The terminal structures of linear plasmids from *Rhodococcus opacus*

Jutta Kalkus, Renate Menne, Michael Reh and Hans G. Schlegel

Author for correspondence: Hans G. Schlegel. Tel: +49 551 39 2364, Fax: +49 551 39 3793, e-mail: hschleg1@gwdg.de

Institut für Mikrobiologie
der Georg-August-
Universität,
Grisebachstrasse 8,
D-37077 Göttingen,
Germany

The telomers of several linear plasmids of *Rhodococcus opacus* (formerly *Nocardia opaca*) were studied. The plasmids pHG201, pHG204 and pHG205 carry proteins bound to their ends, as shown by gel retardation experiments. A sequence hybridizing with the terminal sequence of pHG207, a recombinant linear plasmid consisting of the left part of pHG204 and the right part of pHG205, which was analysed in a previous study by the authors, could be detected in all linear plasmids of the wild-type *R. opacus* strains MR11 and MR22. However, only pHG204 and pHG206 carry terminal inverted repeats (TIRs) like pHG207. Cloning and sequencing of the terminal fragment of pHG204 revealed a nearly perfect TIR of 1016 bp. In contrast, the termini of pHG201 and pHG205 share little homology. Sequence analysis of the two end fragments of pHG201 revealed a similarity of only 65% within the terminal 34/32 bp and a perfect TIR of only 3 bp. The results support the assumption that long TIRs are not absolutely necessary for replication and maintenance of linear plasmids.

Keywords: *Rhodococcus opacus*, linear plasmid, terminal inverted repeat (TIR), terminal protein (TP)

INTRODUCTION

The genus *Rhodococcus* has become a subject of interest in several aspects, including phytopathogenicity (Vantomme *et al.*, 1982), the degradation of xenobiotics (Dabrock *et al.*, 1992), and biological production of hydrogen (Schneider *et al.*, 1984; Zaborosch *et al.*, 1989; Grzeszik *et al.*, 1997a, b). The features mentioned are characteristics associated with and encoded on linear plasmids (Crespi *et al.*, 1992; Dabrock *et al.*, 1994; Kalkus *et al.*, 1990).

The wild-type strains of *R. opacus* MR11 and MR22 each harbour three linear plasmids (Kalkus *et al.*, 1990). The type strain MR11 (Klatte *et al.*, 1994; Rainey *et al.*, 1995) contains the linear plasmids pHG201 (270 kbp), pHG202 (400 kbp) and pHG203 (420 kbp), and strain MR22 contains pHG204 (190 kbp), pHG205 (280 kbp) and pHG206 (500 kbp). In the autotrophic trans-conjugant MR2253 originating from strain MR22 another linear plasmid, pHG207 (225 kbp), was found. The plasmids pHG201, pHG205 and pHG207 were

shown to enable the bacteria to grow chemolitho-autotrophically on gaseous hydrogen and carbon dioxide (Aut character), leading to the name Aut elements (Kalkus *et al.*, 1990). The smallest Aut element, pHG207, has been studied in detail previously (Fig. 1; Kalkus *et al.*, 1993). It was shown to be the product of a single crossover recombination event between the wild-type plasmids pHG204 and pHG205. The left part of pHG207 (more than 120 kbp) originates from pHG204 and the right part (more than 80 kbp) originates from pHG205. The terms left and right refer to the restriction maps of the linear plasmids (Kalkus *et al.*, 1993). Plasmid pHG207 carries an imperfect terminal inverted repeat (TIR) of 583/560 bp and has proteins bound to its ends (terminal proteins, TP); this is consistent with the model proposed for invertrons (Sakaguchi, 1990).

To obtain more information on the general structure of linear plasmids we have investigated some of the other linear plasmids in *R. opacus* cells. These co-existing plasmids can be differentiated by selective markers such as the Aut character mentioned above, thallium resistance (pHG204), and the frequency of transfer. They are not simply derivatives or multimers of a single parental plasmid (Kalkus *et al.*, 1993). In this study we demonstrate that pHG201, pHG204 and pHG205 carry TPs. Hybridization and sequence analysis showed that

Abbreviations: LMP, low-melting-point; TIR, terminal inverted repeat; TP, terminal protein.

The GenBank accession numbers for the nucleotide sequence data reported in this paper are AF001834, AF001835 and AF007825.

these plasmids are of two different types: pHG204 carries long nearly perfect TIRs, whereas in the Aut elements pHG201 and pHG205 the terminal sequences show only a weak similarity to each other.

METHODS

Bacterial strains, phage and plasmids. Sources and references of strains of *Rhodococcus opacus* used in this work are listed in Table 1. *Escherichia coli* XL-1 Blue and plasmid pBluescript SKM13+ were purchased from Stratagene. For preparation of single-stranded DNA, *E. coli* MV1184 and bacteriophage M13KO7 were used (Vieira & Messing, 1987). Strains of *R. opacus* were grown in FM-TYE medium at 30 °C as described previously (Sensfuss *et al.*, 1986). Strains of *E. coli* were routinely grown in LB medium at 37 °C. If required, the medium was supplemented with ampicillin (100 µg ml⁻¹), IPTG (50 µg ml⁻¹) and X-Gal (40 µg ml⁻¹). Cloning of the terminal fragments produced the hybrid plasmids listed in Table 2.

Isolation of linear plasmid DNA. Cells of *R. opacus* were embedded in high concentration in LMP agarose and lysed according to McClelland *et al.* (1987), using lysozyme, N-lauroylsarcosine, SDS and proteinase K as described previously (Kalkus *et al.*, 1990). Isolation of linear plasmid DNA was achieved by electroelution from the agarose plugs, followed by precipitation of the DNA with ethanol and microdialysis against water (Kalkus *et al.*, 1993). DNA of pHG206 was isolated by cutting the relevant band out of LMP agarose gels after separation of total DNA of MR22 from highly concentrated DNA-agarose plugs by PFGE. Treatments of pHG206 with restriction endonucleases were carried out *in*

situ in LMP agarose. For preparation of non-proteolytically-treated plasmid DNA, proteinase K was omitted.

Electrophoresis. PFGE was performed with the Pulsaphor system (Pharmacia) using 1% (w/v) agarose slab gels and 0.5 × TBE buffer (45 mM Tris/45 mM borate/1 mM EDTA, pH 8.5). The buffer was cooled to 13 °C and gels were run at 6 V cm⁻¹. As size standards, *Hind*III- or *Pst*I-cut λ DNA or concatemers of λ DNA (Waterbury & Lane, 1987) were used.

Cloning of the terminal fragments. Terminal fragments were identified by gel retardation experiments (see Results). For isolation, linear plasmid DNA was prepared using proteinase K as described above and was digested with *Sal*I. After separation by electrophoresis, the fragments were extracted from the agarose gel using glass milk (Geneclean, Bio101). The purified end fragments were ligated with the *Sal*I-produced end to the *Sal*I position of the vector (pSKM13+) and with the opposite end to a blunt end produced by *Eco*RV. The DNA was transferred into *E. coli* by electroporation (Ausubel *et al.*, 1987).

Hybridization experiments. DNA was transferred by the Southern blot procedure (Ausubel *et al.*, 1987) to a positively charged nylon membrane (Biodyne B, Pall Filtrationstechnik) and fixed on the membrane by UV irradiation (Khandjian, 1987). If not indicated otherwise, DNA used for hybridization probes originated from the hybrid plasmids listed in Table 2. To eliminate vector DNA, insert DNA was digested with appropriate restriction endonucleases, separated by electrophoresis, and purified from agarose gel. Hybridization probes were labelled with biotin-16-dUTP (Boehringer Mannheim) using a random primer kit (Gibco-BRL) and applying conditions as recommended by the manufacturer. Probes were purified via an exclusion chromatographic column (Sephacrose

Table 1. Strains of *Rhodococcus opacus* used in this work

Strain	Plasmids		Source or reference
	Circular	Linear	
MR11	pHG31-a, pHG31-b	pHG201, pHG202, pHG203	DSM 43205 ^T (= DSM 427); Aggag & Schlegel (1973)
MR22	pHG33	pHG204, pHG205, pHG206	DSM 3346; Sensfuss <i>et al.</i> (1986)
MR2246		pHG201	Sensfuss <i>et al.</i> (1986)
MR2249		pHG204	Kalkus <i>et al.</i> (1993)
MR2253		pHG207	Kalkus <i>et al.</i> (1990)
MR281	pHG33	pHG205	Kalkus <i>et al.</i> (1993)

Table 2. Hybrid plasmids: vector pBluescript SKM13+ with insert

Name	Insert	Description*	Reference
pHG207-L _{KpnI}	2.2 kbp <i>Kpn</i> I fragment	Left end of pHG207	Kalkus <i>et al.</i> (1993)
pHG207-R _{SalI}	2.3 kbp <i>Sal</i> I fragment	Right end of pHG207	Kalkus <i>et al.</i> (1993)
pHG204-R _{SalI}	3.0 kbp <i>Sal</i> I fragment	Right end of pHG204	This work
pHG201-L _{SalI}	1.15 kbp <i>Sal</i> I fragment	Left end of pHG201	This work
pHG201-R _{SalI}	2.3 kbp <i>Sal</i> I fragment	Right end of pHG201	This work

* The terms 'left' and 'right' refer to the macrorestriction maps of the linear plasmids (Fig. 1, Kalkus *et al.*, 1990, 1993).

G-50; coarse). Hybridization and detection of biotinylated probes were carried out as described by Oelmüller *et al.* (1990). Oligonucleotides were radiolabelled at the 5' end with [γ - 32 P]ATP using T4 polynucleotide kinase (Ausubel *et al.*, 1987).

DNA sequence analysis. Sequence determination was carried out by the dideoxynucleotide chain-termination method (Sanger *et al.*, 1977) with single-stranded or alkali-denatured double-stranded plasmid DNA. Radioactive sequencing was performed using a Sequenase version 2.0 sequencing kit (USB) and [35 S]dATP α S. Elimination of compressions was achieved by using 7'-deaza-dGTP or dITP. Oligonucleotides used as primers were synthesized in a Gene Assembler Plus apparatus according to the protocol provided by the manufacturer (Pharmacia LKB). For direct sequencing of pHG207, 10 μ g of proteolytically treated plasmid DNA was alkali-denatured in a standard procedure by adding NaOH to obtain a final concentration of 0.2 M. The solution was incubated for 5 min at 70 °C. The DNA was precipitated with ammonium acetate and ethanol at -20 °C. After washing with 70 % ethanol the denatured DNA was dissolved in water and was immediately used for the annealing procedure with 1 pmol oligonucleotide primer (ATCGACGGCAACGGGAAT). The sequence reactions were done using 7'-deaza-dGTP. Sequence data were analysed using the software package GCG version 8 (Genetics Computer Group).

RESULTS

Detection of regions hybridizing with the terminal sequence of pHG207 in other linear plasmids

Because the linear plasmid pHG207 was shown to carry TIRs and to be a recombination product of the wild-type plasmids pHG204 and pHG205 of strain MR22, we assumed that TIRs are a general feature of the linear plasmids of *R. opacus* (Fig. 1). Furthermore, at least pHG204 and pHG205 should carry sequences homologous to the terminal sequences of pHG207. To examine the possible ubiquity of the terminal sequences of pHG207, the linear plasmids of *R. opacus* MR11 and MR22 were separated by PFGE, transferred to a nylon membrane and hybridized with a biotinylated DNA fragment representing the terminal sequence of the right end of pHG207 (0.6 kbp *Eco*RI fragment) (Fig. 2a, b). The experiment was repeated with less DNA to enable the distinction between pHG202 and pHG203 (data not shown). All linear plasmids from both wild-type strains gave significant signals with this probe.

To investigate this result in more detail, *Sal*I digests of the linear plasmids pHG201, pHG204, pHG205 and pHG206 were hybridized with the same probe (Fig. 3). *Sal*I digests of the control plasmid pHG207 showed two hybridizing fragments, corresponding to the right (2.3 kbp – originating from pHG205) and left (4.0 kbp – originating from pHG204) ends, as expected. Corresponding *Sal*I digests of linear plasmids pHG204 and pHG206 also showed two hybridizing fragments each (Fig. 3), indicating the existence of two regions having homology with terminal sequences of pHG207, and suggesting that TIRs are a general feature of these linear plasmids.

In contrast, patterns of hybridization between the

pHG207 terminal sequence probe and restriction digests of pHG201 and pHG205 showed only one homologous *Sal*I fragment (2.3 kbp) in each (Fig. 3a). The same result – only a single detectable fragment – was obtained in experiments in which the same probe was hybridized against *Kpn*I (7 kbp) and *Bgl*II (0.8 kbp) digests of pHG201 and pHG205 (results not shown). One possible explanation of these results is that long TIRs (more than 7 kbp) resulted in terminal fragments of identical size. However, the macrorestriction maps of pHG201 and pHG205 do not show an extensive symmetry of the termini. In order to discriminate between the two ends, DNA of pHG201 was digested with the rare-cutting enzyme *Asn*I and hybridized with the 0.6 kbp *Eco*RI right-end terminal fragment of pHG207 as probe (data not shown). Only the 21 kbp *Asn*I fragment, representing the right end of pHG201 (Fig. 1; Kalkus *et al.*, 1990), hybridized with the probe. The 190 kbp *Asn*I fragment, representing the left end of pHG201, did not give any signal.

To investigate an alternative possibility, that short TIRs were not detected by the probe under the conditions used for the previous experiments, *Sal*I and *Bgl*II digests of pHG201 and pHG205 were hybridized to an oligonucleotide probe corresponding to the terminal 17 bases of the 3' end of pHG207. Even under conditions of low stringency (55 °C, 6 $\times$ SSC, 1% SDS) no fragments other than the 2.3 kbp *Sal*I fragment and the 0.8 kbp *Bgl*II fragment could be identified in pHG201 and pHG205 carrying the terminal sequence of pHG207 (data not shown).

Identification of the terminal fragments by gel retardation experiments

A general feature of the linear replicons of actinomycetes is that TPs are covalently bound to the 5' end of the DNA. Therefore, gel retardation experiments were done to investigate whether the *Rhodococcus* linear plasmid end fragments homologous to the terminal sequence of pHG207, identified above, had this feature. Restriction fragments were produced by *Sal*I and *Bam*HI of DNA of pHG204 isolated with and without proteolytical treatment (Fig. 4a). The resulting DNA fragments were analysed by Southern hybridization using the 0.6 kbp *Eco*RI fragment of pHG207 as a probe. The indicated sizes of the fragments mentioned below were determined from proteolytically treated DNA samples. The mobility of the 3.0 kbp and the 4.0 kbp *Sal*I fragment, and a 0.9 kbp and a 7.6 kbp *Bam*HI fragment, all of which share homologous sequences with the terminal sequence of pHG207, were retarded by proteins (arrowheads in Fig. 4a). The 1.9 kb *Sal*I fragment, which gave only a faint signal (position marked with an asterisk in Fig. 4a), was not influenced by treatment with proteinase K and was therefore identified as an internal fragment of pHG204. No further retarded fragments could be detected.

Results from the Southern hybridization of *Sal*I-digested DNA simultaneously with the 0.6 kbp right-ended

Fig. 1. Macrorestriction maps of linear plasmids of *R. opacus* strains. Simplified restriction maps of the linear plasmids pHG201, pHG204, pHG205, pHG206 and pHG207 are shown. Black diamonds indicate the termini that have homology to the 0.6 kbp *EcoRI* fragment of pHG207 (right end). White triangles indicate that the left ends of pHG201 and pHG205 are similar by hybridization. Shaded areas indicate the regions of pHG207 which are, with respect to the restriction patterns and hybridizations, homologous to their parental plasmids pHG204 and pHG205 (Kalkus *et al.*, 1993). The non-shaded region of pHG207 has not yet been assigned to pHG204 or pHG205 according to the restriction map. Restriction sites: A, *AsnI*; S, *SpeI*; D, *DraI*. Phenotypes: Aut⁺, Aut character; TI^r, thallium resistance.

Fig. 2. Detection of regions homologous with the terminal sequences of pHG207 and pHG201 in other linear plasmids. (a) PFGE separation of total DNA preparation in agarose plugs of strains MR11 and MR22. (b) Southern blot analysis of (a) with the 0.6 kbp *EcoRI* fragment of pHG207 (right end) as probe. (c) Southern blot analysis of (a) with the 1.15 kbp *SalI* fragment of pHG201 (left end) as probe. Lanes 1 and 4, λ ladder as size standard; lane 2, MR11; lane 3, MR22. PFGE programme: 24 h, pulse time ramp from 20 to 80 s.

terminal *EcoRI* fragment of pHG207 and a 16 kbp left-end terminal *SpeI* fragment of pHG201 as probes, is shown in Fig. 4(b) with and without proteinase K treatment. Only the 2.3 kbp and 1.15 kbp *SalI* fragments of pHG201 appeared to be affected by the protease treatment, suggesting that they contain the plasmid's termini. In a similar experiment, but with only the 0.6 kbp right end of pHG207 as a probe (Fig. 4c), a 2.3 kbp *SalI* fragment of pHG205 was identified as containing one of that plasmid's terminal sequences.

Fig. 3. Detection of regions homologous with the terminal sequence of pHG207 in restriction patterns of the linear plasmids pHG201, pHG204, pHG205 and pHG206. (a) PFGE separation of *SalI*-digested isolated plasmid DNA and Southern blot analysis with the 0.6 kbp *EcoRI* fragment of pHG207 (right end) as probe. (b) Southern blot analysis of *SalI*-digested pHG206 with the 0.6 kbp *EcoRI* fragment of pHG207 (right end) as probe. To the left, the size (kbp) and location of the fragments of the size standard are indicated. To the right, the size (kbp) of the hybridizing fragments is given. PFGE programme: 30 min pulse time 0.1 s, 30 min pulse time 1 s, 11 h pulse time ramp from 1 to 10 s.

The other terminus of pHG205 was localized to a 1.15 kbp *SalI* fragment by hybridization with the 1.15 kbp *SalI* fragment from pHG201 (cloned and labelled) as probe (Fig. 4d).

Fig. 4. Change of the electrophoretic mobility of restriction fragments of pHG204, pHG201 and pHG205 after proteolytic treatment. (a) Restriction pattern of pHG204 produced by *SalI* (S) and *BamHI* (B) and Southern blot analysis using the 0.6 kbp *EcoRI* fragment of pHG207 as a probe. (b–d) Southern blot analysis of: (b) *SalI*-digested pHG201, using the 0.6 kbp *EcoRI* fragment of pHG207 (right end) and the 16 kbp *SpeI* fragment of pHG201 (left end) as probes; (c) *SalI*-digested pHG205, using the 0.6 kbp *EcoRI* fragment of pHG207 (right end) as a probe; (d) *SalI*-digested pHG205, using the 1.15 kbp *SalI* fragment of pHG201 (left end) as a probe. Prot K, plasmid DNA isolated without (–) or with (+) proteinase K treatment. To the left, the size (kbp) and location of the fragments of the size standards are indicated. Arrowheads indicate fragments that showed a changed mobility. The size of these fragments is given in kbp. PFGE programme: 30 min pulse time 0.1 s, 30 min pulse time 1 s, 11 h pulse time ramp from 1 to 10 s.

Validation of the cloning strategy for linear plasmid termini

After restriction digestion the two resulting end fragments of a linear plasmid should have unequal ends – one end produced by the restriction endonuclease used and one end of the linear plasmid. The ends of pSLA2 were shown to be blunt-ended by direct chemical sequencing (Hirochika *et al.*, 1984). However, the 5' ends of pSLA2 and pHG207 were blocked even after proteolytic treatment, probably by remaining amino acid residues (Hirochika & Sakaguchi, 1982; Kalkus *et al.*, 1993). The termini of pHG207 were ligated without a special treatment to the blunt end side of a vector (Kalkus *et al.*, 1993). Because it was crucial to show that the very ends of a linear plasmid can be cloned by this method, direct sequencing was applied to pHG207. A primer was chosen whose sequence represented bases 93–110 of pHG207-*L_{KpnI}* and pHG207-*R_{SalI}*. After annealing of the primer to proteinase K-treated and alkali-denatured pHG207 DNA the sequencing reactions were carried out. The hybrid plasmids pHG207-*L_{KpnI}* and pHG207-*R_{SalI}* were sequenced with the same primer. The sequencing reactions were analysed in parallel (Fig. 5).

Strong bands in all four lanes (A, C, G, T) indicate the abortion of the polymerase reaction at the native linear

plasmid end. The lower bands in these lanes occur at exactly the same position as the last nucleotide of the cloned termini in pHG207-*L_{KpnI}* and pHG207-*R_{SalI}*. The four bands at one position higher might indicate a variable end of pHG207 differing by one nucleotide. Court & Bertrand (1991) reported that T7 DNA polymerase was able to elongate the newly synthesized DNA strand by one nucleotide beyond the end of the template strand. The polymerase used in our experiments is a modified T7 DNA polymerase (Tabor & Richardson, 1989). Therefore, we assume that the upper bands observed in our experiments result from such an additional template-independent elongation.

These experiments indicated that direct ligation of the plasmid ends is a suitable method to clone the very end of a linear plasmid.

Cloning and sequence analysis of terminal fragments of pHG201 and pHG204

For further analysis of the termini of wild-type linear plasmids from strains MR11 and MR22, the 1.15 kbp and the 2.3 kbp *SalI* fragment of pHG201, and the 3.0 kbp *SalI* fragment of pHG204, were isolated and cloned. The left end of pHG204 is assumed to be the same as the left end of pHG207, which has already been sequenced (Kalkus *et al.*, 1993).

Fig. 5. Direct sequencing of pHG207. An autoradiogram of the sequencing ladder of pHG207- L_{KpnI} , pHG207 and pHG207- R_{SalI} is shown. In the lanes of pHG207 only the two bands resulting from termination of the polymerase reaction at the very ends of pHG207 (see text) are visible. Dots mark the first nucleotides belonging to the vector DNA.

The purified fragments were inserted in a *SalI*/*EcoRV*-cut vector (pBluescript SKM13+) and transferred via electroporation into *E. coli* XL1 Blue. Among the few transformants obtained, two positive clones were identified carrying hybrid plasmids containing the 3.0 kbp *SalI* fragment of pHG204, and four and two clones containing the 1.15 kbp and the 2.3 kbp *SalI* fragment of pHG201, respectively. The resulting plasmids are referred to as pHG204- R_{SalI} , pHG201- L_{SalI} and pHG201- R_{SalI} (Table 2). To ensure the uniformity of the clones obtained, the hybrid plasmids were examined by a detailed restriction analysis and by sequencing of the blunt-end junctions between vector and insert DNA. All clones gave identical results.

A 1116 bp length of pHG204- R_{SalI} was sequenced starting at the blunt-end ligation site (data not shown; GenBank/EMBL AF001834). Alignment with the nucleotide sequence of pHG207- L_{KpnI} , which is believed to originate from the left terminal part of pHG204 (Kalkus *et al.*, 1993), revealed a homology of more than 99% within the terminal 1016 bp. The TIR (1016 bp) was clearly separated from the adjoining sequences which do not share significant homology. The only differences within the 'right' and 'left' TIRs of pHG204 were at positions 894 (G in pHG204- R_{SalI} ; C in pHG207- L_{KpnI}), 897 (A in pHG204- R_{SalI} ; G in pHG207- L_{KpnI}), and 902 (T in pHG204- R_{SalI} ; C in

Fig. 6. Alignment of the terminal 100 bases of pHG201. Dots indicate introduced gaps; asterisks mark homologies of the left with the right end. Inverted repeats are marked with converging arrows.

pHG207- L_{KpnI}). The difference at position 894 affected a *Bam*HI site, which is therefore missing in the right terminus of pHG204. This was confirmed by the results obtained in the gel retardation experiment (Fig. 4a): instead of two identical fragments of 0.9 kbp, two differently sized *Bam*HI fragments (0.9 kbp and 7.6 kbp) contained the two termini.

The insert of the hybrid plasmid pHG201- R_{SalI} , carrying the 2.3 kbp *SalI* fragment of pHG201, was partially sequenced starting at the blunt-end ligation site (data not shown; GenBank/EMBL AF007825). Alignment of the nucleotide sequence obtained (1200 bp) with the sequence of pHG207- R_{SalI} revealed 100% homology of the analysed region. Hybridization of the wild-type linear plasmids with pHG201- L_{SalI} as probe revealed the occurrence of the left terminal sequence of pHG201 in pHG201 and pHG205 (Fig. 2c). No other linear plasmid gave a signal. The sequence of the 1.15 kbp insert of pHG201- L_{SalI} was fully determined (not shown; GenBank/EMBL AF001835). Two potential open reading frames (ORF 201L1 [1152–235 (stop)] and 201L2 [247(start)–1152]) were detected. Both have a high probability of coding when checked for codon usage and GC bias of the third position of the triplets by the program CODONPREFERENCE (Devereux *et al.*, 1984). Search in databases (EMBL release 49.0; SWISS-PROT release 34.0) using the search facilities MPsrch (University of Edinburgh) and BLAST (Altschul *et al.*, 1990) did not reveal any significant homology.

Alignment of the right and left terminal sequences of pHG201 revealed a similarity of only 65% for the 34 terminal bases of the left end with the 32 terminal bases of the right end, and a perfect TIR of only 3 bp (Fig. 6).

DISCUSSION

Most of the linear plasmids so far described in bacteria have been found among actinomycetes, especially within the genera *Streptomyces* and *Rhodococcus*. Recently, linear plasmids were also found in the genus *Mycobacterium* (Picardeau & Vincent, 1997). The chromosomes of several *Streptomyces* species have been shown

to be linear as well (Lin *et al.*, 1993; Gravius *et al.*, 1994; Lezhava *et al.*, 1995; Leblond *et al.*, 1996). The common features of the linear replicons in *Streptomyces* are perfect or imperfect TIRs, with proteins bound to their ends. The lengths of TIRs are not uniform: the large linear plasmids SCP1 (350 kbp) and pPZG101 (387 kbp) have very long TIRs of more than 80 kbp (Kinashi *et al.*, 1991; Gravius *et al.*, 1994), and also the linear chromosomes (about 8 Mb) possess TIRs of about 25 kbp (*S. lividans*, *S. griseus*) or even 210 kbp (*S. ambofaciens*) (Lin *et al.*, 1993; Lezhava *et al.*, 1995; Leblond *et al.*, 1996). Smaller linear plasmids like pSCL1 (11.7 kbp) and pSLA2 (17 kbp) have TIRs of less than 1 kbp (Wu & Roy, 1993; Hirochika *et al.*, 1984). The linear plasmid SLP2 (50 kbp) carries a TIR of only 44 bp (Chen *et al.*, 1993). Thus there is not a fixed correlation between the size of the replicon and the length of the TIR, but it could be generalized that large linear replicons carry long TIRs. The chromosome of *S. lividans* and the plasmid pSLA2 were shown to carry a centrally located functional origin of replication, and for some of the linear replicons a conversion into a circular form was reported (Zakrzewska-Czerwinska & Schrempf, 1992; Chang & Cohen, 1994; Shiffman & Cohen, 1992; Lin *et al.*, 1993; Volff *et al.*, 1997). The ends are not necessarily the origin of replication of the linear replicons (Chang & Cohen, 1994). However, in case of the *S. lividans* chromosome, the circularization is accompanied by genetic instability and genomic rearrangements (Volff *et al.*, 1997). The function and essential features of the telomeric region remains unclear.

The aim of this study was to analyse the termini of the linear plasmids in the genus *Rhodococcus*, in order to obtain more information on the general telomeric structure. The first detailed study on a linear plasmid of *R. opacus* was performed using pHG207 (225 kb). With its TIR of 583/560 bp and proteins covalently bound to the 5' ends of the plasmid it possesses all features described for the linear plasmids in *Streptomyces*. However, in comparison to the large linear plasmids SCP1 and pPZG101, the TIR detected is relatively short. Because pHG207 was shown to be the product of a recombination between the wild-type linear plasmids pHG204 and pHG205, it seemed reasonable to suggest that TIRs are a general feature of the linear plasmids of *R. opacus*. Indeed, we could show that all linear plasmids of the wild-type strains MR22 and MR11 carry sequences homologous to the terminal sequence of pHG207. Homologous sequences, detectable by hybridization between different linear replicons, were also reported for *S. lividans*: about 16 kbp of the right end of the linear plasmid SLP2 was identical to the terminal sequences of the chromosome (Chen *et al.*, 1993; Lin *et al.*, 1993). Plasmid SLP2 shares homologous sequences with the linear plasmid pSPA1 from *S. parvulus* as well (Chen *et al.*, 1993).

The sequence analysis of the terminal part of pHG204 reported in this paper revealed a nearly perfect TIR of 1016 bp, with only three mismatches between the right and left ends. Because the alignment was done using the

right end of pHG207 and the left end of pHG204, the differences could have been due to the different sources of the sequences. However, because one of these differences concerned a *Bam*HI restriction site in pHG204 the differences could be verified via different restriction patterns in the TIR of pHG204. A similar feature was described for pSCL1, where the perfect TIR, which is 894 bp in size, is interrupted by a 3 base mismatch after 690 bp. In contrast to the sharp boundaries of the TIRs found in pHG207 and pHG204, the TIRs of pSCL1 and pSLA2 are followed by a less homologous zone of about 25 bp and 180 bp, respectively, with a 'patched' homology (Wu & Roy, 1993; Hirochika *et al.*, 1984). A TIR can also be assumed to be present in pHG206, because the regions which show homology with the terminal sequence of pHG207 are located at the two ends of pHG206 (Fig. 1 and unpublished results).

None of the linear plasmids examined in this study carries TIRs comparable in length to those found in the large linear replicons of *Streptomyces*. Furthermore, the linear plasmid pHG201 was shown to have no real TIR. The first mismatch between the left and the right end appears after the third nucleotide. The homology of the terminal 34/32 bases is only 65%, which is even less than the homology with telomeric sequences of other linear plasmids (e.g. 73% for the left end of pHG201 versus the ends of SLP2 or pSCL). We assume the same is true for the *Aut* element pHG205, because the terminal fragments of pHG201 and pHG205 are homologous and identical in size. These results and the similar restriction patterns of both plasmids (Fig. 3) provoke the assumption of a common ancestor. However, the macro-restriction maps of the two plasmids are different. The finding of a transposable element in MR11 by Grzeszik *et al.* (1997a) offers a reasonable explanation, insofar as transposition events may have caused rearrangements and deletions in the plasmid.

To our knowledge, pHG201 and pHG205 are the only reported examples of actinomycete linear plasmids that do not show a striking homology between their 'right' and 'left' ends. However, the well-studied phage ϕ 29, which serves as a model for TP-primed replication, also carries very short TIR (6 bp) (Yoshikawa *et al.*, 1981; Escarmis & Salas, 1981; Salas *et al.*, 1995). The replication of the genome of ϕ 29 starts with a sliding-back mechanism: the TP-dependent DNA polymerase binds to the single-stranded 3' region of ϕ 29 and catalyses the linkage of the TP and a nucleotide using the second nucleotide of the 3' strand as template (Mendez *et al.*, 1992). After the linkage is established, the TP nucleotide complex slides back to the last nucleotide. Therefore, for a successful replication a repetition of the terminal nucleotide is essential (Mendez *et al.*, 1992). Such a sliding-back mechanism was also reported for the replication of adenoviral DNA and was suggested as a general mechanism for TP-dependent replications. (Graham *et al.*, 1989; Mendez *et al.*, 1992). Looking for a possible template nucleotide within the terminal 10 bases of pHG201 only the G at the second position could

fulfil the function insofar as it is a repetition of the terminal G and it is in the same distance towards the end at both sides.

Our results suggest that it is not the TIRs themselves, but certain structures like inverted or direct repeats at the ends, that are necessary for the complete replication and/or stability of a linear plasmid. TIRs are more likely to be the result of recombination, for example to rescue a linear plasmid after the loss of one telomer. Comparison and alignment of the terminal sequences of pHG201 showed two inverted repeats which have the same central motif (GCTXCGC) and a high GC-content (Fig. 6, positions 16–34, 76–97 in pHG201-L_{SalI} and 16–30, 65–81 in pHG201-R_{SalI}). Analogous inverted repeats were also found in pSCL, pSLA2 and SLP2 (Wu & Roy, 1993; Hirochika *et al.*, 1984; Chen *et al.*, 1993). Chen (1996) described a model, suggested by S. N. Cohen, where the telomers, which are left as a single-stranded 3' overhang after replication of the main part of the plasmid from a central located origin, are folded at inverted repeats to form specific secondary structures. This structure might be the signal for the TP-dependent DNA polymerase to complete the 5' strand. The inverted repeats mentioned above could produce such hairpin structures, which may also function during replication as a termination signal or hindrance for DNA polymerase. A termination of the polymerization hypothetically starting from the TP would restrict the action of the postulated specific TP-dependent DNA polymerase to the telomers. A termination signal for the DNA polymerase which starts from an internal origin would leave a defined 3' overhang. Indeed, Chang & Cohen (1994) detected molecules of pSLA2 with single-stranded parts of around 280 bp at their ends which might be an intermediate form of replication. Another possible role of the conserved inverted repeats is the function as binding sites for proteins which might be essential for the maintenance or replication of the linear plasmid.

Analyses of linear plasmids of other species of *Rhodococcus* are under way. The information gained will contribute to the elucidation of the general telomeric structure of linear wild-type plasmids.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Deutsche Forschungsgemeinschaft (Re 842/1–2). We thank Dr M. Keßeler for critical reading of the manuscript.

REFERENCES

- Aggag, M. & Schlegel, H. G. (1973). Studies on a Gram-positive hydrogen bacterium, *Nocardia opaca* 1b. I. Description and physiological characterization. *Arch Microbiol* **88**, 299–318.
- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403–410.
- Ausubel, F. M., Brent, R., Kingston, R. E., More, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (1987). *Current Protocols in Molecular Biology*. New York: Wiley.
- Chang, P.-C. & Cohen, S. N. (1994). Bidirectional replication from an internal origin in a linear *Streptomyces* plasmid. *Science* **265**, 952–954.
- Chen, C. W. (1996). Complications and implications of linear bacterial chromosomes. *Trends Genet* **12**, 192–196.
- Chen, C. W., Yu, T.-W., Lin, Y.-S., Kieser, H. M. & Hopwood, D. A. (1993). The conjugative plasmid SLP2 of *Streptomyces lividans* is a 50 kbp linear molecule. *Mol Microbiol* **7**, 925–932.
- Court, D. A. & Bertrand, H. (1991). A method for sequencing uncloned termini of linear plasmids. *Nucleic Acids Res* **19**, 1714.
- Crespi, M., Messens, E., Caplan, A. B., Van Montagu, M. & Desomer, J. (1992). Fasciation induction by the phytopathogen *Rhodococcus fascians* depends upon a linear plasmid encoding a cytokinin synthase gene. *EMBO J* **11**, 795–804.
- Dabrock, B., Riedel, J., Bertram, J. & Gottschalk, G. (1992). Isopropylbenzene (cumene) – a new inducer substrate for the isolation of trichloroethene-degrading bacteria. *Arch Microbiol* **158**, 9–13.
- Dabrock, B., Keßeler, M., Averhoff, B. & Gottschalk, G. (1994). Identification and characterization of a transmissible linear plasmid from *Rhodococcus erythropolis* BD2 specifying isopropylbenzene and trichloroethene (TCE) catabolism. *Appl Environ Microbiol* **60**, 853–860.
- Devereux, J., Haeberli, P. & Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res* **12**, 387–395.
- Escaris, C. & Salas, M. (1981). Nucleotide sequence at the termini of the DNA of *Bacillus subtilis* phage ϕ 29. *Proc Natl Acad Sci USA* **78**, 1446–1450.
- Graham, F. L., Rudy, J. & Brinkley, P. (1989). Infectious circular DNA of human adenovirus type 5: regeneration of viral DNA termini from molecules lacking terminal sequences. *EMBO J* **8**, 2077–2085.
- Gravius, B., Glocker, D., Pigac, J., Pandza, K., Hranueli, D. & Cullum, J. (1994). The 387 kbp linear plasmid pPZG101 of *Streptomyces rimosus* and its interactions with the chromosome. *Microbiology* **140**, 2271–2277.
- Grzeszik, C., Lübbers, M., Reh, M. & Schlegel, H. G. (1997a). Genes encoding the NAD-reducing hydrogenase of *Rhodococcus opacus* MR11. *Microbiology* **143**, 1271–1286.
- Grzeszik, C., Roß, K., Schneider, K., Reh, M. & Schlegel, H. G. (1997b). Location, catalytic activity, and subunit composition of NAD-reducing hydrogenases of some *Alcaligenes* strains and *Rhodococcus opacus* MR22. *Arch Microbiol* **167**, 172–176.
- Hirochika, H. & Sakaguchi, K. (1982). Analysis of linear plasmids isolated from *Streptomyces*: association of protein with the ends of the plasmid DNA. *Plasmid* **7**, 59–65.
- Hirochika, H., Nakamura, K. & Sakaguchi, K. (1984). A linear DNA plasmid from *Streptomyces rochei* with terminal repetition of 614 base pairs. *EMBO J* **3**, 761–766.
- Kalkus, J., Reh, M. & Schlegel, H. G. (1990). Hydrogen autotrophy of *Nocardia opaca* strains is encoded by linear megaplasmids. *J Gen Microbiol* **136**, 1145–1151.
- Kalkus, J., Dörrie, C., Fischer, D., Reh, M. & Schlegel, H. G. (1993). The giant linear plasmid pHG207 from *Rhodococcus* sp. encoding hydrogen autotrophy: characterization of the plasmid and its termini. *J Gen Microbiol* **139**, 2055–2065.
- Khandjian, E. W. (1987). Optimized hybridization of DNA blotted and fixed to nitrocellulose and nylon membranes. *Bio/Technology* **5**, 165–167.
- Kinashi, H., Shimaji-Murayama, M. & Hanafusa, T. (1991).

Nucleotide sequence analysis of the unusually long terminal inverted repeats of a giant linear plasmid, SCP1. *Plasmid* **26**, 123–130.

Klatte, S., Kroppenstedt, R. M. & Rainey, F. A. (1994). *Rhodococcus opacus* sp. nov., an unusual nutritionally versatile *Rhodococcus* species. *Syst Appl Microbiol* **17**, 355–360.

Leblond, P., Fischer, G., Francou, F.-X., Berger, F., Guerineau, M. & Decaris, B. (1996). The unstable region of *Streptomyces ambofaciens* includes 210 kbp terminal inverted repeats flanking the extremities of the linear chromosomal DNA. *Mol Microbiol* **19**, 261–271.

Lezhava, A., Mizukami, T., Kajitani, T., Kameoka, D., Redenbach, M., Shinkawa, H., Nimi, O. & Kinashi, H. (1995). Physical map of the linear chromosome of *Streptomyces griseus*. *J Bacteriol* **177**, 6492–6498.

Lin, Y.-S., Kieser, H. M., Hopwood, D. A. & Chen, C. W. (1993). The chromosomal DNA of *Streptomyces lividans* 66 is linear. *Mol Microbiol* **10**, 923–933.

McClelland, M., Jones, R., Patel, Y. & Nelson, M. (1987). Restriction endonucleases for pulsed field mapping of bacterial genomes. *Nucleic Acids Res* **15**, 5985–6005.

Mendez, J., Blanco, L., Esteban, J. A., Bernad, A. & Salas, M. (1992). Initiation of ϕ 29 DNA replication occurs at the second 3' nucleotide of the linear template: a sliding-back mechanism for protein-primed DNA replication. *Proc Natl Acad Sci USA* **89**, 9579–9583.

Oelmüller, U., Krüger, N., Steinbüchel, A. & Friedrich, C. G. (1990). Isolation of prokaryotic RNA and detection of specific messenger-RNA with biotinylated probes. *J Microbiol Methods* **11**, 72–81.

Picardeau, M. & Vincent, V. (1997). Characterization of large linear plasmids in mycobacteria. *J Bacteriol* **179**, 2753–2756.

Rainey, F. A., Burghardt, J., Kroppenstedt, R. M., Klatte, S. & Stackebrandt, E. (1995). Phylogenetic analysis of the genera *Rhodococcus* and *Nocardia* and evidence for the evolutionary origin of the genus *Nocardia* from within the radiation of *Rhodococcus* species. *Microbiology* **141**, 523–528.

Sakaguchi, K. (1990). Invertrons, a class of structurally and functionally related genetic elements that includes linear DNA plasmids, transposable elements, and genomes of Adeno-type viruses. *Microbiol Rev* **54**, 66–74.

Salas, M., Freire, R., Soengas, M. S., Esteban, J. A., Mendez, J., Bravo, A., Serrano, M., Blasco, M. A., Lazaro, J. M., Blanco, L., Gutierrez, C. & Hermoso, J. M. (1995). Protein–nucleic acid interactions in bacteriophage ϕ 29 DNA replication. *FEMS Microbiol Rev* **17**, 73–82.

Sanger, F., Nicklen, H. & Coulson, A. R. (1977). DNA sequencing

with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **77**, 2611–2615.

Schneider, K., Cammack, R. & Schlegel, H. G. (1984). Content and localization of FMN, Fe–S clusters and nickel in the NAD-linked hydrogenase of *Nocardia opaca* 1b. *Eur J Biochem* **142**, 75–84.

Sensfuss, C., Reh, M. & Schlegel, H. G. (1986). No correlation exists between the conjugative transfer of the autotrophic character and that of plasmids in *Nocardia opaca* strains. *J Gen Microbiol* **132**, 997–1007.

Shiffman, D. & Cohen, S. N. (1992). Reconstruction of a *Streptomyces* linear replicon from separately cloned fragments: existence of a cryptic origin of circular replication within the linear plasmid. *Proc Natl Acad Sci USA* **89**, 6129–6133.

Tabor, S. & Richardson, C. C. (1989). Selective inactivation of the exonuclease activity of bacteriophage T7 DNA polymerase by in vitro mutagenesis. *J Biol Chem* **264**, 6447–6458.

Vantomme, R., Elia, S., Swings, J. & De Ley, J. (1982). *Corynebacterium fascians* (Tilford 1936) Dowson 1942, the causal agent of leafy gall on lily crops in Belgium. *Parasitica* **38**, 183–192.

Vieira, J. & Messing, J. (1987). Production of single-stranded plasmid DNA. *Methods Enzymol* **153**, 3–11.

Volff, J.-N., Viell, P. & Altenbuchner, J. (1997). Artificial circularization of the chromosome with concomitant deletion of its terminal inverted repeats enhances genetic instability and genome rearrangement in *Streptomyces lividans*. *Mol Gen Genet* **253**, 753–760.

Waterbury, P. G. & Lane, M. J. (1987). Generation of lambda phage concatemers for use as pulsed field electrophoresis size markers. *Nucleic Acids Res* **15**, 3930.

Wu, X. & Roy, K. L. (1993). Complete nucleotide sequence of a linear plasmid from *Streptomyces clavuligerus* and characterization of its RNA transcript. *J Bacteriol* **175**, 37–52.

Yoshikawa, H., Friedmann, T. & Ito, J. (1981). Nucleotide sequences at the termini of ϕ 29 DNA. *Proc Natl Acad Sci USA* **78**, 1336–1340.

Zaborosch, C., Schneider, K., Schlegel, H. G. & Kratzin, H. (1989). Comparison of the NH₂-terminal amino acid sequences of the four non-identical subunits of the NAD-linked hydrogenases from *Nocardia opaca* 1b and *Alcaligenes eutrophus* H16. *Eur J Biochem* **181**, 175–180.

Zakrzewska-Czerwinska, J. & Schrempf, H. (1992). Characterization of an autonomously replicating region from the *Streptomyces lividans* chromosome. *J Bacteriol* **174**, 2688–2693.

Received 3 July 1997; revised 11 November 1997; accepted 28 January 1998.