A new single-copy mycobacterial plasmid, pMF1, from Mycobacterium fortuitum which is compatible with the pAL5000 replicon

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A 9·2 kb cryptic Mycobacterium fortuitum plasmid, pMF1, was isolated from strain 110 and its restriction map constructed. A 4·2 kb HindIII fragment of pMF1 was found to support replication in mycobacteria and this fragment was cloned and sequenced to characterize the replication elements of the plasmid. Computer analysis identified a putative Rep protein (362 amino acids) with high homology to the putative Rep protein of the Mycobacterium celatum plasmid pCLP and limited homology, mostly in the N-terminal region, to the Rep proteins of Mycobacterium avium pLR7, M. fortuitum pJAZ38 and Mycobacterium scrofulaceum pMSC262. A region containing a putative ori site was located upstream of the rep gene; this region displayed high homology at the nucleotide level with the predicted ori of pCLP and pJAZ38. A plasmid carrying the 4·2 kb HindIII fragment and a kanamycin resistance marker, designated pBP4, was maintained as a single-copy plasmid in Mycobacterium smegmatis and was stably inherited in the absence of antibiotic selection. Plasmid pBP4 was incompatible with the pJAZ38 replicon but was compatible with the widely used pAL5000 replicon, indicating that among the mycobacterial vectors now available there are two incompatibility groups. Significantly, the plasmid was able to replicate in the pathogen Mycobacterium tuberculosis, making it a useful tool for gene expression studies. To provide a choice of restriction sites and easy manipulation, a 2·1 kb fragment containing the minimal replication region was cloned to make the mycobacterial shuttle vector pBP10, which showed similar stability to pBP4.

Keywords: mycobacterial plasmid pMF1, incompatibility, copy number, stability, resolvase

INTRODUCTION

Although Mycobacterium leprae and Mycobacterium tuberculosis were among the first bacteria to be recognized as the aetiological agents of human disease, knowledge of mycobacteria at the molecular level has, until recently, lagged far behind that of other pathogenic bacteria. However, the re-emergence of tuberculosis as a major public health problem, even in highly developed countries, has stimulated the application of modern molecular genetic methods to understanding the molecular basis of pathogenicity of mycobacteria (reviewed by Pelicic et al., 1998), culminating in the sequencing of the entire M. tuberculosis genome (Cole et al., 1998).

The development of transformation techniques permitting the introduction and expression of genes in mycobacteria has been the cornerstone of mycobacterial molecular genetics (Hatfull, 1993). A variety of plasmids have been used, although the most extensively studied of these is pAL5000, isolated from Mycobacterium fortuitum (Labidi et al., 1984, 1985). Other mycobacterial plasmids, such as pMSC262 from Mycobacterium scrofulaceum (Meissner & Falkinham, 1984; Qin et al., 1994), pLR7 from Mycobacterium avium
(Crawford & Bates, 1984; Beggs et al., 1995) and pJAZ38 from M. fortuitum (Gavigan et al., 1997), have been isolated and used to develop additional mycobacterial–Escherichia coli shuttle vectors. Large linear plasmids have also been found in a few mycobacterial species, notably a 25 kb plasmid, pCLP, in Mycobacterium celatum (Picardeau & Vincent, 1997, 1998). Some of these plasmids have a restricted mycobacterial host range, limiting their usefulness; for example pLR7 and pMS262 are unable to replicate in the widely used fast-growing, non-pathogenic Mycobacterium smegmatis mc²155 (Beggs et al., 1995). Thus there is still a need to develop further genetic tools for gene manipulation in mycobacteria.

In this report, we describe the isolation and characterization of a stable, low-copy plasmid, pMF1, from M. fortuitum. This plasmid is able to replicate in M. smegmatis and M. tuberculosis and is compatible with derivatives of pAL5000, but not with those of pJAZ38, making it a useful vector to further advance genetic studies in mycobacteria.

**METHODS**

**Bacterial strains and growth conditions.** E. coli strain XL-1 Blue (Stratagene) was used as a host for all plasmid constructions. Mycobacterial strains used were as follows: M. fortuitum 110 (Labidi et al., 1984) was provided by H. David, Pasteur Institute, France; M. fortuitum 10394 is a plasmid-free strain and was provided by M. J. Garcia, Universidad Autonoma de Madrid, Spain; M. smegmatis mc²155 (Snapper et al., 1990) and M. tuberculosis H37Rv are standard strains maintained at the National Institute for Medical Research, London, UK.

Growth media for E. coli and mycobacteria, and mycobacterial transformation protocols have been described previously (Papavinasasundaram et al., 1998). For mycobacteria, whenever necessary, antibiotics were used at the following concentrations: hygromycin (Hyg), 50 µg ml⁻¹; kanamycin (Kan), 25 µg ml⁻¹ for M. smegmatis and M. tuberculosis and 75 µg ml⁻¹ for M. fortuitum. Plasmids used in this study are listed in Table 1.

**Recombinant DNA techniques.** Plasmid DNA was prepared using Qiaprep spin miniprep or maxiprep kits (Qiagen). When extracting plasmids from mycobacteria, cells were incubated

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**Table 1. Plasmids used in this work**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description/source</th>
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<tbody>
<tr>
<td>pBluescript II KS(+)</td>
<td>Ap', E. coli cloning vector (Stratagene)</td>
</tr>
<tr>
<td>pJ963</td>
<td>Source for hygromycin resistance gene (Lydiate et al., 1989)</td>
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<tr>
<td>pJAZ38</td>
<td>Derived from the M. fortuitum plasmid pJAZ38 (Gavigan et al., 1997)</td>
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<tr>
<td>pKP38</td>
<td>M. smegmatis recA clone (Papavinasasundaram et al., 1997)</td>
</tr>
<tr>
<td>pMV261</td>
<td>Mycobacterial replicating vector (Stover et al., 1991)</td>
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<tr>
<td>pMV306</td>
<td>Mycobacterial integrating vector (Stover et al., 1991)</td>
</tr>
<tr>
<td>pYUB12</td>
<td>E. coli–mycobacterial shuttle plasmid (Snapper et al., 1988, 1990)</td>
</tr>
<tr>
<td>pMF1</td>
<td>Mycobacterial plasmid isolated from M. fortuitum strain 110</td>
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<tr>
<td>pGB9</td>
<td>The entire 9.2 kb fragment of pMF1 obtained by partial HindIII digestion of pMF1 ligated to the pJ166 fragment of pYUB12, thus replacing the mycobacterial origin of replication</td>
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<tr>
<td>pGB4</td>
<td>4.2 kb HindIII fragment of pMF1 ligated to the pJ166 fragment of pYUB12</td>
</tr>
<tr>
<td>pGB5</td>
<td>5 kb HindIII fragment of pMF1 ligated to the pJ166 fragment of pYUB12</td>
</tr>
<tr>
<td>pBP1</td>
<td>4.2 kb HindIII fragment of pGB9 cloned into HindIII site of pBluescript II KS(+)</td>
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<tr>
<td>pBP4</td>
<td>NheI–SpeI fragment of pMV261 containing the Tn903 aph gene inserted into XhoI site of pBP1; confers Kan resistance</td>
</tr>
<tr>
<td>pBP5</td>
<td>Deletion of 1.2 kb KpnI fragment of pBP4; obtained by religation of the remaining 7.2 kb fragment</td>
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<tr>
<td>pBP6</td>
<td>0.5 kb NcoI (end-repaired)–PstI fragment of pKP38 ligated to EcoRV/PstI-digested pBP4</td>
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<tr>
<td>pBP8</td>
<td>1.73 kb BamHI–BglII fragment containing the hygromycin resistance gene of pJ963 cloned into BamHI site of pBP1</td>
</tr>
<tr>
<td>pBP9</td>
<td>pBP4 digested with HindIII and religated to delete the 4.2 kb pMF1 fragment; a pBluescript II KS(+) plasmid carrying the Tn903 aph gene (4.2 kb)</td>
</tr>
<tr>
<td>pBP10</td>
<td>2.1 kb HindIII fragment of pBP1 amplified by PCR and cloned into HindIII site of pBP9; contains minimal region necessary for replication in mycobacteria</td>
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for 30 min at 65 °C after the addition of lysis buffer and then the kit protocol was followed without further modification. Published protocols were used for the isolation of mycobacterial DNA and for Southern hybridization (Papavinasaundaram et al., 1998).

The minimal replication region of pMF1 was amplified by PCR in a reaction mix using the Expand High Fidelity PCR system (Boehringer Mannheim) containing 1·5 mM MgCl2, 200 μM dNTPs and 300 nM each of the primers PMF23 (5'-TTTTattGTGGGACCGGATGGTAGTTG-3') and PMF24 (5'-TTTaatgctCCTCCGGTGCGTAATCGTC-3') (lower case letters in both sequences indicate the HindIII restriction site; see below) and 10 ng template DNA (plasmid pBP1). This PCR reaction was carried out in thin-walled PCR tubes in a GeneAmp PCR system 9700 thermal cycler (PE Applied Biosystems) using the following temperature regime: 1 cycle of denaturation at 94 °C for 3 min; 10 cycles of 94 °C for 15 s, 63 °C for 30 s and 72 °C for 2 min; 30 cycles of 94 °C for 15 s, 63 °C for 30 s and 72 °C for 2 min with a cycle extension of 20 s; and a final elongation step at 72 °C for 7 min.

Nucleotide sequences were determined using an ABI PRISM 377 DNA sequencer with the dRhodamine dye terminator cycle sequencing kit (PE Applied Biosystems). For all other DNA manipulations, standard protocols were followed (Sambrook et al., 1989).

**Nucleotide sequence and data analysis.** The nucleic acid sequences obtained were assembled into a contig and analysed using the biocomputing software Lasergene (DNASTAR) and the University of Wisconsin Genetics Computer Group (GCG) package. Homology searches were performed using the BLAST network service and non-redundant protein and nucleotide sequence databases (Altschul et al., 1990) at the National Centre for Biotechnology Information (NCBI), Bethesda, MD, USA.

**Stability of pMF1 derivatives, plasmid copy number and compatibility with pAL5000.** These experiments were carried out as described by Gavigan et al. (1997) except that the diluted cultures were plated on 7H11 agar supplemented with appropriate concentrations of antibiotics as necessary. Representative copy number of the plasmid pBP4 in *M. smegmatis* mc²155 was determined as single-cell resistance to Kan (Gavigan et al., 1997). For copy number comparisons, *M. smegmatis* mc²155 strains carrying the single-copy integrating vector pMV306 (Stover et al., 1991) and the replicating vector pMV261 (3–5 cell copies) (Stover et al., 1991) were used. Compatibility of the pMF1 derivative pBP8 (similar to pBP4 but carrying a Hyg resistance gene) was tested with the pAL5000-derivative pMV261 and the pJAZ38-derivative pJAZ56 plasmids.

## RESULTS AND DISCUSSION
### Isolation and subcloning of the *M. fortuitum* plasmid pMF1

We isolated a 9·2 kb cryptic plasmid pMF1 from *M. fortuitum* strain 110 and subcloned the entire 9·2 kb fragment obtained by partial HindIII digestion, and the 5 kb and 4·2 kb HindIII fragments to construct the plasmids pGB9, pGB5 and pGB4, respectively. Whereas pGB9 and pGB4 were able to replicate in *M. smegmatis* mc²155 and to produce Kan' colonies after electroporation, pGB5 could not, suggesting that the replication region of pMF1 was located within the 4·2 kb HindIII fragment. This fragment was subcloned into Bluescript II KS (+) to create pBP1. The *aph* gene of pMV261 (conferring Kan resistance) was cloned into pBP1 to construct pBP4, and this plasmid was transformed into electrocompetent cells of *M. smegmatis*, *M. tuberculosis* H37Rv and *M. fortuitum* with high efficiency [approx. 1 × 10⁸ Kan’ colonies (μg plasmid DNA)⁻¹], confirming that all the elements necessary for plasmid replication were located within the 4·2 kb HindIII fragment.

To find out whether the pMF1-derived plasmids integrate into the chromosome, we carried out Southern hybridization analysis of genomic DNA isolated from pBP6 transformants of *M. fortuitum*, *M. smegmatis* and

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**Fig. 1.** Southern analysis of PstI-digested genomic DNA isolated from pBP6 transformants of *M. smegmatis* mc²155 (lane 1), *M. fortuitum* 10394 (lane 3) and *M. tuberculosis* H37Rv (lane 5). DNA from the untransformed parent strains (lanes 2, 4 and 6, respectively) was included for comparison. The 0·5 kb *PstI* fragment, corresponding to the N-terminal region of the *M. smegmatis* recA gene, was used as a probe. Fragment sizes are indicated on the left in kb.

**Fig. 2.** Genetic organization of the 4·2 kb HindIII fragment carrying the elements for replication of the pMF1 plasmid. The ability of the plasmids carrying either the entire fragment or its derivatives to confer replication in *M. smegmatis* mc²155 is indicated on the right. The hatched box indicates the proposed location of the plasmid origin of replication. The ORFs rep and res, encoding proteins with homology to Rep proteins and resolvase, respectively, are indicated. Only relevant restriction sites are shown.
M. tuberculosis. Plasmid pBP6 is derived from plasmids pBP4 and pKP38 and contains the first 500 bp of the mycobacterial recA sequence (see Table 1). The 0.5 kb recA fragment was used as a probe. The Southern analysis of PstI-digested DNA (Fig. 1) and EcoRI-digested DNA (data not shown) confirmed that the plasmid had not randomly integrated into the chromosome in all the three species studied. In addition, resolation of the plasmid from the transformants clearly indicated that the pBP4 and pBP6 plasmids replicated extrachromosomally in mycobacteria.

**Subcloning and sequencing the replication region of pMF1**

The nucleotide sequence of the 4.2 kb HindIII DNA fragment in the pBP1 plasmid was determined on both strands from additional subclones and by primer walking. Computer analysis of the sequence revealed three important elements associated with plasmid replication and stability (Fig. 2): (i) ORF1, encoding a potential replication protein; (ii) ORF2, with homology to the resolvase gene (res), known to play a role in plasmid stability; and (iii) a stretch of conserved DNA present upstream of ORF1 thought to contain the pMF1 site of replication.

ORF1 was located between nucleotides 1787 and 2875 and encoded a protein of 362 amino acids with a predicted molecular mass of 39.3 kDa. A BLAST search of the NCBI database with the translated sequence revealed homology with the Rep proteins of the mycobacterial plasmids pCLP, pJAZ38 and pLR7, but not with the RepA and RepB proteins of pAL5000 (Labidi et al., 1985; Stolt & Stoker, 1996a). The pMF1 Rep protein displayed highest homology with the putative Rep protein of the M. celatum plasmid pCLP (Picardeau et al., 2000; GenBank accession no. AF144883), showing 49% identity through most of the sequence. In contrast, it showed only about 18–20% identity with the Rep proteins of pLR7, pJAZ38 and pMSC262 plasmids. The protein sequence of the pMSC262 Rep is based on the corrected ORF (complement of 812–1654; EMBL accession no. D14416) (Beggs et al., 1995). Alignment of pMF1, pCLP, pJAZ38, pLR7 and pMSC262 Rep proteins using the DNASTAR CLUSTAL analysis software showed that all these Rep proteins had conserved residues, mainly in their N-terminal region (Fig. 3a).

![Fig. 3. Homology of pMF1 (AJ238973; 362 residues) with the mycobacterial plasmids pCLP (AF144883, 350 residues), pJAZ38 (U84216, 368 residues), pLR7 (U18777, 360 residues) and pMSC262 (D14416, 281 residues; ORF as corrected by Beggs et al., 1995) (the EMBL accession numbers of each plasmid sequence and the predicted length of the corresponding Rep protein are given in parentheses). (a) N-terminal region of the deduced replication protein sequences. (b) Conserved DNA region of the plasmids near the putative ori site, present upstream of the rep gene. Residues/nucleotides that are identical to the pMF1 sequence are shown on a black background. The alignment was generated using the CLUSTAL method of the program Megalign (DNASTAR).](image-url)
Mycobacterial plasmid pMF1

**Fig. 4.** pBP10 mycobacterial vector. Ap, ampicillin resistance; Kan, kanamycin resistance; oriE, *E. coli* origin of replication; oriM, mycobacterial origin of replication. Useful restriction sites are also indicated.

contain its *ori* site. Purification of the pMF1 Rep protein will help to clarify whether it also has dual regulatory functions in controlling plasmid replication.

Even though the pMF1 Rep displayed only 18% identity with the pJAZ38 Rep protein, comparison of the DNA sequence present upstream of the *rep* genes indicated that a 235 bp segment of pMF1 (coordinates 1349–1583) was 70% identical to the corresponding region of pJAZ38 (coordinates 388–623) and 67% identical to that of pCLP (coordinates 2162–2383). A 72 bp segment of pMF1 (coordinates 1534–1605) was 62% identical to that of pMSC262 and 57% identical to that of pLR7. Alignment of sequences of the five plasmids in this 72 bp segment revealed high sequence conservation (Fig. 3b), indicating that all these plasmids have similar *ori* sites and therefore are likely to belong to one incompatibility group.

Homology between putative *ori* regions of various plasmids suggested that the pMF1 *ori* is likely to be located between coordinates 1349 and 1605. However, a deletion of the *HindIII–KpnI* fragment (coordinates 1–1131) of pBP4 eliminated the ability of the generated plasmid (pBP5) to replicate in *M. smegmatis* (Fig. 2). The plasmid pBP5 contained the conserved nucleotide region upstream of the *rep* gene, the *rep* gene and the downstream regions. This suggested that the *ori* site or the elements associated with the *ori* required for plasmid replication, are also located upstream of the *KpnI* site. To determine the boundaries of the replication elements, the DNA fragment located between nucleotides 904 and 3000 of pBP1 (Fig. 2) was amplified by PCR and cloned into pBP9 to construct pBP10 (Fig. 4). This plasmid replicated in mycobacteria indicating that the minimal origin of replication and the Rep protein are located between nucleotides 904 and 3000 in the pMF1 sequence.

Database searches also revealed that ORF2 (coordinates 3262–3771) displayed high homology to *res* genes; a 293 bp DNA sequence (coordinates 3351–3644) was found to be 64% identical to the gene encoding the invertase/recombinase-like protein in the *Thiobacillus ferrooxidans* plasmid pTF5 (coordinates 8944–9237) in the ORF 8819–9391; EMBL accession no. U73041) (Dominy et al., 1997). It is thought that the Res proteins help in multimer resolution during plasmid replication, thus ensuring segregation of the plasmids to daughter cells (Dodd & Bennett, 1987). BLAST analysis of the database with the pMF1 Res protein sequence revealed a possible frameshift in the N terminus of the protein (between amino acids 18 and 27). The frameshift mutation was not introduced during the cloning of pBP4 since the parent plasmid, pGB9, had the same frameshift and identical sequences in the remainder of the gene. If this gene is expressed, then it is likely to be missing the first conserved N-terminal region unless the expression occurs by translational frameshifting. The importance of the pMF1 *res* gene with regard to plasmid stability was investigated further (see below).

**Stability of pMF1 derivatives in M. smegmatis**

The pMF1-derivative pGB9 remained stable in *M. smegmatis* for 4 d (40 generations) in the absence of antibiotic selection (Fig. 5). This is similar to the reported stability of other mycobacterial plasmids (Gavigan et al., 1997). The stability of pBP4 and pBP10 was two to three orders of magnitude lower than pGB9 (Fig. 5), indicating that pBP4 and pBP10 may be lacking regions which confer higher plasmid stability. The pGB9 plasmid carried the Tn5 *aph* gene whereas pBP4 and pBP10 plasmids carried the *aph* of Tn903. However, it is reported that both *aph* genes impose a similar burden on the mycobacterial cell and do not alter the stability of.
the plasmid expressing them (Stolt & Stoker, 1996a). It is important to note that although pBP4 included ORF2 encoding the putative res gene (coordinates 3262–3771), it was not found to be more stable in M. smegmatis than pBP10, which lacked this region. This suggests that either the res gene is not expressed or that even if expressed, with translational frameshifting, it alone is not sufficient to confer increased plasmid stability. It will be interesting to see whether correcting the ORF encoding the putative pMF1 Res improves the stability of the pBP4 plasmid in mycobacteria.

Very little is known about stability mechanisms of mycobacterial plasmids. It is interesting to note that the sequenced region of the pCLP plasmid contains parA encoding a putative partitioning protein present about 1.5 kb upstream of the rep gene (EMBL accession no. AF144883). It is suggested that the ATPase activity of ParA is required for an essential energetic step in the orderly segregation of the E. coli P1 plasmid copies to daughter cells (Davis et al., 1996; Bouet & Funnell, 1999). We did not find any parA homologue within the 4.2 kb HindIII fragment of pMF1. It is possible that gene(s) controlling plasmid stabilization functions are located in the additional 5 kb HindIII fragment present in pGB9 which confer increased plasmid stability.

The pMF1-derivative pBP8 is incompatible with M. fortuitum pJAZ38-derivative pJAZ56 but compatible with the pAL5000-derivative pMV261

Competent cells prepared from M. smegmatis carrying pBP8 (identical to pBP4 but expressing Hyg resistance instead of Kan resistance) were electroporated with pMV261 or with pJAZ56 and plated on medium containing either Kan or Hyg or both. Whereas pBP8-containing cells transformed with pMV261 grew readily on all three plates, pBP8 transformants electroporated with pJAZ56 grew only on plates containing either Hyg or Kan but not on plates containing both antibiotics. This demonstrated that the electroporation of pJAZ56 was successful but that pBP8 and pJAZ56 could not coexist in the same cell. Even though the pMF1 and pJAZ38 Rep proteins showed only 18% identity, the plasmids were incompatible presumably due to the additional homology at their replication origins (Fig. 3b). This conservation of nucleotides in the ori region of plasmids pMF1, pCLP, pJAZ38, pLR7 and pMSC262, and the inability of pMF1 and pJAZ38 to coexist suggested that these plasmids can be grouped into one incompatibility unit.

However, unlike the case with pJAZ38, M. smegmatis carrying pBP8 plasmids was successfully transformed with pMV261 (Kan') with high efficiency (~1 × 10^8 colonies (µg DNA)^{-1}) to confer both Hyg and Kan resistance. Single colonies from these double antibiotic resistant (µg DNA)^{-1}) to confer both Hyg and Kan resistance. Single colonies from these double antibiotic resistant plasmids were subcultured into Dubos broth, grown in the absence of antibiotic selection and then plated to determine the proportion of Kan', Hyg' and Kan' + Hyg' colonies. Results from three independent experiments revealed that more than 97% Hyg' bacteria carried both plasmids for at least 30 generations, indicating that the pMF1 origin of replication present in pBP8 was compatible and stably maintained with the pAL5000 origin of pMV261. It has been shown that plasmids pMSC262 and pJAZ38 are also compatible with the pAL5000 replicon (Qin et al., 1994; Gavigan et al., 1997). This suggests that pAL5000 belongs to a second and different mycobacterial plasmid incompatibility group. Whilst the plasmids of the first incompatibility group (pMF1, pCLP, pJAZ38, pMSC262 and pLR7) code for only one Rep protein, plasmid pAL5000 encodes two replication proteins (RepA and RepB) (Stolt & Stoker, 1996a, b, 1997). The pMF1 Rep protein does not show any significant homology with the pAL5000 Rep proteins and there is no homology between their origins of replication. Not surprisingly, these two replicons were compatible.

Copy number of the pMF1 derivatives

Relative plasmid copy number for pBP4 in M. smegmatis was determined using a method based on single cell resistance (SCR) to increasing concentrations of Kan (Gavigan et al., 1997). As shown in Fig. 6, setting the SCR of the strain carrying the pBP4 plasmid (○) was compared to that carrying the single-copy integrating plasmid pMV306 (▲) and the replicating vector pMV261 (3–5 copies; ▲). The experiment was performed in triplicate with consistent results; data from a typical experiment are shown.

![Fig. 6. SCR of M. smegmatis transformants expressed as percentage of cells surviving under increasing concentrations of Kan. The SCR of the strain carrying the pBP4 plasmid (○) was compared to that carrying the single-copy integrating plasmid pMV306 (▲) and the replicating vector pMV261 (3–5 copies; ▲). The experiment was performed in triplicate with consistent results; data from a typical experiment are shown.](image_url)
quantity of the cloned gene products and the stability of the cloned genes, use of single-copy vectors will be more appropriate in complementation studies. To this end, plasmid pBP10 with its smaller oriM will serve as a useful vector for genetic studies in M. smegmatis and M. tuberculosis.

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