Terminal proteins essential for the replication of linear plasmids and chromosomes in *Streptomyces*

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Linear plasmids and chromosomes of the bacterial genus *Streptomyces* have proteins of unknown characteristics and function linked covalently to their 5' DNA termini. We purified protein attached to the end of the pSLA2 linear plasmid of *Streptomyces rochei*, determined the N-terminal amino acid sequence, and used this information to clone corresponding genes from a *S. rochei* cosmid library. Three separate terminal protein genes (here designated as *tpgR1*, *tpgR2*, and *tpgR3*), which map to the *S. rochei* chromosome and to 100-kb and 206-kb linear plasmids contained in *S. rochei*, were isolated and found to encode a family of similar but distinct 21-kD proteins. Using *tpgR1* to probe a genomic DNA library of *Streptomyces lividans* ZX7, whose linear chromosome can undergo transition to a circular form, we isolated a *S. lividans* chromosomal gene (*tpgL*) that we found specifies a protein closely related to, and functionally interchangeable with, TpgR proteins for pSLA2 maintenance in *S. lividans*. Mutation of *tpgL* precluded propagation of the pSLA2 plasmid in a linear form and also prevented propagation of *S. lividans* cells that contain linear, but not circular, chromosomes, indicating a specific and essential role for *tpg* genes in linear DNA replication. Surprisingly, Tpg proteins were observed to contain a reverse transcriptase-like domain rather than sequences in common with proteins that attach covalently to the termini of linear DNA replicons.

[Key Words: Telomere; terminal protein; linear DNA replication; chromosome circularization; *tpgR*; *tpgL*]

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Streptomycetes are a group of eubacteria that are not only the source of more than 60% of the world's antibiotics, but also are believed to be the origin of many of the genes that make bacteria resistant to antibiotics (Davies 1997). Among the vehicles proposed to be involved in the transfer of antibiotic-resistance genes among, and from, *Streptomyces* species are linear extrachromosomal replicons (Davies 1994). How linear plasmids—and also the linear chromosomes found in streptomycetes—replicate their DNA and consequently reproduce the genes they carry has in recent years become a subject of increasing interest (Shiffman and Cohen 1992; Lin et al. 1993; Chang and Cohen 1994; Chen 1996; Chang et al. 1996; Lin and Chen 1997; Huang et al. 1998; Qin and Cohen 1998; Redenbach et al. 1999; Hiratsu et al. 2000; Qin and Cohen 2000; Volff and Altenbuchner 2000; Yamasaki et al. 2000).

Bacillus subtilis phage ϕ 29 DNA has been an extensively studied model for elucidation of the mechanism of linear DNA replication in prokaryotic cells (for review,

see Salas 1991; Salas et al. 1995). A terminal protein (TP) covalently bound to the 5' end of the phage DNA forms a heterodimer with the phage-encoded DNA polymerase, catalyzing covalent linkage of the TP to the first nucleotide of the nascent DNA strand and priming strand-displacing synthesis of full-length ϕ 29 DNA (Salas 1991; Salas et al. 1995; Mendez et al. 1997; Gonzales-Huici et al. 2000). In eukaryotic cells, replication of adenoviruses, which also have protein bound covalently to 5' DNA termini, occurs by a fundamentally similar protein-primed strand-displacement mechanism (van der Vliet 1995; Hay 1996; King et al. 1997; de Jong and van der Vliet 1999; Liu et al. 2000).

Like the ϕ 29 and adenovirus linear replicons, *Streptomyces* linear plasmids and chromosomes have covalently attached protein at their 5' DNA ends (Hirochika and Sakaguchi 1982; Kinashi et al. 1987; Sakaguchi 1990; Lin et al. 1993). However, in contrast to adenovirus and ϕ 29, *Streptomyces* linear DNAs replicate divergently from a site located near the center of the molecule, generating 3' leading-strand overhangs at the telomeres (Chang and Cohen 1994). The recessed 5' ends of the lagging strands produced by the joining together of Okazaki fragments (Kurosawa et al. 1975) are then extended (patched) to produce full-length duplex DNA molecules

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(Chang and Cohen 1994). Because *Streptomyces* chromosomes and plasmids have similar termini (Huang et al. 1998; Qin and Cohen 1998) and *Streptomyces* chromosomes also replicate bidirectionally from internal origins of replication (Musialowski et al. 1994), replication of chromosomes of streptomycetes is presumed also to generate telomeric 3' overhangs that require patching. Sequence analysis of *Streptomyces* telomeres indicates that they have similarities to, and differences with, the telomeres of eukaryotic chromosomes. The 3' overhangs of both types of replicons contain multiple short repeats (Huang et al. 1998; Qin and Cohen 1998; for review of eukaryotes, see Greider 1996; Lingner and Cech 1998; McEachern et al. 2000). However, whereas *Streptomyces* telomeres contain inverted repeats, eukaryote telomeres consist of a long series of tandem direct repeats. Moreover, the proteins that bind to eukaryote telomeres are not covalently attached to the terminus (Greider 1996; Bryan and Cech 1999).

To understand the biological role of the terminal proteins of *Streptomyces* linear replicons, we set out to: (1) isolate and characterize proteins bound covalently to the DNA ends of pSLA2, a 17-kb linear plasmid of *Streptomyces rochei* that has been a paradigm in recent investigations of the structure and function of *Streptomyces* telomeres (Chang and Cohen 1994; Chang et al. 1996; Qin and Cohen 1998, 2000); (2) identify and map the genes that encode terminal proteins in *Streptomyces*; and (3) investigate the role of terminal proteins in the replication of *Streptomyces* chromosomes and plasmids as linear DNA molecules. Our results indicate the existence of a family of structurally distinct, but related and functionally interchangeable, telomere-binding terminal proteins in *Streptomyces*; show that these proteins are encoded by genes carried by chromosomes and plasmids of different *Streptomyces* species; and demonstrate that terminal proteins are essential for the propagation of *Streptomyces* replicons in a linear, but not circular, form.

Results

Identification and characterization of proteins bound covalently to the termini of pSLA2 DNA

Covalent linkage of proteins with the termini of *Streptomyces* linear replicons was first indicated by evidence that pSLA2 linear plasmid DNA is sensitive to 3', but not 5', exonucleases and that DNA mobility in gels is altered by treatment with pronase but not with SDS (Hirochika and Sakaguchi 1982). We found that the protein component of the pSLA2 DNA-terminal protein complex (i.e., pSLA2-TP) was resistant also to treatment with a phenol/chloroform mixture, as are proteins bound covalently to the termini of other linear replicons (Rekosh et al. 1977; Salas et al. 1978; Yehle 1978), and used this property for terminal protein isolation. The pSLA2 DNA-TP complex from gently lysed cells was electroeluted from agarose gels and purified further by phenol/chloroform extraction in the presence of 1% SDS, fol-

lowed by ethanol precipitation. Digestion of this complex with *Bgl*II endonuclease yielded bands migrating in SDS-agarose gels as 8.2-kb, 6.6-kb, 1.4-kb, and 0.65-kb DNA fragments (Fig. 1A, lane 3). Treatment of the *Bgl*II-digested complex by proteinase K prior to electrophoresis led to replacement of the 0.65-kb band by one migrating at the 0.35-kb position (Fig. 1A, lanes 2,3), which is the size expected for both of the terminal fragments of pSLA2 DNA that has been treated with *Bgl*III (Hirochika et al. 1984; Qin and Cohen 1998). Migration of the putative telomeric band was not affected by 8 M urea, 2% SDS, or 4 M guanidine hydrochloride; however, treatment with 0.1 N NaOH and renaturation or treatment with piperidine reproduced the effect of proteinase K treatment (data not shown).

Preparations of the pSLA2-TP complex isolated and purified as described in Materials and Methods were treated with pancreatic DNase I and subjected to polyacrylamide gel electrophoresis, yielding a single band

Figure 1. Analysis of components of the pSLA2-TP Complex. (A) Agarose gel electrophoresis of the *Bgl*II-digested TP complex. A 1-kb DNA ladder was end-labeled with $^{32}\text{PO}_4$ by T4 kinase after dephosphorylation with calf intestinal alkaline phosphatase (CIP): (lane 1) $^{32}\text{PO}_4$ end-labeled 1-kb DNA ladder; (lane 2) pSLA2 complex digested with *Bgl*II and then with proteinase K; (lane 3) pSLA2 complex digested with *Bgl*II. Two sets of this gel were identically prepared for Southern blotting and Western blotting. (B) SDS-PAGE of the DNase I-digested complex. The 12% polyacrylamide gel was stained with Coomassie Brilliant Blue R-250. (Lane 1) Protein size marker (BIO-RAD); (lane 2) DNase I alone shown as control; (lanes 3,4) pSLA2 DNA complex isolated from filter-binding assay (see Materials and Methods) and treated with DNase I. The samples shown are duplicates from independent experiments. (C) Southern blot of lanes shown in A using pSLA2 terminal *Bgl*III fragments as probe. (D) Western blot of lanes shown in A using anti-TpgR1 antibody.

Figure 2. Alignment of the amino acid sequences of proteins isolated from pSLA2–TP complexes and deduced from the nucleotide sequences of PCR products and *tpg* genes. Micro1 and Micro2 indicate two N-terminal amino acid sequences from microsequencing of the 21-kD protein shown in Figure 1. PCR1 and PCR2 indicate the two amino acid sequences deduced from sequence of PCR products amplified using total DNA of *Streptomyces rochei* and degenerate primers corresponding to Micro1 and Micro2. TpgR1: Protein sequence corresponding to the *S. rochei tpgR1* gene on cosmid pBC67. TpgR2: Protein sequence corresponding to the *S. rochei tpgR2* gene on cosmid pBC66. TpgR3: Protein sequence corresponding to the *S. rochei tpgR3* gene on cosmid pBC65. TpgL: Protein sequence corresponding to the *tpgL* gene of *S. lividans* ZX7.

migrating as a 21-kD protein (Fig. 1B, lanes 3,4). N-Terminal microsequencing (Fig. 2) indicated that this band contains at least two closely related proteins differing only at three positions (positions 4, 7, and 14), where similar but not identical amino acids were present (Micro1 and Micro2, Fig. 2). Oligonucleotide primers containing degenerate sequences corresponding to the 40 N-terminal amino acids of Micro1 and Micro2 were designed and used to generate PCR products employing *S. rochei* total DNA as template. This amplification yielded two distinct DNA species (PCR2 and PCR1, Fig. 2) whose nucleotide sequences matched exactly the amino acid sequences of Micro1 and Micro2, respectively, except that PCR1 contains an isoleucine, rather than a proline, at position 20 (Fig. 2).

A family of terminal protein genes carried by plasmids and chromosome of S. rochei

Southern blot hybridization of *Bam*HI-digested total DNA from *S. rochei* using as probe a mixture of PCR1 and PCR2 detected three bands migrating at 5.7-kb, 6.1-kb, and 9.0-kb positions (Fig. 3A). To isolate the DNA sequences represented by these bands, we constructed and cloned a cosmid library of *S. rochei* total DNA in *Escherichia coli* (see Materials and Methods) and analyzed it by colony hybridization using the PCR products as probes. We identified 24 positive clones among 1920 cosmid-bearing colonies. *Bam*HI and *Kpn*I restriction enzyme site mapping of the cosmids plus Southern blot hybridization analysis revealed three distinct groupings, each containing a different *Bam*HI fragment that corresponded in size to one of the bands detected by the PCR probe in *Bam*HI-digested *S. rochei* total DNA.

DNA sequencing of restriction-enzyme-generated fragments of randomly chosen members of the three

groups of cosmids confirmed the existence in *S. rochei* of ORFs encoded by a family of three distinct but closely related genes. The N-terminal region specified by these ORFs corresponds exactly to the N-terminal regions of proteins encoded by PCR products we synthesized using *S. rochei* total DNA as template (see Fig. 2). Analysis of

Figure 3. Southern blot hybridization analysis of *Streptomyces rochei* total DNA probed with a mixture of PCR products corresponding to Micro1 and Micro2. (A) Agarose gel electrophoresis. Left panel shows ethidium-bromide-stained gel. Right panel shows the Southern blot. (Lane 1) 1-kb DNA ladder; (lane 2) *Bam*HI-digested total DNA. The positions of bands about 5.7 kb, 6.1 kb, and 9.0 kb in length are indicated relative to a 1-kb DNA ladder. (B) Pulsed-field gel electrophoresis analysis of *S. rochei* DNA. Left panel shows ethidium-bromide-stained gel where a DNA size marker (yeast chromosomes, lane 1) and *S. rochei* total DNA (lane 2) were separated. Right panel shows Southern blot of the gel.

the sequence by the Frame program (Bibb et al. 1984) indicated that codons in all three ORFs contain a G or a C in the third position, as is typical of *Streptomyces* species, whose DNA contains about 73% (G + C) (Enquist and Bradley 1971). The genes corresponding to the ORFs we identified were assigned the names *tpgR1* (terminal protein gene 1 of *S. rochei*; cosmid pBC67), *tpgR2* (cosmid pBC66), and *tpgR3* (cosmid pBC65). Southern blot hybridization analysis and sequencing of PCR-amplified products corresponding to fractions of *S. rochei* total DNA that had been separated by pulsed-field gel electrophoresis showed that *tpgR1* is contained on a band migrating in the position of chromosomal DNA, whereas *tpgR2* and *tpgR3* are carried, respectively, by 206-kb and 100-kb linear plasmids that were previously identified in this *S. rochei* strain (Fig. 3B; Kinashi et al. 1994). The third plasmid present in this *S. rochei* strain (i.e., the 17-kb SLA2 plasmid) lacks detectable homology with the probe, and no PCR product was generated using *tpg*-specific primers and pSLA2 DNA as template. Analysis of the *tpgR* genes further indicated that the Micro1 and Micro2 proteins attached to pSLA2 telomeres consist of a mix of products encoded by *tpgR1* and the terminal protein gene of at least one of the two large linear plasmids resident in *S. rochei*; the two plasmid-specified proteins are not distinguishable from the N-terminal sequence. The *tpgR1* gene was overexpressed in *E. coli* and confirmed to encode a 21-kD protein (data not shown).

Southern blot analysis (Fig. 1C) of the gel shown in Figure 1A using the 0.35-kb *Bgl*II fragment of pSLA2 as probe confirmed that this DNA fragment, whose mobility is altered by proteinase K digestion, is the terminal fragment of the pSLA2 linear replicon. To show that the *tpgR* gene products can bind to this fragment and consequently slow its migration, we subjected an identically prepared gel to Western blot analysis using as probe a polyclonal antibody generated against the TpgR1 protein that we had expressed in *E. coli* (Fig. 1D). As seen, anti-TpgR1 antibody detects a protein that, when attached in *S. rochei* to the 0.35-kb terminal fragments of pSLA2, caused these fragments to migrate at the 0.65-kb position.

The terminal protein gene of Streptomyces lividans ZX7

Unlike *S. rochei*, the *Streptomyces* species *Streptomyces lividans* can be readily transformed with plasmid DNA (Bibb et al. 1978, 1980), and accordingly has been used widely in studies of plasmid biology (Kieser et al. 1982; Kieser 1984; Hopwood et al. 1987; Deng et al. 1988; Kendall and Cohen 1988; Stein et al. 1989; Stein and Cohen 1990; Qin and Cohen 1998). While certain strains of *S. lividans* have linear chromosomes, others have undergone DNA rearrangements that result in chromosome circularization and the loss of telomeres (Lin and Chen 1997; Volff et al. 1997; Volff and Altenbuchner 2000). Like the telomeres of *S. rochei* linear plasmids, the 5' ends of *S. lividans* linear chromosomal DNA are pro-

ected by covalently attached protein (Lin et al. 1993). We hypothesized that this protein may function in the replication in *S. lividans* of linear DNA of plasmid pSLA2 (Chang and Cohen 1994), which has no detectable *tpg* gene of its own (see above), and thus that the putative *S. lividans* terminal protein may have domain similarities with the Tpg proteins of *S. rochei*. To isolate the postulated *S. lividans tpg* gene, we constructed a cosmid library containing *S. lividans* ZX7 genomic DNA, as described in Materials and Methods, and probed this library with *S. rochei tpgR1* DNA. Four positive cosmid clones were detected among 1920 isolates; *Bam*HI restriction mapping, Southern blot hybridization analysis, and DNA sequencing of cosmids present in these clones showed that all of the cosmids include an ORF (here designated as *tpgL*) encoding a 21-kD protein that shows 61% identity and 77% similarity to TpgR1 of *S. rochei*. The amino acid sequence of TpgL is compared with the sequences of TpgR proteins in Figure 2. Analysis of the genome database of *Streptomyces coelicolor* (http://www.sanger.ac.uk/Projects/S_coelicolor), which is closely related to *S. lividans*, indicates that an ORF identical to *tpgL* and present on cosmid 8D11 of a *S. coelicolor* A3(2) cosmid DNA library (Redenbach et al. 1996) is located ~120 kb from the end of the recently sequenced chromosome of *S. coelicolor* strain M145. There are only two base-pair differences between the *tpg* genes of ZX7 and M145, and no amino acid differences. Southern blot hybridization analysis of *Bam*HI-, *Bgl*II-, *Kpn*I-, and *Pst*I-digested total DNA of *S. coelicolor* strains A3(2) and M145 and of *S. lividans* strains 1326 and ZX7 using the ZX7 *tpgL* gene as probe detected one band for each enzyme digest (data not shown), suggesting that *S. lividans* and *S. coelicolor* each contains only one *tpg* locus.

Tpg protein is required for the propagation of linear plasmids and bacteria containing linear chromosomes

We could not readily inactivate the chromosomal *tpgL* gene in ZX7 cells by a single-gene-replacement strategy using a pBC130 plasmid-borne *tpgL* copy containing an inserted spectinomycin-resistance gene (*spc*^r; Fig. 4A). However, during these experiments, we observed rare *spc*^r *tsr*^s survivors (frequency ~ 10⁻³ versus our normal gene replacement frequency of 10⁻²–3 × 10⁻²); examination of several of these survivors by Southern blot analysis confirmed disruption of the *tpgL* gene, as shown in Figure 4, B and C, for BKKO5. When telomeres are deleted from *Streptomyces* linear replicons, the internal replication origin allows their propagation as circular DNA molecules (Shiffman and Cohen 1992; Lin et al. 1993; Chang and Cohen 1994; Chang et al. 1996; Lin and Chen 1997; Volff et al. 1997; Qin and Cohen 1998; Volff and Altenbuchner 2000). Southern blot analysis of chromosomal DNA isolated from two individual survivors (BKKO5 and BKKO6) indicated that these survivors had, in fact, undergone the telomere loss (Fig. 5A) characteristic of recombinational chromosome circularization in

Streptomyces (Lin and Chen 1997; Volff et al. 1997; Qin and Cohen 1998; Volff and Altenbuchner 2000). Further analysis of BKKO5 after culture for one life cycle in the absence of spectinomycin revealed that spontaneous loss of the *spc*^r gene, which had been inserted into the chromosomal *tpgL* gene, had occurred in 70% of cells. Because *tpgL* is located about 120 kb from one end of the chromosome, the finding suggested that the nonlinear chromosomal replicons formed in the absence of a functional *tpgL* gene undergo further sequence deletion; this conclusion is consistent with previous evidence that the subtelomere regions of *S. lividans* chromosomes that have undergone telomere removal and circularization are structurally unstable (Lin and Chen 1997; Volff et al. 1997; Volff and Altenbuchner 2000). Additionally, Western blot analysis of DNase I-treated preparations obtained from filter-binding of *S. lividans* chromosomal DNA detected a TP only in the preparation derived from ZX7 and not in the preparations from BKKO5, BKKO6, or from five separate *spc*^s BKKO5 derivatives (BKC1–BKC5), all of which had lost their telomeres (Fig. 5B).

pSLA2-derived plasmids that included an *E. coli* plasmid replicon and either lacked or contained the *tpgL* gene (pBC104 and pBC114, respectively, Fig. 6) were constructed as described in Materials and Methods and

Figure 4. Analysis of *Streptomyces lividans* ZX7 chromosomal DNA showing disruption of *tpgL* gene. (A) Schematic representation of *tpgL* gene disruption. *tsr*, thiostrepton-resistance gene; *spc*, spectinomycin-resistance gene. (B) ethidium-bromide-stained agarose gel. (Lane 1) 1-kb DNA ladder; (lane 2) ZX7 total DNA; (lane 3) BKKO5 total DNA; (lane 4) pBC130 plasmid DNA. (C) Southern blot of same gel probed with ³²P-labeled pBC130. All DNAs were digested with *PstI*.

Figure 5. Analysis of telomeres and terminal protein in *S. lividans* ZX7 and its derivatives lacking the *tpgL* gene. (A) Southern blot of *PstI*-digested total DNA of ZX7 (lane 2), BKKO5 (lane 3), BKKO6 (lane 4), and BKC1–BKC5 (lanes 5–9) was hybridized with the 2.6-kb *PstI* chromosome end fragment of ZX7 (Lin et al. 1993). (Lane 1) The 1-kb DNA ladder. (B) Western blot of DNase I-digested filter-binding assay samples of ZX7 (lane 2), BKKO5 (lane 3), BKKO6 (lane 4), and BKC1–BKC5 (lanes 5–9) against TpgR1 antibody. (Lane 1) Protein size marker (BIO-RAD).

cloned in *E. coli*; as seen in Figure 6, each plasmid is cleaved at two locations by *DraI* endonuclease. Previous work has shown that the terminal protein recognizes precisely, and links covalently to, native telomere ends present on plasmid DNA molecules introduced into *S. lividans* by transformation (Shiffman and Cohen 1992; Qin and Cohen 1998, 2000). Whereas DNA of both the pBC104 and pBC114 plasmids yielded transformants at a frequency of $8 \times 10^3/\mu\text{g}$ DNA when treated with *DraI* and introduced into protoplasts of ZX7, only the *tpgL*-containing plasmid pBC114 yielded transformants after *DraI* cleavage and introduction into a host (BKKO5) that lacks a chromosomal *tpgL* gene. Nonlinear DNA from both plasmids yielded transformants (frequency of 2×10^6 transformants/ μg DNA) in bacteria mutated in *tpgL*. Six randomly selected transformants of ZX7, which contains an intact *tpgL* gene, all contained linear plasmids after receiving *DraI*-treated pBC104 or pBC114 DNA (Fig. 6, shown for two transformants in lanes 3 and 10), as did six transformants of BKKO5 that had received *DraI*-cleaved pBC114 (Fig. 6, lane 8). Additionally, 12 randomly selected BKKO5 clones transformed with uncut pBC104 all contained circular plasmids (shown for one transformant in lane 5), as did ZX7 transformed with uncut DNA (data not shown; Qin and Cohen 1998). The linear form of plasmids was confirmed by treatment with NaOH, which causes loss of the double-strand linear DNA band (Fig. 6, lanes 4,9,11; Qin and Cohen 1998, 2000), but does not affect the circular DNA band (lane 6). Collectively, the experiments described in this section indicate that *tpgL* is essential for replication of both chromosomal and plasmid DNA in a linear form in *S. lividans*.

Discussion

Because the 5' ends of *Streptomyces* linear plasmids are known to be attached covalently to terminal protein, these linear DNA molecules were thought until recently to replicate by the protein-primed full-length strand-dis-

Figure 6. Plasmid replicons in *Streptomyces lividans* following *tpgL* gene disruption. (Top) Schematic map of pSLA2 derivatives pBC104 and pBC114. The difference between these two plasmids is that pBC114 contains a functional *tpgL* gene; the site of insertion is shown. *tsr*, thiostrepton-resistance gene; tel, 365-bp pSLA2 telomere end indicated by arrowheads pointing away from the centrally located replication origin of pSLA2. The locations of *DraI*-cleavage sites are indicated. (Bottom) Analysis of plasmid DNA replication of pSLA2 derivatives in *S. lividans* ZX7 and in the *tpgL* disrupted circular chromosomal derivative BKKO5. (chr) Chromosomal DNA; ccc (complete closed circular), linear, and oc (open circular) designate different forms of plasmid DNAs. (Lane 1) The 1-kb DNA-size ladder (Life Technologies); (lane 2) *DraI*-digested pBC104 plasmid DNA isolated from *Escherichia coli*; (lane 3) DNA isolated from ZX7 transformants receiving *DraI*-cleaved pBC104; (lane 4) same DNA as in lane 3, but following treatment with 0.2 N NaOH (30 min at 37°C); (lane 5) DNA isolated from BKKO5 transformants receiving uncleaved pBC104; (lane 6) same DNA as in lane 5 following treatment with 0.2 N NaOH; (lane 7) *DraI*-digested pBC114; (lane 8) DNA isolated from *S. lividans* BKKO5 transformants receiving *DraI*-cleaved pBC114 plasmid DNA isolated from *E. coli*; (lane 9) same as lane 8 following NaOH treatment; (lane 10) same as lane 8 except that host is ZX7; (lane 11) same as lane 10 following NaOH treatment. *Streptomyces* linear plasmid DNA was isolated by treatment with proteinase K and SDS (Qin and Cohen 1998, 2000) from BKKO5 and ZX7 transformants and electrophoresed for 12 hr at 30 V in 0.6% agarose gel.

placement mechanism demonstrated for adenovirus and $\phi 29$ (Salas 1991, 1995) and therefore thought not to require telomere patching. However, because *Streptomyces* linear replicons are now known to replicate bidirectionally from an internal site at the center of the molecule (Shiffman and Cohen 1992; Chang and Cohen 1994; Musialowski et al. 1994), producing replicative intermediates that contain a 3' overhang of leading-strand DNA (Chang and Cohen 1994), their telomeres must un-

dergo patching in order to generate blunt-ended postreplicative DNA molecules. Deletion of widely spaced palindromic DNA sequences in the telomeres of *Streptomyces* linear plasmids precludes plasmid DNA replication in a linear form (Qin and Cohen 1998, 2000), leading to the proposal that pairing of sequences at the terminus with internal sequences near the base of the overhang provides a recognition site for DNA-binding proteins involved in telomere patching. However, the specific proteins that accomplish this task have not been known. *Streptomyces* linear chromosomes (Lin et al. 1993), which contain telomeres similar to those present on *Streptomyces* linear plasmids and also replicate bidirectionally, are presumed to undergo replication and telomere patching by a similar process (Chen 1996; Huang et al. 1998; Qin and Cohen 1998).

The *S. rochei* linear replicon pSLA2, which is 17 kb in length, can use interchangeably the *tpgR* gene products specified by the *S. rochei* chromosome and two large linear plasmids present in this bacterial host. *S. lividans* ZX7, which contains the chromosomal *tpg* gene *tpgL*, related to the *tpgR* genes, requires *tpgL* to propagate its chromosome as a linear replicon and also to propagate pSLA2 derivatives as linear plasmids. *S. lividans* cells that survive after disruption of *tpgL* undergo the telomere loss characteristic of *Streptomyces* chromosomes that have undergone recombinational circularization. Additionally, pSLA2 derivatives, which can replicate as linear DNA in the presence of a functional *tpgL* gene, can be propagated only as circular replicons in *S. lividans* cells lacking *tpgL*. Therefore, *tpg* function is normally required for the replication of either *Streptomyces* chromosomes or plasmids as linear molecules.

A search of GenBank databases for sequence relationships to the family of *tpg* genes and proteins we identified revealed no similarity to terminal proteins linked covalently to 5' DNA termini of previously studied linear replicons. However, the Tpg proteins of *Streptomyces* contain a domain (in TpgL, amino acids 24–83) that includes a predicted Helix–Turn–Helix motif characteristic of DNA-binding proteins (amino acids 37–58, score 9.3 versus threshold of 4.0; SeqWeb version 1.2, HTH Scan). This domain shows 27% identity and 54% similarity to the reverse transcriptase domain of human immunodeficiency virus (HIV; Vergne et al. 2000). The reverse transcriptases (RTs) of hepatitis B and other hepadnaviruses initiate protein-primed synthesis of minus strands of viral DNA on an RNA template by covalent attachment of RT to the first deoxynucleotide of the nascent DNA chain (Wang and Seeger 1992; Beck and Nasal 1997; Lanford et al. 1997). Interestingly, the viral DNA strand remains bound to the polymerase, paralleling the covalent linkage of 5' ends of *Streptomyces* linear plasmid lagging-strand DNA to Tpg proteins. Although the Tpgs we identified are much smaller than known RT proteins, they may participate in protein-primed DNA synthesis at *Streptomyces* linear plasmid telomeres using an RNA template that corresponds in sequence to the 3' leading-strand DNA overhangs generated as replication intermediates (see Chang and Cohen 1994). The

resulting terminal segment of lagging-strand DNA—still carrying its covalently attached Tpg—may then be joined to the 5' end of the most distal Okazaki fragment of that strand. If sequence similarity between Tpg proteins and RT does, in fact, have functional significance, protein-primed telomere patching in *Streptomyces* linear replicons may be mediated by RNA rather than by the modified rolling-hairpin parvoviruslike mechanism considered previously (Huang et al. 1998; Qin and Cohen 1998). For either mechanism, palindrome-mediated fold back of 3' leading-strand overhangs may provide a recognition site, an anchor, for Tpg protein binding, as suggested previously (Qin and Cohen 1998).

Although *S. rochei* and *S. lividans* have widely different phenotypic characteristics (Williams et al. 1983), and the amino acid sequences of the Tpg proteins of these two species show no more than 61% identity (similarity ~77%), nevertheless, pSLA2 can use the *tpg* gene products of either species. This functional interchangeability of chromosomally encoded Tpg proteins of *S. lividans* and *S. rochei* potentially allows the plasmid to continue to replicate in a linear form when transferred in nature between these species. Additionally, the functional interchangeability of Tpg proteins from disparate *Streptomyces* species suggests that the mechanism for telomere patching is similar among different streptomycetes. Finally, we note that the ability of plasmids to acquire *tpg* genes enables such plasmids to replicate in, and thus potentially recombine with, the genomes of streptomycetes whose chromosomes lack *tpg* genes as a result of telomere deletion and circularization.

Materials and methods

Bacterial strains, plasmids, and general methods

Streptomyces rochei 7434-AN₄ (Hirochika and Sakaguchi 1982) and *Streptomyces lividans* ZX7 (Zhou et al. 1988) were kindly provided by K. Sakaguchi and X. Zhou, respectively. The *S. coelicolor* strains A3(2) and M145 and the *S. lividans* strain 1326 (Hopwood et al. 1983) were kindly provided by D.A. Hopwood. The *E. coli* host was *E. coli* DH5 α (Life Technologies), and pSP72 (Promega) was used as the cloning vector. Standard methods were used for culturing cells, DNA cloning, and so on in *E. coli* (Sambrook et al. 1989) and *Streptomyces* (Kieser et al. 2000). Restriction-enzyme-digested DNA fragments were extracted from agarose gel by Qiaquick Gel Extraction Kit (Qiagen). Southern blot hybridization followed the procedure of Church and Gilbert (1984). DNA sequencing was performed using an Applied Biosystems (ABI) Prism 310 Genetic Analyzer and ABI dye terminator sequencing kit. DNA sequencing was carried out for both strands. The degenerate oligonucleotides 5'-GTSGACAGCATCGGSGACGG-3' and 5'-CGSGTSCCCTTSAGCTGCTT-3' (S: G or C) were made (Life Technologies) for amplifying the N-terminal DNA sequences of *tpgR* genes according to the preference for G or C in the third position of codons in *Streptomyces* (see Bibb et al. 1984). Total DNA libraries of *S. rochei* and *S. lividans* ZX7 were constructed by using *E. coli-Streptomyces* shuttle cosmid vectors pHZ1358 (K. Bao and Z. Deng, pers. comm.) and pHZ132 (Hu et al. 1994), respectively. The 30–40-kb inserts for the library were made by *Sau*3AI partial digestion of chromosome DNA and were purified

by sucrose gradient fractionating. The packaging ligation mixture for library construction used the Gigapack III Gold Packaging Extract kit (Stratagene). Pulsed-field gel electrophoresis (PFGE) of *S. rochei* DNA was performed as described by Redenbach et al. (1996). Linear plasmid DNA was isolated as described by Qin and Cohen (1998).

Purification of terminal protein of pSLA2-TP complex

Isolation of the pSLA2 DNA-TP complex followed a *Streptomyces* total-DNA isolation procedure (Kieser et al. 2000), except that the lysozyme solution was changed to 10% sucrose, 2 mg/mL lysozyme, 50 μ g/mL RNase A, 50 mM Tris at pH 8.0, 25 mM EDTA at pH 8.0, and 0.1 mM PMSF. The pSLA2 DNA-TP complex was separated from total DNA by agarose gel electrophoresis and rescued from the gel by electroelution. The pSLA2 DNA-TP complex was further purified by phenol/chloroform extraction and ethanol precipitation. Under optimal extraction conditions the pSLA2 complex represented about 10% of the total DNA of *S. rochei*. Purified pSLA2 DNA-TP complex (in 1 M NaCl, 0.1 mM PMSF) was sonicated to fragment the DNA into small pieces (0.3–5 kb) with a Sonicator W-375 at high output for 10 sec $\times$ 6, and the product was applied to Whatman glass microfiber filter for filter-binding assays (Coombs and Pearson 1978). The filter was washed with 5 volumes of 1 M NaCl, and the DNA-TP complex was eluted from the filter with 0.1 M NaCl containing 0.5% SDS. The eluted DNA-TP complex was precipitated with 0.1 volume of 3 M sodium acetate and 2.5 volumes of ethanol. The pellet was dissolved in TE buffer (containing 0.1 mM PMSF) and treated with protease-free DNase I (Worthington, DPRFS grade) at 37°C for 2 h. The material was concentrated by ultrafiltration with Centricon-10 (Millipore), and TP was separated from the DNase I-digested pSLA2 complex by SDS-PAGE. The gel was transferred onto a PVDF membrane, and the protein band (21 kD) was cut out for amino acid microsequencing of the N terminus (Proseq Microsequencing). For Western blotting the eluates from filters (see above) were digested with DNase I, applied to 12% SDS-PAGE gels and electrophoresed for 2 h at 80 V.

PCR and DNA sequencing analysis of *tpg* gene distribution in *S. rochei*

DNA bands representing the *S. rochei* chromosome and the three linear plasmids (206 kb, 100 kb, and 17 kb) of this species were cut from PFGE low-melting-point agarose gels, and the DNA was extracted by melting the gel for 10 min at 65°C. *Pst*I was added to cut the large DNA molecules after the agarose gel had melted. The rescued DNA was purified by phenol/chloroform extraction and ethanol precipitation. PCR reactions were carried out with Platinum *Taq* HIFI DNA polymerase (Life Technologies), PCR primers 5'-AGCCATATGGTGGACTCGATCGGAGAC-3' and 5'-CCCTCGTAGAACTGGGCCGCTACAG-3', and the above four templates. PCR products rescued from agarose gel were directly sequenced. The sequences of PCR products were compared with the cloned *tpgR* gene sequences from library cosmids.

Overexpression of TpgR1 protein in *E. coli*

A plasmid supplying tRNA genes corresponding to the codons for arginine (two consecutive AGG, AGA, and CGG), proline (CCC), and isoleucine (ATA), all of which are rare in *E. coli* (Kane 1995; Ivanov et al. 1997), was used to overexpress the *tpgR1* gene in *E. coli*. A pACYC-derived plasmid (pPIRL) that encodes tRNAs corresponding to these rare codons was con-

structed with three tRNA genes of isoleucine, arginine, and leucine from Stratagene and a proline tRNA gene from Dr. H. Zhong. The chromosomal *tpgR1* gene sequence was amplified from pBC67 by a PCR using a Platinum *Taq* HIFI DNA polymerase (Life Technologies) (PCR primers: 5'-AGCCATATGGTGGACTCGATCGGAGAC-3' and 5'-CCCTCGTAGAACTGGGCCGCTACAG-3') and cloned into TA cloning vector pCR2.1 (Invitrogen) as plasmid pBC98. The correctness of the construct was confirmed by DNA sequencing analysis. The *tpgR1* gene was further cloned into *NdeI/BamHI*-digested pET28(a) from pBC98 (as plasmid pBC116), which enabled production of a fusion protein containing a histidine tag at the N-terminal end. After IPTG induction, *E. coli* BL21(DE3) containing pBC116 and pPRL overexpressed the TpgR1 fusion protein, which was further purified by Ni²⁺-column chromatography. The fusion protein was confirmed by Western blot analysis using His-tag antibody and used for the production of rabbit anti-TpgR1 polyclonal antibody.

Southern-Western blot analysis of telomere DNA-TP complex

The purified pSLA2 DNA-TP complex was digested with *Bgl*III, and half the mixture was further digested with proteinase K at 50°C in the presence of 0.5% SDS. A 1-kb DNA ladder (Life Technologies) was end-labeled with ³²PO₄ by T4 kinase after dephosphorylation with CIAP. Samples of a labeled 1-kb DNA ladder, the *Bgl*III-digested pSLA2 complex, and the complex digested with both *Bgl*III and proteinase K were loaded in duplicate onto an electrophoresis gel in 1× TAE buffer containing 0.05% SDS. One section of the gel was processed for Southern blotting using the telomere *Bgl*III fragment as probe while the TP was removed from the telomere fragment during the denaturation step of Southern blotting. Western blotting was performed with the adjacent duplicate part of the agarose gel. Double-strand DNA fragments (including terminal DNA-TP complex) in agarose gel were electrophoretically transferred onto Nylon membrane (Hybond-N, Amersham) in 1× TAE buffer containing 0.05% SDS, using a voltage of 100 V for 1 h at 4°C. After the transferred DNA was fixed onto Nylon membrane by UV cross-linking, the membrane was analyzed by Western blotting with anti-TpgR1 antibody.

Terminal protein gene disruption and complementation

A *tpgL* gene disruption plasmid (pBC130; Fig. 4) was constructed by inserting a spectinomycin-resistance gene (*spc*^r) at the *Eco*47III site of the *tpgL* gene following partial digestion of the *tpgL* gene cloned in *E. coli*. To mutate the chromosomal *tpgL* gene of ZX7, protoplasts were transformed with pBC130, which lacks the ability to replicate in *Streptomyces* and contains a gene (*tsr*) encoding thiostrepton resistance; transformants (*spc*^r *tsr*^r) were selected on R5 containing spectinomycin. Clones in which a double crossover leading to replacement of chromosomal *tpgL* by the insertionally mutated gene were isolated by the testing of spores for sensitivity to thiostrepton by replica-plating colonies selected in the presence of spectinomycin and in the absence of thiostrepton (*spc*^r *tsr*^s). Replacement of wild-type *tpgL* by the insertionally mutated gene in *spc*^r *tsr*^s bacteria was confirmed by Southern blot hybridization. For *tpgL* gene complementation, a 4.2-kb *Bgl*III fragment containing the entire *tpgL* gene was cloned into the pQC18-derived replicon pBC104 to generate pBC114 (Fig. 6; Qin and Cohen 1998). ZX7 and its derivative BKK05 (see above) were transformed with the DNA of these two plasmids following digestion with *Dra*I.

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