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 06 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; Lobocka 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
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; Anborghi & 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 DH5a (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 agarose gel electrophoresis and performed a restriction digestions with two restriction enzymes was equal to the sum of the number of fragments 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 M₇ of the fragments obtained by each of several digestions was approximately equal to the M₇ 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.
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 32P-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
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) SacI fragment of pPR1 and the fourth EcoRI 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 ApaLI-SacI 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 EcoRI 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 Nhel, which gives a 1.15 kb fragment containing the left end, and BglII, 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) as a primer; PCR was then performed with poly(dA) as the PLANEND oligonucleotide as primers, the amplified DNA was digested with EcoRI and cloned in EcoRI/SmaI-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 EcoRI 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) as primers. Upon cloning and sequencing the amplified DNA, no A was found at the 5' end of the right pPR2 telomere.
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 GGGGAAC 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.

ACKNOWLEDGEMENTS

We thank Stefano Donadio and Daniela Ghisotti for comments. This work was supported by Gruppo Lepetit S.p.A.; S.P. was recipient of a Fondazione Adriano Buzzati Traverso fellowship sponsored by Gruppo Lepetit S.p.A.

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Received 19 February 1998; revised 28 May 1998; accepted 2 June 1998.