Genetic Analysis of *hrp*-Related DNA Sequences of *Xanthomonas campestris* Strains Causing Diseases of Citrus†

R. P. LEITE, JR., D. S. EGEL,‡ AND R. E. STALL*

*Department of Plant Pathology, Institute of Food and Agricultural Sciences,
University of Florida, Gainesville, Florida 32611*

The *hrp* gene cluster of strains of *Xanthomonas campestris* that cause diseases of citrus was examined by Southern hybridization of genomic DNA and by restriction endonuclease analysis of enzymatically amplified DNA fragments of the *hrp* gene cluster. The *hrp* genes were present in all strains of the pathovars of *X. campestris* tested in this study, including strains of the three aggressiveness groups of the citrus bacterial spot pathogen, *X. campestris* pv. citrumelo. *X. campestris* pv. citri strains in groups A, B, and C, which cause citrus canker A, B, and C, respectively, each produced characteristic restriction banding patterns of amplified *hrp* fragments. The restriction banding patterns of all strains within each group were identical. In contrast, restriction fragment length polymorphism was evident among strains of the moderately and weakly aggressive groups of *X. campestris* pv. citrumelo. *X. campestris* pv. citrumelo strains in the highly aggressive group had a homogeneous restriction banding pattern. The characteristic banding patterns obtained for each bacterial group indicate that *X. campestris* strains causing disease in citrus can be reliably differentiated and identified by restriction analysis of amplified DNA fragments of the *hrp* gene cluster. In addition, the phylogenetic analysis based on the restriction banding patterns of amplified fragments suggests a polyphyletic relationship of the *hrp* genes among the strains of *X. campestris* that cause disease in citrus.

Citrus canker, caused by strains of *Xanthomonas campestris* pv. citri group A, represents an important problem for production of citrus worldwide (7). This disease is characterized by raised lesions on leaves, stems, and fruits. Strains of *X. campestris* pv. citri group A have a relatively wide host range and cause symptoms of various degrees in all commercial citrus varieties (34). In severe cases, abscission of fruits and leaves may result (7, 34). Other xanthomonads that cause similar symptoms on citrus are strains of *X. campestris* pv. citri groups B and C. They are of less importance than strains of *X. campestris* pv. citri group A and have comparatively limited host ranges. Citrus bacterial spot is another bacterial disease of citrus caused by a xanthomonad, and symptoms are similar to citrus canker with a few important differences (31). The pathogen, referred to as *X. campestris* pv. citrumelo (15), causes flat, water-soaked lesions in young leaves. Strains of *X. campestris* pv. citrumelo cause symptoms primarily on trifoliate orange (*Poncirus trifoliata*) and its hybrids, such as Swingle citrumelo (*Citrus paradisi* × *P. trifoliata*) (17).

Although *X. campestris* pv. citri and *X. campestris* pv. citrumelo cause similar diseases of citrus, there is evidence for differences between these pathovars. In addition to the pathogenicity differences listed above, *X. campestris* pv. citrumelo strains appear to be quite heterogeneous both genetically (8, 9) and in aggressiveness (18) compared with *X. campestris* pv. citri. This has resulted in questions about the relationship of the bacterial spot pathogen to other pathovars of *X. campestris*. Several xanthomonads isolated from ornamental plants cause

lesions similar to bacterial spot when artificially inoculated onto young citrus plants (19, 20); they are also genetically similar to some strains of *X. campestris* pv. citrumelo (9, 20). It was suggested that strains of *X. campestris* pv. citrumelo may represent other pathovars of *X. campestris* incidentally isolated from citrus (20) or strains of a xanthomonad that has a wide host range (14); alternatively it was suggested that the most weakly aggressive strains may be "opportunistic" strains which cause symptoms only when associated with injury (8).

These alternatives have not been resolved by studies of the genetics (9, 14, 15, 20–22, 35) or pathogenicity (18, 20) of these strains. The genetics of pathogenicity, however, might favor one of the above hypotheses. An excellent candidate for examination is the hypersensitivity reaction and pathogenicity (*hrp*) gene cluster responsible for pathogenicity reaction on susceptible hosts and a hypersensitive reaction in nonhosts (36). The *hrp* gene cluster has been discovered and characterized in several bacterial phytopathogens, such as *Pseudomonas syringae* pv. phaseolicola (26), *P. solanacearum* (5), *Erwinia amylovora* (2), and *X. campestris* pv. vesicatoria (4). The *hrp* gene cluster of *X. campestris* pv. vesicatoria consists of at least 25 kb of genomic DNA, and polymorphism of restriction fragments of a homologous DNA sequence occurs among pathovars of *X. campestris* (4). Opportunistic xanthomonads, which produce limited symptoms in susceptible hosts and no hypersensitive reaction in nonhosts, do not possess DNA similar to an *hrp* gene cluster (33).

The genomic similarity of strains of *X. campestris* pv. citrumelo has been investigated (9, 14, 15, 20–22, 35), but examination of the similarity between *hrp* clusters of these strains adds information on the comparative genetics of pathogenicity. Similar *hrp* gene clusters among strains with relatively divergent genetic backgrounds might indicate a similar origin of pathogenicity, and dissimilar *hrp* gene clusters would support the hypothesis that many strains of *X. campestris* involved

---

* Corresponding author. Mailing address: Plant Pathology Department, University of Florida, P.O. Box 110680, Gainesville, FL 32611-0680. Phone: (904) 392-7244. Fax: (904) 392-6532. Electronic mail address: REST@gnv.ifas.ufl.edu.
‡ Present address: American Sunmelon, Hinton, OK 73047.

TABLE 1. List of *X. campestris* pathovars used in this study

Pathovar	Disease	Strain	Source designation	Origin	Year of isolation	Source[a]
Alfalfae		82-1		Florida	1982	RES
Bilvae		XCB	X32			ELC
Citrumelo	CBS[b]	F1	084-3048	Florida	1984	DPI
		F6	84-3401	Florida	1984	DPI
		F54	X85-5436-1	Florida	1985	DPI
		F59	X85-6572R1-1	Florida	1985	DPI
		F86	X85-8600-1	Florida	1985	DPI
		F94	X85-6774-3	Florida	1985	DPI
		F100	X85-12689	Florida	1985	DPI
		F228	X84-169	Florida	1984	DPI
		F254	X87-4665-1	Florida	1987	DPI
		F274	X87-5789	Florida	1987	DPI
		F306	X87-6314	Florida	1987	DPI
		F311	X87-6345	Florida	1987	DPI
		F348	X87-7222-1	Florida	1987	DPI
		F361	X88-3851-3	Florida	1988	DPI
		F378	X88-534	Florida	1988	DPI
		3166	84-3166	Florida	1984	DPI
Citri	Canker A	3213	86-3213	Florida	1986	DWG
		3340	86-3340	Florida	1986	DPI
		9760-2	86-9760-2	Florida	1986	DPI
		9771	86-9771	Florida	1986	DPI
		T1		Florida		RES
		115-A		Florida		ELC
	Canker B	B64	XC64	Argentina	1979	ELC
		B69	XC69	Argentina	1979	ELC
		B80	XC80	Uruguay	1983	ELC
		B84	XC84	Uruguay	1983	ELC
		B93	XC93	Argentina		ELC
		B94	XC94	Argentina		ELC
		B148	XC148	Argentina	1988	ELC
	Canker C	70C	XC70	Brazil		ELC
		338	XC338	Brazil	1981	ELC
		339	XC339	Brazil	1981	ELC
		340	XC340	Brazil	1982	ELC
		341	XC341	Brazil	1982	ELC
		342	XC342	Brazil		ELC
Fici		X151				ARC
Maculifoliigardeniae		X22j				DPI
Vesicatoria		75-3				RES
Undetermined		X198				ARC
Undetermined		XCF	X33			ELC

[a] ARC, A. R. Chase, University of Florida, Apopka; DPI, Department of Plant Industry, Gainesville, Fla.; DWG, D. W. Gabriel, University of Florida, Gainesville; ELC, E. L. Civerolo, U.S. Department of Agriculture, Beltsville, Md.; RES, R. E. Stall, University of Florida, Gainesville.

[b] CBS, citrus bacterial spot.

in the citrus bacterial spot disease are diverse. We investigated the presence of the *hrp* gene cluster in strains of *X. campestris* pv. citri and *X. campestris* pv. citrumelo by Southern hybridization of genomic DNA probed with an *hrp* gene cluster from *X. campestris* pv. vesicatoria (33). The similarity of this region was further investigated by amplifying and restricting two DNA fragments of the *hrp* complementation groups B and C/D (4, 25), which are highly conserved among several pathovars of *X. campestris* (3, 25).

MATERIALS AND METHODS

Culture conditions. The strains of *X. campestris* used in this study and their sources are listed in Table 1. All strains had previously been identified as members of *X. campestris* by fatty acid analysis (23). Citrus bacterial spot strains were rated for pathogenicity by Graham and Gottwald (18) and by Graham et al. (20). All strains were streaked onto nutrient agar (Becton Dickinson, Cockeysville, Md.), and single colonies were se-

lected. Nutrient broth cultures were grown for 24 h on a rotatory shaker (150 rpm) at 28°C. Strains of *X. campestris* pv. citri group B were grown on a sucrose-based medium (6). Strains were stored on lima bean agar (Difco, Detroit, Mich.) for short-term storage and in sterile tap water at room temperature for long-term storage.

DNA isolation. The procedure described by Ausubel et al. (1), with minor modifications, was used to extract total genomic DNA. Briefly, bacterial cells were pelleted by centrifugation in an Eppendorf microcentrifuge (Brinkmann Instruments Inc., Westbury, N.Y.) for 2 min at 16,000 × *g*. The pellet was washed in 1 ml of distilled water, pelleted again, and resuspended in 567 μl of TE buffer (10 mM Tris · Cl [pH 8.0], 1 mM EDTA [pH 8.0]). Proteinase K (Boehringer Mannheim, Indianapolis, Ind.) and sodium dodecyl sulfate (SDS) (Sigma, St. Louis, Mo.) were added to final concentrations of 100 μg/ml and 0.5%, respectively. After incubation for 1 h at 37°C, sodium chloride and hexadecyltrimethylammonium bromide (Sigma) were added to each preparation to final concentra-

tions of 0.7 M and 1%, respectively. The preparations were incubated for 10 min at 65°C. DNA was extracted with chloroform-isoamyl alcohol (24:1). The samples were hand shaken continuously and gently for 10 min and centrifuged for 5 min at 16,000 × g. DNA was extracted again by adding phenol-chloroform-isoamyl alcohol (25:24:1) and centrifuging as described above. The DNA was precipitated by adding 0.6 volume of isopropanol and incubating for 30 min at −20°C. The samples were centrifuged for 20 min at 16,000 × g. The DNA pellet obtained was washed with 1 ml of 70% ethanol and centrifuged again. The DNA was dried under vacuum for 20 min, and then the pellet was redissolved in 100 μl of TE buffer and stored at 4°C.

Hybridization analysis. Total genomic DNA was restricted with *Bam*HI (Boehringer Mannheim) for 2 h at 37°C. Samples were then treated with RNase A (type II-A; Sigma) for 30 min and electrophoresed in a 0.7% agarose gel by standard procedures (29). The gel was then denatured in 0.4 N NaOH–0.6 M NaCl for 30 min and neutralized in 0.5 M Tris · Cl–1.5 M NaCl for 30 min. The denatured DNA was transferred by the procedure of Southern (32) to a nylon membrane (Gene Screen Plus; Du Pont, Boston, Mass.). Hybridization was carried out at 68°C with 0.5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% (wt/vol) SDS. Probes were labeled by the random-primed (10) incorporation of digoxigenin-labeled dUTP and detected by the use of the Genius Nonradioactive DNA Labeling and Detection kit (Boehringer Mannheim) as specified by the manufacturer. Plasmid pXV9 containing almost the entire *hrp* gene cluster of *X. campestris* pv. vesicatoria inserted into cosmid pLAFR3 (4, 33) was used as a probe.

DNA amplification. The two sets of oligonucleotide primers used in this study were designed on the basis of nucleotide sequences of the *hrp* gene cluster of *X. campestris* pv. vesicatoria (3, 25). The two primers RST2 and RST3 delineate an 840-bp region and primers RST21 and RST22 delineate a 1,075-bp region of the *hrp* complementation groups B and C/D of *X. campestris* pv. vesicatoria, respectively (25). Oligonucleotide primers were synthesized with a model 394 DNA Synthesizer (Applied Biosystems, Foster City, Calif.) by the DNA Synthesis Laboratory, University of Florida, Gainesville.

DNA was amplified in a total volume of 50 μl. The reaction mixture contained 5 μl of 10× buffer (500 mM KCl, 100 mM Tris · Cl [pH 9.0 at 25°C], 1% Triton X-100), 1.5 mM MgCl$_2$, 200 μM each deoxynucleoside triphosphate (Boehringer Mannheim), 25 pmol of each primer, and 2.5 U of *Taq* polymerase (Promega, Madison, Wis.). The amount of template DNA added was 100 ng of purified total bacterial DNA. The reaction mixture was covered with 50 μl of light mineral oil. A total of 30 amplification cycles were performed in an automated thermocycler (MJ Research, Watertown, Mass.). Each cycle consisted of 30 s of denaturation at 95°C, 30 s of annealing at 62°C, and 45 s of extension at 72°C for primers RST2 and RST3 and 30 s at 95°C, 45 s at 61°C, and 1.5 min at 72°C, respectively, for primers RST21 and RST22. The last extension step was extended to 5 min.

Amplified DNAs were detected by electrophoresis in 0.9% agarose gels in TAE buffer (40 mM Tris acetate, 1 mM EDTA [pH 8.2]) at 5 V/cm of gel (28). After being stained with 0.5 μg of ethidium bromide per ml, the gel was photographed over a UV transilluminator (Fotodyne Inc., New Berlin, Wis.) with type 55 Polaroid film (Polaroid, Cambridge, Mass.). The identity of the amplified DNA fragments was further confirmed by hybridization analysis with an internal DNA probe for each fragment. The internal probes consisted of a 271-bp insert of plasmid pXV840 for the 840-bp fragment and a

335-bp insert of plasmid pXV1075 for the 1,075-bp fragment (25). The hybridization analysis was carried out as described above.

Restriction endonuclease analysis of amplified DNA. Amplified DNAs were restricted with either *Cfo*I, *Hae*III, *Sau*3AI, or *Taq*I under conditions specified by the manufacturer (Promega). The restriction fragments were separated by electrophoresis in 4% agarose gels (3% NuSieve GTG, 1% SeaKem GTG [FMC BioProducts, Rockland, Maine]) in TAE buffer at 8 V/cm. Phage λ *Pst*I-restricted DNA fragments were used as molecular weight standards. After being stained with 0.5 μg of ethidium bromide per ml for 40 min, the gels were destained in 1 mM MgSO$_4$ for 1 h and then photographed over a UV transilluminator with type 55 Polaroid film.

Data analysis. DNA restriction fragment patterns were determined by direct comparison of the electrophoretic patterns of the DNA restricted with each of the four endonucleases. The codes 1 and 0 were assigned according to the presence or absence of each fragment, respectively. The resulting matrix was used to estimate the genetic relationships between strains based on the proportion of shared DNA fragments. The expected proportion of shared fragments (F) was calculated by the equation proposed by Nei and Li (28), $F = 2n_{xy}/(n_x + n_y)$, where n_{xy} is the number of fragments shared between two strains and n_x and n_y are the total number of fragments for each strain. The genetic divergence between strains was calculated as the estimate of the number of nucleotide substitutions per site (δ), based on the proportion of shared DNA fragments (28). The iterative method proposed by Nei (27) was used to estimate the number of nucleotide substitutions per site by using a program written for the SAS system (30).

Relationships among strains were studied by phylogenetic analysis with the BOOT and KITSCH programs from the PHYLIP computer package (12). For the BOOT program, the restriction fragment data encoded 0 or 1 were used as input for reconstruction of an unrooted phylogenetic tree by using the Wagner parsimony criterion. No assumptions were made regarding the ancestral character state, and the pathovar *X. campestris* pv. maculifoliigardeniae X22j was taken as the outgroup to infer the topology of the phylogenetic tree. A total of 100 bootstrap samples were analyzed to determine the confidence intervals of the estimates of the inferred phylogenetic tree (11, 12). The KITSCH program was used to infer a rooted phylogenetic tree by the Fitch-Margoliash method (12, 13). The input data consisted of a distance matrix of pairwise estimates of the number of nucleotide substitutions per site (δ) between strains, and negative branch was not allowed.

RESULTS

Hybridization analysis. The *hrp* gene cluster of *X. campestris* pv. vesicatoria hybridized to genomic DNA of strains of all tested pathovars of *X. campestris*. When genomic DNAs of the strains of different pathovars of *X. campestris* were restricted with *Eco*RI and probed with the *hrp* gene cluster, restriction fragment length polymorphisms occurred (Fig. 1). The banding patterns were very similar for strains of *X. campestris* pv. citrumelo of different aggressiveness groups, and the few polymorphisms that were observed did not correspond to an aggressiveness group. Moreover, the banding patterns of *X. campestris* pv. citrumelo F1 and F100 were very similar to *X. campestris* pv. fici X151 (Fig. 1). Although restriction fragment length polymorphism was not observed for strains within each group of the citrus canker pathogen, the banding patterns for the strains of different groups of *X. campestris* pv. citri were
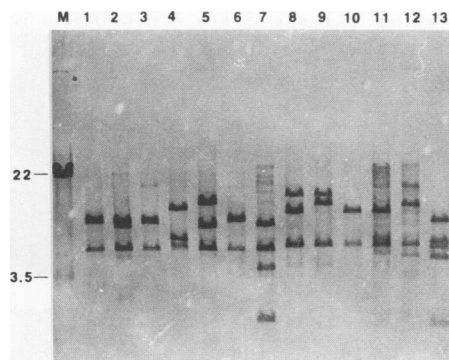
FIG. 1. Hybridization of the *hrp* gene cluster of *X. campestris* pv. vesicatoria to genomic DNA of strains of *X. campestris* causing disease on citrus. Lane M contains phage λ restricted with *Hind*III and *Eco*RI. Lanes 1 to 13 represent genomic DNA restricted with *Eco*RI. Lanes: 1 to 3, strains F1, F6, and F100 of *X. campestris* pv. citrumelo, respectively; 4, *X. campestris* pv. maculifoliigardeniae X22j; 5, *X. campestris* X198 from *S. reginae*; 6, *X. campestris* pv. fici X151; 7, *X. campestris* pv. alfalfae 82-1; 8, *X. campestris* pv. bilvae XCB; 9, *X. campestris* XCF from a *Feronia* sp.; 10 to 12, strains 9771, B84, and 339 of *X. campestris* pv. citri, respectively; 13, *X. campestris* pv. vesicatoria 75-3. Molecular sizes are given in kilobases.

diverse (Fig. 1). Furthermore, the banding patterns of genomic DNA of strains of *X. campestris* pv. citrumelo and the strains of the three groups of *X. campestris* pv. citri were also different when restricted with *Eco*RI and probed with the *hrp* gene cluster (Fig. 1).

DNA amplification. The 840- and 1,075-bp fragments of the *hrp* gene cluster in *X. campestris* pv. vesicatoria were successfully amplified with primers RST2 and RST3 (Fig. 2) and
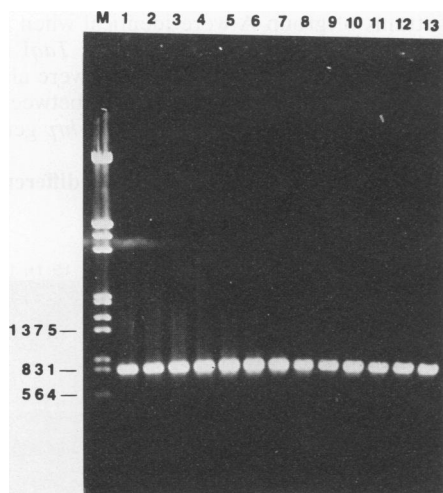

FIG. 2. Amplification of the 840-bp fragment of complementation group B of the *hrp* gene cluster of *X. campestris* pv. vesicatoria from strains of *X. campestris*. Lanes: M, phage λ restricted with *Eco*RI and *Hind*III; 1 to 3, strains F1, F6, and F100 of *X. campestris* pv. citrumelo, respectively; 4, *X. campestris* pv. maculifoliigardeniae X22j; 5, *X. campestris* X198 from *S. reginae*; 6, *X. campestris* pv. fici X151; 7, *X. campestris* pv. alfalfae 82-1; 8, *X. campestris* pv. bilvae XCB; 9, *X. campestris* XCF from a *Feronia* sp.; 10 to 12, strains 9771, B84, and 339 of *X. campestris* pv. citri, respectively; 13, *X. campestris* pv. vesicatoria 75-3. Molecular sizes are given in bases.
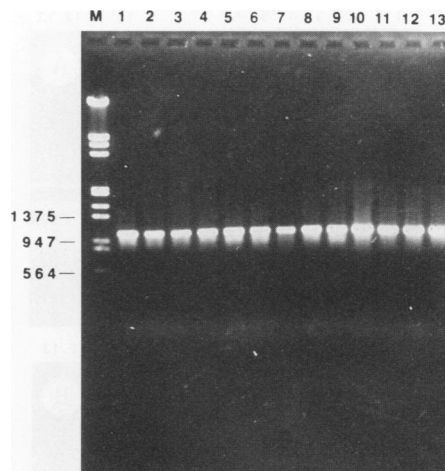

FIG. 3. Amplification of the 1,075-bp fragment of complementation groups C/D of the *hrp* gene cluster of *X. campestris* pv. vesicatoria from strains of *X. campestris*. Lanes: M, phage λ restricted with *Eco*RI and *Hind*III; 1 to 3, strains F1, F6, and F100 of *X. campestris* pv. citrumelo, respectively; 4, *X. campestris* pv. maculifoliigardeniae X22j; 5, *X. campestris* X198 from *S. reginae*; 6, *X. campestris* pv. fici X151; 7, *X. campestris* pv. alfalfae 82-1; 8, *X. campestris* pv. bilvae XCB; 9, *X. campestris* XCF from a *Feronia* sp.; 10 to 12, strains 9771, B84, and 339 of *X. campestris* pv. citri, respectively; 13, *X. campestris* pv. vesicatoria 75-3. Molecular sizes are given in bases.

primers RST21 and RST22 (Fig. 3), respectively. The same size fragments were also successfully amplified from DNA of all strains of the other pathovars of *X. campestris* (Fig. 2 and 3). The DNA fragments were also amplified from 16 strains of *X. campestris* pv. citrumelo representing the three aggressiveness groups and from 19 strains of *X. campestris* pv. citri groups A, B, and C without variation in size (data not shown). The sequence similarity of the two DNA fragments amplified from strains of different pathovars of *X. campestris* to the *hrp* gene cluster of *X. campestris* pv. vesicatoria was further confirmed by Southern hybridization analysis. The amplified DNA fragments of the different strains of *X. campestris* hybridized to the respective internal probes specific for the 840- and 1,075-bp *hrp* gene cluster fragments (data not shown).

Restriction analysis. The 840- and 1,075-bp *hrp* gene cluster fragments amplified from strains of different pathovars of *X. campestris* were each restricted with either *Cfo*I, *Hae*III, *Sau*3AI, or *Taq*I. The banding patterns for each set of fragments from the pathovars of *X. campestris* included in this study were variable. The banding patterns of the 1,075-bp *hrp* gene cluster fragment amplified from strains of different pathovars of *X. campestris* restricted with *Cfo*I and with *Hae*III are presented in Fig. 4. The banding patterns of the strains of *X. campestris* pv. citrumelo were very similar to the patterns obtained for *X. campestris* pv. vesicatoria 75-3 (Fig. 4). Also, *X. campestris* pv. alfalfae, *X. campestris* pv. fici, and *X. campestris* X198 produced patterns similar to those of the strains of *X. campestris* pv. citrumelo (Fig. 4). *X. campestris* pv. bilvae and *X. campestris* pv. citri strains and *X. campestris* XCF also made up a group with a very similar banding pattern (Fig. 4). On the other hand, *X. campestris* pv. maculifoliigardeniae had a more distinct restriction fragment profile (Fig. 4). Although variability was also observed in the banding patterns obtained with the endonucleases *Sau*3AI and *Taq*I, a characteristic pattern for each group or pathovar of *X. campestris* was less evident for these two endonucleases (data not shown). Similarly, restric-
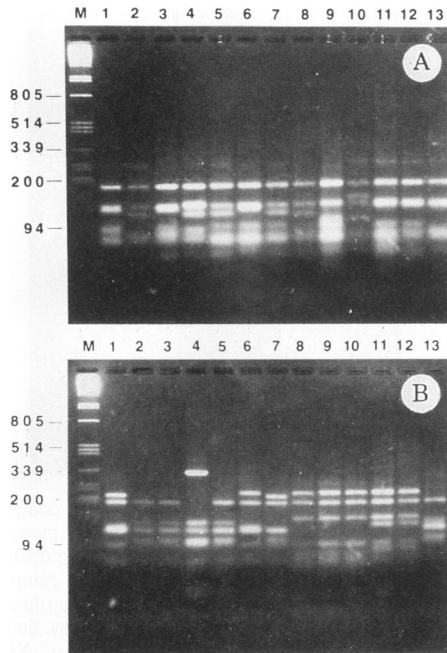
FIG. 4. Restriction analysis of the 1,075-bp DNA fragment of complementation groups C/D of the *hrp* gene cluster amplified from strains of *X. campestris* and restricted with *Cfo*I (A) or *Hae*III (B). Lanes: M, phage λ restricted with *Pst*I; 1 to 3, strains F1, F6, and F100 of *X. campestris* pv. citrumelo, respectively; 4, *X. campestris* pv. maculifoliigardeniae X22j; 5, *X. campestris* X198 from *S. reginae*; 6, *X. campestris* pv. fici X151; 7, *X. campestris* pv. alfalfae 82-1; 8, *X. campestris* pv. bilvae XCB; 9, *X. campestris* XCF from a *Feronia* sp.; 10 to 12, strains 9771, B84, and 339 of *X. campestris* pv. citri, respectively; 13, *X. campestris* pv. vesicatoria 75-3. Molecular sizes are given in bases.

FIG. 5. Restriction analysis of the 1,075-bp DNA fragment of complementation groups C/D of the *hrp* gene cluster amplified from strains of *X. campestris* pv. citrumelo and restricted with *Cfo*I (A) or *Hae*III (B). Lanes: M, phage λ restricted with *Pst*I; 1 to 5, highly aggressive strains F1, F54, F274, F361, and 3166, respectively; 6 to 11, moderately aggressive strains F6, F228, F311, F254, F348, and F378, respectively; 12 to 16, weakly aggressive strains F59, F86, F100, F306, and F94, respectively; 17, *X. campestris* pv. vesicatoria 75-3. Molecular sizes are given in bases.

tion analysis of the 840-bp *hrp* fragment also produced a pattern of variation for the different strains of *X. campestris* (data not shown).

Sixteen strains of *X. campestris* pv. citrumelo, representing all three aggressiveness groups, were analyzed by restriction analysis of the amplified *hrp* fragments. The banding patterns of five strains of the highly aggressive group of *X. campestris* pv. citrumelo were identical to each other when restricted with either *Cfo*I (Fig. 5A), *Hae*III (Fig. 5B), *Sau*3AI, or *Taq*I (data not shown). For certain combinations of fragment and endonuclease, the restriction patterns of the highly aggressive strains were similar to those of some strains of the moderately or weakly aggressive groups; these include the 1,075-bp fragment from the weakly aggressive strain F100 restricted with *Cfo*I (Fig. 5A) and the fragment from the moderately aggressive strain F378 restricted with *Hae*III (Fig. 5B). However, the overall banding patterns of the combinations of two fragments and four endonucleases for the highly aggressive strains were different from the patterns obtained for the strains of the moderately and weakly aggressive groups of *X. campestris* pv. citrumelo. In contrast to the highly aggressive group, restriction fragment length polymorphism was evident for the strains within the moderately and weakly aggressive groups of *X. campestris* pv. citrumelo (Fig. 5). The weakly aggressive strains F94, F100, and F306 had banding patterns almost identical to that of *X. campestris* pv. vesicatoria 75-3 (Fig. 5). Also, strains of the highly aggressive group of *X. campestris* pv. citrumelo

had banding patterns similar to that of *X. campestris* pv. fici X151 (Fig. 4 and 5).

In contrast to the diversity of the moderately and weakly aggressive strains of *X. campestris* pv. citrumelo, strains of *X. campestris* pv. citri of groups A, B, and C each produced characteristic restriction patterns (Fig. 6). The banding patterns of all strains of group A were identical when restricted with either *Hae*III (Fig. 6), *Cfo*I, *Sau*3AI, or *Taq*I (data not shown). Similarly, strains of groups B and C were also homogeneous within each group (Fig. 6), as well as between the two groups for the four endonucleases and the two *hrp* gene cluster fragments.

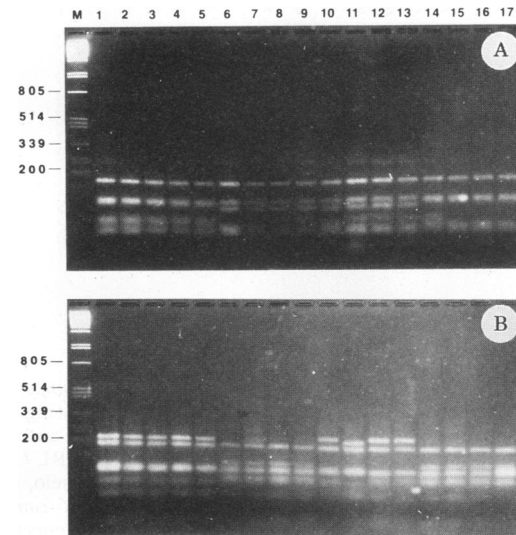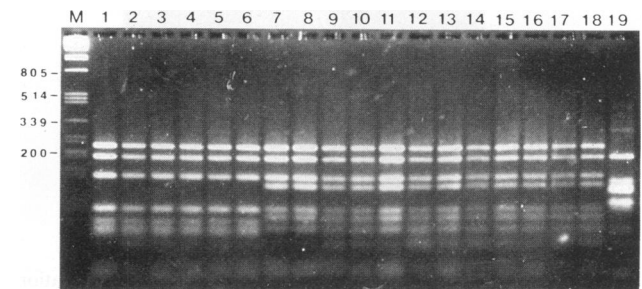**Genetic relationships of the *hrp* genes from different strains**

FIG. 6. Restriction analysis of the 1,075-bp DNA fragment of complementation groups C/D of the *hrp* gene cluster amplified from strains of *X. campestris* pv. citri restricted with *Hae*III. Lanes: M, phage λ restricted with *Pst*I; 1 to 6, group A strains 9771, 3340, 9760-2, 3213, T1, and 115-A, respectively; 7 to 13, group B strains B64, B69, B80, B84, B93, B94, and B148, respectively; 14 to 18, group C strains 338, 339, 340, 341, and 342, respectively; 19, *X. campestris* pv. vesicatoria 75-3. Molecular sizes are given in bases.

TABLE 2. Genetic divergence matrix for 19 strains of X. campestris based on the estimates of the number of nucleotide substitutions per site in two fragments related to the hrp gene cluster

Strain	X. campestris pv. citrumelo[a]									X. campestris pv. citri[b]			Other strains						
	High (F1)	Moderate				Weak				A (9771)	B (B84)	C (339)	82-1	XCB	XCF	X22j	X198	X151	75-3
		F6	F311	F348	534	F86	F94	F100	F306										
F1		0.020	0.009	0.013	0.010	0.014	0.016	0.022	0.020	0.057	0.055	0.055	0.011	0.043	0.041	0.060	0.019	0.004	0.014
F6			0.009	0.015	0.019	0.012	0.012	0.018	0.010	0.057	0.063	0.063	0.007	0.056	0.057	0.052	0.015	0.018	0.018
F311				0.013	0.012	0.008	0.010	0.014	0.012	0.057	0.055	0.055	0.007	0.043	0.044	0.049	0.011	0.007	0.012
F348					0.010	0.010	0.014	0.016	0.016	0.056	0.061	0.061	0.011	0.045	0.046	0.058	0.017	0.009	0.018
534						0.011	0.017	0.017	0.015	0.051	0.050	0.050	0.014	0.036	0.037	0.046	0.018	0.010	0.015
F86							0.007	0.011	0.009	0.050	0.051	0.051	0.006	0.037	0.038	0.048	0.008	0.010	0.012
F94								0.005	0.002	0.057	0.055	0.055	0.012	0.046	0.044	0.052	0.012	0.014	0.005
F100									0.007	0.064	0.058	0.058	0.016	0.049	0.047	0.052	0.016	0.018	0.011
F306										0.057	0.055	0.055	0.014	0.046	0.044	0.052	0.014	0.016	0.023
9771											0.014	0.014	0.059	0.018	0.015	0.082	0.052	0.051	0.057
B84												0.000	0.061	0.012	0.011	0.080	0.051	0.049	0.055
339													0.061	0.012	0.011	0.080	0.051	0.049	0.055
82-1														0.048	0.049	0.065	0.011	0.011	0.018
XCB															0.002	0.074	0.039	0.038	0.046
XCF																0.075	0.040	0.038	0.044
X22j																	0.051	0.060	0.055
X198																		0.017	0.018
X151																			0.012
75-3																			

[a] High, Moderate, and Weak denote aggressiveness groups.

[b] A, B, and C denote canker groups.

of X. campestris. Differences in the number of common restriction fragments from the amplified DNA of the hrp gene cluster indicated that there is variation in the relatedness of the hrp genes of the different strains of X. campestris. The genetic divergence between strains was estimated from the data for 106 restriction fragments obtained with the combination of two hrp gene cluster fragments and four endonucleases. A pairwise matrix of the genetic distances, δ, was calculated for the 18 distinct banding patterns (Table 2). X. campestris pv. citri 339 was included in the genetic analysis as a representative of group C, although the restriction banding patterns of the hrp fragments were identical to those of the strains of X. campestris pv. citri group B. The largest genetic divergence value was 0.082 nucleotide substitution per site between X. campestris pv. maculifoliigardeniae X22j and X. campestris pv. citri 9771 of group A (Table 2). However, most of the estimates of nucleotide substitutions per site are smaller than 0.05, which is considered the upper limit to give accurate estimates of genetic distance based on restriction fragment data (27).

Strains of X. campestris pv. citrumelo that represent all three aggressiveness groups exhibited nine different restriction patterns of the hrp fragments that were divergent from 0.002 to 0.022 nucleotide substitution per site (Table 2). Similarly, strains of X. campestris pv. citri groups A, B, and C showed a low genetic divergence for the hrp genes, ranging from 0.000 to 0.014 nucleotide substitution per site (Table 2). As mentioned above, the banding patterns of the strains of X. campestris pv. citri groups B and C were identical to each other for all combinations of hrp gene cluster fragments and restriction endonuclease digestions. On the other hand, the hrp genes of strains of X. campestris pv. citrumelo were very poorly related to the one of X. campestris pv. citri, with divergence ranging from 0.050 to 0.064 nucleotide substitution per site (Table 2).

The relatedness of the hrp genes of strains of the citrus pathogens X. campestris pv. citrumelo and X. campestris pv. citri to the hrp gene cluster of some other pathovars of X.

campestris was also investigated. The hrp gene clusters of strains of X. campestris pv. alfalfae, X. campestris pv. fici, X. campestris pv. vesicatoria, and X. campestris from Strelitza reginae were closely related to the hrp cluster of X. campestris pv. citrumelo, and the genetic divergence ranged from 0.004 to 0.023 nucleotide substitution per site (Table 2). However, strains of X. campestris pv. citri had hrp genes much less related to those of strains of X. campestris, with genetic divergence ranging from 0.049 to 0.061 nucleotide substitution per site (Table 2). The hrp genes of strains of X. campestris pv. citri were highly related to the hrp genes of X. campestris pv. bilvae and X. campestris XCF from Feronia sp. The genetic divergence of the hrp genes of X. campestris pv. citri from the genes of these strains of X. campestris ranged from 0.011 to 0.018 nucleotide substitution per site (Table 2). Moreover, X. campestris pv. maculifoliigardeniae X22j has hrp genes not highly related to any of the xanthomonads from citrus, with a genetic divergence ranging from 0.046 from X. campestris pv. citrumelo 534 to as high as 0.082 from X. campestris pv. citri group A (Table 2).

The restriction fragment data of the hrp genes, coded 0 or 1, and the distance matrix (Table 2) were used to construct phylogenetic trees based on a parsimony criterion by using the BOOT program and a distance method by using the KITSCH program of the PHYLIP computer package (12), respectively. Although the general topology is slightly different, the phylogenetic trees inferred by using two different approaches of tree reconstruction showed very similar branching patterns for the major clades (Fig. 7 and 8). The branching pattern obtained with the BOOT program is unrooted, although X. campestris pv. maculifoliigardeniae X22j was taken as the outgroup to infer the topology of the tree (Fig. 7). The strains of X. campestris can be divided into three major clades based on the hrp genes, with X. campestris pv. maculifoliigardeniae as the sole member of one clade (Fig. 7). The second clade is the largest one and comprises all strains of X. campestris pv.
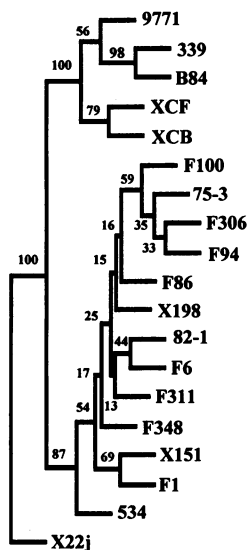
FIG. 7. Unrooted tree for 19 strains of *X. campestris* inferred from restriction analysis data for *hrp* gene cluster fragments from two complementation groups, B and C/D, generated by the BOOT procedure from the PHYLIP computer package by using the Wagner parsimony criterion. Numbers at each node indicate the bootstrap percentages from 100 samples. Bootstrap values less than 50 indicate that the assemblage is not well supported by the data.
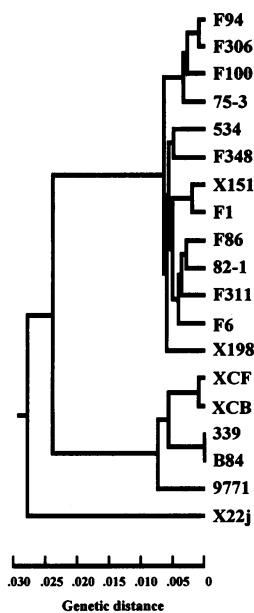

FIG. 8. Rooted tree for 19 strains of *X. campestris* inferred from restriction analysis data for two fragments of complementation groups B and C/D of the *hrp* gene cluster, generated by the KITSCH procedure from the PHYLIP computer package by using the Fitch-Margoliash method.

citrumelo, *X. campestris* pv. alfalfae, *X. campestris* pv. fici, *X. campestris* pv. vesicatoria, and *X. campestris* from *S. reginae* (Fig. 7). The third clade is formed by all the strains of *X. campestris* pv. citri, *X. campestris* pv. bilvae, and a strain of *X. campestris* from *Feronia* sp. (Fig. 7). The assemblage of these phylogenetic clades is highly supported by the bootstrap values (Fig. 7). Further, *X. campestris* pv. maculifoliigardeniae X22j was indeed chosen as an appropriate outgroup to evaluate the relationships among *X. campestris* strains causing disease on citrus on the basis of the *hrp* genes. The rooted tree obtained by the KITSCH program, selected among 1,908 trees examined, also indicates that *X. campestris* pv. maculifoliigardeniae is basal to the remainder of the *X. campestris* strains included in this study (Fig. 8).

The inferred phylogenetic trees seem to support the hypothesis that the *hrp* gene cluster of *X. campestris* pv. citrumelo strains from the three aggressiveness groups are monophyletic and closely related to other pathovars of *X. campestris*, including *X. campestris* pv. alfalfae, *X. campestris* pv. fici, and *X. campestris* pv. vesicatoria. Similarly, the monophyly of the *hrp* genes of *X. campestris* pv. citri, *X. campestris* pv. bilvae, and a strain of *X. campestris* from *Feronia* sp. is also supported (Fig. 7 and 8).

DISCUSSION

Strains of all pathovars of *X. campestris* included in this study have an *hrp* gene cluster on the basis of hybridization of genomic DNA with the *hrp* gene cluster from *X. campestris* pv. vesicatoria and on the basis of amplification of *hrp* fragments with oligonucleotide primers specific for the complementation groups B and C/D of the *hrp* genes. This is particularly significant in regard to the strains associated with citrus bacterial spot disease. Despite differences in the pathogenic characteristics of those strains (18, 20), the presence of an *hrp* gene cluster supports the pathogenic nature of those bacterial

strains. If they were opportunistic xanthomonads (16), they would lack an *hrp* region (4, 33).

The *Eco*RI digests of the genomic DNA that were probed with the *hrp* gene cluster from *X. campestris* pv. vesicatoria revealed polymorphisms within the *hrp* DNA of the strains that caused necrosis on citrus leaves. However, very little polymorphism was observed among the strains of *X. campestris* pv. citrumelo that represented the three aggressiveness groups. Egel (8) also found very little polymorphism of the *hrp* region of the strains of each of the three aggressiveness groups. Also, very little polymorphism occurred among the strains of *X. campestris* pv. citri group A or among strains of *X. campestris* pv. citri groups B and C. Even though other strains of *X. campestris* caused lesions in citrus leaves, only *X. campestris* pv. fici had a very similar banding pattern of the *hrp* region to the pattern for the strains of the highly aggressive group of *X. campestris* pv. citrumelo. This supports the conclusion that the strains obtained from plants with bacterial spot may belong to pathovars from hosts other than citrus. Also, the two xanthomonads isolated from citrus (*X. campestris* pv. bilvae and a strain of *X. campestris* from *Feronia* sp.) that cause lesions similar to bacterial spot seem to have an *hrp* gene cluster different from that in the strains of *X. campestris* pv. citrumelo. Thus, the bacterial spot pathogen appears to be genetically different from the previously described strains of *X. campestris* causing disease in citrus.

Further information about the similarity of the *hrp* genes of the bacteria causing diseases of citrus was obtained from restriction enzyme patterns of amplified fragments of the *hrp* gene cluster. Although the DNA fragments amplified with the two sets of primers were the same size for all the strains of *X. campestris*, characteristic restriction banding patterns for each bacterial group occurred with the 840- and 1,075-bp fragments. Complementation groups B and C/D of the *hrp* gene cluster, from which the fragments were amplified, are considered to be

highly conserved among phytopathogenic xanthomonads (3, 25). Therefore, the homology of the restriction enzyme fragments from amplified *hrp* genes should furnish valid relationships among these pathogens. These relationships were determined by a phylogenetic analysis.

The phylogenetic analysis based on the *hrp* gene cluster showed polyphyletic relationships of the strains of *X. campestris* causing disease in citrus. This suggests that the *hrp* gene cluster may have evolved independently in these strains of *X. campestris*. This evolution could be convergent or parallel. The analysis is based on the assumption that the differences in restriction sites in the *hrp* gene cluster region were due to nucleotide substitutions and not to insertion or deletion of DNA sequences. In fact, this assumption is supported by the fact that no apparent length variation was observed in the two DNA fragments amplified from all strains of *X. campestris*. In support of the phylogenetic analysis presented here is the monophyletic nature of the *hrp* gene cluster to bacterial pathogens with different genetic backgrounds causing disease in different hosts. The phylogenetic grouping presented here also correlates with genetic analyses based on DNA-DNA hybridization, fingerprinting, and conventional restriction fragment length polymorphism (9, 14, 15, 20–22, 24). The data presented support the concept of a group of causal microorganisms of citrus bacterial spot disease which are closely related yet represent a variety of different genotypes.

Two different groups of *X. campestris* pv. citri were distinguished by restriction enzyme analysis of the amplified fragments of the *hrp* gene cluster. All fragment-endonuclease combinations for the strains of group A were uniform. Similarly, the restriction banding patterns for strains of groups B and C were also identical. The banding patterns for each group were different, however. This substantiates other reports of the relative genetic uniformity of the strains of *X. campestris* pv. citri groups A, B, and C based on restriction analysis of the entire genome by using genomic fingerprinting, pulsed-field electrophoresis, or restriction fragment length polymorphisms with random DNA probes (9, 14, 15, 20, 21). The two groups of *X. campestris* pv. citri have about 60% DNA homology (9). The major difference from previous work is that in our studies, specific homologous regions of the bacterial genome, the *hrp* gene cluster, were compared. Nevertheless, the results of the genetic analysis of the *hrp* related regions are consistent with those obtained when the entire genome was randomly examined. From this and the previously cited research, the two groups of strains within *X. campestris* pv. citri should probably be differentiated at some taxonomic level.

Two groups of *X. campestris* pv. citrumelo were also distinguished after restriction enzyme digestion of the amplified *hrp* fragments. Strains of the highly aggressive group were very uniform for all fragment-endonuclease combinations and had a characteristic restriction banding pattern. On the other hand, the moderately and weakly aggressive groups of the bacterium had diverse restriction banding patterns for both amplified *hrp* fragments. This concurs with previous studies of the moderately and weakly aggressive groups of *X. campestris* pv. citrumelo (9, 14, 15, 21, 22, 24). Even with genetic diversity among the moderately and weakly aggressive strains, all strains included under *X. campestris* pv. citrumelo are 80% similar by DNA-DNA hybridization (9). As with strains of *X. campestris* pv. citri, the two groups of strains of *X. campestris* pv. citrumelo should also be distinguished at the taxonomic level.

The results presented in this work demonstrate that strains of *X. campestris* causing disease in citrus can be reliably differentiated and identified by restriction analysis of amplified fragments related to the *hrp* gene cluster. The number of strains examined was small, but the genetic diversity within different pathovars of *X. campestris* could be assessed. The reliable identification of the citrus pathogen by DNA amplification will greatly facilitate disease diagnosis, as well as ecological and epidemiological studies. Further, the use of oligonucleotide primers for the *hrp* gene cluster region provides certainty for identification of strains of *X. campestris* that may not be possible with other methods.

REFERENCES

1. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1987. Current protocols in molecular biology. John Wiley & Sons, Inc., New York.
2. **Beer, S. V., D. W. Bauer, X. H. Jiang, R. J. Laby, B. J. Sneath, Z.-M. Wei, D. A. Wilcox, and C. H. Zumoff.** 1991. The *hrp* gene cluster of *Erwinia amylovora*, p. 53–60. *In* H. Hennecke and D. P. S. Verma (ed.), Advances in molecular genetics of plant-microbe interactions, vol. 1. Kluwer Academic Publishers, Dordrecht, The Netherlands.
3. **Bonas, U. (Institut für Genbiologische Forschung Berlin GmbH).** 1991. Personal communication.
4. **Bonas, U., R. Schulte, S. Fenselau, G. V. Minsavage, B. J. Staskawicz, and R. E. Stall.** 1991. Isolation of a gene cluster from *Xanthomonas campestris* pv. *vesicatoria* that determines pathogenicity and the hypersensitive response on pepper and tomato. Mol. Plant Microbe Interact. **4:**81–88.
5. **Boucher, C. A., F. Van Gijsegem, P. A. Barberis, M. Arlat, and C. Zischek.** 1987. *Pseudomonas solanacearum* genes controlling both pathogenicity and hypersensitivity on tobacco are clustered. J. Bacteriol. **169:**5626–5632.
6. **Canteros de Echenique, B. I., D. Zagory, and R. E. Stall.** 1985. A medium for cultivation of the B strain of *Xanthomonas campestris* pv. *citri*, cause of cancrosis B in Argentina and Uruguay. Plant Dis. **69:**122–123.
7. **Civerolo, E. L.** 1984. Bacterial canker disease of citrus. J. Rio Grande Val. Hortic. Soc. **37:**127–146.
8. **Egel, D. S.** 1991. Ph.D. thesis. University of Florida, Gainesville.
9. **Egel, D. S., J. H. Graham, and R. E. Stall.** 1991. Genomic relatedness of *Xanthomonas campestris* strains causing diseases of citrus. Appl. Environ. Microbiol. **57:**2724–2730.
10. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132:**6–13.
11. **Felsenstein, J.** 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution **39:**783–791.
12. **Felsenstein, J.** 1991. PHYLIP, Phylogeny Inference Package, version 3.4. Department of Genetics, University of Washington, Seattle.
13. **Fitch, W. M., and E. Margoliash.** 1967. Construction of phylogenetic trees. Science **155:**279–284.
14. **Gabriel, D. W., J. E. Hunter, M. T. Kingsley, J. M. Miller, and G. R. Lazo.** 1988. Clonal population structure of *Xanthomonas campestris* and genetic diversity among citrus canker strains. Mol. Plant Microbe Interact. **1:**59–65.
15. **Gabriel, D. W., M. T. Kingsley, J. E. Hunter, and T. Gottwald.** 1989. Reinstatement of *Xanthomonas citri* (ex Hasse) and *X. phaseoli* (ex Smith) to species and reclassification of all *X. campestris* pv. *citri* strains. Int. J. Syst. Bacteriol. **39:**14–22.
16. **Gitaitis, R. D., M. J. Sasser, R. W. Beaver, T. B. McInnes, and R. E. Stall.** 1987. Pectolytic xanthomonads in mixed infections with *Pseudomonas syringae* pv. *syringae*, *P. syringae* pv. *tomato*, and *Xanthomonas campestris* pv. *vesicatoria* in tomato and pepper transplants. Phytopathology **77:**611–615.
17. **Graham, J. H., and T. R. Gottwald.** 1988. Citrus canker and citrus

bacterial spot in Florida: research findings—future considerations. Citrus Ind. **69**:42–51.

18. **Graham, J. H., and T. R. Gottwald.** 1990. Variation in aggressiveness of *Xanthomonas campestris* pv. *citrumelo* associated with citrus bacterial spot in Florida citrus nurseries. Phytopathology **80**:190–196.

19. **Graham, J. H., and T. R. Gottwald.** 1991. Research perspectives on eradication of citrus bacterial diseases in Florida. Plant Dis. **75**:1193–1200.

20. **Graham, J. H., J. S. Hartung, R. E. Stall, and A. R. Chase.** 1990. Pathological, restriction-fragment length polymorphism, and fatty acid profile relationships between *Xanthomonas campestris* from citrus and noncitrus hosts. Phytopathology **80**:829–836.

21. **Hartung, J. S., and E. L. Civerolo.** 1987. Genomic fingerprints of *Xanthomonas campestris* pv. *citri* strains from Asia, South America, and Florida. Phytopathology **77**:282–285.

22. **Hartung, J. S., and E. L. Civerolo.** 1989. Restriction fragment length polymorphisms distinguish *Xanthomonas campestris* strains isolated from Florida citrus nurseries from *X. c.* pv. *citri.* Phytopathology **79**:793–799.

23. **Hodge, N. C. (University of Florida).** 1991. Personal communication.

24. **Kubicek, Q. B., E. L. Civerolo, M. R. Bonde, J. S. Hartung, and G. L. Peterson.** 1989. Isozyme analysis of *Xanthomonas campestris* pv. citri. Phytopathology **79**:297–300.

25. **Leite, R. P., Jr., G. V. Minsavage, U. Bonas, and R. E. Stall.** 1994. Detection and identification of phytopathogenic *Xanthomonas* strains by amplification of DNA sequences related to the *hrp* genes of *Xanthomonas campestris* pv. vesicatoria. Appl. Environ. Microbiol. **60**:1068–1077.

26. **Lindgren, P. B., R. C. Peet, and N. J. Panopoulos.** 1986. Gene cluster of *Pseudomonas syringae* pv. *"phaseolicola"* controls pathogenicity of bean plants and hypersensitivity on nonhost plants. J. Bacteriol. **168**:512–522.

27. **Nei, M.** 1987. Molecular evolutionary genetics. Columbia University Press, New York.

28. **Nei, M., and W.-H. Li.** 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. USA **76**:5269–5273.

29. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

30. **SAS Institute Inc.** 1988. SAS/STAT user's guide, ed. 6.03. SAS Institute Inc., Cary, N.C.

31. **Schoulties, C. L., E. L. Civerolo, J. W. Miller, R. E. Stall, C. J. Krass, S. R. Poe, and E. P. DuCharme.** 1987. Citrus canker in Florida. Plant Dis. **71**:388–395.

32. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.

33. **Stall, R. E., and G. V. Minsavage.** 1990. The use of *hrp* genes to identify opportunistic xanthomonads, p. 369–374. *In* Z. Klement (ed.), Proc. 7th Int. Conf. Plant Pathog. Bacteria 1989. Akadémiai Kiadó, Budapest.

34. **Stall, R. E., and C. P. Seymour.** 1983. Canker, a threat to citrus in the gulf-coast states. Plant Dis. **67**:581–585.

35. **Vauterin, L., P. Yang, B. Hoste, M. VanCanneyt, E. L. Civerolo, J. Swings, and K. Kersters.** 1991. Differentiation of *Xanthomonas campestris* pv. *citri* strains by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of proteins, fatty acid analysis, and DNA-DNA hybridization. Int. J. Syst. Bacteriol. **41**:535–542.

36. **Willis, K., J. J. Rich, and E. M. Hrabak.** 1991. *hrp* genes of phytopathogenic bacteria. Mol. Plant Microbe Interact. **4**:132–138.