Phylogeny of the replication regions of pPT23A-like plasmids from *Pseudomonas syringae*

Ane Sesma,1 George W. Sundin2 and Jesús Murillo1

Author for correspondence: Jesús Murillo. Tel: +34 948 169133. Fax: +34 948 169732. e-mail: jesus@unavarra.es

It was previously shown that most *Pseudomonas syringae* strains contain one or more plasmids with cross-hybridizing replication regions and other areas of homology, and these plasmids were designated the pPT23A-like family. The majority of these plasmids encode genes conferring epiphytic fitness or resistance to antibacterial compounds and those investigated in this study are essential for pathogenicity or increased virulence. The phylogeny of 14 pPT23A-like plasmids from five *P. syringae* pathovars was studied by comparing a fragment of the sequence of their *repA* genes (encoding a replicase essential for replication). In the phylogenetic tree obtained, four groups (≥88.8% identity between their members) could be identified. The first group contained the plasmids from three *P. syringae* pv. *tomato* strains, a *P. syringae* pv. *apii* strain and five out of the seven *P. syringae* pv. *syringae* strains, with identity ranging between 88-8 and 100%. The clustering of the pv. *syringae* strains did not reflect host specialization or previously reported phylogenetic relationships. The second group contained the plasmids from two strains of pv. *glycinea* and pv. *tomato* (95-5% identity), and it also included the previously sequenced replicon of a pathogenicity plasmid from *P. syringae* pv. *phaseolicola*. The plasmids from the remaining two pv. *syringae* strains were distantly related to the other plasmid sequences. Hybridization experiments using different genes or transposable elements previously described as plasmid-borne in *P. syringae*, showed that the gene content of highly related plasmids could be dissimilar, suggesting the occurrence of major plasmid reorganizations. Additionally, the phylogeny of the different native plasmids did not always correlate with the phylogeny of their harbouring strains, as determined by the analysis of extragenic repetitive consensus (ERIC) and arbitrarily primed PCR (AP-PCR) products. Collectively, these results suggest that pPT23A-like plasmids were, in most cases, acquired early during evolution.

Keywords: pathogenicity and virulence plasmids, avirulence gene *avrD*, repeated DNA, *rulAB*, ultraviolet light resistance genes

INTRODUCTION

The phytopathogenic bacterial species *Pseudomonas syringae* is genetically diverse and has traditionally been subdivided into groups of pathovars according to host range (Young et al., 1996). A recent analysis of DNA reassociation rates has allowed the separation of *P. syringae* into nine genomospecies which could contain one to several pathovars with non-overlapping host ranges (Gardan et al., 1999). Likewise, the results of other studies have indicated that the pathovar distinction based on host-range data does not always correlate well with other molecular, biochemical and physiological typing methods (Young et al., 1992; Saunier et al., 1996; Sawada et al., 1999).

Little is known about the specific genetic determinants that affect host range in *P. syringae* or other necro-
genic bacteria, although it is becoming clear that host specificity is defined by the coordinated action of ‘positive’ factors and the antagonistic action of ‘negative’ factors. The negative factors, or avirulence (avr) genes, are the best known and act by restricting the plant cultivars or species that can be infected by a given isolate (Vivian et al., 1997). The positive factors would include determinants that govern the ability to produce disease or increase virulence in a given plant host. In P. syringae, phytoxins and growth regulators generally increase the aggressiveness of the bacterium towards a plant host. However, there is growing evidence to suggest that avr genes could also be the main positive factors determining virulence and/or pathogenicity (Kearney & Staskawicz, 1990; Sparup et al., 1991; Lorang et al., 1994; Ritter & Dangl, 1995; Yang et al., 1996; Jackson et al., 1999). For example, the ability of P. syringae pv. phaseolicola 1449B to infect beans and soybean is conferred by a plasmid-encoded pathogenicity island of about 30 kb which contains three avr genes and four other ORFs with the characteristics of avr genes (Jackson et al., 1999). One of the ORFs, designated virPpBA, confers the ability to infect beans and to cause a hypersensitive response in certain soybean cultivars (Jackson et al., 1999). Avirulence gene avrPpBF, also included within this pathogenicity island, determines the capacity to infect soybean and causes a specific gene-for-gene hypersensitive response on the bean cultivar Red Mexican (Tsiamis et al., 2000).

Many of the determinants involved in virulence and pathogenicity of P. syringae, including several that clearly influence host range, are encoded on native plasmids. Outstanding examples are the genes involved in the biosynthesis of the phytoxin coronatine (Sato, 1988; Bender et al., 1999), auxins (Comai & Kosuge, 1980; Glickmann et al., 1998) and avr genes (Vivian et al., 1997). In some cases, related genes or gene clusters are conserved in unrelated pathovars; examples of this include the coronatine biosynthetic cluster which is present in five pathovars (Mitchell, 1982; Wiebe & Campbell, 1993; Cuppels & Ainsworth, 1995) and avrD sequences which have been detected within a wide pathovar range (Yucel et al., 1994). However, the relationships among different native plasmids from P. syringae have not been studied extensively and authors have reported both similarity and dissimilarity among plasmids from a given pathovar (Curiale & Mills, 1983; Denny, 1988; King, 1989; Sundin et al., 1994). The possibility of plasmid transfer within natural populations via conjugation must also be considered in terms of the introduction of novel genes affecting host interactions into unrelated P. syringae pathovar strains.

The majority of the native plasmids identified in P. syringae belong to the recently described pPT23A-like family; these plasmids share replication sequences and, in most cases, additional areas of homology (Murillo & Keen, 1994; Sundin & Bender, 1996; Glickmann et al., 1998; Sesma et al., 1998; Gibbon et al., 1999). Furthermore, many P. syringae strains contain two to six coexisting pPT23A-like plasmids (Murillo & Keen, 1994; Sesma et al., 1998), suggesting their potential role in the bacterial life cycle and underlying their capacity to overcome incompatibility. In the best characterized case, that of pPT23A and pPT23B, both from P. syringae pv. tomato PT23, approximately 74% of these plasmids consist of repeated sequences (Murillo & Keen, 1994). We are interested in the evolution of the pPT23A-like plasmid family, in particular the molecular evolution of the plasmids and of the sequences they encode with emphasis on the host-selective forces resulting in the compartmentalization of specific plasmid genes within a limited pathovar range. The replication regions from two pPT23A-like plasmids contain a determinant, repA, that is highly homologous to the major replication gene of CoE2 replications, a plasmid group widespread in Escherichia coli and found in other members of the γ-Proteobacteria, and to a plasmid from Thiobacillus intermedius (Gibbon et al., 1999). In this study, we report an analysis of repA sequences as a strategy to examine the phylogeny of native P. syringae plasmids; the repA determinant is essential for replication of the pPT23A-like plasmids and thus is an appropriate locus for comparative study.

**METHODS**

**Bacterial strains, plasmids and growth conditions.** Escherichia coli DH10B was used for cloning purposes. The plasmidless Pseudomonas syringae pv. syringae FFS (Sundin & Bender, 1993) was used to test the replication ability of selected clones. Other P. syringae strains are listed in Table 1. E. coli was grown in LB medium at 37 °C and P. syringae was cultivated in King’s medium B (Hispanlab; King et al., 1954) or in LB at 28 °C. When necessary, media were supplemented with ampicillin or kanamycin at a final concentration of 100 or 25 µg ml⁻¹, respectively.

**Genetic and molecular biology techniques.** Standard molecular biology techniques were followed (Sambrook et al., 1989). Plasmid DNA minipreparations were performed using 1-5 ml of an overnight culture in King’s medium B following a modified alkaline lysis procedure (Zhou et al., 1990), and intact plasmids were separated by electrophoresis on 0.6% agarose (Hispanlab) gels in 1× TAE buffer (40 mM Tris/acetate, 1 mM EDTA, pH 8.0) at 3-2 V cm⁻¹ for 5-6 h at room temperature or for about 16 h at 4 °C (Murillo et al., 1994). Cloning of DNA fragments was done essentially as described by Crouse et al. (1983). DNA was introduced into Pseudomonas by electroporation (Keen et al., 1992).

Fragments to be used as probes were either amplified by PCR or separately cloned in pBluescript (Stratagene) or pK184 (Johnson & Holmes, 1990); in this case, they were excised from the vector and separated in low-melting-point agarose before labelling. Labelling of DNA with digoxigenin and hybridization of uncut plasmid DNA separated by electrophoresis and transferred to nylon membranes (Hybond N+; Amersham) were performed following the manufacturer’s instructions (Boehringer Mannheim).

**Cloning of origins of replication.** The possession of pPT23A-like plasmids among the strains included in this study either was already reported (Murillo & Keen, 1994; Sundin & Bender, 1996; Sesma et al., 1998; Sundin & Murillo, 1999) or was examined by hybridization using the 0.8 kb EcoRI fragment from the replication region of pPT23A (Gibbon et
Table 1. Origin and characteristics of the pPT23A-like plasmids used in this study

<table>
<thead>
<tr>
<th>P. syringae pathovar</th>
<th>Isolate</th>
<th>Host</th>
<th>A-like/total*</th>
<th>Designation</th>
<th>Size (kb)</th>
<th>Known genes</th>
<th>Origin or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>aspe 1089-5</td>
<td>Celer</td>
<td>1/1</td>
<td></td>
<td>p1089A</td>
<td>80</td>
<td>Pathogenicity island with pathogenicity gene avrPphA and other avr genes</td>
<td>Sesma et al. (1998)</td>
</tr>
<tr>
<td>glycinea race 6</td>
<td>Soybean</td>
<td>6/7</td>
<td>pR6C</td>
<td></td>
<td></td>
<td></td>
<td>Sesma et al. (1998)</td>
</tr>
<tr>
<td>Phaseolicola HR11302A</td>
<td>Bean</td>
<td>4/4</td>
<td>pAV505</td>
<td>150</td>
<td></td>
<td></td>
<td>Jackson et al. (1999)</td>
</tr>
<tr>
<td>syringae 5D425</td>
<td>Apricot</td>
<td>1/1</td>
<td>p5D425A</td>
<td>50</td>
<td></td>
<td></td>
<td>D. C. Gross, Washington State University, USA</td>
</tr>
<tr>
<td>B76 Tomato</td>
<td>1/1</td>
<td>pB76A</td>
<td>75</td>
<td></td>
<td></td>
<td></td>
<td>T. P. Denny, University of Georgia, USA</td>
</tr>
<tr>
<td>R86-17 Bean</td>
<td>1/1</td>
<td>pR8617A</td>
<td>75</td>
<td></td>
<td></td>
<td></td>
<td>D. E. Legard, University of Florida, USA</td>
</tr>
<tr>
<td>RBS32-5 Bean</td>
<td>1/1</td>
<td>pRBS325</td>
<td>75</td>
<td></td>
<td></td>
<td></td>
<td>D. E. Legard, University of Florida, USA</td>
</tr>
<tr>
<td>HS191 Milllet</td>
<td>1/1</td>
<td>pCG131</td>
<td>50</td>
<td></td>
<td></td>
<td></td>
<td>A. K. Vidaver, University of Nebraska, USA</td>
</tr>
<tr>
<td>EB48 Ornamental pear</td>
<td>1/1</td>
<td>pEB48</td>
<td>50</td>
<td>Cu² genes</td>
<td></td>
<td></td>
<td>Sunedin et al. (1994)</td>
</tr>
<tr>
<td>7B12 Ornamental pear</td>
<td>1/1</td>
<td>p7B12</td>
<td>70</td>
<td>Cu² and Sm² genes</td>
<td></td>
<td></td>
<td>Sunedin et al. (1994)</td>
</tr>
<tr>
<td>tomato B769</td>
<td>1/1</td>
<td>pB769</td>
<td>100</td>
<td>Coronation synthesis cluster</td>
<td></td>
<td></td>
<td>Sesma (2000)</td>
</tr>
<tr>
<td>tomato B769</td>
<td>1/1</td>
<td>pB769</td>
<td>100</td>
<td>Coronation synthesis cluster</td>
<td></td>
<td></td>
<td>Sesma et al. (1998)</td>
</tr>
<tr>
<td>tomato B769</td>
<td>2/2</td>
<td>pB769</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td>C. L. Rendel, Oklahoma State University, USA</td>
</tr>
<tr>
<td>DC3000 Tomato</td>
<td>1/1</td>
<td>pDC3000</td>
<td>75</td>
<td>Uncharacterized virulence genes†</td>
<td></td>
<td></td>
<td>Cappelli (1986)</td>
</tr>
</tbody>
</table>

* No. of pPT23A-like plasmids present in the strain/total number of plasmids.
† Derivative of P. syringae pv. tomato PT23 that has spontaneously lost plasmid pPT23C.
‡ A derivative of DC3000 cured of the native plasmid is less virulent in its plant host (G. Tsiamis, personal communication).

al., 1999) as probe. repA genes were amplified using primers 532 (5’-GAACGTTGACCTATG-3’) and 1588 (5’-CTCCTGCTGC-3’) which flank a fragment of 1399 bp containing 1279 bp of the repA coding region plus 120 bp upstream of the putative start codon (Gibbon et al., 1999). Total plasmid DNA was used as template for strains containing a single native plasmid and for P. syringae pv. tomato strain OK-1. The origins of replication from pPT23A, pR6C, and pAV505 (see Table 1 for details) were cloned in plasmids pAKC (Murillo & Keen, 1994), pORI601 (Sesma et al., 1998), and pAV505 (see Table 1 for details) which were cloned in plasmids pAKC (Murillo & Keen, 1994), pORI601 (Sesma et al., 1998), and pPPY30 (Gibbon et al., 1999), respectively, which were also used as templates for amplification. Amplifications were carried out in a total volume of 25 μl using 1 μl DNA as template under the following conditions: 4 mM MgCl₂, 75 mM each dNTPs, 1 pmol each primer 532 and 1588 (5’-GAACGGTGGACTTATGG-3’) and 1588 (5’-GAACGGTGGACTTATGG-3’), respectively, which were custom-synthesized primers based on the sequence of the minimal replication regions of plasmids pPT23A and pAV505 from P. syringae (Gibbon et al., 1999). Sequence comparison, amplification bands or bands resulting from digestion of the amplicons were separated by electrophoresis as indicated above and template DNA was purified from appropriate bands excised from the gels using 0.2 μm Nanosep MF columns ( Pall Filtron) as described previously (Sesma et al., 1998).

Analysis of nucleotide sequences. Plasmid DNA was purified using Qiagen columns. Nucleotide sequencing was done using the Big Dye kit (ABI) following the instructions of the manufacturer; sequence reactions were run at the Genetic Technologies Center, Texas A&M University. Both ends of the cloned amplicons were sequenced using universal and custom-synthesized primers based on the sequence of the pPT23A-like plasmid studied. For strains containing two or more pPT23A-like plasmids, these were separated by electrophoresis as indicated above and template DNA was purified from appropriate bands excised from the gels using 0.2 μm Nanosep MF columns (Pall Filtron) as described previously (Sesma et al., 1999). Sequence comparison, amplification bands or bands resulting from digestion of the amplicons were separated by electrophoresis as indicated above and template DNA was purified from appropriate bands excised from the gels using 0.2 μm Nanosep MF columns (Pall Filtron) as described previously (Sesma et al., 1998).

ERIC, arbitrarily primed PCR and data analysis. The genetic relationships among P. syringae strains was examined by PCR using primers for extragenic repetitive consensus (ERIC) and the arbitrarily primed PCR (AP-PCR) techniques. For ERIC analysis, primers ERIC1R and ERIC2 were used for amplification as described by McManus & Jones (1995). AP-PCR was carried out with a single 20 bp oligonucleotide primer complementary to the IS50 portion of Tn5 as described previously (Sundin & Murillo, 1999).

Amplification bands or bands resulting from digestion of amplification products from the origins of replication were scored as 1 (present) or 0 (absent), and a similarity matrix was computed using Dice’s coefficient with the program ntsys-pc.
RESULTS

Conservation of DNA sequences among pPT23A-like plasmids

pPT23A-like plasmids are those that contain sequences that hybridize to the origin of replication of plasmid pPT23A from Pseudomonas syringae pv. tomato PT23 (Gibbon et al., 1999). Although they can share extensive regions of homology (Murillo & Keen, 1994; Sundin et al., 1994; Sesma et al., 1998; Sundin & Murillo, 1999), little is known about particular genes that might be broadly conserved among them. To study the conservation of DNA sequences among 14 pPT23A-like plasmids originating from P. syringae pathovars avirulence gene probes (avirulence gene probes hybridized to all the tested plasmids. Five of the seven probes for sequences that have been previously detected on pPT23A or in the highly related plasmid pPT23B, also from strain PT23 (Table 2). None of the probes hybridized to all the tested plasmids. Five of the probes (avirulence gene avrD, genes involved in the synthesis of coronatine, plasmid stability determinants and the insertion sequence IS1240) hybridized only to 2–4 of the 14 analysed plasmids, indicating their poor conservation. On the other hand, the rulAB genes, which confer resistance to UV light (Sundin et al., 1996; Sundin & Murillo, 1999), hybridized to the native plasmids of three pv. tomato and the seven pv. syringae strains, corroborating its widespread distribution (Sundin et al., 1996; Sesma et al., 1998; Sundin & Murillo, 1999). The insertion sequence IS801, which was originally detected in a P. syringae pv. phaseolicola native plasmid (Romantschuk et al., 1991), was not present in the native plasmids from any of the P. syringae pv. syringae strains, while it hybridized to the plasmids from the remaining seven strains (Table 2). Only in a few cases (rulAB genes, IS1240, IS801 and stbCBAD genes) was hybridization to the probes in more than one pPT23A-like plasmid from the same strain observed (Table 3). For example, five of the pPT23A-like plasmids of P. syringae pv. glycinea race 6 hybridized to IS801, while three of them showed hybridization to the stbCBAD genes.

Phylogeny of the repA sequence of pPT23A-like plasmids

With the availability of the complete sequence of several plasmids from diverse bacterial species, it is apparent that plasmid genomes are mosaics of sequences that can be acquired from varied sources (for example, see Tauch et al., 1996; Sesma et al., 1998; Sundin & Murillo, 1999). Although they can share extensive regions of homology (Murillo & Keen, 1994; Sesma et al., 1998; Sundin & Murillo, 1999), little is known about particular genes that might be broadly conserved among them. To study the conservation of DNA sequences among 14 pPT23A-like plasmids originating from P. syringae pathovars avirulence gene probes (avirulence gene probes hybridized to all the tested plasmids. Five of the seven probes for sequences that have been previously detected on pPT23A or in the highly related plasmid pPT23B, also from strain PT23 (Table 2). None of the probes hybridized to all the tested plasmids. Five of the probes (avirulence gene avrD, genes involved in the synthesis of coronatine, plasmid stability determinants and the insertion sequence IS1240) hybridized only to 2–4 of the 14 analysed plasmids, indicating their poor conservation. On the other hand, the rulAB genes, which confer resistance to UV light (Sundin et al., 1996; Sundin & Murillo, 1999), hybridized to the native plasmids of three pv. tomato and the seven pv. syringae strains, corroborating its widespread distribution (Sundin et al., 1996; Sesma et al., 1998; Sundin & Murillo, 1999). The insertion sequence IS801, which was originally detected in a P. syringae pv. phaseolicola native plasmid (Romantschuk et al., 1991), was not present in the native plasmids from any of the P. syringae pv. syringae strains, while it hybridized to the plasmids from the remaining seven strains (Table 2). Only in a few cases (rulAB genes, IS1240, IS801 and stbCBAD genes) was hybridization to the probes in more than one pPT23A-like plasmid from the same strain observed (Table 3). For example, five of the pPT23A-like plasmids of P. syringae pv. glycinea race 6 hybridized to IS801, while three of them showed hybridization to the stbCBAD genes.

Table 2. Conservation of relevant genetic determinants in different pPT23A-like plasmids as determined by DNA hybridization

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Pathovar</th>
<th>Probe used*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>avrD</td>
</tr>
<tr>
<td>pPT23A</td>
<td>tomato</td>
<td>–</td>
</tr>
<tr>
<td>pOK1B</td>
<td>tomato</td>
<td>–</td>
</tr>
<tr>
<td>p1089A</td>
<td>api</td>
<td>+</td>
</tr>
<tr>
<td>pDC3000A</td>
<td>tomato</td>
<td>–</td>
</tr>
<tr>
<td>pSD425A</td>
<td>syringae</td>
<td>–</td>
</tr>
<tr>
<td>pB8617A</td>
<td>syringae</td>
<td>–</td>
</tr>
<tr>
<td>pBBS325A</td>
<td>syringae</td>
<td>–</td>
</tr>
<tr>
<td>pB76A</td>
<td>syringae</td>
<td>–</td>
</tr>
<tr>
<td>pISR11</td>
<td>syringae</td>
<td>–</td>
</tr>
<tr>
<td>pB120A</td>
<td>tomato</td>
<td>+</td>
</tr>
<tr>
<td>pR6C</td>
<td>glycinea</td>
<td>–</td>
</tr>
<tr>
<td>pAV505</td>
<td>phaseolicola</td>
<td>–</td>
</tr>
<tr>
<td>pPSR14</td>
<td>syringae</td>
<td>–</td>
</tr>
<tr>
<td>pCG131</td>
<td>syringae</td>
<td>–</td>
</tr>
</tbody>
</table>

*avrD, 480 bp HindIII–BglII fragment from the 5’ end of avrD from pPT23B (Kobayashi et al., 1990); cfa, ca 8 kb BamHI fragment from vRVC4 containing ORFs 5 and 6 from the coronatine acid biosynthesis region (Rangaswamy et al., 1998) which is essential for the synthesis of the phytotoxin coronatine; rulAB, internal 0.7 bp HindIII–PstI fragment of the UV-light resistance genes rulAB, isolated from pGWS140 (Sundin & Murillo, 1999); stbCBAD, 833 bp fragment containing the plasmid stability genes stbB and part of stbA, stbC and stbD (positions 4015–4848; accession no. L48985) from pPT23B (Hanekamp et al., 1997); IncC, pPT23A maintenance determinant, isolated as a 0.8 kb EcoRI–KpnI fragment from pAKC (Gibbon et al., 1999); IS1240, 1107 bp internal fragment of IS1240 (positions 2081–3188; accession no. L48985) from pPT23B (Hanekamp et al., 1997); IS801, 1.5 kb EcoRI–HindIII fragment isolated from pOSU801 (Romantschuk et al., 1991).
Phylogeny of *Pseudomonas syringae* plasmids

**Table 3.** Hybridization of different coexisting pPT23A-like plasmids to selected DNA probes

<table>
<thead>
<tr>
<th><em>P. syringae</em> pathovar</th>
<th>A-like/total*</th>
<th>Probe used†</th>
<th>aveD</th>
<th>cfa</th>
<th>rulAB</th>
<th>IncC</th>
<th>stbCBAD</th>
<th>IS1240</th>
<th>IS801</th>
</tr>
</thead>
<tbody>
<tr>
<td>tomato OK-1</td>
<td>2/2</td>
<td>A†</td>
<td>B</td>
<td>B</td>
<td>B</td>
<td>A</td>
<td>A</td>
<td>A, B</td>
<td></td>
</tr>
<tr>
<td>tomato UPN23</td>
<td>3/3</td>
<td>B</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>B</td>
<td>A, B</td>
<td>A, B</td>
<td></td>
</tr>
<tr>
<td>glycinea race 6</td>
<td>6/7†</td>
<td>A</td>
<td>A</td>
<td>A, D</td>
<td>–</td>
<td>A, B</td>
<td>F</td>
<td>–</td>
<td>A, B, C, D, E</td>
</tr>
<tr>
<td>phaseolicola HR11302A</td>
<td>4/4</td>
<td>–</td>
<td>D</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>A</td>
<td>–</td>
<td>A</td>
</tr>
</tbody>
</table>

* Number of pPT23A-like plasmids present in the strain/total number of plasmids.
† See Table 2 for description of probes. Letters are assigned to plasmids in alphabetical order, starting from the largest plasmid, and indicate which plasmids are hybridizing to the probes; –, no hybridization detected.
‡ pR6G is the only plasmid from *P. syringae* pv. glycinea race 6 that does not belong to the pPT23A-like family (Sesma et al., 1998).

---

**Fig. 1.** Replication region from pPT23A. A box shows the position and extent of the replicase (*repA*) gene; vertical black bars inside the box denote nucleotide changes that result in residues that are not conserved between the replicases of pPT23A and pAV505. The putative start and stop codons are shown. Numbers refer to nucleotide positions in the pPT23A sequence (Gibbon et al., 1999). The extent of the fragment amplified and cloned is shown below the replication region; the position of the primers used for amplification (532 and 1588) is shown and the fragments sequenced are denoted as black boxes. E, EcoRI; H, HindIII; V, EcoRV.

---

It is likely that plasmids of the pPT23A-like family are also structural mosaics; for example, PAI recently characterized from *P. syringae* pv. *phaseolicola* is located on a pPT23A-like family plasmid (Jackson et al., 1999). Nevertheless, the absolute conservation of *repA* among pPT23A-like family plasmids and the ability of the cloned *repA* sequence to evict a number of pPT23A-like family plasmids in incompatibility experiments (Sesma et al., 1998) suggests that analysis of *repA* is a good starting point in determining phylogenetic relationships among this plasmid group. However, since we do not have knowledge of the complete sequence of the plasmids under study, we will conservatively refer to our results in terms of gaining an understanding of the phylogeny of the *repA* sequence from pPT23A-like family plasmids.

The minimal fragment able to support the replication of pPT23A was defined as an approximately 1.6 kb fragment that spans gene *repA* (Fig. 1) (Gibbon et al., 1999), which encodes a 437 aa putative replicase that is

---

**Fig. 2.** Restriction analysis of cloned amplicons from different replication regions. DNA was amplified, digested with HaeIII (a) or Sau3AI (b) and separated by gel electrophoresis. DNA amplified from the respective native plasmids showed the same restriction pattern as the cloned amplicons (not shown).
essential for plasmid replication. A 1399 bp fragment containing 1279 bp of the 1311 bp repA coding region plus 120 bp upstream of the putative start codon (Fig. 1; Gibbon et al., 1999) was amplified from the pPT23A-like plasmids and separately cloned in vector pCR2.1 which, in our hands, did not replicate in P. syringae. All the cloned amplicons, except those originating from p5D425A and pB76A, were able to replicate in the plasmidless strain P. syringae pv. syringae FF5 (data not shown), suggesting that they were functional in their parental plasmids. Since in all cases the repA gene was in the opposite orientation with respect to the vector lac promoter, as determined by nucleotide sequencing (data not shown), this suggests that the 120 bp preceding the putative start codon could be sufficient to initiate transcription and for supporting autonomous replication in P. syringae.

The digestion profile of the cloned amplicons with HaeIII and Sma3A1 was identical to the profile of the amplicons obtained from the corresponding native plasmids with primers 532 and 1588 (Fig. 2 and data not shown), indicating that no major changes or reorganizations had occurred during the cloning procedure. Most of the amplicons had a characteristic restriction profile with both enzymes (Fig. 2). DNA bands were used to calculate genetic distance and to construct the tree shown in Fig. 3. In general, plasmids from all the strains of the same pathovar did not cluster together, suggesting their diverse origin.

The RepA proteins from plasmids pPT23A, from P. syringae pv. tomato PT23, and pAV505, from P. syringae pv. phaseolicola HRI1302A, are nearly identical from residues 1 to 373 at their N-terminal ends (96-5% identity), but poorly conserved from positions 374 to 437 in their C termini (50% identity) (Fig. 1; Gibbon et al., 1999). We therefore sequenced both ends of the cloned amplicons to study the phylogenetic relationships of their corresponding native plasmids. The sequence corresponding to the 3’ end of the repA genes contained 290 nt for all the cloned amplicons, while the 5’ sequence comprised 291 or 279 nt (see below), of which 176 nt corresponded to the putative start of the repA gene and the rest to the upstream sequences. In all cases, the sequence of the primers used to amplify the repA genes were excluded. The sequences obtained from the cloned amplicon originating from pPT23A were identical to the previously obtained repA sequence (Gibbon et al., 1999). Three of the amplicons, corresponding to plasmids pPT23A, pOK1B and pPSR14, contained a deletion of 12 nt located 11 (pPT23A and pOK1B) or 19 nt (pPSR14) before the ATG start (Fig. 4). In some of the amplicons, a direct repeat of 9 nt was found bordering the deletion area; since the deleted DNA spanned one of the repeats plus the intervening DNA, it is possible that the deletions were generated by replicative slippage (Hancock, 1995). To avoid overestimating sequence divergence, the 20 nt defined by the ends of the deletions (see Fig. 4) were excluded from all the comparison analyses.

The 3’ and 5’ sequences of each amplicon were treated as a single continuous sequence to obtain the consensus tree shown in Fig. 5. The overall genetic analysis resulted in the clustering of the plasmid amplicons into four distinct groups (identity ≥88.8%; bootstrap values ≥95%; designated A, B, C and D in Fig. 5). Plasmid amplicons from three pv. tomato strains, pv. api and five out of the seven pv. syringae strains formed a well defined group. Among the pv. syringae strains, no obvious correlation was found between host of isolation and phylogenetic proximity. For instance, the sequences of the amplicons from pBBS325A and pBB167A, isolated from beans in Colorado and New York, respectively, were identical and they showed only 13 nt differences with the amplicon from p5D425A, which was isolated

---

**Fig. 3.** Dendrogram derived from restriction data of the amplicons. Cluster analysis was performed using the Dice similarity coefficient as described (Sundin & Murillo, 1999). The scale at the top indicates the degree of genetic relatedness.

**Fig. 4.** Alignment of a portion of the 5’ sequences from the cloned amplicons showing the deletion found upstream of the repA start codon. Numbers on the top refer to nucleotide positions in the pPT23A sequence (Gibbon et al., 1999), while numbers on the right indicate nucleotide positions of the sequenced amplicons. The 20 nt included in the box were excluded from all the phylogenetic analyses. The putative start codon for repA is indicated in bold. Arrows indicate the position of the direct repeats found in the sequences. Alignment was done using the program MULTALIN at IBCP. Dashes indicate gaps introduced to maximize the alignment.

---

A. SESMA, G. W. SUNDIN and J. MURILLO
Phylogeny of *Pseudomonas syringae* plasmids

<table>
<thead>
<tr>
<th>Relative genetic similarity</th>
<th>P. syringae pv. syringae</th>
<th>P. syringae pv. tomato</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BBS32-5</td>
<td>B120</td>
</tr>
<tr>
<td></td>
<td>8B6-17</td>
<td>DC3000</td>
</tr>
<tr>
<td></td>
<td>8B48</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5D425</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HS191</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B76</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7B12</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.60</td>
<td>0.70</td>
</tr>
<tr>
<td></td>
<td>0.80</td>
<td>0.80</td>
</tr>
<tr>
<td></td>
<td>0.90</td>
<td>0.90</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>End</td>
</tr>
</tbody>
</table>

![Fig. 5. Phylogenetic tree generated with the 3' and 5' end sequences of each amplicon treated as a single continuous sequence. The tree was constructed by the neighbour-joining method using the CLUSTAL W Multiple Sequence Alignment interactive program available at EBI and displayed using NJPLOT (Saitou & Nei, 1987). The four plasmid groups are indicated on the right (sequence identity > 88.8%). Only bootstrap percentages that were lower than 95% are shown (numbers in italics).](image)

![Fig. 6. Dendrogram of genetic relatedness among *P. syringae* pv. *syringae* and pv. *tomato* strains. Data from the ERIC and AP-PCR fingerprint patterns generated from each strain were combined and cluster analysis was performed using the Dice similarity coefficient as described (Sundin & Murillo, 1999). The scales at the top indicates the degree of genetic relatedness between the strains tested.](image)

from apricot in California. The second group included the plasmid amplicons from the pv. *glycinea* and pv. *phaseolicola* strains and from the remaining pv. *tomato* strain. Nucleotide sequence identities among the groups were > 92.9% for the pv. *tomato* and pv. *apii* amplicons from Group A, > 91.3% for the *P. syringae* plasmids from Group A and > 90.0% for the amplicons from Group B. Additionally, the amplicons from plasmids pPSR14 and pCG131 from *P. syringae* pv. *syringae* formed two separate groups that showed only 77.1–87.7% identity with the rest of the sequences. pCG131 was the most divergent of the *P. syringae* pv. *syringae* plasmids, perhaps owing to its geographical (New Zealand) or plant (millet) origin, although the closest relatives were the plasmid amplicons from *P. syringae* pv. *phaseolicola* (86.5% identity) and pv. *syringae* 7B12 (85.7% identity).

When analysed separately (data not shown), the 5’ sequences did not allow for a clear separation of the different plasmid groups. However, the 3’ sequences were very discriminative and allowed a similar grouping to that obtained with the complete sequence, except that it was not possible to allocate pAV505 to any of the groups. Also, the 3’ sequences were more conserved than the 5’ sequences among any given plasmid group. For instance, the sequences of plasmid pPT23A showed 92.1/93.8% identity (5’/3’ sequences) to the DC3000 plasmid, which is highly related to pPT23A, while they were 92.8/77.2% identical to the sequences of plasmid from B120, which clustered in a separate plasmid group.

The DNA fingerprint of the strains belonging to pv. *syringae* and pv. *tomato* was determined by using ERIC-PCR and AP-PCR. The position of the strains in the resulting dendrograms and their genetic relatedness (Fig. 6) agreed in general with results obtained by other authors using combinations of these strains (Hendson et al., 1992; Legard et al., 1993; Sundin & Murillo, 1999). In many cases, the relatedness between two strains did not reflect the relatedness between their native plasmids (Figs 5 and 6). For instance, strains 8B48 and B76 showed a low relative genetic similarity, although the plasmids they harboured, pPSR11 and pB76A, respectively, were 98% identical, which is the highest identity value among the native plasmids if we exclude pB8617A and pBBS325A (100% identical).

**DISCUSSION**

The pPT23A-like plasmid family encompasses a large majority of *P. syringae* plasmids of known importance to host–pathogen interactions. This plasmid group is widely distributed among *P. syringae* pathovars; however, inter- and intrapathovar plasmid differentiation is readily apparent regarding the occurrence of specific genes encoded by individual pPT23A-like plasmids. In this study, we observed a limited interpathovar distribution of genes affecting host specificity and virulence (*avrD* and *cfa*, respectively), while the *rulAB* determinant, which is important for survival and population maintenance in the phyllosphere (Sundin & Murillo, 1999), was widely distributed among pathovars. These results corroborate previous examples of the limited...
distribution of host specificity determinants among pathovars; indeed, host range has always been an important determinant of pathovar identification in general.

Knowledge of individual plasmid genotypes may provide important clues in the evolution of the pPT23A-like plasmid family. A determinant such as *rupAB* may have been acquired early in the evolution of this plasmid family and may have been fixed within the plasmid genome because of its importance to phyllosphere fitness, a trait which is universally important to the success of *P. syringae* strains (Beattie & Lindow, 1995). The acquisition of loci such as *avr* genes might have stimulated the emergence of new pathovars and, concomitant with that, the opportunity for continued plasmid evolution within the context of a new host–pathogen interaction. Further analysis will determine if certain plasmid lineages are defined by the presence of additional determinants of importance to host–pathogen dynamics. Likewise, the limited distribution of determinants of importance to plasmid maintenance (IncC and *stbCBAD*) could be illustrative of the requirement of such features only in certain host backgrounds, or may reflect a possibly large diversity of maintenance systems among pPT23A-like plasmids. The distribution of IS elements such as IS801 and IS1240 may also be interesting in terms of the timing of evolutionary acquisition. IS801-like sequences, and those of other transposable elements, have been found to flank *avr* sequences and sequence regions suspected to be pathogenicity islands in several *P. syringae* pathovars (Kim *et al.*, 1998; Jackson *et al.*, 1999). These observations imply that the mobility of these types of sequences among strains is important, possibly from the standpoint of a pathogen adding to its host range or combating a new plant resistance gene. It should be noted that IS801 and *avr* genes were not detected in *pv. syringae* in this study or a previous study (Sundin & Bender, 1996), indicating that the mobility of these sequences may be limited by factors which are currently unknown.

We utilized the *repA* gene as our starting point for large-scale phylogenetic analysis of the pPT23A-like plasmid family. *repA* was appropriate because of its requirement for plasmid replication in *P. syringae* and because it is the only gene currently known to be distributed among all pPT23A-like plasmids. Dendrograms generated from analyses of restriction digest patterns or *S*′ and *S*′′ sequence data of *repA* lacked congruence. This could indicate that the restriction digest data were not substantial enough to resolve relationships among the plasmids, or that additional diversity was present within portions of *repA* that were not sequenced. Nevertheless, similar sequencing analyses have been utilized to examine the diversity present among replication genes of closely related plasmids (Burgos *et al*., 1996; Turner *et al*., 1996) and imply that the relationships generated from our analysis of the *S*′ and *S*′′ sequence data (Fig. 5) would be the most valid. These results indicate that the *repA* sequences from plasmids isolated from different *P. syringae* pathovars were not always clearly distinguished. From these observations, we can infer that ecological factors such as plasmid transfer or similar selection pressures faced by different pathovars could result in the current distribution of *repA* sequences among *P. syringae* pathovars. There have been several examinations of plasmid transfer in planta (Bender & Cooksey, 1986; Burr *et al*., 1988; Sundin *et al*., 1989; Björklöf *et al*., 1995). However, although these studies indicate that plasmid transfer can occur, they do not provide evidence for the natural occurrence of plasmid transfer or if plasmids are mobile among more distantly related strains. In one prospective analysis of plasmid and host genotypes examining a population of *P. syringae pv. syringae* under bactericide (copper and streptomycin) selection pressure, recent plasmid transfer events were inferred, but limited to closely related host strains (Sundin *et al*., 1994). The issue of transfer of pPT23A-like plasmids into *P. syringae* strains that already contain an indigenous pPT23A-like plasmid(s) must also be addressed. In one study utilizing the *P. syringae pv. syringae* strains 4A39 and FF3, each of which contained a single pPT23A-like plasmid, conjugation experiments were done resulting in the transfer of the copper resistance (CuR) plasmid pPSR4 into strain FF3 which contained the streptomycin resistance (SmR) transposon Tn5393 on plasmid pPSR5 (Sundin & Bender, 1996). Further experiments showed that, in the absence of selection, the plasmids were incompatible; however, selection for the CuR and SmR markers enabled the FF3 strain to harbour both pPSR4 and pPSR5 for 32±8 generations, after which pPSR5 was lost, but Tn5393 transposed into pPSR4 (Sundin & Bender, 1996). This experiment showed that surface exclusion or restriction modification systems did not preclude the transfer of a pPT23A-like plasmid into a strain harbouring an incompatible plasmid, at least for these *P. syringae pv. syringae* hosts. Plasmid incompatibility may prevent the establishment of a plasmid in a new *P. syringae* background; however, the extensive homology present on pPT23A-like plasmids may contribute to genetic rearrangements resulting in the acquisition of new sequences by these plasmids. Whether such barriers to plasmid transfer exist in other *P. syringae* pathovars is currently unknown. The results of other studies of *Agrobacterium* and *Rhizobium* spp. have also indicated the long-term stability of plasmid and host genotypes (Young & Wexler, 1988; Orten *et al*., 1996; Wernigreen *et al*., 1997). Our results suggest that most of the pPT23A-like plasmids studied have been maintained for long periods within their respective hosts. Our current analysis of strains HS191, 7B12 and B120, however, does not preclude the possible occurrence of plasmid transfer in nature.

**ACKNOWLEDGEMENTS**

We thank the researchers listed in Table 1 for the gifts of *P. syringae* strains. We are also grateful to C. Manceau for helpful suggestions and to J. L. Jacobs for excellent technical assistance. G.W.S. acknowledges support from the US Department of Agriculture (NRCGP 9702832) and the Texas...
Agricultural Experiment Station. J.M. gratefully acknowledges support from the Spanish CICYT (BI094-0442, BI097-0598) and from Caja Rural de Navarra and Universidad Pública de Navarra for a short stay in Texas A&M University.

REFERENCES


Analysis of genes involved in biosynthesis of coronafacic acid, the polyketide component of the phytotoxin coronatine. J Bacteriol 180, 3330–3338.


Yang, Y., Yuan, Q. & Gabriel, D. W. (1996). Watersoaking function(s) of XcmH1005 are redundantly encoded by members of the Xanthomonas avr/ptb gene family. Mol Plant–Microbe Interact 8, 627–631.


Received 23 March 2000; revised 30 June 2000; accepted 11 July 2000.