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Closely Related Plasmid Replicons Coexisting in the Phytopathogen *Pseudomonas syringae* Show a Mosaic Organization of the Replication Region and Altered Incompatibility Behavior

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Many *Pseudomonas syringae* strains contain native plasmids that are important for host-pathogen interactions, and most of them contain several coexisting plasmids (pPT23A-like plasmids) that cross-hybridize to replication sequences from pPT23A, which also carries a gene cluster coding for the phytotoxin coronatine in *P. syringae* pv. tomato PT23. In this study, three functional pPT23A-like replicons were cloned from *P. syringae* pv. glycinea race 6, suggesting that the compatibility of highly related replicons is a common feature of *P. syringae* strains. Hybridization experiments using three separate incompatibility determinants previously identified from pPT23A and the *rulAB* (UV radiation tolerance) genes showed that the organization of the replication region among pPT23A-like plasmids from several *P. syringae* pathovars is poorly conserved. The putative *repA* gene from four pPT23A-like replicons from *P. syringae* pv. glycinea race 6 was amplified by using specific primers. The restriction profiles of the resulting PCR products for the race 6 plasmids were more similar to each other than they were to that of pPT23A. These data, together with the existence of other cross-hybridizing DNA regions around the replicon among the race 6 pPT23A-like plasmids, suggest that some of these plasmids may have originated from duplication events. Our results also imply that modifications of the *repA* sequences and the poor conservation of putative maintenance determinants contribute to the suppression of incompatibility among members of the pPT23A-like family, thus enhancing the genomic plasticity of *P. syringae*.

Strains of the phytopathogenic bacterium *Pseudomonas syringae* cause economically important losses to crop productivity by inciting disease and, in some cases, by catalyzing frost injury. Isolates of this species are grouped in 46 pathovars depending on the host range. Most *P. syringae* strains contain one or more indigenous plasmids of variable size (1 to >100 kb), some of which are known to be conjugative (10, 29). In many cases, these plasmids are important or essential for host-pathogen interactions, since they contain genes involved in pathogenicity (21), biosynthesis of extracellular virulence factors such as ethylene, indoleacetic acid, and the phytotoxin coronatine (3, 8, 16, 24), determination of host range by avirulence genes (33), competitive fitness (30), resistance to antibacterial compounds, such as copper, streptomycin, and trimethoprim (9), and/or resistance to UV radiation (32). An interesting facet of *P. syringae* plasmid biology is the existence of repeated sequences or areas of homology among different native plasmids of a given strain, which, in the best characterized case of plasmids pPT23A (100 kb) and pPT23B (83 kb) from *P. syringae* pv. tomato PT23, account for an estimated 74% of their total DNA (23).

A 9.2-kb *Kpn*I fragment from pPT23A that could autonomously replicate in *P. syringae* has been previously cloned and characterized, and the minimal region that retained the capacity to replicate (*oriV*-pPT23A) has been defined to a 1.6-kb fragment (23). Hybridization experiments have shown that *oriV*-pPT23A is highly conserved in pPT23B and that as many as six plasmids in a given *P. syringae* strain cross-hybridize with these sequences. Due to the possibility that they originate from a common ancestor, the name pPT23A-like was proposed to designate collectively the family of plasmids that show cross-hybridization with *oriV*-pPT23A (13). The nucleotide sequence of *oriV*-pPT23A (13) has been shown to contain a gene, *repA*, that codes for a protein essential for replication which is highly similar to the replication proteins of pTiK12, from *Thiobacillus intermedius*, and ColE2-like plasmids, among others. The identification of other maintenance determinants of pPT23A was based on the fact that most of them are able to displace their parent plasmids when both are present in the same cell and selection is applied for the cloned determinant. In this way, it was shown that pAKC contains three determinants (IncA, IncB, and IncC) that exert a strong incompatibility with pPT23A: IncA is represented by a 400-bp *Pst*I-*Eco*RI fragment upstream of *oriV*-pPT23A, IncB overlaps *oriV*-pPT23A, and IncC is included in a 0.8-kb *Eco*RI-*Kpn*I fragment located at one end of pAKC. The defined IncC is part of a putative partition system that increases the stability of the cloned *oriV*-pPT23A (6), while the functional significance of the IncA determinant is currently unknown. Partial sequencing has also shown that an operon involved in resistance to UV radiation (*rulAB* genes), which could be conferring a selective advantage for the maintenance of pPT23A, is located immediately downstream of *oriV*-pPT23A and preceding IncC.

It is generally accepted that plasmids that contain related sequences functioning in replication or partition cannot be

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stably maintained in growing populations of bacteria due to incompatibility effects (1, 25, 26). By contrast, the homology of *oriV*-pPT23A sequences among coexisting plasmids within *P. syringae* represents a phenomenon that has not been described for other organisms. The RepA protein of the related ColE2 plasmids functions in *trans* as an activator of replication by binding to the origin sequence in a plasmid-specific manner and synthesizing a primer RNA for initiation of DNA synthesis by DNA polymerase I at the origin (14). Thus, opportunities for the evolution of compatibility among pPT23A-like plasmids having highly homologous RepA proteins may involve sequence divergence within respective *repA* and *ori* sequences maintaining a plasmid-specific method of binding. Alternatively, the observed compatibility could be dependent on the absence or modification of other Inc determinants, such as IncA and IncC. We are interested in identifying the genetic and biochemical mechanisms enabling such related plasmids to coexist and also in determining the effect of homology of *oriV*-pPT23A sequences on the horizontal exchange of plasmids among *P. syringae* strains. In this study, we examined the compatibility of native pPT23A-like plasmids from *P. syringae* pathovars with pPT23A and determined the distribution of specific Inc sequences among these plasmids. To further analyze the compatibility phenomenon of coexisting pPT23A-like plasmids, we cloned and studied the functionality and sequence divergence of the *oriV* sequences from three pPT23A-like plasmids from *P. syringae* pv. glycinea race 6.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *Escherichia coli* DH5α (Life Technologies, Inc., Gaithersburg, Md.) was used for cloning procedures. *P. syringae* pathovars tomato (strains PT17, PT23, PT30 [2], and B120), apii (strain 1089-5), mori (strain 0782-30), morsprunorum (strain 0782-28), and savastanoi (strain 0485-9) were obtained from D. Cooksey (University of California at Riverside). *P. syringae* pathovars tomato (strain DC3000) (12) and glycinea (races 4, 5, and 6) (18) were obtained from N. T. Keen (University of California at Riverside); and *P. syringae* pathovars pisi (strain PN8) (22) and phaseolicola (strain 1302A) were from J. D. Taylor (HRI, Wellesbourne, United Kingdom). The plasmid content of some of these strains has been reported earlier (23). Native plasmids were designated by using an acronym derived from the strain name and a letter in alphabetical order starting from the largest plasmid. *P. syringae* pv. syringae FF5 is a plasmidless strain that shows a high efficiency of electroporation (31).

Plasmids pBluescript SK^+ (Stratagene, La Jolla, Calif.) and pMTL24 (7), which contains a symmetric polylinker, were used for cloning purposes. Plasmid pK184 (Km^r) (15), which does not replicate in *Pseudomonas*, was utilized for testing the functionality of putative origins of replication. pAKC contains a 9.2-kb *Kpn*I fragment from the largest native plasmid of strain PT23 (pPT23A) cloned in pK184 and can replicate autonomously in *P. syringae* (23).

E. coli was grown in Luria broth (LB) medium at 37°C, and *P. syringae* was cultivated in King's medium B (KMB) (19) or in LB medium at 28°C. When necessary, media were supplemented with the following antibiotics at the indicated concentrations: ampicillin, 100 μg/ml; carbenicillin, 100 μg/ml; and kanamycin, 25 μg/ml.

Genetic and molecular biology techniques. Standard molecular biology techniques were used (27). Plasmid DNA minipreparations were prepared from 1.5 ml of an overnight culture in KMB by using a modified alkaline lysis procedure (34). In some instances, plasmids were purified by isopycnic centrifugation in CsCl (27). Intact plasmid DNA was separated by electrophoresis on 0.6% agarose (Pronadisa; Hispanlab S.A., Madrid, Spain) gels in 1× Tris acetate-EDTA (TAE) buffer at 3.2 V/cm for 5 to 6 h at room temperature or 16 to 17 h at 4°C. Plasmids were introduced into *Pseudomonas* by electroporation (17). Cloning of DNA fragments was done essentially as described previously (11).

Amplification of DNA by the PCR was performed with primers RE1.1 (5′-A GTGACGACAAAACCGC-3′) and M553 (5′-GAGAATTCCGTGAGGATGT G-3′), which flank a 933-bp fragment of the *repA* gene from pPT23A corresponding to nucleotides 159 to 1093 of the coding region (13). Native plasmids used as templates for PCR were individually excised from agarose gels and purified by using 0.2-μm Nanosep MF columns as described in the manufacturer's instructions (Pall Filtron, Northborough, Mass.). Conditions for amplification were the following: 1.7 mM $MgCl_2$, 125 μM deoxynucleoside triphosphates, 0.4 μM each primer, and 0.05 U of *Taq* polymerase/μl (BioTaq; Bioline United Kingdom Ltd., London, United Kingdom). The amplification was done on a Linus Autocycler Plus (Corbett Research, Sydney, Australia) with a cycle of 94°C for 2 min, followed by 30 cycles of 1 min per step at 94, 50, and 72°C, and a final extension step of 10 min at 72°C. PCR products digested with *Hae*III were separated on 3% MS8 agarose (Hispanlab S.A.) gels in 1× TAE buffer.

Fragments to be used as probes were cloned separately in pBluescript SK^+ or pK184, excised from the vector, and separated in low-melting-point agarose before being labeled with [α-^{32}P]dCTP (27). DNA separated by electrophoresis and transferred to nylon membranes (Hybond-N+; Amersham) was hybridized at 42°C in 5× SSC (1× SSC is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7])–50% formamide–20 mM $NaPO_4$ (pH 7.0)–1× Denhardt's solution–0.1 mg of herring-sperm DNA ml^{-1} (20). After hybridization, the blots were washed twice, 15 min each, at 42°C in 2× SSC–0.1% sodium dodecyl sulfate and exposed while damp to X-ray film (Biomax; Kodak, Rochester, N.Y.) for 3 to 48 h at −80°C with intensifying screens. When necessary, DNA fragments were labeled with digoxigenin and used as hybridization probes as described in the manufacturer's instructions (Boehringer GmbH, Mannheim, Germany).

Incompatibility assays. The incompatibility between pAKC and related plasmids was assayed by a qualitative test essentially as described previously (25). Briefly, plasmid pAKC was introduced by electroporation, and 3 to 14 transformants for each strain resulting from at least three independent electroporations were selected in KMB plus kanamycin. To evaluate a possible partial incompatibility of a native plasmid with pAKC, isolated colonies of each transformant were serially transferred in KMB plus kanamycin up to four times. The plasmid profile of the clones purified from the selection plate and after the fourth transfer in selective media was examined.

Cloning of origins of replication from native plasmids. Total plasmid DNA from *P. syringae* pv. glycinea race 6 purified by CsCl gradient centrifugation was digested to completion with *Kpn*I and ligated en masse to the *E. coli* vector pK184, which cannot replicate in *Pseudomonas* (15). *Kpn*I cuts *P. syringae* DNA at a low frequency, and previous experiments showed that the homology with *oriV*-pPT23A was located in *Kpn*I fragments larger than 7 kb (data not shown). The ligation mixture was purified by phenol-chloroform extraction (27) and resuspended in sterile distilled water, and aliquots were transformed in *P. syringae* pv. apii 1089-5, which shows a high frequency of electroporation. Plasmid DNA was extracted from transformants growing in KMB plus kanamycin and transformed into DH5α, to analyze the cloned inserts by restriction digestion, and into the plasmidless strain *P. syringae* pv. syringae FF5, to confirm the autoreplicative ability of the recombinant plasmids. We confirmed that the cloned inserts had arisen from race 6 plasmid DNA by using the cloned inserts, or internal fragments thereof, as hybridization probes against double digestions of the corresponding DNAs and comparing the hybridization patterns (see Fig. 3; also data not shown).

RESULTS

Incompatibility of pAKC with native plasmids from several *P. syringae* strains. The presence of closely related plasmids among *P. syringae* strains could limit the potential for horizontal spread. To obtain a measure of the relatedness of plasmids among a range of *P. syringae* strains, pAKC was introduced by electroporation and the resulting transformants were examined to observe the extent of plasmid curing resulting from incompatibility.

The strains tested contained one to six plasmids with homology to *oriV*-pPT23A (Fig. 1 and 2). The Km^r transformants contained pAKC and, in most cases, one or two novel bands of different intensities that showed homology to pAKC (Fig. 1; also data not shown): this was probably due to the formation of multimers of the incoming plasmid. All 14 transformants obtained from *P. syringae* pv. glycinea race 4 lost plasmids pR4C, pR4D, and pR4F, two of which (pR4C and pR4F) cross-hybridized to *oriV*-pPT23A. As expected, since pAKC contains *oriV*-pPT23A, the pPT23A plasmid was evicted from all of the PT23 transformants (Fig. 1). In 5 of 14 transformants analyzed, pPT23B was also evicted, which could be due to the fact that pPT23A and pPT23B have highly homologous origins of replication (23). A plasmid with a size similar to that of pPT23A that cross-hybridizes with *oriV*-pPT23A (Fig. 2) was also evicted in all cases from *P. syringae* pv. tomato PT17 and PT30. Strains PT17, PT23, and PT30 were all isolated in California, display very similar plasmid profiles, and are probably variants of the same strain that differ only in their plasmid content (2). Immediate eviction of the incompatible plasmids was observed, and in all of the cases described above, plasmids were already absent from colonies cultured directly from the selec-

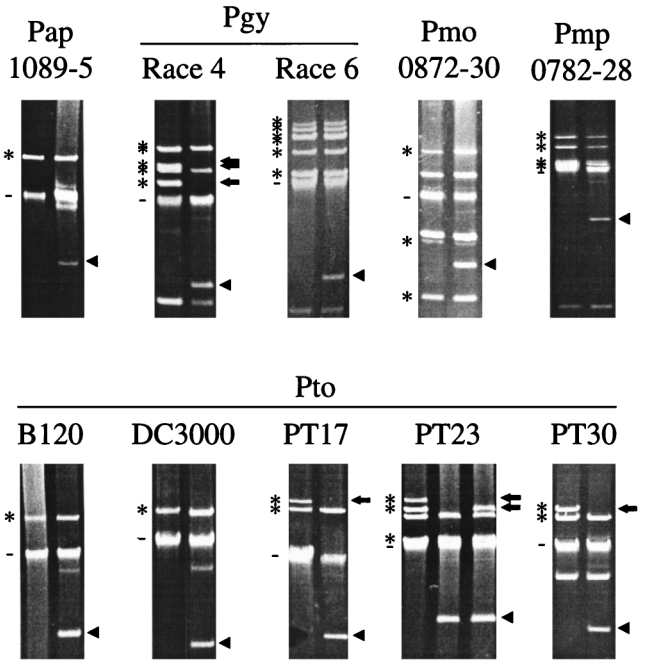

FIG. 1. Incompatibility between pAKC and related native plasmids from *P. syringae* strains. Panels show plasmid profiles of each strain before (left lanes) and after (right lanes) introduction of pAKC (arrowhead). Asterisks indicate plasmids hybridizing to the minimal origin of replication from pPT23A (see Fig. 2), and arrows indicate evicted plasmids. Abbreviations for *P. syringae* pathovars: Pap, apii; Pgy, glycinea; Pmo, mori; Pmp, morsprunorum; Pto, tomato. Short horizontal lines indicate the positions of chromosomal and/or linear plasmid DNA. Gels were scanned (Umax Vista-S6) and labeled by using PowerPoint and a PC Compaq 575e.

tion plates. With the remaining strains (*P. syringae* pathovars apii 1089-5, glycinea race 6, mori 0782-30, morsprunorum 0782-28, and tomato B120 and DC3000), we could not observe specific curing of any of the native plasmids, even after four successive transfers in KMB plus kanamycin.

Conservation of incompatibility determinants and the *rulAB* genes among *P. syringae* native plasmids. pAKC contains three regions that demonstrate strong incompatibility with pPT23A, one of which (IncB) includes the minimal fragment able to replicate autonomously (13). The observed lack of incompatibility between pAKC and other plasmids of the pPT23A-like family suggest that plasmids related to pPT23A could have undergone evolutionary changes in the three Inc regions that allow for their stable coexistence. We therefore analyzed by hybridization the conservation of the determinants linked to *oriV*-pPT23A among plasmids of the 10 strains included in the earlier incompatibility assay (Fig. 1) plus 4 other *P. syringae* strains. We used DNA fragments from the three defined Inc regions and from the *rulAB* operon, which is located adjacent to *oriV*-pPT23A (13) (see Fig. 2), as hybridization probes.

All of the strains analyzed were shown to contain one to six plasmids with homology to the IncB probe (Fig. 2). Based on the intensity of the hybridization signals, IncA-hybridizing sequences were detected in three plasmids in *P. syringae* pv. glycinea race 6 and in two plasmids in *P. syringae* pv. tomato PT17, PT23, and PT30, while *rulAB*-hybridizing sequences were detected in three plasmids in *P. syringae* pv. glycinea race 4. In contrast to the situation regarding IncB, the IncA and *rulAB* probes showed strong hybridization with only one plasmid in five and nine of the strains, respectively, although a few of them also contained one to two plasmids with weak hybridization to the probes. Only five of the strains contained a plasmid that hybridized to the IncC probe, despite the fact that they contained another plasmid(s) of the pPT23A-like family. In all cases, hybridization to the IncA, IncC, or *rulAB* probe was associated only with plasmids that also hybridized to the IncB probe. These results suggest that except for the conservation of IncB, the replication region among plasmids of the pPT23A-like family varies. This is further supported by the fact that only one plasmid in strains *P. syringae* pv. glycinea race 4 and *P. syringae* pv. tomato PT17, PT23, and PT30 cross-hybridized to all four probes.

Cloning of origins of replication from *P. syringae* pv. glycinea race 6. *P. syringae* pv. apii 1089-5, used as a host for cloned origins of replication, contains a single plasmid of 80

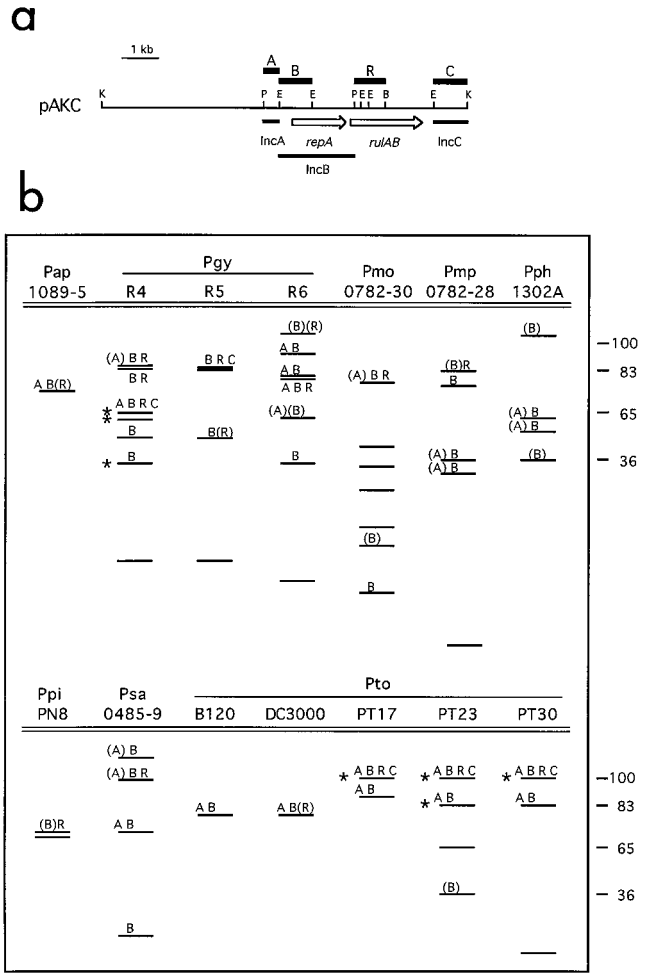

FIG. 2. Conservation of incompatibility determinants and *rulAB* genes in different *P. syringae* pathovars. (a) Structure of the 9.2-kb *Kpn*I insert of pAKC. Arrows indicate the approximate locations of the *rulAB* genes, as defined by partial sequencing, and the gene for the putative replication protein (*repA*). Thick lines under the map show the extent of the incompatibility regions. Solid rectangles labeled A, B, R, and C indicate the restriction fragments used as specific probes for IncA, IncB (minimal origin of replication), *rulAB*, and IncC, respectively. B, *Bam*HI; E, *Eco*RI; K, *Kpn*I; P, *Pst*I. (b) Diagram showing the plasmid profiles of different *P. syringae* strains. Letters above plasmid bands indicate strong or weak (in brackets) cross-hybridization with the probes labeled as shown in panel a. Asterisks indicate plasmids evicted by pAKC in the incompatibility assay (see Fig. 1). Abbreviations for *P. syringae* pathovars: Pap, apii; Pgy, glycinea; Pmo, mori; Pmp, morsprunorum; Pph, phaseolicola; Ppi, pisi; Psv, savastanoi; Pto, tomato. Numbers on the right indicate in kilobases the sizes of the plasmids from strain PT23.

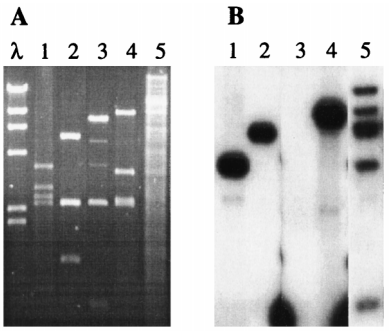

FIG. 3. Hybridization of the cloned origins of replication from *P. syringae* pv. glycinea race 6 with *oriV*-pPT23A. (A) DNAs from pORI601 (lane 1), pORI602 (lane 2), pORI604 (lane 3), and pORI603 (lane 4), and total plasmid DNA from *P. syringae* pv. glycinea race 6 (lane 5) were digested with *Kpn*I and *Eco*RI and separated on an agarose gel. (B) A gel similar to the one shown in panel A was subjected to Southern blot analysis by using a 0.8-kb *Eco*RI fragment from pAKC as a specific *oriV* probe from pPT23A. Lane λ, lambda DNA digested with *Hin*dIII. The gel and autoradiogram were scanned (Umax Vista-S6) and labeled by using PowerPoint and a PC Compaq 575e.

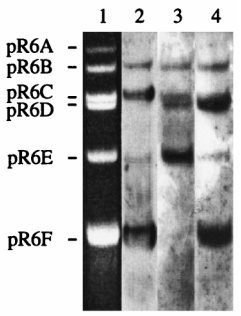

FIG. 4. Assignment of cloned pPT23A-like origins of replication to native plasmids in *P. syringae* pv. glycinea race 6. Native plasmids from *P. syringae* pv. glycinea race 6 were separated on an agarose gel (lane 1) and hybridized by using as probes a 0.8-kb *Eco*RI fragment from pORI601 (lane 2), a 0.8-kb *Hin*dIII fragment from pORI602 (lane 3), or a 0.6-kb *Eco*RI-*Hin*dIII fragment from pORI603 (lane 4). Autoradiograms are overexposed to show weaker hybridization. Plasmid pR6G did not cross-hybridize to any of the probes and was not included to reduce the size of the figure. The gel and autoradiograms were scanned (Umax Vista-S6) and labeled by using PowerPoint and a PC Compaq 575e.

kb (p1089A). Eleven Km^r transformants obtained contained p1089A and an additional plasmid of 11 to 18 kb (data not shown). Total plasmid DNA isolated from these colonies was individually transformed to *E. coli* DH5α and analyzed by restriction digestion. A total of four different plasmids, pORI601 to -604, were identified on the basis of their restriction profiles with *Kpn*I-*Eco*RI and *Kpn*I-*Hin*dIII (Fig. 3A; also data not shown). These plasmids were individually purified from *E. coli* and found to replicate autonomously in the plasmidless strain *P. syringae* pv. syringae FF5, which indicates that they contain a functional origin of replication. Plasmids pORI601, pORI602, and pORI603, but not pORI604, cross-hybridized with the 0.8-kb *Eco*RI fragment from pAKC (Fig. 3B). This fragment contains the first 528 nucleotides of the coding sequence of the putative RepA protein of *oriV*-pPT23A and 308 upstream nucleotides that are also required for replication (13, 23).

To identify the replication determinants in pORI601, -602, and -603, the plasmids were partially digested with *Sau*3AI and separated in a low-melting-point agarose gel and fragments 2 to 3 kb in size were cloned in pMTL24, producing plasmids pORI605, -606, and -607, respectively. These three plasmids contained approximately 3-kb inserts, replicated autonomously in *P. syringae* pv. syringae FF5, and cross-hybridized with the 0.8-kb *Eco*RI fragment from pAKC (data not shown). These results indicate that the DNA homologous to *oriV*-pPT23A in race 6 represents, at least in some cases, functional origins of replication.

Plasmids pORI601, -602, and -603 shared many cross-hybridizing restriction fragments besides the origin of replication (data not shown). In consequence, and to ascertain that the cloned origins of replication were derived from different native plasmids, we performed Southern hybridization analysis with selected non-cross-hybridizing restriction fragments from each plasmid as probes (Fig. 4). Probes derived from pORI602 and pORI603 hybridized strongly to plasmids pR6E and pR6D, respectively, confirming that they originated from different native plasmids. In longer exposures, the probe from pORI602 also hybridized to plasmids B, C, and D, and the probe from pORI603 hybridized to plasmids B, E, and F. The probe derived from plasmid pORI601, however, showed strong hybridization to both pR6C and pR6F and weak hybridization to pR6B and pR6E. These results indicate that DNA adjacent to the replication region is conserved among most of the pPT23A-like plasmids from *P. syringae* pv. glycinea race 6.

Plasmids pORI602 and pORI604 were introduced by electroporation in *P. syringae* pv. glycinea race 6. The results of four independent transformations with pORI602 led to the curing of pR6G (8 kb) in three cases and in one case of pR6E (70 kb), its parental plasmid. On the other hand, pORI604 caused the eviction of pR6G from the five transformants analyzed. Since pR6G is the only plasmid from *P. syringae* pv. glycinea race 6 that does not show homology to *oriV*-pPT23A, it is possible that pORI604 contains the origin of replication from this plasmid. Despite numerous attempts, we were unable to obtain transformants containing pORI601 or pORI603.

PCR amplification of pPT23A-like replication regions. To study the relatedness of the replication regions of pPT23A-like plasmids from *P. syringae* pv. glycinea race 6, we examined the restriction profile of PCR products specifically derived from them. Using primers RE1.1 and M553, we obtained an amplified product of ca. 900 bp in all cases. Reproducible *Hae*III restriction patterns were generated from the 900-bp PCR product obtained from plasmids pAKC, pORI601 and pORI603, and with the native plasmids pR6B, pR6C, and pR6F (Fig. 5).

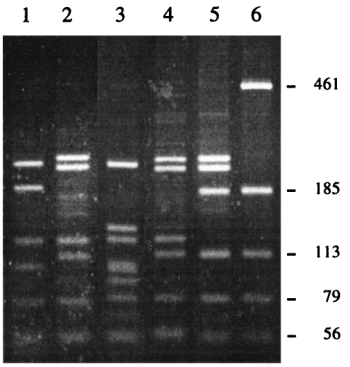

FIG. 5. Diversity of restriction patterns among replication origins related to *oriV*-pPT23A in *P. syringae* pv. glycinea race 6. PCR products digested with *Hae*III were separated on a 3% agarose gel. The primers flank a 933-bp fragment of the coding region of the putative replication protein of *oriV*-pPT23A. Lanes: 1, pR6B; 2, pR6C; 3, pR6F; 4, pORI601; 5, pORI603; 6, pAKC. Numbers on the right indicate the sizes in base pairs of the restriction fragments of pAKC. The gel was scanned (Umax Vista-S6) and labeled by using PowerPoint and a PC Compaq 575e.

All of the plasmids displayed differential restriction patterns, although the patterns of the plasmids from race 6 were more similar to each other than they were to the pattern of pAKC. Taking into account the fragment order in *oriV*-pPT23A, the differences in the restriction pattern of the amplified products could be explained by changes in at least five positions distributed along the entire sequence of this region. The comparison of the restriction patterns also suggests that pORI601 originated from pR6C. No specific amplification products were obtained with pR6A and pORI602, and no reproducible results were observed with pR6D and pR6E.

DISCUSSION

Many *P. syringae* strains contain two or more plasmids with homology to *oriV*-pPT23A (Fig. 2) (23), suggesting that they could have arisen by duplication of preexisting plasmids. The presence of cross-hybridizing origins of replication in up to six plasmids of the same cell could be explained if (i) the origins were no longer functional due to deletion or mutation, (ii) these plasmids contained another functional origin of replication, or (iii) the plasmids had evolved mechanisms that allow them to escape incompatibility. Three cloned origins derived from different plasmids from *P. syringae* pv. glycinea race 6 showed similarity to *oriV*-pPT23A (Fig. 3B), which suggests that, at least in some cases, this similarity identifies functional origins of replication. These origin sequences, however, have undergone modifications that could be responsible for their coexistence: first, only two of them hybridize strongly to the IncB probe and could be amplified with primers specific for the coding sequence for the RepA protein from pPT23A; second, the restriction pattern with *Hae*III is different among the PCR products of these two origins and that of pAKC (Fig. 5). The nucleotide sequences of the *repA* genes of pAV505, from *P. syringae* pv. phaseolicola 1302A, and of pPT23A (13), two pPT23A-like plasmids that are compatible, show ca. 89% identity. Base changes are mainly concentrated in the DNA upstream from the start codons and in the 3′ end of the RepA coding regions. The deletion of 64 nucleotides in this 3′ end in *repA* from pPT23A led to an altered incompatibility phenotype, which supports the idea that small changes in the *repA* sequence would maintain a high degree of homology within *repA* but also allow for coexistence.

The presence of two other strong incompatibility determinants close to *oriV*-pPT23A suggests that these regions should also be modified or absent in coexisting pPT23A-like plasmids to account for the observed compatibility. In hybridization experiments using specific probes for IncA, IncB, IncC, and *rulAB* (Fig. 2), most of the plasmids examined hybridized only to IncB. The hybridization to IncA, IncC, or *rulAB*, when present, was in all cases associated with plasmids hybridizing to the IncB probe. Most of the pPT23A-like replicons did not contain IncC, and in no case did we observe hybridization to this probe in more than one plasmid of the same cell. Since the cloned IncC determinant displays strong incompatibility with pPT23A, its parent plasmid, it is tempting to speculate that this determinant was lost by deletion during the evolution of coexisting pPT23A-like replicons. Although also poorly conserved, IncA is in some cases repeated in up to three plasmids of the same cell. In these cases, some of the copies could no longer be functional or might have undergone specificity changes by mutation. In this respect, pPT23B hybridizes to IncA (Fig. 2), but the sequencing of *oriV*-pPT23B (6) showed that IncA is not located in its vicinity, as it is in *oriV*-pPT23A. Taking into account that pPT23B does not hybridize to IncC or *rulAB*, this suggests that the replication region in this plasmid, and probably in other pPT23A-like plasmids, may have undergone major reorganization events.

The existence of highly related replicons in different *P. syringae* strains could limit the horizontal transfer of plasmids among them. The acquisition of pAKC, which contains three incompatibility determinants, did not result in most cases in the eviction of any native plasmid from the 10 strains analyzed, suggesting that the incompatibility determinants in pAKC are not functionally conserved among plasmids of the pPT23A-like family. Also, this situation could be explained in terms of partial or complete autocompatibility, such as that observed with *oriV*-pPT23B (23) and pORI602. As expected, pAKC led to the eviction from PT23 of pPT23A, its parent plasmid. A plasmid of similar size was also evicted from the related strains PT17 and PT30 but not from other *P. syringae* pv. tomato strains. On some occasions, pPT23B was also evicted, despite the fact that this plasmid is stably maintained with pPT23A. It is possible that the eviction of pPT23B and of three plasmids from *P. syringae* pv. glycinea race 4 was caused by a shift in the balance of *repA* and *ori* sequences due to copy number effects of the pAKC clone.

Taken together, these data suggest that several origins of replication of the pPT23A-like family could coexist in the same cell due to base changes that could alter their specificity plus the incorporation, elimination, and/or functional modification of other strong incompatibility determinants, like IncA or IncC. For example, each of four of the pPT23A-like plasmids from *P. syringae* pv. glycinea race 6 showed a differential hybridization pattern with the Inc probes (Fig. 2) but exhibited *Hae*III digestion patterns of their respective *repA*-PCR fragments which were more similar to each other than to that of pAKC (Fig. 5). This, together with the fact that they share a large amount of repeated DNA, suggests that race 6 plasmids may have originated from a common ancestor and not as a result of horizontal transfer. However, a detailed analysis of pPT23A-like replicons must be performed before the possibility of horizontal transfer can be eliminated. An alternative to explain the coexistence of pPT23A-like plasmids is that some or all of them could contain another functional origin of replication which could alleviate or suppress the incompatibility. We recently determined that plasmids p485C and p485D, from *P. syringae* pv. savastanoi 0485-9, which hybridized with *oriV*-pPT23A (Fig. 2), also hybridize with the unrelated origin of replication cloned in pORI604 (28). This is the first evidence that some *P. syringae* native plasmids can indeed contain more than one origin of replication.

The widespread occurrence of pPT23A-like plasmids among *P. syringae* pathovars is an indication of the evolutionary success of this plasmid family and implies that these plasmids encode determinants of importance to the *P. syringae* life cycle. The study of the replication determinants of pPT23A-like plasmids is of importance in (i) providing molecular tools for their eviction from the host cell, and thus for the evaluation of the role of individual plasmids in pathogenicity or virulence; (ii) providing insights into the mechanisms that allow *Pseudomonas* strains to obtain a greater genomic plasticity; and (iii) yielding information on plasmid speciation and the evolution of new incompatibility groups within a plasmid population. Several determinants involved in pathogenicity, virulence, avirulence, resistance to antibacterial compounds or UV light, and competitive fitness have been located to plasmids in different *P. syringae* strains. The exchange of this information is probably occurring in nature, since the conjugative transfer of plasmids among *P. syringae* strains has been demonstrated in vitro as well as in planta (2, 4, 5). Additionally, it has been shown that several native plasmids in this species can mobilize

chromosomal genes through integration and imperfect excision. The horizontal exchange of genetic information could be, however, hampered by the preexisting incompatibility among highly related replicons in *P. syringae*. Our results show that modifications of the *repA* sequences and the poor conservation of putative maintenance determinants could be contributing to the reduction or suppression of the incompatibility among members of the pPT23A-like family and thus enhancing the genomic plasticity of this species.

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