**Pseudomonas syringae** pv. phaseolicola can be separated into two genetic lineages distinguished by the possession of the phaseolotoxin biosynthetic cluster

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The bean (*Phaseolus* spp.) plant pathogen *Pseudomonas syringae* pv. phaseolicola is characterized by the ability to produce phaseolotoxin (Tox⁺). We recently reported that the majority of the Spanish *P. syringae* pv. phaseolicola population is unable to synthesize this toxin (Tox⁻). These Tox⁻ isolates appear to lack the entire DNA region for the biosynthesis of phaseolotoxin (*argK-tox* gene cluster), as shown by PCR amplification and DNA hybridization using DNA sequences specific for separated genes of this cluster. Tox⁺ and Tox⁻ isolates also showed genomic divergence that included differences in ERIC-PCR and arbitrarily primed-PCR profiles. Tox⁺ isolates showed distinct patterns of IS801 genomic insertions and contained a chromosomal IS801 insertion that was absent from Tox⁻ isolates. Using a heteroduplex mobility assay, sequence differences were observed only among the intergenic transcribed spacer of the five rDNA operons of the Tox⁻ isolates. The techniques used allowed the unequivocal differentiation of isolates of *P. syringae* pv. phaseolicola from the closely related soybean (*Glycine max*) pathogen, *P. syringae* pv. glycinea. Finally, a pathogenicity island that is essential for the pathogenicity of *P. syringae* pv. phaseolicola on beans appears to be conserved among Tox⁺, but not among Tox⁻ isolates, which also lacked the characteristic large plasmid that carries this pathogenicity island. It is proposed that the results presented here justify the separation of the Tox⁺ and Tox⁻ *P. syringae* pv. phaseolicola isolates into two distinct genetic lineages, designated Pph1 and Pph2, respectively, that show relevant genomic differences that include the pathogenicity gene complement.

**INTRODUCTION**

*Pseudomonas syringae* pv. phaseolicola is a seed-borne pathogen of bean (*Phaseolus vulgaris*) worldwide that causes the halo blight disease. Disease symptoms are typically watersoaked lesions that eventually develop a surrounding yellow halo produced by the release of the non-specific toxin, phaseolotoxin (Mitchell, 1978). Based on their virulence to a range of bean cultivars, nine races of *P. syringae* pv. phaseolicola have been distinguished (Taylor et al., 1996). Recently, the ability of this pathogen to produce disease in bean has been shown to be based on the possession of a pathogenicity island (PAI), localized to a 150 kb plasmid, that includes genes that are either essential for pathogenicity on bean and soybean or that contribute to aggressiveness in an additive fashion (Jackson et al., 1999; Tsiamis et al., 2000). In addition to the PAI, *P. syringae* pv. phaseolicola strains are defined by possession of the *argK-tox* gene cluster, which directs phaseolotoxin...
biosynthesis and appears to increase virulence (Patil et al., 1974; Mitchell, 1978; de la Fuente-Martinez et al., 1992). Additionally, phaseolotoxin has been considered a useful determinative character unique to \textit{P. syringae} pv. phaseolicola among the bacterial bean pathogens. It is generally believed that only isolates able to synthesize phaseolotoxin (Tox$^+$ isolates) are of epidemiological significance and, hence, this DNA region is commonly used as a target for PCR detection and identification of \textit{P. syringae} pv. phaseolicola (Schaad et al., 1995).

\textit{P. syringae} pv. phaseolicola can readily be distinguished from other pathovars of \textit{P. syringae} pathogenic to beans, such as pathovars syringae and glycinea, by nutritional characteristics and because only \textit{P. syringae} pv. phaseolicola isolates produce water-soaked lesions on bean pods (Palleroni, 1984; Völksch & Weingart, 1997; Marques et al., 2000). In general, \textit{P. syringae} pv. phaseolicola appears to be a more or less homogeneous pathovar, although it displays a degree of genetic and phenotypic variation that overlaps with isolates from \textit{P. syringae} pv. glycinea (Marques et al., 2000). On the basis of phenotypic characteristics and ERIC-PCR-generated profiles, strains of \textit{P. syringae} pv. glycinea, \textit{P. syringae} pv. phaseolicola isolated from bean and \textit{P. syringae} pv. phaseolicola isolated from kudzu (\textit{Pueraria lobata}), can be divided into three distinct groups (Völksch & Weingart, 1997). Additionally, intratohvar variation in \textit{P. syringae} pv. phaseolicola can be linked, in some cases, to the host plant species of isolation (Marques et al., 2000). Isolates that produce natural infections on kudzu vine are distinguished, among other characters, for carrying a plasmid-borne \textit{efe} gene (Nagahama et al., 1994) and, similar to isolates from \textit{Vigna radiata}, by their REP-PCR profile with ERIC primers (Völksch & Weingart, 1997; Marques et al., 2000).

Most isolates of \textit{P. syringae} pv. phaseolicola are reported to be Tox$^+$ and naturally occurring isolates unable to synthesize phaseolotoxin (Tox$^-$ isolates), which usually possess the corresponding \textit{argK}-tox gene cluster region, are rare (Rudolph, 1995; Schaad et al., 1995). We reported recently, however, that over 60\% of the Spanish field isolates of \textit{P. syringae} pv. phaseolicola were Tox$^-$ and did not produce the expected PCR amplification using a primer pair specific for ORF6 (Rico et al., 2003), which is essential for phaseolotoxin biosynthesis and is routinely used as a target for the detection of this pathogen (Schaad et al., 1995). Additionally, Tox$^-$ isolates did not show hybridization to an ORF6-specific DNA probe (Rico et al., 2003), suggesting the absence of part or of the entire \textit{argK}-tox gene cluster. This raised the possibility that the Spanish Tox$^-$ isolates were genetically separable from the more common isolates that synthesize phaseolotoxin. In this study, we analyse the genetic variability within the Spanish \textit{P. syringae} pv. phaseolicola population, in comparison with \textit{P. syringae} pv. phaseolicola and \textit{P. syringae} pv. glycinea isolates from international collections. Collectively, our results allowed the differentiation of two genetic lineages in \textit{P. syringae} pv. phaseolicola and suggest the separate evolution of their pathogenicity gene complement.

**METHODS**

**Bacterial strains and growth conditions.** \textit{Escherichia coli} DH5$\alpha$ was used for cloning purposes and was propagated in LB at 37$^\circ$C (Sambrook et al., 1989). The type races of \textit{P. syringae} pv. phaseolicola 1281A (race 1), 1301A (race 3), 1302A (race 4), 1449B (race 7), 2656A (race 8) and 2709A (race 9) have been described elsewhere (Taylor et al., 1996). Strains Hb-1b and M2/1 of \textit{P. syringae} pv. phaseolicola were isolated from beans in an unknown place and Germany, respectively, and do not produce phaseolotoxin (Völksch & Weingart, 1997). Another 13 Tox$^+$ and 24 Tox$^-\textit{P. syringae}$ pv. phaseolicola isolated in Spain were characterized previously (Rico et al., 2003). \textit{P. syringae} pv. phaseolicola CFBP1390 and \textit{P. syringae} pv. glycinea CFBP2214 are the pathotype strains and were obtained from C. Manceau (INRA, Angers, France). \textit{P. syringae} pv. glycinea strains PG4180 and 49a/90 (both race 4) were obtained from M. Ullrich (Bremen University, Bremen, Germany). \textit{P. syringae} strains were routinely grown on King’s medium B (KMB) (King et al., 1954) at 25–28$^\circ$C.

**PCR analysis.** Genetic variability among \textit{P. syringae} strains was examined by PCR fingerprinting of repetitive DNA sequences using primers for extragenic repetitive consensus (ERIC), repetitive extragenic palindromic (REP) and the arbitrarily primed PCR (AP-PCR) techniques. For ERIC and REP analyses, primers and reaction conditions were as described by McManus & Jones (1995). AP-PCR was carried out using the universal M13 reverse primer (5$^-\text{AGCGGA-TAACAAATTTCAGACG}-3^-$) or a single 20 bp oligonucleotide primer (5$^-\text{GGTTCCGTTCAGGACGCTAC}-3^-$) complementary to the IS50 portion of Tn5, as described by Sundin & Murillo (1999). For the amplification of phaseolotoxin biosynthetic genes, we assessed two different primer pairs which are specific for DNA regions separated in the genome that are essential for phaseolotoxin biosynthesis. Primers PHA19 and PHA95 amplify a 480 bp internal fragment from the amnidinotransferase gene \textit{amtn} (Marques et al., 2000; Hernández-Guzmán & Alvarez-Morales, 2001) and primers OCTF-03 and OCT-R amplify a 632 bp DNA fragment of the ornithine carbamoyltransferase gene \textit{argK} (Sawada et al., 2002), which confers resistance to phaseolotoxin. Amplification of genes included in the pathogenicity island was performed with primers DL-04523 (5$^-\text{AGCCCGCGCGCGCTCTG-3^-$} and DR-05216 (5$^-\text{GAAGTGAA-}
\text{GGACCGCAAGC-3^-$} for \textit{avrD}) and primers CL-19541 (5$^-\text{GATCG-}
\text{TAAAGAAGGGCGATTT-3^-$} and CR-20852 (5$^-\text{CGTCGATGTAG-}
\text{CATGTATAAA-3^-$} for \textit{avrPHC}). The exchangeable effector locus (EEL) region of the \textit{hrp} pathogenicity island (Alfano et al., 2000) was also assayed using primers \textit{avrvPhe-}FOR (Stevens et al., 1998) and \textit{queA-2} (5$^-\text{AATCACGGGAAATCGGAGCT-3^-$} within the coding regions of the \textit{hrpK} and \textit{queA} genes, respectively. A 627 bp fragment from the insertion sequence element \textit{IS801} (Romantschuk et al., 1991) was amplified from \textit{P. syringae} pv. phaseolicola strain 1449B using primers \textit{IS801F} (5$^-\text{AGTCGCTGAACACCTCAG-3^-}$) and \textit{IS801R1} (5$^-\text{GCCTTCTTTGAGGAACGACAG-3^-}$). The occurrence of a chromosomal insertion of \textit{IS801} in \textit{P. syringae} pv. phaseolicola was tested by amplification with primers RP-1 and RP-2 (González et al., 1998). For amplifications, bacterial cell suspensions of isolates grown on KMB were prepared in 500 ml sterile distilled water and subjected to freeze–thaw lysis. Standard PCR reactions were performed in a final volume of 25 ml containing as template 50 ng total genomic DNA or 5 ml bacterial lysates, using either \textit{Taq} DNA polymerase (Biotaq Bioline) or Ready To Go PCR Beads (Amersham Pharmacia Biotech).

**General molecular techniques.** Total DNA was extracted using a Puregene DNA isolation kit (Genta Systems), according to the
manufacturer’s instructions. 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 x TAE as described previously (Murillo et al., 1994). PCR products were purified using the GFX PCR DNA purification kit (Amerham Pharmacia Biotech). DNA sequencing was performed by MWG-Biotech AG. Nucleotide sequences were aligned using CLUSTALW (Thompson et al., 1997) and database comparisons were made via the BLASTN, BLASTP and TBLASTX algorithms (Altschul et al., 1997). Preliminary sequence data from *P. syringae* pv. tomato DC3000 and *P. syringae* B728a genome projects were obtained from The Institute for Genomic Research (http://www.tigr.org) and the DOE Joint Genome Institute (http://www.jgi.doe.gov) websites, respectively.

For Southern blots, chromosomal DNA was routinely digested with appropriate restriction enzymes, and DNA fragments separated by electrophoresis in 1% agarose gels were transferred to a nylon membrane (Roche Diagnostics). For the preparation of DNA probes, specific DNA fragments were gel-extracted and cloned into the pGEM-T Easy vector (Promega). After restriction digestion, the inserts were separated by electrophoresis, excised from the gels and used as probes. Preparation of labelled probes with digoxigenin, Southern hybridization and detection of the hybridized DNA were carried out with the DIG DNA labelling and detection kit (Roche Diagnostics).

**Heteroduplex mobility assay (HMA).** The sequence polymorphism of the internal transcribed spacer (ITS) region was amplified by 16S and 23S rRNA genes was analysed using a DNA HMA (Delwart et al., 1993). The ITS region was amplified by primers D21 and D22 (Manceau & Horvais, 1997) and PCR products were migrated in 5% polyacrylamide gels (Delwart et al., 1993).

**RESULTS**

**A group of *P. syringae* pv. phaseolicola isolates lack the phaseolotoxin biosynthetic gene cluster**

In a previous study (Rico et al., 2003), 94 Spanish Tox− isolates lacked ORF6, which is contained in the argK-tox gene cluster and used for detection purposes (Schaad et al., 1995; Zhang & Patil, 1997). By PCR amplification and DNA hybridization we tested the conservation of the argK-tox gene cluster among a collection of six *P. syringae* pv. phaseolicola type races, 13 Tox+ Spanish isolates, 24 Spanish Tox− isolates and the two Tox− strains Hb-1b and M2/1, isolated elsewhere. We focused on genes *argK* and *amtA*, which currently define the ends of the cluster, for their importance in the detection of this pathogen (Schaad et al., 1995; Hernández-Guzmán & Alvarez-Morales, 2001).

PCR amplification using primers internal to *amtA* (Fig. 1a) and *argK* (not shown) yielded the expected 480 and 632 bp amplification products, respectively, for all the Tox+ isolates tested, as well as for the Tox− isolates Hb-1b and M2/1. Conversely, no strong specific amplicons were observed for any of the Spanish Tox− isolates or for *P. syringae* pv. glycinea strains PG4180 and 49a/90 (Fig. 1a). It determined that the published sequence of primer PHA19 (Marques et al., 2000) showed two mismatches in its 5′ end with the sequence of the *amtA* gene deposited in the databases (accession no. AF186235; Hernández-Guzmán & Alvarez-Morales, 2001). Although the *argK* gene was shown to be highly conserved (Sawada et al., 1999), these results suggest that the observed lack of amplification observed for some of the Tox− isolates might be due to possible sequence variations in their argK-tox gene cluster with respect to the primers used. We therefore examined the conservation of this cluster by DNA hybridization.

Internal fragments of *amtA* and *argK* were amplified as above from the *amtA* gene using primers PHA19/PHA95 (Marques et al., 2000; Hernández-Guzmán & Alvarez-Morales, 2001). Lanes: 1, *P. syringae* pv. phaseolicola (Pph) isolate 1281A; 2, 2709A; 3, 1449B; 4, CYL215; 5, CYL281; 6, CYL285; 7, CYL207; 8, CYL283; 9, CYL286; 10, CYL233; 11, CYL275; 12, CYL325; 13, CYL352; 14, CYL309; 15, CYL314; *P. syringae* pv. glycinea (Pgy) isolates 16, 49a/90; 17, PG4180. Pph1 and Pph2 correspond to the two genetic lineages of *P. syringae* pv. phaseolicola. (b) Southern hybridization of EcoRI-digested total DNA. An internal fragment of the *amtA* gene was amplified from *P. syringae* pv. phaseolicola strain 1449B with primers PHA19/PHA95, labelled with digoxigenin and used as probe. Lanes are as described above. Sizes are indicated to the left in kb.
hybridize with either of the two probes, suggesting that they may lack the entire argK-tox gene cluster. We propose to designate the group of strains containing the argK-tox gene cluster Pph1, and the group of strains lacking this cluster Pph2.

**Isolates containing or lacking the argK-tox gene cluster can be differentiated into two groups by REP-PCR**

The phaseolotoxin biosynthetic cluster appears to have been acquired by horizontal gene transfer (Sawada et al., 1997, 1999) and, as a consequence it is possible that the *P. syringae* pv. phaseolicola isolates containing this DNA and those lacking it might represent distinct genetic lineages. We used PCR fingerprinting of repetitive DNA sequences (REP-PCR) to assess the genetic diversity among the above 21 Pph1 and 24 Pph2 isolates. We also analysed two strains of *P. syringae* pv. glycinea, because strains of this pathovar also lack the argK-tox gene cluster and are closely related phylogenetically to *P. syringae* pv. phaseolicola (Gardan et al., 1999; Marques et al., 2000; Yamamoto et al., 2000).

The REP-PCR amplification profiles were similar among all isolates examined (Fig. 2), although strains of *P. syringae* pv. phaseolicola showed several strong differential bands that allowed their distinction from the *P. syringae* pv. glycinea isolates. One of these was a 1700 bp band present in the ERIC profile (Fig. 2). Additionally, strains belonging to Pph1 and Pph2 could be distinguished on the basis of significant differences in their REP-PCR banding profiles (Fig. 2). Besides several minor differential bands, a strong 734 bp band was present in the IS50 profile of all the Pph1 strains (Fig. 2), independently of their place of isolation. Hybridization experiments showed that the 45 *P. syringae* pv. phaseolicola isolates examined contained several fragments with homology to the sequences included in the 734 bp fragment (not shown). However, the pattern of hybridization to the probe showed significant differences between Pph1 and Pph2 isolates (not shown), indicating the existence of more dissimilarities than those revealed by REP-PCR. The analysis of the nucleotide sequence of the 734 bp band, obtained in this work, indicated that it is a mosaic (Table 1) that probably resulted from a reorganization event. Comparison with the databases showed that parts of this sequence are also repeated and scattered in different positions of the *P. syringae* pv. tomato DC3000 genome and plasmid pDC3000A (Table 1).

All the Pph2 isolates showed a characteristic REP-PCR profile that included two strong differential bands: a 1289 bp band present in the ERIC profile and a 295 bp band amplified by the IS50 primer (Fig. 2). The nucleotide sequences of the 1289 and 295 bp bands were also determined and analysed. The 1289 bp band appears to be well conserved, since its nucleotide sequence was highly conserved in the genomes of *P. syringae* pv. tomato DC3000 and pv. syringae B728a (Table 1) and because the *P. syringae* pv. glycinea strains contained a co-migrating band (Fig. 2). All Pph1 and Pph2 isolates showed a unique 10 kb EcoRI hybridization band in Southern experiments using the 1289 bp fragment as a probe (not shown). In contrast, the 295 bp band showed strong hybridization only to genomic DNA from Pph2 isolates, and the homologous DNA was localized to a native plasmid of 40–50 kb (not shown). The comparison of the nucleotide sequence of the 295 bp band with the databases suggests that it is a chimera of sequences that are separated in other *P. syringae* strains (Table 1).

**Conservation of the exchangeable effector loci**

The *hrp* cluster encodes a type III secretion system that injects specialized proteins, or effectors, into the plant host cell; these effectors appear to be the main host range determinants, promoting pathogenicity or defence reactions of the plant. In *P. syringae*, the *hrp* cluster is bordered by two DNA regions containing diverse effector genes (Alfano et al., 2000).
The 150 kb virulence plasmid of Pph1 is not present in Pph2

Strains of *P. syringae* pv. phaseolicola usually contain a large native plasmid of around 150 kb that, in the race 7 strain 1449B, was shown to carry the PAI (Jackson et al., 1999). We therefore decided to evaluate the conservation and physical location of the PAI between groups Pph1 and Pph2 by examination of the plasmid profiles and by Southern hybridization with probes specific for effector genes *avrD* and *avrPphC*, which are located in the leftmost border and in the centre of the PAI, respectively (Yucel et al., 1994; Jackson et al., 1999). *avrD* is widely distributed in *P. syringae* and restricts infection on certain soybean cultivars by triggering a defence response, as does *avrPphC*. Additionally, *avrPphC* also behaves as a virulence gene on bean cultivar Canadian Wonder (Tsiamis et al., 2000).

The profiles of Pph1 isolates showed diverse native plasmids and all of them contained a large plasmid similar to the 150 kb virulence plasmid present in strain 1449B (Fig. 3a). In contrast, the Pph2 isolates contained one or two native plasmids of 30–50 kb, with absence of the typical 150 kb plasmid present in Pph1 (Fig. 3a). DNA probes specific for genes *avrD* and *avrPphC* showed hybridization with the large plasmid present in strain 1449B and in all the other Pph1 isolates (Fig. 3b), indicating that the physical location of the PAI is conserved in Pph1. Conversely, *avrD* did not show hybridization with any of the plasmids of the Pph2 isolates (Fig. 3b), although it hybridized to a 5-6 kb *HindIII* fragment when digested total genomic DNA was used instead of intact native plasmids (not shown). The *avrPphC* probe, however, hybridized to a single plasmid of 40–50 kb in each Pph2 isolate (Fig. 3b). These results suggest a different organization of the pathogenicity genes included in the PAI among Pph1 and Pph2 isolates.

### IS801 insertion patterns are different for Pph1 and Pph2

The 1512 nt insertion sequence element IS801 has a limited distribution among *P. syringae* (Romantschuk et al., 1990, 1991) and is thought to produce permanent insertions due to its putative replicative transposition mechanism (Mendiola et al., 1994; Richter et al., 1998). Therefore, we examined the profile of IS801 insertions as a potential method of fingerprinting strains of *P. syringae* pv. phaseolicola. Genomic and plasmid DNA of selected *P. syringae* pv. phaseolicola strains were digested with *HinDIII* and hybridized with a single-stranded probe specific for IS801.

### Table 1. Features of the ERIC and IS50 profile bands that differentiated strains of Pph1 and Pph2

<table>
<thead>
<tr>
<th>Band specificity/ size (bp)*</th>
<th>Primer</th>
<th>Position</th>
<th>Relevant nucleotide homologies (nucleotide position/accession no.)</th>
<th>Identity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pph1/734</td>
<td>IS50</td>
<td>321–466</td>
<td><em>P. syringae</em> pv. tomato DC3000 genome (5 346 878–5 347 023)</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td></td>
<td>365–466</td>
<td><em>P. syringae</em> pv. syringae B728a plasmid pDC3000A (3 047–3 148)</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td></td>
<td>466–734</td>
<td><em>P. syringae</em> pv. syringae tomato DC3000 genome (908 368–908 100)</td>
<td>95</td>
</tr>
<tr>
<td>Pph2/295</td>
<td>IS50</td>
<td>19–276</td>
<td>Plasmid pIAA1, DNA region downstream IAA lysine synthetase gene</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td></td>
<td>124–249</td>
<td>DNA region upstream type III effector HopPmaD gene; <em>P. syringae</em> pv. savastanoi (M35373)</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td></td>
<td>251–277</td>
<td><em>P. syringae</em> pv. tomato DC3000 genome (16 683–16 709)</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1–1289</td>
<td>DNA IS801 insertion sequence element; <em>P. syringae</em> (X57269)</td>
<td>100</td>
</tr>
<tr>
<td>Pph2 and Pgy/1289</td>
<td>ERIC</td>
<td>1–1289</td>
<td><em>P. syringae</em> pv. tomato DC3000 genome (3 101 476–3 100 187)</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>P. syringae</em> pv. syringae B728a genome Pyr_7 (NZ_AABP020 00007)</td>
<td>84</td>
</tr>
</tbody>
</table>

*Ppy, *P. syringae* pv. glycinea.
pv. phaseolicola strains was digested with PstI and subjected to Southern hybridization using the leftmost 627 bp fragment of IS\textit{801} as probe. Strains of Pph1 and Pph2 could be unequivocally differentiated by their IS\textit{801} hybridization fingerprint (Fig. 4a), although there was some variation in the number and size of bands within each group. IS\textit{801} hybridization patterns were used to calculate genetic distances and to construct a tree (Fig. 4b) that clearly clustered together isolates of each group. The hybridization pattern of \textit{P. syringae} pv. phaseolicola strain RW60, which lacks the 150 kb virulence plasmid (Jackson \textit{et al.}, 1999), and of PstI-digested total plasmid DNA from several \textit{P. syringae} pv. phaseolicola strains (not shown), indicate that four to seven hybridizing bands per strain could correspond to chromosomal DNA. Since most of these chromosomal insertions were present in both Pph1 and Pph2 isolates, we explored the conservation of previously described IS\textit{801} insertions.

Race 2 isolates of \textit{P. syringae} pv. phaseolicola, but not race 1 isolates, were reported to harbour an IS\textit{801} insertion in a putative avirulence gene, and primers RP-1 and RP-2 were
designed to amplify a 2.7 kb-specific fragment from the strains containing this insertion and a 1.2 kb-specific fragment from the strains lacking it (González et al., 1998). In our hands, however, amplification with RP-1 and RP-2 produced a 2.7 kb band from each of the 21 Pph1 strains utilized in this study (not shown), as well as with another 111 Tox+ P. syringae pv. phaseolicola strains from our collection, irrespective of their race assignment. In contrast, 94 P. syringae pv. phaseolicola isolates that lacked tox-specific DNA (Rico et al., 2003), including the 24 examined here and P. syringae pv. glycinea strain PG4180, produced a 1.2 kb band after PCR amplification with primers RP-1 and RP-2 (not shown). No amplification was observed for strains P. syringae pv. glycinea 49a/90, pv. tomato DC3000 or pv. syringae B728a. However, the genome sequences of DC3000 and B728a show high homology to the DNA amplified from P. syringae pv. phaseolicola with RP-1 and RP-2 (accession no. Y09452), although they do not contain an IS801 insertion in this fragment, suggesting the occurrence of primer mismatches that prevented PCR amplification.

**Pph1 and Pph2 can be differentiated by HMA analysis of the ITS sequences**

The ITS is a non-coding sequence located between the 16S and 23S rRNA genes that is frequently used for taxonomic studies (Gurtler & Stanisich, 1996). HMA is a PCR-based technique (Delwart et al., 1993) that facilitates the analysis of even minor sequence differences among the five rDNA operons present in P. syringae and it has been successfully used for the establishment of phylogenetic relationships among P. syringae pathovars and other species of bacteria (Sutra et al., 2001; Catara et al., 2002).

Different electrophoretic HMA profiles obtained by direct migration of PCR-amplified ITS, indicated a clear diversity between P. syringae pv. glycinea and P. syringae pv. phaseolicola and allowed the differentiation of groups Pph1 and Pph2 (Fig. 5). The HMA profiles of all the Pph1 isolates presented a unique homoduplex band, indicating that the ITS copies in the different rDNA operons were identical within each strain. Conversely, the Pph2 isolates showed a homoduplex band that co-migrated with that observed for Pph1 isolates, but also showed two supplementary bands with reduced mobility that correspond to heteroduplexes, indicating sequence differences between the ITS copies in the different rDNA operons within each strain. For the three P. syringae pv. glycinea strains analysed, the ITS sequences were identical within each strain and shorter than the ITS sequences of P. syringae pv. phaseolicola (Fig. 5).

**DISCUSSION**

Our results show that P. syringae pv. phaseolicola is composed of at least two genetic lineages which present important differences in their virulence gene complement and other genetic determinants. We propose to designate these lineages as two genomic groups, Pph1 and Pph2. The groups differ in possession of the phaseolotoxin biosynthetic gene cluster, REP-PCR profiles, plasmid content, the conservation of a PAI, the insertion pattern of IS801 and the HMA profile of ITS sequences. Rico et al. (2003) previously demonstrated that Pph2 isolates did not cross-react with a commercial polyclonal antibody raised against the more common Pph1 isolates, suggesting the existence of other significant differences between these groups. All Pph2 isolates can utilize mannitol as sole carbon source (Rico et al., 2003) while the majority of Pph1 isolates cannot (Palleroni, 1984). Pph1, Pph2 and P. syringae pv. glycinea strains share identical gyrB and rpoD gene sequences (Yamamoto et al., 2000; Rico et al., 2003), although P. syringae pv. phaseolicola and P. syringae pv. glycinea can be readily distinguished and each pathovar has a characteristic, although overlapping, host range (Palleroni, 1984; Völkisch & Weingart, 1997; Marques et al., 2000). Additionally, all the techniques used in this study revealed genomic differences that allowed the clear separation of Pph1 and Pph2, and confirmed the separation between P. syringae pv. phaseolicola and P. syringae pv. glycinea. ERIC- and AP-PCR have been used successfully with P. syringae for intrapathovar strain differentiation (Louws et al., 1994; Sundin et al., 1994; Little et al., 1998), including P. syringae pv. phaseolicola (Völkisch & Weingart, 1997; Marques et al., 2000). AP-PCR was very discriminating in this study and produced two strong bands that were specific for each of the groups. Although these bands are composed of highly repetitive DNA (see Table 1), they could be used as potential markers for the rapid identification of Pph1 and Pph2 strains by PCR.
The hybridization patterns of genomic DNA to IS801 clearly distinguished Pph1 and Pph2, but the phylogenetic significance of this observation is uncertain because most of the hybridizing bands corresponded to plasmid DNA. However, a chromosomal insertion of IS801 that was present in all Pph1 strains, but absent from Pph2 and strains of other P. syringae pathovars, could be a reliable marker for identification. This is because IS801 belongs to a family of insertion elements that follow a replicative rolling-circle transposition (Mendiola et al., 1994; Richter et al., 1998), making it likely that IS801 insertions would be permanent. Also, the relatively relaxed target specificity of IS801 (Richter et al., 1998) makes the independent occurrence of two insertions in the fragment amplified by RP-1 and RP-2 rather improbable, even more so if we take into account the limited occurrence of IS801 chromosomal insertions in P. syringae pv. phaseolicola. In contrast to a previous report (González et al., 1998), our results show that this IS801 insertion is not race-specific.

Additional evidence for the separation of groups Pph1 and Pph2 is provided by the different HMA patterns of the ITS sequences, indicating the existence of sequence differences among the ITS copies only in the Pph2 genomes. This is significant because sequence differences in the ITS among pathovars of P. syringae are strongly correlated with significant genomic differences (Manceau & Horvais, 1997; Sawada et al., 1997). By using DNA hybridization, several restriction fragment length polymorphisms have been described among different P. syringae pv. phaseolicola strains (González et al., 2000), suggesting further variation associated to the rDNA operons of this bacterium. We do not know, however, if there are similar restriction site variations that could distinguish Pph1 and Pph2.

Our results concerning genes involved in pathogenicity also suggest the separate evolution of at least part of the pathogenicity gene complement for Pph1 and Pph2. The differential capacity to synthesize phaseolotoxin, which is a putative virulence factor, is accompanied by differences in the genomic organization of the effector genes avrD and avrPphC. However, the EEL sequences adjacent to the hrp cluster are highly conserved among Pph1, Pph2 and P. syringae pv. glycinea, indicating that the genes responsible for host range have a different genomic location.

Among many other plant-pathogenic bacteria, including several pathovars of P. syringae, only strains of P. syringae pv. phaseolicola and P. syringae pv. actinidiae, as well as a single isolate of P. syringae pv. syringae, were found to produce phaseolotoxin and contain DNA specific for this biosynthetic gene cluster (Tourte & Manceau, 1995; Sawada et al., 1997; Tamura et al., 2002). The complete conservation of the argK coding sequence, as compared to the phylogeny of the chromosomal genes gyrB and rpoD, and the pathogenicity-related genes hrpL and hrcP, suggests that the argK-tox gene cluster was horizontally transferred after the divergence of the ancestor of P. syringae into the modern pathovars (Sawada et al., 1999). In support of this, we showed that the internal organization of the argK-tox gene cluster was highly conserved among diverse Pph1 strains. Moreover, all the Pph2 isolates appear to lack the entire argK-tox gene cluster, because they failed to hybridize to two specific probes that correspond to well separated genes (amtA and argK) within this cluster. Therefore, it seems likely that the capacity to infect beans was acquired by P. syringae pv. phaseolicola earlier than the capacity to synthesize phaseolotoxin. The role of this toxin in pathogenicity is not clear, although there is some evidence that it might increase the virulence of the infection (de la Fuente-Martínez et al., 1992) or allow it to become systemic on bean plants (Patil et al., 1974). However, the production of phaseolotoxin is considered a defining characteristic of P. syringae pv. phaseolicola and strains unable to synthesize it are only rarely reported (Rudolph, 1995; Schaad et al., 1995; Volksch & Weingart, 1998; Marques et al., 2000), suggesting that the production of phaseolotoxin, or the activity of other gene(s) that might have been co-transferred with the argK-tox gene cluster, could confer an important selective advantage.

The PAI in P. syringae pv. phaseolicola strain 1449B spans around 30 kb of contiguous DNA located in the 150 kb native plasmid and includes several effector genes, some of which are involved in pathogenicity and virulence (Jackson et al., 1999; Tsiamis et al., 2000). Other pathovars of P. syringae contain homologues of one or more of the genes included in this PAI, although the PAI itself is not conserved among them and the number of genes and their physical location (plasmid versus chromosome) is highly variable (Jackson et al., 2002). However, the PAI would appear to be conserved among the Pph1 group of strains since all of them contained a large plasmid that hybridized to both avrD- and avrPphC-specific probes. By contrast, in all the Pph2 isolates the DNA homologous to avrD was located in the chromosome while a plasmid smaller than 30 kb contained the avrPphC homologue.

It was suggested that kudzu strains could represent a different group because they can also be differentiated from other P. syringae pv. phaseolicola strains by their REP- and ERIC-PCR fingerprints, esterase zymotypes, O-serogroup, capacity to utilize mannitol, ethylene production and infection of kudzu plants (Goto & Hyodo, 1987; Volksch & Weingart, 1997; Marques et al., 2000). In our opinion, it seems more likely that the kudzu strains could represent a subdivision of the Pph1 group, because they also harbour the argK-tox gene cluster and it is unlikely that this group of genes has been independently acquired by P. syringae pv. phaseolicola twice during evolution. In addition, strains isolated from Vigna spp., which also possess the argK-tox gene cluster, can also be differentiated by their ERIC-PCR pattern and their O-serogroup (Marques et al., 2000). Nevertheless, given the existence of several independent characters that separate the currently delineated Pph1 and Pph2, there is a likelihood that other possible genomic groups may exist within P. syringae pv. phaseolicola, showing...
characteristics intermediate between the different groups. We have clearly demonstrated the existence of two *P. syringae* pv. phaseolicola genetic lines and provided a basis for a clearer understanding of the mechanisms behind the acquisition of virulence genes and their clustering in pathogenicity islands.

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