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

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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 *Pst*I digestion and ligation with a fragment encoding Km^R. 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

The GenBank accession number for the sequence reported in this paper is AF078924.

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 (pJa4733) (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.*, 1975). pM3 showed symmetric incompatibility with IncP-9 plasmid R2 (Kawakami *et al.*, 1972) indicating that pM3 contains just one replicon, which was assigned to 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 hsdR met trpR*) (W. Brammar, University of Leicester), *E. coli* TG1 [$\Delta(lac-proAB)$ *supE hsd* Δ 5 (F' *traD36 thi proAB⁺ lacI^a lacZ* Δ M15) *r⁻m⁻*] (F. C. H. Franklin, University of Birmingham) and *E. coli* C2110 (*polA1 his rha* P2^s) (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 (1.5%, w/v) when necessary. Antibiotics and components used for selection were added as appropriate at the following concentrations: kanamycin sulphate (25 μ g ml⁻¹), ampicillin

(100 μ g ml⁻¹), chloramphenicol (25 μ g ml⁻¹), tetracycline hydrochloride (10–1500 μ g ml⁻¹), streptomycin sulphate (10–3000 μ g ml⁻¹), penicillin G, sodium salt (300 μ g ml⁻¹ in agar, 120 μ g ml⁻¹ in liquid medium), X-Gal (0.02%) and IPTG (1 mM). IPTG (0.1 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 COMPOSITION. WINDOW and STATPLOT were used to create a G + C profile. Alignments were constructed with PILEUP and PRETTY. ORFs were identified using CODONREFERENCE. 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 were transformed into competent *E. coli* TG1 and recombinants were 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 *Eco*RI–*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 Δ *rep*, was created by removing the 223 bp fragment between the two *Bam*HI sites in the *rep* ORF. pAG4.1 was constructed by inserting into pUC18 the 903 bp *Bam*HI–*Pst*I fragment of pAG4, containing the putative *par* promoter region. To facilitate manipulation and study of pMT2 (by replacing Km^R with Ap^R and adding a second replicon) we digested pMT2 with *Xho*I, which cuts in the *aph* gene, and ligated it to pBR322 digested with *Sal*I. This created pAG10.

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

Table 1. Plasmids used in this study

Plasmid	Description	Source/reference
pACYC184	Cm ^R Tc ^R , Tra ⁻	Chang & Cohen (1978)
pAG1	Ap ^R , pMT2 <i>Pst</i> I fragment 1* cloned into pUC18; contains <i>rep</i> , <i>oriV</i> and part of <i>orf1</i>	This study
pAG2	Ap ^R , pMT2 <i>Pst</i> I fragment 2* cloned into pUC18; contains <i>orf3</i> (<i>res</i>), <i>orf4</i> (<i>tolA</i>) and <i>orf5</i> (<i>korA</i>)	This study
pAG3	Ap ^R , pMT2 <i>Pst</i> I fragment 3* cloned into pUC18; does not contain any complete ORFs	This study
pAG4	Ap ^R , pMT2 <i>Pst</i> I fragment 4* cloned into pUC18; contains putative <i>par</i> and <i>mpf</i> promoters and <i>orf8</i> and <i>orf9</i> (<i>mpfA</i> and <i>B</i>)	This study
pAG5	Ap ^R , pMT2 <i>Pst</i> I fragment 5* cloned into pUC18; does not contain any complete ORFs	This study
pAG7	Km ^R Cm ^R , pMT2 with pACYC184 inserted into <i>Hind</i> III site within <i>orf4</i> (<i>tolA</i>)	This study
pAG8	Km ^R Cm ^R , pMT2 with pACYC184 inserted into <i>Bgl</i> II site within <i>orf5</i> (<i>korA</i>)	This study
pAG9	Km ^R Cm ^R , pMT2 with pACYC184 inserted into <i>Bgl</i> II site directly upstream of <i>orf8</i> (<i>mpfA</i>)	This study
pAG1.1	Ap ^R , pAG1 deletion derivative; contains <i>rep</i> and <i>oriV</i>	This study
pAG1.1 Δ <i>rep</i>	Ap ^R , <i>rep</i> deletion mutant derived from pAG1.1; contains <i>oriV</i>	This study
pAG10	Ap ^R Tc ^R , pBR322 inserted into pMT2 <i>Xho</i> I site	This study
pAG11	Cm ^R , pMT2/pACYC184 hybrid with <i>Sal</i> I fragment removed; creates a smaller pMT2 mini replicon	This study
pAG11 Δ <i>rep</i>	Cm ^R , <i>rep</i> deletion mutant derived from pAG11	This study
pAG4.1	Ap ^R , pAG4 deletion derivative; contains <i>par</i> promoter	This study
pAGB1	Ap ^R , pMT2 <i>Bgl</i> II fragment 1 cloned into pUC18	This study
pAGB2	Km ^R , pMT2 <i>Bgl</i> II fragment 2 cloned into pUC18	This study
pBR322	Ap ^R Tc ^R , Tra ⁻	Bolivar <i>et al.</i> (1977)
pGBT30	Ap ^R , <i>tacp</i> ; pMB1-based expression vector	Jagura-Burdzy <i>et al.</i> (1991)
pM3	Tc ^R Sm ^R , Tra ⁺	This study
pME6000	Tc ^R , derivative of plasmid pBBR1	Maurhofer <i>et al.</i> (1998)
pMT2	Km ^R , Mob ⁺	This study
pMT2 Δ <i>res</i>	Km ^R , <i>res</i> mutant of pMT2	This study
pREN1	Km ^R , pMT2 <i>repp-xylE</i> promoter probe	This study
pREN2	Ap ^R , <i>tacp</i> -pMT2 <i>rep</i> ; based on pGBT30	This study
pPT01	Km ^R , <i>xylE</i> promoter probe	Thorsted <i>et al.</i> (1996)
pUC18	Ap ^R , Tra ⁻	Yanisch-Perron <i>et al.</i> (1985)

* Fragments are illustrated in Fig. 1a.

Recombinants were selected with kanamycin and chloramphenicol. pAG9 has pACYC184 inserted into the *Bgl*II 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 *Sal*I 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. pAG11 Δ *rep* was created by digesting pAG11 with *Sal*I and *Nde*I which each cut once. Both *Sal*I-*Nde*I fragments were purified. The smaller of the two DNA fragments, containing

the *rep* gene, was digested with *Bam*HI, which cuts twice within the *rep* ORF. The three fragments were run on a gel and the *Sal*I-*Bam*HI and *Nde*I-*Bam*HI purified and ligated resulting in a deletion within the *rep* ORF. The Δ *rep* DNA fragment was then ligated to the larger *Sal*I-*Nde*I fragment to create a *rep* deletion mutant of pAG11. An *orf3* (the putative *res* gene) mutant of pMT2, pMT2 Δ *res*, was constructed by digesting pMT2 with *Bsr*GI and *Afl*III (positions 3812 and 4035 respectively), purifying the large fragment, and religating it in the presence of two 14 bp linkers (5'-GTACAGCTCGAGGA-3' and 5'-TTAATCCTCGAGCT-3') designed to create an extra *Xho*I 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); ▽, insertion; △, 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 *Bam*HI, was ligated to the 594 bp *Bam*HI 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 *Eco*RI and *Sal*I, 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 (10^4 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 carrying cloned fragments from pMT2) or tetracycline (when testing pME-6000), 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-hydroxymuconic 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 pMT2Δ*res*, 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 3×10^9 c.f.u. ml⁻¹ and in 150 min three generations of growth have been completed). Aliquots (5 ml) of the samples containing approximately 1×10^6 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 *Eco*RI and *Sal*I 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 Hybaid 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 Tc^R variant of pM3 was partially digested with *Pst*I and ligated with a 1.24 kb *Pst*I fragment from pUC4K (Km^R). 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 *Pst*I and both of the *Bgl*II fragments were inserted into pUC18 (Fig. 1a). The complete nucleotide sequence of pMT2 was determined by sequencing all of the cloned *Pst*I fragments and across the *Pst*I sites in the cloned *Bgl*II fragments. Both DNA strands were sequenced to eliminate any errors. pMT2 was found to be 9766 bp in size including the *Pst*I fragment carrying the Km^R 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 CODONPREFERENCE 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-

Table 2. pMT2 ORFs with clearly identified putative functions in replication

Name*	Co-ordinate	Putative Shine–Dalgarno sequence†	No. residues	Molecular mass (Da)	Isoelectric point	Identity (%)	Overlap (aa)	Sequence from database‡	GenBank accession no.
<i>orf1</i>	2618–1956	<u>GGAGGGTGCTTT</u>	162	18 292	4.61	–§	–	–	–
<i>orf2 (rep)</i>	3197–2643	<u>TAGGAGGCCCA</u>	183	19 872	6.69	44	223	Rep (pBBR1)	X66730
<i>orf3 (res)</i>	4289–3654	<u>TACGGTAACACT</u>	211	23 599	9.79	35	196	ParA (resolvase, RK2)	P22996
						34	170	TnpR (resolvase, Tn21)	P04130
<i>orf4 (tolA)</i>	4929–4504	<u>CAAGGACGTGACCC</u>	141	16 002	5.75	30	81	TolA (<i>P. putida</i>)	P44678
<i>orf5 (korA)</i>	5276–4926	<u>TGGAAAGACTCGGA</u>	116	13 466	8.14	25	93	KorA (R751)	Q57423
<i>orf6 (parB)</i>	6217–5273	<u>TGAGGATTGAATC</u>	314	34 766	5.99	30	224	Spo0J (<i>Bacillus subtilis</i>)	P26497
						24	270	KorB (RK2)	P07674
<i>orf7 (parA)</i>	6995–6230	<u>TAGGAAGGTCACC</u>	255	28 251	4.79	36	258	IncC (R751)	Q52312
						29	231	Soj (<i>B. subtilis</i>)	P37522
<i>orf8 (mpfA)</i>	7998–8459	<u>AAGAGGCAATGGT</u>	153	16 851	10.80	30	105	VirB2 (Ti plasmid)	P17792
<i>orf9 (mpfB)</i>	8468–8764	<u>AAGTGAGGTAAGCG</u>	98	10 948	9.32	36	56	VirB3 (Ti plasmid)	P17793
<i>orf10 (mpfC)</i>	8751–end	<u>TGGGGGTATCGCAGT</u>	Unknown	Unknown	Unknown	24	191	VirB4 (Ti plasmid)	P17794

* 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 identified as described below, 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 *Hind*III site (position 4872 on the pMT2 map) and both *Bgl*II sites of pMT2 (Fig. 1a; pAG7, pAG8 and pAG9). pACYC184 was chosen because it does not carry Km^R 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. *Hind*III 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 Tn1/3-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 pMT2Δ*res* 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 pMT2Δ*res*, 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 *Bgl*II site of pMT2 *Pst*I fragment 4 – with no effect in pMT2 plasmid replication) was used in further manipulation of the pMT2 replicon. pAG11 was constructed by a *Sal*I deletion, leaving only 5340 bp of pMT2 DNA from the region between the *Bgl*II site (position 7729) upstream of the *parA* promoter and the *Sal*I site (position 2389) downstream of the *rep* ORF (Fig. 1a). pAG11 was as stably maintained as pMT2. We constructed a further derivative, pAG11Δ*rep*, 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 0.40 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. pAG1.1Δ*rep*, with an internal *Bam*HI deletion in-

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* were involved in plasmid maintenance. Indeed, the effect of an insertion into the *Hind*III 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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