Mycobacterial linear plasmids have an invertron-like structure related to other linear replicons in actinomycetes

Mathieu Picardeau and Véronique Vincent

Author for correspondence: Véronique Vincent. Tel: +33 1 45 68 83 60. Fax: +33 1 40 61 31 18.
e-mail: vvincent@pasteur.fr

Laboratoire de Référence
des Mycobactéries, Institut
Pasteur, 75724 Paris Cedex
15, France

The authors previously identified large plasmids in *Mycobacterium xenopi*, *M. branderi* and *M. celatum* which appeared to have a linear topology. This study has confirmed the presence of such linear plasmids in mycobacteria, including *M. avium*, and demonstrated that the ends of these replicons are covalently bound with protein(s), suggesting an invertron-like structure. The termini of one 25 kb plasmid, designated pCLP, from *M. celatum* were cloned and the first 500 bp of each terminus were sequenced. The termini of this plasmid show the characteristic features of invertrons with terminal inverted repeats of 45 bp (with imperfect matches) and several palindromic sequences. Moreover, similarity existed in the structure and terminal nucleotide sequence of pCLP and the termini of linear replicons of *Streptomyces* and *Rhodococcus* species, indicating a conservation of these linear extrachromosomal elements within the *Actinomycetales*.

Keywords: mycobacteria, actinomycetes, linear plasmids, invertron

INTRODUCTION

Our understanding of the organization of bacterial genomes has been greatly modified over the past few years. Many reports have revealed a wide variety of bacterial chromosomes. For example, *Agrobacterium tumefaciens* contains both a linear and a circular chromosome, *Rhodobacter sphaeroides* has two circular chromosomes, while *Streptomyces* and *Borrelia* species have linear chromosomes (Hinnebusch & Tilly, 1993). Extrachromosomal elements also consist of molecules of both linear and circular topology.

The first bacterial linear plasmid, pSLA2, was isolated in 1979 from *Streptomyces rochei* (Hayakawa *et al.*, 1979). Among the linear elements, Sakaguchi has defined a class of extrachromosomal elements with inverted repeats at their ends and protein bound to their 5' ends, termed invertrons (Sakaguchi, 1990). These structural characteristics have been found in some actinomycetes, viruses and bacteriophages, as well as in eukaryotic cells. A second class of bacterial linear plasmids, having covalently closed ends at the termini of the DNA, was reported in the genus *Borrelia* (Hinnebusch *et al.*, 1990).

In a previous study, we demonstrated the presence of extrachromosomal elements in *Mycobacterium xenopi*, *M. celatum* and *M. branderi* that migrated in pulsed-field gel electrophoresis (PFGE) like linear molecules. The susceptibility of these elements to exonucleases also supported their linear topology and suggested an invertron-like structure (Picardeau & Vincent, 1997). Therefore, we have undertaken a physical study of the ends of these linear plasmids to determine whether they are linked with proteins and contain inverted repeats. We also studied the relationships of the mycobacterial linear plasmids with those of other actinomycetes.

METHODS

Mycobacterial strains. The *M. celatum* strains used in this study were clinical isolates kindly provided by W. R. Butler (Centers for Disease Control, Atlanta, USA). As previously described, *M. celatum* strain 4 contains two extrachromosomal elements of about 25 and 320 kb, and *M. celatum* strain 9 contains one extrachromosomal element of 180 kb (Picardeau & Vincent, 1997). *M. avium* strain 951849 was a clinical isolate from our laboratory (Centre National de Référence des Mycobactéries, Institut Pasteur).

PFGE. Mycobacterial isolates were cultivated in Middlebrook 7H9 broth, and cells were embedded in agarose and lysed as previously described (Picardeau *et al.*, 1996). For preparation of non-proteolytically treated plasmids, proteinase K was

Abbreviation: TIR, terminal inverted repeat.

The GenBank accession numbers of the nucleotide sequences of the left and right termini of pCLP are AF044971 and AF044972, respectively.

Fig. 1. PFGE of DNA genomic preparations obtained either by the standard isolation procedure, including proteinase K (PK) treatment, or without proteinase K treatment, as indicated. (a) PFGE and Southern blot analysis of *M. celatum* strain 9 performed with the radiolabelled 180 kb plasmid of the host strain. Lanes 1 and 2, undigested DNA; lanes 3 and 4, DNA digested with *DraI*. (b) PFGE and Southern blot analysis of *M. celatum* strain 4 performed with the radiolabelled 25 kb plasmid of the host strain. Lanes 1 and 2, undigested DNA; lanes 3 and 4, DNA digested with *DraI*; lanes 5 and 6, DNA digested with *XbaI*. (c) PFGE and Southern blot analysis of *M. avium* strain 951849 performed with the radiolabelled 145 kb plasmid of the host strain. Lanes 1 and 2, undigested DNA. Lanes M, 50 kb DNA concatemers of the bacteriophage λ genome (sizes are indicated in kb beside the gel).

omitted and plugs were incubated in 1% (w/v) SDS, 0.05 M EDTA instead of N-lauroylsarcosinate, 0.5 M EDTA. For digestion, plugs were extensively washed in TE buffer (10 mM Tris/HCl, pH 8.0, 1 mM EDTA) and incubated with *XbaI* or *DraI* for 2 h at 37 °C. PFGE was performed by zero-integrated-field electrophoresis (ZIFE; Vysis, Les Ulis, France) using the 8–200 kb and 8–500 kb ROM cards.

Isolation of linear plasmid DNA. Separation of plasmid from chromosomal DNA was achieved by PFGE of highly concentrated DNA agarose plugs. DNA bands were excised from PFGE agarose gels and the plasmids were recovered from the gel by using a nucleic acid extraction kit (Nucleotrap, Macherey-Nagel). The linear plasmid DNA was eluted with TE as recommended by the manufacturer.

Southern blotting. DNA bands separated by PFGE, performed as described above, were blotted onto nylon membranes. The membranes were hybridized overnight at 65 °C in Rapid hybridization buffer (Amersham) with the linear plasmid DNA probe radiolabelled with [α -³²P]dCTP using a commercial kit (Megaprime, Amersham). The membranes were then washed as described previously (Picardeau *et al.*, 1996).

Cloning and sequencing of terminal DNA fragments. The 25 kb linear plasmid DNA of *M. celatum* strain 4, designated pCLP, was digested with *XbaI*. The *XbaI* was inactivated at 65 °C, and the digested DNA was purified into distilled sterile water and ligated into the *HincII/XbaI* sites of pBluescript II KS⁺ (Stratagene). The ligation mixture was transformed into *Escherichia coli* XL-1 Blue MRF' (Stratagene) by electro-

poration (Gene Pulser unit, Bio-Rad). Recombinant colonies were selected on Luria-Bertani solid medium [10 g Bactotryptone l⁻¹ (Difco), 5 g yeast extract l⁻¹, 10 g NaCl l⁻¹, pH 7.5] supplemented with 100 μ g ampicillin ml⁻¹, 2 mM IPTG and 0.04% X-Gal. Recombinant plasmids were isolated by alkaline lysis (Birnboim & Doly, 1979) or a Qiagen Midi kit. The presence of inserts into the recombinant plasmids was determined by restriction analysis.

The sequences of the double-stranded plasmid DNAs were determined by the dideoxy chain-termination method (Sanger *et al.*, 1977) with universal and reverse primers by using a *Taq* DyeDeoxy terminator cycle sequencing kit (Applied Biosystems), a model 9600 GeneAmp PCR system (Perkin-Elmer), and a model 373 stretch DNA analysis system (Applied Biosystems). Nucleotide sequences were analysed using the GCG package (University of Wisconsin, Madison, WI, USA) and The DNA Inspector IIe software (Textco, Lebanon, NH, USA).

PCR assays. Amplification reactions were performed in volumes of 50 μ l containing 1 $\times$ *Taq* polymerase buffer [50 mM Tris/HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.01% (w/v) gelatin], 5 μ l dimethyl sulfoxide, 200 μ M of each deoxynucleoside triphosphate, 1 μ M of each primer, and 2 U *Taq* polymerase (Perkin-Elmer) for 38 cycles: 1 min at 95 °C, 1 min at 55 °C, and 1 min at 72 °C, followed by a 10 min extension at 72 °C.

We selected primers Com (5'-CCC CCC CCC CGGCTT CGC CG-3'), V3 (5'-AAG AAA TAC AGA TAC CCG CC-3'),

Ec4.2 (5'-ATT TTT CGT GAT GCT TGC CG-3'), L2 (5'-GCA CAA CGA TTT AGA GAC GC-3') and L3B (5'-GTC AGG TTG CTC CAC GCT GG-3') from the nucleotide sequences of the linear plasmid pCLP (see Fig. 2 for the locations of primers). Primers were tested on crude DNA extract of *M. celatum* strain 4 and on the two isolated plasmids, of 25 kb and 320 kb. The amplified products (the 403 bp product of Com/V3, the 333 bp product of Com/Ec4.2 and the 246 bp product of L2/L3B) were cloned into pGEM-T (Promega) as recommended by the manufacturer and the recombinant plasmids were isolated and sequenced as described above.

RESULTS

Association of protein(s) with the DNA of the linear plasmids

To investigate whether the plasmids contained a covalently bound protein, proteinase K was omitted from the lysis treatment of the cells during the preparation of the plugs. The objective was to preserve the putative terminal protein, which could be expected to retard the linear plasmid DNA during electrophoresis as shown for invertrons (Hirochika & Sakaguchi, 1982; Kinashi & Shimaji-Murayama, 1991). The result is shown in Fig. 1. The preparation of mycobacterial genomic DNA without proteinase K did not allow the extrachromosomal elements to enter the gel (Fig. 1a, b, c, lanes 2). Proteinase-K-treated and -untreated DNA samples were also electrophoresed after digestion with

DraI and *XbaI*. The restriction patterns of the chromosomal DNAs were identical in proteinase-K-treated and -untreated DNAs, suggesting that the chromosomal DNAs were released and migrated into the gel in the absence of proteinase K treatment (Fig. 1a, lanes 3 and 4; Fig. 1b, lanes 3–6). Southern blot analysis following PFGE of undigested DNA probed with the host plasmids (the 180 kb plasmid detected in *M. celatum* strain 9, the 25 kb plasmid detected in *M. celatum* strain 4, and the 145 kb plasmid in *M. avium* strain 951849, respectively) revealed that the extrachromosomal elements remained trapped in the wells (Fig. 1a, b, c, lanes 2). The same results were obtained for all the linear plasmids tested in other species including *M. xenopi* and *M. branderi* (data not shown).

Cross-hybridization of the 25 kb linear plasmid with genomic DNA

Southern blot analysis following PFGE of *DraI*- and *XbaI*-digested DNA probed with the plasmids revealed that the extrachromosomal elements did not cross-hybridize with any DNA fragments of the chromosome of *M. celatum* strain 9 (Fig. 1a, lane 4). Our data also demonstrated that both plasmids of *M. celatum* strains 4 and 9 used as probes were not digested with *DraI* (Fig. 1). One element, the 25 kb plasmid from *M. celatum* strain 4, hybridized with the larger plasmid (Fig. 1b, lane 1). The size of this plasmid was estimated to be 320 kb by comparison with linear standards using migration

Fig. 2. Cloning and sequence analysis of plasmid pCLP of *M. celatum* strain 4. (a) The inserts of 10 kb and 14.5 kb of recombinant plasmids pMPV7 and pMPV3, respectively, corresponding to the whole length of pCLP. Regions which have been sequenced are indicated by hatched boxes. The *XbaI* site, terminal inverted repeats (TIRs) and primers used for PCR analysis are indicated. (b) Alignment of the first 100 bp of the ends of pCLP showing the nucleotide sequences of the left and right termini. Identical (—) and different (*) nucleotides are indicated. TIR sequences are in bold and palindromic sequences are boxed, with their short inverted repeats indicated by arrows.

conditions which resolved higher molecular mass fragments (Picardeau & Vincent, 1997). The plasmid estimated at 25 kb was designated pCLP, while the other, of 320 kb, was designated pLLP. Interestingly, we found hybridizations with several restriction fragments larger than the 25 kb plasmid (Fig. 1b, lanes 3–6). The presence of two hybridization signals with proteinase-K-treated and -untreated DNA digested with *Dra*I (Fig. 1b, lanes 3 and 4) suggest either the homology of pCLP with internal fragments of pLLP and/or cross-hybridization with the chromosomal DNA. Southern blot analysis also suggested that pCLP was restricted by *Xba*I and that the two restriction fragments obtained, corresponding to the two hybridization signals in the lower part of the blot, were retarded in PFGE by omission of proteinase K treatment (Fig. 1b, lane 5).

Characteristic features of the termini of pCLP

In comparison with linear replicons belonging to the genus *Streptomyces*, we hypothesized that free ends of mycobacterial linear plasmids, after proteinase K treatment, were blunted. For determining the DNA nucleotide sequence of the terminal fragments, pCLP was isolated and digested with *Xba*I. As Southern blot analysis after *Xba*I digestion was consistent with the presence of a single *Xba*I restriction site within pCLP, both restriction fragments could include the termini of this plasmid. The *Xba*I fragments were cloned into a Bluescript vector restricted with *Xba*I and *Hinc*II. Thus, ligation of *Xba*I fragments could only occur with the *Xba*I end of the vector and its opposite blunt end produced by the *Hinc*II cut. Among the transformants isolated, the restriction analysis of the cloned inserts showed two large fragments, of 10 kb and 14.5 kb, within the recombinant plasmids. These recombinant plasmids, designated pMPV7 and pMPV3, were selected for sequencing. The nucleotide sequence of 500 bp at each extremity of both inserts was determined (Fig. 2a).

Sequence analysis of the cloned end fragments revealed a high homology between the two terminal nucleotide sequences. The termini of pCLP contained 29 bp imperfect inverted repeats (93% base identity). This similarity could be extended for a further 16 bp at somewhat lower levels of identity (80% base identity). Surprisingly, we found that the first 10 bp of termini contain only guanines (Fig. 2b). Palindromic sequences, consisting of a cluster of two short inverted repeats, were located in the first 100 bp. Only a few other palindromes were found outside the first 100 bp of the termini. Although the terminal nucleotide sequences were not completely homologous to each other, the palindromic sequences had the same locations (Fig. 2b).

Due to the cloning strategy employed, the possible loss of a small internal *Xba*I fragment could not be excluded. Thus, we selected primers L3B and L2 from the inserts of recombinant plasmids pMPV3 and pMPV7, respectively, to check that the whole length of pCLP had been cloned. Moreover, to detect any rearrangement during

Fig. 3. Base composition analysis of the first 400 bp of the terminal DNA fragments of plasmid pCLP of *M. celatum* strain 4. (a) Nucleotide sequence of the left terminus of pCLP corresponding to the insert of recombinant plasmid pMPV7. (b) Nucleotide sequence of the right terminus of pCLP corresponding to the insert of recombinant plasmid pMPV3.

the cloning procedures, we synthesized primers based on the known sequences: Com, corresponding to terminal nucleotide sequences shared by the two inserts; V3, from the pMPV3 insert; and Ec4.2 using the pMPV7 insert sequence (Fig. 2). PCR results on crude DNA extract of *M. celatum* strain 4 using primer couples Com/V3, Com/Ec4.2 and L2/L3B were consistent with the expected size. The sequence analysis of these PCR products revealed no sequence rearrangements (data not shown). Furthermore, preparative PFGE was used to isolate the 25 and the 320 kb plasmids, which were subsequently purified and used for PCR analysis. In both plasmids, we obtained the same results with the three primer couples as with the total genomic DNA as template (data not shown).

We also studied the base composition of the first 400 bp of the two terminal fragments. The first 60 bp of the termini had a high GC content. The mean GC content was 62 mol% in the left arm of pCLP and 49 mol% in the right terminal fragment. Within the right terminal sequence we found three regions of low GC content (less than 30%), alternating with regions of high GC content. Within the left terminal fragment, except for the first 60 bp, the variations in GC content were significantly less pronounced (Fig. 3).

Comparison of the DNA sequences of pCLP, comprising the terminal fragments and one internal region spanning both inserts (Fig. 2a), with the EMBL and GenBank

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1  -----C C C C G G C A - G C G G G T A C C C C . . . S l y d
2  -----C C C C G G C A - G C G G G T A C C C C . . . S c l a
3  -----C C C C G G C A - G C G G G T A C C C C . . . S r o c
4  G G G G G G G G G G G C C A A C C G C C T C C C T . . . M c e l
5  --G G G G G G G G G G C C A A C C G C C C A C C T . . . M c e l
6  --G G G G G G G G G G C C G G C C A G G C C T A A C G . . . S c o e
7  -----C C C C G G C - G C T G C T C C G G . . . R h o d

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Fig. 4. Terminal nucleotide sequences of linear plasmids from actinomycetes. 1, SLP2 from *S. lividans* (Chen *et al.*, 1993); 2, pSCL from *S. clavuligerus* (Wu & Roy, 1993); 3, pSLA2 from *S. rochei* (Hirochika *et al.*, 1984); 4, left arm of pCLP from *M. celatum* (this study); 5, right arm of pCLP from *M. celatum* (this study); 6, SCP1 from *S. coelicolor* (Kinashi *et al.*, 1991); 7, pHG207 from *Rhodococcus* sp. (Kalkus *et al.*, 1993). Nucleotide sequences identical in at least four sequences are shown on a black background.

databases revealed no significant homologies (data not shown). However, when the terminal nucleotide sequences of pCLP were compared with those of previously known sequences of linear plasmids from actinomycetes, homology was observed (Fig. 4). The first 10 bp of all the termini were quite similar, with a high GC content: only one adenine or one thymine was present (Fig. 4). Interestingly, the six terminal bases of SCP1 from *S. coelicolor* were all guanines.

DISCUSSION

The linearity of some plasmids in mycobacteria suggested by electrophoretic mobility, nuclease treatment and hybridization experiments (Picardeau & Vincent, 1997) has been confirmed in this study. The mycobacterial linear plasmids characterized in this study have common features with those of *Streptomyces* and *Rhodococcus* and with other linear replicons designated as invertrons. They contain terminal inverted repeats (TIRs) with proteins linked to their ends (Sakaguchi, 1990).

Structure of mycobacterial linear plasmids

In this study, we demonstrated that proteins were covalently bound to the mycobacterial linear plasmids. These proteins were removed after proteinase K treatment. On the basis of the retardation of the terminal fragments of plasmids during PFGE and of their susceptibility to exonuclease III (Picardeau & Vincent, 1997), the structure of the mycobacterial linear plasmids can be assigned to the class of invertron (Sakaguchi, 1990). Taking into account that all invertrons described in prokaryotes and eukaryotes have proteins covalently attached at their 5' ends (Sakaguchi, 1990), one may assume that this is also the case for the mycobacterial linear plasmids. Further investigation will include the characterization of the DNA-protein complex.

The fact that the termini of pCLP can be ligated to the blunt ends of a cloning vector indicates that these termini are blunt. The existence of TIRs confirmed the invertron-like structure. In actinomycetes, the size of

these TIRs is quite variable, ranging from 44 bp in SLP2 to 9500 bp in pPZG101 (Chen *et al.*, 1993; Gravius *et al.*, 1994). There is no correlation between plasmid size and the length of the TIR sequence (see Table 1). Analysis of the integration of sex factor SCP1 of *Streptomyces coelicolor* into the chromosome showed that the free ends of SCP1 had been lost, suggesting a role of these TIRs in the integration of linear replicons similar to that of the TIRs of transposons (Hanafusa & Kinashi, 1992). Thus, the presence of TIR sequences within mycobacterial linear plasmids raises the possibility of their integration into the chromosome. However, we did not find any evidence of such events.

The palindromic sequences that we identified at the plasmid ends are also a characteristic feature of linear replicons. Interestingly, the sequence alignment of termini of other actinomycete linear plasmids (Fig. 4) showed a highly conserved region within the first 20 bp which corresponds to the first palindrome detected in mycobacterial plasmids (Fig. 2). In pCLP palindromes, hairpin sequences are often trinucleotides. These trinucleotides are GNA, which has been shown to form a stable single-residue loop closed by G and A, constituting a so-called sheared pairing (Chou *et al.*, 1997). For linear replicons of streptomycetes, they are thought to be potential hairpins required for the replication of the termini (Chen, 1996). The left arm of pCLP is noticeable because of its region of very low GC content. AT-rich regions were also observed in the terminal sequences of pHG207 from *Rhodococcus* sp. (Kalkus *et al.*, 1993). Such an AT-rich region might facilitate strand separation at the initial stage of replication termini and/or could be the evidence of a foreign origin of the mycobacterial linear plasmids from micro-organisms with a low GC content.

Hybridization experiments with pCLP as a probe (Fig. 1), and PCR analysis of the termini and of an internal region of pCLP, suggest the presence of pCLP within the 320 kb plasmid of *M. celatum* strain 4 in at least one copy.

PCR analysis confirmed that the whole length of the plasmid has been cloned. Sequence analysis and sub-cloning experiments can now be undertaken for further investigation of the replication mechanisms and specially for the identification of a putative DNA polymerase gene, detected in almost all linear plasmids sequenced to date (Chang *et al.*, 1996; Wu & Roy, 1993).

Distribution of linear plasmids in Actinomycetales

Initially, mycobacterial linear plasmids were found in *M. xenopi*, *M. celatum* and *M. branderi*. An investigation of possible hosts for linear replicons has allowed the detection of other linear plasmids in *M. avium*.

In Table 1, we report all the linear replicons described to date within the actinomycetes except derivatives of pSLA2 and other plasmids whose structures and

Table 1. Distribution of linear plasmids in *Actinomycetales*

Species*	Plasmid designation	Length (kb)	Blunt ends and/or terminal proteins	TIR (bp)	Conjugative transfer	Functions/genes carried	References
<i>M. celatum</i> 4†	pCLP	25	yes	45	ND	ND	This study
<i>R. fascians</i>	pFID188	200	ND	ND	yes	Development of fasciation in plants	Crespi <i>et al.</i> (1992, 1994)
<i>R. erythropolis</i>	pBD2	208	ND	ND	yes	Degradation of isopropylbenzene and co-oxidation of trichloroethene	Dabrock <i>et al.</i> (1994); Kebeler <i>et al.</i> (1996)
<i>R. opacus</i> ‡	pHG207	225	yes	583	yes	Hydrogen autotrophy	Kalkus <i>et al.</i> (1993, 1990)
<i>R. erythropolis</i>	pTA421	500	ND	ND	ND	Biphenyl degradation	Kosono <i>et al.</i> (1997)
<i>R. globerulus</i>	pSP6/pLP6	350/650	ND	ND	ND	Biphenyl degradation	Kosono <i>et al.</i> (1997)
<i>S. lividans</i>	SLP2	50	yes	44	yes	ND	Chen <i>et al.</i> (1993)
<i>S. lividans</i>	pBL1§	43	yes	ND	yes	ND	Zotchev <i>et al.</i> (1992)
<i>S. clavuligerus</i>	pSCL1	12	yes	900	ND	Regulatory proteins	Keen <i>et al.</i> (1988); Wu & Roy (1993)
<i>S. rochei</i>	pSLA2	17	yes	614	ND	Lankacidin group antibiotics	Hirochika <i>et al.</i> (1984); Hayakawa <i>et al.</i> (1979)
<i>S. rimosus</i>	pSRM	43	yes	ND	ND	ND	Chardon-Loriaux <i>et al.</i> (1986)
<i>S. ambofaciens</i>	pSAM	80	ND	ND	ND	ND	Leblond <i>et al.</i> (1990, 1996)
<i>S. avermitilis</i>	pSA1/pSA2	100/250	ND	ND	ND	ND	Evans <i>et al.</i> (1994)
<i>S. rimosus</i>	pPZG101	387	ND	9500	ND	ND	Gravius <i>et al.</i> (1994)
<i>S. coelicolor</i>	SCP1	350	yes	7000	yes	Methylenomycin; spore formation	Kinashi (1994); Kinashi <i>et al.</i> (1987); Kinashi & Shimaji-Murayama (1991)
<i>S. lasaliensis</i>	pKSL	520	ND	ND	ND	Lasalocid and echinomycin	Kinashi (1994)

ND, Not determined.

* *M.*, *Mycobacterium*; *R.*, *Rhodococcus*; *S.*, *Streptomyces*.† Other linear plasmids have been identified in *M. avium*, *M. xenopi*, *M. celatum* and *M. branderi* (Picardeau & Vincent, 1997; this study).‡ Formerly *Nocardia opaca*.§ pBL1 was isolated from a plasmidless strain after its mating with *S. bambergensis* S712 harbouring the 640 kb plasmid PSB1.

|| pSAM1 is found as either a linear or a circular molecule.

functions have not been studied (Kinashi, 1994). In studies where the existence of terminal proteins and/or TIR sequences has not been described, the linearity of the molecules was demonstrated by their electrophoretic behaviour, the restriction analysis of the plasmid (in this case the sum of restriction fragments was in agreement with the molecular mass obtained by PFGE), by their susceptibility to exonucleases treatment or by electron microscopy (Chardon-Loriaux *et al.*, 1986; Hayakawa *et al.*, 1979; Kosono *et al.*, 1997). We can assume that all these linear replicons have an invertron-like structure.

Within the genera *Streptomyces* and *Rhodococcus*, belonging, like the genus *Mycobacterium*, to the order

Actinomycetales (Stackebrandt *et al.*, 1997), several linear plasmids as carriers of genetic information have been described (Table 1). The genus *Rhodococcus* contains micro-organisms that exhibit broad metabolic diversity (Finnerty, 1992). Most of these metabolic properties are encoded on circular or linear plasmids (Dabrock *et al.*, 1994; Finnerty, 1992; Kalkus *et al.*, 1993; Kebeler *et al.*, 1996). The species *Nocardia opaca*, where the pHG207 linear plasmid was identified (Kalkus *et al.*, 1990), has been renamed as a *Rhodococcus* species. The genus *Rhodococcus* also includes a plant-pathogenic species, *R. fascians*, harbouring a plasmid responsible for development of fasciation in plants (Crespi *et al.*, 1992, 1994) (Table 1). The genus *Strepto-*

myces comprises many antibiotic-producing species, accounting for over 70% of naturally occurring antibiotics (Berdy, 1980). Most of the antibiotic biosynthesis genes have been found to be chromosomal. However, some of these genes are located on linear plasmids (Hayakawa *et al.*, 1979; Kinashi, 1994; Kinashi *et al.*, 1987).

Streptomyces, *Rhodococcus* and *Mycobacterium* spp. contain plasmids of both circular and linear topology (Kinashi, 1994; Zotchev *et al.*, 1992). However, it is interesting that while the chromosomes of both *Streptomyces* and *Rhodococcus* species appear linear (Crespi *et al.*, 1992; Lin *et al.*, 1993), the chromosome of the *Mycobacterium* species investigated so far is circular (Philipp *et al.*, 1996; Picardeau & Vincent, 1997). The linearity of the chromosome of streptomycetes and nocardioforms could derive from the integration of linear plasmids (Gravius *et al.*, 1994; Lin *et al.*, 1993). Among prokaryotes, only a few phages have the same structure as the *Streptomyces* linear plasmids (Hinnebusch & Tilly, 1993). As suggested by Chang *et al.* (1996), *Streptomyces* linear plasmids may occupy an evolutionarily intermediate position between circular plasmids and linear phage replicons. Little information is available on extrachromosomal elements of other genera belonging to the order *Actinomycetales*. The study of their basic genetics has been relatively neglected, and thus it will not be surprising if linear plasmids are identified in other actinomycetes.

In actinomycetes, almost all the extensively studied linear replicons have been found to be transmissible plasmids (Table 1). These plasmids may have played an important role in the evolution of the catabolic pathway for some compounds and for the dissemination of antibiotic biosynthesis genes. Some of these plasmids can integrate into the chromosome (Gravius *et al.*, 1994; Kinashi *et al.*, 1992). To date, it is unknown whether mycobacterial plasmids, including both circular and linear molecules, are capable of conjugative transfer. It is relevant that mycobacteria are not competent for transformation and their cell walls are considered as a barrier. However, there is evidence that recombination occurs in nature. Recently, intragenetic transfer of mercury resistance between *Mycobacterium fortuitum* and *M. smegmatis* has been observed in mating experiments (Gavigan *et al.*, 1997). However, the conjugational fertility was not attributed to any identified plasmids. Sequence homologies were found between circular plasmids from rapidly growing and slow-growing mycobacteria (Gavigan *et al.*, 1997) and between plasmids from *Rhodococcus* species and plasmid pAL5000 from *M. fortuitum* (DeMot *et al.*, 1997; Kulakov *et al.*, 1997). Further evidence of genetic exchanges is also supported by the existence of homologous insertion sequences within mycobacteria and related micro-organisms (Kato *et al.*, 1994; Picardeau *et al.*, 1997). Moreover, conjugative transfer of a shuttle plasmid from *E. coli* to *M. smegmatis* has been reported (Lazrak *et al.*, 1990). Although plasmids are suggested to play a role in genetic exchanges, no experimental

evidence has been obtained in mycobacteria. It could be of interest to investigate the conjugative ability of the mycobacterial linear plasmids. Genetic flux may have occurred between members of these genera, which are common throughout nature.

Further studies of mycobacterial linear plasmids will allow the development of new genetic tools, the utilization of these plasmids as epidemiological markers and a better understanding of genetic exchange in actinomycetes.

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REFERENCES

- Berdy, J. (1980). Recent advances in and prospects for antibiotic research. *Process Biochem* 15, 28–35.
- Birnboim, H. C. & Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res* 7, 1513–1523.
- Chang, P. C., Kim, E. S. & Cohen, S. N. (1996). *Streptomyces* linear plasmids that contain a phage-like, centrally located, replication origin. *Mol Microbiol* 22, 789–800.
- Chardon-Loriaux, I., Charpentier, M. & Percheron, F. (1986). Isolation and characterization of a linear plasmid from *Streptomyces rimosus*. *FEMS Microbiol Lett* 35, 151–155.
- 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 kb linear molecule. *Mol Microbiol* 7, 925–932.
- Chou, S. H., Zhu, L. & Reid, B. R. (1997). Sheared purine pairing in biology. *J Mol Biol* 267, 1055–1067.
- Crespi, M., Messens, E., Caplan, A. B., vanMontagu, 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.
- Crespi, M., Vereecke, D., Temmerman, W., vanMontagu, M. & Desomer, J. (1994). The *fas* operon of *Rhodococcus fascians* encodes new genes required for efficient fasciation of host plants. *J Bacteriol* 176, 2492–2501.
- Dabrock, B., Kebeler, M., Averhoff, B. & Gottschalk, G. (1994). Identification and characterization of a transmissible linear plasmid from *Rhodococcus erythropolis* BD2 that encodes isopropylbenzene and trichloroethene catabolism. *Appl Environ Microbiol* 60, 853–860.
- DeMot, R., Nagy, I., DeSchrijver, A., Pattanapitpaisal, P., Schoofs, G. & Vanderleyden, J. (1997). Structural analysis of the 6 kb cryptic plasmid pFAJ2600 from *Rhodococcus erythropolis* NI86/21 and construction of *Escherichia coli*–*Rhodococcus* shuttle vectors. *Microbiology* 143, 3137–3147.
- Evans, M., Kaczmarek, F. S., Stutzman-Engwall, K. & Dyson, P. (1994). Characterization of a *Streptomyces lividans*-type site-specific DNA modification system in the avermectin producer *Streptomyces avermitilis* permits investigation of two novel giant linear plasmids, pSA1 and pSA2. *Microbiology* 140, 1367–1371.

- Finnerty, W. R. (1992). The biology and genetics of the genus *Rhodococcus*. *Annu Rev Microbiol* 46, 193–218.
- Gavigan, J. A., Ainsa, J. A., Pérez, E., Otal, I. & Martin, C. (1997). Isolation by genetic labeling of a new mycobacterial plasmid, pJAZ38, from *Mycobacterium fortuitum*. *J Bacteriol* 179, 4115–4122.
- Gravius, B., Glocker, D., Pigac, J., Pandza, K., Hranueli, D. & Cullum, J. (1994). The 387 kb linear plasmid pPZG101 of *Streptomyces rimosus* and its interactions with the chromosome. *Microbiology* 140, 2271–2277.
- Hanafusa, T. & Kinashi, H. (1992). The structure of an integrated copy of the giant linear plasmid SCP1 in the chromosome of *Streptomyces coelicolor* 2612. *Mol Gen Genet* 231, 363–368.
- Hayakawa, T., Tanaka, T., Sakaguchi, K., Otake, N. & Yonehara, H. (1979). A linear plasmid-like DNA in *Streptomyces* sp. producing lankacidin group antibiotics. *J Gen Appl Microbiol* 25, 255–260.
- Hinnebusch, J. & Tilly, K. (1993). Linear plasmids and chromosomes in bacteria. *Mol Microbiol* 10, 917–922.
- Hinnebusch, J., Bergström, S. & Barbour, A. G. (1990). Cloning and sequence analysis of linear plasmid telomeres of the bacterium *Borrelia burgdorferi*. *Mol Microbiol* 4, 811–820.
- 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 an inverted 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.
- Kato, K., Ohtsuki, K., Mitsuda, H., Yomo, T., Negoro, S. & Urabe, I. (1994). Insertion sequence IS6100 on plasmid pOAD2, which degrades nylon oligomers. *J Bacteriol* 176, 1197–1200.
- Kebeler, M., Dabbs, E. R., Averhoff, B. & Gottschalk, G. (1996). Studies on the isopropylbenzene 2,3-dioxygenase and the 3-isopropylcatechol 2,3-dioxygenase genes encoded by the linear plasmid of *Rhodococcus erythropolis* BD2. *Microbiology* 142, 3241–3251.
- Keen, C. L., Mendelovitz, S., Cohen, G., Aharonowitz, Y. & Roy, K. L. (1988). Isolation and characterization of a linear DNA plasmid from *Streptomyces clavuligerus*. *Mol Gen Genet* 212, 172–176.
- Kinashi, H. (1994). Linear plasmids from Actinomycetes. *Actinomycetologica* 8, 87–96.
- Kinashi, H. & Shimaji-Murayama, M. (1991). Physical characterization of SCP1, a giant linear plasmid from *Streptomyces coelicolor*. *J Bacteriol* 173, 1523–1529.
- Kinashi, H., Shimaji, M. & Sakai, A. (1987). Giant linear plasmids in *Streptomyces* which code for antibiotic biosynthesis genes. *Nature* 328, 454–456.
- Kinashi, H., Shimaji-Murayama, M. & Hanafusa, T. (1992). Integration of SCP1, a giant linear plasmid, into the *Streptomyces coelicolor* chromosome. *Gene* 115, 35–41.
- Kosono, S., Maeda, M., Fuji, F., Arai, H. & Kudo, T. (1997). Three of the seven *bphC* genes of *Rhodococcus erythropolis* TA421, isolated from a termite ecosystem, are located on an indigenous plasmid associated with biphenyl degradation. *Appl Environ Microbiol* 63, 3282–3285.
- Kulakov, L. A., Larkin, M. J. & Kulakova, A. N. (1997). Cryptic plasmid pKA22 isolated from the naphthalene degrading derivative of *Rhodococcus rhodochrous* NCIMB 13064. *Plasmid* 38, 61–69.
- Lazrak, R., Clavel-Sérés, S., David, H. L. & Roulland-Dussoix, D. (1990). Conjugative transfer of a shuttle plasmid for *Escherichia coli* to *Mycobacterium smegmatis*. *FEMS Microbiol Lett* 69, 135–138.
- Leblond, P., Francou, F. X., Simonet, J. M. & Decaris, B. (1990). Pulsed-field gel electrophoresis analysis of the genome of *Streptomyces ambofaciens* strains. *FEMS Microbiol Lett* 72, 79–88.
- Leblond, P., Fischer, G., Francou, F. X., Berger, F., Guérineau, M. & Decaris, B. (1996). The unstable region of *Streptomyces ambofaciens* includes 210 kb terminal inverted repeats flanking the extremities of the linear chromosomal DNA. *Mol Microbiol* 19, 261–271.
- 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.
- Philipp, W. J., Poulet, S., Eiglmeier, K. & 7 other authors (1996). An integrated map of the genome of the tubercle bacillus, *Mycobacterium tuberculosis* H37Rv, and comparison with *Mycobacterium leprae*. *Proc Natl Acad Sci USA* 93, 3132–3137.
- Picardeau, M. & Vincent, V. (1997). Characterization of large linear plasmids in mycobacteria. *J Bacteriol* 179, 2753–2756.
- Picardeau, M., Varnerot, A., Rauzier, J., Gicquel, B. & Vincent, V. (1996). *Mycobacterium xenopi* IS1395, a novel insertion sequence expanding the IS256 family. *Microbiology* 142, 2453–2461.
- Picardeau, M., Bull, T. J. & Vincent, V. (1997). Identification and characterization of IS-like elements in *Mycobacterium goodii*. *FEMS Microbiol Lett* 154, 95–102.
- 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.
- Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* 74, 5463–5467.
- Stackebrandt, E., Rainey, F. A. & Ward-Rainey, N. L. (1997). Proposal for a new hierarchic classification system, *Actinobacteria* classis nov. *Int J Syst Bacteriol* 47, 479–491.
- Wu, X. & Roy, K. L. (1993). Complete nucleotide sequence of a linear plasmid from *Streptomyces clavuligerus* and characterization of its RNA transcripts. *J Bacteriol* 175, 37–52.
- Zotchev, S. B., Soldatova, L. I., Orekhov, A. V. & Schrempf, H. (1992). Characterization of a linear extrachromosomal DNA element (pBL1) isolated after interspecific mating between *Streptomyces bambergensis* and *S. lividans*. *Res Microbiol* 143, 839–845.

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