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Restriction Fragment Length Polymorphism Evidence for Genetic Homology within a Pathovar of *Pseudomonas syringae*

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***Pseudomonas syringae* pv. phaseolicola NPS3121 *hrp* sequences were used as hybridization probes in a restriction fragment length polymorphism (RFLP) analysis of 24 *P. syringae* pv. tabaci strains as a means to evaluate the genetic and taxonomic relationship of pathovars of *P. syringae*. Southern blot analyses of genomic restriction digests, with *hrpA-S* sequences as hybridization probes, and restriction analyses of PCR-amplified DNA of regions within *hrpD* were conducted. The resulting RFLP patterns were uniform for 23 of the 24 isolates tested, with strain BR2R having a unique pattern. BR2R is a pathogen of bean which was classified as pathovar tabaci because of its ability to produce tabtoxin, but unlike the other 23 tabaci strains in this study, it does not incite disease symptoms on tobacco. When a DNA fragment containing *hrpM* sequences was used as a hybridization probe, the tabaci isolates could be divided into three groups on the basis of the RFLP patterns: BR2R, Pt11528R and Pt113R, and the remaining strains. For all of the above analyses, BR2R shared identical RFLP patterns with *P. syringae* pv. phaseolicola NPS3121, also a bean pathogen which does not cause disease on tobacco. However, BR2R and NPS3121 could be differentiated from each other on the basis of the RFLP patterns from restriction analysis of PCR-amplified DNA of *argF*, while the remaining tabaci strains had a third pattern. These studies indicate that *hrp* genes and *argF* are conserved in strains of *P. syringae* pathogenic to tobacco, suggesting that *P. syringae* strains pathogenic to specific hosts may have a high level of genetic similarity. We believe that these analyses have shown that distinct identifiable genetic differences may be correlated with host range and suggest that such information may be useful for assigning pathovar designations.**

The genetic and taxonomic relationships that exist between strains of *Pseudomonas syringae* are not well understood. Although these bacteria are primarily foliar pathogens, members of this species produce diverse types of disease symptoms including necrosis, chlorosis, galls, and cankers (4, 31). Significantly, they induce hypersensitive reactions on nonhost plants (16). This response is characterized by a rapid localized necrosis of plant tissue at the site of pathogen infection and is believed to be a mechanism of plant disease resistance which limits the multiplication and spread of incompatible pathogens. At least 40 biotypes of *P. syringae* have been differentiated by the host plant on which they cause disease; these biotypes have been designated pathovars (4, 37). Although these pathovars are specialized with respect to host range, they are difficult to differentiate by standard bacterial taxonomic tests, leading to their classification within the single species *P. syringae* (30, 31). However, *P. syringae* pathovars differ not only in their ability to cause disease on different plant hosts and the types of symptoms they incite but also in disease etiology, virulence, and ability to produce chemically distinct phytotoxins (31). This phenotypic diversity suggests that there are genetic differences among many of the *P. syringae* pathovars which cannot be detected by bacterial taxonomic tests.

Mutational analysis of *P. syringae* pathovars has led to the identification of hypersensitive reaction and pathogenicity (*hrp*) genes (35). These genes control the ability of *P. syringae* strains to elicit hypersensitive reactions on nonhost plants and resistant cultivars of susceptible plants, as well as the ability to produce disease symptoms on susceptible host plants. Initially, *hrp* genes were identified in *P. syringae* pv. phaseolicola (20); however, they have been found to be present in a large number of *P. syringae* pathovars, including glycinea, tabaci, syringae, tomato, and pisi (35). *hrp* genes have also been found in other bacterial plant pathogens including *Erwinia amylovora*, *Xanthomonas campestris* pathovars, and *Pseudomonas solanacearum* (35). The precise functions of *hrp* genes have not been determined; however, recent studies suggest that they encode products specifically required for the export of metabolites involved in plant-bacterium interactions across the bacterial cell membrane (5, 9), while others have been shown to encode products which function as elicitors of the hypersensitive reaction on nonhost plants (12, 34).

P. syringae pv. phaseolicola NPS3121 *hrp* genes are located within two linkage groups (Fig. 1). The first is approximately 22 kb in size and consists of seven complementation groups: *hrpL*, *hrpAB*, *hrpC*, *hrpD*, *hrpE*, *hrpF*, and *hrpSR* (26, 27). The second group, designated *hrpM*, is approximately 3.7 kb in size and is not linked to the *hrpL-R* cluster (7, 22). Previous hybridization studies showed DNA homology between NPS3121 *hrp* sequences and the genomes of a number of *P. syringae* pathovars (19). Gene replacement experiments also indicated that the NPS3121 *hrp* genes are functionally conserved in *P. syringae* pv. glycinea and pv. tabaci (19). This indicated that DNA sequences from the NPS3121 *hrp* cluster could be used as hybridization probes to detect diversity within homologous regions of other *P. syringae* pathovars.

We have been using strains of *P. syringae* pv. tabaci in studies of host-pathogen interactions and were interested in studying the genetic variability that exists within this pathovar. While a

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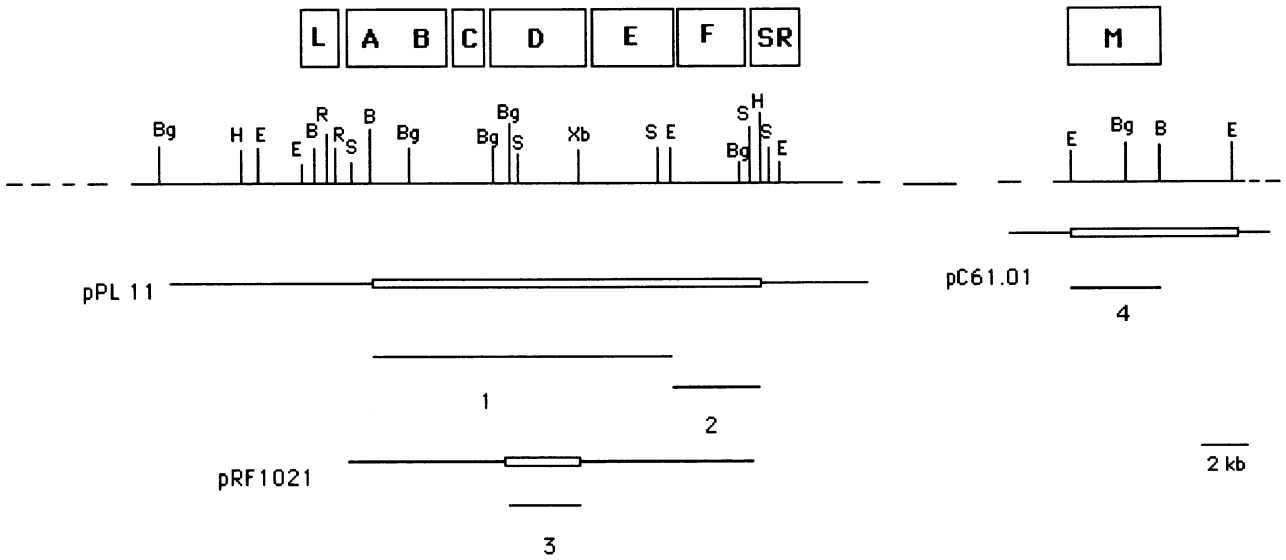

FIG. 1. *P. syringae* pv. phaseolicola NPS3121 *hrp* regions, plasmids, and DNA probes used in this study. The open rectangles at the top and letters within them depict complementation groups as defined previously (7, 26). B, Bg, H, E, S, Xb, and R designate cleavage sites for the restriction enzymes *Bam*HI, *Bgl*II, *Hin*dIII, *Eco*RI, *Sac*I, *Xba*I, and *Eco*RV, respectively. Probes 1 and 2 are the 15.5-kb *Bam*HI-*Eco*RI fragment (*hrpA-E*) and the 4.5-kb *Eco*RI-*Hin*dIII fragment (*hrpF-S*), respectively, from pPL11; probe 3 is the 3.2-kb *Bgl*II-*Xba*I fragment (*hrpD*) from pRF1021; and probe 4 is the 3.7-kb *Bam*HI-*Eco*RI fragment (*hrpM*) from pC61.01. On plasmid diagrams, open regions correspond to cloned *hrp* sequences, and lines represent vector sequences.

number of laboratories have studied the genetic diversity which exists among the *P. syringae* pathovars, only limited information is available about the genetic diversity within each pathovar (17, 23, 24, 31). In this work, we describe a restriction fragment length polymorphism (RFLP) analysis of *P. syringae* pv. tabaci with *hrp* sequences from the closely related pathovar *P. syringae* pv. phaseolicola as hybridization probes. We present data which indicate that strains of *P. syringae* pv. tabaci pathogenic on tobacco are genetically homologous within *hrpA-S* and *argF*, indicating that host range is correlated with a high degree of genetic uniformity within this pathovar of *P. syringae*.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and media. The bacterial strains, plasmids, and phage used in this study are presented in Table 1. The *P. syringae* strains were cultured at 28°C in King's medium B (14), and the *Escherichia coli* strains were grown at 37°C in Luria-Bertani medium (29). Bacto Agar (Difco, Detroit, Mich.) at 1.5% (wt/vol) was added to media for plate cultures. Antibiotics (Sigma, St. Louis, Mo.) were used for selection at the following concentrations (micrograms per milliliter): rifampin, 100; tetracycline, 15; and ampicillin, 50.

DNA extraction. Total genomic DNA was extracted from 100-ml overnight broth cultures of *P. syringae* strains with hexadecyltrimethylammonium bromide (CTAB) as described in Ausubel et al. (1). Plasmid DNA was extracted by an alkaline lysis procedure (29). All DNA samples were further purified by cesium chloride-ethidium bromide density gradient centrifugation (29).

Southern hybridization. Total genomic DNA (4 μg) was digested with the restriction endonucleases *Eco*RI, *Bgl*II, *Xba*I, *Eco*RV, *Hin*dIII, and *Sac*I, in single or double digests according to the manufacturer's specifications (Boehringer Mannheim, Indianapolis, Ind.). Restricted DNA was analyzed by electrophoresis through 0.7% SeaKem agarose (FMC Bioproducts, Rockland, Maine) in 1× TBE buffer (1× TBE = 89 mM Tris-HCl [pH 8.0], 89 mM boric acid, and 2 mM EDTA) at 22 V for 18 h. The DNA was denatured and transferred to GeneScreen Plus membranes according to the manufacturer's protocol (NEN Dupont, Boston, Mass.).

The membranes were prehybridized and hybridized in a solution consisting of 50% formamide, 5× Denhardt's solution (1× Denhardt's solution = 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 1% sodium dodecyl sulfate (SDS), 1 M NaCl, 0.05 M Tris-HCl (pH 7.5), and 100 μg of denatured salmon sperm DNA per ml at 42°C. Hybridization probes (Fig. 1) included the 15.5-kb *Bam*HI-*Eco*RI fragment and the 4.5-kb *Eco*RI-*Hin*dIII fragment from pPL11 containing *P. syringae* pv. phaseolicola *hrpA-E* and *hrpF-S* sequences, respectively (18, 26); the 3.2-kb *Xba*I-*Bgl*II fragment from pRF1021 containing *P. syringae* pv. phaseolicola *hrpD* sequences (7); and the 3.7-kb *Eco*RI-*Bam*HI fragment from pC61.01 containing *P. syringae* pv. phaseolicola *hrpM* sequences (7). These fragments were labeled with [α-^{32}P]dCTP by random hexamer labeling (6) and added to the hybridization buffer at a final concentration of 10^6 cpm/ml. The membranes were hybridized for 14 to 18 h and washed with constant agitation twice in 2× SSC (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) at room temperature for 5 min, once in 2× SSC plus 1% SDS at 65°C for 30 min, once in 1.5× SSC plus 1% SDS at 65°C for 30 min, and, finally, once in 0.1× SSC at room temperature for 30 min. The membranes were exposed to AIF/RX X-ray film (Fuji) for 10 h with one Cronex Lightning Plus intensifying screen (NEN Dupont) at −80°C.

PCR analysis. PCR primers were constructed from *P. syringae* pv. phaseolicola NPS3121 *hrpD* (7) and *argF* sequence data (10) with the computer program PC Gene (IntelliGenetics Inc., Mountain View, Calif.). Primers, 20 nucleotides in length, were matched by T_m that varied between 62 and 64°C depending upon the primer set. Two primer sets were constructed to amplify two consecutive DNA segments within the

TABLE 1. Bacterial strains, plasmids, and phage

Strain, plasmid, or phage	Relevant characteristics[a]	Reference or source
Strains		
Pseudomonas syringae pv. tabaci		
BR2R	Rif^r; isolated in Brazil, 1979	28
JS78-45R	Rif^r; isolated in Florida, 1978	J. Skoog via H. Spurr
JS78-45-1R	Rif^r; isolated in Florida, 1978	J. Skoog via H. Spurr
JS78-45-3R	Rif^r; isolated in Florida, 1978	J. Skoog via H. Spurr
JS78-46R	Rif^r; isolated in Florida, 1978	J. Skoog via H. Spurr
JS78-51R	Rif^r; isolated in Pennsylvania, 1978	J. Skoog via H. Spurr
JS78-53R	Rif^r; isolated in Pennsylvania, 1978	J. Skoog via H. Spurr
JS78-64W#2R	Rif^r; unknown origin, 1978	J. Skoog via H. Spurr
JS81-70R	Rif^r; isolated in Maryland, 1981	J. Skoog via H. Spurr
JS81-75R	Rif^r; isolated in Maryland, 1981	J. Skoog via H. Spurr
JS82-89R	Rif^r; isolated in Maryland, 1982	J. Skoog via H. Spurr
JS82-90R	Rif^r; isolated in Maryland, 1982	J. Skoog via H. Spurr
JS82-93R	Rif^r; isolated in Kentucky, 1982	J. Skoog via H. Spurr
JS82-107R	Rif^r; isolated in Maryland, 1982	J. Skoog via H. Spurr
JS84-116R	Rif^r; isolated in Pennsylvania, 1984	J. Skoog via H. Spurr
JS84-117R	Rif^r; isolated in Pennsylvania, 1984	J. Skoog via H. Spurr
JS84-118R	Rif^r; isolated in Maryland, 1984	J. Skoog via H. Spurr
JS84-119R	Rif^r; isolated in Maryland, 1984	J. Skoog via H. Spurr
JS88-63R	Rif^r; origin unknown, 1988	J. Skoog via H. Spurr
JSWF-1-1-1R	Rif^r; unknown origin and date of isolation	J. Skoog via H. Spurr
Psa45R	Rif^r; isolated in Florida, unknown date of isolation	R. Durbin
Psa52R	Rif^r; isolated in Pennsylvania, unknown date of isolation	R. Durbin
Pt113R	Rif^r; isolated in Europe, 1965	28
Pt11528R	Rif^r; origin unknown, 1923	American Type Culture Collection
Pseudomonas syringae pv. phaseolicola		
NPS3121	Rif^r; unknown origin and date of isolation	25
Escherichia coli		
DH5α	F$^-$ *endA1 hsdR17* ($r_k^- m_k^+$) *supE44 thi recA1 gyrA* φ80*lacZ*ΔM15 *relA1* Δ(*lac-proAB-argF*) λ$^-$	Bethesda Research Laboratories
HB101	F$^-$ *hsdS20* (*hsdR hsdM*) *recA13 thi leu arg-14 proA2 lacY1 galK2 rpsL xyl-5 mtl-1 supE44* λ$^-$	3
JM101	*supE thi* Δ(*lac-proAB*) (F′ *traD36 proAB lacI*^{q} *lacZ*ΔM15)	36
MIKE	Wild-type isolate from sewage	15
Plasmids		
pC61.01	Tet^r; carries the 3.4-kb *Bam*HI-*Eco*RI fragment containing NPS3121 *hrpM* in pLAFR3	7
pPL11	Tet^r; carries the 20-kb *Bam*HI-*Hin*dIII fragment containing NPS3121 *hrpA-S* sequences cloned in the synonymous sites of pWB5A	18
pRCP36	Tet^r; carries the 2.1-kb *Bst*EII fragment containing the NPS3121 *argF* in pLAFR3	25
Phage		
pRF1021	Amp^r; M13mp18 carrying the 3.2-kb *Bgl*II-*Xba*I fragment containing NPS3121 *hrpD*	7

[a] Rif, rifampin; Tet, tetracycline; Amp, ampicillin; r, resistant.

4.4-kb *hrpD* region. The *hrpD* set 1 (5′-GATCAGCAAGG TTTCGGCGG-3′ [upstream] and 5′-CAGTGCTGTTCAG TCGTCGG-3′ [downstream] amplified an estimated 1,100-bp fragment (nucleotide positions 253 to 1352 of *hrpD* coding region), and the *hrpD* set 2 (5′-CTGAACAGCACTGGTCC GAA-3′ [upstream] and 5′-CGACCAGAAACATGCCTTG C-3′ [downstream]) amplified an estimated 1,023-bp fragment (nucleotide positions 1340 to 2362 of *hrpD* coding region). The *argF* primer set (5′-CATCGAGGTCATTGAGGCGG-3′ [upstream] and 5′-CAGCAACTGACTCATGCGCG-3′ [downstream]) amplified an approximately 1,470-bp fragment containing the ornithine carbamoyltransferase (OCTase) coding region. (Please note: in addition to the 918-nucleotide OCTase coding region, this amplified fragment contains approximately 540 nucleotides 5′ to the *argF* initiation codon and 11 nucleotides 3′ of the termination codon). Amplification of DNA was performed in 100-μl volumes containing 500 mM deoxynucleoside triphosphates, 0.5 μM each primer, 2.5 U of *Taq* DNA polymerase (Boehringer Mannheim or Promega [Madison, Wis.]), 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 1.0 mM $MgCl_2$, and 2 ng of DNA template. Amplifications were performed in an MJR Programmable Thermal Controller model PTC-100 (MJ Research, Inc., Waterstown, Mass.) with an initial three-step cycle of 2 min at 95°C, 2 min at 55°C, and 2 min at 72°C, followed by 30 cycles of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C. Positive controls consisting of pPL11 or pRCP36 (containing *argF*) and negative controls lacking DNA were included with each experiment.

The amplified products were extracted in phenol-chloroform-isoamyl alcohol (25:24:1), precipitated with 0.3 M sodium acetate and 95% ethyl alcohol at −20°C for 24 h, washed with 70% ethyl alcohol, dried, and resuspended in TE (10 mM

TABLE 2. Tabtoxin production, disease symptoms, and summary of RFLP groupings observed for *P. syringae* pv. phaseolicola NPS3121 and strains of *P. syringae* pv. tabaci

P. syringae strains	Tabtoxin[a]	Disease symptoms on:		RFLP grouping		
		Tobacco	Bean	*hrpA-S*	*hrpM*	*argF*
P. syringae pv. phaseolicola NPS3121	−	HR[b]	Halo blight	A	A	A
P. syringae pv. tabaci						
BR2R	+	HR	Wildfire	A	A	B
Pt113R, Pt11528R	+	Wildfire	HR	B	B	C
JS78-64W#2R, JS82-107R, JS84-116R, JS84-117R, JS84-118R, JS84-119R, JS88-63R, JSWF-1-1-1R	+	Wildfire	HR	B	C	C
Psa45R, Psa52R, JS78-45R, JS78-45-1R, JS78-45-3R, JS78-46R, JS78-51R, JS78-53R, JS81-70R, JS81-75R, JS82-89R, JS82-90R, JS82-93R	−	Angular leaf spot	HR	B	C	C

[a] Tabtoxin production determined by biological assay as described in Materials and Methods.
[b] HR, hypersensitive reaction.

Tris-Cl [pH 8.0], 1 mM EDTA) buffer. PCR products were digested with various restriction endonucleases according to the manufacturer's specifications (Boehringer Mannheim) and electrophoresed through 4% agarose consisting of 0.8% SeaKem and 3.2% Nusieve agarose (FMC Bioproducts) in 1× TBE buffer at 54 V for 12 h. Gels were stained with 10^{-4} mg of ethidium bromide per ml and photographed over a UV transilluminator. All experiments were repeated at least two times.

Growth and inoculation of plants. *Nicotiana tabacum* (tobacco) cv. Judy's Pride was grown from seed in the greenhouse. Seeds were germinated in Metro-Mix 220 planting mix and transplanted 2 to 4 weeks after germination into clay pots containing a 1:2 mixture of Metro-Mix 220 planting mix and clay. Inoculation of plants was 10 to 16 weeks after transplanting. *P. syringae* strains were grown overnight, pelleted by centrifugation, and washed in sterile distilled water. Cell suspensions were adjusted to approximately 10^5 CFU/ml (for analysis of compatible interactions) and 10^8 CFU/ml (for analysis of incompatible interactions) with sterile distilled water and infiltrated into tobacco leaves with a disposable plastic Pasteur pipette as described previously (19). Standard plate count procedures were used to verify inoculum concentrations. Plants were incubated in the greenhouse after inoculation, and observations of symptom development were made daily. All inoculations were repeated three times.

Phaseolus vulgaris L. (bean) cv. Red Kidney plants were grown and inoculated, by vacuum infiltration, as previously described (13).

Tabtoxin bioassay. *P. syringae* pv. tabaci strains were assayed for tabtoxin production by the procedure of Gasson (8). Briefly, *E. coli* MIKE (15) was grown overnight in Luria-Bertani medium. A 100-μl aliquot was introduced into 5 ml of melted 0.9% M9 agar (29) at 50°C with 0.4% glycerol substituted for glucose, overlaid onto Turner's minimal agar (33), and allowed to dry for 30 min. Overnight *P. syringae* pv. tabaci cultures (5 ml) were centrifuged and washed with sterile distilled water. Cells were resuspended in 5 ml of sterile distilled water, and 5 μl of each isolate was spotted onto plates previously overlaid with strain MIKE and allowed to dry. Four test isolates were spotted per plate, with *P. syringae* pv. phaseolicola and *E. coli* as controls. The plates were incubated at 28°C and observed 24 and 48 h after plating. Clear zones surrounding *P. syringae* strains indicated tabtoxin production. Each strain was tested twice by this procedure.

RESULTS

Disease reactions on tobacco and bean. Disease phenotypes for all *P. syringae* strains were determined by infiltration into tobacco and bean plants; the results of these analyses are summarized in Table 2. Except for strain BR2R, all *P. syringae* pv. tabaci strains, at a concentration of 10^5 CFU/ml, caused either wildfire or angular leaf spot on tobacco plants. BR2R, like *P. syringae* pv. phaseolicola NPS3121, did not cause disease on tobacco but elicited a hypersensitive reaction instead. Wildfire of tobacco has been associated with *P. syringae* tabtoxin production (21); therefore, we used an in vitro assay to determine tabtoxin production for each strain used in this study (8, 15). All *P. syringae* pv. tabaci strains that caused wildfire also produced tabtoxin, while strains that caused angular leaf spot did not (Table 2). BR2R, which did not cause wildfire of tobacco, but instead elicited a hypersensitive reaction, also produced tabtoxin in vitro (Table 2). *P. syringae* pv. phaseolicola NPS3121 did not produce tabtoxin in vitro. Again with the exception of BR2R, all *P. syringae* pv. tabaci strains, when infiltrated into bean at a concentration of 10^8 CFU/ml, induced a hypersensitive reaction within 24 h after infiltration.

Although strain BR2R produced tabtoxin, the plant reactions on tobacco and bean were distinct from those induced by the other *P. syringae* pv. tabaci isolates and more closely resembled those of *P. syringae* pv. phaseolicola NPS3121 with subtle differences. Like NPS3121, BR2R induced a hypersensitive reaction on tobacco within 24 h after infiltration with a concentration of 10^8 CFU/ml. When infiltrated into bean, both BR2R and NPS3121 incited disease but differed in the extent of tissue chlorosis and time required for lesion formation. When bean was infiltrated with BR2R at an inoculum concentration of 10^5 CFU/ml, sunken chlorotic lesions developed 48 h after infiltration; these lesions spread rapidly, and infiltrated leaves became completely necrotic 5 to 7 days after infiltration. In contrast, bean infiltrated with an identical inoculum of NPS3121 produced water-soaked lesions between 72 and 96 h after infiltration. The degree of chlorosis on bean, observed with NPS3121, was also much reduced compared with BR2R and was observed primarily on young secondary leaves rather than on the primary leaves that had been infiltrated. Thus, BR2R produced reactions on tobacco which were very different from those of the other *P. syringae* pv. tabaci isolates and also distinct from those of *P. syringae* pv. phaseolicola NPS3121.

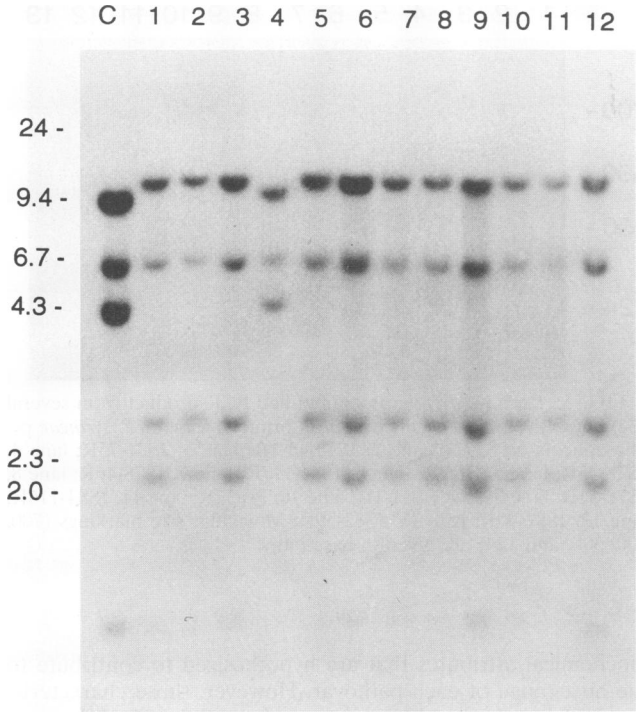

FIG. 2. Southern blot autoradiograph of *Bgl*II-*Eco*RI-digested total genomic DNA from several *P. syringae* pv. tabaci strains probed with the 15.5-kb *Bam*HI-*Eco*RI fragment (*hrpA-E*) from pPL11. Lane C, *P. syringae* pv. phaseolicola NPS3121; lane 1, *P. syringae* pv. tabaci JS78-45-1R; lane 2, JS81-75R; lane 3, JS78-45R; lane 4, BR2R; lane 5, JS84-117R; lane 6, JS78-46R; lane 7, JS78-51R; lane 8, JSWF-1-1-1R; lane 9, Psa52R; lane 10, JS84-116R; lane 11, JS82-90R; lane 12, JS82-89R. Molecular size markings are given in kilobase pairs.

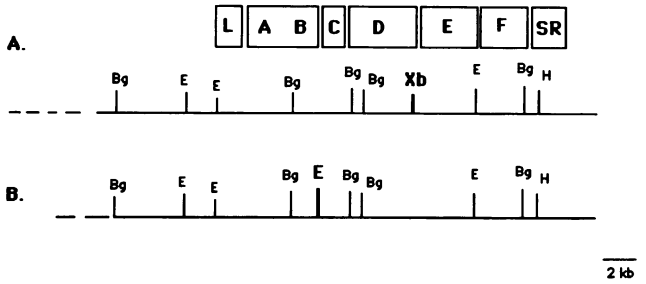

FIG. 3. Diagrammatic comparison of partial restriction maps of the *hrpL-R* cluster of *P. syringae* pv. phaseolicola NPS3121 (A) as reported by Rahme et al. (26) and *P. syringae* pv. tabaci (B). The restriction sites in boldface represent unique sites present in each pathovar. E, Xb, Bg, and H designate cleavage sites for the restriction enzymes *Eco*RI, *Xba*I, *Bgl*II, and *Hin*dIII, respectively. The restriction map of *P. syringae* pv. tabaci is identical for all isolates used in this study except for BR2R.

Conservation of *hrp* genes in *P. syringae* pv. tabaci. Southern blot analysis of genomic DNA from each bacterial strain was conducted with the fragments containing *hrpA-E* sequences and *hrpF-S* sequences from pPL11 as hybridization probes (Fig. 1). With the exception of BR2R, the RFLP patterns detected within *hrpA-E* and *hrpF-S* regions were identical for all *P. syringae* pv. tabaci strains when *Bgl*II, *Eco*RI, *Eco*RV, *Hin*dIII, *Sac*I, and *Xba*I were used in single and double restriction digests (the *Bgl*II-*Eco*RI digest probed with *hrpA-E* sequences is shown in Fig. 2). Interestingly, the banding patterns of BR2R and NPS3121 were identical in all cases. When DNA restricted with *Eco*RI, *Bgl*II, *Xba*I, *Eco*RV, and *Sac*I was probed with the fragment containing *hrpA-E* sequences, two RFLP patterns were observed, one for BR2R and NPS3121 and another for the remaining *P. syringae* pv. tabaci isolates. Identical groupings were made on the basis of DNA restricted with *Eco*RI, *Bgl*II, *Eco*RV, and *Sac*I and probed with the fragment containing *hrpF-S* sequences (data not shown).

With the aid of a previously published restriction map of the NPS3121 *hrpL-R* region (26), a partial restriction map of the corresponding *P. syringae* pv. tabaci *hrp* region was constructed (Fig. 3). Genomic DNA digested with *Eco*RI, *Bgl*II, and *Xba*I, in single and double restriction digests, was probed with fragments containing *hrpA-E* and *hrpD* sequences. Within the region corresponding to *hrpD* and *hrpE* in BR2R and NPS3121 (Fig. 3), an *Xba*I site that was not present in the remaining 23 *P. syringae* pv. tabaci strains was observed. Conversely, these 23 tabaci strains contained an *Eco*RI site within the region corresponding to *hrpAB* and *hrpC*, which was not observed in BR2R or NPS3121.

Southern analysis of *Eco*RV-digested DNA, probed with a fragment containing *hrpM* sequences, detected three distinct RFLP patterns: NPS3121 and BR2R lacked *Eco*RV restriction sites in *hrpM*, Pt11528R and Pt113R had two sites, and the remaining *P. syringae* pv. tabaci strains had one site (data not shown). A summary of the RFLP groupings for all strains used in this study is shown in Table 2.

PCR analysis of *hrpD*. To increase the sensitivity of the RFLP analysis, segments of *hrpD* were amplified by PCR and analyzed with restriction endonucleases that recognize 4-base sequences and thus cleave the DNA more frequently than the enzymes used above that recognize 6-base sequences. Two sets of PCR primers, described in Materials and Methods, were constructed from *hrpD* sequence data (7) and used to amplify two approximately 1-kb-DNA, consecutive segments within *hrpD*. The PCR products were subsequently digested with *Sau*3A and *Taq*I and analyzed by gel electrophoresis.

Gel electrophoresis demonstrated that the resulting PCR products from all *P. syringae* pv. tabaci strains and NPS3121 were the predicted size, based upon sequence information, with both sets of primers. When the amplified products were digested with *Sau*3A or *Taq*I and analyzed by gel electrophoresis, the banding patterns for NPS3121 and BR2R were identical and distinct from the other *P. syringae* pv. tabaci strains (Fig. 4).

PCR analysis of *argF*. Two genes which code for OCTase have been cloned from *P. syringae* pv. phaseolicola NPS3121 (11, 25). The first is *argK*, which encodes an OCTase that is produced specifically during phaseolotoxin biosynthesis; *argK* is linked to the phaseolotoxin biosynthetic genes and allows *P. syringae* pv. phaseolicola to survive in the presence of this toxin. The second gene, *argF*, encodes a form of OCTase that is inactivated by phaseolotoxin; *argF* is required for the biosynthesis of arginine during normal metabolism and is not directly involved in pathogenicity or the elicitation of the hypersensitive reaction. To address the possibility that the *hrp* regions might be conserved to a higher degree than other gene sequences in *P. syringae* pv. tabaci, PCR analysis of *argF* was conducted by a strategy similar to that used for *hrpD*.

A primer set was constructed from sequence information (10) and used to amplify an approximately 1.5-kb fragment containing the *argF* coding region. This primer set produced

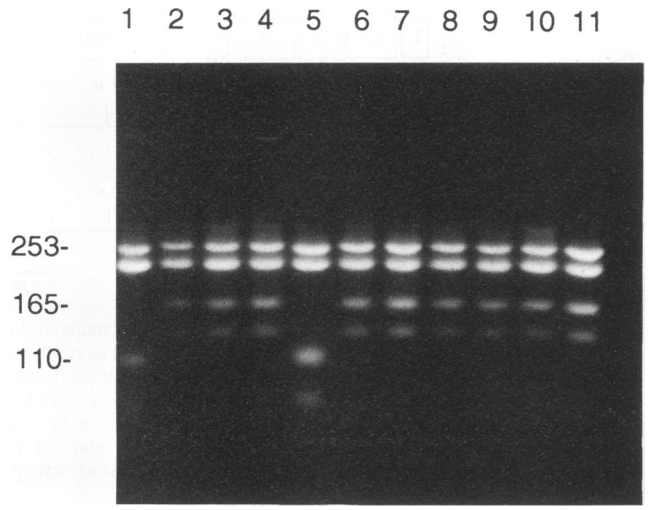

FIG. 4. *Sau*3A-digested amplified PCR product from several *P. syringae* pv. tabaci strains with *hrpD* primer set 2. Lane 1, pPL11 (containing NPS3121 *hrp* sequences); lane 2, JS78-45-1R; lane 3, JS81-75R; lane 4, JS78-45R; lane 5, BR2R; lane 6, JS84-117R; lane 7, JS78-46R; lane 8, JS78-51R; lane 9, JSWF-1-1-1R; lane 10, Psa52R; lane 11, JS84-116R. Molecular size markings (253, 165, and 110) are given in base pairs. Please note that the NPS3121 *hrp* region is represented by plasmid pPL11 in this figure and that banding patterns observed with pPL11 were identical to those for NPS3121 (data not shown).

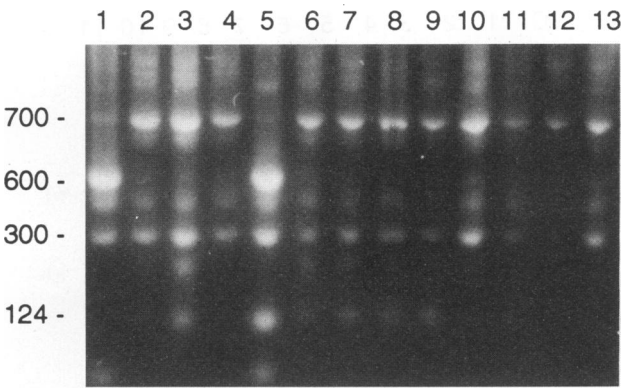

FIG. 5. *Eco*RI-*Bgl*II-digested amplified PCR product from several *P. syringae* pv. tabaci strains with *argF* primers. Lane 1, *P. syringae* pv. phaseolicola NPS3121; lane 2, JS78-45-1R; lane 3, JS81-75R; lane 4, JS78-45R; lane 5, BR2R; lane 6, JS84-117R; lane 7, JS78-46R; lane 8, JS78-51R; lane 9, JSWF-1-1-1R; lane 10, Psa52R; lane 11, JS84-116R; lane 12, JS82-90R; lane 13, JS82-89R. Molecular size markings (700, 600, 300, and 124) are given in base pairs.

the expected amplification product with all *P. syringae* pv. tabaci strains and NPS3121. When the amplification products were digested with the restriction enzyme *Sau*3A and analyzed by gel electrophoresis, all *P. syringae* pv. tabaci strains and NPS3121 produced the same DNA banding pattern (data not shown). However, when the amplification products were double-digested with *Bgl*II and *Eco*RI, three different groups were identified: NPS3121, BR2R, and the other *P. syringae* pv. tabaci strains (Fig. 5). The BR2R DNA banding pattern was similar to that of NPS3121, but in addition contained a DNA fragment which was similar in size to a fragment amplified in the other *P. syringae* pv. tabaci strains.

DISCUSSION

There has been much interest and confusion regarding the genetic relationships and taxonomy of *P. syringae* (30, 31). Many strains of *P. syringae* were originally classified as separate species on the basis of the host plant from which they were isolated. In 1980, the International Committee of Systematic Bacteriology reviewed the classification of pseudomonads and reduced the accepted number of species from over 100 to 23 (32, 37). Many of the strains previously classified as unique species were consequently grouped into *P. syringae*, on the basis of determinative tests designed primarily for the identification of saprophytic bacteria and members of the family *Enterobacteriaceae*. Host range, however, is a very useful criterion to differentiate plant-pathogenic bacteria; therefore, strains of *P. syringae* have been further differentiated into subspecies groupings, referred to as pathovars, on the basis of host-plant interactions (31, 37).

For scientists studying plant-bacterium interactions, the most significant feature of *P. syringae* strains is the fact that they are plant pathogens. It may be difficult to design biochemical tests to differentiate the unique physiological and/or biochemical attributes that are hypothesized to contribute to the host range of each pathovar. However, those characteristics that contribute to host range may be reflected in differences in genome organization within each pathovar. Consequently, genetic techniques may offer more data for characterization and identification of these bacteria than do determinative tests.

In this study, 24 *P. syringae* pv. tabaci strains and 1 *P. syringae* pv. phaseolicola strain were compared by RFLP analysis. The hybridization probes used included *P. syringae* pv. phaseolicola NPS3121 DNA sequences containing *hrpA-S* (*hrpA-E*, *hrpF-S*, and *hrpD*) and *hrpM*. On the basis of the RFLP patterns within the *hrpA-S* cluster, the strains were divided into two groups, 23 of the *P. syringae* pv. tabaci isolates forming one group and BR2R and NPS3121 forming the other (Table 2). Thus, except for BR2R, these studies indicate that within the *hrpA-S* region the restriction sites examined are conserved within *P. syringae* pv. tabaci. For many of the restriction endonucleases analyzed, the DNA banding patterns of *P. syringae* pv. tabaci and *P. syringae* pv. phaseolicola NPS3121 were similar. However, and with the exception of BR2R, *P. syringae* pv. tabaci contained unique *Sac*I, *Eco*RI, *Eco*RV, and *Xba*I restriction sites producing DNA banding patterns distinct from those for NPS3121. These same restriction enzymes resulted in DNA banding patterns for BR2R that were distinct from those for the *P. syringae* pv. tabaci strains but identical to those for NPS3121. Similar results were also seen in the PCR analysis of *hrpD*.

Three RFLP groups were observed when genomic DNA was digested with *Eco*RV and probed with *hrpM*. These groups consisted of Pt11528R and Pt113R, BR2R and NPS3121, and the remaining 21 *P. syringae* pv. tabaci strains. Although the RFLP pattern of Pt11528R and Pt113R was unique, no differences in disease symptoms were observed with these strains in comparison with other *P. syringae* pv. tabaci strains causing wildfire of tobacco. A previous study of the DNA fingerprint patterns of *P. syringae* pv. tabaci also found that Pt11528R and Pt113R were unique in comparison with 29 other *P. syringae* pv. tabaci strains (2). The significance of this is currently not understood.

Strain BR2R, originally isolated from *Phaseolus vulgaris*, is the causal agent of bean wildfire and is distinct from the other

P. syringae pv. tabaci strains that elicit a hypersensitive reaction on bean (28). Although BR2R produces tabtoxin, it does not incite disease symptoms on tobacco. The disease produced by BR2R on bean is similar to halo blight caused by *P. syringae* pv. phaseolicola; both induce lesions and bright chlorosis 48 to 72 h after inoculation. But BR2R, unlike *P. syringae* pv. phaseolicola, causes only foliar symptoms and is unable to infect bean pods in the field or produce phaseolotoxin (28). In this study, BR2R possessed a unique RFLP pattern in comparison with the *P. syringae* pv. tabaci strains that are pathogenic on tobacco. Interestingly, the RFLP patterns observed in BR2R during the analysis of *hrpA-S*, *hrpD*, and *hrpM* were identical to the banding patterns observed in *P. syringae* pv. phaseolicola NPS3121.

PCR analysis of *argF* produced an identical DNA banding pattern for the *P. syringae* pv. tabaci strains (including BR2R) and *P. syringae* pv. phaseolicola when the amplified product was digested with the restriction enzyme *Sau*3A. However, the DNA banding patterns for BR2R, *P. syringae* pv. phaseolicola, and the other *P. syringae* pv. tabaci strains were distinct from each other when the amplified product was restricted with *Eco*RI and *Bgl*II. The banding pattern for BR2R contained all the DNA bands observed for *P. syringae* pv. phaseolicola NPS3121 and *P. syringae* pv. tabaci. Thus, *P. syringae* pv. tabaci strains pathogenic on tobacco had a high degree of sequence conservation within *argF* similar to that observed for the *hrp* regions. These results suggest that the *hrp* sequences are not conserved to a greater extent than other genes in *P. syringae* pv. tabaci.

Although there was some variation in *hrpM*, this study has shown 23 of the *P. syringae* pv. tabaci strains to be highly conserved within *hrpA-S* and to be distinct from strain BR2R and from *P. syringae* pv. phaseolicola NPS3121. A recent study indicates that *hrp* genes of *P. syringae* pv. syringae are also highly conserved (17). The RFLP patterns observed for BR2R were similar to those of the bean pathogen *P. syringae* pv. phaseolicola NPS3121. This RFLP data in conjunction with host range suggest that the classification of BR2R within pathovar tabaci should be reexamined. Further studies, such as comparative genetic analyses of BR2R with other strains of *P. syringae*, combined with host range and disease etiology analyses need to be completed before a pathovar classification of BR2R may be determined.

Although additional studies are required, we believe that these results raise interesting questions concerning the importance of host range and disease etiology in our understanding of the genetic and taxonomic relationships of *P. syringae*. Our studies have shown that distinct and identifiable genetic differences may be correlated with host range and suggest that information from such analyses of *P. syringae* strains may be useful for assigning pathovar designations.

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