The replication and stable-inheritance functions of IncP-9 plasmid pM3

Alicia Greated,1 Marina Titok,2 Renata Krasowiak,1 Rebecca J. Fairclough1 and Christopher M. Thomas1

Author for correspondence: Christopher M. Thomas. Tel: +44 121 414 5903. Fax: +44 121 414 5925. e-mail: c.m.thomas@bham.ac.uk

Little is known of the transfer and maintenance machinery of the IncP-9 plasmids, which are found in *Pseudomonas* spp. and include both degradative and resistance plasmids. One such plasmid, pM3, which confers resistance to streptomycin and tetracycline, was found repeatedly in *Pseudomonas* species from numerous locations in Belarus. pM3 has a broad host range, but is unable to replicate in enterobacteria at 37 °C and above. A mini derivative, pMT2, was constructed by partial PstI digestion and ligation with a fragment encoding KmR. The complete sequence of pMT2 was determined. Analysis of its 8526 bp of pM3 DNA revealed several ORFs whose predicted polypeptide products were found to have similarity to previously analysed proteins involved in plasmid replication (*rep* gene), transfer (*mpf*; mating-pair formation gene) and stable maintenance (*par*, *mrs* genes). The organization of these genes showed similarity to several plasmid systems including the Ti and pSYM plasmids as well as IncP-1 plasmids. Subcloning narrowed down the region required for replication, and identified the putative *rep* gene and putative *par* promoter region as able to express incompatibility. *rep* deletion mutants were lost from the cell line, and expression of the *rep* gene was shown to be controlled by negative autoregulation. A pMT2 derivative with an insertion between the *rep* and *par* genes showed very weak, if any, ability to replicate autonomously, suggesting that plasmid maintenance may depend on a close interaction of *rep* and *par* functions.

Keywords: IncP-9 plasmids, broad-host-range replicon, plasmid incompatibility, antibiotic resistance, *Pseudomonas putida*

**INTRODUCTION**

Very little is known about the plasmids that are characteristic of *Pseudomonas* species, despite the fact that these bacteria and their mobile elements play a vital role in both environmental and clinical contexts (Boronin, 1992a). Fourteen plasmid groups have been identified (IncP-1 to IncP-14), but these include groups that have also been classified in *Escherichia coli*; for example IncP-1 is IncP in *E. coli*, while similarly IncP-4 is IncQ. The *Pseudomonas* IncP-9 group consists of both degradative, for example pWW0 (~117 kb), SAL (~68 kb) and NAH (~81 kb), and resistance plasmids, for example R2 (~73 kb) and pMG18 (~100 kb) (Bayley et al., 1979; Boronin, 1992b; Lehrbach et al., 1983). The host range of at least some of these plasmids is clearly wider than *Pseudomonas* species because pWW0, when tagged with the transposon Tn401 has been shown to transfer to, and be maintained in, *Escherichia coli* (Benson & Shapiro, 1978). In the case of the degradative plasmids however, expression of the catabolic functions may limit the species in which it can confer a selective advantage.

With renewed interest in environmental spread of bacterial traits, additional IncP-9 plasmids have been isolated. One of these, carrying tetracycline and streptomycin resistance, and estimated to be 75 kb in size, is pM3 (Titok et al., 1991a). pM3 was found in 16 isolates of *Pseudomonas* species originating from: sewage and soil from a pharmaceutical production plant in Minsk, Belarus; soil in the region of several industrial locations
in Azerbaijan and Belarus; and farmland in Belarus. The isolation of an antibiotic-resistance plasmid from industrial locations is interesting, as one would usually associate isolates from such sites with catabolic phenotypes and degradative plasmids. Standard conjugation tests showed the ability of pM3 (from *Pseudomonas putida* M) to transfer to a range of Gram-negative bacteria (Titok et al., 1991a). pM3 was completely stable in *Pseudomonas aeruginosa, Pseudomonas stutzeri* and *Pseudomonas syringae*, but could not replicate at 37 °C in the other species tested, most of which were enterobacteria (Escherichia coli, Salmonella typhimurium and Serratia marcescens), and was even lost spontaneously during growth at 28 °C. Incompatibility experiments demonstrated the transfer and retention of pM3 in recipients harbouring plasmids belonging to several *Pseudomonas* incompatibility groups: IncW (Sa) (Watanabe et al., 1968), IncN (pJ4733) (Arai & Ando, 1980), IncP-1 (RP4) (Ingram et al., 1973), IncP-3 (R40a) (Shapiro, 1979), IncP-4 (RSF1010) (Barth, 1979) and IncP-6 (Rms149) (Sagai et al., 1973). pM3 was found to be independent of an homologous replicon plasmid, pMT2, derived from pM3. We present molecular, genetic and functional analyses of the transfer replicon plasmid, pMT2, derived from pM3. We present yet been confirmed experimentally.

The existence of these elements in pM3 has not published observation). This suggested that pM3 may contain transposon-like elements, a typical feature of plasmids belonging to the same incompatibility group which argues in favour of the surface exclusion of and pM3 plasmids into strains with another IncP-9. In addition, the frequency of transfer of R2 and pM3 plasmids into strains with another IncP-9 plasmid is three orders of magnitude lower than the transfer of these plasmids into plasmid-free recipients, which argues in favour of the surface exclusion of plasmids belonging to the same incompatibility group and thus conservation of transfer functions. The appearance of tetracycline-sensitive deletion derivatives of pM3 was found to be independent of an homologous recombination system in the recipient (M. Titok, unpublished observation). This suggested that pM3 may contain transposon-like elements, a typical feature found in plasmids belonging to the IncP-9 group (Tsuda, 1996). The existence of these elements in pM3 has not yet been confirmed experimentally.

This paper describes the characterization of a mini-replicon plasmid, pMT2, derived from pM3. We present the complete nucleotide sequence of pMT2, and molecular, genetic and functional analyses of the transfer and maintenance functions with the view to using this information to underpin predictions about IncP-9 plasmid behaviour.

METHODS

**Bacterial strains and plasmids.** *Escherichia coli* NEM259 (*supE supF bsdR met trpR*) (W. Brammar, University of Leicester), *E. coli* TG1 (*Δlac–proAB supE bsdΔ5* (F *traD36 thi proAB* lac* proph* lacZΔM15) *r m*) (F. C. H. Franklin, University of Birmingham) and *E. coli* C2110 (*polA1 his rfa P2*) (D. R. Helinski, University of California, San Diego) were used in this study. Plasmids are listed in Table 1. Bacteria were routinely grown in Luria broth (Kahn et al., 1979) or minimal M9 medium (Gerhardt et al., 1994) supplemented with agar (15%, w/v) when necessary. Antibiotics and components used for selection were added as appropriate at the following concentrations: kanamycin sulphate (25 µg ml⁻¹), ampicillin (100 µg ml⁻¹), chloramphenicol (25 µg ml⁻¹), tetracycline hydrochloride (10–1500 µg ml⁻¹), streptomycin sulphate (10–3000 µg ml⁻¹), penicillin G, sodium salt (300 µg ml⁻¹ in agar, 120 µg ml⁻¹ in liquid medium), X-Gal (0.02%) and IPTG (1 mM). IPTG (0.01 mM) was used to induce the tac promoter.

**Plasmid DNA isolation and manipulation.** Plasmids were isolated on a small scale according to the methods of Birnboim & Doly (1979) or Holmes & Quigley (1981). Large-scale plasmid DNA isolation was performed using the alkaline-lysis method followed by a CsCl purification step and removal of ethidium bromide as described by Sambrook et al. (1989). DNA was manipulated and analysed using standard techniques (Sambrook et al., 1989). In general, enzymes were from MBI Fermentas, Boehringer Mannheim or Northumbria Biological and were used according to the manufacturer’s instructions. DNA fragments, including PCR products, were purified using a GeneClean kit (bio101).

**DNA-sequence determination and analysis.** Sequence was generated using ‘universal’ primers for the flanking vector sequences and custom oligonucleotides to ‘walk’ along intervening DNA. Both strands were sequenced. Dye-terminator methods (Alta Bioscience) were used in the automated sequencing of the cloned fragments using kits from Applied Biosystems on ABI373 or 377 machines. The sequence generated was submitted to GenBank and has the accession number AF078924. Sequence was analysed using programs from the Genetics Computer Group, Madison, WI, USA (Devereux et al., 1984). G + C content was calculated using the *composition, window and statplot* were used to create a G + C profile. Alignments were constructed with *pileup* and *pretty*. ORFs were identified using *codonpreference*. Comparison of new sequences to those in the database was performed using the *fasta* program (Pearson & Lipman, 1988).

**Plasmids constructed during this work.** Plasmids are listed in Fig. 1a. Purified pM3 DNA was partially digested, ligated with a purified *Pst*I DNA fragment from pUC4K (encoding a kanamycin-resistance gene) and transferred into competent *E. coli* TG1. The smallest plasmid obtained was designated pMT2. DNA fragments from pMT2 cut with *Pst*I and *Bgl*II were cloned into pUC18 cut with *Pst*I and *Bam*HI respectively. Ligation products are transferred into competent E. coli TG1 and recombinants are identified by plating onto Luria agar with penicillin, IPTG and X-Gal giving pAG1–pAG5, pAGB1 and pAGB2 (Fig. 1a). To construct pAG1.1, the 905 bp *EcoRI–Pst*I fragment of pAG1, containing the putative *rep* and *oriV* region, was inserted into pUC18. A *rep* deletion variant of pAG1.1, pAG1.1_Δrep, was created by removing the 223 bp fragment between the two *BamH*I sites in the *rep* ORF. pAG4.1 was constructed by inserting into pUC18 the 903 bp *BamHI–Pst*I fragment of pAG4, containing the putative *par* promoter region. To facilitate manipulation and study of pMT2 (by replacing Km* with Ap*) and adding a second replication) we digested pMT2 with *Xho*I, which cuts in the *aph* gene, and ligated it to pBR322 digested with Sall. This created pAG10.

pMT2/pACYC184 hybrids were created as follows. Partial digests of pMT2 DNA were carried out with limiting amounts of HindIII or *Bgl*II. Reactions were terminated by incubation at 65 °C (for 30 min) or the addition of EDTA (final 10 mM) at time intervals between 0 and 15 min. The products were ligated to pACYC184 (linearized with HindIII or *BamHI*) and transformed into competent *E. coli* NEM259.
Table 1. Plasmids used in this study

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<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Source/reference</th>
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<td>pACYC184</td>
<td>Cm&lt;sup&gt;+&lt;/sup&gt;, Tc&lt;sup&gt;+&lt;/sup&gt;, Tra&lt;sup&gt;−&lt;/sup&gt;</td>
<td>Chang &amp; Cohen (1978)</td>
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<td>pAG1</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt;, pMT2 PstI fragment 1* cloned into pUC18; contains rep, oriV and part of orf1</td>
<td>This study</td>
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<td>Ap&lt;sup&gt;+&lt;/sup&gt;, pMT2 PstI fragment 2* cloned into pUC18; contains orf3 (res), orf4 (tolA) and orf5 (korA)</td>
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<td>Ap&lt;sup&gt;+&lt;/sup&gt;, pMT2 PstI fragment 3* cloned into pUC18; does not contain any complete ORFs</td>
<td>This study</td>
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<td>pAG4</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt;, pMT2 PstI fragment 4* cloned into pUC18; contains putative par and mpf promoters and orf8 and oriV (mpfA and B)</td>
<td>This study</td>
</tr>
<tr>
<td>pAG5</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt;, pMT2 PstI fragment 5* cloned into pUC18; does not contain any complete ORFs</td>
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<td>pAG7</td>
<td>Km&lt;sup&gt;+&lt;/sup&gt; Cm&lt;sup&gt;−&lt;/sup&gt;, pMT2 with pACYC184 inserted into HindIII site within orf4 (tolA)</td>
<td>This study</td>
</tr>
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<td>pAG8</td>
<td>Km&lt;sup&gt;+&lt;/sup&gt; Cm&lt;sup&gt;−&lt;/sup&gt;, pMT2 with pACYC184 inserted into BgIII site within orf5 (korA)</td>
<td>This study</td>
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<td>pAG9</td>
<td>Km&lt;sup&gt;+&lt;/sup&gt; Cm&lt;sup&gt;−&lt;/sup&gt;, pMT2 with pACYC184 inserted into BgIII site directly upstream of orf8 (mpfA)</td>
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<td>pAG1.1</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt;, pAG1 deletion derivative; contains rep and oriV</td>
<td>This study</td>
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<td>pAG1.1Δrep</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt;, rep deletion mutant derived from pAG1.1; contains oriV</td>
<td>This study</td>
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<td>pAG10</td>
<td>Ap&lt;sup&gt;−&lt;/sup&gt; Tc&lt;sup&gt;−&lt;/sup&gt;, pBR322 inserted into pMT2 XhoI site</td>
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<td>pAG11</td>
<td>Ap&lt;sup&gt;−&lt;/sup&gt; Tc&lt;sup&gt;−&lt;/sup&gt;, pBR322/pACYC184 hybrid with SalI fragment removed; creates a smaller pMT2 mini replicon</td>
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<td>This study</td>
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<td>pBR322</td>
<td>Ap&lt;sup&gt;−&lt;/sup&gt; Tc&lt;sup&gt;−&lt;/sup&gt;, Tra&lt;sup&gt;−&lt;/sup&gt;, pMB1-based expression vector</td>
<td>Bolivar et al. (1977)</td>
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<tr>
<td>pGBT30</td>
<td>Ap&lt;sup&gt;−&lt;/sup&gt;, tacp; pMB1-based expression vector</td>
<td>Jagura-Burdzey et al. (1991)</td>
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<tr>
<td>pM3</td>
<td>Tc&lt;sup&gt;−&lt;/sup&gt; Sm&lt;sup&gt;+&lt;/sup&gt;, Tra&lt;sup&gt;−&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pME6000</td>
<td>Tc&lt;sup&gt;−&lt;/sup&gt;, derivative of plasmid pBRR1</td>
<td>Maurhofer et al. (1998)</td>
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<tr>
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<td>Km&lt;sup&gt;−&lt;/sup&gt;, Mob&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
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<td>pMT2Δres</td>
<td>Km&lt;sup&gt;−&lt;/sup&gt;, res mutant of pMT2</td>
<td>This study</td>
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<td>pREN1</td>
<td>Km&lt;sup&gt;−&lt;/sup&gt;, pMT2 rep–xyE promoter probe</td>
<td>This study</td>
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<td>pREN2</td>
<td>Ap&lt;sup&gt;−&lt;/sup&gt;, tacp–pMT2 rep; based on pGBT30</td>
<td>This study</td>
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<tr>
<td>pPT01</td>
<td>Km&lt;sup&gt;−&lt;/sup&gt;, xyE promoter probe</td>
<td>Thorsted et al. (1996)</td>
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<td>pUC18</td>
<td>Ap&lt;sup&gt;−&lt;/sup&gt;, Tra&lt;sup&gt;−&lt;/sup&gt;</td>
<td>Yanisch-Perron et al. (1985)</td>
</tr>
</tbody>
</table>

*Fragments are illustrated in Fig. 1a.

Recombinants were selected with kanamycin and chloramphenicol. pAG9 has pACYC184 inserted into the BgIII site at position 7729 on the pMT2 map. Since pAG9 retained a functional IncP-9 replication system it was used as the starting point for further dissection. The orientation of the components of pAG9 places the SalI sites in pACYC184 and pMT2 close together. pAG11 was constructed by deleting this fragment, giving a kanamycin-sensitive and chloramphenicol-resistant plasmid that retains 5340 bp of pMT3 DNA. pAG11Δrep was created by digesting pAG11 with SalI and NdeI which each cut once. Both SalI–NdeI fragments were purified. The smaller of the two DNA fragments, containing the rep gene, was digested with BamHI, which cuts twice within the rep ORF. The three fragments were run on a gel and the SalI–BamHI and NdeI–BamHI purified and ligated resulting in a deletion within the rep ORF. The Δrep DNA fragment was then ligated to the larger SalI–NdeI fragment to create a rep deletion mutant of pAG11. An orf3 (the putative res gene) mutant of pMT2, pMT2Δres, was constructed by digesting pMT2 with BsrGI and AflII (positions 3812 and 4035 respectively), purifying the large fragment, and religating it in the presence of two 14 bp linkers (5′-GTACAGCTCGAGGAG-3′ and 5′-TTAATCTCGAGCT-3′) designed to create an extra XhoI site when successfully ligated with pMT2.
Fig. 1. (a) Linear restriction map and corresponding ORFs of pMT2 (excluding Km\(^R\)). White arrows show direction of transcription. Below are shown the regions of pMT2 used to construct plasmids described in this study. Unless otherwise stated the vector was pUC18. Alternative vectors (or inserted plasmids) were as follows: *, pBR322; †, pACYC184; ‡, pPT01; †, pGBT30. Cloned DNA fragments are shown as arrows; the PCR product is shown by a line (without arrowheads); \(\triangleright\), insertion; \(\subseteq\), deletion. Note that the XhoI site used to construct pAG10 is not shown, as it occurs within the Km\(^R\) gene. (b) G + C profile of pMT2 (excluding Km\(^R\)). WINDOW and STATPLOT were used to plot the values using the GCG package with a window size of 100 nt and shift increment of 3 nt.
To produce pREN1, pPT01, digested with BamHI, was ligated to the 594 bp BamHI fragment purified from pAG1, containing the putative rep promoter. pREN2 was created by digesting both pGBT30 and the rep PCR product (see ‘amplification of pMT2 rep using PCR’ section) with EcoRI and SalI, and ligating the products together (selecting on ampicillin plates) to create a plasmid harbouring the complete pMT2 rep ORF under control of the tac promoter.

**Determination of plasmid stability.** Plasmid-containing bacteria (10⁶ cells ml⁻¹) were inoculated into liquid rich medium without antibiotic selection and grown at optimal temperature until stationary phase, after which the percentage of plasmid-free cells was determined by plating on antibiotic medium. Loss of plasmid was confirmed by agarose gel electrophoresis.

**Determination of incompatibility.** Competent *E. coli* NEM259 cells containing pMT2 were transformed with the plasmid to be tested and grown overnight at 30 °C in Luria broth containing antibiotic to select for the incoming plasmid. After serial dilution, aliquots (20 μl) were spotted onto penicillin (when testing pUC18 or pUC18 derivatives cloning cloned fragments from pMT2) or tetracycline (when testing pME6000), and kanamycin plates to determine the presence of the test plasmid and pMT2, respectively.

**Assay of catechol 2,3-oxygenase activity.** XyLE assays were conducted using the procedure described by Zukowski *et al.* (1983). One unit of catechol 2,3-oxygenase (the product of *xylE*) activity is defined as the amount required to convert 1 μmol catechol to 2-hydroxyxymuconic semialdehyde in 1 min under standard conditions. Protein concentrations were determined by the biuret method (Gornall *et al.*, 1949).

**UV irradiation to study effect of res mutation.** Single colonies obtained from the transformation of *E. coli* NEM259 with pMT2 and pMT2ares, were used to inoculate Luria broth containing kanamycin. Overnight cultures were subcultured into new selective medium (1:100 dilution) and grown at 30 °C for 150 min. Serial dilutions of the cultures were carried out and plated onto kanamycin and non-selective agar so the presence of the plasmids could be determined (assuming the stationary-phase culture contains 3 x 10⁶ c.f.u. ml⁻¹ and in 150 min three generations of growth have been completed). Aliquots (5 ml) of the samples containing approximately 1 x 10⁶ c.f.u. ml⁻¹ were distributed into sterile glass Petri dishes. Samples were irradiated for 0, 5, 20 and 60 s with a broad-spectrum UV lamp known from previous experiments to have both DNA damaging and mutagenic properties. Following irradiation, all samples were maintained in the dark. UV-treated samples were grown for approximately 20 generations to reach stationary phase. The cultures were then a) harvested for small-scale isolation of plasmid DNA, and b) serially diluted and spotted onto kanamycin and non-selective agar to assess any changes in plasmid content.

**Amplification of pMT2 rep using PCR.** pMT2 orf2 (putative rep gene) was amplified using PCR. A pair of primers was designed to contain the rep start and stop codons, and include EcoRI and SalI restriction sites, respectively. Primers used were as follows: 5’-GGA ATT CAT GGC CAA TGA CAA AAA CGA G-3’ and 5’-GTC GAC TCA GTT ACC GTG GGG AAT A-3’. PCR was performed on a Hybrid thermal cycler; a 5 min denaturing step at 94 °C was followed by 2 cycles of 94 °C (30 s), 50 °C (30 s), 72 °C (1 min) and then 25 cycles of 94 °C (30 s), 58 °C (30 s), 72 °C (1 min) and a final extension step at 72 °C for 5 min. Reactions consisted of 0.1 μg pMT2 template, 0.6 μM each primer, 200 μM each dNTP and 1 U *Taq* DNA polymerase (Expand High Fidelity PCR System; Boehringer Mannheim) and reaction buffer as recommended by the manufacturer (Boehringer Mannheim).

**RESULTS**

**Isolation and DNA sequence analysis of the pM3 mini-replicon pMT2**

As reported previously in Russian (Titok *et al.*, 1991b) DNA of a Tc³ variant of pM3 was partially digested with *PstI* and ligated with a 1-24 kb *PstI* fragment from pUC4K (Km³). The resulting plasmid, pMT2, remained incompatible with the parent plasmid pM3. pMT2 was unable to replicate in enterobacteria at 37 °C but was stably maintained in *P. putida* M under the same conditions as the parent plasmid pM3. However, pMT2 was lost from the other *Pseudomonas* species at a significant rate at both 28 °C and 37 °C, suggesting that some component of the stable-inheritance mechanism has been lost or altered. Attempts to create smaller derivatives using common restriction enzymes were unsuccessful.

Each of the *PstI* and both of the *BglII* fragments were inserted into pUC18 (Fig. 1a). The complete nucleotide sequence of pMT2 was determined by sequencing all of the cloned *PstI* fragments and across the *PstI* sites in the cloned *BglII* fragments. Both DNA strands were sequenced to eliminate any errors. pMT2 was found to be 9766 bp in size including the *PstI* fragment carrying the Km³ determinant (GenBank accession no. AF078924). The DNA derived from pM3 was 8526 bp. It has a G+C content of 55.6 mol% compared to approximately 60 mol% G+C for DNA of its host *P. putida*.

ORFs were identified using the CodonPrefer program with the *Pseudomonas* species usage table. The co-ordinates of the putative ORFs of pMT2 are listed in Table 2. Nomenclature is based on similarity to sequences in the database. All the putative start codons were AUG, and eight of the known stop codons were UGA, the remaining being UAA—the stop codon for orf1. Shine–Dalgarno sequences were identified as purine-rich regions with complementarity to the 3’ end of 16S rRNA. The distance between the Shine–Dalgarno site and the upstream start codon varied from 4 to 9 nt (Table 2). Overlap between putative stop and start signals was evident in the case of orf6 and orf5.

A G+C profile of pMT2 was constructed using a window of 100 nt and a shift increment of 3 nt (Fig. 1b). To identify a possible replication origin, we scanned the G+C profile for the largest A+T rich regions. Minor troughs correlated with putative promoter regions, but a major trough occurred between co-ordinates 3223 and 3468, upstream of the putative rep gene. Analysis of this region revealed direct and inverted repeats and potential methylation sites. Direct repeats included the sequences GATATC (occurs three times within 120 bp), GGG-TRGCTCAT (repeated five times within 150 bp), TAT-

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**Table 2. Nomenclature is based on similarity to se-**
Table 2. pMT2 ORFs with clearly identified putative functions in replication

<table>
<thead>
<tr>
<th>Name*</th>
<th>Co-ordinate</th>
<th>Putative Shine-Dalgaro sequence†</th>
<th>No. residues</th>
<th>Molecular mass (Da)</th>
<th>Isoelectric point</th>
<th>Identity (%)</th>
<th>Overlap (aa)</th>
<th>Sequence from database‡</th>
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<td>orf1</td>
<td>2618–1956</td>
<td>GAGGGTTGCTTT</td>
<td>162</td>
<td>18292</td>
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<td>-5</td>
<td>-</td>
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<tr>
<td>orf2</td>
<td>3197–3434</td>
<td>TACAGCACCC</td>
<td>183</td>
<td>19872</td>
<td>6.69</td>
<td>44</td>
<td>223</td>
<td>Rep (pBBR1) (E. coli)</td>
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<td>4289–3524</td>
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<td>211</td>
<td>23199</td>
<td>9.79</td>
<td>35</td>
<td>196</td>
<td>ParA (resolvase, RK2)</td>
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<td>4929–5160</td>
<td>CAGGCGTTGACC</td>
<td>141</td>
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<td>31</td>
<td>81</td>
<td>TOLA (P. putida)</td>
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<td>5276–4926</td>
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<td>orf8</td>
<td>7998–8459</td>
<td>AAGGGCCTATGT</td>
<td>153</td>
<td>16851</td>
<td>10.80</td>
<td>30</td>
<td>105</td>
<td>VirB2 (T. plasmid)</td>
<td>P17792</td>
</tr>
<tr>
<td>orf9</td>
<td>8468–8764</td>
<td>AAGGGGTTAAGC</td>
<td>98</td>
<td>10948</td>
<td>9.32</td>
<td>36</td>
<td>56</td>
<td>VirB1 (T. plasmid)</td>
<td>P17793</td>
</tr>
<tr>
<td>orf10</td>
<td>8751–end</td>
<td>TACGGGCTATCCGAG</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
<td>24</td>
<td>191</td>
<td>VirB4 (T. plasmid)</td>
<td></td>
</tr>
</tbody>
</table>

* ORF names in parentheses refer to implied function based on database comparisons.
† Underlined sequences match the consensus.
‡ Sequence similarity of pMT2 putative translation products with related sequence in the database (values from FASTA search).
§ No significant identity to known bacterial proteins.
∥ ORF continues past the end of available sequence.

CTCWCA (directly repeated and inverted within 35 bp), TATGAGCTA (inverted once) and two potential methylation sites (GATC) (R = A/G and W = A/T). Combined with the location of the putative rep gene as described above, we therefore propose this site to be the pMT2 origin of replication.

Stable-inheritance functions: par, korA and res

The vector pACYC184 was inserted into one HindIII site (position 4872 on the pMT2 map) and both BglII sites of pMT2 (Fig. 1a; pAG7, pAG8 and pAG9). pACYC184 was chosen because it does not carry Km and relies on a DNA polymerase I-dependent replicon so that hybrids with pMT2 can be tested in a PolA strain (C2110) to determine whether they still have a functional IncP-9 replicon. HindIII preferentially cut the site at position 4872, so we were not able to obtain inserts at the other sites (positions 660 and 6159). pAG9 (Fig. 1a) transformed C2110 as efficiently as pAG10, and gave similar plasmid DNA yield. However, pAG7 and pAG8 (Fig. 1a) would not transform C2110, indicating that the region encoding the putative partitioning and control functions must remain intact for a functional replication system.

The predicted product of orf3 showed significant similarity to para of RK2 that encodes a resolvase mediating site-specific recombination at the mrs (multimer resolution system) site and belonging to the resolvase family from the TnI/3-like transposons (Gerlitz et al., 1990; Eberl et al., 1994). To test whether orf3 was necessary for plasmid stability, we compared the loss rate and plasmid profile of pMT2 and pMT2Δres during repeated batch culturing. No difference was observed. This negative result may have been due to the normally low level of dimer formation. We repeated the experiment with a UV irradiation step to induce DNA damage, and thus recombination repair, which should stimulate dimer formation (Kuzminov, 1999). However, once again we observed no difference between the behaviour of pMT2 and the res mutant pMT2Δres, providing no evidence that pMT2 Res (the product of orf3) functions in plasmid multimer resolution.

Replication function

pAG9 (the pACYC184–pMT2 hybrid created by inserting pACYC184 into the BglII site of pMT2 PstI fragment 4—with no effect in pMT2 plasmid replication) was used in further manipulation of the pMT2 replicon. pAG11 was constructed by a SalI deletion, leaving only 3340 bp of pMT2 DNA from the region between the BglII site (position 7729) upstream of the parA promoter and the SalI site (position 2389) downstream of the rep ORF (Fig. 1a). pAG11 was as stably maintained as pMT2. We constructed a further derivative, pAG11αrep, with an internal deletion in the rep gene (orf2), which in contrast to pAG11, could not be established in C2110.

To study regulation of the pMT2 rep gene (orf2), the region containing the rep promoter was cloned into a xylE promoter probe vector, pPT01, to create pREN1. XylE activity measured on exponential cultures was variable, apparently influenced by exact growth conditions and will be reported elsewhere. XylE activity measured on stationary-phase cultures gave consistent results. pREN1 in the presence of pUC18 vector gave 0.4 XylE units (mg protein)−1. pAG10, pAG1 and pAG1.1 (Fig. 1), which carry the whole of pMT2, the oriV–rep–orf1 region and the oriV–rep region respectively, all gave approximately 2.2-fold repression. pAG1.1αrep, with an internal BamHI deletion in-
activating the rep ORF, gave no repression. We amplified the rep ORF by PCR and placed it under the control of the tac promoter in vector pGBT30. Under the same conditions as described above, this plasmid, pREN2, gave 2.5-fold repression of the promoter activity detected from pREN1, when induced with IPTG. We concluded that the product of orf2 is responsible for regulating the transcription from the oriV region in pREN1.

**Incompatibility determinants**

Incompatibility is normally a consequence of plasmids sharing one or more of the plasmid-replication or partitioning functions. Each of the pMT2 PstI fragments (cloned into pUC18) was tested for incompatibility with intact pMT2. Two clones, pAG1 and pAG4, displaced resident pMT2. To further localize the regions responsible for the incompatibility effect we constructed plasmids pAG1.1 and pAG4.1 (Fig. 1a), both of which retained an incompatibility phenotype. pAG4.1 does not encode any complete ORFs, whereas pAG1.1 encodes the intact pMT2 rep ORF. When this ORF was inactivated (pAG1.1∆rep) the ability to displace pMT2 was lost.

The only Rep protein with similarity to the product of orf2 was that of pBBR1 and its derivatives (Antoine & Locht, 1992) (Table 2). pME6000, a tetracycline-resistant derivative of pBBR1, was tested against pMT2 and the plasmids were found to be compatible, implying that pBBR1 does not belong to the IncP-9 plasmid family.

**DISCUSSION**

In this paper we have presented a preliminary characterization of a mini replicon derived from the IncP-9 plasmid pM3. This mini replicon, pMT2, retains the general replication properties of the parent plasmid – ability to replicate in enteric bacteria at 30 °C but not 37 °C – but is less stable, suggesting that it lacks certain stability determinants present in pM3. Nevertheless, our analysis revealed a number of genes which seem likely to be largely responsible for the replication and stable maintenance of IncP-9 plasmids. PCR primers designed on the basis of the putative rep gene found in pMT2 (Greater & Thomas, 1999) also gave a product for another IncP-9 plasmid, pWW0, suggesting that this replication system is an element of other IncP-9 plasmids. Since IncP-9 plasmids have been mainly isolated from *Pseudomonas* species, the temperature sensitivity in enteric bacteria may simply reflect the lack of selection to retain a functional rep system for these species. Indeed, studies with the *Pseudomonas* plasmid pPS10 revealed that a similar sensitivity could be suppressed by single amino acid changes in Rep (Fernandez-Tresguerres et al., 1995).

The complete nucleotide sequence of pMT2 has revealed a number of ORFs with probable functions in pM3 replication and maintenance. Since nothing was previously known about the rep/par genes of IncP-9 plasmids, it was striking that the nucleotide sequence revealed ORFs that, almost without exception (9/10), had a function that could be predicted on the basis of similarity to previously characterized proteins. However, the level of similarity is sufficiently low to explain why currently used plasmid replicon-based probes (Couturier et al., 1988) should not give a positive signal. Perhaps of greatest interest was the putative rep gene which was identified by its similarity to the rep gene of pBBR1 from *Bordetella bronchiseptica* (Antoine & Locht, 1992) (Fig. 2). We showed that the region containing this ORF was a major incompatibility determinant and that an internal deletion in the gene led to loss of ability to replicate, strengthening the idea that this is indeed the rep gene. Although pBBR1 is a small, medium-copy-number plasmid, it does, like pM3, have a broad host range. But we showed that pMT2 and a pBBR1 derivative are able to co-exist within the same cell line, demonstrating that pBBR1 does not belong to the IncP-9 plasmid group.

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**Fig. 2.** Alignment of pM3/pMT2 and pBBR1 putative Rep polypeptide sequences. The alignments were compiled using PILEUP and PRETTY. Identical conserved amino acids are in bold print.
We expect replication of pM3 and other IncP-9 plasmids to occur by a theta intermediate since a rolling-circle replication strategy should not be viable for a large plasmid. Apart from the Rep of pBBR1, we found no other proteins with significant similarity to the predicted product of orf2. Both pMT2 Rep and pBBR1 Rep are smaller than other known replication proteins, which are between 25 and 40 kDa (Del Solar et al., 1998). However, like these other replication-initiator proteins, Rep from pMT2, while slightly acidic overall (Table 2), has a basic N terminus and an acidic C terminus, and may consist of two domains. A similar charge distribution has also been observed for pBBR1 Rep (Antoine & Locht, 1992). As demonstrated for many replication proteins, pMT2 Rep appears to autoregulate its own synthesis, and may have domains for transcriptional repression and origin activation (Del Solar et al., 1998; Giraldo et al., 1998).

The roles of the other genes in this region are yet to be determined. We could not establish a phenotype for the putative resolvase identified on the basis of its similarity to ParA from the IncP-1 plasmid, RK2 (which has been shown to contribute to the stable inheritance of RK2) (Gerlitz et al., 1990).

We have not yet dissected out the partitioning functions provided by orf6 and orf7 although they are clearly related to the parA and parB families of active partitioning genes (Williams & Thomas, 1992). A significant relationship was evident between the putative translation product of orf7 and other ParA-related proteins. Regions of high similarity corresponded to the three distinct conserved motifs of members of the ATPase superfamily identified by Koonin (1993), which function in cell-division processes including plasmid and chromosomal partitioning (Motallebi-Veshareh et al., 1990; Ogasawara & Yoshikawa, 1992). Often, plasmids carrying the same active partitioning machinery will be incompatible (Williams & Thomas, 1992). We showed the putative par promoter region of pMT2, i.e. the candidate cis-acting sequence of the partitioning system, to be an incompatibility determinant. It is probable that the regulation of the par genes involves orf5 (pMT2 korA) since its product is similar to the global regulator KorA of IncP-1 plasmids R751 and RK2 (Jagura-Burdzy & Thomas, 1994; Thomas et al., 1995), and insertions in orf5 resulted in the loss of pMT2 from the cell line. The final ORF in this block is orf4, whose predicted product shows similarity to the transmembrane protein TolA (Sen et al., 1996). On the basis of its cotranscription with parA, parB and korA, it would make sense if the product of orf4 was involved in plasmid maintenance. Indeed, the effect of an insertion into the HindIII site within orf4 is consistent with this hypothesis. It is possible that this defect is due to polar effects on the downstream ORF (orf3, encoding a putative resolvase) since we cannot identify an obvious transcription terminator downstream of orf4. However, there are a number of candidate promoter sequences for orf3 so it is unlikely to depend on transcription through orf4. The basis for the alignment of the putative polypeptide product of orf4 with TolA appears to be that both proteins contain a predicted region of extended alpha helix rather than the product of orf4 being highly hydrophobic.

The remaining set of pMT2 genes contains the beginning of an operon with ORFs demonstrating similarity to a variety of mating-pair-formation genes involved in creation of a membrane channel for the transport of DNA (Kuldau et al., 1990; Pansegrau et al., 1994; Thorsted et al., 1998). We therefore propose that this region is the first part of a complete operon present in pM3 required for conjugative transfer. The organization of back-to-back transfer and replication/partitioning systems is similar to the pSYM/Ti plasmids of Agrobacterium and Rhizobium (Li & Farrand, 2000).

In conclusion, our analysis of pMT2, the minimal replicon of pM3, shows that the replication, stable-inheritance and transfer functions appear to be a novel mosaic of genes previously identified in other systems. The sequence and associated analysis should allow development of tools to monitor IncP-9 plasmids in the environment (Greated & Thomas, 1999).

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