Identification of two linear plasmids in the actinomycete *Planobispora rosea*

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Two linear plasmids (pPR1, 27·5 kb, and pPR2, 16 kb) were identified in *Planobispora rosea*, an actinomycete that produces the antibiotic GE2270, an inhibitor of the elongation factor Tu. Strains lacking both plasmids still produce and are resistant to GE2270. The two plasmids share an internal region of high similarity, but no cross-hybridization was detected between their telomeric regions or between plasmid and chromosomal DNA. The 5′ ends of the plasmids appear to be linked to terminal proteins. The telomeric regions of pPR2 were cloned after 3′-end homopolymer tailing and PCR amplification. The approximately 650 nt telomeric DNA sequences of pPR2 are repeated in inverted orientation and are rich in direct and inverted repeats; the 350 bp terminal region is less G C-rich than the rest of the plasmid. The structural organization of these plasmids appears to be similar to *Streptomyces* linear replicons.

Keywords: telomeres, terminal protein, *Actinomycetales*, replication, inverted repeats

INTRODUCTION

Reports of linear DNA molecules in prokaryotes have become increasingly frequent and linear bacteriophages, plasmids and bacterial chromosomes no longer appear an exception to the paradigm of the circular bacterial replicon (for reviews see Hinnebusch & Tilly, 1993; Chen, 1996).

Numerous linear plasmids have been found in several *Streptomyces* species (Kinashi *et al*., 1987; Sakaguchi, 1990), and occasionally in other *Actinomycetales* (Crespi *et al*., 1992; Kalkus *et al*., 1990, 1993). These extrachromosomal replicons range in size from 12 to more than 600 kb (Sakaguchi, 1990; Kinashi *et al*., 1987), and more than one plasmid may coexist in the same host. Interestingly, the *Streptomyces* chromosome is also linear (Lin *et al*., 1993; Chen *et al*., 1993; Lezhava *et al*., 1995; Leblond *et al*., 1996), although circular forms have been described for some deletion mutants in *Streptomyces lividans* (Lin *et al*., 1993).

All the *Streptomyces* linear plasmids characterized thus far share structural features: the telomeric regions are long inverted repeats from 0·6 to 81 kb in length containing direct and inverted repeated sequences (Hirochika *et al*., 1984; Kinashi *et al*., 1991; Wu & Roy, 1993). In most cases analysed, a terminal protein is covalently linked to the 5′ ends of the DNA molecule. In a few instances the presence of such plasmids has been associated with the production of antibiotics (Kinashi *et al*., 1987; Davies, 1994).

Two main types of telomeric organization have been described in bacterial linear replicons: (i) covalently closed palindromic hairpin loops, as exemplified by the plasmid prophage form of coliphage N15 (Malinin *et al*., 1992; Svarchevsky & Rybchin, 1984; Łobocka *et al*., 1996) and the linear plasmids and chromosome of *Borrelia* (Hinnebusch & Barbour, 1991; Barbour *et al*., 1996; Casjens *et al*., 1997; Fraser *et al*., 1997); and (ii) terminal inverted repeats with 5′ ends covalently linked to a terminal protein, as exemplified by the well characterized *Bacillus subtilis* phage Φ29 (reviewed by Salas, 1991) and by several *Streptomyces* plasmids (Sakaguchi, 1990). The mechanism of replication of the former type of linear replicon has not yet been detailed, whereas two different replication strategies have been described in the latter type: (i) Φ29 replication starts at both ends, using the terminal protein as the primer, and proceeds throughout the whole DNA molecule; (ii) several streptomycete plasmids and linear chromosomes, as well as the linear plasmids of *Borrelia*, appear to replicate bidirectionally from a unique internal origin; in such instances, telomere-promoted replication appears to be confined to the terminal regions of the

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linear molecule (Casjens & Huang, 1993; Chang & Cohen, 1994; Chang *et al.*, 1996).

Planobispora rosea is an actinomycete that produces the antibiotic GE2270, a thiazolyl peptide inhibitor of the translation elongation factor Tu (Goodfellow, 1992; Selva *et al.*, 1991; Anborgh & Parmeggiani, 1991; Tavecchia *et al.*, 1995). Here, we report the identification and preliminary characterization of two linear plasmids (pPR1, 27·5 kb long, and pPR2, 16 kb) from this strain. They were found to share structural features with other *Streptomyces* linear replicons.

METHODS

Bacterial strains and plasmids. *P. rosea* (ATCC 53733) strains Pbr 1435, Pbr 1438, Pbr 1439, Pbr 1440, Pbr 1441 and Pbr 1832 were obtained from the Biosearch Italia (formerly Lepetit Research Center) strain collection and were cultivated as described by Selva *et al.* (1991). GE2270 production was measured as described by Selva *et al.* (1991). Plasmid pGEM-3Z (Promega) was used for cloning pPR1 and pPR2 restriction fragments. *Escherichia coli* DH5α (Hanahan, 1983) and JM109 (Yanisch-Perron *et al.*, 1985) were used as recipients in transformations with the recombinant plasmids.

DNA preparation. Total DNA of *P. rosea* was prepared from 5 g wet-packed mycelia according to Fishman & Hershberger (1983) with some modifications. In brief, the mycelium resuspended in 10 mM Tris, 0·125 M EDTA, 12·5% (w/v) sucrose was treated with lysozyme (10 mg ml^{-1}) for 15 min at room temperature. Proteinase K was added to a final concentration of 50–150 μg ml^{-1} and the lysed suspension was incubated at 65 °C for 1 h before precipitation with 0·5 M NaCl for 2 h on ice. After centrifugation at 42000 ***g***, the DNA in the supernatant was precipitated with 2-propanol and pelleted by centrifugation.

Linear plasmid DNA was purified from total *P. rosea* DNA by 10–30% sucrose/1 M NaCl gradient centrifugation in a Beckman SW40 ultracentrifuge rotor. About 50–150 mg total DNA per 12 ml sucrose gradient was centrifuged for 10 h at 36000 r.p.m. at 18 °C, collected in 0·7 ml fractions, precipitated with ethanol and pelleted by centrifugation. The dried pellet was dissolved in Tris/EDTA pH 7·2 and analysed by electrophoresis in agarose gels. The fractions that contained predominantly plasmid DNA were pooled. Alternatively, pPR1 and pPR2 DNA was purified from chromosomal DNA by inverted-field agarose gel electrophoresis with a Bio-Rad Pulsewave 750 apparatus and electroeluted from the gel. Ethidium bromide/CsCl equilibrium gradient centrifugation was performed as described by Maniatis *et al.* (1982).

DNA manipulations. Poly(dT) or poly(dA) tailing of DNA fragments was performed using calf thymus terminal transferase (Boehringer) under the conditions suggested by the manufacturer. The tailed DNA was then copied for 10 cycles (1 min, 95 °C; 1 min, 53 °C; 1 min, 72 °C) with *Taq* DNA polymerase (Promega) and PCR-amplified for 30 cycles (1 min, 95 °C; 1 min, 45 °C; 1 min, 72 °C) using either $poly(dA)_{35}$ or $poly(dT)_{35}$ and the oligonucleotide AGAATGGCCACCATAGAAATCCGAT (named PLANEND, from base 336 to 312 on the complementary strand of pPR2 left end; see Fig. 4) as primers.

Southern blot hybridization was performed as described by Maniatis *et al.* (1982) on Hybond-N (Amersham) nylon membranes. ^{32}P-labelled pPR1 and pPR2 DNA probes were prepared by random primer extension with the Prime-a-Gene labelling kit (Promega).

DNA sequencing and sequence analysis. pPR2 restriction fragments cloned in pGEM3-Z were sequenced by the method of Sanger *et al.* (1977) using synthetic oligodeoxynucleotides that primed replication either within the vector plasmid sequence or in previously sequenced pPR2 regions. Sequences were analysed with the GCG software package (Devereux *et al.*, 1984). The GenBank accession numbers for the sequenced regions of pPR2 are AF041863, AF041864 and AF041865 for the internal sequence spanning the region of homology with pPR1, the left telomeric region and the right telomeric region, respectively.

RESULTS AND DISCUSSION

Identification and characterization of pPR1 and pPR2

Six strains of *P. rosea* from the Lepetit strain collection were examined for the presence of extrachromosomal elements. When the total bacterial DNA was extracted by procedures in which proteinase K treatment was performed prior to phenol extraction (either according to Fishman & Hershberger, 1983 or Hintermann *et al.*, 1981), and fractionated by either standard or inverted-field agarose gel electrophoresis, two discrete bands (see Fig. 3), in addition to the chromosomal DNA, could be detected in strain Pbr 1435 and Pbr 1832, but not in the other strains. The slow- and the fast-migrating bands, named pPR1 and pPR2, respectively, could not be detected when the DNA was obtained by procedures in which phenol extraction was performed prior to proteinase K treatment. This suggested that the plasmid DNA was tightly associated with proteins and was thus lost in the organic phase.

Ethidium bromide/CsCl equilibrium gradient centrifugation of Pbr 1435 DNA indicated that both pPR1 and pPR2 had the same buoyant density as chromosomal DNA: by agarose gel electrophoresis of the gradient fractions, plasmid DNA was detected only in the fractions containing chromosomal DNA (data not shown). This suggested that pPR1 and pPR2 could be linear DNA molecules.

We purified the pPR1 and pPR2 DNA either by sucrose gradient centrifugation or by preparative inverted-field agarose gel electrophoresis and performed a restriction analysis of the two plasmids (Fig. 1). These experiments provided conclusive evidence that the plasmids were linear since: (i) none of the 13 enzymes used produced a single fragment that migrated more slowly than the undigested plasmid, as expected for a single cut of a CCC DNA molecule; (ii) the sum of the M_r of the fragments obtained by each of several digestions was approximately equal to the M_r of the undigested plasmid; (iii) the number of fragments obtained by digestion with two restriction enzymes was equal to the sum of the number of fragments obtained by the two single digestions minus one, which is diagnostic of a linear molecule. In these experiments the size of pPR1 and pPR2 was estimated to be 27·5 and 16 kbp, respectively.

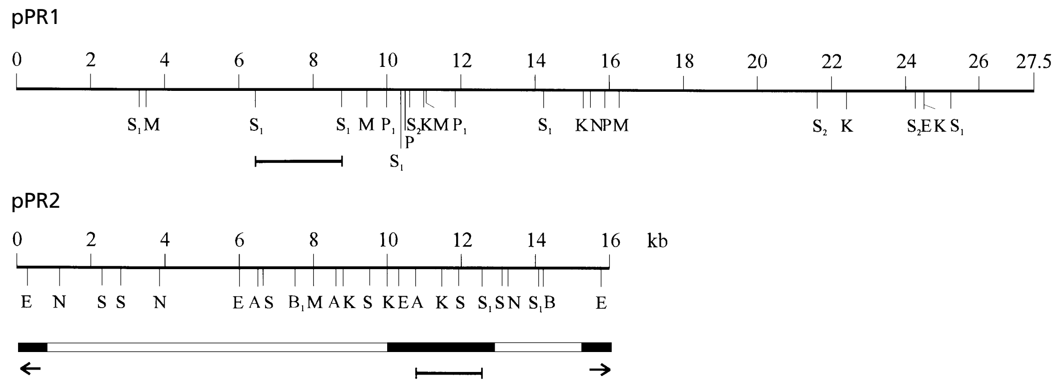

Fig. 1. Restriction maps of pPR1 and pPR2. The maps were established in part by restriction analysis of purified pPR1 or pPR2 DNA and in part by Southern blot analysis of total *P. rosea* DNA digests using ^{32}P-labelled pPR1 or pPR2 DNA as probes. Ambiguities were resolved by restriction analysis of cloned fragments and by inspection of sequenced regions. A, *Apa*LI; B, *Bgl*II; B_1, *Bam*H1; E, *Eco*RI; K, *Kpn*I; M, *Mlu*I; N, *Nhe*I; P, *Pvu*II; P_1, *Pst*I; S, *Sph*I; S_1, *Sac*I; S_2, *Sal*I. *Xho*I did not cut either plasmid. To confirm plasmid linearity, pPR1 was double-digested with *Nhe*I/*Mlu*I and *Sal*I/*Pst*I, and pPR2 with *Bgl*II/*Bam*HI, *Bgl*II/*Mlu*I and *Nhe*I/*Mlu*I. Bars indicate the regions of homology between the two plasmids; black rectangles indicate the sequenced regions; arrows indicate the terminal inverted repeats at the pPR2 ends.

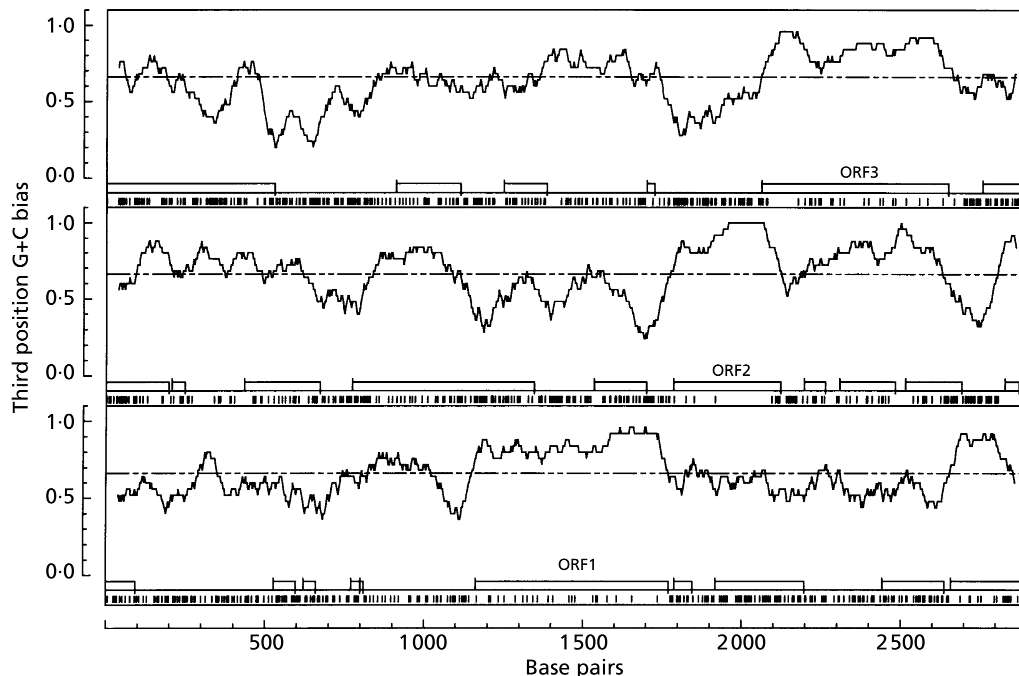

Fig. 2. Identification of potential protein encoding sequences in the homologous region of pPR2. The pPR2 sequence encompassing the region of homology between pPR2 and pPR1 was examined using the CODONPREFERENCE algorithm of the Wisconsin package, with the codon preference table Sererme2 (GenBank). In the plots, which report the results for each reading frame, the straight broken line shows the mean G+C content, whereas the irregular curve gives the G+C bias in the third position of the reading frame. The horizontal boxes indicate reading frames starting either with AUG or outside the sequence; in the strip below, each vertical line marks the occurrence of a rare codon. Plots of the complementary strand did not show open reading frames with a high G+C bias or infrequent occurrence of rare codons.

DNA extracted from the six *P. rosea* strains and digested with *Bam*HI and *Pvu*II was analysed by Southern blot hybridization using either pPR1 or pPR2 ^{32}P-labelled DNA as a probe. Only Pbr 1435 and Pbr 1832 gave all the hybridization signals expected for each linear plasmid; the other strains did not hybridize with either plasmid probe (data not shown), ruling out the presence of integrated forms. Since all six *P. rosea* strains both produce and are resistant to the GE2270 antibiotic, these traits are likely to be chromosomally encoded. This is supported by the following observations: (i) recently it has been shown that the *tuf* gene of *P. rosea* encodes an

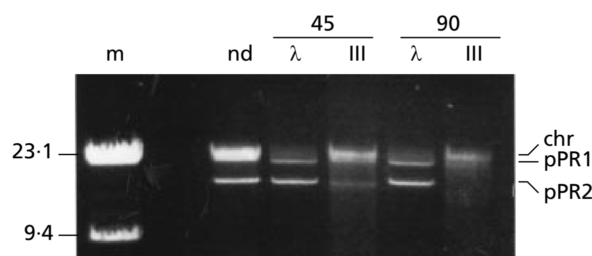

Fig. 3. Exonuclease analysis of *P. rosea* plasmid DNA. Total DNA of Pbr 1435 prepared as described in Methods was treated at 37 °C with λ exonuclease (lanes λ) or exonuclease III (lanes III). Non-digested DNA (lane nd) or DNA digested for 45 and 90 min (lanes labelled 45 and 90, respectively) was fractionated by 0·5 % agarose gel electrophoresis and visualized under UV light after ethidium bromide staining. Under such conditions the pPR1 DNA migrates at the lower edge of the chromosomal DNA band (chr). Lane m contains molecular mass markers with sizes in kb indicated on the left.

elongation factor Tu highly resistant *in vitro* to GE2270 (Sosio *et al*., 1996); (ii) a chromosomal gene cluster seemingly encoding a biosynthetic pathway for this antibiotic peptide has been cloned and sequenced (M. Sosio & S. Donadio, unpublished).

Southern blot analysis of plasmid-carrying *P. rosea* DNA also revealed cross-hybridization at high stringency between the third (from the left) *Sac*I fragment of pPR1 and the fourth *Eco*RI fragment of pPR2. By Southern blot hybridization using subcloned fragments of pPR2 as probes, the region of homology between the two plasmids was restricted to the *Apa*LI–*Sac*I fragment of pPR2 shown in Fig. 1 (data not shown).

The DNA fragment encompassing the homologous region was sequenced and the sequence was analysed by the CODONPREFERENCE program (Gribskov *et al*., 1984), applying the codon usage table compiled for *Streptomyces*. The analysis revealed the presence of three open reading frames (ORF1, ORF2 and ORF3; Fig. 2), with a high third-position G+C bias and infrequent usage of rare codons. Such ORFs are good candidates for coding genes (Bibb *et al*., 1984). BLAST analysis (Altschul *et al*., 1990) found similarities between deduced ORF1, ORF2 and ORF3 proteins and the RecF of *Mycobacterium tuberculosis* (SWISS-PROT Q59586), a guanylyltransferase of *Xanthomonas campestris* (SWISS-PROT P29956) and several MutT proteins (Michaels & Miller, 1992), respectively. ScanProsite search against the PROSITE database (Appel *et al*., 1994) found the MutT signature (Koonin, 1993) at aa 169–188 of the ORF3-encoded protein. The homologous region of pPR1 and pPR2 might thus code for functions related to recombination and/or DNA repair.

The presence of a common region on two otherwise non-homologous plasmids might suggest the presence of a transposable element. However, we could not detect by sequence analysis diagnostic features of transposons, such as inverted repeats bracketed by direct repeats or ORFs with similarity to known transposases. Also, we did not detect upon Southern blot analysis the presence of additional plasmids that could have arisen by homologous recombination between the two plasmids.

The 5′ ends of pPR1 and pPR2 are protected from λ exonuclease digestion

Many linear plasmids and viruses have a terminal protein linked to their 5′ ends (Salas, 1991; Chen, 1996); their DNA is therefore insensitive to specific 5′–3′ DNA exonuclease digestion, even after proteinase K treatment (Ito, 1978). To determine whether this was true for pPR1 and pPR2, we tested the plasmid DNA for sensitivity to *E. coli* exonuclease III and phage λ exonuclease, which degrade dsDNA 3′–5′ and 5′–3′, respectively. As shown in Fig. 3, DNA exonuclease III degraded both chromosomal and plasmid DNA, whereas λ exonuclease degraded chromosomal DNA but not pPR1 and pPR2. These data, together with the observation reported above that in cell lysates not treated with protease the plasmids are lost in the organic phase during DNA extraction, suggest that the 5′ ends of pPR1 and pPR2 are covalently linked to a terminal protein.

Cloning and sequence analysis of the telomeric regions of pPR2

Attempts to directly clone restriction fragments containing either end of the plasmid failed, even after proteinase K or piperidine treatment of the plasmid DNA. To clone the telomeric regions of pPR2 we thus adopted the following strategy: first we cloned the subtelomeric *Eco*RI fragments and determined the sequence of both left and right telomere-proximal regions. It turned out that such sequences were nearly perfect inverted repeats about 350 nt long (see Fig. 4). Then proteinase-K-treated pPR2 DNA was doubly digested with *Nhe*I, which gives a 1·15 kb fragment containing the left end, and *Bgl*II, which gives a 1·55 kb fragment containing the right end (Fig. 1). The digested DNA was fractionated by agarose gel electrophoresis and the bands corresponding to the left and right ends were excised and purified. The DNA was treated with terminal transferase in the presence of dTTP to tail the 3′ ends, denatured and replicated with *Taq* polymerase for ten cycles using poly$(dA)_{35}$ as a primer; PCR was then performed with poly$(dA)_{35}$ and the PLANEND oligonucleotide as primers, the amplified DNA was digested with *Eco*RI and cloned in *Eco*RI/*Sma*I-digested pGEM-3Z. Four clones containing the left end and two containing the right end were obtained and sequenced. All such clones gave identical sequences 310 nt long from the *Eco*RI site to the run of As at the 5′ end corresponding to the plasmid extremities. To define whether any such A belonged to the pPR2 5′ termini, we repeated the above procedure with the right end DNA except that we added a poly(dA) tail at the 3′ end with terminal transferase and amplified the DNA with poly$(dT)_{35}$. Upon cloning and sequencing the amplified DNA, no A was found at the 5′ end of the right pPR2 telomere.

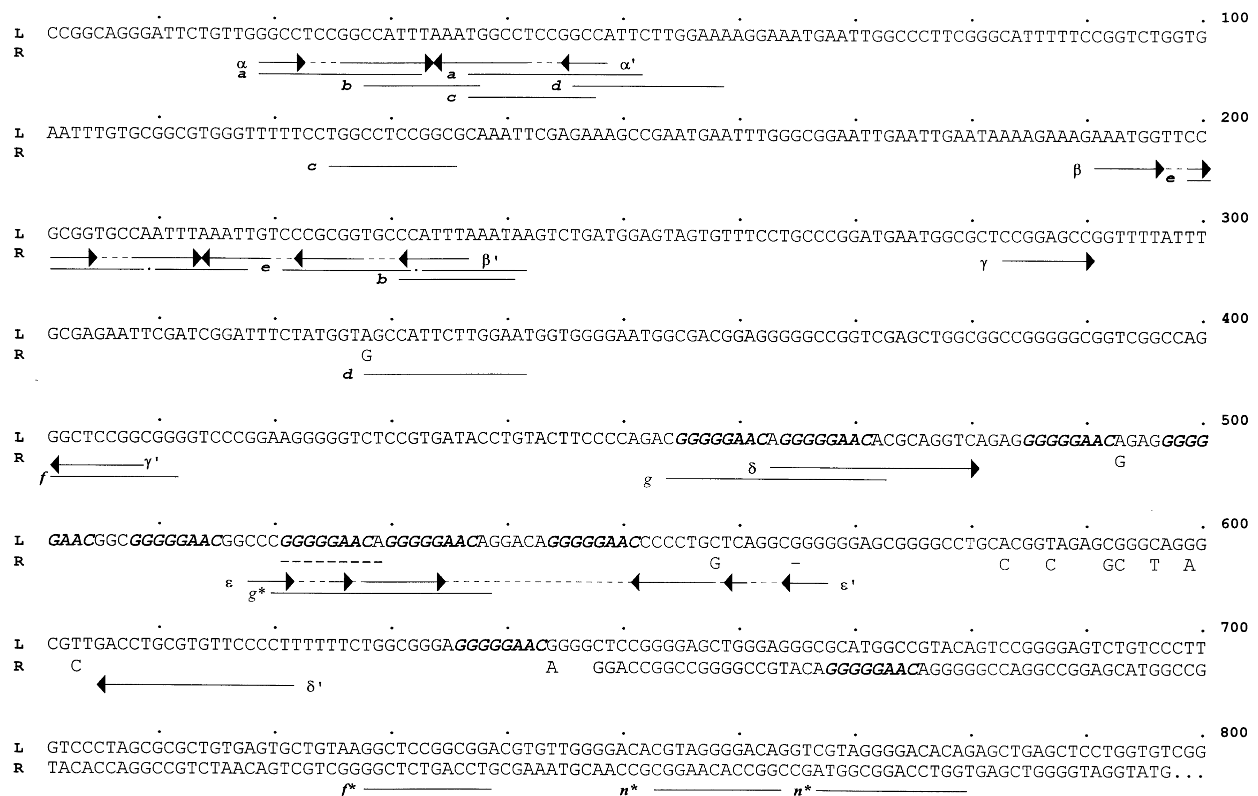

Fig. 4. Sequence of the pPR2 telomeric regions. The 5′–3′ sequence of the left end, starting from the terminal base, is reported on the upper line marked L. Differences observed in the right end sequence are indicated in the lower line (R). Major inverted repeats (arrows labelled with Greek letters) and direct repeats longer than 9 nt (lines labelled with Roman letters) are shown beneath the sequence; an asterisk indicates repeats present only in the left end. The GGGGGAAC repeat is in boldface italics.

The pPR2 telomeric regions (Fig. 4) are composed of two nearly perfect inverted repeats approximately 650 nt long, rich in direct and inverted repeats. The 350 nt long terminal domain has a low G + C content (50 mol%), followed by 250 nt rich in G + C (74 mol%) and then by DNA with a base composition (66 mol% G + C) similar to the other sequenced regions of the plasmid. The G + C-rich region is characterized by multiple direct repetitions of the GGGGGAAC sequence.

Southern blot analysis of restriction-enzyme-digested total *P. rosea* DNA using pPR2 telomeric DNA as a probe gave only hybridization signals corresponding to the pPR2 termini, even at low stringency (data not shown). Thus pPR2 telomeres do not seem to share any significant sequence similarity with those of pPR1 or any chromosomal sequence of *P. rosea*. In addition, a computer search did not detect any significant sequence similarity to the telomeric regions of other linear DNA molecules.

In conclusion, we have identified two linear plasmids of *P. rosea* that add to the growing list of prokaryotic linear replicons. Both exhibit structural similarities with the *Streptomyces* linear plasmids and chromosomes in that they appear to have terminal proteins bound to the 5′ ends of their DNA; moreover, the pPR2 telomeres are composed of palindromic sequences rich in direct and inverted repeats. A preliminary analysis of pPR2 replication by two-dimensional gel electrophoresis (data not shown) suggests that pPR2 is not fully replicated starting from the ends but contains an internal origin(s). Although pPR1 and pPR2 share a common central region, they have different telomeres. This suggests that their terminal proteins may also be different.

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