Pathological, Restriction-Fragment Length Polymorphism, and Fatty Acid Profile Relationships Between *Xanthomonas campestris* from Citrus and Noncitrus Hosts

J. H. Graham, J. S. Hartung, R. E. Stall, and A. R. Chase

Associate professor, University of Florida, IFAS, Citrus Research and Education Center, Lake Alfred 33850; research plant pathologist, U.S. Department of Agriculture, ARS, Plant Science Institute, Beltsville, MD 20705; professor, University of Florida, IFAS, Department of Plant Pathology, Gainesville 32611; and professor, University of Florida, IFAS, Central Florida Research and Education Center, Apopka, 32703, respectively.

Florida Agricultural Experiment Station Journal Series R-00308. This research was supported by USDA-ARS-AAD/IFAS Cooperative Agreement 58-43YK-5-3.

The authors express their appreciation to D. L. Drouillard, D. Fardelmann, N. C. Hodge, and A. S. O'Conner for their expert technical assistance. We also thank P. V. Oudemans and T. R. Gottwald for assistance in data analysis. Accepted for publication 16 February 1990.

ABSTRACT

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

Pathogenic strains of *Xanthomonas campestris* from noncitrus hosts were screened for their ability to cause necrosis on wounded, detached leaves of the citrus cultivars Swingle citrumelo and Duncan grapefruit. Thirteen of 56 noncitrus strains produced reactions that were similar to those caused by *X. c.* pv. *citrumelo* strains isolated from several outbreaks of citrus bacterial spot in Florida nurseries. Noncitrus strains of the weakly to moderately aggressive type, including strains of *X. c.* pv. *alfalfae*, *X. c.* pv. *fici*, *X. c.* pv. *maculifoliigardeniae*, and three strains from *Strelitzia*, elicited necrotic spots on spray-inoculated immature foliage of both Swingle citrumelo and Duncan grapefruit. When noncitrus strains were injection-infiltrated into Swingle citrumelo leaves, they multiplied and reached populations as high as those attained by a weakly aggressive strain from citrus. Strains of *X. c.* pv. *campestris*, *X. c.* pv. *phaseoli*, and *X. c.* pv. *malvacearum* that did not elicit necrosis on detached leaves failed to multiply in leaves. The group of weakly to moderately aggressive strains from noncitrus hosts was compared with aggressive and less aggressive strains from citrus by restriction-fragment length polymorphism analysis of genomic DNA and by cellular fatty-acid profiles. Most of the weakly to moderately aggressive strains of noncitrus origin could not be separated from the group of citrus strains by either analysis. Other *X. campestris* strains that did not grow in planta and give a disease reaction were less related to the citrus and the other noncitrus strains by these analyses. These findings raise doubts as to the role of less aggressive strains as primary pathogens of citrus and their inclusion within a separate pathovar, *X. c.* pv. *citrumelo*.

Strains of *Xanthomonas campestris* have been identified as the cause of 55 outbreaks of a leaf, fruit, and stem spotting disease in Florida citrus nurseries since 1984 (11,12,23). Because the nursery strains were isolated from citrus, and because *X. campestris* strains are classified based on the host from which first isolated (26), these strains were provisionally included as group E within pathovar *citri* (7,9,13). Groups A, B, C, and D of *X. c.* pv. *citri* cause citrus canker, which is characterized by corky, erumpent-to-sunken lesions on leaves, stems, and fruit (4). Subsequently, the nursery strains were distinguished from strains of *X. c. citri* by several independent analyses as well as host symptomatology (2,5,7,8,12–16,24). This led to the use of the name citrus bacterial spot (CBS) for the nursery disease characterized by flat-spreading foliar lesions (11). Gabriel et al (8) proposed that strains associated with CBS be named as a new pathovar, *citrumelo*, based on within-group similarities in restriction-fragment length polymorphisms (RFLPs) and dissimilarities with other strains of *X. c. citri* (8). However, the strains included under *X. c.* pv. *citrumelo* are characterized by variation in aggressiveness (6,12), serological reactions (2), fatty acid profiles (24), isozymes (15), genomic fingerprints (13), and RFLP relationships (7,8,14). Differences in disease incidence and severity in the field (10,12) and cytopathology (5,16) have also been identified.

The aggressive strains, found in four different locations in Florida, produce foliar lesions with extensive water-soaking and are apparently spread naturally in citrus nurseries (10,12). This aggressive pathotype represents a group of highly related strains by RFLP analysis (7,8,14; J. S. Hartung, *unpublished data*). The less aggressive strains vary in their host reaction (12) and appear to be only transmitted mechanically (10). The less aggressive pathotypes are heterogeneous by RFLP and fingerprinting analyses of genomic DNA, although all of the strains are somewhat related to each other and to the group of aggressive strains (7,8,14; J. S. Hartung, *unpublished data*). Gabriel et al (7,8) suggested that at least some strains of *X. c. citrumelo* are closely related to pathovars of *X. campestris* that attack legumes, e.g., *X. c.* pv. *alfalfae*. Because of the variability of the weakly aggressive strains of *X. c. citrumelo*, we hypothesized that these strains represent several different pathovars of *X. campestris* which attack citrus as a secondary host only under the conducive conditions frequently present in nurseries.

This study was initiated to determine whether strains of *X. campestris* that are pathogens of hosts other than citrus in Florida (and by definition are not pathovar *citrumelo*) have the ability to grow in citrus leaves and cause reactions like the weakly aggressive pathotype in citrus nurseries. Furthermore, we sought to evaluate whether *X. campestris* strains which cause leaf spots on citrus are related regardless of host origin (i.e., pathovar type) through comparisons of RFLPs of genomic DNA and profiles of cellular fatty acids.

MATERIALS AND METHODS

Bacterial strains. Strains of *X. campestris* are listed in Table 1 by pathovar and/or host of origin. Most are pathogens of ornamental plants from Florida nurseries and were isolated between 1984 and 1987 (1,3,18). All strains were tested for pathogenicity on the host plant of origin and closely related host species (A. R. Chase, *unpublished data*). In most cases, three host plants per strain were inoculated with 1×10^8 colony-forming units (cfu)/ml in 0.1 M $MgSO_4$ (determined turbidimetrically)

© 1990 The American Phytopathological Society

TABLE 1. *Xanthomonas campestris* pathovars and strains, their host origin, and reaction on wound-inoculated detached leaves of Swingle citrumelo and Duncan grapefruit

Strain I.D. (source)[a]	Pathovar	Host	Detached leaf rating[b]	
			Swingle citrumelo	Duncan grapefruit
82-1 (RES)	*alfalfae*	*Medicago sativa*	2	2
X6 (ELC)	*campestris*	*Brassica oleracea*	0	0
F1 (ELC)	*citrumelo*	*Poncirus trifoliata* × *Citrus sinensis*	3	3
F6 (ELC)	*citrumelo*	*Citrus paradisi*	2	2
F59 (ELC)	*citrumelo*	*P. trifoliata* × *C. paradisi*	1	1
F86 (ELC)	*citrumelo*	*P. trifoliata* × *C. paradisi*	1	1
F94 (ELC)	*citrumelo*	*P. trifoliata* × *C. paradisi*	1	1
F100 (ELC)	*citrumelo*	*P. trifoliata* × *C. paradisi*	1	1
F306 (ELC)	*citrumelo*	*P. trifoliata* × *C. paradisi*	1	1
X13 (ARC)	*dieffenbachiae*	*Anthurium* sp.	0	0
X82 (ARC)	*dieffenbachiae*	*Anthurium andreanum*	0	0
X268 (ARC)	*dieffenbachiae*	*Anthurium* sp.	0	0
X175 (ARC)	*dieffenbachiae*	*Dieffenbachia* sp.	0	0
X183 (ARC)	*dieffenbachiae*	*Dieffenbachia* sp.	0	0
X185 (ARC)	*dieffenbachiae*	*Dieffenbachia* sp.	0	1
X151 (ARC)	*fici*	*Ficus benjamina*	2	1
X207 (ARC)	*fici*	*F. benjamina*	0	0
X209 (ARC)	*fici*	*F. benjamina*	0	0
X212 (ARC)	*fici*	*F. benjamina*	0	0
X217 (ARC)	*fici*	*F. benjamina*	0	0
X224 (ARC)	*fici*	*F. benjamina*	0	0
X25 (ARC)	*hederae*	*Schefflera arboricola*	0	0
X37 (ARC)	*hederae*	*Brassaia actinophylla*	0	0
X200 (ARC)	*hederae*	*S. arboricola*	0	0
X300 (ARC)	*hederae*	*Hedera helix*	0	0
X22J (DPI)	*maculifoliigardeniae*	*Gardenia* sp.	2	2
X10 (ARC)	*malvacearum*	*Hibiscus rosa-sinensis*	0	0
X203 (ARC)	*malvacearum*	*H. rosa-sinensis*	0 (TC)[c]	0 (TC)
X204 (ARC)	*malvacearum*	*H. rosa-sinensis*	0 (TC)	0 (TC)
X323 (ARC)	*malvacearum*	*H. rosa-sinensis*	0	0
X128 (ARC)	*pelargonii*	*Pelargonium hortulanum*	1	0
X231 (ARC)	*pelargonii*	*P. hortulanum*	0	0
X244 (ARC)	*pelargonii*	*P. hortulanum*	0	0
X302 (ARC)	*pelargonii*	*P. hortulanum*	0	0
X303 (ARC)	*pelargonii*	*P. hortulanum*	1	1
X34 (ELC)	*phaseoli*	*Phaseolus vulgaris*	0	0
X45 (ATCC)	*phaseoli*	*P. vulgaris*	0	0
X87 (ARC)	*poinsettiicola*	*Codiaeum variegatum*	0	0
X349 (ARC)	*poinsettiicola*	*Euphorbia pulcherrima*	0	0
X155 (ARC)	*syngonii*	*Syngonium podyphyllum*	0	0
X161 (ARC)	*syngonii*	*S. podyphyllum*	0	0
X166 (ARC)	*syngonii*	*S. podyphyllum*	0	0
X172 (ARC)	*syngonii*	*S. podyphyllum*	0	0
X192 (ARC)	*syngonii*	*S. podyphyllum*	0	0
XV1 (JBJ)	*vesicatoria*	*Lycopersicon esculentum*	0	0
X180 (ARC)	undetermined (1)	*Fittonia verschaffeltii*	0	0
X257 (ARC)	undetermined (1)	*F. verschaffeltii*	0	0
X294 (ARC)	undetermined (1)	*F. verschaffeltii*	0	0
X295 (ARC)	undetermined (1)	*F. verschaffeltii*	1	1
X30 (ARC)	undetermined (17)	*Pellionia* sp.	0	0
X33 (ARC)	undetermined (17)	*P. pulchra* sp.	0	0
X53 (ARC)	undetermined (17)	*Pilea cadierei*	0	0
X229 (ARC)	undetermined (17)	*P. spruceana*	0	0
X264 (ARC)	undetermined (17)	*P. cadierei*	0	0
X22 (ARC)	undetermined (3)	*Strelitzia reginae*	0	0
X137 (ARC)	undetermined (3)	*S. reginae*	1	0
X142 (ARC)	undetermined (3)	*S. reginae*	1	0
X143 (ARC)	undetermined (3)	*S. reginae*	2	1
X144 (ARC)	undetermined (3)	*S. reginae*	1	0
X154 (ARC)	undetermined (3)	*S. reginae*	0	0
X198 (ARC)	undetermined (3)	*S. reginae*	2	0
X199 (ARC)	undetermined (3)	*S. reginae*	1	0
X270 (ARC)	undetermined (3)	*S. reginae*	0	0

[a] Sources of strains: RES = R. E. Stall, University of Florida, Gainesville; DPI = Division of Plant Industry, Gainesville, FL; ELC = E. L. Civerolo, USDA, Beltsville, MD; ARC = A. R. Chase, University of Florida, Apopka; ATCC = American Type Culture Collection; JBJ = J. B. Jones, University of Florida, Bradenton.

[b] Disease severity ratings of wound-inoculation sites were made 14–21 days after inoculation as follows: 0 = no reaction; 1 = weakly aggressive, water-soaking indistinct (<1 mm wide), necrosis distinct but limited, both ± around wound; 2 = moderately aggressive, water-soaking distinct (<1 mm wide) from necrosis, both completely around wound; 3 = aggressive, water-soaking extensive (>1 mm wide) around wound, necrosis extensive and indistinct from water-soaking. Ratings are of 20 and 10 inoculation sites on detached Swingle citrumelo and Duncan grapefruit leaves, respectively.

[c] TC = Tissue collapse 48 hr after inoculation.

by gently spraying the foliage and then intermittently misting for 14–21 days after inoculation. All strains were rated as pathogenic on the host of origin. Strains from citrus were isolated from outbreaks of CBS from 1984 to 1988, and their pathogenicity was evaluated by one or more of the following tests: detached leaf assay (12), growth rate in attached leaves of Swingle citrumelo and Duncan grapefruit (6), spray inoculation of unwounded leaves (J. H. Graham, *unpublished data*), and greenhouse and field inoculations of wounded leaves (12).

All strains were confirmed to be *X. campestris*, either by their color and appearance on yeast extract-dextrose-calcium carbonate agar, growth on selective kasugamycin-cephalexin-chlorothalonil medium (KCB) (12), or xanthomonadin pigment analysis (22) compared to well-characterized strains of *X. campestris*. Strains were stored in sterile tap water at 5 C.

Characterization of strain reactions on citrus. A detached-leaf assay was used to screen strains of *X. campestris* for their ability to elicit water-soaking and necrosis on citrus (Table 1). Strains from citrus which give a range of reactions (Table 1, F1, F6, F100) were included as controls for comparative purposes. The detached leaf assay was performed as previously described (12) with modifications. The disease severity ratings were made 14–21 days after inoculation as follows: 0 = no reaction; 1 = weakly aggressive, water-soaking indistinct (< 1 mm wide), necrosis distinct but limited, both ± around wound; 2 = moderately aggressive, water-soaking distinct (< 1 mm wide) from necrosis, both completely around the wound; 3 = aggressive, water-soaking extensive (> 1 mm wide), around wound, necrosis extensive and indistinct from water-soaking. The assay was performed on two Swingle citrumelo leaves and one Duncan grapefruit leaf per strain (10 inoculation sites per leaf). In cases where positive reactions were obtained, the assay was repeated at least once. Strains were assigned an aggressiveness type based on the ratings from the two inoculation tests.

Spray inoculation of unwounded leaves. Six noncitrus strains that gave weakly to moderately aggressive reactions on wounded detached leaves (X22J, 82-1, X151, X137, X143, and X198) and one strain that did not (X6) were further tested by spray inoculation of attached, nonwounded leaves of Swingle citrumelo and Duncan grapefruit in the greenhouse. Bacteria were grown to late log phase, centrifuged, and resuspended in 0.075 M phosphate buffer (pH 7.0). The suspension was adjusted turbidimetrically to 10^8 cfu/ml, and the inoculum concentration of each strain was confirmed by serial dilution plating on Difco nutrient agar. Seedlings (50 cm in height) with 6–12 immature leaves were covered with a plastic bag for 24 hr prior to treatment in the greenhouse. After removal of the bag, the bacterial suspension was gently sprayed onto the adaxial surfaces of the leaves until runoff, and the bag was replaced immediately to maintain leaf wetness for 4 days. Bags were removed and the

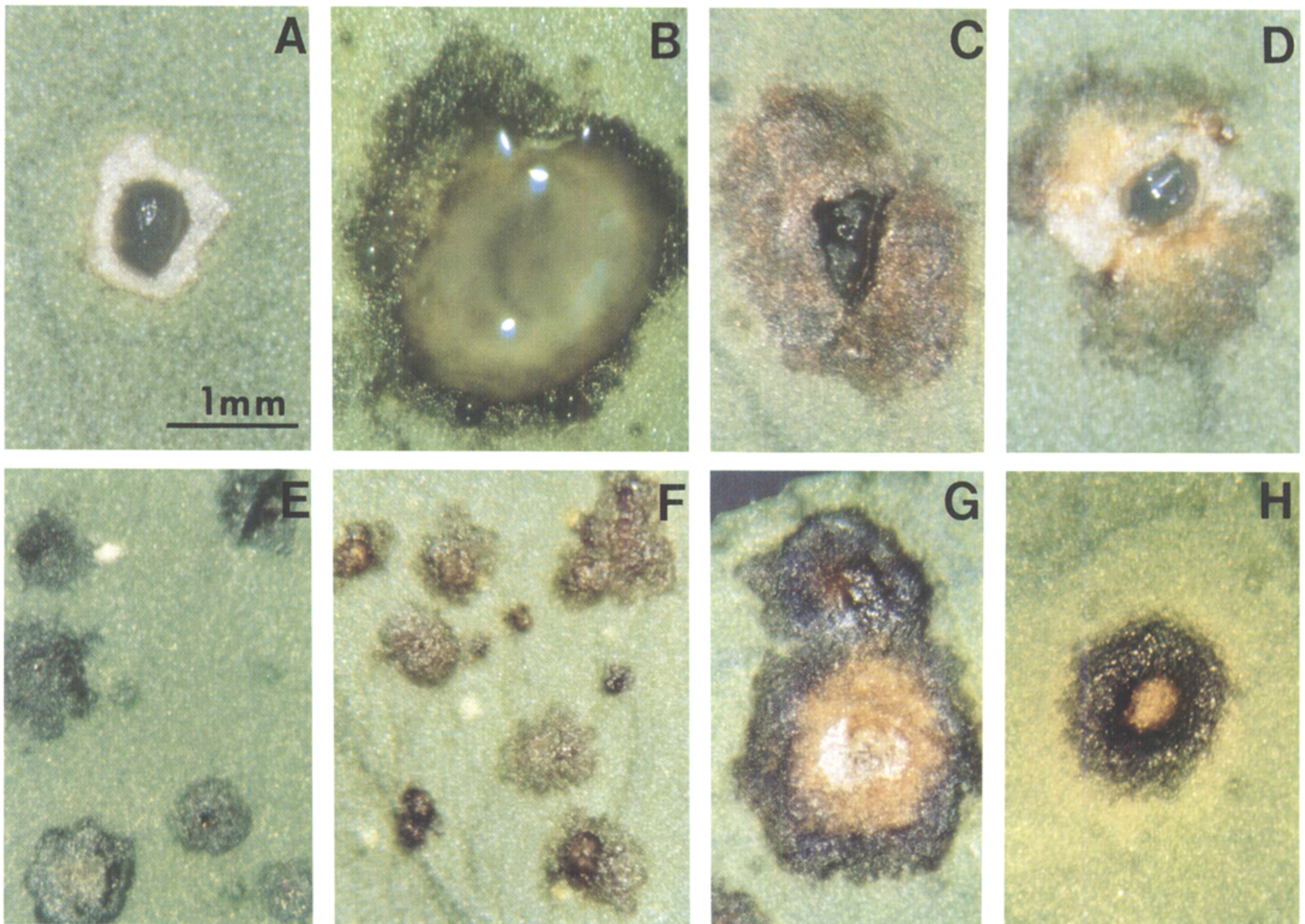

Fig. 1. Reactions of wound-inoculated detached leaves and spray-inoculated attached leaves of Swingle citrumelo to *Xanthomonas campestris* from citrus and noncitrus hosts. A–D = reactions of detached leaves 21 days after inoculation. **A,** Wound reaction of uninoculated leaf; **B,** aggressive reaction of citrus strain F1 with persistent water-soaking and indistinct necrosis covered with bacterial ooze; **C,** weakly aggressive reaction of citrus strain F100 with necrosis limited to the periphery of the wound and no water-soaking at the perimeter; and **D,** weakly aggressive reaction of noncitrus strain X143 of *X. campestris* from *Strelitzia*. E–G = lesions on nonwounded sprayed leaves 30–40 days after inoculation. **E,** Citrus strain F100 with a cluster of necrotic spots of varying sizes; **F,** noncitrus strain X22J of *X. c.* pv. *maculifoliigardeniae* gives a reaction similar to F100; **G,** expanded lesions of X22J have a gray appearance at the margin of the necrosis under the dew-forming conditions. **H,** A citrus bacterial spot lesion on Swingle citrumelo from a commercial greenhouse nursery that was associated with weakly aggressive strains. All magnifications are relative to the scale in panel A, except panel B, which is 0.75X.

plants left under greenhouse conditions (23/30 C night/day) without overhead watering for the next 4 wk. Five plants of each variety were treated with each strain tested, and three uninoculated plants were sprayed with buffer. Spray inoculations were repeated on a new flush of leaves on the same set of seedlings, except plants were moved to dew chamber conditions (23/27 C, 97/90% RH night/day, and 12-hr photoperiod) where dew formed on leaves each night for 4 wk after inoculation. A parallel test of similar design was conducted with a weakly aggressive strain from citrus (F100) in a quarantine greenhouse in Gainesville, FL. One noncitrus strain that did not give a reaction on detached leaves (XV-1) was included as a control.

In each test, bacterial populations in lesions elicited by selected strains (X22J, X198, and X6) were determined 45 days after inoculation. Leaf disks of 0.37 cm in diameter were removed from five leaves on five different plants ($n = 5$). Three leaves without lesions on inoculated plants were sampled as a check for epiphytic or endophytic populations in the absence of lesions ($n = 3$). Leaf disks were ground in sterile phosphate buffer and dilution plated on KCB. Populations were expressed as cfu/cm^2 leaf area.

In-leaf bacterial growth. Five strains from noncitrus hosts that gave reactions on detached and spray-inoculated leaves (X22J, X151, X137, X143, and X198) were examined for their ability to grow in Swingle citrumelo leaves. These strains were compared with three strains that gave an atypical reaction or gave no reaction (X6, X45, and X203) and one weakly aggressive strain from citrus (F100). Suspensions of each strain in sterile tap water (10^5 cfu/ml) were injection-infiltrated into fully expanded but immature leaves of Swingle citrumelo with a 26-gauge needled syringe at several points. Each strain was inoculated into one leaf of each of five plants. Bacterial populations were assayed at 0, 1, 5, 10, 20, and 30 days after time of inoculation by removing 0.37-cm-in-diameter leaf disks from within the infiltrated area. Leaf disks were ground in 1 ml of sterile phosphate buffer and dilution plated onto nutrient agar amended with chlorothalonil (Bravo 720, 12 mg/L). Populations were expressed as cfu/cm^2 of leaf area and were subjected to the General Linear Model procedure (SAS, Cary, NC) for repeated measures of analysis of variance with time as a repeated measure of leaf populations. Linear contrasts of strains were made in the univariate mode after adjustment for correlation among repeated measures. The results of three experiments are reported.

Restriction-fragment length polymorphism analysis. Genomic DNA was isolated as described previously (14) from the following: strains from citrus (F1, F6, F59, F86, F94, F100, and F306); noncitrus strains that multiplied in leaves and caused a reaction on citrus (X22J, X151, X137, X143, and X198); and strains that did not grow and cause reactions on citrus (X6, X45, and X203). Electrophoresis of DNA was in 0.8% agarose gels in TPE buffer (0.08 M Tris-phosphate, 0.002 M ethylenediaminetetraacetic acid) at 1.5 V/cm for 16 hr. Restriction endonuclease digestions, Southern blotting, and hybridizations with biotin-11-dUTP-labeled DNA probes were done as described previously (14). Cosmid probes were constructed from a complete genomic library of the group A strain XC62 of *X. c. citri* (14). For each of six probes, the similarity coefficient (F) of strains X and Y was calculated as:

$$F = \frac{2n_{xy}}{n_x + n_y},$$

where $2n_{xy}$ is the number of fragments shared between two strains and $n_x + n_y$ is the total number of hybridization bands (19). The F values in Table 2 are the mean from six hybridization probes. Cluster analysis was done by the unweighted pair-group method for the F means of all strain comparisons (NTSYS-pc program, Exeter Pub. Ltd., Setauket, NY). Analysis of variance of the F values and Duncan's multiple range test was used to compare each strain with the group of citrus strains. For this analysis, the F values for citrus strains were treated as replications to determine the mean similarity to the group of citrus strains for each strain.

Fatty acid analysis. Fatty acids were extracted by the method of Miller and Berger (18) with minor modifications. Single colonies of each strain grown on nutrient agar were inoculated onto fatty acid-free Difco Trypticase Soy Broth agar. After 24 hr of growth at 28 ± 1 C, approximately 40 mg of wet weight of cells (one 4-mm loopful) was transferred to a 13 × 100 mm glass test tube fitted with a Teflon-lined screw cap. For fatty acid saponification and derivatization to methyl esters, 1.0 ml of 1.2 N NaOH in 50% methanol was added to the test tube, which was capped tightly and heated in a 100 C water bath for 5 min. The tube was vortexed 5–10 seconds and heated at 100 C for 25 min. When cooled, 2 ml of 6.0 N HCl in methanol (325:275 v/v) was added, the mixture vortexed and heated in a 80 ± 1 C water bath for 10 min and immediately cooled to room temperature. For extraction, 1.25 ml of hexane:anhydrous methyl-t-butyl ether (1:1, v/v) was added and the tube mixed end-over-end on a rotator for 10 min. The lower aqueous phase was removed and 3.0 ml of 0.3 N NaOH was added to the remaining organic phase and mixed for 5 min on the rotator. Two-thirds of the upper organic layer was transferred to a gas chromatography autosampler vial.

Fatty-acid methyl esters were separated with a Hewlett Packard 5890 gas-liquid chromatograph fitted with a capillary column (Ultra 2, crosslinked 5% phenyl methyl silicone, 25 m × 0.2 mm i.d.) and a flame ionization detector. Temperature was programmed to begin at 170 C and increase 5 C/min to a final temperature of 270 C for 2 min. Nitrogen carrier gas flow rate was 20 ml/min. A sample volume of 2.0 μl was automatically injected (HP 7673A autosampler) with a column-head split ratio of 100:1.

Profiles of fatty acids were stored in the computerized Microbial Identification (MIDI) System (Microbial ID, Inc., Newark, DE). The averages of four individual profiles from four separate extractions of each strain were generated, which enabled the development of a unique library for each bacterial strain. Pattern recognition within the MIDI Library Generation Software statistically compared the peak area and the profile of the test strain to each library to determine the relationship of that strain to the others. A similarity index based upon Gaussian distance expresses how nearly the profile of the test strain matches that of the library (21). Analysis of variance and cluster analysis of similarity indices of all strains were performed as described above for RFLP analysis.

RESULTS

Characterization of *X. campestris* reactions on citrus. Fifty-eight pathogenic strains of *X. campestris* from noncitrus hosts were screened on wounded, detached leaves of Swingle citrumelo and Duncan grapefruit and their reactions compared to those of seven strains from citrus (Table 1). As previously reported (12), strains F1 and F6 gave disease ratings of 3 (Fig. 1B) and 2, respectively, which are considered standard aggressive and moderately aggressive reactions. Of the remaining strains from citrus, F100 gave a weakly aggressive reaction of 1 on Swingle citrumelo (Fig. 1C) and Duncan grapefruit. Strains F59, F86, F94, and F306 also gave weakly aggressive ratings of 1 on both cultivars.

Thirteen strains from noncitrus hosts varied from moderately to very weakly aggressive on Swingle citrumelo and Duncan grapefruit. Ratings were usually higher on Swingle citrumelo than on Duncan grapefruit. Often, only Swingle citrumelo reacted (Table 1, X128, X142, X144, X198, and X199), but X185 reacted only with grapefruit. The noncitrus strains that elicited very weakly aggressive reactions were *X. c.* pv. *dieffenbachiae* X185; *X. c.* pv. *pelargonii* X128 and X303; *X. campestris* from *Fittonia* X295; and three strains of *X. campestris* from *Strelitzia*, X142, X144 and X199. Six strains elicited weakly to moderately aggressive reactions on Swingle citrumelo and Duncan grapefruit comparable to citrus strains F100 (Fig. 1C) and F6. These included *X. c. alfalfae* 82-1, *X. c.* pv. *fici* X151, and *X. c.* pv. *maculifoliigardeniae* X22J; and three strains of *X. campestris* from *Strelitzia* X137, X143, and X198. All of these strains were

associated with necrosis with a thin margin (< 1 mm wide) of water-soaking on Swingle citrumelo (Fig. 1D) and less necrosis and no water-soaking on grapefruit. In several cases, only one or two strains of a given pathovar elicited a response: one of six strains of *X. c. dieffenbachiae*, one of six strains of *X. c. fici*, two of five strains of *X. c. pelargonii*, and one of four strains of *X. campestris* from *Fittonia*. However, six of nine strains of *X. campestris* from *Strelitzia* gave reactions ranging from weakly aggressive to moderately aggressive. Of the four strains of *X. c.* pv. *malvacearum*, two (X203, X204) elicited a rapid response. The tissue around the wound site collapsed within 48 hr, which resulted in an extensive water-soaked area with a band of tan necrotic tissue at the margin.

Six noncitrus strains (X22J, 82-1, X151, X137, X143, and X198) and one citrus strain (F100) that were weakly to moderately aggressive on wounded, detached citrus leaves and two strains (X6 and XV-1) that gave no reaction were sprayed onto nonwounded Swingle citrumelo and Duncan grapefruit. Ten to 14 days later, small necrotic lesions of varying size (0.1–2.0 mm) and density (3.1–9.8 lesions/cm^2 leaf) developed on the immature leaves of both varieties (Fig. 1E and F). Strains X6 of *X. c. campestris* and XV-1 of *X. c.* pv. *vesicatoria* failed to elicit a reaction, both on detached leaves and after spray inoculation. Forty-five days after inoculation, populations of noncitrus strains X198 and X22J in the leaves with lesions were comparable to those of the citrus strain F100 (10^7 to 10^8 cfu/cm^2 leaf). No bacteria were detected from leaves without lesions. When plants inoculated with noncitrus strains were maintained under conducive conditions (dew formed on the foliage every night cycle), the lesions expanded further than under nonconducive conditions (Fig. 1F and G). The size and appearance of the lesions were similar to lesions on Swingle citrumelo in a commercial greenhouse nursery that received overhead irrigation (Fig. 1G and H). In all cases, the necrotic tissue appeared dry with little or no water-soaking at the margin.

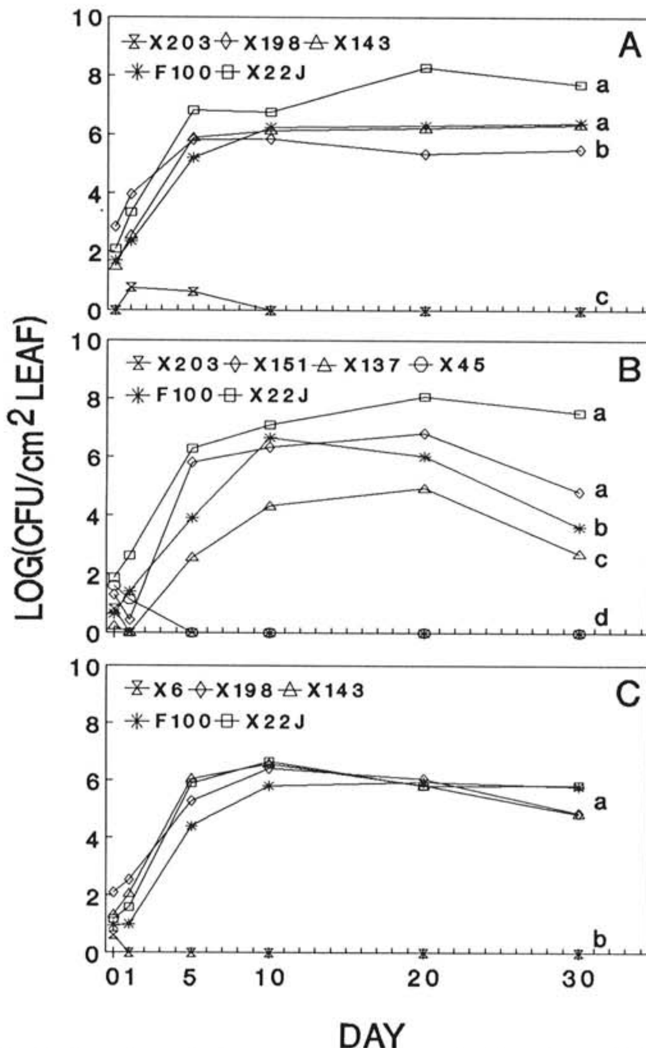

Fig. 2. Growth of *Xanthomonas campestris* strains from citrus (F100) and from noncitrus hosts (prefixed X) in Swingle citrumelo leaves after injection-infiltration (description of strains in Table 1). Each point represents five observations. Curves followed by unlike letters have significantly different ($P \leq 0.05$) population development with time according to linear contrast analysis (see text).

In-leaf bacterial growth. To test whether the ability of noncitrus strains to cause a reaction on citrus leaves was associated with multiplication of the bacterium in planta, low inoculum levels (10^5 cfu/ml) were used for injection-infiltration of leaves. Within 10 days, strains that grew in leaves elicited small, necrotic lesions similar to those observed after spray inoculation (Fig. 1F and G). Strains that did not grow in leaves from low inoculum levels did not cause spots. High inoculum levels (10^8 cfu/ml) of several *X. campestris* pathovars elicited diffuse chlorosis after 7 days and necrotic flecks by 14 days after injection-infiltration of Swingle citrumelo leaves (Graham, *unpublished data*). This reaction was considered to be artifactual, due to the high inoculum dosage and the method of inoculation.

In three tests, populations of F100 reached 10^6–10^7 cfu/cm^2 in Swingle citrumelo leaves after 10 days and either were maintained or decreased 10- to 100-fold (Fig. 2A, B, and C). In the first test (Fig. 2A), the population growth of *X. c. maculifoliigardeniae* X22J and X143 from *Strelitzia* were similar to F100 over the 30 days after inoculation according to linear contrast analysis. Populations of X198 from *Strelitzia* were 10^5–10^6 cfu/cm^2, which was significantly lower than that of F100. The recovery of *X. c. malvacearum* X203 was erratic, and bacteria were not detected after 5 days. In the second test (Fig. 2B), the populations in planta of strain X22J and strain X151 of *X. c. fici* were significantly greater than that of strain F100, which reached 5×10^6 cfu/cm^2. Populations of strain X137 from *Strelitzia* were substantially lower and reached only 5×10^6 cfu/cm^2. Neither *X. c. malvacearum* X203 or *X. c. phaseoli* X45 was recovered after 24 hr. In the third test (Fig. 2C), population development of strains X22J, X198, X143, and F100 was similar. Strain X6 of *X. c. campestris* was not detected after the initial recovery from the leaf at time of inoculation.

RFLP analysis. To determine if there was genetic relatedness among strains with the ability to multiply and cause reactions on citrus leaves irrespective of their original host, similarity coefficients were derived by RFLP analysis (Table 2). As

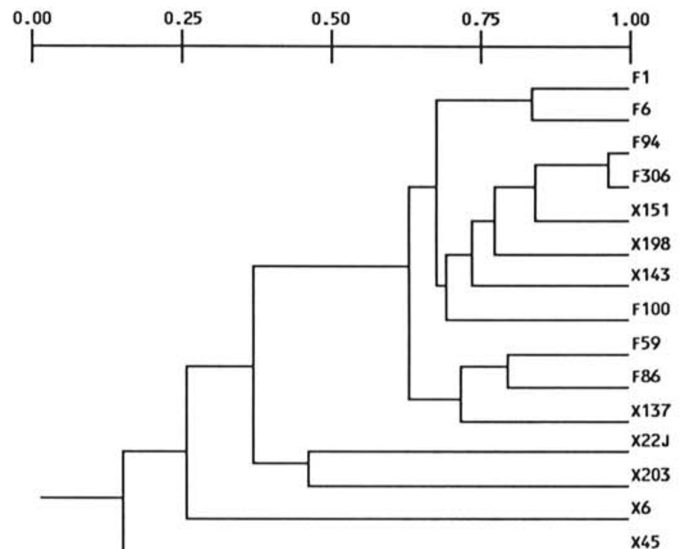

Fig. 3. Dendogram obtained by cluster analysis of similarity coefficients, F, derived from RFLP analysis of strains of *Xanthomonas campestris* from citrus (prefixed F) and from noncitrus hosts (prefixed X) (see Table 1). Scale refers to the similarity index (see text).

previously shown (14), aggressive strain F1 and moderately aggressive strain F6 from citrus were strongly related ($F = 0.85$) but not identical (Table 2). Weakly aggressive strain F100 was moderately related to strains F1 and F6 ($F = 0.63$), as were the other strains from citrus (F59, F86, F94, F306); similarity coefficients ranged from 0.58 to 0.75. Within the citrus group, strains varied from highly related (F94 vs. F306, $F = 0.97$) to weakly related (F100 vs. F59 or F86, $F = 0.52$). As a group, the less aggressive strains from citrus were no more closely related to the more aggressive strains and to each other than the group of noncitrus strains that grew in leaves and caused reactions (e.g., X151, X137, X143, and X198). The mean similarity coefficients between these noncitrus strains and citrus strains ranged from 0.66 to 0.74. Of the noncitrus strains that grew well in citrus leaves, X22J was the only strain that was significantly less related (mean $F = 0.41$) to the citrus strains. Strains that failed to multiply in leaves, *X. c. campestris* X6, *X. c. phaseoli* X45, and *X. c. malvacearum* X203 were even less related to the spotting strains based on RFLP analyses. The mean similarity coefficients between X6, X45, and X203 and citrus strains were 0.11, 0.28, and 0.34, respectively.

Cluster analysis of the similarity coefficients yielded one possible dendogram with a matrix correlation coefficient of 0.97 ($P \leq 0.001$) (Fig. 3). Strains F1 and F6 formed one group, followed by a group of citrus (F94, F100, F306) and noncitrus strains including *X. c. fici* X151 and two strains of *X. campestris* from *Strelitzia*, X143 and X198. Strain X137 of *X. campestris* from *Strelitzia* was clustered with a less related group of citrus strains (F59 and F86) to the F1–F6 group. *X. c. maculifoliigardeniae* X22J was the only strain that grew in citrus leaves that was not grouped with the citrus strains. Strain X22J was grouped with *X. c. malvacearum* X203, a strain which caused tissue collapse

TABLE 2. Similarity coefficients, F[w], derived from restriction fragment length polymorphism (RFLP) analysis for strains of *Xanthomonas campestris* from citrus and noncitrus hosts[x]

	Citrus strains								
	F1	F6	F59	F86	F94	F100	F306	Mean x[y]	DLR[z]
Citrus strains									
F1	1.00	0.85	0.62	0.58	0.65	0.63	0.65	0.66 a	3
F6	0.85	1.00	0.62	0.65	0.75	0.63	0.74	0.71 a	2
F59	0.62	0.62	1.00	0.80	0.63	0.52	0.63	0.64 a	1
F86	0.58	0.65	0.80	1.00	0.63	0.52	0.63	0.64 a	1
F94	0.65	0.75	0.63	0.63	1.00	0.77	0.97	0.73 a	1
F100	0.63	0.63	0.52	0.52	0.77	1.00	0.97	0.67 a	1
F306	0.65	0.74	0.63	0.63	0.97	0.78	1.00	0.73 a	1
Noncitrus strains									
X137	0.57	0.67	0.72	0.73	0.68	0.58	0.67	0.66 a	1
X143	0.62	0.69	0.59	0.56	0.79	0.63	0.75	0.66 a	2
X198	0.62	0.78	0.59	0.70	0.81	0.66	0.80	0.71 a	2
X151	0.63	0.77	0.71	0.70	0.87	0.65	0.84	0.74 a	2
X22J	0.49	0.41	0.35	0.35	0.41	0.46	0.41	0.41 b	2
X45	0.28	0.25	0.27	0.27	0.29	0.34	0.29	0.28 c	0
X203	0.39	0.44	0.36	0.31	0.41	0.38	0.36	0.38 b	0
X6	0.14	0.09	0.09	0.09	0.12	0.12	0.09	0.11 d	0

[w]The derivation of F is described in the text.
[x]Description of strains in Table 1.
[y]Mean of citrus strains. When self-comparisons, i.e., F1 → F1, are excluded, $n = 6$. Mean separation by Duncan's multiple range test. Those followed by unlike letters differ significantly at $P \leq 0.01$.
[z]Detached leaf rating on Swingle citrumelo from Table 1.

TABLE 3. Similarity indices[v] of fatty acid profiles for strains of *Xanthomonas campestris* from citrus and noncitrus hosts[w]

	Citrus strains[x]								
	F1	F6	F59	F86	F94	F100	F306	Mean x[y]	DLR[z]
Citrus strains									
F1	0.92	0.55	0.36	0.67	0.61	0.65	0.56	0.57 ab	3
F6	0.69	0.86	0.50	0.75	0.75	0.56	0.43	0.61 a	2
F59	0.39	0.45	0.92	0.63	0.71	0.52	0.26	0.49 bc	1
F86	0.68	0.55	0.53	0.93	0.74	0.77	0.46	0.62 a	1
F94	0.63	0.61	0.65	0.78	0.93	0.74	0.40	0.64 a	1
F100	0.65	0.56	0.52	0.77	0.74	0.93	0.50	0.62 a	1
F306	0.62	0.37	0.25	0.50	0.50	0.49	0.93	0.46 c	1
Noncitrus strains									
X137	0.05	0.00	0.01	0.03	0.04	0.01	0.00	0.02 d	1
X143	0.59	0.27	0.46	0.55	0.65	0.71	0.22	0.49 bc	2
X198	0.70	0.47	0.35	0.57	0.61	0.66	0.55	0.56 abc	2
X151	0.69	0.47	0.41	0.70	0.66	0.71	0.59	0.60 a	2
X22J	0.47	0.34	0.50	0.61	0.56	0.54	0.38	0.49 bc	2
X45	0.50	0.43	0.61	0.61	0.76	0.73	0.45	0.58 ab	0
X203	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.01 d	0
X6	0.06	0.01	0.01	0.01	0.01	0.01	0.04	0.02 d	0

[v]The derivation of similarity indices is described in the text.
[w]Description of strains in Table 1.
[x]Strain designations represent libraries which were the mean of four individual profiles from four separate extractions of each strain.
[y]Mean of strains when self-comparisons are excluded ($n = 6$). Mean separation by Duncan's multiple range test. Those followed by unlike letters differ significantly at $P \leq 0.01$.
[z]Detached leaf rating on Swingle citrumelo from Table 1.

but did not multiply in planta. Strains X6 of *X. c. campestris*, a vascular pathogen, and X45 of *X. c. phaseoli* were singularly unrelated to the other groups of spotting strains.

Fatty acid analyses. Comparisons of fatty acid profiles of strains with their respective library yielded similarity indices from 0.86 to 0.93. The indices were less than 1.0 because libraries were the mean of four individual profiles from four separate extractions of each strain. The profiles from each extraction differed slightly due to variation in the culture conditions, the amount of bacteria extracted, and extraction procedures. Cluster analysis of the reciprocal test strain to library comparisons (e.g., strain F1 with library F6 = 0.55 vs. strain F6 with library F1 = 0.69) yielded a matrix correlation coefficient of 0.92 ($P \leq 0.01$).

Similarity indices between groups of citrus and noncitrus strains followed a pattern similar to that of RFLP analysis (Tables 2 and 3). In general, there was a moderate degree of similarity among weakly aggressive strains from citrus, and these strains were likewise related to the more aggressive strains F1 and F6. Citrus strains F59 and F306 were, however, significantly less related to several of the other citrus strains. Fatty acid analysis also confirmed that most of the noncitrus strains were related to the aggressive and less aggressive citrus strain, except for strain X137 of *X. campestris* from *Strelitzia.* Notably, this strain multiplied to a lower level in planta than other spotting strains (Fig. 2). Conversely, *X. c. maculifoliigardeniae* X22J, which grew well in leaves, was somewhat more related to other spotting strains according to fatty-acid profiles (Table 3) than by RFLP analyses (Table 2). Fatty-acid profiles of nonspotting strains X6 and X203 were very dissimilar to the spotting strains, whereas *X. c. phaseoli* strain X45 was related by fatty acid analyses. The dendogram generated by cluster analysis of similarity indices from fatty acid profiles contrasted with that from the RFLP analysis (Figs. 3 and 4). This was confirmed by a low correlation (0.42) between the two similarity matrices. Nevertheless, there were groupings of weakly aggressive strains from citrus and noncitrus hosts by fatty acid analysis. Nonspotting strains with the exception of *X. c. phaseoli* were again the least related group as was revealed by RFLP analysis.

DISCUSSION

The weakly aggressive strains associated with the majority of the nursery outbreaks of CBS (12) were confirmed in this study to be pathologically (10,12) and genetically (7,8,13,14) distinguishable from the aggressive type. However, the weakly aggressive strains were not as highly related to one another as

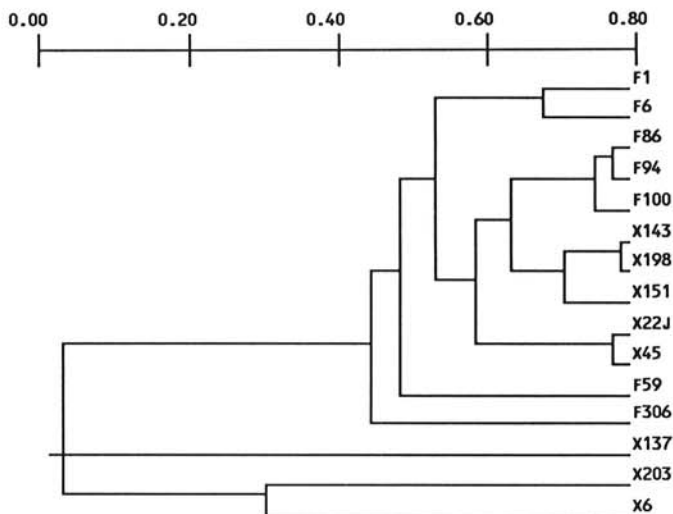

Fig. 4. Dendogram obtained by cluster analysis of similarity indices derived from fatty acid profiles of strains of *Xanthomonas campestris* from citrus (prefixed F) and from noncitrus hosts (prefixed X) (see Table 1). Scale refers to the similarity index (see text).

the aggressive strains were related to each other according to similarity coefficients derived from RFLP analysis (8,14; J. S. Hartung, *unpublished data*). Whereas the most aggressive strains may be a clonal group (e.g., Group E2 and E; 7,8,14), less aggressive strains apparently are not. Nevertheless, all weakly aggressive strains are somewhat related to each other and to the aggressive strains. This was confirmed not only by RFLP analysis but also by similarities in profiles of cellular fatty acids.

The genetic and biochemical affinities among these strains may account for their ability to grow in citrus leaves and cause reactions. In a previous study of the population dynamics of nursery strains in citrus leaves, the aggressive and moderately aggressive strains multiplied more rapidly than the weakly aggressive strains (6). The reactions produced by the weakly aggressive pathotype were associated with varying degrees of necrosis without water-soaking (12). This may be indicative of an ability for multiplication in tissue (25) but not for the sustained growth required for lesion expansion (6; J. H. Graham, *unpublished data*).

Strains from other hosts (e.g. *Gardenia, Strelitzia, Ficus*) of *X. campestris* were found to elicit necrosis in leaves of citrus, particularly Swingle citrumelo. The ability of *X. campestris* from noncitrus hosts to cause reactions was consistently related to the ability of the strain to multiply and attain population levels in leaves that were similar to that of a weakly aggressive strain from citrus. Strains of *X. campestris* (pathovars *campestris, malvacearum,* and *phaseoli*) which did not give reactions on citrus leaves did not multiply in planta. Similar differences in population development of *X. c.* pv. *oryzae*, *X. c.* pv. *poae*, and *X. c. campestris* in nonhost plant species also have been described (20).

The strains of *X. campestris* with the ability to grow in leaves and produce lesions were related by RFLP and fatty acid analyses, regardless of whether the strains were originally isolated from citrus or from other hosts (i.e., pathovar type). Strains which did not grow in citrus leaves and cause necrosis were not closely related to strains that did by these analyses. Thus, genetic, biochemical, and pathological relationships among the weakly aggressive strains originating from citrus and from other hosts were such that separation of these strains by these criteria was impossible.

Previously, weakly and moderately aggressive pathotypes were almost exclusively associated with mechanical transmission on Swingle citrumelo and grapefruit cultivars in citrus nurseries during late summer and fall after the most conducive period for epiphytic and endophytic growth of bacteria (12). The present survey of pathogens of *X. campestris* from Florida demonstrated that over 20% of strains are capable of causing reactions, primarily on Swingle citrumelo, and less so on Duncan grapefruit. Thus, several outbreaks of CBS on Swingle citrumelo may have been caused by resident strains of other pathovars. Recently, moderately aggressive strains from a citrus nursery outbreak were identified as moderately to highly related (0.75–0.91 similarity indices) to a combined fatty acid library of strains from *Strelitzia* (R. E. Stall, *unpublished data*). When spray inoculated onto *Strelitzia* plants, the nursery strains elicited necrosis that was indistinguishable from that caused by X143 and X198 (see 3; J. H. Graham and A. R. Chase, *unpublished data*).

Finally, we have demonstrated that *X. campestris* strains from a wide variety of ornamentals have an even broader host range than previously reported (1,3,17), i.e., including citrus. Apparently, the host ranges of a number of strain groups from ornamental plants are wider than previously recognized for pathovars of *X. campestris.* The existence of strains with broad host ranges presents difficulties for the pathovar concept of *X. campestris.* A weakly parasitic group of xanthomonads that has a wide host range would account for the variability of strains that cause lesions on citrus as well as other hosts in Florida. Hence, we find populations of strains in citrus nurseries that vary from weakly to moderately aggressive (12) and are mechanically transmitted. However, these strains do not multiply to levels in planta that are sufficient for epidemic development.

By contrast, the aggressive strains represent a closely related

group that produces extensive lesions and that is capable of natural spread (10,12). These strains appear to be most aggressive on Swingle citrumelo and its parent trifoliate orange and much less aggressive on grapefruit and other commercial citrus cultivars (J. H. Graham, *unpublished*). The status of aggressive strains as primary or secondary pathogens of citrus remains to be resolved. We did not encounter xanthomonads from other hosts that elicited an aggressive reaction. While our survey does not preclude the possibility that citrus is an alternative host for the aggressive strains, they are apparently pathologically and genetically unique. From the etiological and taxonomic standpoint, they may represent the only true CBS pathogens capable of epidemic development on citrus in the field. Since the less aggressive strains are genetically very diverse in contrast with several other *Xanthomonas* pathovars (7,8,14), it may be appropriate to limit the definition of strains to be included within the newly proposed pathovar *citrumelo* (8) to the aggressive type only. Then, the pathovar will be comprised of a single, genetically homogeneous group (e.g., Group E2 and E; 7,8,14).

LITERATURE CITED

1. Blake, J. H., Chase, A. R., and Simone, G. W. 1989. A foliar disease of *Fittonia verschaffeltii* caused by a pathovar of *Xanthomonas campestris*. Plant Dis. 73:269-272.
2. Brlansky, R. H., Lee, R. F., and Civerolo, E. L. 1986. Detection of *Xanthomonas campestris* from citrus by membrane entrapment and immunofluorescence. (Abstr.) Phytopathology 76:1101.
3. Chase, A. R., and Jones, J. B. 1987. Leaf spot and blight of *Strelitzia reginae* (bird-of-paradise) caused by *Xanthomonas campestris*. Plant Dis. 71:845-847.
4. Civerolo, E. L. 1984. Bacterial canker disease of citrus. J. Rio Grande Vall. Hortic. Soc. 37:127-146.
5. Dieneldt, M. M., and Lawson, R. H. 1989. Histopathology of *Xanthomonas campestris* pv. *citri* from Florida and Mexico in wound-inoculated detached leaves of *Citrus aurantifolia*: Transmission electron microscopy. Phytopathology 79:336-348.
6. Egel, D. S., Graham, J. H., and Stall, R. E. 1989. Growth in leaves of *Xanthomonas campestris* of different aggressiveness types isolated from citrus bacterial spot in Florida. (Abstr.) Phytopathology 78:1551.
7. Gabriel, D. W., Hunter, J. E., Kingsley, M. T., Miller, J. W., and Lazo, G. R. 1988. Clonal population structure of *Xanthomonas campestris* and genetic diversity among citrus canker strains. Mol. Plant-Microbe Interact. 1:59-65.
8. Gabriel, D. W., Kingsley, M. T., Hunter, J. E., and Gottwald, T. R. 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.
9. Gottwald, T. R., Civerolo, E. L., Garnsey, S. M., Brlansky, R. H., Graham, J. H., and Gabriel, D. W. 1988. Dynamics and spatial distribution of *Xanthomonas campestris* pv. *citri* group E strains in simulated nursery and new grove situations. Plant Dis. 72:781-787.
10. Gottwald, T. R., and Graham, J. H. 1990. Spatial pattern analysis of citrus bacterial spot in Florida citrus nurseries. Phytopathology 80:181-190.
11. Graham, J. H., and Gottwald, T. R. 1988. Citrus canker and citrus bacterial spot in Florida: Research findings–future considerations. Citrus Ind. 69:42-45, 48-51.
12. Graham, J. H., and Gottwald, T. R. 1990. Variation in aggressiveness of *Xanthomonas campestris* pv. *citrumelo* associated with citrus bacterial spot in Florida citrus nurseries. Phytopathology 80:190-196.
13. Hartung, J. S., and Civerolo, E. L. 1987. Genomic fingerprints of *Xanthomonas campestris* pv. *citri* strains from Asia, South America, and Florida. Phytopathology 77:282-285.
14. Hartung, J. S., and Civerolo, E. L. 1989. Restriction fragment length polymorphisms distinguish *Xanthomonas campestris* strains isolated from Florida citrus nurseries from *X. c.* pv. *citri*. Phytopathology 79:793-799.
15. Kubicek, Q. B., Civerolo, E. L., Bonde, M. R., Hartung, J. S., and Peterson, G. L. 1989. Isozyme analysis of *Xanthomonas campestris* pv. *citri*. Phytopathology 79:297-300.
16. Lawson, R. H., Dienelt, M. M., and Civerolo, E. L. 1989. Histopathology of *Xanthomonas campestris* pv. *citri* from Florida and Mexico in wound-inoculated detached leaves of *Citrus aurantifolia*: Light and scanning microscopy. Phytopathology 79:329-335.
17. Miller, J. W., and Chase, A. R. 1986. A new disease of *Pellionia* and *Pilea* spp. caused by *Xanthomonas campestris*. Plant Dis. 70:346-348.
18. Miller, L., and Berger, J. 1985. Bacteria identification by gas chromatography of whole cell fatty acids. Hewlett-Packard Application Note 228-241. 8 pp.
19. Nei, M., and Li, L.-H. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Nat. Acad. Sci. USA 76:5269-5273.
20. Robinson, J. N., and Callow, J. A. 1986. Multiplication and spread of pathovars of *Xanthomonas campestris* in host and non-host plants. Plant Pathol. 35:169-177.
21. Roy, M. A. 1988. Use of fatty acids for identification of phytopathogenic bacteria. Plant Dis. 72:460.
22. Schaad, N. W., ed. 1988. Laboratory guide for identification of plant pathogenic bacteria. 2nd ed. American Phytopathological Society, St. Paul, MN. 164 pp.
23. Schoulties, C. L., Civerolo, E. L., Miller, J. W., Stall, R. E., Krass, C. J., Poe, S. R., and DuCharme, E. P. 1987. Citrus canker in Florida. Plant Dis. 71:388-395.
24. Stall, R. E., and Hodge, N. C. 1989. Use of fatty acid profiles to identify strains of *Xanthomonas campestris* pv. *citri* from the citrus canker epidemic in Florida. (Abstr.) Phytopathology 79:376.
25. Young, J. M. 1973. Development of bacterial populations in vivo in relation to plant pathogenicity. N.Z. J. Agric. Res. 17:105-113.
26. Young, J. M., Dye, D. W., Bradbury, J. F., Panagopaulos, C. G., and Robbs, C. F. 1978. A proposed nomenclature and classification for plant pathogenic bacteria. N.Z. J. Agric. Res. 21:153-157.