# Phylogenetic Evidence for a Diversification of *Pseudomonas syringae* pv. *pisi* Race 4 Strains into Two Distinct Lineages

B. Cournoyer, D. Arnold, R. Jackson, and A. Vivian

Department of Biological Sciences, University of the West of England, Bristol BS16 1QY, United Kingdom.
The current address of B. Cournoyer: Université Blaise Pascal–Clermont–Ferrand II, URA CNRS 1940–BIOMOVE, 63 177 Aubière, France.
This research was supported by the Natural Sciences and Engineering Research Council of Canada, the Gatsby Foundation (United Kingdom), and the Biotechnology and Biological Sciences Research Council (United Kingdom) and was carried out under Ministry of Agriculture, Fisheries, and Food license PHF 678/81 (91) issued under the Plant Health (Great Britain) Order 1987.
We thank M.-C. Grosjean for comments on the manuscript and the SERC Seqnet (Daresbury, United Kingdom) and BIOMSERV (Lyon, France) services for use of their computer network. We thank J. D. Taylor for the cultures of *Pseudomonas syringae* pv. *pisi* from the HRI-Wellesbourne (United Kingdom) collection.
Accepted for publication 19 June 1996.

## ABSTRACT

Cournoyer, B., Arnold, D., Jackson, R., and Vivian, A. 1996. Phylogenetic evidence for a diversification of *Pseudomonas syringae* pv. *pisi* race 4 strains into two distinct lineages. Phytopathology 86:1051-1056.

The emergence of race variants among *Pseudomonas syringae* pv. *pisi* was investigated. *P. syringae* pv. *pisi hrpL*, intergenic sequences between *hrpKL* and *hrpJL*, and partial *hrpK* DNA sequences were used to establish the origin of a race 4-like derivative (PT10) obtained as an IncP1 plasmid transconjugant from a race 6 strain (1704B). DNA sequence comparisons of the *hrpJKL* region from these two strains show 10 substitutions, including silent and nonsilent substitutions in *hrpL*, and others in intergenic regions. Phylogenetic analysis of these sequences (DNA and deduced amino acids), including homologs from other *P. syringae* pathovars, group the lineages corresponding to the *P. syringae* pv. *pisi* sequences and cluster them with the *P. syringae* pv. *syringae* sequence outside a cluster corresponding to the *P. syringae* pv. *phaseolicola*/*P. syringae* pv. *morsprunorum* sequences. The race 4 derivative (PT10) is part of a lineage different from the race 6 strain. A *hrpJKL* polymerase chain reaction/restriction fragment length polymorphism method was used to identify environmental strains that correspond to the PT10 race 4-like population. Phylogenetic analysis including partial *hrpL* sequences from the race 4-type strain (895A), the race 4-like seed strains, and all the other *P. syringae* homologous sequences available show a divergence of the race 4 strains into two distinct lineages: a type-strain lineage and a PT10-like lineage.

## RÉSUMÉ

Le phénomène de changement/modification du statut des races chez *Pseudomonas syringae* pv. *pisi* a été étudiée. Le gène *hrpL*, les intergènes *hrpKL* et *hrpJL*, et une séquence partielle du gène *hrpK* ont été utilisés dans l'objectif d'expliquer l'émergence d'une souche bactérienne de race 4 (PT10) après transfert conjugatif d'un plasmide IncP1 chez un isolat de race 6 (1704B). La comparaison des séquences d'ADN *hrpJKL* entre ces souches montre la présence de dix substitutions incluant des substitutions silencieuses et nonsilencieuses au sein de *hrpL*, et des substitutions dans les régions intergènes. Les analyses phylogénétiques faisant usage de ces séquences (ADN et acides aminés déduits), et des gènes homologues disponibles chez les autres pathovars de *P. syringae*, regroupent les séquences de *P. syringae* pv. *pisi* et les groupent avec la séquence de *P. syringae* pv. *syringae* à l'extérieur d'un groupe correspondant aux séquences de *P. syringae* pv. *phaseolicola* et *P. syringae* pv. *morsprunorum*. La souche de race 4 (PT10) fait partie d'une lignée différente de celle de la souche mère (1704B). Une méthode d'amplification de la région *hrpJKL* par la réaction en chaîne de la polymérase suivi d'une analyse du polymorphisme de longueur de fragments de restriction pour l'identification d'isolats environnementaux semblables à PT10 a été élaborée. Les analyses phylogénétiques effectuées avec des séquences *hrpL* partielles des isolats identifiés selon cette méthode comme étant de type PT10, de la souche type de la race 4 (895A), et des autres séquences de *P. syringae* homologues disponibles ont permis d'observer la diversification de la race 4 en lignées distinctes: une lignée correspondant à la souche type et une de type PT10.

*Pseudomonas syringae* pv. *pisi* is the causal agent of bacterial blight of pea. This disease is characterized by spots on the aboveground plant tissues that are initially shiny and water-soaked and later become darker and necrotic. *P. syringae* pv. *pisi* is a seedborne pathogen that can be carried internally or externally. The use of contaminated seeds can result in severe bacterial blight outbreaks under wet conditions. Differential interactions between strains of the pathovar and cultivars of pea demonstrated the occurrence of physiologic races among *P. syringae* pv. *pisi*. Seven races are currently recognized (2). The pattern of interactions may be interpreted in terms of a gene-for-gene model involving six pairs of genes for avirulence in the pathogen and resistance in the host. This model predicts the outcome of the interactions to be either compatible when disease symptoms, typified by water-soaking, develop or incompatible when a host defense reaction, termed the hypersensitive response (13), prevents development of the disease.

The use of resistant cultivars occasionally appear to favor the emergence of races without a detectable avirulence phenotype or with modified avirulence activities (28). Introduction of plasmid vectors of the IncP1 family was also shown to be associated with the emergence of variants among what were thought to be *P. syringae* pv. *pisi* pure cultures (17). We, thus, decided to study race evolution using IncP1 plasmid-generated variants as models, since their emergence conditions could be duplicated. Analysis of genomic and plasmid DNA restriction fragment length polymorphisms (RFLP) of variants obtained after the introduction of IncP1 plasmids showed massive differences between the profiles, suggesting either extensive gene

Corresponding author: B. Cournoyer
E-mail addresses: cournoyer@cicsun.univ-bpclermont.fr
cournoye@ecosol.univ-lyon1.fr

Publication no. P-1996-0816-01R

rearrangements or selection of previously undetected subpopulations (17).

Here, we present *hrpL* DNA sequences to establish the genetic proximity of *P. syringae* pv. *pisi* race variants and parent cultures. The gene, *hrpL*, codes for a putative sigma factor phylogenetically related to the $\sigma^{54}$ RNA polymerase subunit (29). This gene is essential for both pathogenicity and induction of the plant hypersensitive response. The presence of relatively conserved and variable domains between these sigma factors make *hrpL* a good candidate for the study of phylogenetic relationships between phytopathogenic bacteria. Furthermore, the broad distribution of this gene among *P. syringae* suggests a common origin. The rRNA sequences are too conserved to study closely related individuals (19). Phylogenetic analysis using *hrpL* sequences from *P. syringae* pv. *pisi* variants and field strains clearly showed that race 4 derivatives obtained from apparently pure cultures were subpopulations rather than genetically rearranged strains. This suggests the presence of subpopulations among cultures that can live in intimate association without affecting the interaction with pea plants.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** *P. syringae* pv. *pisi* strains used are described in Table 1. *Escherichia coli* strain DH5α is described elsewhere (5). *P. syringae* pv. *pisi* cultures were either grown in Luria-Bertani's broth (16) or on King's B plates (12). Fluorescence phenotype was determined according to Moulton et al. (17).

**DNA manipulations.** All DNA manipulations were performed according to Sambrook et al. (25). *E. coli* DH5α (5) was used for all pBluescript- (26) and pUC18-derived (20) DNA clonings. Polymerase chain reaction (PCR) was performed according to Simonet et al. (27). Primers L7 (ATTTTCATAGGACGATTCTG of the *hrpJKL* nucleotide sequence of Xiao et al. [29]; position 341 to 368) and L8 (GGAGAACTGGATATACGCAT; position 1,238 to 1,219) were used to amplify the full *hrpL* gene. Annealing was performed at 55°C. The L1 (ACCTGGTTGTGTGGCATTGC; position 807 to 826) and L2 (CCGTGAGCGGACGGTGCC; position 1,094 to 1,077) primers were used to amplify an internal region of *hrpL*. Annealing was performed at 60°C. The *hrpL* PCR fragments were visualized after agarose gel electrophoresis (according to Simonet et al. [27]) and were extracted and purified using the Qiaex kit (Qiagen, Hybaid, Cambridge). The PCR fragments were then cloned in DH5α using a PCR cloning kit (Pharmacia, St. Albans, United Kingdom). Plasmid extractions and plasmid DNA sequencing were performed in both directions according to Jones and Schofield (9).

**DNA sequence analyses.** DNA and deduced amino acids (AA) sequences were aligned using the multiple alignment clustal algorithm (7). Sites involving gaps were excluded from analysis. Evolutionary distances between DNA sequence pairs corrected for multiple substitutions were computed using Kimura's 2-parameter model (10), allowing for unequal rates of transitions and transversions. Evolutionary distances between AA sequence pairs were computed according to Kimura (11). Phylogenetic trees were inferred using both the neighbor-joining (NJ) (24) and maximum-parsimony (MP) (4) methods. Bootstrap analyses were performed to estimate the significance level of the NJ tree internal branches (6). The basic local alignment search tool (BLAST) of Altschul et al. (1) was used to search for similarity with the GenBank database.

## RESULTS

**Full-length *hrpL* DNA sequences and molecular phylogeny.** The *hrpL* gene of *P. syringae* pv. *pisi* race 6 strain 1704B and the derived race 4-like variant strain PT10 was PCR amplified using the L7/L8 primers. PCR was performed directly on bacterial cells (frozen stock), and the annealing temperature was adjusted to obtain good PCR amplification specificity. These PCR products were purified, cloned, and sequenced. Ambiguities were not observed between the various PCR subclones sequenced. The DNA sequences obtained (Fig. 1) for 1704B and PT10 shared 98.9% identity and extended from the complementary DNA sequence of the conserved *P. syringae hrpJ* putative ribosome-binding site and harp box to the beginning of *hrpK*, including the Met-Arg-Ile-Ser-Ser-Ser codons that are conserved among all *hrpK* genes characterized so far. BLAST analysis confirmed the DNA sequence similarities with the *hrpJKL* genes of *P. syringae* (accession numbers U03854, U16817, L36536, L11582, and U03855). Among the 10 substitutions that differentiated the 1704B and PT10 DNA sequences, three were silent, three were nonsilent, and four were intergenic (Figs. 1 and 2). The two *hrpJL* intergenic substitutions were outside the harp box and $\sigma^{54}$ promoter DNA sequences, whereas one of the two substitutions in the *hrpKL* intergene was inside the harp box sequence (Fig. 1). However, this harp box substitution (C for 1704B and T for PT10) was within the tolerance limit of the harp box consensus sequence (3). These observations clearly showed that 1704B and PT10 were different strains, since substitutions at *hrpL* silent sites or in the intergenic spacer were observed, and these could not be related to changes in *hrpL* gene expression nor protein activity that could generate a race 4-like strain from race 6.

Phylogenetic relationships between these *hrpJKL* DNA sequences or their deduced *hrpL* AA sequences and the GenBank *P. syringae* database homologs were inferred by the NJ method using Kimura's estimated evolutionary distances (Table 2) and the MP method. The significance of internal branches of NJ phylogenetic trees was assessed by applying the NJ algorithm to 1,000 bootstrap replicates. Among the DNA and *hrpL* AA sequence data sets, 142 and 20 informative sites, respectively, were observed. Both tree-building methods were applied to both data sets, including DNA and AA sequences, and produced the same tree topology. DNA/AA sequences of PT10 and 1704B *P. syringae* pv.

TABLE 1. *Pseudomonas syringae* pv. *pisi* strains

| Strain[a] | Relevant characteristics[b] | Reference/Source[c] |
|---|---|---|
| 895A | Wild-type race 4 isolated on cv. Martus seed (United States); F | HRI (1975) |
| 1554A | Race 4 isolated on cv. Sprite (United Kingdom); F | MAFF Harpenden (1986) |
| 1629 | Race 4 isolated on cv. Astara (United Kingdom); F | MAFF Harpenden (1986) |
| 1788 | Race 4 isolated on cv. Countess seed (United Kingdom); F | MAFF Cambridge (1987) |
| 1792 | Race 4 isolated on cv. Progreta seed (United Kingdom); F | MAFF Cambridge (1987) |
| 1811A | Race 4 isolated on cv. Green Pearl seed (United Kingdom); F | MAFF Cambridge (1987) |
| 1812A | Race 4 isolated on cv. Spain (United States); non-F | MAFF Cambridge (1987) |
| 1892 | Race 4 isolated on cv. Avola seed (United States); non-F | MAFF Cambridge (1987) |
| PT10 | Race 4-like rifampicin-resistant putative derivative of 1704B ; non-F | Moulton et al. 1993 (17) |
| 1704B | Wild-type race 6 isolated on cv. Stehgolt seed (France); F | MAFF Cambridge (1987) |

[a] *P. syringae* pv. *pisi* designations, except PT10, are Horticulture Research International Culture Collection numbers.
[b] Avirulence phenotypes were determined using pea differential cultivars inoculated according to Moulton et al (17). F = fluorescent.
[c] HRI = Horticulture Research International, Wellesbourne, United Kingdom. MAFF = Ministry of Agriculture, Fisheries, and Food, United Kingdom.

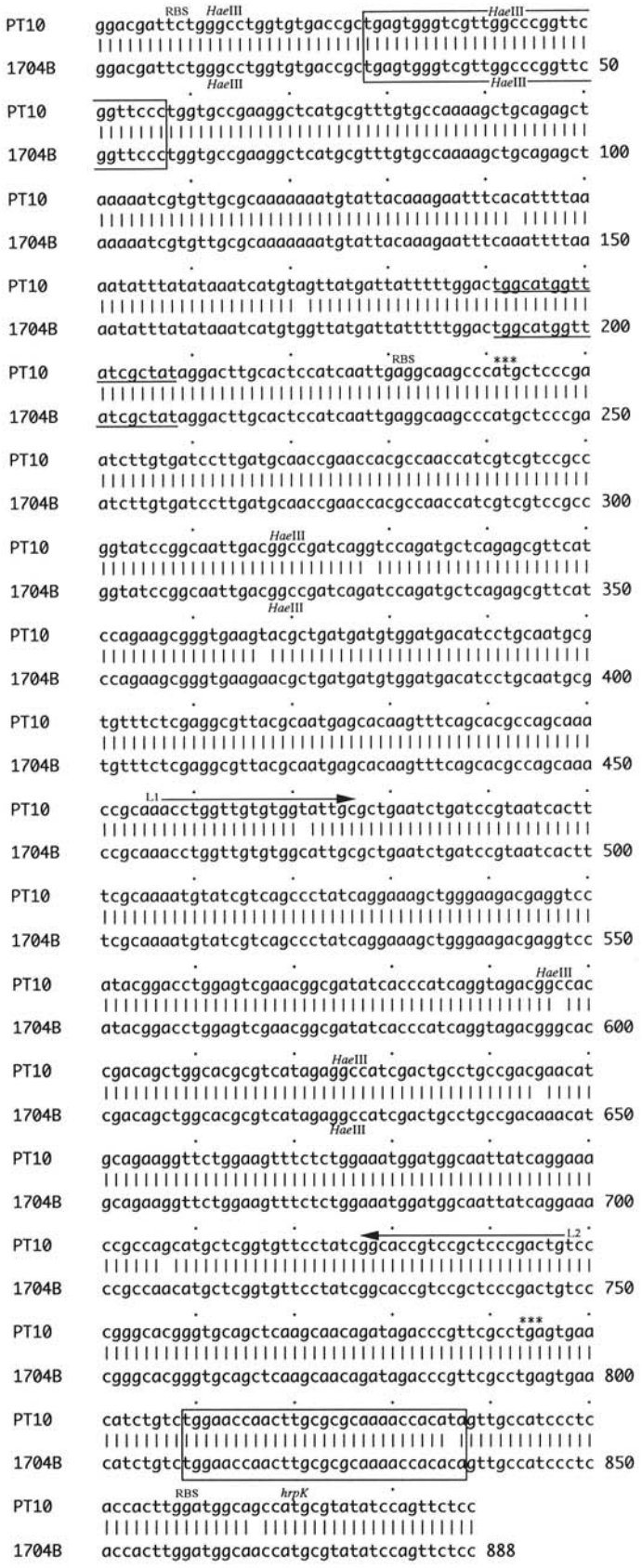


**Fig. 1.** Bestfit analysis of *Pseudomonas syringae* pv. *pisi* PT10 and 1704B *hrpJKL* DNA sequences. The *hrpL* start and stop codons are shown (***). The beginning of *hrpK* is shown. The harp box DNA sequences are boxed, a putative $\sigma^{54}$ promoter sequence is underlined, and putative ribosome-binding sites are shown (RBS). The position of L1 and L2 polymerase chain reaction primers are shown (arrows). Identities are illustrated by vertical lines. These DNA sequences share 98.9% identity. The *Hae*III restriction sites are given. The PT10 and 1704B GenBank accession numbers are U52919 and U52918, respectively.

*pisi* strains grouped together and were clustered with the *P. syringae* pv. *syringae* Pss61 DNA/AA sequence outside the *P. syringae* pv. *phaseolicola* and *P. syringae* pv. *morsprunorum* lineages (Fig. 3). The MP analysis identified only one most parsimonious tree for each data set requiring 155 substitutions for the DNA sequence analysis and 22 for the AA sequence analysis. This most parsimonious tree was the same for each data set and was identical to the

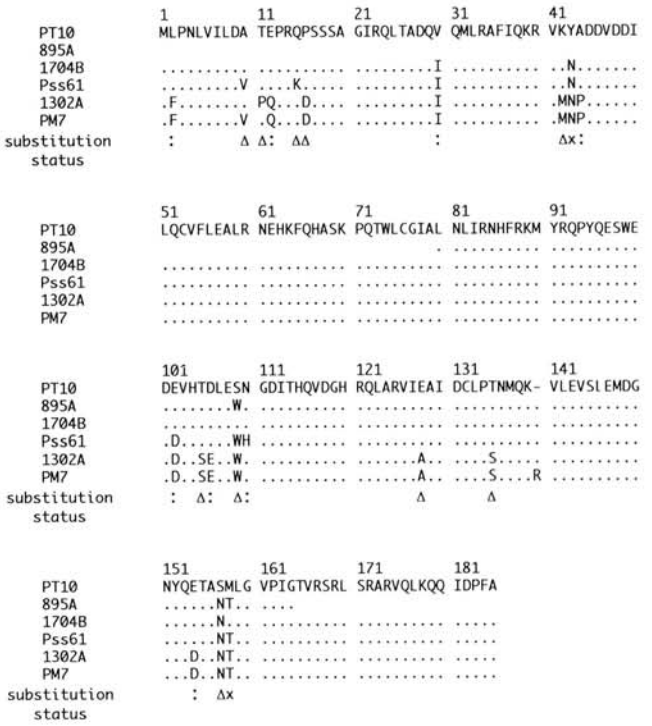


**Fig. 2.** Alignment of deduced *hrpL* amino acids (AA) sequences from *Pseudomonas syringae* pv. *pisi* strains PT10, 1704B, and 895A, *P. syringae* pv. *syringae* Pss61, *P. syringae* pv. *phaseolicola* 1302A, and *P. syringae* pv. *morsprunorum* PM7. Identities are represented by dots. Conservative (:), less-conservative (Δ), and nonconservative (×) substitutions between PT10 *hrpL* AA sequence and the other sequences are shown. A gap was introduced to correctly align all sequences. The PT10, 1704B, and 895A GenBank accession numbers are U52919, U52918, and U52920, respectively. The GenBank accession numbers for the other DNA/AA sequences are as follows: Pss61 numbers are U03854 and U03855 (29); 1302A number is U16817 (15), but was revised according to R. Jackson, A. Vivian, and J. Mansfield (*unpublished data*); and PM7 number is L36536 (14). *P. syringae* pv. *pisi* AA sequences were deduced from Figures 1 and 5.

TABLE 2. Kimura's 2-parameters distances (below diagonal) between *Pseudomonas syringae hrpJKL* DNA sequence pairs and Kimura's empirical method corrected distances (above diagonal) between full-length deduced *hrpL* amino acids sequence pairs[a]

| Bacterial strain | PT10 | 1704B | Pss61 | 1302A | PM7 |
|---|---|---|---|---|---|
| PT10 | – | 1.6 | 5.1 | 9.9 | 9.9 |
| 1704B | 1.3 | – | 3.3 | 8.0 | 8.0 |
| Pss61 | 9.5 | 8.3 | – | 8.0 | 6.8 |
| 1302A | 15.9 | 15.3 | 15.5 | – | 1.1 |
| PM7 | 15.8 | 15.2 | 15.2 | 0.5 | – |

[a] A total of 793 and 184 sites were used for the DNA and the amino acid (AA) data sets, respectively. Homologous sites 1 to 87 and 884 to 888, according to the nucleotide numbering of Figure 1, were excluded from the DNA data set, since they were not available for the *P. syringae* pv. *morsprunorum* strain (PM7). Distances are expressed per 100 sites. PT10 and 1704B are *P. syringae* pv. *pisi* strains and their sequences are presented in Figures 1 and 2. The GenBank accession numbers for the other DNA/AA sequences are as follows: *P. syringae* pv. *syringae* (Pss61) numbers are U03854 and U03855 (29); *P. syringae* pv. *phaseolicola* (1302A) number is U16817 (15), but was revised according to R. Jackson, A. Vivian, and J. Mansfield (*unpublished data*); and *P. syringae* pv. *morsprunorum* (PM7) number is L36536 (14).

NJ tree topology shown in Figure 3. Only the NJ tree from the DNA sequence data set is shown, since its topology was identical to the NJ tree from the derived *hrpL* AA data set. The length of the AA-derived NJ tree branches were different from the DNA-derived NJ tree (Table 2 has a relative estimation), but the same groups were observed: Pss61-1704B-PT10 sequences grouped together outside the other lineages (supported by 100% bootstrap replicates) and the 1704B-PT10 sequences grouped together inside the Pss61-1704B-PT10 cluster for 98% of the bootstrap replicates.

**Characterization of wild-type PT10-like strains.** The above phylogenetic analysis showed that the *hrpL* gene can be used to differentiate closely related strains within *P. syringae*. Analysis of restriction sites among the 1704B and PT10 sequences showed that *Hae*III could differentiate *hrpL* sequences (Fig. 1). A screening for variability among *P. syringae* pv. *pisi* race 4 wild-type strains was, thus, performed to identify a PT10-like strain coming from naturally infected pea seeds or plants. The L7/L8 PCR amplified fragments, digested by *Hae*III, showed the presence of two PCR/RFLP profiles among these strains (Fig. 4). The profiles divided the strains into two groups: group A comprised race 4 895A-like strains (i.e., 895A, 1554A, 1629, 1788, 1792, and 1811A) and group B comprised race 4 PT10-like strains (i.e., PT10, 1812A, and 1892). The race 6 1704B strain had a profile identical to the 895A-like strain (data not shown). To establish more clearly the genetic proximity of these strains, PCR with the L1/L2 primers was performed on 895A, 1812A, and 1892 bacterial cells to amplify an internal *hrpL* segment containing the *Hae*III *hrpL*-differentiation site. These segments were cloned and sequenced. The 1812A and 1892 DNA sequences were identical and shared 95.2% identity with the 895A sequence because of 12 substitutions (Fig. 5), whereas there were only three substitutions differentiating the 1812A/1892 sequences from the 1704B DNA sequence at this *hrpL* region. No difference was observed between PT10 and 1812A/1892 partial *hrpL* sequences.

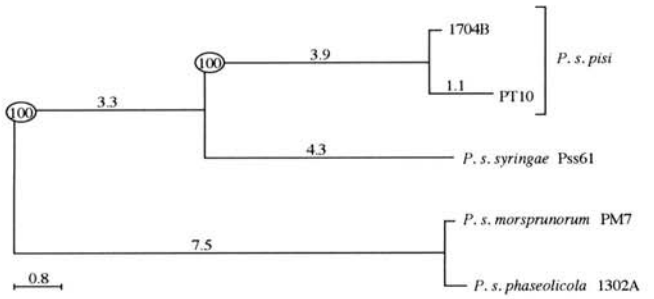


**Fig. 3.** *Pseudomonas syringae hrpJKL* DNA sequences-derived neighbor-joining (NJ) phylogenetic tree. The NJ method (24) was used to construct a tree from Kimura's 2-parameters estimated distances (Table 2). Horizontal distances are proportional to phylogenetic distances expressed in substitutions per 100 sites. Vertical separations are for clarity only. The root-containing branch is arbitrarily divided into two parts. Bootstrap values are indicated in circles.

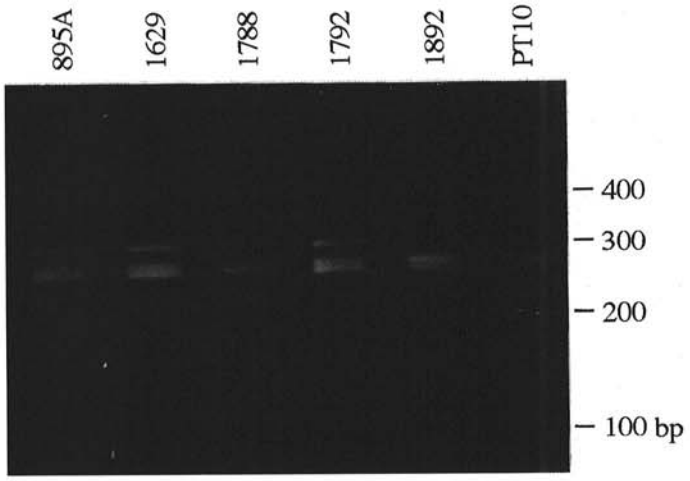


**Fig. 4.** *Hae*III restriction fragments length polymorphisms (RFLP) of a polymerase chain reaction (PCR)-amplified *hrpJKL* region of *Pseudomonas syringae* pv. *pisi* race 4 strains. The PCR primers L7 and L8 were used. Corresponding positions of the first four bands of a 100-bp DNA ladder are shown. Light PCR products below the 250-bp limit are PCR artifacts. The 1554A/1811A and 1812A strains *hrpJKL* PCR/RFLP profiles are not shown but were found identical to those of 895A and PT10, respectively.

Phylogenetic relationships between these partial *hrpL* DNA sequences and their deduced *hrpL* amino acids (AA) sequences and the GenBank *P. syringae* database homologs were inferred as previously described. Kimura's estimated evolutionary distances were used (Table 3) to infer the NJ trees. Among the DNA and partial *hrpL* AA sequence data sets, 47 and 10 informative sites were observed, respectively. Both tree-building methods, NJ and MP, applied on both data sets, DNA and AA, produced the same tree topology. The *P. syringae* pv. *pisi* race 4 PT10, 1812A, and 1892 strains and the race 6 1704B strain sequences grouped together and were clustered with the race 4 895A sequence, confirming the *hrpL* PCR/RFLP division of race 4 into two groups, A (895A-like) and B (PT10-like). This *P. syringae* pv. *pisi* cluster grouped with the *P. syringae* pv. *syringae* Pss61 *hrpL* lineage outside the *P. syringae* pv. *phaseolicola* 1302A and *P. syringae* pv. *morsprunorum* PM7 lineages. However, the MP analysis identified three most parsimonious trees for each data set, DNA and AA, requiring 57 and 10 substitutions, respectively. These MP trees differed only at the PT10-1812A-1892 cluster, because it represented a trichotomy that involves identical sequences. These most parsimonious trees were the same ones for each data set and were identical to the NJ tree shown in Figure 6, considering that the PT10-1812A-1892 sequences were not differentiated by any substitution. Only the DNA data set-derived NJ phylogenetic tree is shown, since its topology was identical to the AA-derived NJ tree and its lineages were resolved at a higher bootstrap level. The length of the AA-derived NJ tree branches were different from the DNA-derived ones (Table 3 has a relative estimation), but the same groupings were observed: i) *P. syringae* pv. *syringae* Pss61 and *P. syringae* pv. *pisi* strains partial *hrpL* AA sequences grouped together and apart from the *P. syringae* pv. *phaseolicola* and *P. syringae* pv. *morsprunorum* sequences (for 100% bootstrap replicates); ii) the *P. syringae* pv. *pisi* PT10-1812A-1892-1704B-895A sequences grouped together inside this *P. syringae* pv. *syringae* and *P. syringae* pv. *pisi* cluster (for 69% bootstrap replicates); and iii) inside the *P. syringae* pv. *pisi* group of sequences, the 895A *hrpL* sequence clearly diverged from the others (for 92% boot-

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                .         .         .         .         .
895A            gctgaatctgatccgcaatcacttccgcaaaatgtatcgccagccctatc
                ||||||||||||||| |||||||| ||||||||||||||| ||||||||||
1812A/1892      gctgaatctgatccgtaatcactttcgcaaaatgtatcgtcagccctatc 50
                .         .         .         .         .
895A            aggaaagctgggaagacgaagtccataccgatctggagtggaacggcgat
                |||||||||||||||||| |||||||| || ||||||| ||||||||||
1812A/1892      aggaaagctgggaagacgaggtccatacggacctggagtcgaacggcgat 100
                .         .         .         .         .
895A            atcacccatcaggtagacggtcaccgacagctggcacgcgtcatagaggc
                |||||||||||||||||||| ||||||||||||||||||||||||||||
1812A/1892      atcacccatcaggtagacggccaccgacagctggcacgcgtcatagaggc 150
                .         .         .         .         .
895A            catcgactgcctgccgacaaacatgcagaaggttctggaagtttctctgg
                |||||||||||||||||| ||||||||||||||||||||||||||||||
1812A/1892      catcgactgcctgccgacgaacatgcagaaggttctggaagtttctctgg 200
                .         .         .         .         .
895A            aaatggacggcaattatcaggaaaccgccaacacgctcggtgttccgatc
                ||||||| |||||||||||||||||||||||||| |||||||||||| |||
1812A/1892      aaatggatggcaattatcaggaaaccgccaacatgctcggtgttcctatc 250
```

**Fig. 5.** Bestfit analysis of *Pseudomonas syringae* pv. *pisi* 895A, 1812A, and 1892 partial *hrpL* DNA sequences. The 1812A and 1892 sequences are identical. Identities are illustrated by vertical lines. 95.2% identity is observed between the 895A and 1812A/1892 sequences. The 895A, 1812A, and 1892 GenBank accession numbers are U52920, U52921, and U52922, respectively.

strap replicates), and the PT10-1812A-1892 sequences were clustered outside the 1704B lineage (for 97% bootstrap replicates).

## DISCUSSION

The *hrpL* gene was proposed to encode an alternative sigma factor that is required for the expression of the *hrp* gene cluster in *P. syringae* (29). These genes are thought to specify components of a type III secretion system essential for pathogenicity and host range (8). Therefore, this essential function must be conserved among *P. syringae* pathovars and the gene sequence should be highly similar (if not identical) between strains that would have emerged from a recent and common parent. Analysis of the full-length *hrpL* sequences and the upstream and downstream intergenes from the parent strain (race 6, 1704B) and the IncP1 plasmid-driven putative variant (race 4-like, PT10) was, thus, performed. These 888 nucleotide-long DNA sequences were differentiated by 10 substitutions: three silent *hrpL* substitutions, three nonsilent *hrpL* substitutions, and four intergenes substitutions located outside the conserved consensus promoter and ribosome-binding site sequences. These major differences showed that these *hrpL* sequences must have diverged prior to the IncP1 plasmid-driven selection that allowed detection of the race 4-like strain or subpopulation. To add further support to this conclusion, the molecular phylogeny of *hrpL* was investigated among *P. syringae*, and phylogenetic inferences about the evolution of this species were drawn.

The molecular phylogeny of *hrpL* DNA sequences combined with the *hrpJK* and *hrpKL* intergenes and of the deduced *hrpL* amino acids sequences using all the available homologs of *P. syringae* were inferred using the NJ and MP methods. The same tree topology was obtained using these methods. The NJ-lineages inferred from both data sets were recovered at high confidence levels. The recovered lineages were as follows: the PT10 and 1704B *P. syringae* pv. *pisi* strains reported DNA/AA sequences grouped together and were clustered with the *P. syringae* pv. *syringae* Pss61 DNA/AA sequences outside the *P. syringae* pv. *phaseolicola* and *P. syringae* pv. *morsprunorum* lineages. These groupings are in line with those reported by Palleroni (21) and based on several observations including rRNA homologies and extensive DNA/DNA hybridization studies among *P. syringae* strains by Palleroni et al. (22) and Pecknold and Grogan (23). Therefore, *hrpL* gene genealogy can at least be considered not to have been affected by lateral transfer events since the emergence of these *P. syringae* pathovars. This genealogy can, thus, be used to investigate the evolution of strains within this species.

Here, we investigated the relationship between a *P. syringae* pv. *pisi* race 6 strain (expressing no avirulence genes), designated 1704B, and a variant strain (PT10) previously thought to be derived from it, designated as race 4-like because of its avirulence phenotype (17). This derivation involved the initial isolation of a spontaneous rifampicin-resistant (Rif-r) mutant (strain PT2) from 1704B and its subsequent mating with *E. coli* carrying the IncP1 plasmid RP4 specifying tetracycline-resistance (Tc-r) to obtain transconjugants (Tc-r and Rif-r). The RP4-containing transconjugants included strain PT4, which was subsequently cured of its RP4 to give strain PT10, which remained Rif-r and avirulent towards *R4*-carrying pea cultivars (17).

Considerable care was taken to ensure that all propagative steps involved the single, nonsectored colonies that should have ensured the genetic homogeneity of the cultures (P. J. Moulton and A. Vivian, *unpublished data*). In consequence, Moulton et al. (17)

TABLE 3. Kimura's 2-parameters distances (below diagonal) between *Pseudomonas syringae* partial *hrpL* DNA sequence pairs and Kimura's empirical method corrected distances (above diagonal) between partial deduced *hrpL* amino acids sequence pairs[a]

| Bacterial strain | PT10, 1812A, 1892 | 895A | 1704B | Pss61 | 1302A | PM7 |
|---|---|---|---|---|---|---|
| PT10, 1812A, 1892 | – | 3.7 | 1.2 | 6.3 | 11.7 | 11.7 |
| 895A | 5.4 | – | 2.5 | 2.5 | 7.6 | 7.6 |
| 1704B | 1.2 | 4.5 | – | 5.0 | 10.3 | 10.3 |
| Pss61 | 12.3 | 8.1 | 11.9 | – | 7.6 | 7.6 |
| 1302A | 17.7 | 16.1 | 17.2 | 14.2 | – | 0.0 |
| PM7 | 17.2 | 15.6 | 16.7 | 14.2 | 0.4 | – |

[a] A total of 250 and 83 sites were used for the DNA and the AA data sets, respectively. Distances are expressed per 100 sites. *P. syringae* pv. *pisi* DNA/amino acid sequences are from Figures 1, 2, and 5. The GenBank accession numbers for the other sequences are as follows: Pss61 numbers are U03854 and U03855 (29); 1302A number is U16817 (15), but was revised according to R. Jackson, A. Vivian, and J. Mansfield (*unpublished data*); and PM7 number is L36536 (14). PT10, 1812A, 1892, 895A, and 1704B are *P. syringae* pv. *pisi* strains. Pss61 is a *P. syringae* pv. *syringae* strain, 1302A is a *P. syringae* pv. *phaseolicola* strain, and PM7 is a *P. syringae* pv. *morsprunorum* strain. The 1812A and 1892 strains partial *hrpL* sequences are identical to the corresponding PT10 *hrpL* sequence.

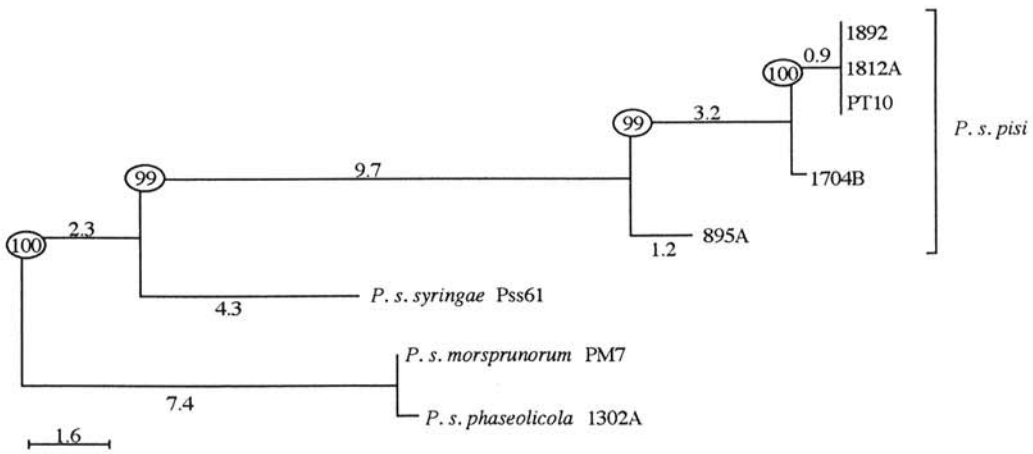


**Fig. 6.** *Pseudomonas syringae* partial *hrpL* DNA sequences-derived neighbor-joining (NJ) phylogenetic tree. The NJ method (24) was used to construct a tree from Kimura's 2-parameters estimated distances (Table 3). Horizontal distances are proportional to phylogenetic distances expressed in substitutions per 100 sites. Vertical separations are for clarity only. The root-containing branch is arbitrarily divided into two parts. Bootstrap values are indicated in circles.

concluded that the race 4-like strain was derived either by spontaneous genomic rearrangement to yield a small subpopulation of race 4-like cells or by some undefined agency of the plasmid RP4. The results presented here show that different *hrpL* genes in 1704B and PT10 refute the hypothesis of a recent and massive IncP1-driven genome rearrangement. Instead, these two strains most likely came from different natural field populations. Because the PT10 strain race 4-like population was shown to inherit IncP1 plasmids at a higher frequency than 1704B (17), this ability presumably favored the emergence of the previously undetected race 4-like population.

These results suggest that PT10-like strains should be observed in the environment and might have already been isolated from pea seeds or plants. A number of *P. syringae* pv. *pisi* race 4 strains were screened and found to be divided into two groups using *Hae*III PCR/RFLP profiles of *hrpJKL*: group A comprised race 4 895A-like strains (including the type-race strain) and group B contained race 4 PT10-like strains. This division matched the fluorescent properties of the strains, group A containing fluorescent strains and group B nonfluorescent ones. To clearly demonstrate that PT10-like strains were observed in the environment and were part of a common phylogenetic lineage that seems to be different from the race 4-type strain, a segment of *hrpL* was amplified for 895A (group A), 1812A (group B), and 1892 (group B) and sequenced. The 1812A and 1892 partial sequences were identical, but showed more overall differences with the 895A sequence than observed between the PT10 and 1704B full-length *hrpL* DNA sequences. No nucleotide difference was observed between PT10, 1812A (PT10-like), and 1892 (PT10-like) partial *hrpL* DNA sequences, showing that these strains are phylogenetically closer to each other than to the other *P. syringae* pv. *pisi* strains characterized so far. Phylogenetic relationships between these partial *hrpL* DNA sequences or their deduced *hrpL* AA sequences and the GenBank database homologs were inferred as previously described. These analyses confirm the groups previously obtained using the full-length *hrpL* DNA sequences and show the presence of at least two race 4 phylogenetic lineages among *P. syringae* pv. *pisi*: the race 4 895A and the race 4 PT10-like (PT10, 1812A, and 1892) lineages. Race 4 PT10-like sequences grouped with the race 6 1704B sequence outside race 4 895A lineage.

It is now clear that PT10 was a subpopulation among the race 6 strain. However, it is quite surprising that this subpopulation could never be isolated without using an IncP1 plasmid. It is also not clear why, if 1704B (which is Rif-sensitive [Rif-s]) was a mixture of two separate Rif-s races (the PT10-like isolates were also Rif-s), it was possible to obtain at the expected frequency a Rif-r culture requiring presumably independent spontaneous mutations in both organisms. Since such mutations can be obtained at a frequency of about $10^{-9}$ Rif-r mutants per Rif-s bacterial cell (18), the expected frequency of two mutations in the mixture assuming approximately equal numbers of cells of each race would presumably be about $10^{-18}$, precluding recovery on plates containing about $10^{10}$ cells. Investigations are underway to attempt to clarify the biology of the relationship between race 4-like strains that are associated with field strains.

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