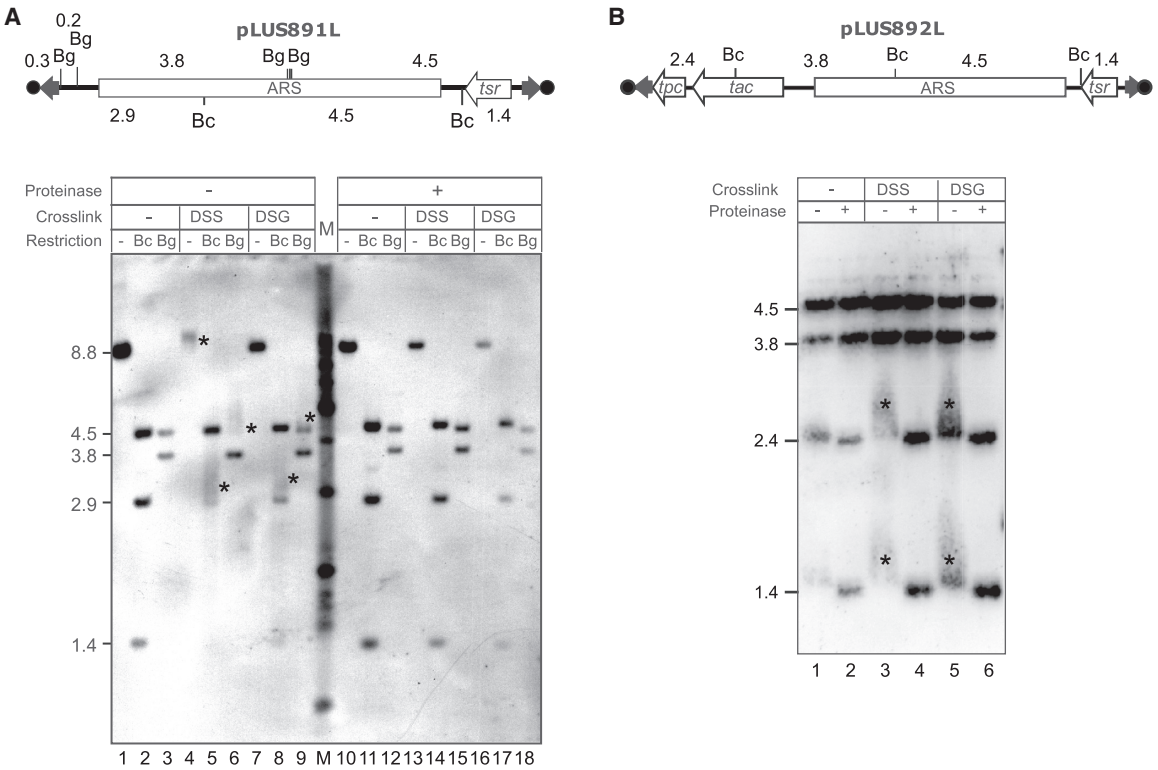

Figure 1. *In vivo* cross-linking of telomeres. Mycelium from liquid cultures was treated with DSS or DSG. Total DNA was isolated, digested with BclI (Bc) or BglII (Bg), fractionated by gel electrophoresis and hybridized to labeled plasmid DNA. (**A**) pLUS891L. The physical map of the plasmid DNA is shown above. The sizes of the restriction fragments are indicated (in kb). *tsr*, thiostrepton resistance gene; ARS, autonomously replicating sequence of pSLA2; filled arrows, SCP1 telomeres; filled circles, Tpc proteins. The DNA linked by the cross-linker is indicated by an asterisk. 'M', DNA size markers. The size of the DNA fragments is depicted on the left (in kb). A set of samples was treated with proteinase K ('+') before electrophoresis (right panel). (**B**) pLUS892L. The symbols and analyses are as in (A).

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 lyzed by dilution into 10 volumes of TE buffer. Five microliter 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 add 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 × 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.

Table 2. Oligonucleotides used in this study

L primer for pLUS892L (Figure 3)	TCGACGGCAGTTCCC
L primer for pLUS971 (Figure 4A)	AGGAGGTCCCGCATG
L primer for pLUS890L (Figure 4B)	CCAGGAGGTCCCGCA
R primer for all three (Figures 3 and 4)	GTCCGGTTCCTCGAC
a primer for *S. coelicolor* chromosomal ends (Figure 5)	TGGCAGAGGGCGGGTGCG
b primer for SCP1 ends (Figure 5)	GGAGCCGCTGACACAGCG

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 $MgCl_2$) 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 5and 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, these heterogeneous DNA species disappeared and the terminal fragments reappeared (Figure 1A, right, lanes 14, 15, 17 and 18). In comparison, the internal (4.5-kb BclI and 3.8-kb BglII) fragments were not affected by either cross-linking or proteinase K treatment.

The same results were seen in pLUS892L DNA (Figure 1B). Cross-linking with DSS and DSG also affected only the 1.4- and 2.4-kb terminal BclI fragments (lanes 3 and 5), and the effect of the cross-linking was negated by proteinase K treatment (lanes 4 and 6).

These results indicated that the TPs interacted with 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*.

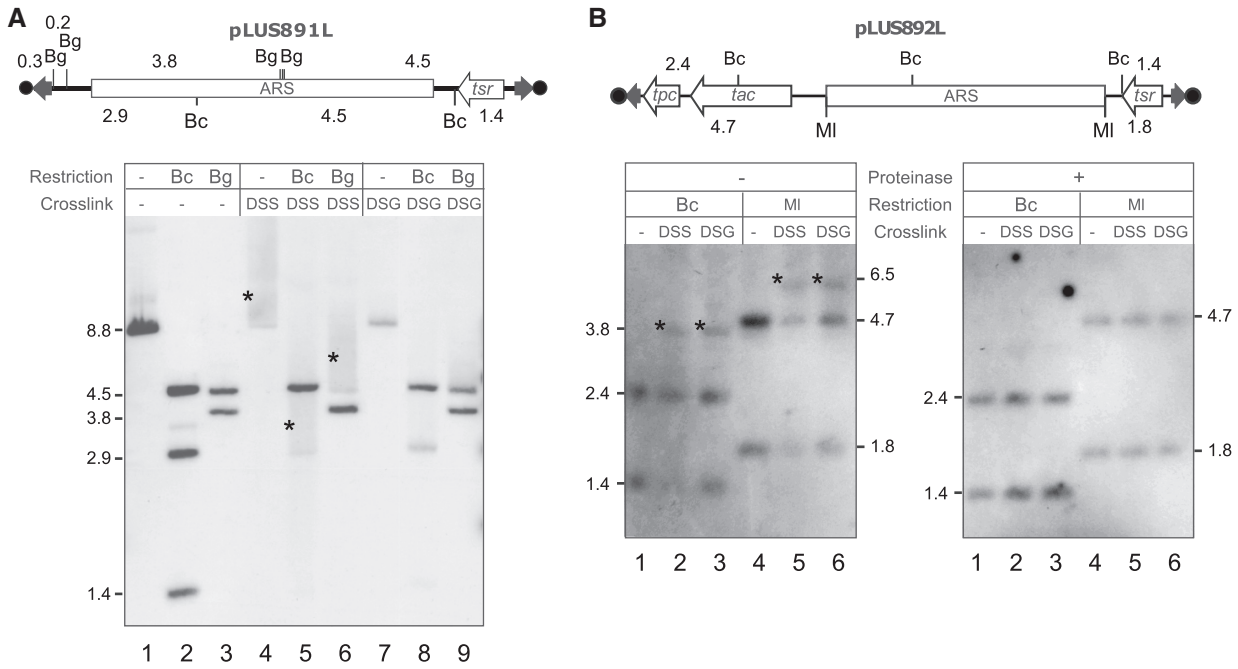

Figure 2. *In vitro* cross-linking of telomeres. (**A**) Without GnHCl treatment. Total DNA of *S. lividans* 3200/pLUS891L was treated with DSS or DSG, digested with BclI (Bc) or BglII (Bg), fractionated by gel electrophoresis and hybridized with pLUS891L DNA probe. (**B**) With GnHCl treatment. Genomic DNA of *S. lividans* 3200/pLUS892L was isolated by GnHCl–CsCl gradient centrifugation and treated with DSS or DSG. The treated DNA was digested with BclI or MluI, fractionated by gel electrophoresis and hybridized to the SCP1 terminal DNA probe. The physical map of pLUS892L is shown above with the BclI and MluI (Ml) sites and fragments sizes indicated. In the control experiment (right panel), the samples were treated with proteinase K before electrophoresis. The cross-linked DNA is indicated by an asterisk.

Telomere proteins interact intermolecularly

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 3B, lane 4), and no PCR product was found using the L or R primers singly (lanes 2 and 6). 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).

The 1.6-kb PCR product might have resulted from intermolecular as well as intramolecular cross-linking. It was reasonable to assume that at least half of it was produced from intramolecularly cross-linking. This assumption was supported in subsequent studies.

When the DNA was purified with a GnHCl–CsCl centrifugation purification step before cross-linking and 3C analysis, no PCR product was produced with the L or R primer singly, and only the combined L and R primers gave a 1.6-kb product (Figure 3C, lane 10). This indicated the absence of intermolecular TP–TP interactions, supporting the interpretation of the restriction-hybridization analysis (Figure 2; last section). By inference, the 1.6-kb PCR product in the latter reflected only intramolecular TP–TP interactions.

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

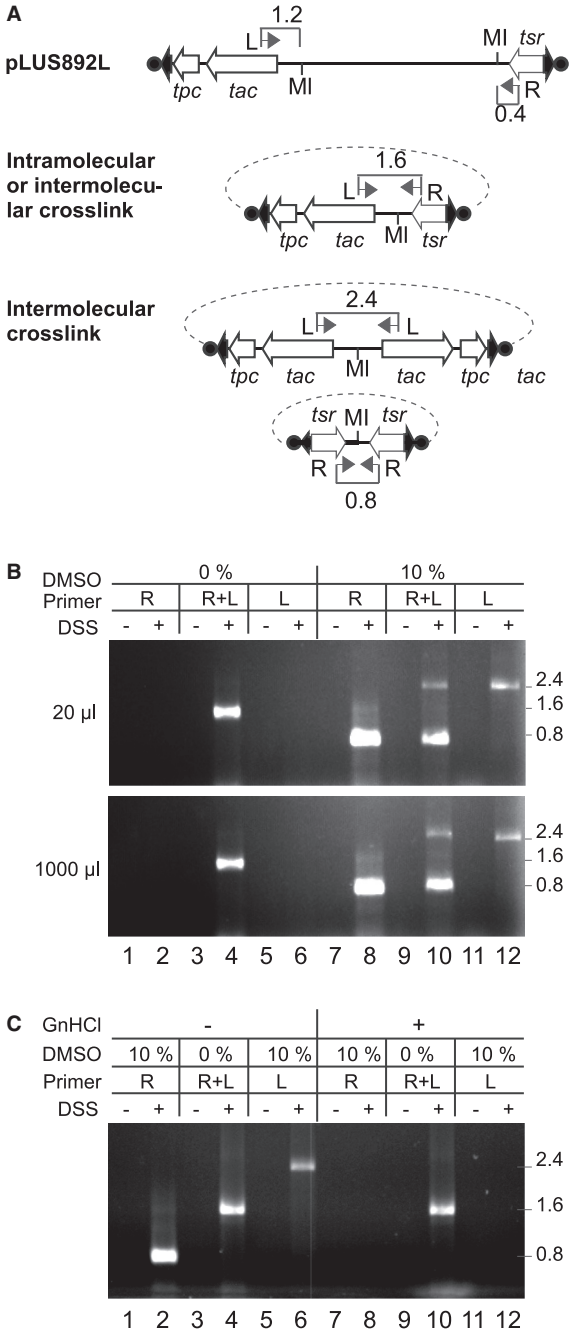

Figure 3. Specificity of telomere interactions analyzed by the 3C procedure. (**A**) Physical map of pLUS892L and the predicted cross-linked, ligated products. On pLUS892L (top), the positions of the L and R primers and their distances to the nearest MluI ('MI') sites are indicated (in kb). On the expected intramolecular (middle) and intermolecular (bottom) products, the expected PCR products and their sizes (in kb) are indicated. Cross-linking is depicted by the dashed arcs. The other symbols are as in Figure 1. (**B**) Detection of intermolecular and intramolecular cross-linking. *S. lividans* 3200/pLUS892L mycelium was treated with DSS, and lysed by protoplasting and osmotic shock. DNA in the lysate was digested with MluI, and ligated at two different concentrations (top panel, 20-µl ligation reaction; bottom panel, 1-ml ligation reaction) followed by LM-PCR using the R, L or both primers ('R + L') in the absence ('0%') or presence ('10%') of DMSO. The PCR products were analyzed by gel electrophoresis. The sizes of the PCR products are indicated (in kb). (**C**) Detection of intramolecular cross-linking after GnHCl centrifugation. Cross-linking and detection were performed as in B except for an additional GnHCl–CsCl gradient centrifugation step (right half) DNA before cross-linking. The volume of the ligation reaction was 1 ml.

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 1× and 50× 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 in the absence of any proteolytic and denaturation agent. 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

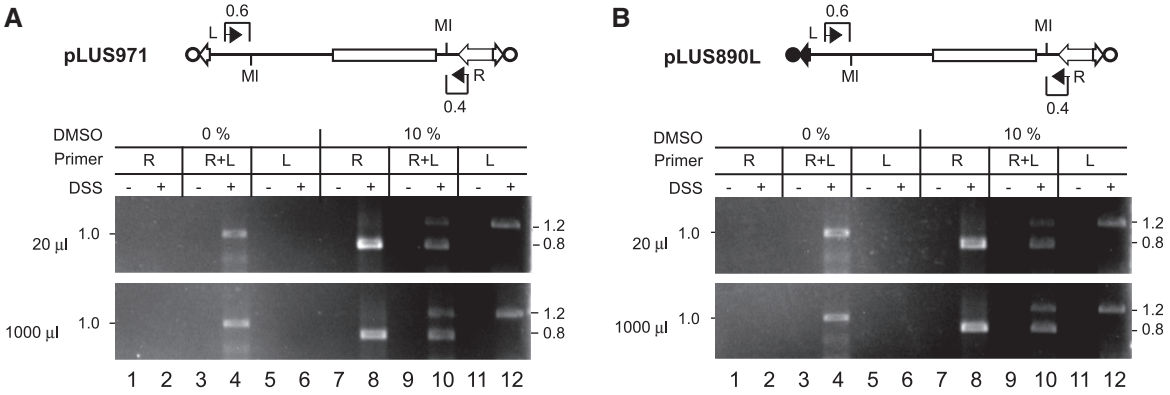

Figure 4. Interactions between chromosomal telomeres and between a chromosomal and plasmid telomere. (**A**) Top, physical map of pLUS971 is shown above. The open arrows and open circles represent the telomeres and TPs of the *S. lividans* chromosome, respectively. The distances between the primers and the nearest MluI ('MI') sites are indicated (in kb). Middle and bottom, 3C analysis as described in Figure 3. (**B**) Top, physical map of pLUS890L. The filled arrow and filled circle represent the SCP1 telomere and terminal protein, respectively. Middle and bottom, 3C analysis as described in Figure 3.

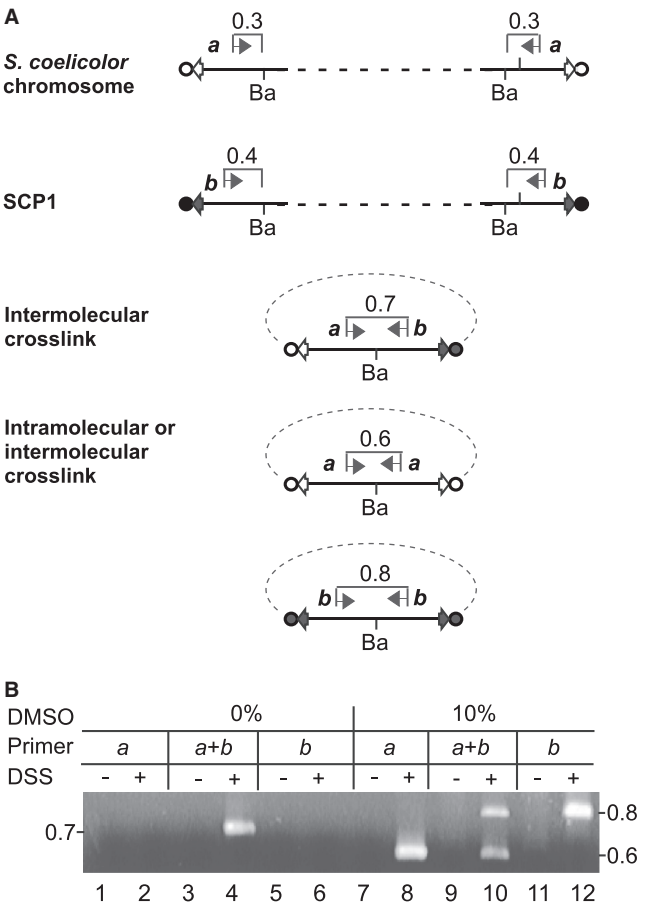

Figure 5. Interactions between the telomeres of the *S. coelicolor* chromosome and the SCP1 plasmid. (**A**) Shown from the top are the physical maps of the termini of the *S. coelicolor* chromosome and SCP1 with the distances between the primers (***a*** and ***b***, respectively) and the nearest BamHI ('Ba') sites indicated (in kb), and the expected products of ligation between the telomeres of the chromosome and SCP1, between the chromosomal telomeres and between the SCP1 telomeres. The other symbols are as in Figures 3 and 4. (B) 3C analysis as described in Figure 3.

linear form (lane 5). These results indicate that the linear plasmids were negatively supercoiled *in vivo*.

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

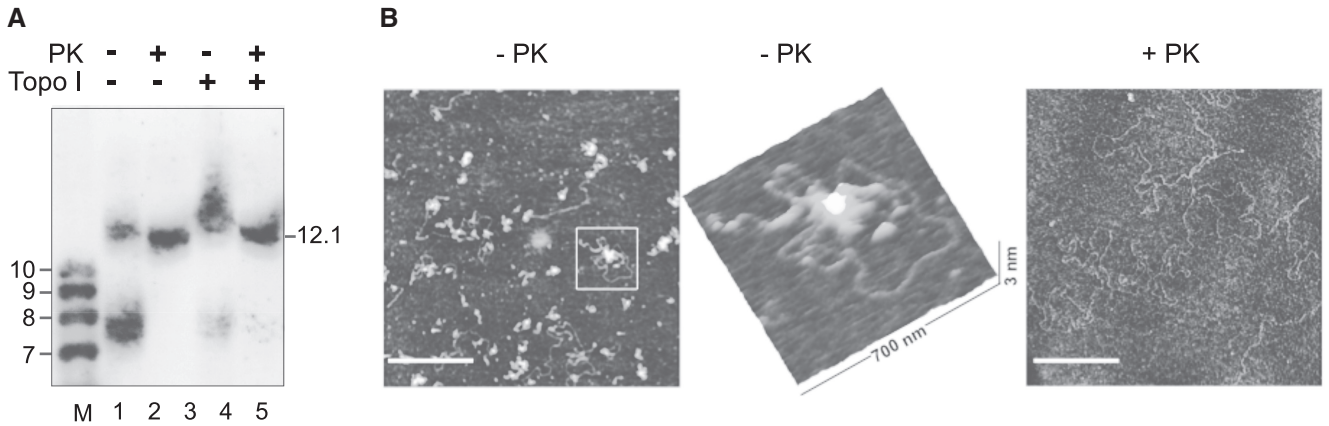

Figure 6. Superhelical structures formed by linear plasmid DNA. (**A**) *S. lividans* 3200/pLUS892L mycelium was osmotically lysed. The cell lysate was treated with *E. coli* Topoisomerase I ('Topo I'), fractionated by gel electrophoresis and hybridized to the pLUS892L DNA probe. In the control experiments, samples were treated with proteinase K ('PK') before electrophoresis, or the topoisomerase treatment was omitted, or both. The sizes of the (proteinase K-treated) linear pLUS892L DNA and the marker DNAs ('M') are indicated (in kb). (**B**) AFM examination of isolated DSS-cross-linked linear plasmid DNA (pLUS891L) without proteinase K-treatment ('−PK') shows supercoiled DNA structures that are held together by telomere–telomere interactions (left and center images). The center image shows a zoomed-in surface plot of the coiled DNA molecule boxed in the left image. Proteolytic digestion of the DSS-cross-linked DNA by proteinase K ('+PK') transformed it into relaxed, linear structures (right). The scale bar is 1 μm.

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 replication and post-replication segregation of these replicons. For instance, C.-C. Hsu and C. W. Chen (unpublished data) recently discovered that the partitioning defect (Δ*parAB*) 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 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-replicational 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 recombination, 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

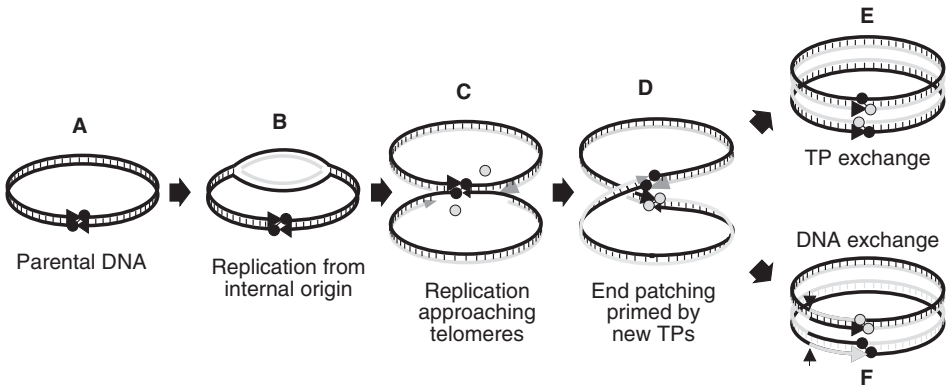

Figure 7. Post-replicational Möbius strip-like structure. (**A**) The parental linear DNA molecule forms a circular configuration through associations between the TPs (black disks). The 3′-ends are indicated by the arrowheads. The association of the two 5′-ends is analogous to the half twist in a classical Möbius strip in the simplest form. (**B**) Replication is initiated at an internal origin and proceeds bidirectionally. The newly synthesized DNA strands are in gray. (**C**) Replication reaches the telomeres, and results in single-stranded gaps at the 3′-ends. 'New' TPs (gray disks) are posed to prime the patching synthesis. (**D**) The gaps are patched by DNA synthesis primed by new TPs. In this diagram, the 'old' TPs on the parental strands remain associated to each other, and the 'new' TPs on the daughter strands become associated with each other. This pseudo-dimeric structure resembles the single long strip that is produced, when a Möbius strip is cut along the centerline. It cannot be readily resolved into two monomers without rearranging non-covalent or covalent bonds through one of the two alternative pathways described below. (**E**) In the 'TP exchange' pathway, the parental TPs dissociate from each other and each pairs with the proper 'daughter' TP on the distal telomere of the same DNA. (**F**) In the 'DNA exchange' pathway, the structure is resolved by (presumably site-specific) recombination at the arbitrarily designated site (indicated by the arrows) without disruption of the TP associations. [Modified from reference (6)].

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* possess 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.

ACKNOWLEDGEMENTS

We thank Dr Ming-Ta Hsu for helpful advice and suggestions and Dr David Hopwood for critical reading of the manuscript. C.W.C. is the recipient of National Professorship from Ministry of Education, R. O. C.

FUNDING

National Science Council, R. O. C. (NSC95-2321-B-010-002, NSC96-2321-B-010-001); Ministry of Education, R. O. C. (Aim for the Top University Plan). Funding for open access charge: The publication charges will be paid by our research grant from National Science Council, R. O. C.

Conflict of interest statement. None declared.

REFERENCES

1. Lin,Y.-S., Kieser,H.M., Hopwood,D.A. and Chen,C.W. (1993) The chromosomal DNA of *Streptomyces lividans* 66 is linear. *Mol. Microbiol.*, **10**, 923–933.
2. Sakaguchi,K. (1990) Invertrons, a class of structurally and functionally related genetic elements that include linear plasmids, transposable elements, and genomes of adeno-type viruses. *Microbiol. Rev*, **54**, 66–74.
3. Chang,P.C. and Cohen,S.N. (1994) Bidirectional replication from an internal origin in a linear *Streptomyces* plasmid. *Science*, **265**, 952–954.
4. Chaconas,G. and Chen,C.W. (2005) In Higgins,N.P. (ed.), *The Bacterial Chromosome*. American Society for Microbiology, Washington, DC, pp. 525–539.
5. Huang,C.-H., Tsai,H.-H., Tsay,Y.-G., Chien,Y.-N., Wang,S.-L., Cheng,M.-Y., Ke,C.-H. and Chen,C.W. (2007) The telomere system of the *Streptomyces* linear plasmid SCP1 represents a novel class. *Mol. Microbiol.*, **63**, 1710–1718.
6. Chen,C.W. (2007) In Meinhardt,F. and Klassen,R. (eds), *Microbial Linear Plasmids*. Springer-Verlag, Berlin, Heidelberg, pp. 33–61.
7. Yang,C.-C., Huang,C.-H., Li,C.-Y., Tsay,Y.-G., Lee,S.-C. and Chen,C.W. (2002) The terminal proteins of linear *Streptomyces* chromosomes and plasmids: A novel class of replication priming proteins. *Mol. Microbiol.*, **43**, 297–305.
8. Bao,K. and Cohen,S.N. (2001) Terminal proteins essential for the replication of linear plasmids and chromosomes in *Streptomyces*. *Genes Dev.*, **15**, 1518–1527.
9. Bao,K. and Cohen,S.N. (2003) Recruitment of terminal protein to the ends of *Streptomyces* linear plasmids and chromosomes by a novel telomere-binding protein essential for linear DNA replication. *Genes Dev.*, **17**, 774–785.
10. Kinashi,H., Shimaji-Murayama,M. and Hanafusa,T. (1991) Nucleotide sequence analysis of the unusually long terminal inverted repeats of a giant linear plasmid, SCP1. *Plasmid*, **26**, 123–130.

11. Zhang,R., Yang,Y., Fang,P., Jiang,C., Xu,L., Zhu,Y., Shen,M., Xia,H., Zhao,J., Chen,T. *et al.* (2006) Diversity of telomere palindromic sequences and replication genes among *Streptomyces* linear plasmids. *Appl. Environ. Microbiol.*, **72**, 5728–5733.
12. Ohnishi,Y., Ishikawa,J., Hara,H., Suzuki,H., Ikenoya,M., Ikeda,H., Yamashita,A., Hattori,M. and Horinouchi,S. (2008) Genome sequence of the streptomycin-producing microorganism *Streptomyces griseus* IFO 13350. *J. Bacteriol.*, **190**, 4050–4060.
13. Goshi,K., Uchida,T., Lezhava,A., Yamasaki,M., Hiratsu,K., Shinkawa,H. and Kinashi,H. (2002) Cloning and analysis of the telomere and terminal inverted repeat of the linear chromosome of *Streptomyces griseus*. *J. Bacteriol.*, **184**, 3411–3415.
14. Bentley,S.D., Brown,S., Murphy,L.D., Harris,D.E., Quail,M.A., Parkhill,J., Barrell,B.G., McCormick,J.R., Santamaria,R.I., Losick,R. *et al.* (2004) SCP1, a 356,023 base pair linear plasmid adapted to the ecology and developmental biology of its host, *Streptomyces coelicolor* A3(2). *Mol. Microbiol.*, **51**, 1615–1628.
15. Tsai,H.H., Huang,C.H., Lin,A.M. and Chen,C.W. (2008) Terminal proteins of Streptomyces chromosome can target DNA into eukaryotic nuclei. *Nucleic Acids Res*, **36**, e62.
16. Bao,K. and Cohen,S.N. (2004) Reverse transcriptase activity innate to DNA polymerase I and DNA topoisomerase I proteins of *Streptomyces* telomere complex. *Proc. Natl Acad. Sci. USA*, **101**, 14361–14366.
17. Huang,T.W. and Chen,C.W. (2008) DNA polymerase I is not required for replication of linear chromosomes in *Streptomyces*. *J. Bacteriol.*, **190**, 755–758.
18. Hopwood,D.A. (1967) Genetic analysis and genome structure in *Streptomyces coelicolor*. *Bacteriol. Rev.*, **31**, 373–403.
19. Wang,S.-J., Chang,H.-M., Lin,Y.-S., Huang,C.-H. and Chen,C.W. (1999) *Streptomyces* genomes: circular genetic maps from the linear chromosomes. *Microbiology*, **145**, 2209–2220.
20. Stahl,F.W. and Steinberg,C.M. (1964) The theory of formal phage genetics for circular maps. *Genetics*, **50**, 531–538.
21. Robinson,A.J., Younghusband,H.B. and Bellett,A.J. (1973) A circular DNA-protein complex from adenoviruses. *Virology*, **56**, 54–69.
22. Wong,M. and Hsu,M. (1989) Linear adenovirus DNA is organized into supercoiled domains in virus particles. *Nucleic Acids Res.*, **17**, 3535–3550.
23. Ortin,J., Vinuela,E., Salas,M. and Vasquez,C. (1971) DNA-protein complex in circular DNA from phage φ29. *Nat. New Biol.*, **234**, 275–277.
24. Arnberg,A. and Arwert,F. (1976) DNA-protein complex in circular DNA from *Bacillus* bacteriophage GA-1. *J. Virol.*, **18**, 783–784.
25. Banerjee,S. and Spector,D.J. (1992) Differential effect of DNA supercoiling on transcription of adenovirus genes *in vitro*. *J. Gen. Virol.*, **73(Pt 10)**, 2631–2638.
26. Qin,Z. and Cohen,S.N. (1998) Replication at the telomeres of the *Streptomyces* linear plasmid pSLA2. *Mol. Microbiol.*, **28**, 893–904.
27. Chen,C.W., Yu,T.-W., Lin,Y.S., Kieser,H.M. and Hopwood,D.A. (1993) The conjugative plasmid SLP2 of *Streptomyces lividans* is a 50 kb linear molecule. *Mol. Microbiol.*, **7**, 925–932.
28. Dekker,J., Rippe,K., Dekker,M. and Kleckner,N. (2002) Capturing chromosome conformation. *Science*, **295**, 1306–1311.
29. Wang,H., Yang,Y., Schofield,M.J., Du,C., Fridman,Y., Lee,S.D., Larson,E.D., Drummond,J.T., Alani,E., Hsieh,P. *et al.* (2003) DNA bending and unbending by MutS govern mismatch recognition and specificity. *Proc. Natl Acad. Sci. USA*, **100**, 14822–14827.
30. Qin,Z., Shen,M. and Cohen,S.N. (2003) Identification and characterization of a pSLA2 plasmid locus required for linear DNA replication and circular plasmid stable inheritance in *Streptomyces lividans*. *J. Bacteriol.*, **185**, 6575–6582.
31. Wong,S.S. and Wong,L.-J.C. (1992) Chemical crosslinking and the stabilization of proteins and enzymes. *Enzyme Microb. Technol.*, **14**, 866–874.
32. Müller,D.R., Schindler,P., Towbin,H., Wirth,U., Voshol,H., Hoving,S. and Steinmetz,M.O. (2001) Isotope-tagged cross-linking reagents. A new tool in mass spectrometric protein interaction analysis. *Anal. Chem.*, **73**, 1927–1934.
33. Pomp,D. and Medrano,J.F. (1991) *Organic Solvents as Facilitators of Polymerase Chain Reaction*. Eaton, Natick, MA, ETATS-UNIS.
34. Newton,C.R. and Granham,A. (1997) *PCR*. BIOS Scientific Publishers, Oxford, UK.
35. Huang,C.H., Chen,C.Y., Tsai,H.H., Chen,C., Lin,Y.S. and Chen,C.W. (2003) Linear plasmid SLP2 of *Streptomyces lividans* is a composite replicon. *Mol. Microbiol.*, **47**, 1563–1576.
36. Huang,T.W. and Chen,C.W. (2006) A *recA* null mutation may be generated in *Streptomyces coelicolor*. *J. Bacteriol.*, **188**, 6771–6779.
37. Hopwood,D.A., Kieser,T., Wright,H.M. and Bibb,M.J. (1983) Plasmids, recombination, and chromosomal mapping in *Streptomyces lividans* 66. *J. Gen. Microbiol*, **129**, 2257–2269.