Replication regions from plant-pathogenic *Pseudomonas syringae* plasmids are similar to ColE2-related replicons

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Many strains of the phytopathogen *Pseudomonas syringae* contain mutually compatible plasmids that share extensive regions of sequence homology and essential replication determinants. The replication regions of two compatible large plasmids involved in virulence or pathogenicity, pPT23A from *P. syringae* pv. *tomato* strain PT23 and pAV505 from *P. syringae* pv. *phaseolicola* strain HRI1302A, were isolated. DNA sequencing of the origins of replication revealed homologous ORFs, designated ORF-Pto and ORF-Pph, respectively. Both ORFs are 1311 bp long and encode peptides of 437 amino acids with predicted molecular masses of 48259 (Pto) and 48334 (Pph) Da. Expression of the two ORFs in *Escherichia coli* produced peptides of 50 kDa (Pto) and 56 kDa (Pph). The predicted peptides showed an overall identity of 89.7%, being highly conserved from residues 1 to 373, but showing considerable variation in their C-terminal regions (50% identity over the last 64 aa). The two ORFs had significant similarity with the putative replication protein from plasmid pTiK12 of *Thiobacillus intermedius* and other ColE2-related plasmids. However, both peptides were 100 residues longer than any of the known ColE2-related rep sequences. Subcloning of fragments from the replication region of pPT23A revealed the presence of at least three incompatibility determinants, designated IncA, IncB and IncC. Partial sequencing of the region downstream of ORF-Pto revealed homology to the *ruAB* genes, involved in UV resistance, from plasmid pPSR1. It is proposed that the replication origin of pPT23A serves as the prototype of a family of related plasmids.

Keywords: incompatibility, UV light resistance, *Pseudomonas syringae* pvs *tomato* and *phaseolicola*, virulence, pPT23A-like plasmids

INTRODUCTION

A common feature of *Pseudomonas syringae* strains is the presence of one or several native plasmids, ranging in size from 2 to >200 kb (Curiale & Mills, 1983; Coplin, 1989; Sundin *et al.*, 1994; Sesma *et al.*, 1998). Whilst many of these plasmids remain cryptic, functions such as

avirulence (Vivian & Gibbon, 1997), resistance to copper, streptomycin, or both (Cooksey, 1990; Sundin *et al.*, 1994), and biosynthesis of extracellular virulence factors (Comai & Kosuge, 1980; Bender *et al.*, 1989; Nagahama *et al.*, 1994; Kamiuntun, 1995) have been identified.

P. syringae pv. *tomato* strain PT23 has four native plasmids, designated pPT23A–pPT23D and ranging in size from 36 to 100 kb (Bender & Cooksey, 1986). Plasmid pPT23A (100 kb) carries the gene cluster for the synthesis of the phytotoxin coronatine (Bender *et al.*, 1989). pPT23B (83 kb) contains avirulence gene *avrD* (Kobayashi *et al.*, 1990), which contributes to host range, and an as yet uncharacterized DNA region that putatively participates in the synthesis of a new toxin

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Abbreviations: ORF-Pph, putative replicase gene from pAV505; ORF-Pto, putative replicase gene from pPT23A.

The EMBL accession numbers for the sequences reported in this paper are AJ222648 (*oriV*-pAV505), AJ224509 (*oriV*-pPT23A, *ruIA*), AJ224510 (partial *ruIA*B), and AJ006933 (partial *ruIB*).

(Sesma *et al.*, 1997; A. Sesma, M. T. Aizpun, A. Ortiz, D. Arnold, A. Vivian & J. Murillo, unpublished). These two plasmids carry the main virulence determinants of strain PT23 since a strain devoid of both did not induce chlorosis, produced very small necrotic lesions and was greatly reduced in its ability to colonize tomato leaves (Bender *et al.*, 1989; Sesma *et al.*, 1997). Plasmid pPT23C (65 kb) is cryptic and pPT23D (36 kb) confers resistance to copper, a widely used bactericide. pPT23A and pPT23C were shown to be conjugative, and pPT23D could be mobilized by cointegration with pPT23C (Bender *et al.*, 1989).

Southern hybridization experiments showed that many *P. syringae* plasmids share a large amount of DNA (Murillo & Keen, 1994; Sesma *et al.*, 1998), and it has been estimated that in strain PT23, 74% of plasmid pPT23B is repeated in pPT23A. Some of the repeated DNA includes putative transposable elements, replication and maintenance determinants, genes for UV resistance (Murillo & Keen, 1994; Sesma *et al.*, 1998) and genes involved in conjugation (A. Canal, A. Sesma & J. Murillo, unpublished). The replication region of plasmid pPT23A was identified by hybridization with a 1.2 kb *Hind*III fragment containing an uncharacterized origin of replication from the *P. syringae* pv. *syringae* plasmid pOSU900 (Mukhopadhyay *et al.*, 1990; Murillo & Keen, 1994). The defined minimal replication region of pPT23A hybridized to the co-resident plasmids pPT23B and pPT23D in strain PT23 and to six different plasmids that were co-resident in a strain of *P. syringae* pv. *glycinea* (Murillo & Keen, 1994; Sesma *et al.*, 1998). This region was shown to be highly conserved among plasmids in *P. syringae* pathovars. These results suggest that most of the plasmids in *P. syringae* may be derived from a common ancestor through accumulation of mutations that permit the observed compatibility of highly related plasmids.

P. syringae pv. *phaseolicola* race 4 strain HRI1302A has four native plasmids, designated pAV505 (150 kb), pAV506 (50 kb), pAV507 (47 kb) and pAV508 (42 kb) (Jackson, 1997). Plasmid pAV505 is essential for the production of disease on bean plants and other hosts (Jackson, 1997). Additionally, an avirulence phenotype, which matched a resistance gene in pea (*Pisum sativum*), was shown to require the presence of two cloned regions of DNA, designated regions I and II. The two regions were separated by approximately 4 kb and located on pAV505 (Wood *et al.*, 1994). The cloned region II displayed a strong incompatibility with pAV505 (Jackson, 1997) and hybridized with the minimal replication region of pPT23A. This suggested that it could be part of the pAV505 replication region and that it could participate in avirulence (M. J. Gibbon, D. L. Arnold & A. Vivian, unpublished).

In this work we characterize the replication regions of these two plasmids with the aim of gaining a better understanding of the determinants and mechanisms that allow for the coexistence of highly related replicons in *P. syringae*, and thus for the horizontal distribution of pathogenicity determinants.

METHODS

Bacterial culture conditions. Strains and plasmids used in this work are listed in Table 1 and Fig. 5. *Escherichia coli* was grown on Luria-Bertani (LB) medium (Sambrook *et al.*, 1989) at 37 °C, and *P. syringae* on King's medium B (KMB) (King *et al.*, 1954) or LB at 25–28 °C. When necessary, media were supplemented with antibiotics at the following concentrations ($\mu\text{g ml}^{-1}$): ampicillin, 100; kanamycin, 25; tetracycline, 12.5.

Genetic manipulations. Standard molecular biology techniques were performed as described by Sambrook *et al.* (1989). Plasmids were isolated by a modified alkaline lysis procedure (Zhou *et al.*, 1990) and intact native plasmids were separated by electrophoresis on 0.6% agarose gels in 1× TAE as previously described (Murillo *et al.*, 1994). DNA fragments separated in low-melting-point agarose (Life Science, Gibco-BRL) were ligated as described by Crouse *et al.* (1983). DNA was introduced into *P. syringae* cells by triparental conjugation or electroporation (Keen *et al.*, 1992).

Double-stranded DNA, purified by extraction on mini columns (Qiagen), was sequenced by PCR using the Ampli-Cycle kit (Perkin-Elmer), following the manufacturer's recommendations. Primers for sequencing were pUC M40 forward and reverse, and custom-synthesized oligonucleotides (15 to 20-mer) based on previously determined sequences within individual inserts. Clones for sequencing were either previously obtained by exonuclease III digestion of appropriate plasmids (Murillo & Keen, 1994) or by subcloning of specific restriction fragments in pBluescript or pK184. The sequence was analysed using the University of Wisconsin GCG package, available through the SEQNET facility at Daresbury, UK (Devereux *et al.*, 1984). Homology searches were performed using the BLAST network service at the NCBI, Bethesda, MD, USA, and FASTA at Daresbury, and alignments were done using CLUSTAL W at the EBI, Cambridge, UK, or the IBCP, Lyon, France. Phylogenetic trees were constructed using the neighbour-joining method at the CLUSTAL W server at the EBI, and viewed using TREEVIEW software (Page, 1996).

Plasmid pAori60 was subjected to mutagenesis with Tn3HoKmGus (Bonas *et al.*, 1989) as described by Dangel *et al.* (1992). Expression of the Tn3Gus fusions was analysed using 4-methylumbelliferyl- β -D-glucuronide (Biosynth) following published methodology (Gallagher, 1992). The precise location of selected insertions was determined by sequencing using specific primers for the terminal repeats of the transposon. To determine the effect of individual transposon insertions on the replication ability of *oriV*-pPT23A, inserts of selected clones were excised from the vector by digestion with *Kpn*I, which does not cut inside the transposon, and cloned in pK184. The replication ability of the resulting clones was then assessed by their capacity to transform the plasmidless strain *P. syringae* pv. *syringae* FF5.

In vivo expression and labelling of proteins in *E. coli*. High-level expression of appropriate constructs was performed with a T7 RNA polymerase-promoter system using *E. coli* BL21(DE3), and proteins were specifically labelled with L-[³⁵S]methionine (Amersham) essentially as described by Studier & Moffatt (1986) and Hidalgo *et al.* (1992). The labelled proteins were resolved by electrophoresis in 12% SDS-polyacrylamide gels and analysed by autoradiography (Sambrook *et al.*, 1989).

Incompatibility assays. The identification of determinants in pAKC showing incompatibility against the parent plasmid pPT23A was assayed by a qualitative test essentially as

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Reference or source
Strains		
<i>Escherichia coli</i>		
BL21(DE3)	<i>hsdS gal (λcIts857 ind1 Sam7 nin5 lacUV5-T7 gene 1)</i>	Studier & Moffatt (1986)
DH5α	F ⁻ <i>ΔlacU169 (φ80 lacZΔM15) endA1 recA1 hsdR17 supE44 thi-1 gyrA96 relA1</i>	Bethesda Research Laboratory, Gaithersburg, MD, USA
<i>Pseudomonas syringae</i>		
pv. <i>phaseolicola</i> HRI1302A	Contains four plasmids: pAV505 (150 kb), pAV506 (50 kb), pAV507 (47 kb) and pAV508 (42 kb)	Jackson (1997)
pv. <i>syringae</i> FF5	Plasmidless	Sundin & Bender (1993)
pv. <i>tomato</i> PT23	Contains four plasmids: pPT23A (100 kb), pPT23B (83 kb), pPT23C (65 kb) and pPT23D (36 kb)	Bender & Cooksey (1986)
Plasmids		
pAKC	9.2 kb <i>KpnI</i> fragment containing the minimal region necessary for replication from pPT23A cloned in pK184	Murillo & Keen (1994)
pAori1	2.4 kb partial <i>EcoRI</i> fragment from pAKC cloned in pK184; contains the minimal replication region from pPT23A	Murillo & Keen (1994)
pAori2	As pAori1, but with an additional 0.2 kb <i>EcoRI</i> fragment, and opposite orientation; ORF- <i>Pto</i> is transcribed from the <i>lac</i> promoter	Murillo & Keen (1994)
pAori23	Obtained by religation of pAori2 digested with <i>PstI</i> , blunted and digested with <i>EcoRV</i> ; contains an 1.5 kb <i>EcoRI</i> (partial)- <i>EcoRV</i> fragment that can replicate in <i>P. syringae</i> ; ORF- <i>Pto</i> is truncated by 67 nt	This work
pAori41	2 kb <i>BglII</i> - <i>EcoRV</i> from pAori1 cloned in <i>BamHI</i> - <i>EcoRV</i> of pBluescript; ORF- <i>Pto</i> is truncated by 67 nt and is under the control of the T7 promoter of the vector	This work
pAori42	As pAori41, but opposite orientation	This work
pAori44	9.2 kb <i>KpnI</i> fragment from pAKC joined to the Sm ^r cassette from pSmUC	This work
pAori60	9.2 kb <i>KpnI</i> fragment from pAKC cloned in pRK415	This work
pAV514	4.3 kb <i>PstI</i> fragment from pPPY40 cloned in pK184; orientation of ORF- <i>Pph</i> in opposition to the vector <i>lac</i> promoter	This work
pAV515	As pAV514, but opposite orientation	This work
pBluescript KS(+), pBluescript SK(-)	Ap ^r , <i>E. coli</i> cloning vectors containing T7 promoter	Stratagene, La Jolla, CA, USA
pHoKmGus	Ap ^r Km ^r <i>tnpA</i> , pWB15a replicon, promoter-less β-glucuronidase gene in Tn3	Bonas <i>et al.</i> (1989)
pSShe	Cm ^r <i>tnpA</i> ⁺ , pACYC184 replicon	Stachel <i>et al.</i> (1985)
pK184	Km ^r , <i>E. coli</i> cloning vector, p15a replicon	Jobling & Holmes (1990)
pPPY40	Tc ^r , pLAFR3-based cosmid containing a 25 kb fragment from pAV505,	Wood <i>et al.</i> (1994)
pPPY50	4.3 kb <i>PstI</i> fragment from pPPY40 cloned in pBluescript KS(+); ORF- <i>Pph</i> under the control of the T7 promoter of the vector	This work
pPPY51	As pPPY50, but opposite orientation	This work
pRK415	Tc ^r , broad-host-range cloning vector, IncP replicon	Keen <i>et al.</i> (1988)
pSmUC	Ap ^r Sm ^r Sp ^r , contains a Sm/Sp cassette symmetrically flanked by diverse restriction sites	Murillo <i>et al.</i> (1994)

described by Nordström (1993). Clones constructed in pK184 (pAori23 and pAori25) or pRK415 were introduced into PT23 by electroporation. Resulting transformants were selected on

KMB plus the appropriate antibiotic and, after three sequential single colony purifications on the same medium, the plasmid profiles were analysed. At least two transformants

from at least three separate electroporation experiments were analysed for each clone.

RESULTS

The minimal origins of replication encode a putative replication protein

The origin of replication from plasmid pPT23A, from *P. syringae* pv. *tomato* PT23 was cloned as a 9.2 kb *KpnI* fragment in pK184, which does not replicate in *P. syringae*, to give pAKC (Murillo & Keen, 1994). Using exonuclease III deletion clones in an *in vivo* replication assay, the minimal fragment from pAKC able to support replication in *P. syringae* (*oriV*-pPT23A) was defined as a 1.6 kb fragment. The nucleotide sequence of this fragment (1662 nt) contains a large ORF (ORF-*Pto*; Fig. 1a) that could encode a polypeptide of 437 aa, with a

predicted molecular mass of 48259 Da. Further experiments reduced the minimal fragment which retained the capacity for replication in *P. syringae* to a 1556 bp *EcoRI* (partial)-*EcoRV* fragment (designated pAori23; Fig. 1a and data not shown), which contained ORF-*Pto*, C-terminally truncated by 21 aa.

A 4.3 kb *PstI* fragment from *P. syringae* pv. *phaseolicola* HRI1302A cosmid clone pPPY40, involved in an avirulence phenotype (Wood *et al.*, 1994), was found by hybridization to be similar to *oriV*-pPT23A (data not shown). Plasmids pAV514 and pAV515, which contain the 4.3 kb *PstI* fragment from pPPY40 in pK184 in different orientations, replicated autonomously in strain PT23 and were compatible with plasmid pPT23A, even after four transfers in media with kanamycin. The nucleotide sequence of a 1675 bp fragment internal to the 4.3 kb *PstI* fragment (Fig. 1a) showed a high overall

Fig. 1. (a) Genetic organization of the replication regions of pAKC, derived from pPT23A, and pPPY40, derived from pAV505. The enlargements show the sequenced fragments, and the position and orientation of the putative replicase genes (ORF-*Pto* and ORF-*Pph*) are indicated by arrows. The loops represent major repeats found upstream of both ORFs. Selected Tn3HoKmGus insertions in pPT23A DNA are shown by vertical lines with the corresponding insertion number. The position and orientation of the partially sequenced *rulAB* homologue are indicated. The length of *rulAB* was proportionately extended beyond the sequenced DNA based on the homology with the *rulAB* genes from pPSR1. E, *EcoRI*; H, *HindIII*; K, *KpnI*; P, *PstI*; V, *EcoRV*. (b) The major repeated sequence found upstream of the putative replicases. Boxes indicate nucleotides that could be pairing to form a stem-and-loop structure.

pPT23A	MSHDNAISLA	LATSSTANAD	PLAISTHPP	ARFFEDGTAL	NRLLEAPYM	50
pAV505	MSHDNAISLA	LATSSTANAD	PLAISTHPP	ARFFEDGTAL	NRLLEAPYL	50
pPT23A	SFLKQGFSEN	LPLSSIRATV	KSVGRWTWDR	YTGDRRCHRG	AMQLDLSLSL	300
pAV505	SFLKQGFSEN	LPLSSIRATV	KSVGRWTWDR	YTGDRRCHRG	AMQLDLSLSL	300
pPT23A	TERQSLAAKR	TNELRHKATE	SKIRAACRQL	QDQKTLVRS	AIATLAGISV	350
pAV505	TERQSLAAKR	TNELRHKATE	SKIRAACRQL	QDQKTLVRS	AIATLAGISV	350
pPT23A	RTVASYPHIL	TEVSQPSVTS	VLRGTRPAK	AATGAHQVD	STIPCFARQQ	400
pAV505	RTVASYPHIL	TEVSQPSVTS	VLRGTRPAK	AATGAHQVD	STIPCFARQQ	400
pPT23A	AANRRAPGVQ	SGVHQISACP	KSPQAGEELK	KEHDDCS		437
pAV505	AANRRAPGVQ	SGVHQISACP	KSPQAGEELK	KEHDDCS		437

Fig. 2. Comparison of the putative Rep proteins from pPT23A and pAV505. Non-conservative substitutions are in white lettering on a black background and conservative substitutions are boxed. For simplicity, the sequence from position 51 to 250, where only a conservative substitution was found at position 180, has been omitted. The filled arrowhead indicates the start of the deletion on pAori23.

similarity (88.8% identity in the putative coding regions, and 84.4% identity in the preceding DNA) to the sequence of *oriV*-pPT23A and was designated *oriV*-pAV505. A large ORF, ORF-*Pph*, was present in *oriV*-pAV505 between nt 323 and 1636. ORF-*Pph* is predicted to encode a polypeptide of 437 aa, with a predicted molecular mass of 48334 Da.

The polypeptides deduced from ORF-*Pto* and ORF-*Pph* showed an overall identity of 89.7% and were almost identical from position 1 to 373 (96.5% identity) (Fig. 2). The sequences, however, were different from position 374 to 437 (50% identity), with changes concentrated in a region of 35 residues (positions 374–408) where only seven identical residues could be found. The overall G + C content of *oriV*-pPT23A and *oriV*-pAV505 was 58.2 and 59.1 mol%, respectively, which is at the lower limit of the mean content of the *P. syringae* chromosome (Palleroni, 1984).

Approximately 250 bp upstream of the putative ATGs of both ORF-*Pto* and ORF-*Pph* there is a perfect inverted repeat with a 13 or 14 bp stem, respectively, and a 7 or 6 bp loop, respectively, surrounded by short (3–5 nt) poly(A) stretches (Fig. 1b). The change of a G in the left branch of the stem in *oriV*-pPT23A for a C in *oriV*-pAV505 is accompanied by a corresponding complementary change in the right arm, so that the stem in *oriV*-pAV505 still forms a perfect repeat. Additional inverted or direct repeats, of nine or more nucleotides, conserved in both sequences, were not found, nor did we detect features common to origins of replication containing iterons, which are present, for instance, in the origins of plasmids pPS10 from *P. syringae* pv. *savastanoi* (Nieto *et al.*, 1992) and pRO1600 from *Pseudomonas aeruginosa* (West *et al.*, 1994).

The sequence of three non-contiguous fragments (totalling 728 nt) downstream of *oriV*-pPT23A (Fig. 1a), showed a high degree of similarity to the *rulAB* genes of plasmid pPSR1 of *P. syringae* pv. *syringae* A2 (Sundin *et al.*, 1996). A 16 bp sequence, highly homologous to the

Fig. 3. Analysis of polypeptides produced from ORF-*Pto* and ORF-*Pph*. Autoradiogram of [³⁵S]methionine-labelled polypeptides separated by SDS-PAGE from cell extracts of *E. coli* BL21(DE3) bearing pPPY51 (lane 1), pPPY50 (lane 2), pBluescript KS(+) (lane 3), pAori41 (lane 4) and pAori42 (lane 5). Numbers on the left indicate the size of the specifically labelled products.

consensus binding site for LexA and located –45 to –30 relative to the start codon of *rulA* in pPSR1, was conserved in sequence and position in pPT23A. The putative start codons of *rulA* and *rulB*, and the stop codon of *rulA*, were also conserved in pPT23A.

ORF-*Pto* and ORF-*Pph* produce proteins of the expected size

Plasmids pAori41, pAori42, pPPY50 and pPPY51 were introduced into *E. coli* BL21(DE3). Appropriate cultures induced with IPTG were pulse-labelled with [³⁵S]methionine for 5 min, and total cell lysates were separated by SDS-PAGE (Fig. 3). Plasmids pAori41 and pPPY50, in which ORF-*Pto* and ORF-*Pph* were under the control of the T7 promoter, directed the specific labelling of approximately 50 and 56 kDa polypeptides, respectively. The direction of transcription of the genes and the sizes of the products, as deduced from the sequences (both 48.3 kDa), correlate with these results, since although the insert in pAori41 [fragment 1.5 kb *EcoRI* (partial)–*EcoRV*] lacks the last 21 residues of ORF-*Pto*, the construction adds 19 residues from the vector sequences to the ORF. We cannot account for the difference in size between the deduced product of ORF-*Pph* and the expressed product in *E. coli*.

Transposon mutants in ORF-*Pto* do not replicate

Plasmid pAori60, which contains a 9.2 kb *KpnI* fragment including *oriV*-pPT23A in pRK415, was subjected to mutagenesis with Tn3HoKmGus. The *KpnI* fragments

containing transposon insertions 37, 95, 204, 250, 293 and 368 (Fig. 1a) were cloned in pK184 and their capacity to replicate was assayed by transforming the individual clones in *P. syringae* pv. *syringae* FF5. Clones carrying insertions 37, 95 and 204, located in the coding region of ORF-*Pto*, did not yield any transformants in three separate electroporation experiments, which suggests that they inactivate a function essential for replication. Clones carrying insertions 250, 293 and 368 consistently yielded 10^3 – 10^5 transformants ($\mu\text{g DNA}$) $^{-1}$ when introduced into strain FF5. Insertion number 368 is located in IncA, 603 bp upstream of the putative start codon of ORF-*Pto*, whilst insertions 293 and 250 are located 56 and 143 bp, respectively, downstream of the putative stop codon of this ORF (data not shown). We did not detect β -glucuronidase activity in PT23 cells carrying any of the transposon insertions, either in exponential or stationary phases.

ORF-*Pto* and ORF-*Pph* are similar to other replication proteins

Searches of the databases revealed that the nucleotide sequences of ORF-*Pto* and ORF-*Pph* showed a high similarity (52.8% overall identity) with the coding region of the putative replication protein of plasmid pTiK12 (65 kb) from *Thiobacillus intermedius* K12 (English *et al.*, 1995). The polypeptides deduced from ORF-*Pto* and ORF-*Pph* (Fig. 2) showed a 45.9% overall identity with the putative replication protein of pTiK12 and a significant similarity with the replication proteins of the ColE2-related plasmids and several other plasmids from Gram-negative and Gram-positive bacteria, as well as with the PspPI DNA methyltransferase, associated with the PspPI restriction-modification system, from *Psychrobacter* sp. (Rina *et al.*, 1997) (Fig. 4 and data not shown). The degree of homology among all these proteins is higher in the first two-thirds of the sequence, and they are poorly conserved in the C-terminal part, both in sequence and in length. The proteins deduced from ORF-*Pto* and ORF-*Pph* were more than 100 residues larger than any of the proteins in this group. Two putative adjacent ORFs of the streptomycin-resistance plasmid pCPP519 from *Pseudomonas* sp. PyR19 (T. C. Huang & T. J. Burr, EMBL accession no. AF029751) also showed a high similarity to the first (71% identity over 138 aa) and second (53% identity over 131 aa) half of ORF-*Pto* (Fig. 4 and data not shown). This could imply either a mutational event or a sequencing error, leading to the apparent frameshift in the pCPP519 sequence.

Given the similarity to the ColE2-related replicons, we were interested to see whether the pPT23A-like replicons were maintained in *E. coli*. However, we consistently failed to obtain transformants of *E. coli* DH5 α using pAori44, which contains the 9.2 KpnI fragment from pAKC joined to a Sm/Sp cassette, either at 28 or 37 °C. This result suggests that the similarity among ORF-*Pto* and the replicases from the ColE2-related plasmids is not sufficient to allow the replication of *oriV*-pPT23A in *E. coli*.

Fig. 4. Dendrogram of genetic relatedness of (putative) replication proteins and a methyltransferase. The tree was constructed by the neighbour-joining method using the CLUSTAL W Multiple Sequence Alignment interactive program available at the EBI, Cambridge, UK, and displayed using TREEVIEW. Replication proteins are from the following plasmids: pMB1 from *Bifidobacterium longum* (Rossi *et al.*, 1996); pXZ10142 (S. L. Shi, Z. X. Wang, Y. Deng, H. Zhu, B. He, & Z. X. Zheng, EMBL accession no. X72691) and pXZ10145.1 (Z. Zheng, S. Shi, X. Jiang, Z. Wang & L. Caro, accession no. U85507) from *Corynebacterium glutamicum*; pFAJ2600 from *Rhodococcus erythropolis* (De Mot *et al.*, 1997); pJ1 from *Neisseria gonorrhoeae* (Korch *et al.*, 1985); pTiK12 from *Thiobacillus intermedius* K12 (English *et al.*, 1995); pPT23A, deduced from ORF-*Pto*, from *P. syringae* pv. *tomato* PT23; pAV505, deduced from ORF-*Pph*, from *P. syringae* pv. *phaseolicola* HRI1302A; ColE2-P9 from *Shigella* sp. and ColE3-CA38 from *E. coli* (Hiraga *et al.*, 1994); pAL5000 from *Mycobacterium fortuitum* (Rauzier *et al.*, 1988); pRBL1 (Ankri *et al.*, 1996) and pBL-A8 (V. Leret, accession no. Y11902) from *Brevibacterium linens*. pCPP519 denotes the sequence of ORF2, which could encode a 157 aa protein of unknown function, from the streptomycin resistance plasmid pCPP519 from the non-fluorescent *Pseudomonas* sp. PyR19 (T. C. Huang & T. J. Burr, accession no. AF029751; see text), and *pim* is the gene for a methyltransferase from *Psychrobacter* sp. (Rina *et al.*, 1997). For simplicity, other plasmids of the ColE2-family were not included in the tree.

Identification of two putative maintenance determinants in the pPT23A replication region

Low-copy-number bacterial plasmids are generally stably maintained because they encode several systems that minimize plasmid loss during cell division. As a result of their activity, the majority of these maintenance systems express incompatibility toward their parent plasmids (Novick, 1987; Austin & Nordström, 1990; Nordström, 1993). Taking advantage of this, we sought to identify other replication or maintenance determinants in pAKC by testing the ability of different restriction fragments from it to evict pPT23A by incompatibility.

Fig. 5. Mapping of incompatibility regions in pAKC. Restriction fragments listed in the third column and represented by horizontal arrows were cloned in pK184 or in pRK415, as indicated, and introduced by electroporation into strain PT23. After three successive single colony purifications on KMB plus kanamycin for pK184, or KMB plus tetracycline for pRK415, the plasmid profile of transformants was analysed. Incompatibility with pPT23A was scored as + or - if at least half or less than half, respectively, of the transformants analysed had lost pPT23A, as determined by gel electrophoresis. The number of transformants that lost pPT23A and the total number analysed are shown in parentheses. Arrows indicate the direction of transcription from the *lac* promoter in the vector. Transformants came from at least three different electroporations. B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; P, *Pst*I; V, *Eco*RV.

Restriction fragments from pAKC, cloned in pRK415 or pK184, were introduced individually into strain PT23 by electroporation and the plasmid profile of the resulting transformants was analysed to check for the loss of pPT23A. The results (Fig. 5) indicate that at least three separable incompatibility determinants are included in pAKC, which, due to the incompatibility phenotype, could contain replication or maintenance determinants. The first of them was specified on the 0.4 kb *Pst*I-*Eco*RI fragment located immediately upstream of *oriV*-pPT23A and has been designated IncA. The second determinant (IncB) was contained in a 2 kb *Eco*RI-*Pst*I fragment that spans the minimal fragment with capacity to replicate. In ColE2-related plasmids, the incompatibility displayed by the minimal replicating fragment was due to either the origin of replication, a sequence of around 30 bp, or to a small regulatory antisense RNA (Hiraga *et al.*, 1994). In this case, we were unable to locate IncB more precisely since pAori31 only showed a partial incompatibility, whilst pAori19 did not lead to the curing of pPT23A in any of the eight transformants analysed. Moreover, pAori23, which contains the first 1556 bp of *oriV*-pPT23A and could replicate autonomously in *P. syringae*, did not displace pPT23A in 15

transformants analysed, although in most of them both pPT23A and pPT23B showed alterations in size after five consecutive transfers under kanamycin selection on KMB (data not shown). The third determinant, designated IncC, comprised the 0.8 kb *Eco*RI-*Kpn*I fragment of pAKC, which showed a strong incompatibility against pPT23A.

DISCUSSION

In *P. syringae* strains, it is common to find one or several plasmids cross-hybridizing to an uncharacterized origin of replication from the *P. syringae* pv. *syringae* plasmid pOSU900 (Mukhopadhyay *et al.*, 1990; Murillo & Keen, 1994; Sundin *et al.*, 1994). The origin of replication of plasmid pPT23A is now the best-characterized member of this family of plasmids, and in consequence we propose to designate it the pPT23A-like family. pPT23A and pAV505 are compatible pPT23A-like plasmids from *P. syringae* pv. *tomato* PT23 and *P. syringae* pv. *phaseolicola* HRI1302A, respectively. Both plasmids are important or essential for the interaction of these bacteria with their host plants since they carry virulence

and/or pathogenicity determinants (Bender *et al.*, 1987; Jackson, 1997; Sesma *et al.*, 1997) and are stably maintained in spite of coexisting with other similar replicons in the same cell. The results presented here provide a preliminary characterization of the closely related replication regions of these two plasmids from different pathovars of *P. syringae*.

The minimal regions required for replication of pPT23A and pAV505 are contained on approximately 1.6 kb DNA fragments. The most prominent features of the nucleotide sequences of these fragments are a repeated sequence that could form a stem-and-loop structure and a large ORF (1311 nt), which are separated by around 250 nt (Fig. 1). Although the role of the repeated sequence is currently unknown, it is possible that it plays an important role in replication since it is well-conserved in the two replication regions, and similar putative stem-and-loop structures are essential elements of many other replicons (del Solar *et al.*, 1998). The nucleotide sequences of the putative ORFs (ORF-*Pto* and ORF-*Pph*) are very similar to the sequence of the corresponding replication protein of plasmid pTiK12 from *T. intermedius*. We do not currently have a satisfactory explanation for this fact, since although it could suggest the horizontal exchange of replicons between the two genera, *T. intermedius* is a chemolithotroph that does not colonize the same niches as *P. syringae* (Holt *et al.*, 1994). Also, the deduced polypeptides of the two ORFs are highly similar to the replication proteins (RepA) found in the origins of replication of other plasmids (Figs 2, 4). The synthesis of polypeptides of the expected size from these regions in an *in vivo* system, abolition of the replication phenotype by transposon insertions on the putative RepA coding region from pPT23A, and the high homology of the deduced polypeptides with characterized RepA proteins from other plasmids further support the idea that ORF-*Pto* and ORF-*Pph* indeed encode replication proteins. It is interesting to note that these two putative RepA proteins are more than 100 aa larger than any other homologous RepA.

The proteins deduced from ORF-*Pto* and ORF-*Pph* are nearly identical from residues 1 to 373 in their N-terminal ends (96.5% identity), but are different from positions 374 to 437 in their C-termini (50% identity) (Fig. 2). The lower conservation of the C-terminal part of the putative RepA proteins also seems to occur among other pPT23A-like plasmids, since a DNA probe corresponding to the 3' half of ORF-*Pto* did not hybridize to all the plasmids that showed hybridization to the rest of *oriV*-pPT23A (Murillo & Keen, 1994). These differences in the C-terminal parts of the putative RepA proteins suggest that this end might be involved in specific recognition of the origin. Furthermore, a plasmid clone containing *oriV*-pPT23A with a modified ORF-*Pto* lacking the wild-type DNA for the last 21 residues (pAori23) is still able to replicate, but shows an altered incompatibility phenotype (Fig. 5). It has been suggested that the C-terminal regions in RepA proteins of the ColE2-related plasmids, which are highly related to the pPT23A-family (Fig. 4), play an important role in

their interaction with the origin and, thus, in incompatibility (Hiraga *et al.*, 1994).

pAKC contains at least three regions, designated IncA, IncB and IncC, that show a strong incompatibility with pPT23A (Fig. 5). In general, the determinants that express strong incompatibility are those involved in replication, partition, determination of copy number and stabilization through killing (Novick, 1987; Austin & Nordström, 1990; Nordström, 1993). As expected, the replication region corresponded to one of the identified Inc determinants, IncB. Although we were unable to further define the location of the determinant inside the minimal region required for replication, it is likely to lie at its 3' end since pAori23 showed an altered incompatibility phenotype (Fig. 5). The size of IncA (0.4 kb) and its position in the immediate vicinity of the minimal replicating region (Nordström, 1993) suggests that it could be involved in copy number control. Partition systems usually consist of two loci and a *cis*-acting partition site, which generally acts as a strong incompatibility determinant (Nordström & Austin, 1989). IncC was shown to be included in a 0.8 kb *EcoRI*-*KpnI* fragment. Taking into account its size and that pAKC is rapidly lost upon subculturing in non-selective media (Murillo & Keen, 1994), it is possible that the defined IncC includes a *cis*-acting partition site and that pAKC does not contain a complete *par* system. pAV505 and many other pPT23A-like plasmids did not cross-hybridize with the 0.8 kb *EcoRI*-*KpnI* fragment containing IncC (Sesma *et al.*, 1998), which suggests that this region is poorly conserved in *P. syringae*.

Partial sequencing around *oriV*-pPT23A disclosed the presence of a putative operon involved in UV resistance, termed *ruAB* (Sundin *et al.*, 1996), located immediately after ORF-*Pto* (Fig. 1a). Since *ruAB* genes are present in many pPT23A-like plasmids (Sundin & Bender, 1996; Sesma *et al.*, 1998), it is possible that genes for UV resistance could contribute to the fitness of this plasmid family, and perhaps favour the accumulation of several of its members in a given cell.

Wood *et al.* (1994) described a gene library cosmid, pPPY40, and showed that an avirulence phenotype, which matched a resistance gene in pea, required two regions, I and II, as shown by subcloning and transposon mutagenesis. Subsequently, it was shown that cosmids with Tn3Gus insertions in region II were very unstable in *P. syringae* pv. *ptisi*, with only 1–2% of cells retaining the cosmid; this contrasted with 95% stability over a 7 d period for cells carrying pPPY40 and 30% stability for the parent cosmid, pLAFR3 (M. J. Gibbon & A. Vivian, unpublished). This suggested that region II was involved in the stable maintenance of pPPY40 in *P. syringae* pv. *ptisi* and probably accounts for its apparent involvement in the avirulence phenotype.

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