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DNA Sequence Variation and Phylogenetic Relationships among Strains of *Pseudomonas syringae* pv. syringae Inferred from Restriction Site Maps and Restriction Fragment Length Polymorphism

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We evaluated the restriction fragment length polymorphism of genomic DNA among 53 strains of the phytopathogenic bacterium *Pseudomonas syringae* pv. syringae. Twenty-nine strains were isolated from beans, and the rest were isolated from 11 other hosts. Southern blots of DNA digested with *Eco*RI or *Hin*dIII were hybridized to two random probes from a cosmid library of *P. syringae* pv. syringae and a *hrp* (hypersensitive reaction and pathogenicity) cluster cloned from *P. syringae* pv. syringae. The size of hybridizing fragments was determined, and a similarity matrix was constructed by comparing strains on a pairwise basis for the presence or absence of fragments. The proportion of shared fragments was then used to estimate sequence divergence. Dendrograms were produced by using the unweighted pair group method with averages and the neighbor-joining method. For the *hrp* region, *Bam*HI, *Eco*RI, *Eco*RV, and *Hin*dIII restriction sites were mapped for six representative bean strains and used to construct *Eco*RI and *Hin*dIII restriction maps for all 30 strains pathogenic on beans. Restriction mapping revealed the presence of a 3-kb insertion in nine bean strains and a probable second insertion or deletion event on the left-hand side of the *hrp* cluster that biased estimates of nucleotide sequence divergence from fragment comparisons. This demonstrated that the determination of phylogenetic relationships among bacteria by using restriction fragment length polymorphism data requires mapping restriction sites to remove the effect of insertion or deletion events on the analysis. Our limited data provide support for the hypothesis that strains of *P. syringae* pv. syringae pathogenic to beans belong in a taxonomic group distinct from strains of *P. syringae* pv. syringae pathogenic on other hosts. However, additional independent loci need to be assayed for DNA variation to refine the relationships among such closely related bacteria.

The phytopathogenic bacterium *Pseudomonas syringae* pv. syringae has a host range that includes legumes, cereals, grasses, fruit trees, and woody ornamentals (3). Some diseases caused by this pathogen are bacterial brown spot of bean, bacterial canker of cherry and plum, leaf blight of wheat, and holcus spot of corn. However, it appears that *P. syringae* pv. syringae exhibits host specificity (4, 8, 13, 34, 35). Saad and Hagedorn (35) found that strains of *P. syringae* pv. syringae isolated from *Phaseolus vulgaris* L. (bean) and *Phaseolus lunatus* L. (lima bean) caused disease on greenhouse-grown beans, whereas strains isolated from other hosts produced pod reactions indicative of nonpathogens. Other researchers have also reported that only strains of *P. syringae* pv. syringae isolated from beans induced typical disease symptoms in greenhouse bioassays (8, 34). Because of this, Rudolph (34) proposed naming the pathogen *P. syringae* pv. phaseoli. Cheng et al. (4) found that strains of *P. syringae* pv. syringae isolated from beans caused pathogenic reactions in detached bean pods, whereas strains from other hosts, including lima beans, caused nonpathogenic reactions. However, a strain of *P. syringae* pv. syringae isolated from almond also caused a pathogenic reaction on bean pods.

Determining relationships between strains of *P. syringae* pv. syringae isolated from different hosts is difficult. Greenhouse testing of the host range of phytopathogenic bacteria is often cumbersome because of the many hosts that need to be tested and the ambiguity of disease symptoms in these assays. Pathogenicity tests with *P. syringae* pv. syringae are particularly difficult because strains that do not cause disease on beans in the field can cause symptoms that are indistinguishable from those caused by the bean pathogen in greenhouse tests (4). Although biochemical tests are useful in distinguishing pathovars of *P. syringae* (16), they are not useful in distinguishing strain differences within a pathovar.

Analysis of restriction fragment length polymorphism (RFLP) in chromosomal DNA has been used to determine the phylogenetic relationships among several phytopathogenic bacteria. Lazo et al. (25) reported that groupings of *Xanthomonas campestris* pathovars based upon RFLP data correlated well with classification based upon pathogenicity. Grahman et al. (12) found that strains of *X. campestris* with the ability to grow on citrus leaves and produce lesions were closely related by analysis of RFLPs. Strains that did not grow in citrus leaves and cause necrosis were not closely related to those that did. Strongly aggressive strains of *X. campestris* pv. cirumelo, the cause of citrus bacterial spot, were distinguished from less aggressive strains by RFLP

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TABLE 1. Strains of *P. syringae* pv. syringae used in this study

Host of origin	Location collected	Strain	Source
Bean	New York[a]		
	Erie	B86-17, B86-20-3, BBS 9-7, BBS 13-9	D. E. Legard
	Genesee	B86-3, B86-4, B86-24, BBS 6-3, BBS 102-6, BBS 104-1	D. E. Legard
	Oneida	B86-28, B86-29	D. E. Legard
	Ontario	B86-13, BBS 16-5, BBS 22-5, BBS 100-4	D. E. Legard
	Orleans	BBS 36-5	D. E. Legard
	Wayne	B86-11, B86-14, BBS 2-7	D. E. Legard
	Yates	B86-2	D. E. Legard
	Colorado	BBS 32-5	D. E. Legard
	Wisconsin	B86-7, B86-31, B86-37	D. E. Legard
	New York	Pss 6	J. E. Hunter
	Wisconsin	48	S. S. Hirano
	New Zealand	PDDCC 3462	
	France	PDDCC 4916	
Lima bean	New York	B86-33, BBS 108-7, BBS 112-5, Lima 1-1, Lima 2-2, Lima 3-3, Lima 4-4	D. E. Legard
	New York	PDDCC 3890	D. E. Legard
Pear	?	Ps-8	R. S. Dickey
	New York	Ps-40	T. J. Burr
	England	B301D	D. C. Gross
Corn	?	Ps-30, Ps-SD-10	R. S. Dickey
	South Dakota	464	D. C. Gross
Pea	New Zealand	190-38, 209-50-2	M. L. Powelson
Peach	North Carolina	Ps-1, Ps-10	D. F. Ritchie
Almond	California	B-15+	J. E. DeVay
Apple	Washington	W4N95	D. C. Gross
Apricot	California	5D425	D. C. Gross
Cherry	California	5D433	D. C. Gross
Lilac	?	Ps-9	R. S. Dickey
Wheat	Delaware	61	M. Sasser

[a] Strains were collected in the counties listed under New York.

analysis (11, 14). Denny et al. (7) reported that RFLP data on strains of *P. syringae* pv. tomato, a pathogen with a limited host range, revealed that they are closely related, whereas strains of *P. syringae* pv. syringae from different hosts were more distantly related. Hendson et al. (15) tested host range, nutritional characteristics, and RFLP variation of *P. syringae* pathovars tomato, antirrhini, maculicola, and apii. They concluded that these pathovars formed a mosaic of closely related strains in which each subgroup of strains possessed certain characteristics of one or more other subgroups. Cook et al. (5) studied genetic diversity in *Pseudomonas solanacearum* and were able to distinguish races and biovars through analysis of RFLP data. Demezas et al. (6), by using RFLPs, reported that *Rhizobium leguminosarum* bv. trifolii had a high level of genetic diversity within the biovar.

Here we use both RFLP and restriction site map analysis of chromosomal DNA to evaluate the relatedness of strains of *P. syringae* pv. syringae isolated from different hosts to gain insight into the evolution of host specificity.

MATERIALS AND METHODS

Bacterial strains. The strains used in this study are listed in Table 1. All strains were identified as *P. syringae* pv. syringae by biochemical tests described previously (4) and in most cases had been recovered from diseased plants. Most strains used in this study were oxidase negative (24), ice nucleation active at −3.0°C, utilized mannitol, sorbitol, L-lactate, and inositol, produced levan and acid from sucrose; they did not utilize L-tartrate, homoserine, or anthranilate. However, some strains of *P. syringae* pv. syringae were not ice nucleation active (strains PS-30, PS-SD-7, PS-SD-10, Lima 2-2, and Lima 3-3) at −3.0°C, did not utilize L-tartrate (strains PS-8, PS-9, and 190-38), or did not produce levan (strain 61). The 29 strains of *P. syringae* pv. syringae isolated from beans were selected from a large culture collection previously described and confirmed as pathogens on beans by an excised bean pod assay (4). Bean strains with the preface B86 were collected in 1986, those with the preface BBS were collected in 1987 (BBS 2-7 through BBS 36-5) and 1988 (BBS 100-4 through 104-10), and strain Pss 6 was collected in 1985. Cultures were grown on Kings' medium B (21) and stored in 15% glycerol at −80°C.

Preparation of total genomic and plasmid DNA. Bacteria were grown overnight in LB medium in a shaking water bath at 28°C. DNA was prepared by a modification of the method of Lazo et al. (25) as follows: 500 to 600 μl of overnight culture was transferred to a 2.0-ml Eppendorf microcentrifuge tube. Cells were pelleted by spinning for 3 min in a microcentrifuge, and the supernatant was poured off. The cell pellet was resuspended in 950 μl of TEN (50 mM Tris, 50 mM EDTA, 150 mM sodium chloride [pH 8.0]) with proteinase K (150 μg/ml), and then 45 μl of 20% sodium dodecyl sulfate (SDS) was added. This suspension was incubated at 50°C for 2 to 3 h, extracted twice with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1; phenol equilibrated to pH 7.5 with 0.1 M Tris), and then extracted twice

with an equal volume of chloroform-isoamyl alcohol (24:1). The chloroform-isoamyl alcohol extractions were heated to 56°C for 5 min before being emulsified.

Emulsified suspensions were separated by microcentrifugation at 10,000 × *g* for 8 min, and the supernatant was transferred to a clean Eppendorf microcentrifuge tube. DNA was precipitated by adding 2 volumes of ice-cold 95% ethanol, mixing gently, and incubating at −20°C for 20 min. DNA was spooled out of the ethanol with a sterile capillary tube (with one flame-sealed end), rinsed with 70% ethanol, and then air dried for 10 min. The DNA was resuspended in TE (10 mM Tris, 1 mM disodium EDTA [pH 8.0]) with 10 μg of DNase-free RNase A (United States Biochemical Corp., Cleveland, Ohio) per ml.

Plasmid DNA was prepared by the rapid alkaline extraction method of Birnboim (2).

Digestion of DNA, separation of restriction fragments, and Southern transfer. Total genomic DNA was digested to completion with restriction endonucleases *Bam*HI, *Eco*RI, *Eco*RV, or *Hin*dIII (5 U of enzyme per μg of DNA for 18 h at 37°C) as recommended by the supplier (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). Approximately 4 μg of restriction endonuclease-digested DNA was separated by gel electrophoresis on 0.6% agarose gels in Tris-acetate buffer (40 mM Tris, 20 mM sodium acetate, 2.0 mM EDTA [pH 7.5]) at 1.3 V/cm for 17 to 18 h. Size standards of lambda DNA digested with *Eco*RI or *Bst*eII were also run on each gel. The gels were stained with ethidium bromide (0.5 μg/ml) and photographed. Gels were then irradiated for 2 min on a UV transluminator (Fotodyne, Inc., New Berlin, Wis.), and the DNA was transferred under alkaline conditions for 4 to 6 h to nylon membranes as suggested by the manufacturer (CUNO, Inc., Meriden, Conn.) before the membranes were baked at 80°C for 2 h.

DNA probes. The three DNA probes used in this study were constructed by using the cosmid vector pLAFR3. One DNA probe, pHIR11, has a 30-kb insert that contains the *hrp* region from *P. syringae* pv. syringae 61 (20). The other DNA probes, pDAN7 and pDAN25, were selected at random from a genomic library we constructed of *P. syringae* pv. syringae B86-7. The total genomic DNA of strain B86-7 was partially digested with *Sau*3A as described by Maniatis et al. (31), and DNA fragments in the 20- to 40-kb range were selected for cloning. Linker arms of the cosmid vector pLAFR3 were prepared by digesting an aliquot of the vector DNA to completion with *Hin*dIII and another with *Eco*RI. Both digests were then dephosphorylated with calf intestine alkaline phosphatase as described by Maniatis et al. (31) and ligated to the partially digested genomic DNA. The recombinant molecules were packaged by using commercial lambda extracts (Gigapack Plus; Stratagene, La Jolla, Calif.) and transduced into *Escherichia coli* DH5α. Lambda and probe DNA were radiolabeled with 50 μCi of [^{32}P]dCTP by the random primer method (9). Unincorporated nucleotides were removed by using a minicolumn of Sephadex G50 (1), and the activity of radiolabeled DNA was determined with a scintillation counter.

DNA hybridization. Southern blots were prehybridized at 42°C overnight in hybridization solution (50% formamide, 5× SSC [1× SSC is 0.15 M NaCl plus 0.15 M sodium citrate, pH 7.0], 0.05 M $NaPO_4$, 1% SDS, 100 μg of denatured salmon sperm DNA per ml, 10% polyethylene glycol [average molecular weight, 8,000]). The prehybridization solution was discarded, and hybridization was carried out at 42°C for 18 to 24 h with fresh solution containing the radiolabeled probe added to achieve an activity of 2 × 10^6 cpm/ml. Hybridized membranes were washed by soaking in two changes of 2× SSC–0.1% SDS at room temperature for 15 min each and then soaked in two changes of 0.1× SSC–0.1% SDS at 60°C for 30 min each. After the final wash, membranes were rinsed in 0.1× SSC, blotted dry, wrapped in plastic wrap, and exposed to X-Omat AR film (Eastman Kodak Co., Rochester, N.Y.) at −70°C with intensifying screens (Cornex Lightning-Plus; E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.) or at room temperature without screens. The use of control strains and lambda DNA size standards on each membrane allowed comparisons to be made between membranes.

Each probe was individually hybridized to the membranes, and fragments were scored and then removed (by washing at 42°C for 30 min each in 0.4 M NaOH and then 0.1× SSC and then briefly rinsing in 0.1× SSC at room temperature) before reprobing.

Restriction mapping. Restriction maps of the *hrp* region in strains of *P. syringae* pv. syringae isolated from beans were constructed. The *Bam*HI, *Eco*RI, *Eco*RV, and *Hin*dIII restriction sites were mapped for six strains (B86-3, B86-7, B86-13, BBS 2-7, BBS 9-7, and BBS 22-5) representative of the different RFLP patterns observed. Southern blots of single and double restriction digests of genomic DNA of each strain were hybridized to three individual subclones of pHIR11 (pHIR11BB8 [20], pSH9, and pSH14; provided by S. W. Hutcheson of the University of Maryland). Restriction maps of the *Hin*dIII and *Eco*RI sites for the 30 strains pathogenic on beans were derived by inference from the original fragment data compared with the restriction site information for the six strains that were mapped by single and double digests.

Estimation of sequence divergence and phylogeny. The size of hybridized restriction fragments was determined by comparison with lambda DNA size standards, and strains were evaluated for the presence or absence of hybridized fragments of specific sizes. Data from separate hybridizations of *Eco*RI and *Hin*dIII digests with the three DNA probes were combined for analysis. For fragment data, pairwise comparisons were made by the NTSYS program (Exeter Publishing Ltd., Setauket, N.Y.) by using the following equation: $F = 2n_{xy}/(n_x + n_y)$, where n_{xy} is the number of fragments shared by the two strains and n_x and n_y are the numbers of fragments in strain x and y, respectively. A similarity matrix of F values was constructed using the NTSYS program and converted to base substitutions per nucleotide as described by Nei and Li (32). Sequence divergence among strains of *P. syringae* pv. syringae pathogenic on beans was also estimated from restriction site data by using the following equation of Nei and Li (32): sequence divergence = $-\ln(s)/r$, where s is equal to twice the number of shared restriction sites in the compared strains divided by the sum of the number of restriction sites in the two strains and r is equal to the number of nucleotides in the recognition site of the restriction enzymes used. Cluster analysis was conducted on fragment-derived estimates of sequence divergence, and dendrograms were constructed with the NTSYS program by using the unweighted pair group method with averages (UPGMA) and with the NJTREE program (20a) by using the neighbor-joining method of Saitou and Nei (36).

RESULTS

The two DNA probes randomly selected from the cosmid library of *P. syringae* pv. syringae B86-7 each contained approximately 30 kb of chromosomal DNA (no plasmids

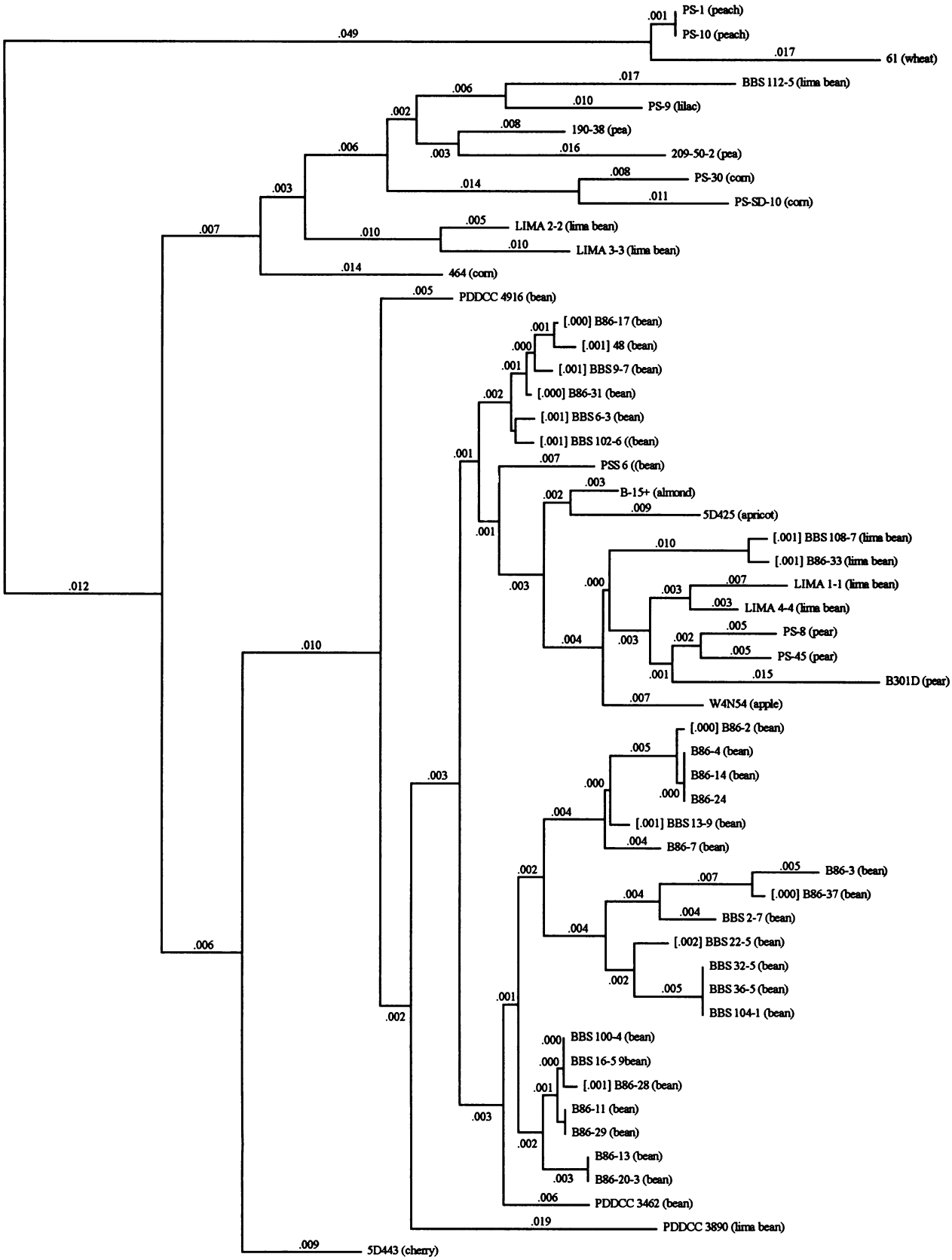

FIG. 1. Dendrogram obtained from cluster analysis of restriction fragments by using the neighbor-joining algorithm. The branch lengths are listed above each branch and are the estimated sequence divergence. Each strain of *P. syringae* pv. syringae was digested with the restriction enzymes *Eco*RI and *Hin*dIII and hybridized to three homologous DNA probes. Strains are listed with the host from which they were originally isolated.

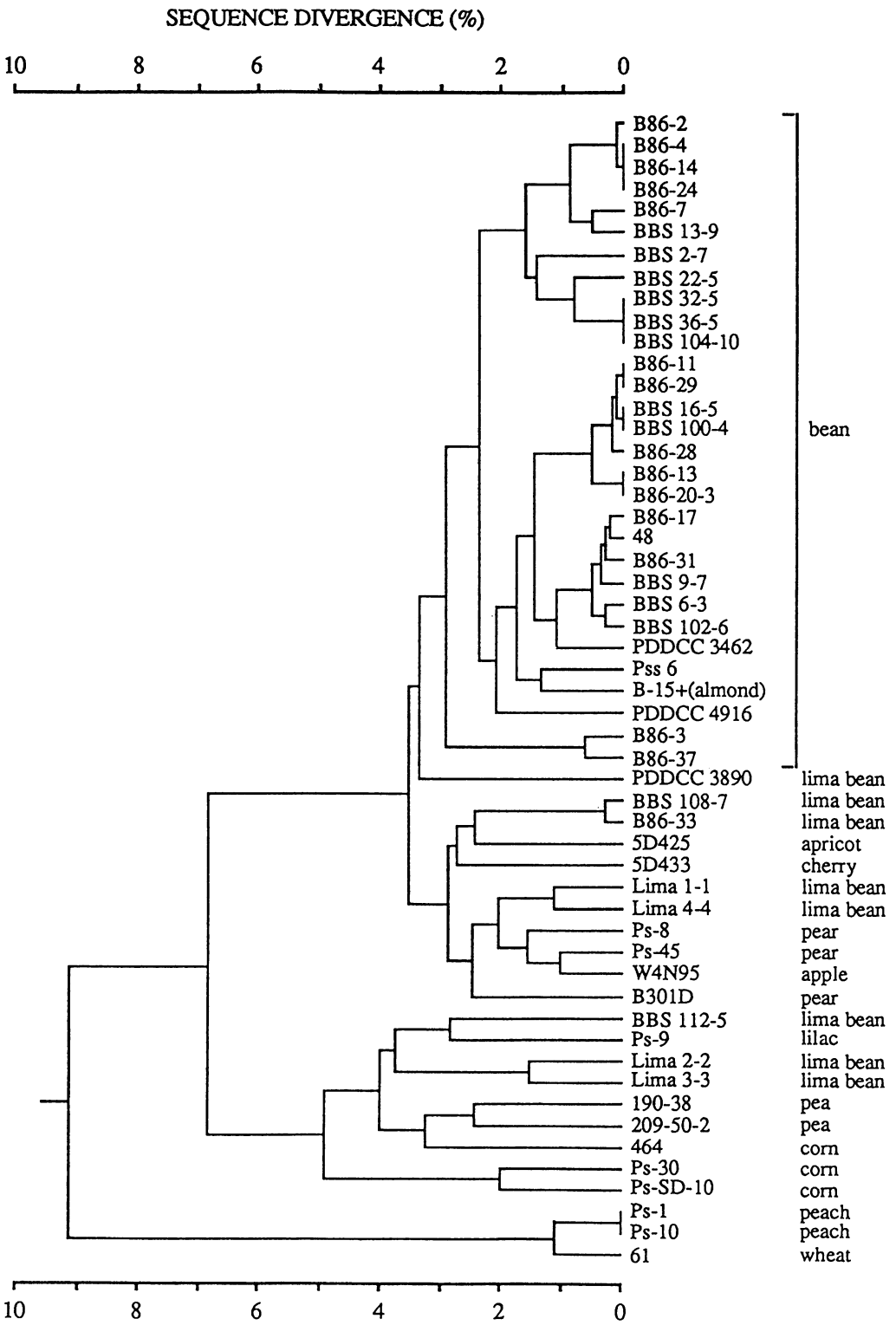

FIG. 2. Dendrogram obtained from cluster analysis of restriction fragments by using the UPGMA. Each strain of *P. syringae* pv. syringae was digested with the restriction enzymes *Eco*RI and *Hin*dIII and hybridized to three homologous DNA probes. Strains are listed with the host from which they were originally isolated.

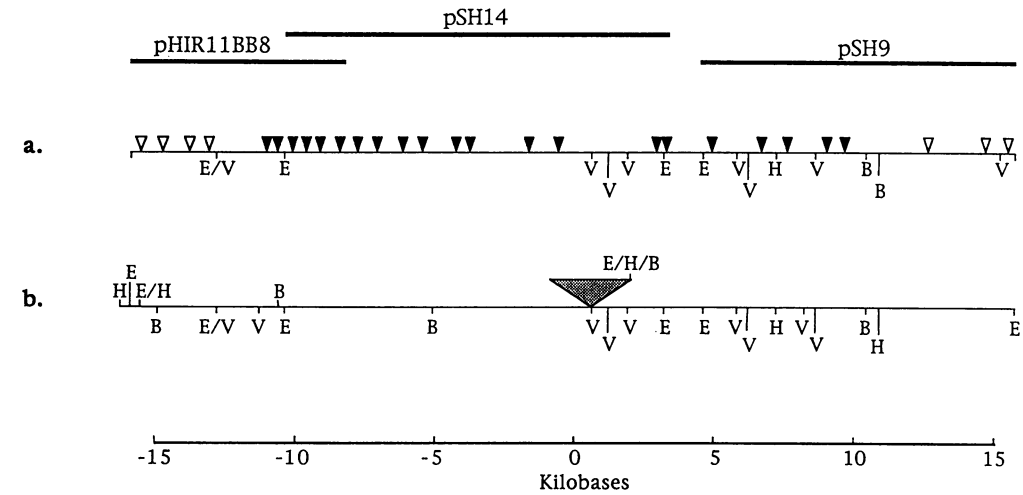

FIG. 3. (a) Restriction map of pHIR11, which contains a *hrp* cluster cloned from *P. syringae* pv. syringae 61. The locations of the three subclones (pHIR11BB8, pSH14, and pSH9) used as probes for mapping restriction sites in this region (solid bars at top), the locations of transposon insertions that abolished *hrp* activity (solid triangles), and the locations of transposon insertions that failed to affect *hrp* activity or only partially abolished *hrp* activity (11) (empty triangles) are indicated. Restriction enzyme site abbreviations: B, *Bam*HI; E, *Eco*RI; V, *Eco*RV; H, *Hin*dIII. (b) Composite restriction map for the 30 strains of *P. syringae* pv. syringae pathogenic on bean. Restriction sites below the plane of the map are conserved in all strains, and sites above the plane are variable. The shaded triangle represents a 3-kb insertion or deletion event found in nine strains.

were found in this strain [26]). The random clones as well as the *hrp* region clone pHIR11 all readily hybridized with *P. syringae* pv. syringae under the hybridization conditions used. For each strain, 22 to 31 *Eco*RI and *Hin*dIII restriction fragments hybridized to the three probes (average, 27 fragments). A complete table of the RFLP character states has been published elsewhere (27). A similarity matrix of proportion of shared fragments was used to estimate (32) base substitutions per nucleotide (sequence divergence) for construction of the dendrograms. Strains of *P. syringae* pv. syringae pathogenic on beans cluster together in the neighbor-joining dendrogram (Fig. 1). However, a cluster containing several strains from other hosts, including lima beans, pears, apples, and apricots, cluster with one group of strains pathogenic on beans before these strains join the main cluster of bean strains. Except for strains isolated from lima beans that formed two divergent clusters, strains from other hosts clustered with other strains from the same host or closely related hosts. In the UPGMA dendrogram (Fig. 2), strains of *P. syringae* pv. syringae isolated from bean clustered together exclusive of strains recovered from other hosts with the exception of strain B-15+, a pathogen of beans originally isolated from almonds.

A composite restriction map of the pHIR11 region for the bean strains B86-3, B86-7, B86-13, BBS 2-7, BBS 9-7, and BBS 22-5 is presented in Fig. 3b. These strains were selected for mapping because their hybridized restriction fragments accounted for the RFLP variation observed among the bean strains. Nineteen restriction sites were conserved among the six bean strains of *P. syringae* pv. syringae. Most restriction site variation was located to the left of the region associated with *hrp* functions (19). Five of the six strains (not BBS 2-7) contained a *Bam*HI site to the left of the 14-kb *Eco*RI fragment, and BBS 9-7 contained a 3-kb insertion with *Eco*RI, *Hin*dIII, and *Bam*HI sites on the right-hand side of the 14-kb *Eco*RI fragment.

The *Hin*dIII and *Eco*RI restriction fragment data for the *hrp* region were also used to extrapolate restriction sites for all 30 strains of *P. syringae* pv. syringae pathogenic on beans. It was found that *Eco*RI and *Hin*dIII restriction sites within the *hrp* functional domain, except for the 3-kb insertion containing single *Eco*RI and *Hin*dIII sites occurring in 9 of 30 strains (B86-17, B86-31, BBS 6-3, BBS 9-7, BBS 102-6, PDDCC 4916, Pss 6, B-15+, and 48), were conserved. In the neighbor-joining dendrogram, these nine strains formed the group of bean strains that clustered separately from the other bean strains in the dendrogram (Fig. 1). In addition, one *Eco*RI and one *Hin*dIII site to the right of the *hrp* region were conserved. However, sites to the left of the *hrp* region were not highly conserved, with two *Hin*dIII sites and four *Eco*RI sites being variable.

Most of the observed variation in the restriction site maps (Fig. 3b) occurred to the left of the *hrp* region. Additional mapping of restriction sites suggested that most if not all of the variation in this region is probably due to insertion or deletion events (26). We were unable to fully resolve the restriction map in this region because most of it lies outside the area of the probe and no adjacent DNA probes were available.

The most parsimonious phylogenetic trees were constructed from restriction site data for the 6 bean strains mapped with four enzymes (Fig. 4a) and for all 30 bean strains mapped with two enzymes (Fig. 5a). The *Hin*dIII (−17.0) and *Eco*RI (−16.5) restriction sites described in these trees could not be scored in most cases because of insertion or deletion events in that region. Estimates of sequence divergence from these site data are presented in Fig. 4b and 5b and ranged from 0.4 to 2.0%. The effects of insertion or deletion events were not included in the calculations of the estimates of sequence divergence.

DISCUSSION

Transposable elements are normal constituents of most bacterial genomes (22), and the mobile nature of these elements causes insertion or deletion events. One basic

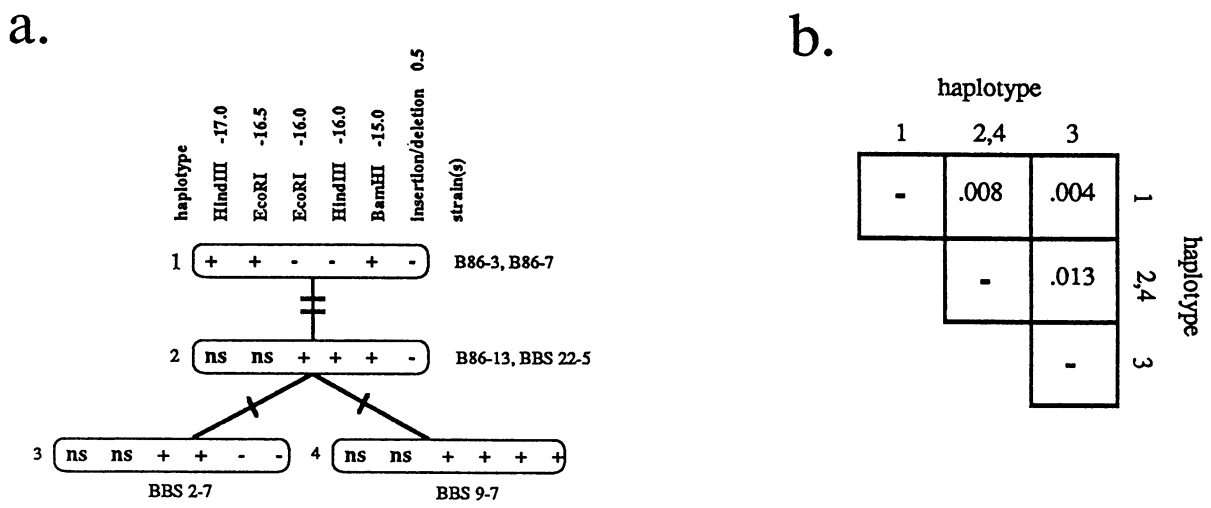

FIG. 4. (a) The most parsimonious phylogenetic tree for six strains of *P. syringae* pv. syringae pathogenic on beans that were mapped for four enzymes in the *hrp* region. Each haplotype is composed of map variants of the listed restriction and insertion or deletion sites corresponding to locations detailed in Fig. 3b. ns, not scored, since outside probed region. (b) Estimates of sequence divergence between the haplotypes described in panel a by using the method of Nei and Li (22). Haplotypes 2 and 4 differ only by an insertion or deletion and are combined for this analysis.

assumption made when restriction endonuclease fragment data are used to estimate sequence divergence and phylogenetic relationships is that changes in fragment size are caused only by base-pair substitutions and not by insertion or deletion events (32). Insertions and deletions can be useful for determining phylogenetic relationships. However, when used in studies to estimate sequence divergence from fragment data, undetected length variation can lead to dramatic overestimation of divergence. In the *hrp* region, we mapped an internal 3-kb insertion or deletion and possibly a second one on the left-hand side that inflated the estimates of sequence divergence based on fragment data. Insertion or deletion events can cause strains with otherwise identical restriction site maps to appear highly divergent when only restriction fragment data are analyzed. While several studies have used fragment data to investigate phylogenetic relation-

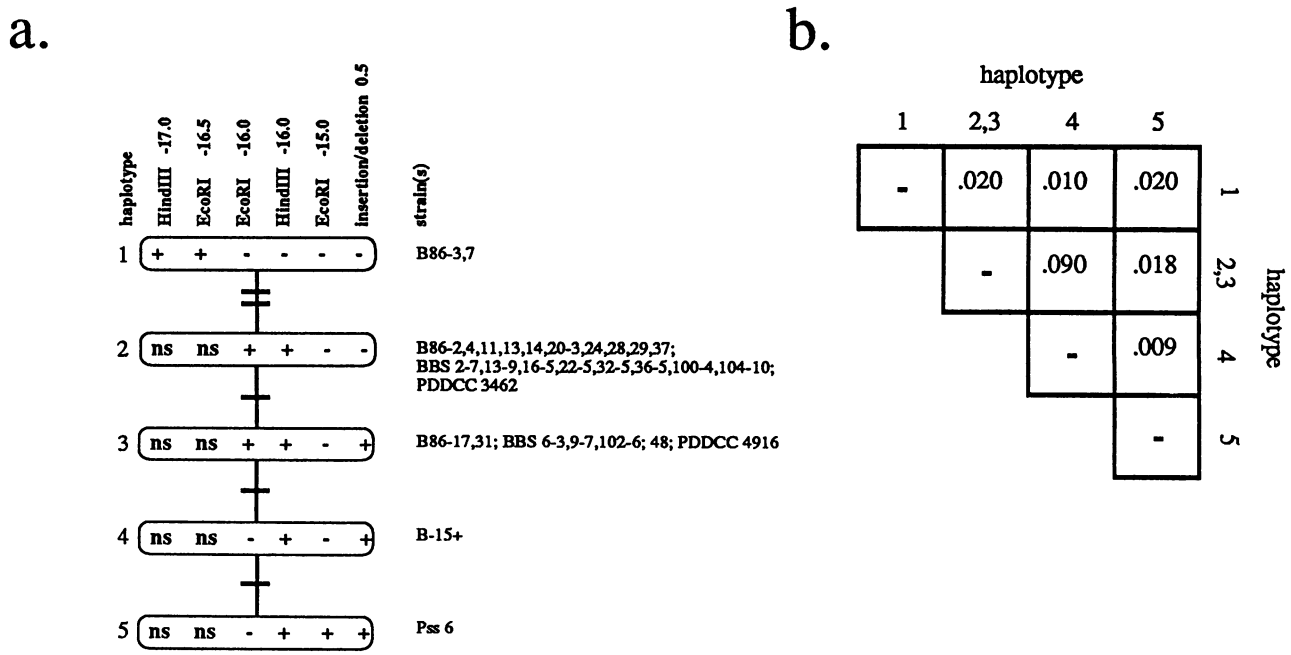

FIG. 5. (a) The most parsimonious phylogenetic tree for the *hrp* region of 30 strains of *P. syringae* pv. syringae pathogenic on beans from which maps were inferred from fragment data with *Eco*RI and *Hin*dIII. The loss of *Eco*RI-16.0 is the result of either a convergent loss or a recombination event. Our data do not allow the explanation to be determined. ns, not scored, since outside probed region. (b) Estimates of sequence divergence between the haplotypes described in panel a by using the method of Nei and Li (22). Haplotypes 2 and 3 differ only by an insertion or deletion event and are combined for this analysis.

ships between bacterial strains (7, 10, 15), our data clearly show the importance for mapping restriction sites when RFLP data are used to estimate sequence divergence and phylogenetic relationships in bacteria.

Despite the limitations in using fragment data to determine phylogenetic relationships, we believe that these results do provide insights into the evolution of strains of *P. syringae* pv. syringae pathogenic on beans. The grouping of bean strains into a single cluster in the UPGMA dendrogram or into three clusters in the neighbor-joining dendrogram supports the hypothesis that the evolution of a pathogenic relationship between beans and *P. syringae* pv. syringae was probably the result of one or a few founding events rather than a large series of founding events that would have occurred if strains of *P. syringae* pv. syringae from other hosts had periodically shifted to a host range on beans. If a large series of founding events had occurred, then we would expect no clustering of the bean strains in the dendrograms. In the neighbor-joining dendrogram, all of the bean strains cluster together with one heterogeneous group of strains from other hosts within the cluster. It is possible that the presence of this group within the bean grouping may be due to the use of fragment data. The low level of sequence divergence among these closely related strains contributes to the difficulty in determining the true phylogeny of this group. The nine strains pathogenic on bean that group with strains from other hosts before clustering with the other bean strains had a 3-kb insertion that mapped inside the *hrp* locus. Insertion or deletion events have also undoubtedly affected our divergence estimates for strains from other hosts. Additional data from direct sequencing of selected representatives identified in this study hopefully would allow the resolution of a single origin for the bean strains. However, unlike the bean strains, the phylogeny of lima bean strains appears to be very scattered (i.e., it may have moved to or from other hosts multiple times), and a single origin may not have occurred.

We found that restriction sites within the *hrp* region of strains of *P. syringae* pv. syringae pathogenic on beans were highly conserved. This is not surprising since this region is associated with pathogenicity functions (18–20), and variation might affect the ability of the bacterium to infect its host(s). Scholz et al. (37) also found that restriction sites in the *hrp* gene region were highly conserved among strains of *P. syringae* pv. tabaci.

Our estimates of sequence divergence inferred from the restriction map data for the *hrp* region (0.4 to 2.0%) are in the same range as those reported by Nelson et al. (33) for isolates of *Salmonella* spp. and *E. coli*. They found an average pairwise difference of 0.24% within serovar groups of *Salmonella* spp. (3.8% between serovar groups) and 0.2% within *E. coli* for the *gapA* gene. Our fragment length data for the three genomic regions together (including *hrp*) suggest greater levels of strain divergence (Fig. 1 and 2), but these estimates may be inflated by insertion or deletion variation.

Because of the influence of insertion or deletion events on the estimation of sequence divergence from fragment data, we constructed dendrograms by using both the neighbor-joining method and the UPGMA. Saitou and Nei (36) reported that the neighbor-joining algorithm is less sensitive to unequal rates of sequence evolution than the commonly used UPGMA. When we used the fragment data, the UPGMA algorithm produced a dendrogram very similar to the neighbor-joining tree, with the exception that in the UPGMA dendrogram the bean strains clustered together exclusive of strains from other hosts. Sorting of ancestral polymorphism among these closely related strains and the resulting potential for different phylogenies among different genes (e.g., 23) will always present a problem when analyzing closely related, polymorphic taxa. Nevertheless, we believe our RFLP data support a previous suggestion (34) that strains of *P. syringae* pv. syringae pathogenic on beans belong in their own pathovar grouping distinct from strains of *P. syringae* pv. syringae pathogenic on other hosts. This is supported by observations on the biology of the organism. Although *P. syringae* pv. syringae is a ubiquitous epiphyte on plants (28, 30), epiphytic *P. syringae* pv. syringae recovered from plants outside bean production regions are not pathogenic on beans (28, 29), and strains of *P. syringae* pv. syringae isolated from other hosts are rarely pathogenic on beans (4). These data support the view that the bean pathogen has evolved a specialized ecological association with the common bean (*P. vulgaris*). However, it will be necessary to assay additional DNA polymorphism at independent loci to more accurately determine phylogenetic relationships between strains of *P. syringae* pv. syringae from beans and those from other hosts.

Strains of *P. syringae* pv. syringae pathogenic on beans from the same geographic region were not more closely related to each other than strains recovered from different locales. For example, of three strains with identical restriction fragment haplotypes, one strain (BBS 32-5) was collected in Colorado, whereas the other two were collected in different regions of New York (BBS 36-5 and BBS 104-1 from Orleans County and Genesee County, respectively). Also, the four strains from Wisconsin (B86-7, B86-31, B86-37, and 48) were as closely related to strains from New York as they were to each other. The lack of a relationship between haplotype and collection location is not unexpected since the pathogen is seed-borne (17), and companies distribute seeds throughout the United States. Therefore, the recovery of strains of *P. syringae* pv. syringae from different locations with an identical haplotype, and different haplotypes from within one region, may be attributed to the introduction of the pathogen on bean seed into these areas. No association was found between the year a bean strain was collected and its haplotype.

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