Long palindromes formed in *Streptomyces* by nonrecombinational intra-strand annealing

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Long inverted repeats (palindromes) are ubiquitous among prokaryotic and eukaryotic genomes. Earlier work has implicated both DNA breaks and short inverted repeats (IRs) in the formation of long palindromes in yeast and Tetrahymena by a proposed mechanism of intramolecular recombination. Here we report that long-palindromic linear plasmids are formed in *Streptomyces* following double strand DNA breakage by a nonrecombinational intra-strand annealing process that also involves IRs. By modification of palindrome-generating linear plasmids and development of a novel procedure that enables the sequencing of palindrome junctions, we show that long-palindrome formation occurs by unimolecular intra-strand annealing of IRs followed by 3′ extension of the resulting DNA fold-back. The consequent hairpin structures serve as templates for synthesis of duplex linear plasmids containing long palindromes. We suggest that this model for long-palindrome formation in *Streptomyces* may represent a generally applicable mechanism for generating DNA palindromes.

[*Key Words*: Palindrome; inverted repeat; linear plasmid; telomere; DNA damage]

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Long inverted repeat sequences (DNA palindromes) are a ubiquitous feature of chromosomal and extrachromosomal DNAs of plants, animals, and bacteria where they have been found to have a prominent role in genetic instability and gene amplification (for reviews, see Fried et al. 1991; Rayko 1997). Long palindromes constitute the limbs of bacterial transposable elements (Kopecko and Cohen 1975; for reviews, see Berg and Howe 1989), the telomeres of some linear replicons (Sakaguchi 1990), and the arms of amplified DNA segments associated with drug resistance (Beverley et al. 1984; Walton et al. 1986) or neoplasia (Bishop 1991; Brison 1993; Stark 1993). They are the substrates for multiple pathways of DNA recombination in *Escherichia coli* (Cromie et al. 2000) and the products of processes as diverse as the repair of chromosome breaks (Windle et al. 1991; Coquelle et al. 1997) and programmed cell differentiation (Coyne et al. 1996) in mammalian cells.

Long DNA palindromes have been studied extensively in both prokaryotes and eukaryotes and earlier investigations have led to multiplicity of suggestions about the mechanism of their formation (Kikuchi et al. 1985; Ellis and Day 1986; Ford and Fried 1986; Passananti et al. 1987; Hyrien et al. 1988; Kunes et al. 1990; Fried et al. 1991; Ma et al. 1993; Butler et al. 1995, 1996; Lin et al. 1997; Lyu et al. 1999). Short inverted repeats (IRs) have been implicated in two particularly appealing models considered by Butler et al. (1995, 1996); these involve (1) intramolecular homologous recombination at cruciform structures formed by short IRs located adjacent to double-strand DNA breaks (Butler et al. 1995, 1996), or (2) the formation of palindromic DNA by single strand annealing (SSA) of IRs followed by removal of nonhomologous DNA and gap-filling DNA replication (Butler et al. 1995). Investigations in the ciliate Tetrahymena have led to the conclusion that formation of DNA palindromes by the latter mechanism is unlikely (Butler et al. 1995), and subsequent work in yeast has provided experimental support for a model of palindrome formation by intramolecular homologous recombination (Butler et al. 1996).

Although most plasmids isolated from prokaryotic and eukaryotic cells exist as DNA circles, some replicate as linear DNA (Kinashi et al. 1987; Sakaguchi 1990; Hinnebusch and Tilly 1993). *Streptomyces* is a Gram-positive filamentous spore-forming eubacterial genus that contains linear chromosomes (Lin et al. 1993) as well as a variety of stably-inherited linear plasmids ranging in length from 12 kb to >1000 kb (Kinashi et al. 1987; Keen et al. 1988; Sakaguchi 1990; Pandza et al. 1998). pSLA2 is a 17-kb high-copy-number linear *Streptomyces rochei* plasmid (Hirochika and Sakaguchi 1982; Hirochika et al. 1984) whose replication is initiated internally and proceeds bidirectionally from the origin towards the telomeres, where a leading strand 3′ overhang is filled in to produce blunt-ended duplex molecules (Chang and Co-

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hen 1994; Chang et al. 1996; Qin and Cohen 1998). As the termini of *Streptomyces* linear chromosomes closely resemble those of pSLA2 and other linear *Streptomyces* plasmids, chromosomal DNA replication is presumed to occur by a similar mechanism (Fischer et al. 1998; Huang et al. 1998; Qin and Cohen 1998).

Whereas lengthy deletions within one of the two pSLA2 telomeres normally prevent pSLA2 replication as a linear plasmid (Qin and Cohen 1998), we observed that such damaged plasmids can survive by becoming large palindromic linear DNA replicons containing a normal telomere at each end. The ability to create collections of independently formed palindromic derivatives of the same molecular species in *Streptomyces* provided an opportunity to investigate in detail the mechanism of palindrome formation. Our findings indicate that palindromic linear replicons can be produced in *Streptomyces* by unimolecular nonrecombinational events involving (1) intra-strand annealing of short IRs located on a single strand DNA segment, and (2) replication/extension from the 3′ terminus using a strand of the folded back DNA molecule as a template.

Results

Formation of palindromic linear replicons following telomere damage in linear plasmids

During investigations of sequence requirements for propagation of the *S. rochei* plasmid pSLA2 (Qin and Cohen 1998), we observed that constructs having only one intact telomere occasionally yielded viable plasmid replicons. To determine the nature of such plasmids, we constructed pSLA2 derivatives containing a functionally defective telomere that includes only the terminal 56 bp (Qin and Cohen 1998) (pQC110; Fig. 1); these were linearized with *Dra*I endonuclease and introduced by transformation into *Streptomyces lividans* strain ZX7. As seen in Table 1, using >1 μg plasmid DNA and ZX7 protoplasts transformable at high efficiency, we obtained stable transformants; these were observed at 0.01× the transformation frequency seen for pSLA2 replicons containing two functional telomeres. Similar results were obtained for constructs containing a telomere damaged at other sites or lacking one telomere entirely (data not shown).

As seen in Figure 1, the distance between the telomere termini of pQC110 is ~15 kb. However, gel analysis of plasmid DNA isolated from 14 individual pQC110-derived transformants showed bands corresponding to DNA species 14–30 kb in length (Fig. 2A); these DNAs were resistant to treatment with bacteriophage λ exonuclease but sensitive to *E. coli* exonuclease III (Fig. 2B), as described by Hirochika et al. (1984), Hirochika and Sakaguchi (1982), and by Chang and Cohen (1994), indicating that the rescued replicons are linear plasmids whose 5′ ends, like those of pSLA2, are protected by covalently bound terminal protein. Electrophoresis of these DNAs following treatment with 0.2 N NaOH and 1% SDS and neutralization by addition of acid-phenol-

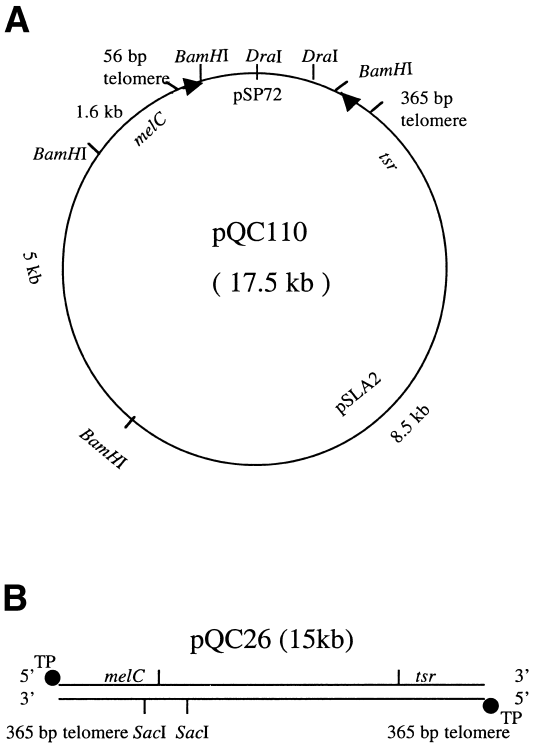

Figure 1. Schematic map of pSLA2-derived circular and linear plasmids. (*A*) pQC110, showing the *E. coli* pSP72 plasmid-derived segment and a pSLA2-derived DNA segment containing one functional 365-bp telomere (Qin and Cohen 1998) and one defective telomere containing only the terminal 56-bp sequence. Telomere ends are indicated by arrowheads. Relevant restriction endonuclease cleavage sites are shown. (*B*) pQC26, a pSLA2-derived linear plasmid isolated from *S. lividans* ZX7. Terminal protein (TP) is attached covalently to 5′ DNA termini. The locations of the *mel*C (melanin-producing) and *tsr* (thiostrepton resistance) genes are shown, as are the locations of *Sac*I cleavage sites. The plasmid contains two functional telomeres. Plasmid DNA was cloned in *E. coli*, linearized by treatment with *Dra*I enconuclease, and introduced into *ZX7* by transformation, as indicated in Materials and Methods.

chloroform (Hopwood 1985) showed bands migrating in each instance as DNA molecules containing half the original mass (Fig. 2, cf. A with C). The conversion of the DNAs to faster migrating molecules implies that they consist of palindromic duplex linear replicons that yield single-strand hairpins on denaturation/renaturation (Ford and Fried 1986; Walton et al. 1986). Consistent with this notion, we found that transformation of ZX7 with gel-purified 14.5-kb molecules from lane 7 of Figure 2C produced 29-kb linear replicons identical in size to those seen in the corresponding lane of Figure 2A.

Native and denatured/renatured linear plasmid DNA preparations isolated from 14 pQC110-derived transformants were compared by Southern blotting following digestion with *Bam*HI endonuclease (Fig. 2D,E). No preparation from any transformant showed a DNA fragment corresponding to the distance between the end of the damaged telomere and the nearest internal *Bam*HI site (see Fig. 1), implying that the damaged telomere was ab-

Table 1. *Transformation of* Streptomyces lividans ZX7 *using pSLA2-derived circular and linear plasmids*

Plasmids	Treatment[a]	Transformation frequency[b]	No. of transformant clones examined[c]
*Dra*I-linearized pQC110 (from *E. coli*)	NT	1×10^1	16
	λ *exonuclease*	2×10^2	6
	exonuclease III	3×10^1	8
	NaOH/HCl	5×10^2	10
*Sac*I-cleaved linear pQC26 (from ZX7)	NT	8×10^1	8
	λ *exonuclease*	1×10^3	19
	exonuclease III[d]	9×10^1	8
	NaOH/HCl	5×10^3	35

[a]NaOH/HCl treatment (see Materials and Methods). Partial digestion of DNA by λ *exonuclease* or *exonuclease* III. (NT) No treatment.
[b]Relative transformation frequency obtained by comparing to transformation frequency of *Dra*I-linearized pQC101 (5×10^3 transformants/μg DNA).
[c]Linear plasmid DNA replicons were isolated from individual colonies by adding proteinase K/SDS or NaOH/SDS (see Materials and Methods). In all instances, 100% of clones examined contained long-palindromic DNA.
[d]Treated with Klenow fragment of DNA *polymerase* I before *exonuclease* III treatment.

sent in every case. However, bands ~8.5 kb in length, which is the distance from the end of the intact telomere to the proximal internal *Bam*HI site (see Fig. 1), were observed for both types of DNA preparations in all plasmid survivors (Figs. 2D,E). Additionally, *Bam*HI treatment of both native and denatured DNA preparations

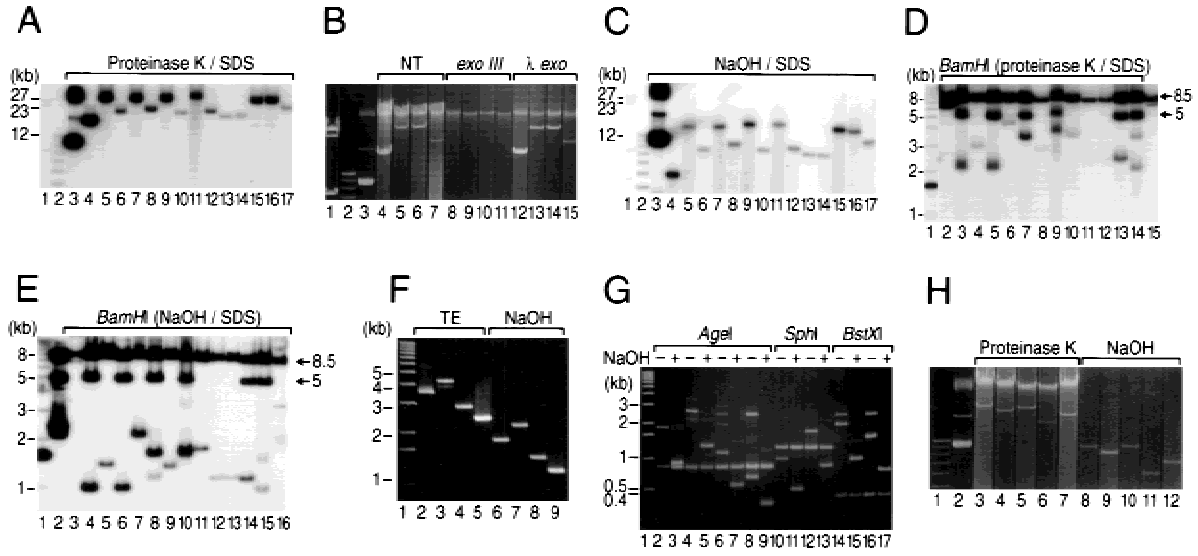

Figure 2. Southern blot of pQC110 and pQC26-derived DNAs isolated from ZX7 transformants. (*A*) DNA isolated by treatment with proteinase K and SDS (Qin and Cohen 1998) from transformants receiving *Dra*I-cleaved pQC110 DNA. DNAs were electrophoresed for 13 hr at 40 v in 0.5% agarose gel and probed with ^{32}P-labled pQC110 DNA (lanes *4–17*). Molecular lengths were calculated relative to *Hin*dIII-treated bacteriophage λ DNA (lane *1*), a 1-kb DNA-size ladder (Life Technologies, Inc.) (lane *2*), or covalently closed circular pQC110 DNA isolated from *E. coli* (lane *3*). (*B*) Surviving replicons are linear plasmids. Lanes *4–7* (NT) show DNA isolated from 4 randomly selected transformants by proteinase K/SDS treatment. Aliquots of the same DNAs were treated with 100 units exonuclease III (lanes *8–11*) or 10 units λ exonuclease (lanes *12–15*) at 37°C for 4 hr and electrophoresed for 18 hr at 38 v in 0.5 % agarose gel. λ *Hin*dIII-treated DNA (lane *1*), 1-kb DNA ladder (lane *2*), and pQC110 DNA (from *E. coli*, lane *3*) are molecular size markers. (*C*) Electrophoresis of pQC110-derived DNAs shown in *A* after treatment with NaOH and renaturation. Lane designations are as in *A*. (*D*) *Bam*HI digestion of pQC110-derived DNAs from *A*. Lane *1* contains a 1-kb ladder. Lanes *2–15* correspond to DNAs in lanes *4–17* of *A*. The 8.5-kb and 5-kb DNA bands discussed in the text are indicated. (*E*) *Bam*HI digestion of pQC110-derived DNAs following denaturation and renaturation. Lanes *2–16* correspond to DNAs in lanes *3–17* of *A*. (*F*) Effect of denaturation on migration of *Bam*HI fragments containing putative palindrome apices of linear plasmids. Agarose gel analysis of inserts recovered from agarose gel following *Bam*HI digestion of pQC143–pQC146. The banding position of DNAs dissolved in TE (lanes *2–5*) or analyzed following treatment with NaOH and neutralization is shown in lanes *6–9*. (*G*) Endonuclease analysis of *Bam*HI fragments containing putative palindrome apices. DNAs were digested by the enzymes indicated and electrophoresed for 3 hr at 80 v on 1% agarose gel. Lanes *2*, *4*, *6*, *8*, and lanes *10*, *12*, *14*, *16* correspond to lanes *2–5* from *F*. Lanes *3*, *5*, *7*, *9*, and lanes *11*, *13*, *15*, *17* correspond to lanes *6–9* from *F*. (*H*) Effect of denaturation on migration of *Sac*I-cleaved pQC26 DNA isolated from four transformants by adding proteinase K/SDS (lanes *3–7*) or NaOH/SDS (lanes *8–12*), and electrophoresed for 20 hr at 36 v in 0.5% agarose gel. Lane *1* (1-kb ladder) and 2 (pQC26, from *E. coli*) are markers.

from some plasmids released an ~5-kb band. When absent, this band was replaced by one that differed in length among different transformants and also differed in length for native versus denatured/renatured DNA. Importantly, *Bam*HI treatment of all of the native plasmid DNA preparations also generated an additional variable-length band that dropped to half its original mass following denaturation and neutralization. One plasmid DNA (Fig. 2E, lane 3) had undergone an additional rearrangement that resembled the central deletions produced by palindrome resolution and recombination in mouse germ-line cells (Akgun et al. 1997).

Together, the above observations suggested that deletions of different lengths had occurred in the damaged arm of pQC110 and that a process leading to the formation of palindromic replicons had duplicated the undamaged arm. To determine the correctness of this interpretation, we recovered the variable length *Bam*HI fragments from the gel shown in Figure 2D and cloned these fragments in *Bam*HI-cleaved pSP72, yielding the plasmids pQC143, pQC144, pQC145, and pQC146, respectively, from lanes 10, 6, 4, and 11. The *Bam*HI pQC110-derived insert of each of these plasmids was reduced to half the original duplex DNA mass following denaturation and renaturation (Fig. 2F), indicating that the inserts are palindromic. These inserts were recovered from the Figure 2F gel, treated further with *Bst*XI, *Age*I, or *Sph*I, and analyzed again by electrophoresis (Fig. 2G). The resulting cleavage patterns established that these fragments contain the apices of palindromes, each limb of which includes a copy of the replication origin of pSLA2 (Fig. 3).

The above results indicate that rescue of telomerically damaged *Streptomyces* linear plasmid replicons had occurred by deletion of the plasmid arm containing the defective telomere and duplication of the plasmid arm containing a functional telomere. The longest deletion in the plasmids we analyzed extended to a position just short of the pSLA2 *rep2* gene (Chang et al. 1996), an essential component of the replication origin, indicating that no genes between *rep2* and the damaged telomere are required for propagation of pSLA2.

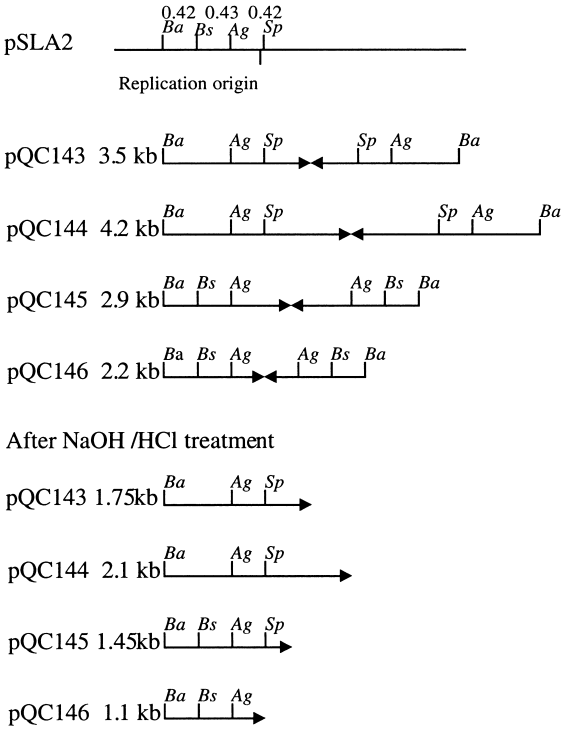

Figure 3. Diagrammatic interpretation of enzyme digestion pattern in Figure 2G. The apices of long palindromes are shown by pairs of arrows. Cleavage sites are (*Ba*) *Bam*HI; (*Ag*) *Age*I; (*Sp*) *Sph*I; (*Bs*) *Bst*XI. The sizes determined for *Bam*HI fragments determined by gel migration prior to or following denaturation/renaturation are shown for each plasmid. A restriction map of the replication origin region is shown at the *top*.

Mechanism of long-palindrome formation

Butler et al. (1995, 1996) have proposed a model for the formation of long DNA palindromes in both *Saccharomyces cerevisiae* and Tetrahymena by intramolecular recombination when a double strand DNA break is introduced adjacent to short IRs. To better understand the mechanism of long-palindrome formation in *Streptomyces* linear plasmids, the pSLA2-derived plasmid pQC26 (Fig. 1; Qin and Cohen 1998) isolated from *S. lividans* ZX7 was treated with *Sac*I endonuclease to intentionally produce a site-specific double-strand DNA break between one telomere and the plasmid's replication origin. As seen in Figure 2H, transformation of ZX7 by pQC26 DNA molecules that had been cleaved site-specifically by *Sac*I yielded a series of different-sized plasmid replicons, which were reduced to half their original mass following denaturation/renaturation-as had been observed for pQC110. This finding, which is consistent with the view that double-strand breaks promote long-palindrome formation (Butler et al. 1995), also suggested that the apices of the palindromes produced in different plasmids were located at varying distances (calculated to be ≤3.6 kb) from the *Sac*I-generated DNA break.

Previously, it has not been practical to directly determine the DNA sequence at the junctions of long palindromes (>100 bp) with adjoining nonpalindromic DNA (Butler et al. 1996; Devine et al. 1997), largely because rapid fold-back and base-pairing between palindrome arms prevent the binding of sequencing primers (Devine et al. 1997). To address this problem, we devised a strategy (Fig. 4A) that uses selective single-strand digestion within palindromes followed by annealing of undigested strands to produce hybrid DNAs that can be cloned and sequenced, enabling the identification of palindrome/spacer junctions and also the detection of nucleotide sequence differences between the two limbs of palindromes. We sequenced the palindrome junctions in the variable-length *Bam*HI fragments from four independent palindromic replicons derived from pQC110 replicons (pQC143, pQC144, pQC145, and pQC146) and of two pQC26-derived replicons (pQC383 and pQC384), and compared these sequences with those of the parental

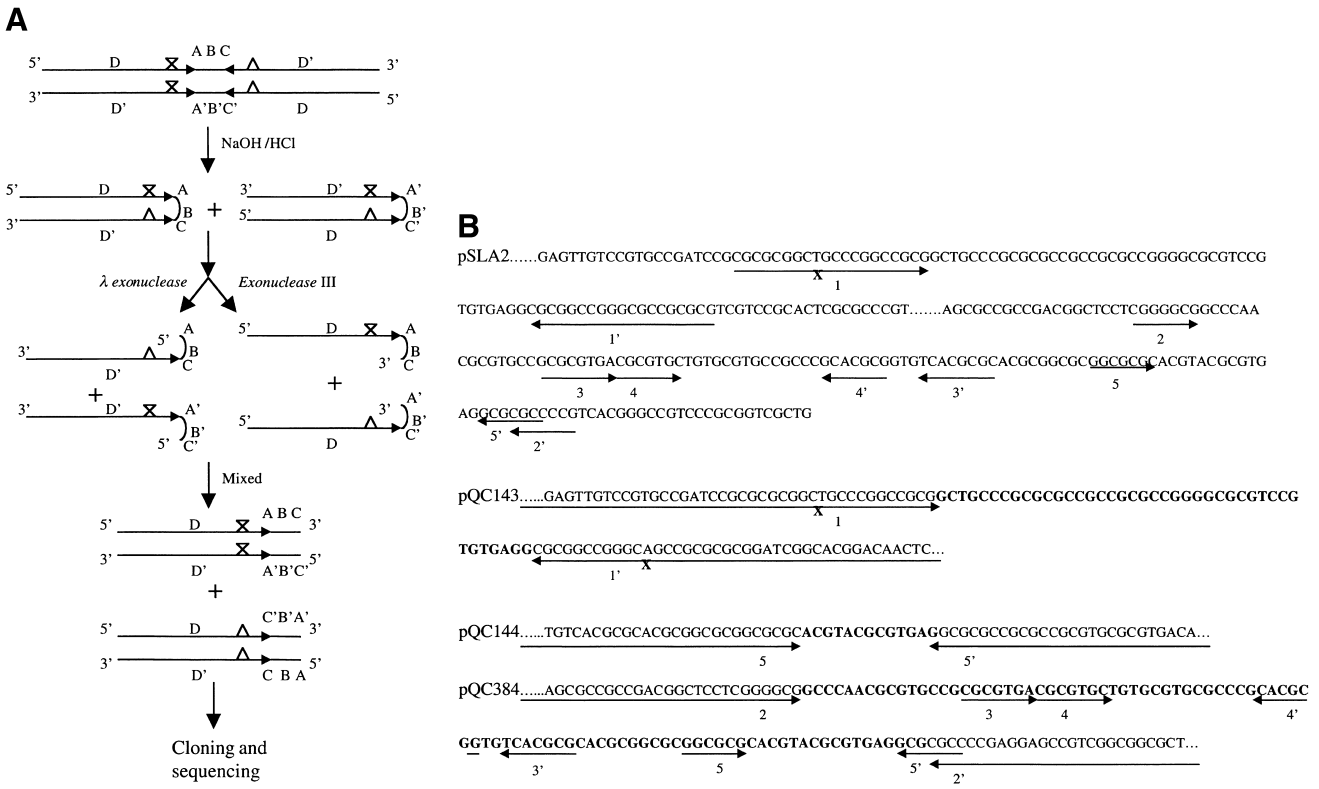

Figure 4. Strategy for cloning and sequencing long-palindromic DNA and sequencing results. (*A*) Schematic diagram of a strategy for cloning and sequencing of the junctions of palindromes with adjacent nonpalindromic DNA. DNA fragments containing long palindromes were released from a circular plasmid DNA by restriction enzyme digestion and treated as described in Materials and Methods. X and Δ indicate the position of base-pair differences between the two arms of palindromes. (*B*) Sequence analysis of the junctions of long palindromes with nonpalindromic DNA. T7 or SP6-specific primers complementary to vector sequences were used. The junctions of long palindromes from pQC143, pQC144, and pQC384 with pSLA2 DNA sequences are shown along with the corresponding segment of the parental pSLA2 plasmid. Short- and long-palindromic sequences are indicated by pairs of arrows. X designates a one base-pair difference between the two arms of a pair of short IRs of pSLA2 that was harmonized in pQC143 and another plasmid, but persistent in the long palindromes of other replicons (data not shown). Pairs of short IRs are numerically designated. Sequences between the limbs of long-palindromes (i.e., spacer regions) of pSLP2-derived plasmids are shown in bold.

pSLA2 plasmid. In all six instances, we found that a short IR sequence of pSLA2 was incorporated into the arms of the long palindrome; the results for plasmids pQC143, pQC144, and pQC384 are shown in Figure 4B. These short IRs, which differed in different palindromic replicons and ranged from 7–19 bp in length, were located at junctions of the arms of the long palindrome with a central spacer region that corresponded to the sequence present in pSLA2 between short IRs. In each palindromic plasmid, the sequence adjoining one limb of the short IR was replaced by the complement of the corresponding pSLA2 sequence, as was reported also for palindromes formed in yeast (Butler et al. 1996). The longest distances between the sequenced palindrome apices and the site of *Sac*I cleavage that produced the double-strand DNA break removing the plasmid telomere were 2.1 kb (pQC383) and 2.6 kb (pQC384).

The presence of a *mel*C (melanin-producing) gene near one telomere of pQC26 enabled us to also identify instances of palindrome formation by native linear plasmid DNA introduced into ZX7 by transformation. Whereas cells containing pQC26 normally produce black-colored colonies of tsr-resistant transformants, we observed rare Mel$^-$ *tsr*-resistant colonies (frequency <1%) after transformation by linear pQC26. Gel analysis of linear plasmids isolated from two such colonies indicated that they had deleted the telomere adjacent to *mel*C and had become large palindromic replicons that included the *tsr* telomere (Fig. 1) at both ends.

The finding of pairs of short IRs at the apices of long palindromes in palindromic linear plasmid replicons of *Streptomyces* is consistent with previous evidence that short IR sequences are an essential ingredient for long-palindrome formation in other species (Yasuda and Yao 1991; Butler et al. 1995, 1996). These IRs were proposed to form cruciform structures that promote intramolecular recombination in yeast and Tetrahymena (Butler et al. 1995, 1996). To investigate the mechanism by which short IRs may promote palindrome formation in *Streptomyces*, we tested the ability of pQC26 DNA that had been cleaved by *Sac*I and then denatured, and also of similarly-treated total DNA from pQC26-containing cells, to yield palindromic linear plasmids. We found that all of 35 randomly selected transformants receiving

denatured *Sac*I-cleaved DNA, and all 8 transformants that received undenatured *Sac*I-cleaved DNA, contained palindromic linear plasmid replicons and that the transformation frequency increased 50-fold following denaturation (see Table 1). Additionally, trimming back the *Sac*I-generated 5′ end of pQC26 DNA by λ exonuclease (Chang and Cohen 1994; Qin and Cohen 1998) increased the frequency of formation of palindromic linear replicons 10-fold (Table 1), whereas 3′–5′ single-strand digestion by *E. coli* exonuclease III had no effect on this frequency. These results indicate that the formation of long DNA palindromes is favored by conditions that promote single-strandedness of 3′ termini. Additionally, our finding that *Sac*I-generated DNA fragments that have been denatured can generate palindromic linear replicons in *S. lividans* (Table 1) indicates that palindrome formation in this species does not require a cruciform substrate nor intramolecular homologous recombination.

Discussion

The investigations reported here indicate that giant palindromic replicons in *Streptomyces* linear plasmids can be produced by intra-strand annealing of, and turnaround replication at, short IR sequences following a double-strand DNA break telomeric to both a short IR and replication origin. The unimolecular intra-strand annealing (ISA) mechanism proposed here for the formation of long DNA palindromes in *Streptomyces* differs from the cruciform/recombination model proposed by Butler et al. (1995,1996) as the mechanism for palindrome formation in yeast and Tetrahymena. We show that neither cruciform formation nor recombination is essential to generate long DNA palindromes in *Streptomyces*: Single-stranded linear plasmid DNA molecules containing a replication origin, one functional telomere, short IRs, and *Sac*I-generated end can form palindromic replicons. A second intramolecular recombination model based on the single-strand annealing mechanism (SSA) developed from work with mammalian cells (Lin et al. 1984; Fishman-Lobell et al. 1992; Jeggo 1998) was also considered by Butler et al. (1995) but discarded as a plausible mechanism for long-palindrome formation. Like the nonrecombinational ISA model of palindrome formation suggested by the data we have obtained for *Streptomyces*, the SSA mechanism of Butler et al. (1995) involves duplex formation between short IRs located on the same strand.

DNA breakage, which can occur at a distance of several kilobases from short IRs produced by intrastrand annealing of sequences that can be as short as 7 nucleotides in length, may result from restriction endonuclease cleavage, defective DNA replication, or environmental insult by chemical or physical agents (e.g., Jeggo 1998). We suggest that the annealing of randomly located short IR limbs can generate a substrate for long palindrome formation when present on single-strand 3′ overhangs protruding from duplex DNA or on entirely single-stranded molecules (Fig. 5). According to this model, trimming back of unpaired sequences 3′ to the DNA

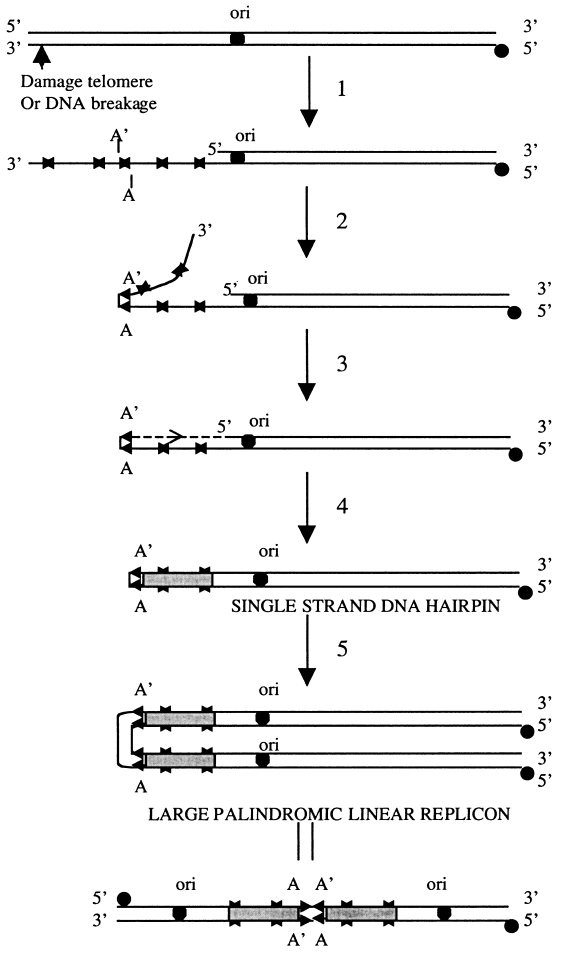

Figure 5. Model for long-palindrome formation in *Streptomyces*. Following double-strand breakage of the DNA of linear replicons, the 5′ end at the site of breakage is digested by a single-strand-specific 5′–3′ exonuclease to make a long 3′ single-stranded overhang (step 1). Step 2 is fold-back annealing of 3′ single-stranded overhang at an internally located pair of short IRs (marked by arrowheads A and A′). This is followed by exonucleolytic removal of unpaired sequences distal to the paired IR and extension of the 3′ end to produce a giant hairpin molecule (steps 3 and 4). After bidirectional replication, a large-palindromic linear replicon is formed (step 5).

segment formed by IR intra-strand annealing generates a 3′ end that is extended on a template consisting of the parental DNA sequence adjacent to the IR limb. Such extension of the folded-back DNA strand duplicates the functional arm of the parental plasmid. Thus, long-palindrome formation by the ISA mechanism involves single-strand DNA digestion and replication of an intermediate hairpin structure rather than DNA fusion. As occurs in long palindromes of yeast and Tetrahymena, the limbs of the short IR form the apex of the long *Streptomyces* palindrome and the sequence located between the short IR becomes the central spacer of the long palindrome. We suggest that the mechanism proposed for the formation of long DNA palindromes in *Streptomyces* may be applicable to other organisms as well. Neverthe-

less, there potentially may be multiple mechanisms by which long palindromes can be formed in different species and our data do not exclude other possible mechanisms more complicated than the one we have described.

Replication of hairpin DNA molecules generated by extension of the 3′ DNA terminus produces giant palindromic duplex linear *Streptomyces* plasmids containing two copies of the replication origin. Our results indicate that on denaturation and neutralization of the DNA of such plasmids, base-pairing of complementary sequences regenerates hairpin molecules containing an intact telomere sequence at each end. When these are introduced into cells, they replicate to again produce duplex linear plasmids containing long palindromes.

Potentially, intra-strand annealing of other pairs of short IRs located in the single-strand fold-back segment of pSLA2 may assist in stabilizing annealing of the apical IR by dividing the fold-back loop into multiple duplex and single-strand segments. Consistent with this notion is our finding by analysis of GenBank sequences that the 215-bp central spacer regions of naturally occurring linear plasmid DNA molecules from the yeast *Kluyveromyces lactis* (Kitada and Gunge 1988) also contain multiple short IRs, which may promote stabilization of the structure as hypothesized for pSLA2. We suggest that the novel sequencing strategy that has enabled us to examine the palindrome spacer junctions of multiple molecules and consequently to arrive at these conclusions may be useful in studying long-palindromic DNA from other organisms.

Materials and methods

Strains, plasmids, and general methods

S. lividans strain ZX7 (Zhou et al. 1988) was used as the *Streptomyces* host strain. *E. coli* DH5α (Life Technologies, Inc.) and pSP72 (Promega, Inc.) were the *E. coli* host and cloning vector, respectively. Standard methods were used for culturing cells and DNA cloning in *E. coli* (Sambrook et al. 1989), and *S. lividans* (Hopwood 1985). Long-palindromic DNA was cloned in strain DH5α or JC7623 (Gibson et al. 1992) and DNA was extracted from agarose gel by using the Qiaquick Gel Extraction Kit (Qiagen, Inc.). Linear plasmid DNA was isolated from colonies grown on R5 plates or liquid culture as described by Qin and Cohen (1998). Southern blot hybridization used the procedure of Church and Gilbert (1984). Sequencing of DNA was done by using an Applied Biosystem (ABI, Inc.) Prism 310 Genetic Analyzer and the ABI dye terminator sequencing kit.

Cloning and sequencing the junctions of long-palindromic DNA

Cloned plasmids containing *Bam*HI DNA fragments that included long palindromes were digested with the indicated restriction endonuclease and the projecting single-strand segment filled in using the Klenow fragment of DNA polymerase I (Life Technologies, Inc.) in the presence of dNTPs to make blunt-ended DNA. DNA fragments recovered from low-melting-point agarose gels using a Qiaquick Gel Extraction were denatured by adding 0.2 N NaOH at 37°C for 10 min, then neutralized at 65°C by adding 0.2 N HCl and 0.1 N TrisHCl (pH 8.0) and incubating for 10 min. After precipitation by addition of isopropanol and ethanol (Hopwood 1985), DNA was dissolved in 50 µl TE (10 mM TrisHCl, pH 8.0; 1 mM EDTA). Aliquots of DNA were incubated with 100 units of *E. coli* exonuclease III or 10 units of bacteriophage λ exonuclease (either purchased from Life Technologies, Inc. or a gift of Drs. Deb Chatterjee and Per Harbury) at 37°C for 1 hr and the completeness of their digestion was confirmed by gel electrophoresis. DNA samples digested by λ exonuclease or exonuclease III were mixed and annealed at 65°C for 2 hr following addition of 2× SSC. DNAs were precipitated by addition of isopropanol and ethanol, dissolved in TE, and treated with 0.5 µ Klenow fragment of DNA polymerase I at 37°C for 5 min. One µl 0.1 mM dNTP mix was added and samples were incubated for another 5 min. DNAs were electrophoresed in agarose gel (0.7%–1.4%, depending on expected length of annealed DNA fragments) and bands of the expected sizes were recovered and ligated into pSP72 treated with *Eco*RV. Ligated DNA was introduced into DH5α by transformation. Clones containing plasmids were identified and sequenced by PCR using a T7 or SP6 primer complementary to pSP72 sequences.

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