Diversity of Telomere Palindromic Sequences and Replication Genes among *Streptomyces* Linear Plasmids

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Streptomyces sp. linear plasmids and linear chromosomes usually contain conserved terminal palindromic sequences bound by the conserved telomeric proteins Tap and Tp, encoded by the *tap* and *tpg* genes, respectively, as well as plasmid loci required for DNA replication in circular mode when the telomeres are deleted. These consist of iterons and an adjacent *rep* gene. By using PCR, we found that 8 of 17 newly detected linear plasmids in *Streptomyces* strains lack typical telomeric *tap* and *tpg* sequences. Instead, two novel telomeres in plasmids pRL1 and pRL2 from the eight strains and one conserved telomere in pFRL1 from the other strains were identified, while multiple short palindromes were also found in the plasmids. The complete nucleotide sequence of pRL2 revealed a gene encoding a protein containing two domains, resembling Tap of *Streptomyces* and a helicase of *Thiobacillus*, and an adjacent gene encoding a protein similar to Tpg of *Streptomyces* and a portion of the telomere terminal protein pTP of adenoviruses. No typical iterons-*rep* loci were found in the three plasmids. These results indicate an unexpected diversity of telomere palindromic sequences and replication genes among *Streptomyces* linear plasmids.

Streptomyces species are gram-positive, high-G+C, mycelium-producing eubacteria. Unlike the case for most eubacteria, linear plasmids and linear chromosomes are common in *Streptomyces* species (3, 8, 11, 19, 20, 28). The linear plasmids vary in size between 12 kb (16) and 1,700 kb (19). Their telomeres contain long inverted repeat sequences of 44 bp (7) to 180 kb (21), and the 5' telomeric ends are linked covalently to terminal proteins (Tp) (1, 31). The telomeres of the ~8-Mb linear *Streptomyces* chromosomes are 46 bp to 1 Mb long (14, 29). With the exceptions of the telomeres of the large linear plasmid SCP1 and the *Streptomyces griseus* linear chromosome (9, 18), *Streptomyces* linear plasmids and linear chromosomes usually contain conserved palindromic DNA sequences at their telomeres (13).

Unlike the terminal protein-capped linear replicons of adenoviruses and bacteriophage Φ 29 (25), replication of *Streptomyces* linear plasmids starts at centrally located loci (27) and proceeds bidirectionally toward the telomeres (5). This leaves an ~280-nucleotide (nt) single-strand overhang at the 3' telomeric end of pSLA2 as a replication intermediate (5). To convert the 3' overhang to a double strand, the terminal 144 nt of the telomere contain short palindromes 1 to 5 (22), with palindromes 2/3 being bound by the conserved telomere-associated protein (Tap) to recruit the conserved telomere terminal protein (Tp) (1, 2).

Streptomyces linear plasmids can also propagate in circular mode when the telomeres are deleted (5, 10, 24, 27). The

centrally located locus for replication of pSLA2 consists of a *rep-2* gene (encoding a DNA helicase) and its adjacent iterons within a transcribed *rep-1* gene (6). The replication loci of plasmids SCP1 and pSLA2-L also consist of *rep* genes and different iteron sequences (10, 24). Such iterons-*rep* loci were revealed by complete nucleotide sequencing of other *Streptomyces* linear plasmids (such as pSCL1 and SLP2 [12, 30]).

Do the conserved telomere palindromic sequences, *tap/tpg* genes, and iterons-*rep* loci exist widely among larger populations of *Streptomyces* linear replicons? By investigating ~100 *Streptomyces* strains, we show here that nearly half of the linear plasmid-harboring *Streptomyces* strains lack highly conserved *tap/tpg* sequences. Consistent with this, two novel and one conserved telomere palindromic sequence were identified on plasmids in the corresponding *Streptomyces* strains. The complete nucleotide sequences of the three plasmids showed no typical iterons-*rep* loci, and one plasmid carried two genes encoding proteins resembling Tap of *Streptomyces* and a portion of the telomere terminal protein pTP of adenoviruses.

MATERIALS AND METHODS

Bacterial strains, plasmids, and general methods. *Escherichia coli* strain DH5 α (Life Technologies Inc.) and plasmid pBluescript II SK (Stratagene) were used as the cloning host and vector, respectively. Plasmid isolation, transformation, and PCR amplification were done following the methods of Sambrook et al. (26). *Streptomyces lividans* ZX7 was the host for propagating plasmids. *Streptomyces* culture, plasmid isolation, pulsed-field gel electrophoresis, preparation of protoplasts, and transformation were done following the methods of Kieser et al. (17). Isolation of nondenatured plasmid DNA followed the method of Qin and Cohen (22). The ~80 *Streptomyces* strains, isolated from Yunnan Province, China, and identified by the standard procedures of actinomycete classification, were provided by Jiang Chenglin and Xu Lihua. The ~20 *Streptomyces* strains, isolated from Hunan Province, China, and identified by PCR se-

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quencing of the 16S rRNA gene, were provided by Fang Ping. The sequences of the telomere terminal proteins (Tp) of *Streptomyces coelicolor*, *S. lividans*, and *Streptomyces rochei* were aligned, and the highly conserved amino acid regions were chosen to design a pair of oligonucleotides (for *tpgC* nt 77 to 96, 5' CGCAGATGCGGTACCTGGTC 3'; for *tpgC* nt 281 to 262, 5' GTGGT TGCCGCCTTCTGCCG 3'). Similarly, oligonucleotides from the conserved sequences of the telomere-associated protein (Tap) were for *tapC* nt 566 to 585 (5' GCGGCCTGGTCCTGGACGTG 3') and *tapC* nt 1676 to 1657 (5' AGGTCGGACATCGTGGCGAG 3').

DNA sequencing and analysis. About 10 µg DNA of pRL1 and pRL2 was purified from pulsed-field gels. After shotgun cloning, PCR sequencing was done on an Applied Biosystems model 377 genetic analyzer at the Chinese Human Genome Center in Shanghai. Analysis of protein coding regions was performed with FramePlot 3.0 beta (15; <http://watson.nih.gov/jun/cgi-bin/frameplot-3.0b.pl>). Sequence comparisons and protein domain searching were done with software from the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/BLAST/>). DNA secondary structures (e.g., direct repeats and inverted repeats) were predicted with DNA Folder (www.bioinfo.rpi.edu/applications/mfold/old/dna/) and Clone Manager, version 5.

Nucleotide sequence accession numbers. The GenBank accession numbers for the complete nucleotide sequences of pRL1, pRL2, and pFRL1 are DQ322649, DQ322650, and DQ322651, respectively.

RESULTS

Identification of linear plasmids among a population of *Streptomyces* strains. To detect indigenous linear plasmids among a population of *Streptomyces* strains, mycelia of ~100 randomly selected strains were embedded in plugs of low-melting-point agarose gel and subjected to pulsed-field gel electrophoresis (17). Both fast-migrating and very large bands were detected (Fig. 1A), suggesting the presence of linear plasmids and linear chromosomes. As summarized in Table 1, various sizes (20 to 500 kb) of linear plasmids were detected; among the 17 strains, most harbored one plasmid, while 3 strains harbored three or four plasmids.

To verify the conformations of the plasmids, genomic DNA of strain 44030 (harboring the 32-kb plasmid pRL1) was treated with *E. coli* exonuclease III and λ exonuclease (22) and then electrophoresed in an agarose gel. As shown in Fig. 1B, the 32-kb DNA band was sensitive to *E. coli* exonuclease III but resistant to λ exonuclease, suggesting that pRL1 DNA was linear, with a free 3' end but a blocked 5' end. The same result was obtained with the 20-kb pRL2 plasmid of strain 44414 (Fig. 1C).

PCR testing for conserved telomere *tap* and *tpg* sequences in *Streptomyces* strains. To investigate if the conserved telomere *tpg* and *tap* genes exist among the 17 *Streptomyces* strains harboring linear plasmids, oligonucleotides designed from the highly conserved amino acid sequences of the Tap and Tp proteins were used for PCR (see Materials and Methods). As shown in Fig. 2, PCR-amplified DNA bands of expected sizes were detected for strains FR1 (harboring linear plasmids pFRL1, pFRL2, and pFRL3) and *S. lividans* ZX7 (as a positive control containing chromosomal *tap* and *tpg* sequences) but not for strains 44414 (containing pRL2, pRL3, and pRL4) and 44030 (containing pRL1). As summarized in Table 1, such PCR-amplified products were detected in 9 of 17 strains, indicating that they might contain conserved *tpg/tap* genes, but not in the other 8 strains, suggesting that they might contain novel *tpg/tap* genes.

Cloning and sequencing of the novel telomeres of linear plasmids pRL1 and pRL2 and the conserved telomere of pFRL1. Previous work indicated a correlation between con-

FIG. 1. Identification of linear plasmids among *Streptomyces* strains. (A) Linear plasmids were detected by pulsed-field gel electrophoresis (17). The plug-embedded mycelium was electrophoresed in a 1.0% agarose gel at 120 V with a 50- to 90-s switch time at 14°C for 20 h. The DNA marker is in lane M, and linear plasmids pRL2, pRL1, and pFRL1 are indicated by arrows. DNA bands of possible linear chromosomes are indicated (L Chr). (B and C) Confirmation of linearity of pRL1 and pRL2. Nondenatured plasmid DNA was isolated from strains 44030 and 44414 (22). Aliquots of the DNA (in Tris-EDTA buffer) were treated with 100 U of *E. coli* exonuclease III or 10 U of λ exonuclease at 37°C for 1 h and electrophoresed in a 0.5% agarose gel at 25 V for 12 h. λ HindIII DNA was used as a size marker. The position of residual chromosomal DNA (Chr) detected in the lanes after treatment with either λ exonuclease or exonuclease III is indicated. Lp, linear plasmid.

served telomere palindromic sequences and conserved telomere binding proteins in *Streptomyces* (1, 2, 31). To clone putatively novel telomeres of the linear plasmids containing the proposed novel *tpg/tap* genes, telomere treatment and forced cloning were employed (20). The gel-purified, 32-kb

TABLE 1. Summary of linear plasmids detection among ~100 *Streptomyces* strains and PCR results for the conserved regions of the *tpg* and *tap* genes

Strains	Size of linear plasmid(s) detected (kb)	PCR result for conserved regions of the <i>tpg</i> and <i>tap</i> genes
44030	32	—
44414	20, 160, 260	—
44463	30	—
44476	90	—
44549	50	—
44380	35	+
44432	20, 30, 50, 300	+
44524	280	+
44554	130	+
44577	170	+
44598	500	+
44628	350	+
FR1	54, 100, 450	+
F2	360	+
F8	370	—
F9	150	—
F11	500	—

DNA of pRL1 was digested with EcoRI, ligated with *E. coli* pBluescript II SK treated with EcoRI and SmaI (blunt end cut), and then introduced by transformation into *E. coli* DH5 α . A plasmid, pZR82, containing a 7-kb insert was obtained. The nucleotide sequence extending from the SmaI site of pZR82 (Fig. 3A), presumed to be the telomere of pRL1, contained multiple short palindromes different from those of conserved *Streptomyces* telomeres (13).

Similarly, a 1.3-kb XhoI fragment of pRL2 was cloned into the XhoI and EcoRV (blunt end cut) sites of pBluescript II SK to obtain pZR11. The corresponding nucleotide sequence of pZR11 (Fig. 3B) also contained multiple short palindromes which were different from those of both pRL1 and conserved *Streptomyces* telomeres.

A 1.2-kb EcoRI fragment of pFRL1 similarly yielded pZR6. The corresponding sequence closely resembled conserved

FIG. 2. Detection of highly conserved *tpg* and *tap* sequences among *Streptomyces* strains by PCR. The genomic DNAs of *Streptomyces* strains ZX7 (positive control), 44030, 44414, and FR1 were prepared, and PCR amplifications were performed using pairs of oligonucleotides from the *tpg/tap* sequences (see Materials and Methods). The PCR products were electrophoresed in a 1% agarose gel at 100 V for 2 h. A 100-bp DNA ladder was used as a size marker.

FIG. 3. Telomere sequences (150 nt) of linear plasmids pRL1 (A), pRL2 (B), and pFRL1 (C). The short palindromes (numbered) are shown by pairs of arrows.

Streptomyces telomeres [e.g., those of *Streptomyces coelicolor* A3(2), with an identity of 42/43 bp], especially in the first 43 bp (Fig. 3C).

The complete nucleotide sequence of linear plasmid pRL2 shows that two genes encode proteins, with one resembling *Tap* of *Streptomyces* and the helicase of *Thiobacillus* and one resembling *Tpg* of *Streptomyces* and a portion of the telomere terminal protein pTP of adenoviruses. pRL2 was the smallest linear plasmid containing a novel telomere sequence found among *Streptomyces* strains. PCR sequencing following the “shotgun cloning” of linear plasmid DNA (see Materials and Methods) would miss the end sequences. After combining the internal and telomeric sequences, the complete nucleotide sequence of pRL2 (GenBank accession number DQ322650) consisted of 20,252 bp, with a 70.4% G+C content and a telomere of 427 bp. The 26 open reading frames (ORFs) on pRL2 were predicted by FramePlot 3.0 beta. Interestingly, one ORF, pRL2.4c, encoded a protein of 1,100 amino acids containing two domains which resembled the telomere-associated protein TapR of the *Streptomyces rochei* linear plasmid pSLA2-L (from aa 560 to 1080; expectation value, 2×10^{-8} ; identity, 134/640 bp [20%]) and the superfamily II helicase of *Thiobacillus denitrificans* (from aa 175 to 520; expectation value, 2×10^{-4} ; identity, 77/325 bp [23%]). As for the cotranscribed *Streptomyces tpg* and *tap* genes (2), the spacer between pRL2.4c and the adjacent pRL2.3c ORF was only 3 bp (Fig. 4A). The pRL2.3c-encoded protein of 216 amino acids resembled the telomere terminal protein TpgR of the *Streptomyces rochei* linear plasmid pSLA2-L (expectation value, 1×10^{-4} ; identity, 56/184 bp [30%]) and part of the telomere terminal protein pTP of simian adenovirus (Fig. 4B) (expectation value, 3.7; identity, 17/55 bp [30%]). These results indicated that the two genes, pRL2.3c and pRL2.4c, of pRL2 might be cotranscribed and involved in telomere replication.

There was one possible predicted iteron near one telomere (Fig. 5A). However, this iteron and its adjacent ORFs (pRL2.1 and pRL2.2) did not propagate when cloned into an *E. coli* plasmid, pQC156, containing *Streptomyces melC* and *tsr* selection markers (23), yielding pZR81, and introduced into *S.*

FIG. 4. Characteristics of the two genes of pRL2. (A) Comparison between proteins pRL2.4c and pRL2.3c and the TapR/TpgR proteins encoded by *Streptomyces* linear plasmid pSLA2-L (2). The two functional protein domains (TapR and helicase) of pRL2.4c are indicated. (B) Alignment of the pRL2.3c protein sequence and the telomere terminal protein (pTP) sequence of simian adenovirus.

lividans ZX7 by transformation, suggesting that there was no typical iteron-*rep* locus in pRL2 for DNA replication (Fig. 5C).

Complete nucleotide sequences of linear plasmids pRL1 and pFRL1 revealed no common iterons-*rep* loci for replication. The pRL1 sequence (DQ322649) consisted of 32,759 bp, and its telomere was 3,269 bp long, containing two hypothetical genes. The G+C content was 66.9%, which was lower than that of typical *Streptomyces* genomes (e.g., 72.1% for *S. coelicolor*) (4). Thirty-three ORFs were predicted on pRL1, with no homology

to conserved *tpg/tap* or *rep* genes of *Streptomyces* linear plasmids. Four different iteron sequences at different positions on pRL1 were predicted (Fig. 5B). As for pRL2, the pRL1 iterons and the adjacent ORFs did not allow propagation when cloned into *E. coli* plasmid pQC156 to yield pZR23 and pZR99, respectively, and introduced into *S. lividans* ZX7 by transformation, suggesting a lack of a typical iteron-*rep* locus on pRL1 for replication (Fig. 5C).

The pFRL1 sequence (DQ322651) consisted of 54,288 bp,

FIG. 5. Iterons of pRL1 and pRL2 and their comparison with typical iterons-*rep* loci of *Streptomyces* linear plasmids pSLA2 and SCP1. (A and B) Iterons (indicated by arrows) at the positions (numbered sequences) of pRL2 and pRL1 predicted by DNA Folder (see Materials and Methods). (C) Comparison of typical iterons-*rep* loci of pSLA2 (6) and SCP1 (24) with the iterons-ORFs of pRL2 and pRL1. The telomere, iterons, and adjacent genes of pRL2 were cloned into pBluescript II SK to obtain pZR30, treated with BamHI and BglII to release the pRL2 fragment, and cloned into the BamHI site of pQC156 to yield plasmid pZR81. The HindIII fragment (7.0 kb) and EcoRI fragment (9.1 kb) of pRL1 were cloned into pQC156 to yield pZR99 and pZR23, respectively. Iterons are indicated by striped boxes, the telomere is indicated by a dotted arrow, and genes are indicated by thick arrows. The genes encoding known proteins (in parentheses) are shown.

FIG. 6. Characteristics of telomere sequences of linear plasmids pFRL1 and pRL2. (A) Secondary structure of the 80 nt of 3' pRL2 telomere terminal sequence predicted by DNA Folder. Folding back and base pairing of short palindrome 1 to one arm of palindrome 4 are shown. (B) Multiple sequence alignment of telomere sequences from *Streptomyces* linear plasmids. The positions of telomere palindromes 1 to 4 are shown.

with a 72.1% G+C content and a telomere of 421 bp. Fifty-nine ORFs were predicted on pFRL1, including two conserved *tpg* genes and one *tap* gene. However, as for pRL1 and pRL2, there was no typical iteron-*rep* locus on pFRL1.

DISCUSSION

With the exceptions of the large linear plasmid SCP1 and the *Streptomyces griseus* linear chromosome (9, 18), the telomeres of *Streptomyces* linear plasmids (e.g., pSLA2, pSLA2-L, pSCL1, SLP2, pSPA1, and SAP1) and linear chromosomes (e.g., those of *S. coelicolor*, *S. lividans*, *Streptomyces parvulus*, *Streptomyces lipmanii*, and *Streptomyces avermitilis*) contain conserved palindromic sequences (10, 13, 14, 22, 30). Correspondingly, conserved telomere replication genes (e.g., *tap* and *tpg*) are found on several *Streptomyces* linear plasmids and linear chromosomes (1, 2, 31). However, by investigating a population of *Streptomyces* strains, we found that nearly one-half (8/17) of the linear plasmid-harboring strains lacked highly conserved *tap/tpg* sequences. We further identified two novel and one conserved telomere palindromic sequence from the corresponding *Streptomyces* species, supporting the hypothesis of specificity between telomere palindromic sequences and telomere-binding proteins.

Although the two novel telomere sequences identified here differ from the conserved telomere sequences, they also contain multiple short palindromes. Like the case for linear plasmids pSLA2 and SLP2 (4, 13, 22), the 3'-telomeric palindrome

1 of pRL2 may “fold back” internally (folding energy, -29.1 kcal/mol) to one arm of palindrome 4 (Fig. 6A), suggesting that the “fold-back” is an important step for telomere replication. Interestingly, like the conserved Tap/Tpg proteins, pRL2 encodes two proteins, one of which resembles Tap of *Streptomyces* and the helicase of *Thiobacillus* and the other of which resembles Tpg of *Streptomyces* and the telomere terminal protein pTP of adenoviruses. Recent experiments indicated that the two pRL2 genes are involved in linear plasmid replication (our unpublished data). These results suggest that *Streptomyces* linear replicons share similar mechanisms of telomere replication, even though the telomere sequences differ, and that the process of “filling in” the 3' overhang by the proposed telomere terminal protein-priming replication in *Streptomyces* might be similar to telomere replication in adenoviruses. Replication of linear replicons of adenoviruses starts at the telomeres and undergoes a strand displacement mechanism (25). Although *Streptomyces* linear plasmids usually contain centrally located origins for starting replication (5, 10, 24, 27), the possibility that replication starts at the telomeres in some linear replicons may not be ruled out. We found that besides the double-stranded DNA band, one high-mobility band could also be detected during the replication of a pRL2-derived plasmid, pZR108, in *S. lividans* ZX7 (our unpublished data), supporting this suggestion.

Previous studies showed that the telomeres of several *Streptomyces* linear chromosomes and linear plasmids contain conserved palindromes (palindromes 1 to 7) within the first 180 nt

(13). Our data on the telomere sequence of linear plasmid pFRL1 and the recently published telomere sequences of linear plasmids pSLA2-L, SLP2 (left telomere), and SAP1 (9, 12, 14) show that the conserved palindromes lie within palindromes 1 to 3 (Fig. 6B). Consistent with this result, Bao and Cohen (2) showed that palindromes 2/3 of the telomere sequence are bound specifically by the *Streptomyces* telomere-associated protein (Tap).

Even though the telomere of linear plasmid SCP1 differs from those of pSLA2 and pSLA2-L (10, 18), these plasmids all contain typical iterons-*rep* loci for DNA replication in circular mode when the telomeres are deleted (6, 10, 24). We found that linear plasmid pFRL1, containing the conserved telomere sequence, lacks typical iterons-*rep* loci, indicating no correlation between the telomere sequences and the iterons-*rep* loci. Additionally, except for the plasmid transfer gene *tra* and the partition gene *parA*, no common genes are found among the three linear plasmids sequenced here, suggesting that the centrally located loci for DNA replication in circular mode are diverse in *Streptomyces* linear plasmids pRL1, pRL2, and pFRL1.

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