Strains of *Pseudomonas syringae* pv. *syringae* from Pea Are Phylogenetically and Pathogenically Diverse

Alberto Martín-Sanz, Marcelino Pérez de la Vega, Jesús Murillo, and Constantino Caminero

First and fourth authors: Instituto Tecnológico Agrario, Consejería de Agricultura y Ganadería de la Junta de Castilla y León, Ctra. Burgos, km 119, 47071 Valladolid, Spain; second author: Área de Genética, Departamento de Biología Molecular, Universidad de León, 24071 León, Spain; and third author: Departamento de Producción Agraria, ETS Ingenieros Agrónomos, Universidad Pública de Navarra, 31006 Pamplona, Spain.

Current address of A. Martín-Sanz: Laboratory of Plant Breeding, Graduate School for Experimental Plant Sciences, Wageningen University, Droevendaalsesteeg 1, 6708 PB Wageningen, The Netherlands.

Accepted for publication 25 January 2013.

ABSTRACT

Martín-Sanz, A., Pérez de la Vega, M., Murillo, J., and Caminero, C. 2013. Strains of *Pseudomonas syringae* pv. *syringae* from pea are phylogenetically and pathogenically diverse. *Phytopathology* 103:673–681.

Pseudomonas syringae pv. *syringae* causes extensive yield losses in the pea crop worldwide, although there is little information on its host specialization and its interactions with pea. A collection of 88 putative *P. syringae* pv. *syringae* strains (including 39 strains isolated from pea) was characterized by repetitive polymerase chain reaction (rep-PCR), multilocus sequence typing (MLST), and *syrB* amplification and evaluated for pathogenicity and virulence. rep-PCR data grouped the strains from pea into two groups (1B and 1C) together with strains from other hosts; a third group (1A) was formed exclusively with strains isolated from non-

legume species. MLST data included all strains from pea in the genomospecies 1 of *P. syringae* pathovars defined in previous studies; they were distributed in the same three groups defined by rep-PCR. The inoculations performed in two pea cultivars showed that *P. syringae* pv. *syringae* strains from groups 1A and 1C were less virulent than strains from group 1B, suggesting a possible pathogenic specialization in this group. This study shows the existence of genetically and pathogenically distinct *P. syringae* pv. *syringae* strain groups from pea, which will be useful for the diagnostic and epidemiology of this pathogen and for disease resistance breeding.

Additional keywords: pea bacterial blight, *Pisum sativum*.

Pseudomonas syringae can be found both as a harmless commensal on leaves and as a plant pathogen, causing disease (mostly foliar necroses) in practically every cultivated plant species (44). *P. syringae* has traditionally been divided into at least 60 pathovars, which are characterized by their host range (6,8,44); as a whole, the species has an extremely large host range, while individual pathovars or strains are typically restricted to one or a small number of host species. *P. syringae* is a highly heterogeneous species, and DNA-DNA hybridization experiments allowed its reclassification, together with a few other *Pseudomonas* spp., into at least nine different genomospecies (12), which can also be differentiated by multilocus sequence typing (MLST) (5,30). This heterogeneous group of bacteria is commonly known as *P. syringae* sensu lato (12,44). Evolutionary genetic analyses reveal that, with respect to the core genome, *P. syringae* is a highly clonal and stable species that is endemic within plant populations, yet the genetic variation seen in these genes only weakly predicts host association (35).

Pea (*Pisum sativum* L.) bacterial blight can be caused by both *Pseudomonas syringae* pv. *pisi* and *P. syringae* pv. *syringae* (18). Both pathovars induce identical symptoms in the field and belong to the same genomospecies (genomospecies 1) (12); additionally, MLST analyses indicate that *P. syringae* pv. *pisi* can be separated into two phylogenetic groups, group 1 and group 2, of which

group 1 clusters with strains of *P. syringae* pv. *syringae* from diverse hosts and other pathovars from genomospecies 1 (27,34). However, there are no comprehensive studies that evaluate the biological characteristics and phylogenetic relationships of *P. syringae* pv. *syringae* strains isolated from pea; therefore, it is not clear whether or not these populations represent a homogeneous group of host-specialized strains or, alternatively, if pea is a general plant host for this pathogen. *P. syringae* pv. *pisi* is considered the main causal agent of bacterial blight of field pea and the most damaging, being able to cause disease over a wider range of environmental conditions than *P. syringae* pv. *syringae* (14). However, *P. syringae* pv. *syringae* can be as frequent and damaging as *P. syringae* pv. *pisi* in regions with frost risk when the crop is at seedling stage and under certain circumstances, especially in early sowings (24,33). Disease symptoms are more evident after frost periods, heavy rains, or mechanical damage to the crop, all of which cause plant injuries that facilitate entry of the pathogen and infection initiation. Disease usually starts with the appearance of small water-soaked areas on any aerial plant part that, favored by humid and warm conditions, extend and eventually can lead to plant wilting and death (18). Control of pea bacterial blight is difficult and is mainly based on cultivation of resistant cultivars, crop rotation, and, because the pathogen is transmitted by seed, the use of pathogen-free seed (14). The use of pea cultivars combining bacterial blight resistance and frost tolerance has been described as a useful preventive control strategy (26).

P. syringae pv. *syringae* is a heterogeneous pathovar whose members are collectively able to cause disease in >200 different plant species (4,17). It was described by Bradbury (4) as “a complicated mosaic of slightly different taxa from a large number of different host plants”. One characteristic in common for most

Corresponding author: C. Caminero; E-mail address: camsalco@itacyl.es

*The e-Xtra logo stands for “electronic extra” and indicates that the online version contains three supplemental figures and two supplemental tables.

of the *P. syringae* pv. *syringae* strains is the production of toxic lipodepsinona peptide metabolites, such as syringomycin (38). However, there is a certain degree of host specialization within this pathovar and there are strains classified as *P. syringae* pv. *syringae* that are not pathogenic to lilac or lemon fruit, which are considered as common hosts for strains of pv. *syringae* (37). *P. syringae* pv. *syringae* as a causal agent of pea bacterial blight was first described in 1966 in Wisconsin (13). The information for this taxon as a pea pathogen is still scarce, and pathogenic races have not been differentiated. In fact, there have been limited studies in relation to the presence or absence of host plant resistance, the genetic diversity of *P. syringae* pv. *syringae* strains isolated from pea, or their pathogenic variability and virulence. The knowledge of the structure and diversity of populations of this pathogen within the different countries is crucial for epidemiological studies, to define quarantine risks posed by exotic strains, and to facilitate the design of efficient strategies for pea bacterial blight control, including disease resistance breeding.

Several culture media as well as chemotaxonomic, metabolic, and molecular tests have been used to ascertain the relationships within *P. syringae* sensu lato (44). Biochemical tests have been traditionally used to differentiate pathovars of *P. syringae* and to evaluate inter- and intrapathovar variability (36). However, such tests cannot accurately discriminate between strains of *P. syringae* pvs. *pisi* and *syringae* pathogenic to pea (24). Diverse polymerase chain reaction (PCR)-generated genetic markers have also been used to distinguish these two pathovars. The most useful are a marker derived from the *syfB* gene, which is present in almost all the *P. syringae* pv. *syringae* strains (24,37), and the AN3 and AN7 markers, found in repulsion in all the *P. syringae* pv. *pisi* strains analyzed (2,24,27). Repetitive DNA PCR-based genomic fingerprinting (rep-PCR) with repetitive extragenic palindromic (REP), enterobacterial repetitive intergenic consensus (ERIC), and BOX primer sets is a sensible, reproducible, and highly discriminatory methodology to assess bacterial diversity at the strain and pathovar level (21,22). Several studies have successfully used rep-PCR for genetic characterization of different pathovars of *P. syringae* (10,26,29,37). On the other hand, MLST focuses strictly on the bacterial core genome. This highly accurate and reproducible approach uses the DNA sequences from housekeeping genes to differentiate strains and clonal lineages. MLST has been used to study genetic diversity and evolution in *P. syringae* sensu lato and to identify pathovars and strains within this species (5,10,16,26,35,39).

The objectives of this study were to (i) assess the genetic and pathogenic diversity of a collection of *P. syringae* pv. *syringae* strains from different countries and hosts, including a representative set of strains isolated from pea; (ii) investigate the relationships between *P. syringae* pv. *pisi* and *P. syringae* pv. *syringae* strains isolated from pea, and also in relation to other pathovars and *P. syringae* pv. *syringae* strains isolated from different host species; (iii) analyze the relationship between the genetic patterns and the virulence of the strains in two pea cultivars; and (iv) clarify whether the strains isolated from pea in Spain originate from one or several inoculum sources.

MATERIALS AND METHODS

Bacterial strains. A collection of 88 putative *Pseudomonas syringae* pv. *syringae* isolates and strains was used, including 29 isolates obtained from infected fields in the main pea-producing area of Spain (24) and 10 strains isolated from pea in other countries. The remaining 49 strains were included as a representation of the diversity of this pathovar, including the pathotype strain (CFBP 1392, the same as LMG 1247). The strains and isolates used in this study include a representation of 19 countries and 30 hosts (both woody and herbaceous host plants), and they are described in detail in Supplemental Table 1. Bacteria were

routinely grown on King's B medium (KB) plates and preserved in media with 15% glycerol at -75°C for long-term storage. For simplicity, all strains from collections and Spanish isolates will be hereafter referred as strains.

Strain identification. Strains were identified according to a classical methodology (36), including grouping by gram reaction using the KOH test; Hugh-Leifson oxidation/fermentation test; fluorescence on KB medium; levan production, oxidase reaction, potato soft rot, arginine dihydrolase activity, and tobacco hypersensitivity (LOPAT) test (19); the use of homoserine, betaine, D-tartrate, erythritol, and trigonelline as carbon sources; and aesculin and gelatine hydrolysis. Duplicate tubes or plates were used for each test, and tests were repeated at least twice.

The AN3 (132 bp) and AN7 (272 bp) markers and the *syfB* gene marker (752 bp) were PCR amplified using specific primer pairs (2,38) and DNA from well-isolated colonies as template. Each amplification reaction was carried out at least twice. Negative controls (sterile deionized water) and positive controls (*P. syringae* pv. *syringae* pathotype strain CFBP 1392 for *syfB*; type strains of race 1, HRI-W 299A, and race 2, HRI-W 202, of *P. syringae* pv. *pisi* for AN3 and AN7, respectively) were included. Gene *syfB*, whose product is involved in the production of cyclic lipodepsinona peptides, is present in *P. syringae* pvs. *atrofaciens*, *aptata*, and *syringae* but not in others such as *P. syringae* pvs. *pisi* or *phaseolicola*. AN3 and AN7 markers are specific to *P. syringae* pv. *pisi*.

rep-PCR. Strains were grown on KB plates at 25°C for 48 h and genomic DNA was purified using the Qiagen DNeasy Tissue Kit following the manufacturer's instructions. The concentration of DNA was estimated by electrophoresis of 2 μl of DNA suspensions, together with a DNA marker, on a 1% agarose gel with $1\times$ Tris-acetate-EDTA (TAE) buffer and stained with ethidium bromide. Total genomic DNA was adjusted to a final concentration of 20 ng/ μl and stored at -20°C before use. rep-PCR with REP-PCR, ERIC-PCR, and BOX-PCR primers was performed as described previously (32). PCR products were separated by electrophoresis in 1.5% agarose gels in $0.5\times$ TAE and visualized under UV light after staining with ethidium bromide. Strains were tested at least three times with each rep-PCR method to ensure reproducibility of the DNA fingerprints.

MLST. MLST was performed with partial sequences of *gapI*, *gyrB*, and *rpoD* genes coding for glyceraldehyde-3-phosphate dehydrogenase, DNA gyrase B, and σ factor 70, respectively. Gene fragments were amplified from genomic DNA of 16 *P. syringae* pv. *syringae* strains, which represented the rep-PCR variability of the collection, with the primers described by Sarkar and Guttman (35) and according to the PCR procedure described previously (10). PCR products were purified with the QIAquick PCR purification kit (Qiagen, Hilden) following the manufacturer's instructions and sequenced using a Megabace 500 (Amersham, Freiburg) sequencer. Sequences were edited, aligned, and compared with other *Pseudomonas* sp. sequences (35) obtained from the National Center for Biotechnology Information database and the Plant-Associated Microbes Database (1) (Supplemental Table 2) by using the MEGA5 program (41). Additional *P. syringae* sequences were selected to include other groups of strains and genomospecies from *P. syringae* sensu lato (5,35). Furthermore, *P. syringae* pv. *pisi* sequences described in previous research (27,35) were included for comparison of the two main *P. syringae* pathovars that infect pea.

Data analysis. The size of the fragments amplified by rep-PCR was estimated using a DNA 100-bp size-ladder (DNA Molecular Weight Marker XIV; Roche, Mannheim). Band presence or absence was scored as a 1 or 0, respectively. Only clear and repeatable (in three replicas) bands were scored. The strains were grouped in dendrograms using distance derived from Jaccard similarity indices. Distances were calculated as 1-similarity. Unweighted pair-group method with arithmetic means (UPGMA)

and neighbor-joining (NJ) clustering methods were used to obtain dendrograms by means of the PHYLIP 3.63 package (9), and confidence was estimated by bootstrap with 1,000 resamplings. Population structure was analyzed by means of the Structure program 2.3 (31). For MLST analysis a concatenated data set (1,181 aligned nucleotides) was used to construct dendrograms in MEGA5 using the Tamura 3-Parameter distance (40) and the NJ method to infer the genetic relationships between *P. syringae* strains; confidence levels of the branching points were determined using 1,000 bootstrap replicates. *P. syringae* pv. *pisi* strains with identical concatenated sequences were represented by a single sequence in the dendrogram analyses. The MLST sequences generated were deposited in the EMBL GenBank database with the following accession numbers: *gapA*, HE604351 to HE604365 and HE565700; *gyrB*, HE604381 to HE604395 and HE565702; and *rpoD*, HE604396 to HE604410 and HE565703. These sequences were also submitted to the Plant Associated and Environmental Microbes Database (1).

Pathogenicity tests. Bacterial strains were inoculated on immature lemon fruit (*Citrus lemon* 'Primofioro'), excised bean pods (*Phaseolus vulgaris* 'Ancha Lisa'), and excised pea pods (*Pisum sativum* 'Ucero') according to a previous methodology (37) to assess their host range. Plant material was first surface sterilized with 70% ethanol and rinsed in distilled sterilized water; then, strains were inoculated by depositing 20 µl of a bacterial suspension (10⁸ CFU/ml) onto punctures made with sterilized entomological needles at 10 sites per strain. Ten sites within two fruit or pods were inoculated with each strain. Inoculated lemon fruit, bean pods, and pea pods were kept in a humid chamber at 20°C for 7 days and the infection response was recorded daily,

scoring the presence (water-soaked, necrotic lesions, and so on) or absence of symptoms. Positive controls inoculated with strains P684 (*Pseudomonas syringae* pv. *phaseolicola* race 7; for bean pods), HRI-W 895A (*P. syringae* pv. *pisi* type race 4; for pea pods), and CFBP 1769 (*P. syringae* pv. *syringae*; for lemon fruit) were included.

Subsequently, bacterial strains were inoculated in the stem of 2-week-old seedlings of 'Gracia' and 'Iceberg' pea, susceptible and resistant, respectively, to *P. syringae* pv. *syringae* (24,26), to evaluate their virulence. The inoculation methodology and growing conditions of pea seedlings under controlled conditions were essentially as described previously (26). Stem inoculations were carried out by scraping freshly grown bacteria from the surface of KB plates with the tip of a sterile entomological mounting pin and then stabbing it into the main stem at its junction with the stipules at the youngest node. Nine seedlings per pea cultivar were inoculated with each bacterial strain in three independent replicates (three plants per replica). The response was scored 10 days after inoculation using an arbitrary scale, as follows: 0, small localized necrosis in the infection point; 1, necrotic spots around the infection point not longer than 0.5 cm; 2, necrotic lesions of 0.5 to 1.5 cm; 3, lesions longer than 1.5 cm; and 4, wilting of the seedlings from the inoculation point to the apex; an example of these reactions is shown in Supplemental Figure 3. Strains were classified into five arbitrary virulence categories depending on the averaged values for each cultivar-strain combination, as follows: 0 to 0.5, nonpathogenic (NP); 0.6 to 1.5, weakly virulent (W); 1.6 to 2.5, moderately virulent (M); 2.6 to 3.5, highly virulent (H); and 3.6 to 4, extremely virulent, causing apical death (AD).

TABLE 1. Pathogenic and metabolic variability among and within the groups established by repetitive polymerase chain reaction (rep-PCR) and multilocus sequence typing (MLST) for *Pseudomonas syringae* pv. *syringae* strains isolated from pea and other hosts

Host, reaction ^a	<i>P. syringae</i> pv. <i>syringae</i> strain groups ^b							
	Group 1B				Group 1C			
	Group 1A	Pea	Other hosts	Total	Pea	Other hosts	Total	Total
Pea seedlings								
'Gracia'								
NP	1	0	0	0	0	2	2	3
W	4	3	2	5	8	7	15	24
M	0	8	4	12	3	2	5	17
H	0	10	8	18	0	2	2	20
AD	0	6	11	17	0	0	0	17
'Iceberg'								
NP	5	0	0	0	0	1	1	6
W	0	11	10	21	11	10	21	42
M	0	11	9	20	0	2	2	22
H	0	5	3	8	0	0	0	8
AD	0	0	3	3	0	0	0	3
Lemon fruit								
No reaction	0	0	0	0	3	1	4	4
Lesions >50%	5	7	4	11	3	7	10	26
Lesions >80%	0	20	21	41	5	5	10	51
Bean pods								
Necrotic lesions	3	27	23	50	11	12	13	76
Water-soaked lesions	2	0	2	2	0	1	1	5
Pea pods								
Necrotic lesions	5	18	25	43	10	13	23	71
Water-soaked lesions	0	0	0	0	1	0	1	1
Both lesion types	0	9	0	9	0	0	0	9
<i>syrB</i> amplification	0	27	25	52	10	13	23	75
Assimilation of homoserine ^c	0	24	10	34	0	1	1	35
Total number of strains	5	27	25	52	11	13	24	81

^a Gracia and Iceberg were the cultivars used in the inoculations. Categories of arbitrary virulence were NP, nonpathogenic; WA, weakly virulent; MA, moderately virulent; HA, highly virulent; and AD, extremely virulent, causing apical death. Lesions >50%, progressive lesions on >50% of inoculated sites; Lesions >80%, progressive lesions on >80% of inoculated sites.

^b Classification of the strains in groups 1A, 1B, and 1C was done according to the results obtained in the genetic characterization carried out by rep-PCR (groups A, B, and C) and MLST (genomespecies 1). Columns indicate number of strains showing each trait; strains from groups B and C are shown in two columns, depending on the host from which they were originally isolated.

^c Utilization of homoserine as the sole carbon source.

RESULTS

Identification and characterization of bacterial strains. The 88 strains analyzed in this study were gram-negative, oxidative in Hugh-Leifson medium, positive for levan production and induction of the hypersensitivity response on tobacco leaves, and negative for the presence of oxidase and arginine dihydrolase and for potato rotting (LOPAT IA). Moreover, the majority of the strains (>90%) hydrolyzed aesculin; liquefied gelatin; utilized D-tartrate, trigonelline, and erythritol; and were fluorescent on KB medium. There was variability in the assimilation of homoserine

(42% of positive responses) and betaine (59%). Most of the strains that could utilize homoserine as the sole carbon source were isolated from pea (from Spain, New Zealand, and Australia) and common vetch (*Vicia sativa*) and *Lathyrus* spp. (Spain). Finally, in total, 75 strains amplified the 752-bp fragment of the *syrB* gene and none of them amplified the specific markers for *P. syringae* pv. *psii* (AN3 and AN7).

Inoculation of lemon fruit, bean and pea pods, and pea seedlings showed that 81 strains were pathogenic to at least one of these plant hosts. The inoculation in lemon fruit showed that 77 strains (87.5% of the total) were pathogenic: 26 strains were classified as + (progressive lesions on more than 50% of inoculated sites), and 51 were ++ (progressive lesions on more than 80% of inoculated sites). The most common response observed in the inoculation of bean pods was progressive necrosis (76 strains, 86.4% of the total), followed by nonreaction (7 strains, 7.9%) and water-soaking (5 strains, 5.7%). In the case of the pea pods, the main response was progressive necrosis (71 strains, 80.7%), then a mixture of water-soaking and necrotic symptoms (9 strains, 10.2%), nonpathogenic reaction (7 strains, 7.9%), and water-soaking (1 strain, 1.1%). Seven strains (CFBP 2337, CFBP 6886, NCPPB 2747, NCPPB 2805, NCPPB 3163, NCPPB 3185, and NCPPB 3259) did not amplify *syrB* and were nonpathogenic on lemon fruit, pea and bean pods, and pea seedlings. For this reason, they were not considered to be *P. syringae* pv. *syringae* and not included in further analysis. A summary of the results of some tests for *P. syringae* pv. *syringae* strains isolated from pea and other host species is shown in Table 1. The individual response of the strains to all these tests (biochemical tests, inoculations, and *syrB* amplification) is shown in Supplemental Table 1.

rep-PCR. The rep-PCR analyses yielded a total of 91 clearly observable polymorphic bands of 0.1 to 2.2 kb (Fig. 1). Strains showed a high level of heterogeneity, generating 54 different rep-PCR patterns. REP-, ERIC-, and BOX-PCR generated 31, 34, and 26 polymorphic bands, respectively. The NJ and UPGMA dendrograms elaborated individually with BOX-, REP-, or ERIC-PCR bands and, with all the rep-PCR polymorphic bands, showed consistent trees with a similar topology (Fig. 2; Supplemental Figure 1) but with low bootstrap values. According to these dendrograms and the Structure analysis (Supplemental Figure 2), the strains could be grouped into three main clusters. They have been called as groups 1A, 1B, and 1C, where the letters differentiate the three clusters and the number refers to genomospecies 1. Group 1A is formed by only five strains from three countries (Spain, Australia, and Brazil), and isolated from three hosts (*Solanum lycopersicum*, *Mangifera indica*, and *Piper nigrum*). Group 1B contains 52 strains, which is the majority of the strains evaluated in this study, having a representation of 15 host species from 10 countries. It is worth mentioning that this group includes 27 strains isolated from pea from South Africa (1 strain), Australia (2 strains), New Zealand (3 strains), and Spain (21 strains). Finally, group 1C includes 24 strains from 10 hosts and 10 countries. In total, 11 strains from this group were isolated from pea (8 in Spain, 2 in the United Kingdom, and 1 in France). The pathotype strain CFBP 1392 is located in this cluster. The rep-PCR fingerprints of the nonpathogenic and *syrB*-negative strains (CFBP 2337, CFBP 6886, NCPPB 2747, NCPPB 2805, NCPPB 3163, NCPPB 3185 [isolated from pea], and NCPPB 3259), whose inclusion in *P. syringae* pv. *syringae* was described previously as questionable, were very different from those observed for the remaining strains. These strains were not included in the dendrograms generated.

This assay revealed genetic variability among strains collected from the same plant species. This is the case for the 2 strains isolated from *Syringa vulgaris* (CFBP 1392 and CFBP 1766, placed in groups 1C and 1B, respectively) and the strains from pea, with 11 located in group 1C and 27 in group 1B. In all, the 38 pathogenic *Pseudomonas syringae* pv. *syringae* strains isolated from

Fig. 1. Representative repetitive polymerase chain reaction (rep-PCR) patterns of *Pseudomonas syringae* pv. *syringae* strains performed by repetitive extragenic palindromic-PCR. A 100-bp ladder is included; * indicates pathotype strain.

pea represent 21 rep-PCR patterns: 11 placed in group 1C and 10 in group 1B.

MLST analysis. A subset of 16 strains (10 of them isolated from pea), selected to cover the genetic diversity revealed by rep-PCR, was analyzed by MLST (1,181 aligned nucleotides from three partial gene sequences). Each of these strains showed a different MLST sequence profile. This analysis also clearly discriminated the three groups identified by rep-PCR, and confirmed the existence of a high genetic variability among *P. syringae* pv. *syringae* strains isolated from pea (Fig. 3). Subsequently, in order to study the relation of the *P. syringae* pv. *syringae* strains used in this work with *P. syringae* pv. *pisi* and other *P. syringae* strains, a MLST dendrogram was also generated with the sequences from those 16 *P. syringae* pv. *syringae* strains plus *P. syringae* pv. *pisi* strains from Martín-Sanz et al. (27), other *P. syringae* strains, and pathovars employed in previous studies (5,35) (Fig. 3). All *P. syringae* pvs. *syringae* and *pisi* strains were grouped within a cluster which agreed with genomospecies 1 and the related genomospecies 7 (12), except the strain Ps 9220. Additionally, the *P. syringae* pv. *syringae* strains could be separated into three subclusters, supported by high bootstrap values, which are in agreement with the groups defined previously by rep-PCR (Fig. 2). Subcluster A included two *P. syringae* pv. *syringae* strains clearly discriminated from all other *P. syringae* pvs. *syringae* and *pisi* strains in genomospecies 1; subcluster B was formed exclusively by *P. syringae* pv. *syringae* strains, all of them except one isolated from legume crops; while subcluster C included all the *P. syringae* pv. *pisi* strains and some *P. syringae* pv. *syringae* strains. Within subcluster C, *P. syringae* pv. *pisi* sequences are clearly differentiated into two sets, one including nine strains with the sequence designated *Ppi* 1 (see strain names

in Figure 3 caption) and closely similar sequences, all of which amplified the AN7 marker, and a second one with five strains with sequences designated *Ppi* 2 (Fig. 3), which amplified the AN3 marker and had an MLST nucleotide sequence identical to that of strain *P. syringae* pv. *syringae* P285, also isolated from pea.

Pathogenicity on pea cultivars. A classification of the strains used in this work according to their pathogenic responses in the two tester pea cultivars is shown in Table 1. Stem inoculation of Gracia and Iceberg pea showed that strain NCPPB 2264 and CFBP 1679 were not pathogenic on pea seedlings. Likewise, strain CFBP 1894 was not pathogenic to Gracia but, in contrast was pathogenic to Iceberg. Conversely, strains CFBP 1789, UMAF 2802, UMAF 4002, and UMAF 0081 were pathogenic to Gracia but not to Iceberg. The remaining strains were pathogenic, producing brown necrotic lesions in the inoculation points that moved upward to the apex, sometimes producing AD. There was an important variability in the length of the lesions depending upon the combination of *P. syringae* pv. *syringae* strain–pea cultivar evaluated. The virulence spectrum of the *P. syringae* pv. *syringae* strain collection evaluated in this work is visually represented in Supplemental Figure 3, whereas the response elicited by each strain upon stem inoculation in both pea cultivars is shown in Supplemental Table 1. First, it is clear that the strain collection behaved more aggressively in Gracia than in Iceberg, as was expected, because the first pea genotype was more susceptible to *P. syringae* pv. *syringae* in other studies (24,26). Second, it is interesting that the virulence of the strains toward pea is also correlated with their grouping by rep-PCR and MLST analyses. Thus, strains from groups 1C and 1A seem to be much less aggressive than strains from group 1B; however, a wider strain sample would be needed to confirm this result. This trend was

Fig. 2. Neighbor-joining tree of 81 *Pseudomonas syringae* pv. *syringae* strains based on the combined repetitive polymerase chain reaction (rep-PCR) data. Figures indicate the bootstrap supporting values >50; figures within parentheses indicate the number of strains with the same rep-PCR pattern included in a given branch (2-1 = CFBP 1763 and CFBP 1768; 2-2 = UMAF 6016 and UMAF 6582; 2-3 = NCPPB 3505 and NCPPB 3506; 2-4 = P59 and CRD 02/Z14B; 8 = P74, P125, P207, P275, P487, P488, P526, and P528; 17 = CRD 02/231, CRD 03/199, CRD 04/167, NCPPB 2688, NCPPB 2689, P144, P151, P185, P204, P243, P262, P271, P277, P523, P524, and P525). Each set of repeated rep-PCR patterns was computed as a single pattern. The nomenclature was chosen to coincide with the multilocus sequence typing groups defined for strains of genomospecies 1 (Fig. 3) and, for this reason, the three groups detected by rep-PCR are named 1A, 1B, and 1C.

also observed when only strains isolated from pea were considered. Finally, it is worth mentioning that the most aggressive strains (AD response in Gracia and AD/H in Iceberg) are those isolated not only from pea but also from other hosts such as common lilac, common bean, hairy vetch, and melon.

DISCUSSION

rep-PCR has been widely applied to classify and analyze the genetic differentiation of species within bacterial genera (21,22) and has been useful to assess the diversity of *P. syringae* patho-

Fig. 3. Neighbor-joining tree of the *Pseudomonas* spp. multilocus sequence typing concatenated data set (1,181 bp). *Pseudomonas syringae* pvs. *syringae* and *pisi* are abbreviated as *Psy* and *Ppi*, respectively, and the host of isolation is given next to each strain designation. The four groups and the genomospecies refer to those defined by Sarkar and Guttman (35), Bull et al. (5), and Gardan et al. (12). *P. syringae* pv. *pisi* strains with identical concatenated sequences are represented by a single sequence, as follows: *Ppi* 1 corresponds to *P. syringae* pv. *pisi* strains H5E1, P123, P134, P282, CFBP 4767, CFBP 4485, HRI-W 202, HRI-W 870A, and HRI-W 1704B; *Ppi* 2 corresponds to *P. syringae* pv. *pisi* strains H5E3, P68, CFBP 4746, CFBP 4484, and HRI-W 299A. Vertical lines on the right indicated the three subgroups described within group 2. AN3 and AN7 indicate the specific marker amplified by each set of *P. syringae* pv. *pisi* strains. Type and pathotype strains are indicated by T or PT, respectively. Bold numbers within parenthesis on the right indicated the genomospecies of pathovars other than *syringae* and *pisi*. Bootstrap node support values are indicated. More detailed strain identification is shown in Supplemental Table 2.

vars (10,27,29,37). In the present study, rep-PCR allowed differentiating 54 genetic patterns that clustered 81 *P. syringae* pv. *syringae* strains into three groups (1A, 1B, and 1C) on NJ (Fig. 2) and UPGMA dendrograms and Structure analysis. *P. syringae* pv. *syringae* strains isolated from pea clustered in group 1B (27 strains) and 1C (11 strains), showing a relatively high genetic diversity. However, this probably has a very limited significance because the large majority of the analyzed strains were pathogenic to pea independently of their host of isolation, reflecting the typical wide host range of *P. syringae* pv. *syringae* (4) and indicating that strains of this pathovar might survive in the environment, moving among compatible plant hosts. The very different rep-PCR fingerprints of the seven strains which were nonpathogenic to pea, lemon, or bean supports the possibility that they were misclassified as *P. syringae* pv. *syringae*; therefore, they were not included in further analyses.

The MLST analysis was mainly carried out using sequences from diverse strains isolated from field epidemics in Spain and sequences previously used in other phylogenetic analyses (5,35,39). Our data agree with the general dendrogram topologies described in those works: the *P. syringae* pathovars analyzed in this work are distributed in four clades. All *P. syringae* pv. *syringae* strains are included in genomospecies 1 except strain Ps 9220 which, in our dendrogram and in previous ones (16,35), is included in genomospecies 4 (Fig. 3). It is possible, therefore, that this strain does, indeed, belong to a different pathovar and has not been correctly identified. Likewise, *P. syringae* pv. *syringae* strains are distributed into three clusters (A, B, and C) within genomospecies 1, and *P. syringae* pv. *pisi* strains are included in one of these clusters (C in our case), splitting into two clearly different genetic lineages.

The MLST analysis also agrees with the rep-PCR results and supports the existence of at least three different genetic lineages in *P. syringae* pv. *syringae*. Most of the *P. syringae* pv. *syringae* strains from pea cluster in group B together with other strains of this pathovar isolated from other legume crops. The *P. syringae* pv. *syringae* strains from group C are the most similar to *P. syringae* pv. *pisi* but, although strains from this pathovar amplifying the AN7 marker are discriminated in a subgroup supported with a 100% bootstrap value (Fig. 3), our results showed a very high genetic similarity between *P. syringae* pv. *syringae* strains in cluster C and strains of *P. syringae* pv. *pisi* amplifying marker AN3. For instance, the MLST sequences from strain *P. syringae* pv. *syringae* P285 were identical to those from strains in group Ppi 2 (Fig. 3). Additionally, these sequences cluster very close to those from the recently sequenced strain *P. syringae* pv. *syringae* FF5, isolated from ornamental pear and the pathotype strains from pvs. *aptata* and *atrofaciens*, although these last three pathovars might be synonymous (44). Lack of correlation between genetic proximity and pathovar assignation has already been shown for pvs. *avellanae*, *lachrymans*, and *morsprunorum*, which contain strains belonging to two different genomospecies (12,28), showing that evolutionary convergence of pathogenicity is somewhat frequent in *P. syringae*. However, the occurrence of two genetic lineages of *P. syringae* pv. *pisi* within genomospecies 1 seems to be due, instead, to two independent events of host specialization. Indeed, certain phytopathogenic *P. syringae* with a narrow host range and infecting current monoculture agricultural crops appear to have evolved from ancestors with a broader host range that were adapted to natural mixed plant communities (7). In support of our hypothesis, the widespread distribution of the ability to infect pea among the three *P. syringae* pv. *syringae* genetic lineages suggests that this pathogenic capability is ancestral to genomospecies 1. Remarkably, *P. syringae* pv. *pisi* 1704B (belonging to the AN7 group) has gained numerous type III effectors that are not present within the genomes of five strains from other pathovars of genomospecies 1 (3,20), and at least two of these genes (*hopC1* and *hopZ2*) (3,43) act negatively by pre-

venting infection of bean plants, a common host for the three lineages of *P. syringae* pv. *syringae* (34). *P. syringae* pv. *pisi* and *P. syringae* pv. *syringae* shared 12 type III effector families (3) but there are 7 specific families for *P. syringae* pv. *pisi* and 3 for *P. syringae* pv. *syringae*. The effectors in common could act as general virulence factors in pea while the specific ones could play roles in the diversification of these two closed pathovars. Moreover, these two pathovars are able to produce different phytotoxins, with syringomycin, syringopeptin, and syringolin being exclusively produced by *P. syringae* pv. *syringae* (20,42). Therefore, it is possible that a gain in effector genes by strains of *P. syringae* pv. *pisi* has resulted in a reduction in host range, effectively limiting the occurrence of this pathovar in agricultural ecosystems to the pea crop, although it is known that *P. syringae* pv. *pisi* can affect other plant hosts such as lentil (*Lens culinaris* L.) in controlled conditions, with which it displays a race structure (15). However, it is not known whether there has been a similar massive gain of effector genes in the *P. syringae* pv. *pisi* AN3 group.

Given the complex phylogenetic relationships among the diverse genetic lineages in pvs. *pisi* and *syringae*, is it justified that they are considered different pathovars? Pathovars are defined as a "...set of strains with the same or similar characteristics, differentiated at infrasubspecific level from other strains of the same species...on the basis of distinctive pathogenicity to one or more plant hosts" (9). Although strains of both pathovars produce indistinguishable symptoms in natural field infections, they induce distinctive symptoms in pea and other hosts in inoculations in controlled conditions: *P. syringae* pv. *syringae* produces necrotic symptoms on pea while *P. syringae* pv. *pisi* generates water-soaked lesions (4,24,26,27,37). Likewise, *P. syringae* pv. *pisi* is a pathogen restricted to pea whereas *P. syringae* pv. *syringae* can cause disease in additional hosts. Therefore, we believe that their differential pathogenicity fully justifies the separation of these two pathovars.

The existence of different genetic lineages of *P. syringae* pv. *syringae* and *P. syringae* pv. *pisi* also significantly impact our ability to efficiently differentiate strains from both pathovars. The use of homoserine as the sole carbon source has been traditionally evaluated to distinguish between *P. syringae* pvs. *syringae* and *pisi* strains isolated from pea, because the first pathovar was described as unable to use this amino acid while the second was described as positive (36). However, at least several *P. syringae* pv. *syringae* strains utilized homoserine as the sole carbon source (24; this study). Thus, in total, 34 of the 52 strains included in group 1B (65.4% of the total of this group and 88.9% of the strains from pea) and CFBP 4110, included in group 1C, were positive for this test. The positive strains included in group 1B were isolated from pea and other legume crops in several countries, although group 1B also included strains unable to use homoserine isolated from pea in different countries. Therefore, these data indicate that the assimilation of homoserine is not a reliable test and should not be used as the only criterion to differentiate *P. syringae* pvs. *syringae* and *pisi* strains from pea. In fact, the ability to use homoserine seems to be associated with other genetic characteristics because all pv. *syringae* strains included in group A and 23 of the 24 in group 1C were negative, whereas most of them in B were positive. Thus, we can conclude that inoculation in pea and amplification of AN3/7 (amplified in repulsion from all *P. syringae* pv. *pisi* strains) and *syrB* (amplified from most *P. syringae* pv. *syringae* strains) markers are the most important criteria to differentiate between these two pathovars. Nevertheless, further studies are needed, including the identification of host-specific virulence factors, for the rapid and efficient differentiation of these two pathovars.

MLST and rep-PCR results support a possible linkage between genetic lineages and virulence in two pea cultivars. However, the degree of virulence toward two pea cultivars was not correlated

with the host of isolation, with certain strains isolated from non-legumes showing high levels of virulence to pea. According to the symptoms observed in both lemon fruit and pea seedlings (Table 1), strains in group 1A were the least aggressive and, incidentally, none of them produced a specific amplicon with the *sydB* primers, whereas those in group 1C showed an intermediate virulence. This trend was observed when considering all the strains or only strains isolated from pea. In turn, group 1B appears to contain a larger percentage of highly virulent strains than groups 1A and 1C, although this could be biased by the limited sample size of these two last groups. Additionally, it is possible that the degree of virulence against the two pea cultivars tested is not broadly representative of the intrinsic virulence potential of each strain. In support of this, a few strains show high virulence toward one of the cultivars but only weak virulence toward the other.

Knowledge about the genetic diversity of *P. syringae* pv. *syringae* strains from pea and other hosts and their virulence is pivotal for the selection of appropriate strains to be used in disease resistance testing. The high level of pathogenic and genetic variability found in the populations evaluated in the present study can provide a basis for selecting useful strains to identify and characterize resistance genes in pea. Very aggressive strains from group 1B are good candidates for resistance selection screening in pea germplasm collections and to study host specialization of this pathovar toward pea. Indeed, the highly virulent strain P262 has been used in the search of resistance to *P. syringae* pv. *syringae* in pea and in the genetic mapping of resistance genes (11,26). P262 has the same rep-PCR genetic pattern as 12 other strains isolated from pea (10 from Spain and 2 from New Zealand) and 4 from chickling pea (Spain), and it is also very close to another 5 strains (3 isolated from pea in Spain and 2 in Australia). Considering these data and the information about the resistance to this pathovar in pea described by Martín-Sanz et al. (26), it is clear that future studies are needed to detect more significant pea cultivar-*P. syringae* pv. *syringae* strain interactions, and to know whether it is possible to differentiate pathogenic races, which will be very useful for disease resistance breeding and to provide a clearer picture of the pathogenic specialization of this pathogen.

It is not possible to establish with complete certainty the origin of the *P. syringae* pv. *syringae* strains collected from pea bacterial blight epidemics during the last 10 years in Spain, when growers started to use early (autumn-winter) sowing for the pea crop. However, these results suggest that seed batches contaminated with *P. syringae* pv. *syringae* contributed to the initiation or expansion of these epidemics, when the area devoted to pea increased considerably. This hypothesis is supported by the fact that Spanish strains present a genetic diversity that is comparable with that of strains from other countries, and because this seed-borne pathogen was unknown as a pea pathogen in Spain until approximately the turn of the century. A similar hypothesis has been proposed for the origin of Spanish *P. syringae* pv. *pisi* isolates (27). Because *P. syringae* pv. *syringae* is a polyphagous pathogen, the possibility that cross contamination from other hosts could increase the genetic diversity of the Spanish *P. syringae* pv. *syringae* population in pea cannot be ruled out. This is supported by the fact that this pathogen was recently identified in other grain legumes crops such as common vetch, chickling pea, and grass pea in the same area of Spain (23,25); because four of the five *P. syringae* pv. *syringae* strains from chickling pea have rep-PCR patterns identical to strains from pea; and because strains from common vetch and grass pea are identical to each other and to strains from pea isolated in Spain. All these data stress the need to increase the phytosanitary controls of seed lots and fields of all these crop species to avoid the expansion of the pathogen.

Results from this work provide the foundations for a better understanding of the genetic structure and diversity among strains of *P. syringae* pv. *syringae*; in particular, those isolated from pea.

It is clear that there are genetically and pathogenically distinct strain groups isolated from pea. Furthermore, this study also contributes to further our knowledge on the relationships between the two most important pea bacterial pathogens (*P. syringae* pv. *syringae* and *P. syringae* pv. *pisi*), to design more accurate detection and identification protocol, to identify a possible pathogenic specialization in *P. syringae* pv. *syringae* strains from pea, and to identify appropriate strains for disease resistance testing.

ACKNOWLEDGMENTS

This research was supported by projects ITACYL 2004/845 and GR113, from the Junta de Castilla y León, and RTA 2006-00077-00-00, from the INIA, as well as by an INIA personal Ph.D. fellowship to A. Martín-Sanz. We thank CFBP (France), NCPPB (United Kingdom), A. de Vicente (Universidad de Málaga, Spain), and J. L. Palomo (Centro Regional de Diagnóstico, Junta de Castilla y León, Spain) for providing bacterial strains; and two anonymous reviewers for their valuable comments and suggestions on the manuscript.

LITERATURE CITED

- Almeida, N. F., Y. S., R. Cai, C. R. Clarke, C. E. Morris, N. W. Schaad, E. L. Schuenzel, G. H. Lacy, X. Sun, J. B. Jones, J. A. Castillo, C. T. Bull, S. Leman, D. S. Guttman, J. C. Setubal, and Vinatzer, B. A. 2010. PAMDB, a multilocus sequence typing and analysis database and website for plant-associated microbes. *Phytopathology* 100:208-215.
- Arnold, D. L., Athey-Pollard, A., Gibbon, M. J., Taylor, J. D., and Vivian, A. 1996. Specific oligonucleotide primers for the identification of *Pseudomonas syringae* pv. *pisi* yield one of two possible DNA fragments by PCR amplification: evidence for phylogenetic divergence. *Physiol. Mol. Plant Pathol.* 49:233-245 (Erratum: 1997, 51:213).
- Baltrus, D. A., Nishimura, M. T., Romanchuk, A., Chang, J. H., Mukhtar, M. S., Cherkis, K., Roach, J., Grant, S. R., Jones, C. D., and Dangl, J. L. 2011. Dynamic evolution of pathogenicity revealed by sequencing and comparative genomics of 19 *Pseudomonas syringae* isolates. *PLoS Pathog.* 7:e1002132.
- Bradbury, J. F. 1986. Guide to Plant Pathogenic Bacteria, 1st ed. International Mycological Institute, Kew, UK.
- Bull, C. T., Clarke, C. R., Cai, R., Vinatzer, B. A., Jardini, T. M., and Koike, S. T. 2011. Multilocus sequence typing of *Pseudomonas syringae* sensu lato confirms previously described genomospecies and permits rapid identification of *P. syringae* pv. *coriandricola* and *P. syringae* pv. *apii* causing bacterial leaf spot on parsley. *Phytopathology* 101:847-858.
- Bull, C. T., De Boer, S. H., Denny, T. P., Firrao, G., Fischer-Le Saux, M., Saddler G. S., Scortichini, M., Stead, D. E., and Takikawa, Y. 2010. Comprehensive list of names of plant pathogenic bacteria, 1980-2007. *J. Plant Pathol.* 92:55-592.
- Cai, R., Yan, S., Leman, S., and Vinatzer, B. A. 2011. Reconstructing host range evolution of bacterial plant pathogens using *Pseudomonas syringae* pv. *tomato* and its close relatives as a model. *Infect. Genet. Evol.* 11:1738-1751.
- Dye, D. W., Bradbury, J. F., Goto, M., Hayward, A. C., Lelliott, R. A., and Schroth, M. N. 1980. International standards for naming pathovars of phytopathogenic bacteria and a list of pathovar names and pathotype strains. *Rev. Plant Pathol.* 59:153-168.
- Felsenstein, J. 1989. PHYLIP—Phylogeny Inference Package (version 3.2). *Cladistics* 5:164-166.
- Ferrante, P., and Scortichini, M. 2010. Molecular and phenotypic features of *Pseudomonas syringae* pv. *actinidiae* isolated during recent epidemics of bacterial canker on yellow kiwifruit (*Actinidia chinensis*) in central Italy. *Plant Pathol.* 59:954-962.
- Fondevilla, S., Martín-Sanz, A., Satovic, Z., Fernández-Romero, M. D., Rubiales, D., and Caminero, C. 2012. Identification of quantitative trait loci involved in resistance to *Pseudomonas syringae* pv. *syringae* in pea (*Pisum sativum* L.). *Euphytica* 186:805-812.
- Gardan, L., Shafik, H., Belouin, S., Broch, R., Grimont, F., and Grimont, P. A. D. 1999. DNA relatedness among the pathovars of *Pseudomonas syringae* and description of *Pseudomonas tremiae* sp. nov. and *Pseudomonas cannabina* sp. nov. (ex Sutic and Dowson 1959). *Int. J. Syst. Bacteriol.* 49:469-478.
- Hoitink, H. A., Hagedorn, D. J., and McCoy, E. 1967. Survival, transmission, and taxonomy of *Pseudomonas syringae* van Hall, the causal organism of bacterial brown spot of bean (*Phaseolus vulgaris* L.). *Can. J. Microbiol.* 14:437-441.
- Hollaway, G. J., Bretag, T. W., and Price, T. V. 2007. The epidemiology and management of bacterial blight (*Pseudomonas syringae* pv. *pisi*) of

- field pea (*Pisum sativum*) in Australia: A review. *Aust. J. Agric. Res.* 58:1086-1099.
15. Hunter, P. J., and Taylor, J. D. 2006. Patterns of interaction between isolates of three pathovars of *Pseudomonas syringae* and accessions of a range of host and nonhost legume species. *Plant Pathol.* 55:46-53.
 16. Hwang, M. S. H., Morgan, R. L., Sarkar, S. F., Wang, P. W., and Guttman, D. S. 2005. Phylogenetic characterization of virulence and resistance phenotypes of *Pseudomonas syringae*. *Appl. Environ. Microbiol.* 71:5182-5191.
 17. Kennelly, M. M., Cazorla F. M., de Vicente, A., Ramos, C., and Sundin, G. W. 2007. *Pseudomonas syringae* diseases of fruit trees. Progress toward understanding and control. *Plant Dis.* 91:4-17.
 18. Lawyer, A. S., and Chun, W. 2001. Foliar diseases caused by bacteria. Pages 22-24 in: *Compendium of Pea Diseases*, 2nd ed. J. M. Kraft and F. L. Pfleger, eds. American Phytopathological Society, St. Paul, MN.
 19. Lelliot, R. A., Billing, E., and Hayward, A. C. 1966. A determinative scheme for the fluorescent plant pathogenic *Pseudomonas*. *J. Appl. Bacteriol.* 29:470-489.
 20. Lindeberg, M., Cunnac, S., and Collmer, A. 2012. *Pseudomonas syringae* type III effector repertoires: Last words in endless arguments. *Trends Microbiol.* 4:199-208.
 21. Louws, F. J., Dennos, W. F., Taylor, C., and Bruijn, F. J. 1994. Specific genomic fingerprints of phytopathogenic *Xanthomonas* and *Pseudomonas* pathovars and strains generated with repetitive sequences and PCR. *Appl. Environ. Microbiol.* 60:2286-2295.
 22. Louws, F. J., Rademarker, J. L. W., and de Bruijn, F. J. 1999. The three Ds of PCR-based genomic analysis of phyto-bacteria: diversity, detection and diseases diagnosis. *Annu. Rev. Phytopathol.* 37:81-125.
 23. Martín-Sanz, A., Palomo, J. L., and Caminero, C. 2009. First report of bacterial blight caused by *Pseudomonas syringae* pv. *syringae* on vetch in Spain. *Plant Dis.* 93:1348.
 24. Martín-Sanz, A., Palomo, J. L., Pérez de la Vega, M., and Caminero, C. 2011. Identification of pathovars and races of *Pseudomonas syringae*, the main causal agent of bacterial diseases in pea in North-Central Spain, and the search for resistance. *Eur. J. Plant Pathol.* 129:57-69.
 25. Martín-Sanz, A., Palomo, J. L., Pérez de la Vega, M., and Caminero, C. 2012. Characterization of *Pseudomonas syringae* pv. *syringae* isolates associated with bacterial blight in *Lathyrus* spp. and sources of resistance. *Eur. J. Plant Pathol.* 134:205-216.
 26. Martín-Sanz, A., Pérez de la Vega, M., and Caminero, C. 2012. Resistance to *Pseudomonas syringae* in a collection of pea germplasm under field and controlled conditions. *Plant Pathol.* 61:375-387.
 27. Martín-Sanz, A., Pérez de la Vega, M., Murillo, J., and Caminero, C. 2012. Genetic, biochemical and pathogenic diversity of *Pseudomonas syringae* pv. *pisi* strains. *Plant Pathol.* 61:1063-1072.
 28. O'Brien, H. E., Thakur, S., Gong, Y., Fung, P., Zhang, J., Yuan, L., Wang, P. W., Yong, C., Scortichini, M., and Guttman, D. S. 2012. Extensive remodeling of the *Pseudomonas syringae* pv. *avellanae* type III secretome associated with two independent host shifts onto hazelnut. *BMC Microbiol.* 12:141.
 29. Oguiza, J. A., Rico, A., Rivas, L. A., Sutra, L., Vivian, A., and Murillo, J. 2004. *Pseudomonas syringae* pv. *phaseolicola* can be separated into two genetic lineages distinguishable by the possession of the phaseolotoxin biosynthetic cluster. *Microbiology* 150:473-482.
 30. Parkinson, N., Bryant, R., Bew, J., and Elphinstone, J. 2010. Rapid phylogenetic identification of members of the *Pseudomonas syringae* species complex using the *rpoD* locus. *Plant Pathol.* 60:338-344.
 31. Pritchard, J. K., Stephens, M., and Donnelly, P. 2000. Inference of population structure using multilocus genotype data. *Genetics* 155:945-959.
 32. Rademarker, J. L., and de Bruijn, F. J. 1997. Characterization and classification of microbes by rep-PCR. Pages 151-171 in: *DNA Markers: Protocols, Applications and Overview*. G. Caetano-Anolles and P. M. Gresshoff, eds. John Wiley & Sons, New York.
 33. Richardson, H. J., and Hollaway, G. J. 2011. Bacterial blight caused by *Pseudomonas syringae* pv. *syringae* shown to be an important disease of field pea in south eastern Australia. *Australas. Plant Pathol.* 40:260-268.
 34. Sarkar, S. F., Gordon, J. S., Martin, G. B., and Guttman, D. S. 2006. Comparative genomics of host-specific virulence in *Pseudomonas syringae*. *Genetics* 174:1041-1056.
 35. Sarkar, S. F., and Guttman, D. S. 2004. Evolution of the core genome of *Pseudomonas syringae*, a highly clonal, endemic plant pathogen. *Appl. Environ. Microbiol.* 70:1999-2012.
 36. Schaad, N. W., Jones, J. B., and Chun, W. 2001. *Laboratory Guide for Identification of Plant Pathogenic Bacteria*, 3rd ed. American Phytopathological Society, St. Paul, MN.
 37. Scortichini, M., Marchesi, U., Dettori, M. T., and Rossi, M. P. 2003. Genetic diversity, presence of the *sybB* gene, host preference and virulence of *Pseudomonas syringae* pv. *syringae* strains from woody and herbaceous host plants. *Plant Pathol.* 52:277-286.
 38. Sorensen, K. N., Kim, K. H., and Takemoto, J. Y. 1998. PCR detection of cyclic lipodepsinonapeptide-producing *Pseudomonas syringae* pv. *syringae* and similarity of strains. *Appl. Environ. Microbiol.* 64:226-230.
 39. Studholme, D. J. 2011. Application of high-throughput genome sequencing to intrapathovar variation in *Pseudomonas syringae*. *Mol. Plant Pathol.* 12:829-838.
 40. Tamura, K. 1992. Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G+C content biases. *Mol. Biol. Evol.* 9:678-687.
 41. Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* 28:2731-2739.
 42. Vinatzer, B. A., Gail, M. T., Lee, M., Jelenska, J., Hotton, S., Fairfax, K., Jenrette, J., and Greenberg, J. T. 2006. The type III effector repertoire of *Pseudomonas syringae* pv. *syringae* B728a and its role in survival and disease on host and non-host plants. *Mol. Microbiol.* 62:26-44.
 43. Vivian, A., and Arnold, D. L. 2000. Bacterial effector genes and their role in host-pathogen interactions. *J. Plant Pathol.* 82:163-178.
 44. Young, J. M. 2010. Taxonomy of *Pseudomonas syringae*. *J. Plant Pathol.* 92:S1.5-S1.14.