Mycobacteriophage D29 contains an integration system similar to that of the temperate mycobacteriophage L5

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A mycobacteriophage D29 DNA fragment cloned in pRM64, a shuttle plasmid that transforms *Mycobacterium smegmatis*, was sequenced. The determined sequence was 2592 nucleotides long and had a mean G+C content of 63.7 mol%, similar to that of mycobacterial DNA. Four ORFs were identified: one with strong homology to dCMP deaminase genes; one homologous to mycobacteriophage L5 gene 36, whose function is unknown; one encoding a possible excisase; and one encoding an integrase. The intergenic region between the putative excisase gene and the integrase gene had a lower than average G+C content and showed the presence of the same *attP* core sequence as mycobacteriophage L5. Transformation experiments using subclones of pRM64 indicated that the integrase gene and all the intergenic region were essential for stable transformation. A subclone containing the integrase gene and the core *attP* sequence was able to transform but recombinants were highly unstable. Southern analysis of total DNA from cells transformed with pRM64 and its derivatives showed that all the plasmids were integrated at one specific site of the bacterial chromosome. A recombinant exhibiting a high level of resistance to the selective drug kanamycin had two plasmids integrated at different sites. These results demonstrated that the D29 sequences contained in pRM64 were integrative, indicating that the generally held view of D29 as a virulent phage must be reviewed.

Keywords: *Mycobacterium*, mycobacteriophage D29, integrase, site-specific integration, dCMP deaminase

INTRODUCTION

Mycobacterial diseases, namely tuberculosis and leprosy, still remain important world-wide public health problems. This has been seriously aggravated during the last decade by the increase of drug-resistant tuberculosis affecting AIDS patients (Snider *et al.*, 1994). The understanding of pathogenesis and drug resistance, the search for new therapeutic drugs, and the preparation of a new generation of vaccines require further advances in the present knowledge of the molecular biology of mycobacteria. This strongly depends on the availability of efficient tools for genetic analysis and manipulation

of mycobacteria. The majority of shuttle vectors used in molecular biology of mycobacteria (Snapper *et al.*, 1988; Lazraq *et al.*, 1990; Stover *et al.*, 1991; Barletta *et al.*, 1992) derive from a *Mycobacterium fortuitum* plasmid, pAL5000 (Labidi *et al.*, 1985). Because vectors containing the origin of pAL5000 are not always stable and transform inefficiently (Snapper *et al.*, 1988; Lazraq *et al.*, 1990), Lazraq *et al.* (1991) reasoned that the origin of replication (*oriR*) of a broad-spectrum virulent mycobacteriophage could be an alternative to pAL5000 in constructing stable multicopy plasmids. For this purpose, a plasmid (pRM64) was constructed containing the kanamycin-resistance gene from Tn903 and an origin, *oriR*, present in a *Pst*I insert from mycobacteriophage D29 (Froman *et al.*, 1954).

Mycobacteriophage D29 has been extensively studied (reviewed by Mizuguchi, 1984). It is apparently related to another well-known mycobacteriophage, L5, because

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lysogens for L5 show immunity to superinfection with D29 (Donnelly-Wu *et al.*, 1993). However, there is an important difference between the two phages, i.e. L5 is a temperate phage (Lee *et al.*, 1991) whereas D29 has been traditionally considered as a virulent phage (White *et al.*, 1962).

Soon after the construction of pRM64, David *et al.* (1992) reported the preparation of a similar plasmid, pBL415. The sequence of this second plasmid has not been published but was deposited in the GenBank database (accession number X70352). The observation that this sequence contained an integrase-like gene was unexpected, considering that D29 is a virulent bacteriophage. Therefore, we thought that it was necessary to examine further the D29 fragment cloned into pRM64. In this paper, we report the full sequence of the mycobacteriophage D29 region inserted in plasmid pRM64 and the characterization of the minimal regions required for stable transformation of mycobacteria. We have also identified an ORF with strong homology to dCMP deaminases and three other ORFs, one encoding an integrase with strong similarity to L5 integrase and another encoding a putative excisase.

METHODS

Bacterial strains and media. *Escherichia coli* XL1-Blue (Stratagene) was grown in Luria-Bertani (LB) medium. When necessary, the medium was supplemented with 100 µg ampicillin ml⁻¹. The high-frequency transformation strain *Mycobacterium smegmatis* mc²155 (Snapper *et al.*, 1990) was cultivated in liquid medium RV10b (Redmond & Ward, 1966) or on LB agar. Resistance to kanamycin was assayed by adding the desired concentration of the drug to the medium.

DNA extraction. Plasmid DNA was extracted from *E. coli* using a Qiagen plasmid midi kit, as recommended by the manufacturer. For minipreparation of *M. smegmatis* total DNA, cells were incubated until the late-exponential phase of growth and a loopful of cells was suspended in 500 µl 10 mM Tris/HCl (pH 8.0), 1 mM EDTA (TE), heated at 80 °C for 20 min and treated with 1 mg lysozyme ml⁻¹ for 1 h at 37 °C. After 10 min incubation at 65 °C with 100 µg proteinase K ml⁻¹ and 1% SDS, NaCl and cetyltrimethylammonium bromide were added to final concentrations of 0.7 M and 33 mM, respectively. The mixture was incubated at 65 °C for 10 min and DNA was extracted with chloroform/isoamyl alcohol, precipitated with cold ethanol, dried under vacuum, and dissolved in a small volume of TE.

DNA manipulations and hybridization. All DNA manipulations, restriction enzyme digestions, agarose gel electrophoresis and Southern hybridizations were performed following standard procedures (Sambrook *et al.*, 1989).

Bacterial transformation. Competent *E. coli* cells were transformed by a classical calcium chloride technique (Sambrook *et al.*, 1989). *M. smegmatis* was transformed by electroporation, as follows. A 100 ml culture was grown to OD₆₅₀ 0.6, cooled on ice for 30 min and centrifuged at 6500 g for 15 min at 4 °C. The pellet was washed with cold water and then with cold 10% (v/v) glycerol, and finally resuspended in a small volume of 10% glycerol, to obtain a final concentration of about 10⁹ c.f.u. ml⁻¹. This cell suspension was distributed in small

volumes and stored at -70 °C. A 40 µl aliquot was thawed, transferred to an electroporation cuvette and mixed with 1 µl DNA solution containing 100 ng DNA. The cells were cooled on ice for 10 min and subjected to a 4.5 ms pulse using a Gene Pulser electroporator (Bio-Rad) set at 1.25 µF, 200 Ω, 25 kV cm⁻¹. Cells were immediately diluted in 1 ml RV10b liquid medium and incubated at 37 °C for 3 h under mild agitation. They were then spread on LB agar plates containing 10 µg kanamycin ml⁻¹.

DNA sequencing. pRM64 and the subclones containing pRM64 *Bam*HI or *Sal*I subfragments were sequenced by the dideoxy chain-termination method (Sanger *et al.*, 1977). Both strands were completely sequenced. For sequencing, 1 pmol of double-stranded plasmid DNA was denatured in 0.2 M NaOH and 2 mM EDTA, neutralized and precipitated with ethanol. The denatured DNA was used as template for sequencing, using a Sequenase (version 2.0) kit from United States Biochemical as recommended by the manufacturer. Sequencing primers were standard primers corresponding to both flanking regions of the vector DNA or specific primers synthesized on the basis of sequence already read. To confirm the sequence of regions with GC stretches resulting in band compression, dITP was used in some experiments instead of dGTP. Sequencing was also performed by cycle reactions using *Taq* DNA polymerase and fluorescent-dye-coupled dideoxy terminators. The products were analysed with a 373A automated DNA sequencer (Applied Biosystems). Analysis of DNA and protein sequences was performed with the programs of the University of Wisconsin Genetics Computer Group (Devereux *et al.*, 1984).

Assay of plasmid stability. Three independent *M. smegmatis* transformants from each plasmid were inoculated into RV10b medium and grown to saturation. Cultures were diluted 1:100 in medium without kanamycin and grown again to saturation. Additional passages were done for a total of 160 generations. At 3 d intervals, aliquots of the same volume were plated on solid medium with or without 10 µg kanamycin ml⁻¹ and the percentage of drug-resistant cells was calculated 4 d later.

RESULTS

Nucleotide sequence of the mycobacteriophage D29 integration region

We determined the sequence of the 2592 nucleotide mycobacteriophage D29 *Pst*I insert in pRM64. The phage insert had a high G+C content (63.7 mol%) typical of mycobacterial DNA. However, there was a central region of about 600 nucleotides showing a moderately lower G+C content of 60.2 mol%. Direct or inverted repeats of significant length were not detected. The sequence showed several possible ORFs on both strands. We considered four of these ORFs as putative genes because their codons corresponded to frequently used codons in a table compiled from known mycobacterial and mycobacteriophage genes and because they were preceded by consensus ribosome-binding sequences (Kempesell *et al.*, 1992). The positions and orientation of these four ORFs, designated g1, g2, g3 and g4, are indicated in Fig. 1. The D29 insert comprises only the 3'-part of ORF g1, which starts in an adjacent DNA fragment. All the ORFs were oriented in the same direction. The first three genes mapped closely

Fig. 1. (a) Representation of the DNA fragment from mycobacteriophage D29 cloned in pRM64 as well as of the subcloned derivatives described in the text. Restriction enzyme sites: B, *Bam*HI; Bs, *Bst*XI; E, *Eco*RI; H, *Hind*III; N, *Nae*I; P, *Pst*I; S, *Sal*I; Sp, *Sph*I; X, *Xba*I. (b) Plasmid loss under non-selective conditions from cells transformed with pRM64 (○), pGR30 (●) and pGRB1 (■). The experiments were done in triplicate with consistent results. The generation time of *M. smegmatis* under our experimental conditions was estimated to be 3 h.

in the left third of the sequence but the fourth gene was separated from the others by a large intergenic region of 620 nucleotides, corresponding to the region with lower G+C content. The two left-most genes were contiguous, with the termination codon of g1 immediately upstream of the ATG codon of g2. Therefore, the putative translation start signal of g2 was part of the coding sequence of g1.

We could only assign a probable function to three of the genes, as described below. The other putative gene, g2, encodes a small protein of 56 amino acids, with a predicted M_r of 6322. This hypothetical protein showed 89% similarity to the product of mycobacteriophage L5 gene 36 (Hatfull & Sarkis, 1993), whose function is unknown.

Fig. 2. Multiple alignment of the predicted product of ORF g1 to dCMP deaminases from *Caenorhabditis elegans*, *Homosapiens*, bacteriophages T2 and T4, and *Saccharomyces cerevisiae* (SWISS-PROT accession numbers P30648, P32321, P00814, P16006 and P06773, respectively). Perfect matches are indicated by black boxes and conservative substitutions by grey boxes. The first 100 amino acids of the yeast enzyme were omitted from this alignment.

D29 probably has a dCMP deaminase gene

A FASTA search of the deduced product of g1, the leftmost ORF of the D29 insert, revealed a strong homology to dCMP deaminase genes. Fig. 2 shows a multiple alignment of dCMP deaminases (EC 3.5.4.12) from bacteriophages T2 and T4, *Saccharomyces cerevisiae*, *Homo sapiens*, *Caenorhabditis elegans* and the predicted product of the D29 g1 gene. These proteins share two conserved central domains separated by a spacer of variable length and flanked by amino and carboxyl-terminal tails that have little or no homology. The partial D29 gene sequence found in the pRM64 insert comprised only part of the left conserved domain, which was contiguous to the other conserved domain. In this sense, the D29 gene was more related to the eukaryotic genes than to the T-even genes.

The D29 genome encodes an integrase and possibly an excisase

The region of the D29 insert in pRM64 showing the highest level of similarity to mycobacteriophage L5 was the 3'-half of the sequence, covering gene g4 in D29 and gene 33 in L5. This gene encodes an integrase (Lee *et al.*,

D29	MDAEAWLASEKRLIDNEEWTPPAEREKKAASAITVEEYTKKWIAERDLA	50
L5	MDAEAWLAGEKRLIEMETWTPPQDRAKKAAASAITLEEYTRKWLVDERDLA	50
D29	GGTKDLYSTHARKRIYPVLGDTVAEMTPALVRAWWAGMGKQYPTARRHA	100
L5	DGTRDLYSGHAERRIYPVLGEVAVTEMTALVRAWWAGMRKHPTARRHA	100
D29	YNVLRAVMNTAVEDKLVSENPCRIEQKAPAEERDVEALTPEELDVVAGEVF	150
L5	YNVLRAVMNTAVEDKLIAENPCRIEQKAADERDVEALTPEELDIVAAEIF	150
D29	EHYRVAVYILAWTSLRFGELIEIRRKDIVDDGETMKLRVRRGAAARVGEKI	200
L5	EHYRIAAYILAWTSLRFGELIELRRKDIVDDGMTMKLRVRRGASRVGNKI	200
D29	VVGNTKTVRSKRFPVTPPHVAAMIREHMDRTKMNGPEALLVTTTRGQR	250
L5	VVGNAKTVRSKRFPVTPPHVAEMIRAHMKDRTKMNGPEAFVLTTCQNR	250
D29	LSKSAFTSRSLKKGYAKIGRPDLRIHDLRAVGATLAAQAGATTKELMVRIG	300
L5	LSKSAFTKSLKRGYAKIGRPDLRIHDLRAVGATFAAQAGATTKELMARIG	300
D29	HTTPRMAMKYQMASAARDEEIAARRMSELGITP	333
L5	HTTPRMAMKYQMASEARDEATAEAMSLAKTS	332

Fig. 3. Alignment of the amino acid sequence of the putative D29 integrase with the sequence of mycobacteriophage L5 integrase. Vertical bars indicate perfect homology and colons or dots indicate conservative substitutions.

1991; Hatfull & Sarkis, 1993; Lee & Hatfull, 1993). Correspondingly, the predicted product of D29 gene g4, 333 amino acids long, had a high degree of similarity (83.5%) to the L5 integrase (Fig. 3). Most of the sequence divergence was due to conservative substitutions.

Comparison of the DNA sequences in this region of both bacteriophages also disclosed the presence in D29 of the same *attP* core sequence as in mycobacteriophage L5 (Lee *et al.*, 1991; Hatfull & Sarkis, 1993). The 42 nucleotide *attP* core sequence mapped in the lower G + C content region and was oriented to the right (Fig. 1).

After identifying the integrase gene, we considered the hypothesis that D29 also encodes an excisase gene. The presence of L5 DNA to the right of the *attP* site confers instability to plasmids, which led to the suggestion that an excisase gene, probably gene 34, is present in that region (Lee *et al.*, 1991; Hatfull & Sarkis, 1993). Like L5 gene 34, the D29 ORF g3 is adjacent to the *attP* site at its 5'-side and corresponds to that L5 gene. Comparison of the D29 ORF g3 and L5 gene 34 revealed 60% similarity. Therefore, g3 might be a candidate excisase gene. FASTA searches using g3 or its predicted product as query sequences did not result in significant homology to any known protein. As an alternative approach, we performed a multiple alignment of the deposited sequences of excisases from coliphages lambda, P2, P22, HK022 and ϕ 80, from *Staphylococcus* phage L54a, from transposon Tn1545 from *Streptococcus pneumoniae*, and from *Saccharopolyspora erythraea*. From this multiple alignment no conserved motifs were detected, but a homology profile was extracted using program ProfileMaker. We compared this profile to the predicted product of gene g3, using the program ProfileGap, and observed a functional homology of 61%. However, in

the absence of experimental evidence, the role of g3 as a putative excisase is conjectural.

Functional dissection of the D29 integration region

If those two genes actually encode an integrase and an excisase, it would be interesting to know their role in the replication and stability of vectors based on the D29 integration region. To elucidate this issue, we prepared a set of subclones from pRM64 spanning different subregions of the original D29 insert (Fig. 1a).

Deletions in the 5'-region of the insert were prepared by digestion of pRM64 with *Bam*HI and re-ligating to generate pGRB1, or by double digestion with *Bst*XI and *Xba*I, treating with S1 nuclease and Klenow enzyme to generate blunt ends and re-ligating to generate pGR30. Both recombinants contain the putative integrase gene and lack the possible excisase gene. Clone pGR30 contains all the intergenic region, whereas pGRB1 contains only the *attP* sequence, without its upstream region. A much shorter recombinant, pGRS3, was constructed by excision of the *Sal*I fragment of pRM64. To prepare deletions in the other end of pRM64, we constructed pRM64c, which contained the same *Pst*I fragment from D29 but in the opposite orientation. From this recombinant, we constructed subclone pGRBP by digestion with *Bam*HI and re-ligating. A shorter deletion was prepared by digestion of pRM64c with *Eco*RI and *Nae*I, repairing the ends and blunt end ligation, generating pGRN8. Subclone pGRBP contained the putative excisase gene and the 5'-end of the intergenic region but lacked the *attP* sequence and the integrase gene. The other subclone, pGRN8, lacked only the integrase gene. Finally, we constructed a recombinant, pGR30N, lacking both genes but retaining all the intergenic region. To construct this recombinant, we first excised the *Bst*XI-*Sph*I fragment of pRM64c and then digested the resulting recombinant with *Eco*RI and *Nae*I. The protruding ends were filled by treatment with Klenow enzyme and re-ligated.

Recombinant pGR30 transformed *M. smegmatis* as efficiently as pRM64 or pRM64c, yielding about 10^5 kanamycin-resistant transformants (μ g plasmid DNA)⁻¹. Recombinant pGRB1 was much less efficient, yielding only 600 transformants (μ g DNA)⁻¹. No kanamycin-resistant transformants were observed after transformation with any of the other plasmids.

Subclone pGR30 was quite stable, as demonstrated by determination of the proportion of cells that had conserved kanamycin resistance (Fig. 1b). After 160 generations of non-selective growth, 53% of descendants from pGR30-transformed cells still maintained the plasmid. The original plasmid pRM64 was less stable, since only 17.4% of descendants were resistant to kanamycin. Recombinant pGRB1, which was also able to transform *M. smegmatis*, although much less efficiently, was very unstable. The proportion of kanamycin-resistant descendants decreased progressively with cultivation and was lower than 0.01% when

Fig. 4. Hybridization of cellular DNA from *M. smegmatis* transformed with pRM64, pGR30, pGRB1 or pHLD69 and from pGR30-transformed cells showing a high level of resistance to kanamycin (pGR30+) with probes P1 (a) and P2 (b). DNA was digested with *XmnI* (X) or *PvuII* (P). Lane m is a *BstEII* digest of lambda labelled DNA (dimensions in kb: 8.4, 7.2, 6.4, 5.7, 4.8, 4.3, 3.7, 2.3, 1.9, 1.4 and 1.3).

the culture reached 160 generations. No spontaneous kanamycin-resistant clone was observed in control cultures of non-transformed cells.

Taken together, these results indicated that the integrase gene and the complete intergenic region around the *attP* sequence were required for efficient transformation and for stability of plasmids based on the D29 origin. In addition, the removal of the putative excisase gene seemed to confer a higher degree of plasmid stability in successive cell generations.

Chromosomal integration of D29-derived plasmids

The presence of an integrase gene and its role in transformation and plasmid stability suggest that plasmids containing this D29 region may be integrated in the bacterial chromosome. To test this hypothesis, we performed a Southern analysis of pRM64 and its derivatives in transformed cells.

Total DNA from transformed cells was extracted and digested with *XmnI* or *PvuII*. *XmnI* cuts pRM64 once, in the pUC19 sequence, and there are two *PvuII* sites in pRM64, also in the pUC19 sequence. The digests were separated by agarose gel electrophoresis and hybridized with two different probes. One probe, P1, was the 1.5 kb *Bam*HI–*Pst*I fragment of pRM64 containing the integrase gene. The other probe, P2, was a 1.6 kb *Xmn*I–*Aat*II fragment of pRM64 containing the kanamycin-resistance gene and the 5'-region of the *amp* gene. Both fragments were electroeluted from gels and labelled.

As shown in Fig. 4, probe P1 revealed a single *XmnI* band, of 6 kb, and a single *PvuII* band, of 11 kb, in DNA from cells transformed with all tested plasmids, pRM64, pGR30 and pGRB1. A faint *XmnI* band of 3.7 kb and another faint *PvuII* band of 8.9 kb were also detected in the pGR30 sample. The *XmnI* and *PvuII* bands detected

in plasmid DNA extracted from *E. coli* had different sizes from those described above and corresponded to the sizes deduced from the plasmid sequences. Fragments with sizes larger than those expected from plasmid DNA were also detected when transformed cell DNA was hybridized with the P2 probe. In all tested DNAs, except those containing pHLD69, this probe detected a single *XmnI* band of 9.4 kb and a *PvuII* band of 3.6 kb. As expected, the *PvuII* band had the size of the larger *PvuII* plasmid fragment, since this enzyme cuts the plasmid twice, but the *XmnI* band had a much larger size than the plasmid. In contrast to these results, two bands in each enzyme digest were detected when we assayed DNA from a cell culture derived from a pGR30 recombinant resistant to high doses of kanamycin, up to 1000 µg ml⁻¹. Besides the bands described above, an equally strong *XmnI* band of 3.5 kb and a *PvuII* band of 6.4 kb were detected with the P1 probe. Probe P2 also detected additional bands of 6.6 and 10 kb in digests of *XmnI* and *PvuII*, respectively. As control we used DNA from cells transformed with the autonomous plasmid pHLD69 (Houssaini-Iraqi *et al.*, 1992), containing the pAL5000 origin. As expected, pHLD69 was not detected by probe P1. Probe P2 detected single *XmnI* and *PvuII* bands with the sizes expected for unintegrated plasmid bands.

DISCUSSION

Mycobacteriophages have provided replication origins or integration systems for the construction of vectors for genetic manipulation of mycobacteria (Lazraq *et al.*, 1991; Lee *et al.*, 1991; Stover *et al.*, 1991; David *et al.*, 1992). Mycobacteriophages may also provide active promoters driving the expression of foreign genes in recombinant bacille Calmette–Guérin. The insert that we sequenced, pRM64, is a plasmid shuttle vector containing a DNA fragment from mycobacteriophage D29 that was originally considered to be an origin of

replication (Lazraq *et al.*, 1991). A similar vector, pBL415, has also been described (David *et al.*, 1992). The sequence of the D29 fragment inserted in this second vector was deposited in the GenBank database (accession number X70352) but to our knowledge it has not been published. Some differences in the two determined sequences result in considerable changes in the translation maps deduced from the sequences, mainly in the 5'-region of the fragment. Significant differences were found at position 175 in ORF g1, resulting in the introduction of a stop codon and a frameshift in the pBL415 sequence, and at position 664 in ORF g3, where the pBL415 sequence shows the absence of two nucleotides in a GC compression region. This explains why the authors did not describe the dCMP deaminase gene and the putative excisase gene that we have identified.

The dCMP deaminase gene is a salvage pathway gene which is also found in T-even bacteriophages (Hall & Tessman, 1966; Maley *et al.*, 1983) but not in lambdoid phages. Comparison of the putative D29 gene with known dCMP deaminase genes showed that the gene in the D29 insert is almost complete, probably lacking only the first 36 codons. The dCMP deaminase gene has not been identified in the D29-related mycobacteriophage L5, whose complete sequence is known (Hatfull & Sarkis, 1993). Both phages show considerable similarity in a region corresponding to L5 gene 36 but the region immediately upstream, where the dCMP deaminase gene maps, shows high divergence between the two viruses.

A gene closely related to the L5 integrase gene (Lee & Hatfull, 1993) was also identified. The D29 gene is functioning, since transformed *M. smegmatis* are very stable recombinants containing a single-copy insert of the plasmid, as demonstrated by Southern analysis of transformed cell DNA using two probes mapping diametrically opposed in the plasmid. If the plasmids were integrated, it would be expected that the probes would hybridize to different DNA fragments of cell DNA joined to plasmid sequences to each side of the *attP* sequence. In contrast, hybridization of autonomous plasmid DNA would only detect the corresponding plasmid sequence. Our results indicate that pRM64 and derivatives are integrated in the cell chromosome and we were not able to detect even trace amounts of free plasmid in the hybridization experiments. Transformation and plasmid integration were not observed when pRM64 derivatives without the integrase gene were tested. Single-copy vector integration may result in low levels of foreign gene expression but has the important advantage of ensuring a high stability of recombinants. This is critically required in situations where it is not possible to select directly for maintenance of the foreign gene or where selection by drug resistance is undesirable, as in the case of recombinant vaccines.

Plasmid pBL415, which is apparently identical to pRM64, is present in multiple copies in transformed cells (David *et al.*, 1992). We do not have a plausible explanation for the discrepancy between these results

and our data. This issue is difficult to discuss because the data on the copy number of pBL415 are not presented in that article.

In accordance with the integrase function, D29 also has an *attP* core sequence identical to that described for L5 (Lee *et al.*, 1991), with the exception of the last nucleotide. The homology between L5 DNA and *M. smegmatis* DNA at this terminal position is probably only coincidental. However, the core sequence is not sufficient for efficient viral DNA integration and stability, as shown by the rapid plasmid loss by cells transformed with pGRB1. This plasmid has the core *attP* sequence but lacks the intergenic region upstream from the core sequence. All the D29 sequence 5' of this intergenic region is not essential for stable transformation. This allows the construction of vectors like pGR30, with a much smaller insert than pRM64.

Our results on the dissection of the *attP* region agree with the recently published characterization of the mycobacteriophage L5 *attP* site (Peña *et al.*, 1997). The L5 *attP* core sequence is flanked on both sides by arm-type sites, designated P1–P7. Sites P1–P3, located between the *int* gene and the *attP* core sequence, and site P4, at the other side of the core sequence, are essential for transformation. Peña *et al.* (1997) compared the sequences of the *attP* region of L5 and D29 and found similar arm-type sites in D29. The sequence from the left end of the D29 insert in pRM64 to the *Bam*HI site that was excised in pGRB1 contains the arm-type sites P4–P7. Therefore, at least site P4 is essential for efficient transformation, as in L5.

The *attB* site partially overlaps a tRNA^{gly} gene (Lee *et al.*, 1991) and the points of strand exchange are located at the anticodon loop of the tRNA gene at *attB* (Peña *et al.*, 1996). Therefore, integration is probably highly specific. The faint bands detected in Southern blots of DNA derived from transformed cells may correspond to alternative integration sites. Since the exact copy number of tRNA genes in *M. smegmatis* is not known, it is possible that other copies of *attB* provide alternative integration sites. In the same way, the high level of kanamycin resistance of a pGR30-transformed clone can be explained by integration and expression of two plasmid copies, as indicated by the hybridization results. The sizes of the additional bands detected in this experiment were not compatible with tandem integration in the same site, either in the same or in the inverted orientation. The integration within a tRNA gene can also explain the broad host range of mycobacteriophage D29, because tRNA genes are highly conserved. This may also indicate that D29 integrase can function in other mycobacterial species, thus enabling the construction of vectors that can be used for genetic manipulation of other species besides *M. smegmatis*. This possibility is currently being tested.

Hatfull & Sarkis (1993) suggested that L5 gene 34, located 3' from the *attP* region, encodes an excisase, but experimental evidence for this assignment is still lacking. This gene corresponds to the g3 ORF that we identified

in D29. As compared to D29, the L5 putative excisase gene is truncated at its 3'-end and extends further to the 5'-side. This makes the D29 homologue even more related to the homology profile of phage excisases than the L5 gene. If the g3 ORF actually encodes an excisase, pRM64 derivatives with this gene deleted will be expected to show increased stability. Although pGR30 was apparently more stable than pRM64, the difference in stability was not striking. Therefore, it is possible that D29 does not encode a functional excisase. Alternatively, excision may require accessory virus-induced functions coded by genes mapping in other D29 DNA fragments. Curiously, the most unstable of the plasmids that we have constructed was pGRB1, which lacks the putative excisase gene. A plasmid containing the integration region of mycobacteriophage L5 but lacking the arm-type sites P4-P7, like pGRB1, also showed low transformation efficiency and high instability (Peña *et al.*, 1997). It is possible that the absence of those sites favours an excisase-independent excision, perhaps mediated by the integrase or by a cell factor.

Unlike L5, which is a temperate phage, D29 has been traditionally considered as a lytic phage (Froman *et al.*, 1954; Bowman, 1958; Mizuguchi, 1984; Donnelly-Wu *et al.*, 1993). Different plaque morphologies were observed within the original isolate of D29 but this was due to differences in adsorption (Bowman, 1958) and lysogeny could not be demonstrated in infected colonies (White *et al.*, 1962). Consistent with these facts and in the absence of sequencing data, the D29 fragment cloned in shuttle vectors pRM64 and pBL415 was previously considered to be an origin of replication (Lazraq *et al.*, 1991; David *et al.*, 1992). Our results clearly indicate that this is not the case and that the cloned sequence represents an integration region. Site-specific integration is a key feature of temperate phages, but induction and maintenance of lysogeny also require other functions, such as a repressor of productive infection. It is possible that D29 has evolved from a temperate phage and become defective in one or more functions required for establishment of lysogeny. Alternatively, DNA integration may be an intermediate step in the phage replication cycle.

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