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Genetic Relationship among Worldwide Strains of *Xanthomonas* Causing Canker in Citrus Species and Design of New Primers for Their Identification by PCR†

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Partial sequence analysis of the ribosomal operon in *Xanthomonas axonopodis* allowed discrimination among strains causing the A, B, and C types of citrus bacterial canker (CBC) and quantification of the relationship of these organisms with other species and pathovars in the same genus. Sets of primers based on sequence differences in the internally transcribed spacer and on a sequence from the plasmid gene *pthA* involved in virulence were designed for specific identification of xanthomonads causing CBC diseases. The two sets were validated with a collection of *Xanthomonas* strains associated with citrus species. The primer set based on ribosomal sequences had a high level of specificity for *X. axonopodis* pv. citri, whereas the set based on the *pthA* gene was universal for all types of CBC organisms. Moreover, the relationships among worldwide *Xanthomonas* strains causing CBC were analyzed by amplification of repetitive sequences (enterobacterial repetitive intergenic consensus and BOX elements). Under specific conditions, pathotypes of these *Xanthomonas* strains could be discerned, and subgroups of the pathotypes were identified. Subgroups of strains were associated with certain geographic areas of the world, and on this basis the origin of type A strains introduced into Florida could be inferred.

Xanthomonas axonopodis pv. citri and *X. axonopodis* pv. aurantifolii cause citrus bacterial canker (CBC), a serious disease of most citrus species and cultivars in many citrus-producing areas worldwide (1). Distinct forms of CBC based on different pathogenicities of the bacteria have been described. The Asiatic form or A type, caused by *X. axonopodis* pv. citri, is the most virulent form and affects the widest range of hosts, including *Citrus* spp. and many closely related rutaceous plants. The CBC B and C types are caused by *X. axonopodis* pv. aurantifolii. Pathotype B strains affect primarily lemon (*Citrus limon* (L.) Burm f.), but they also affect some other citrus species and are found only in Argentina and neighboring Uruguay and Paraguay. Pathotype C strains have been isolated in a small area of Sao Paulo State in Brazil and infect Key or Mexican lime (*Citrus aurantifolia* (Christm.) Swingle) (21). The symptoms induced by the three forms of canker organisms are similar and consist of erumpent and corky lesions surrounded by oily or water-soaked margins on leaf, fruit, and stem tissues (1). A new strain of *X. axonopodis* pv. citri, designated A*, was identified in southwest Asia and has a host range restricted naturally to Key or Mexican lime; this strain is closely related to type A strains (24). Another strain (type A^w), which behaves similarly, has recently been discovered in Florida; this strain has a restricted host range that includes Key or Mexican lime and alemow (*Citrus macrophylla* Wester) (X. Sun, R. E. Stall, J. Cubero, T. R. Gottwald, J. H. Graham, W. D. Dixon, T. S. Schubert, M. E. Peacock, E. R. Dickstein, and P. H. Chaloux, Citrus Canker Research Workshop, abstr., p. 15, 2000 [http://doacs.state.fl.us/~pi/workshop.html]). However, the A^w strain has a slightly different disease phenotype and host range than A* strains (http://doacs.state.fl.us/~pi/workshop.html).

Several approaches are currently being used in studies of the taxonomy and epidemiology of plant-pathogenic bacteria. Sequence variation in the ribosomal DNA (rDNA) operon has been used for classification of bacterial species and for strain discrimination within species (14). The sequence corresponding to the rRNA of the small 16S ribosomal subunit has been used to study relationships among all *Xanthomonas* species but appears to be highly conserved within a genomic species (10) and therefore not useful for strain discrimination. However, the internally transcribed spacer (ITS) between the 16S and 23S genes is sufficiently variable that it can be used to compare closely related strains and to identify genotypes in the same species (15, 17). In this study we utilized rDNA from the 16S subunit and ITS sequences to evaluate the relationship of *Xanthomonas* species causing canker in citrus to other species in this genus.

Families of repetitive DNA sequences, like repetitive extragenic palindromic, enterobacterial repetitive intergenic consensus (ERIC), and BOX elements, which are present in all prokaryotes, can be used for bacterial fingerprinting. PCRs based on these repetitive sequences (rep-PCRs) have been used effectively for analysis of several genera and species of bacteria (12, 14). For *Xanthomonas* species, rep-PCRs have been used to assess variation among pathovars and have revealed low levels of intrapathovar diversity (12, 13, 16, 18, 23).

The variability among strains of *Xanthomonas* causing CBC in citrus has been characterized by using different methods, including physiological tests, phage typing, plasmid profile and probe analysis, restriction analysis, and serological approaches (4, 5, 6, 7, 19, 24). In this paper, we describe the use of

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TABLE 1. Strains of *Xanthomonas* spp. used to evaluate three set of primers, based on plasmid (2/3), virulence (*J-pth*), and ribosomal (*J-Rx*) sequences, for PCR identification

Taxon	Strain(s)	Origin	CBC type	PCR result		
				2/3 primers[a]	*J-pth* primers[b]	*J-Rx* primers[c]
X. axonopodis pv. citri	12971	Argentina	A	+[e]	+	+
	12758, 12789	Bolivia	A	+	+	+
	12834, 12917	Brazil	A	+	+	+
	JH410-5, JH35, JH81.2, JJ36.2	People's Republic of China	A	+	+	+
	LW	Florida	A[w]	−	+	+
	MA, MI	Florida	A	+	+	+
	CFBP1209	Hong Kong	A	+	+	+
	CFBP2851, CFBP2852	India	A	+	+	+
	X169	India	A*	+	+	+
	X406	Iran	A*	+	+	+
	CFBP2854, JJ201.2, X62, X63	Japan	A	+	+	+
	JJ37.2, JK146.4	Malaysia	A	+	+	+
	JJ9.3, JJ9.8, JK176-1	Mauritius Island	A	+	+	+
	X205	Oman	A*	+	+	+
	X100	Pakistan	A	+	+	+
	08946	Paraguay	A	+	+	+
	JJ223.2, JK148.4	Philippines	A	+	+	+
	JA151.1, B24, C31, C40	Reunion Island	A	+	+	+
	JJ10.1, JJ10.3, JJ10.6	Rodrigues Island	A	+	+	+
	X271, X292, X322, X289	Saudi Arabia	A*	+	+	+
	CFPB2548, CFBP2549, JJ53-3	Taiwan	A	+	+	+
	JK144.6, JK20.3	Thailand	A	+	+	+
	12970	Uruguay	A	+	+	+
	JN551.2	Vietnam	A	+	+	+
X. axonopodis pv. aurantifolii	X148, X69	Argentina	B	−	+	−
	X64	Argentina	B	−	−	−
	X80, X84	Uruguay	B	−	+	−
	X2905, X2906, X341, X70	Brazil	C	−	+	−
X. axonopodis pv. citrumelo	F1	Florida	NA[d]	−	−	−
X. axonopodis pv. dieffenbachiae	X462, X175a, X175b, X376, X696	Florida	NA	−	−	−

[a] The 2/3 primers were designed to amplify inside the *X. axonopodis* pv. citri plasmid (8).
[b] Primers for the *pth* gene (primers *J-pth1* and *J-pth2*).
[c] Primers for rDNA (primers *J-RXg* and *J-RXc2*).
[d] NA, not applicable.
[e] +, fragment of expected size amplified; −, fragment of the expected size not amplified.

rep-PCR with BOX and ERIC primers that, under specific conditions, are used not only to separate pathotypes but also to differentiate strains of the same pathotype. This method allowed us to evaluate the diversity of *Xanthomonas* strains causing CBC in Florida and to relate *X. axonopodis* pv. citri strains to a worldwide collection in order to establish the possible geographic origin of these strains.

PCR-based methods for diagnosis of CBC have been described previously (2, 8, 9). These methods are based on primers designed to amplify a fragment in a plasmid of *X. axonopodis* pv. citri. However, the primers do not detect all strains of *X. axonopodis* pv. aurantifolii and certain *X. axonopodis* pv. citri strains, including some of those recently isolated in Florida (http://doacs.state.fl.us/~pi/workshop.html). Because of the deficiencies of previously described primers, we developed two new sets of primers for detection and identification of different pathotypes of *Xanthomonas* causing CBC. One set is based on the sequence of *pthA*, one of the primary genes involved in virulence of *X. axonopodis* pv. citri (22, 25), and was designed to be universal for all citrus canker strains. The other set is based on a sequence in the ITS region of the rDNA operon and was designed for specific identification of CBC type A strains. These sets of primers were tested with a worldwide collection of *Xanthomonas* strains isolated from citrus.

MATERIALS AND METHODS

Bacterial strains and DNA isolation. The bacterial strains used in this study are listed in Table 1. All canker-producing *Xanthomonas* strains were provided as heat-killed bacteria in water suspensions (absorbance at 600 nm, 0.1 to 0.4) to comply with quarantine restrictions. Strains of other *Xanthomonas* pathovars were grown on nutrient agar plates for 48 h before water suspensions were prepared as described above. DNA was extracted with a single phenol-chloroform-isoamyl alcohol step, precipitated in ethanol, and resuspended in ultrapure water. DNA was stored at −20°C until it was used.

rDNA sequencing. The following six *X. axonopodis* strains causing citrus canker were chosen for sequence analysis: three *X. axonopodis* pv. citri strains (one strain belonging to each of the three genotypes presently found in Florida [strains LW, MI, and MA]), two strains of *X. axonopodis* pv. aurantifolii (one type B strain from Argentina [strain X84] and one type C strain from Brazil [strain X341]), and one strain of *X. axonopodis* pv. citrumelo isolated in Florida (strain F1). Primers FGPS6 and FGPL132′ (17) were used to amplify sequences including the 16S rDNA gene, the ITS, and a small fragment of the 23S rDNA gene. PCRs were performed in 50-μl (total volume) mixtures containing 1× *Taq* buffer, 3 mM $MgCl_2$, each primer at a concentration of 1 μM, each deoxynucleoside triphosphate at a concentration of 0.2 mM, 1 U of *Taq* polymerase (Promega, Madison, Wis.), and DNA extracted as described above as the template. The amplification reaction conditions consisted of 93°C for 1 min, 55°C for 1 min, and 72°C for 2.5 min for 40 cycles plus an initial step of 94°C for 5 min and a final step of 72°C for 10 min. The amplified product (about 2,400 bp) was cloned into the pGEM-T vector (Promega), and competent *Escherichia coli* cells were transformed. Purified plasmids were sequenced at the DNA sequencing core laboratory of the University of Florida (Gainesville, Fla.). Results were confirmed by sequencing the complete region in both the forward and reverse directions and

TABLE 2. Levels of sequence similarity and numbers of nucleotide differences for 16-23S rDNAs of various *Xanthomonas* species and strains

Organism	% Sequence similarity or no. of nucleotide differences[a]								
	X. axonopodis pv. citrumelo F1	*X. axonopodis* pv. vesicatoria	*X. axonopodis* pv. aurantifolii X84	*X. axonopodis* pv. aurantifolii X341	*X. axonopodis* pv. citri MA	*X. axonopodis* pv. citri LW	*X. axonopodis* pv. citri MI	*X. compestris* pv. campestris	*X. gardneri*
X. axonopodis pv. citrumelo F1		99.8	99.6	99.4	99.4	99.4	99.3	98.8	98.8
X. axonopodis pv. vesicatoria	4		99.8	99.6	99.6	99.6	99.5	99.0	99.0
X. axonopodis pv. aurantifolii X84	9	5		99.6	99.4	99.4	99.3	98.8	98.8
X. axonopodis pv. aurantifolii X341	12	8	9		99.3	99.3	99.2	98.7	98.7
X. axonopodis pv. citri MA	13	9	12	15		99.8	99.7	99.1	99.0
X. axonopodis pv. citri LW	13	9	12	15	4		99.7	99.1	99.0
X. axonopodis pv. citri MI	15	11	14	17	6	6		99.0	98.9
X. campestris pv. campestris	26	22	25	28	19	19	21		99.8
X. gardneri	26	22	25	28	21	21	23	4	

[a] The values on the upper right are levels of sequence similarity, expressed as percentages; the values on the lower left are numbers of nucleotide differences.

using two sets of primers, one set for pGEMT and the other set for the rDNA operon.

Sequence analysis. The sequences were aligned and compared with those available in the GenBank database for *X. axonopodis* pv. vesicatoria (accession number AF123090), *Xanthomonas campestris* pv. campestris (AF123092), and *Xanthomonas gardneri* (AF123093). The analytical program MEGA version 2.0 (S. Kumar, K. Tamura, I. B. Jakobsen, and M. Nei, submitted for publication) was used to construct a dendrogram using the neighbor-joining method (20) and the Jukes-Cantor model (11).

Primer design and PCR conditions for citrus canker strains. The design of oligonucleotide primers *J-pth1* (5′-CTTCAACTCAAACGCCGGAC) and *J-pth2* (5′-CATCGCGCTGTTCGGGAG) was based on the nuclear localization signal in virulence gene *pthA* (25) (accession number U28802.1). DNAs from xanthomonad strains shown in Table 1 were used as targets in PCRs performed in 25-μl mixtures containing 1× *Taq* buffer, 3 mM $MgCl_2$, 0.1 μM primer *J-pth1*, 0.1 μM primer *J-pth2*, each deoxynucleoside triphosphate at a concentration of 0.2 mM, and 1 U of *Taq* polymerase (Promega). The amplification reaction conditions consisted of 93°C for 30 s, 58°C for 30 s, and 72°C for 45 s for 40 cycles plus an initial step of 94°C for 5 min and a final step of 72°C for 10 min. The PCR products were visualized under UV light in 2% agarose gels stained with ethidium bromide. PCRs were also performed by using the DNA extracts and reaction conditions described above with primer set 2/3 (8). Primers *J-RXg* (5′-GCGTTGAGGCTGAGACATG) and *J-RXc2* (5′-CAAGTTGCCTCGGAG CTATC) were designed by using the rDNA sequences described above to amplify type A strain sequences. PCRs were carried out in 25-μl mixtures containing 1× *Taq* buffer, 1.5 mM $MgCl_2$, 0.04 μM primer *J-RXg*, 0.04 μM primer *J-RXc2*, each deoxynucleoside triphosphate at a concentration of 0.2 mM, and 1 U of *Taq* polymerase (Promega); the PCR amplification conditions were the same as those used with the other primers. The products were visualized under UV light in 2% agarose gels stained with ethidium bromide.

DNA amplification conditions for BOX and ERIC PCRs. BOX PCRs were carried out in 25-μl mixtures containiug 1× *Taq* buffer, 6 mM $MgCl_2$, 2.4 μM primer BOX1R (12), each deoxynucleoside triphosphate at a concentration of 0.2 mM, and 2 U of *Taq* polymerase (Promega); the amplification conditions consisted of 94°C for 30 s, 48°C for 30 s, and 72°C for 1 min for 40 cycles plus an initial step of 94°C for 5 min and a final step of 72°C for 10 min, and DNAs from strains listed in Table 1 were used as the templates. ERIC PCRs were carried out in 25-μl mixtures containing 1× *Taq* buffer, 3 mM $MgCl_2$, 1.2 μM primer ERIC1R, 1.2 μM primer ERIC2 (12), each deoxynucleoside triphosphate at a concentration of 0.2 mM, and 2 U of *Taq* polymerase (Promega); the amplification conditions were the same as those used for the BOX PCRs. The PCR products were analyzed by 3% agarose gel electrophoresis in 1× TAE buffer for 2 h at 110 V and were stained with ethidium bromide.

A total of 84 strains isolated from the three main areas of Florida affected by CBC (21) were analyzed to evaluate the reproducibility of BOX and ERIC PCRs. For each of the *X. axonopodis* pv. citri strains studied, both the DNA extraction and PCR procedures were repeated.

Data analysis. BOX and ERIC PCR fingerprint results were converted to binary form (0 = absence or negative; 1 = presence or positive), and similarity coefficients for pairs of strains were calculated with the program NTSYS, version 2.1 (Exeter Software, Setauket, N.Y.), using SIMQUAL with the Dice coefficient (3) and were subjected to unweighted pair group method (UPGMA) cluster analysis with the program MEGA, version 2.0 (Kumar et al., submitted).

Nucleotide sequence accession numbers. The nucleotide sequences determined in this study have been deposited in the GenBank database under accession numbers AF442739 to AF442744.

RESULTS AND DISCUSSION

Analysis of rDNA sequences. Partial sequence analysis of rDNA revealed high levels of similarity among the *Xanthomonas* strains studied; the mean level of sequence similarity was 99.3% (Table 2). A dendrogram based on pairwise comparisons of all the strains contained two main clusters (Fig. 1). All of the strains except the *X. campestris* and *X. gardneri* strains grouped in one cluster. The mean level of sequence similarity for the clusters was 99.0%, and the maximum number of base differences was 28. In the larger cluster, one group included *X. axonopodis* pv. vesicatoria, *X. axonopodis* pv. citrumelo, and both strains of *X. axonopodis* pv. aurantifolii, and the second group included all *X. axonopodis* pv. citri strains. The mean level of sequence similarity between these two groups was 99.4%, and the maximum number of base differences was 17. The first group contained two subgroups, one that included *X. axonopodis* pv. vesicatoria and *X. axonopodis* pv. citrumelo and one that included *X. axonopodis* pv. aurantifolii types B and C. The mean level of sequence similarity between these subgroups was 99.6%, and the maximum number of base differences was 12.

In a previous study, analysis of the 16S rDNA sequences of all *Xanthomonas* species revealed very limited diversity (10). Because of the high levels of sequence similarity in this region, the 16S gene is considered unsuitable for discriminating and identifying closely related strains. Nevertheless, variability in ITS sequences has been reported previously and was considered useful for discriminating bacterial strains (17). The variability in rDNA among the different strains analyzed in this study was sufficient to identify two groups in the species *X. axonopodis*. *X. axonopodis* pv. citri strains causing type A CBC were placed in one group, while *X. axonopodis* pv. aurantifolii

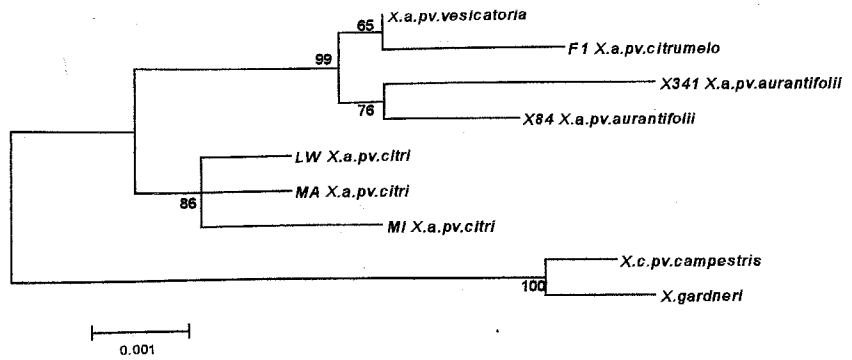

FIG. 1. Neighbor-joining dendrogram showing phylogenetic relationships based on pairwise comparisons using the Jukes-Cantor parameter. The distance between two strains is obtained by adding the lengths of the connecting horizontal branches using the scale at the bottom (percentage of sequence difference). Bootstrap values (based on 1,000 replicates) are indicated at the nodes. *X.a.*, *X. axonopodis*; *X.c.campestris*, *X. campestris* pv. campestris.

strains causing type B and C CBC in citrus species were more closely related to a strain of *Xanthomonas* that causes citrus bacterial spot, a noncanker disease, and a strain of *X. axonopodis* pv. vesicatoria pathogenic for tomato. The canker strain with a limited host range isolated in Florida (type A^w) was clearly placed in the group of type A strains, which supports the conclusion concerning the relationship based on fatty acid analysis (http://doacs.state.fl.us/~pi/workshop.html) and on the rep-PCR analysis described below.

Identification by PCR of *Xanthomonas* causing canker in citrus. Results of PCR amplifications performed with the three sets of primers tested are shown in Table 1. Primers *J-pth1* and *J-pth2* were used to amplify target sequences in DNAs extracted from 58 strains that cause CBC and had different geographic origins. The expected 197-bp amplification product was produced with DNAs of 57 strains that included *X. axonopodis* pv. citri and *X. axonopodis* pv. aurantifolii type B and C strains. Only one strain of *X. axonopodis* pv. aurantifolii (strain X64) did not produce an amplification product from the target sequence. At this time, the B type is considered nearly extinct or masked by the presence of the A type in South America, the only place where this kind of CBC has been detected. Furthermore, this set of primers was able to generate the specific amplified product from type A^w strains (e.g., strain LW) that were isolated in Florida and cause canker in a limited range of hosts. A^w strains were not detected with primers described previously (18; http://doacs.state.fl.us/~pi/workshop.html). The specific 197-bp PCR product was not obtained with DNAs extracted from other *Xanthomonas* strains previously associated with citrus, such as strains of *Xanthomonas dieffenbachiae* and *X. axonopodis* pv. citrumelo (5). Although *pthA* belongs to a family of *Xanthomonas* avirulence-pathogenicity genes (25) and the primers generate the same specific PCR product with other *Xanthomonas* spp. (results not shown), these xanthomonads do not attack citrus and therefore should not present a problem in diagnosis and identification of CBC.

Plasmids responsible for virulence in *X. axonopodis* pv. citri are apparently stable (9), but accurate identification of different *X. axonopodis* pv. citri strains required the use of an additional genomic rDNA character. After a partial DNA sequence of the rDNAs of several *Xanthomonas* spp. was obtained, a variable region in the ITS was identified (Fig. 2). Based on this region, primers *J-RXg* and *J-RXc2* were designed to specifically amplify DNA from *X. axonopodis* pv. *citri*. *J-RXg* was a primer that annealed with DNAs from all of the *Xanthomonas* strains studied, while the 3′ end of *J-RXc2* annealed only to DNAs from strains of *X. axonopodis* pv. citri. This set of primers yielded the specific 179-bp PCR product with *X. axonopodis* pv.

J-RXc2

3′ CTATCGAGGCTCCGTTGAACC 5′

F1	1992	ACACAACACG-GCATATGACCCTGAGGCAACTTGGGGTTATATGGTCAAGCGAATAA	2047
X.a.v.	1992	ACACAACACG-GCATATGACCCTGAGGCAACTTGGGGTTATATGGTCAAGCGAATAA	2047
X341	1992	ACACAACACG-ACATATGATCCTGAGGCAACTTGGGGTTATATGGTCAAGCGAATAA	2047
X84	1991	ACACAACACG-ACATATGATCCTGAGGCAACTTGGGGTTATATGGTCAAGCGAATAA	2046
MI	1991	ACACAACACG-GCAGATAGCTCCGAGGCAACTTGGGGTTATATGGTCAAGCGAATAA	2046
LW	1992	ACACAACACG-GCAGATAGCTCCGAGGCAACTTGGGGTTATATGGTCAAGCGAATAA	2047
MA	1993	ACACAACACG-GCAGATAGCTCCGAGGCAACTTGGGGTTATATGGTCAAGCGAATAA	2048
X.c.c.	1992	ACACAACACGTACATGTAGCTCCGAGGCAACTTGGGGTTATATGGTCAAGCGAATAA	2048
X.gardneri	1991	ACACAACACG-ACATGTAACTCCGAGGCAACTTGGGGTTATATGGTCAAGCGAATAA	2046

FIG. 2. ITS sequences used to design primer *J-RXc2* specific for PCR amplification of *X. axonopodis* pv. citri rDNA. Variable regions are indicated by boldface type. F1, *X. axonopodis* pv. citrumelo F1; *X.a.v.*, *X. axonopodis* pv. vesicatoria; X84 and X341, *X. axonopodis* pv. aurantifolii X84 and X341; MI, LW, and MA, *X. axonopodis* pv. citri MI, LW, and MA; *X.c.c.*, *X. campestris* pv. campestris. The sequence of primer *J-RXc2* is shown at the top.

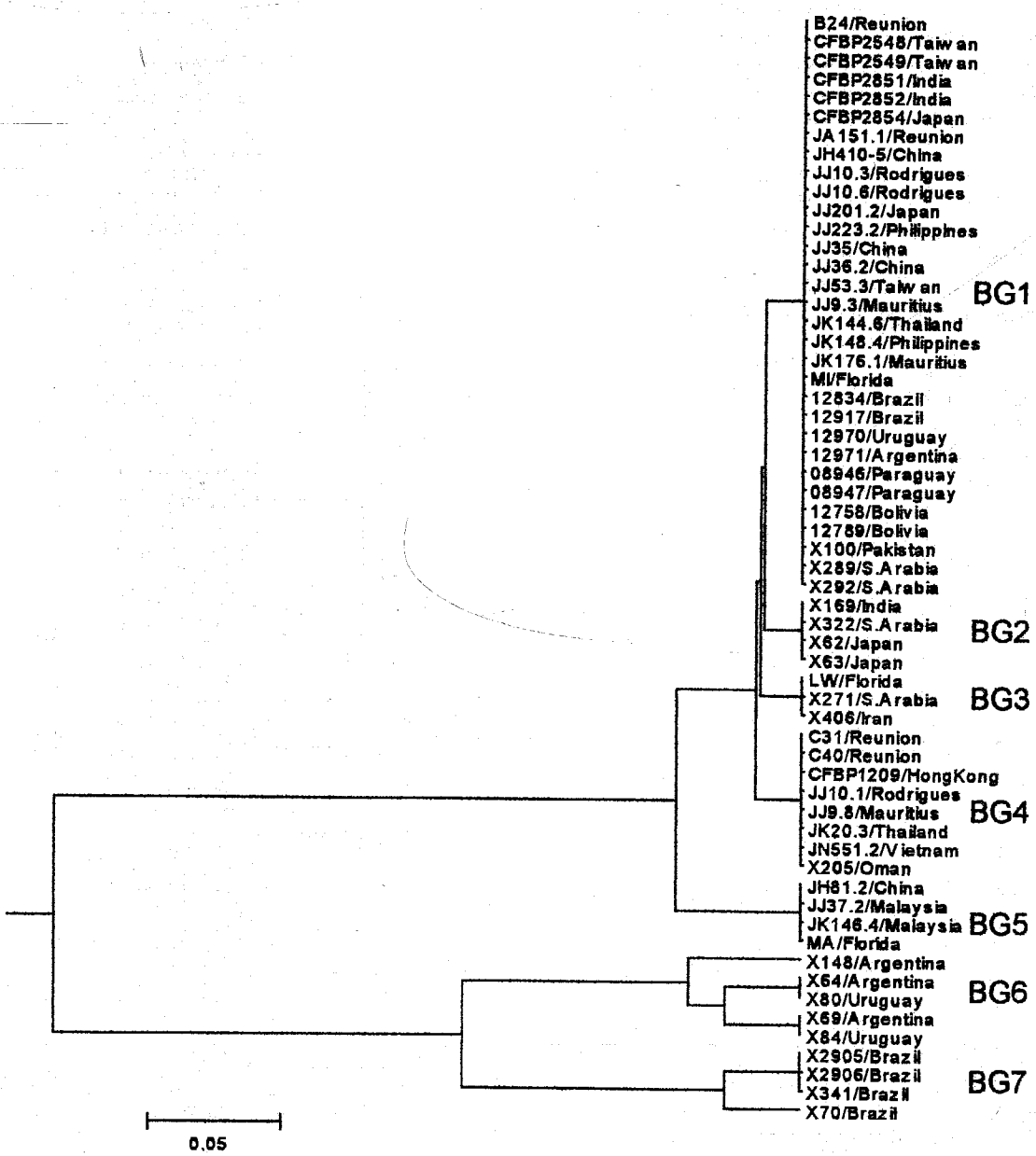

FIG. 3. Dendrogram showing relationships among *Xanthomonas* strains causing CBC based on BOX PCR analysis. Similarities were calculated by using the Dice coefficient, and clustering was achieved by UPGMA using the NTSYS version 2.1 and MEGA version 2.0 programs. BG1 to BG5 strains are *X. axonopodis* pv. citri strains, while BG6 and BG7 strains are *X. axonopodis* pv. aurantifolii strains.

citri but not with *X. axonopodis* pv. aurantifolii, *X. axonopodis* pv. citrumelo, or *X. axonopodis* pv. dieffenbachiae (Table 1).

Although the target sequence for the ribosomal primers was detected occasionally in other *Xanthomonas* spp. (results not shown), this finding should not interfere with the efficacy of the primers for detection of canker strains because the other xanthomonads tested are not isolated from citrus. Special conditions for this set of primers based on rDNA would make them unsuitable for use in the detection of *X. axonopodis* pv. citri on plant material because of the possibility of unpredicted products if the primers annealed to rDNAs from other bacteria. Thus, the rDNA primers should be used only under the amplification conditions described above and in combination with another PCR analysis for identification of particular strains.

An example of the utility of the two sets of primers described here is the case of an A^w strain recently isolated in Florida (http://doacs.state.fl.us/~pi/workshop.html). Capable of inducing symptoms in Key or Mexican lime and alemow, this A^w strain gave negative PCR results when the 2/3 primers were used for diagnosis (8) but positive results when the two sets of primers described in this paper were used. The *J-pth1–J-pth2* primer set may be used to detect *Xanthomonas* causing CBC directly on plant material by using protocols described previously (2) and also the internal control constructed for these

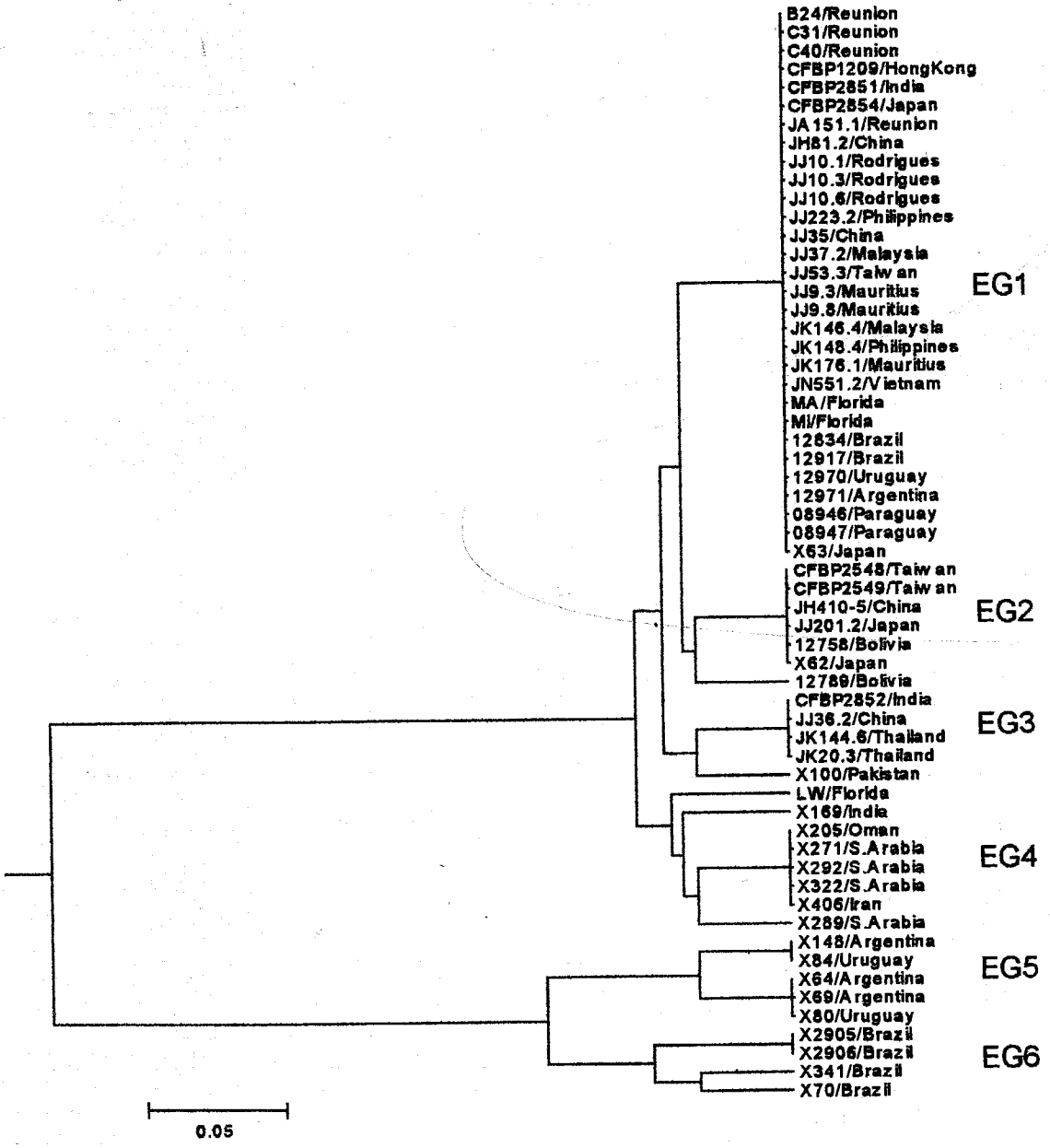

FIG. 4. Dendrogram showing relationships among *Xanthomonas* causing CBC based on ERIC PCR analysis. Similarities were calculated by using the Dice coefficient, and clustering was achieved by UPGMA using the NTSYS version 2.1 and MEGA version 2.0 programs. EG1 to EG4 strains are *X. axonopodis* pv. citri strains, while EG5 and EG6 strains are *X. axonopodis* pv. aurantifolii strains.

primers (results not shown). Using the two sets of primers based on rDNA and *pthA* together is ultimately the most robust approach for identification of *X. axonopodis* pv. citri and *X. axonopodis* pv. aurantifolii strains for diagnosis of CBC in quarantine situations. These primer sets overcome the deficiencies of primers presently used (8) for identification of strains such as the A^w strain.

Classification by BOX and ERIC PCRs of *Xanthomonas* strains causing CBC. Different fingerprints were generated by the products of BOX and ERIC PCRs. Under our reaction conditions, primers corresponding to conserved sequences of BOXA units annealed to *Xanthomonas* DNAs and yielded PCR products ranging in size from approximately 100 to 1,000 bp. Cluster analysis of the data obtained from BOX PCRs revealed two major clusters that correlated with the two pathovars of *Xanthomonas* causing CBC (Fig. 3). One cluster included all *X. axonopodis* pv. citri strains, and the other cluster contained all *X. axonopodis* pv. aurantifolii strains; the mean level of similarity between the clusters was 43%. Within the *X. axonopodis* pv. citri cluster the levels of similarity between strains were always >85%. In the *X. axonopodis* pv. aurantifolii cluster, two groups were distinguished at a mean level of similarity of about 75%. One group corresponded to the B type (group BG6), and the other corresponded to the C type (group

BG7); for each group the levels of similarity between strains were >90%.

Although the levels of similarity within the two major clusters were high, the presence and absence of certain bands allowed differentiation into groups and subgroups. Within the *X. axonopodis* pv. citri cluster, the main group included all strains from South America and strains from several locations in Asia, the Indian Ocean, and the Philippine Sea. Four additional groups of highly related strains (groups BG1, BG2, BG3, and BG4) were also identified within the *X. axonopodis* pv. citri cluster. BG2 and BG3 contained most of the *X. axonopodis* pv. citri strains with restricted host ranges, including the strain recently described in Florida (the A^w strain). The remaining *X. axonopodis* pv. citri group, group BG5, was defined by a limited number of strains originally isolated from a more restricted geographic area in the People's Republic of China and Malaysia and included one of the strains present in Florida (strain MA). Within the *X. axonopodis* pv. aurantifolii cluster separation between pathotype B (group BG6) and pathotype C (group BG7) strains was clearly discerned, and some variation among strains of each type was found.

The 84 strains isolated in Florida produced three different fingerprints. All of the strains from southern Florida characterized produced a pattern indistinguishable from that of our reference strain for this area, designated pattern MI (Miami). Most of the CBC strains from the west coast of Florida produced a pattern identical to pattern MA (Manatee) but distinct from pattern MI. Strains from a third area, in which the host range was limited (A^w type), produced a fingerprint identical to the strain LW (Lake Worth) pattern.

Like the BOX elements, the ERIC primers yielded PCR products that ranged from 100 to 1,000 bp long (Fig. 4). Analysis of the fingerprints yielded two main clusters, one that included all *X. axonopodis* pv. citri strains and one that included all *X. axonopodis* pv. aurantifolii strains; the mean level of similarity between the main clusters was about 47%. In the *X. axonopodis* pv. citri cluster, two groups could be defined, both at a similarity level of >87%. One of the groups contained most of the strains, which could be separated into two subgroups. Most of the *X. axonopodis* pv. citri strains studied clustered in one subgroup that included groups EG1 and EG2. A few *X. axonopodis* pv. citri strains, including one strain from the People's Republic of China, two strains from Thailand, one strain from India, and one strain from Pakistan, clustered in group EG3. All strains described as having a restricted host range, including the A^w strain isolated in Florida, were in group EG4. The other main cluster included pathotype B (group EG5) and pathotype C (group EG6) strains, and the mean level of similarity between the groups was about 80%. The diversity of strains in the B and C groups was also determined by the presence of particular bands. Among the 84 strains isolated in Florida two groups were differentiated with ERIC elements. Most of the strains were indistinguishable from MI and MA, which produced the same fingerprint. However, a different fingerprint was obtained for A^w strains from Florida with a restricted host range.

The utility of the repetitive BOX and ERIC sequences for characterization of plant-pathogenic bacteria has been widely demonstrated (12, 13, 16, 18). PCR conditions described in other studies did not generate enough variability to discriminate among *Xanthomonas* strains of the same pathotype. However, modification of the conditions used for the PCR allowed discrimination of strains belonging to the same pathotype. This was achieved by using amplification conditions that favored the generation of low-molecular-weight PCR products in order to discern more polymorphisms.

Both ERIC and BOX-PCR clearly separated strains causing CBC into two main clusters corresponding to *X. axonopodis* pv. citri and *X. axonopodis* pv. aurantifolii. This confirmed the analysis of the rDNA and is in accordance with other studies using different techniques of xanthomonads causing diseases in citrus (4, 7, 19, 24). Moreover, both PCR methods allowed discrimination within the A, B, and C groups of different subgroups related to some characteristic of the strains. Both rep-PCR and rDNA analysis confirmed that the A^w strains should be included with the A strains. Although rep-PCR revealed limited variability of *X. axonopodis* pv. citri, this technique also allowed delineation of groups of strains within the CBC A type, which potentially could result in association of the groups with certain geographic areas of the world. For example, BOX PCR distinguished a group from a specific area in Asia that included strains from the People's Republic of China and Malaysia, as well as the MA strain isolated in Florida. Thus, a possible origin of the MA outbreak in Florida may be inferred. The same fingerprint was obtained for the MA strain now in that area of Florida and isolates from the original outbreak that occurred in 1986, an infestation that was supposedly eradicated in 1994 (21). The presence of the same genotype confirms that there was a reemergence of the same strain and not an introduction of a new *X. axonopodis* pv. citri strain in that area of Florida. In some areas having the MA strain, the MI genotype from South Florida was introduced, probably as a consequence of movement of plant material from the southern part of the state into the central area. On the other hand, A^w strains in Florida were related to the A* strain, which suggests a common origin in southwest Asia. However, there are apparent differences because PCR analysis revealed plasmid sequence differences between A^w and A* strains. Moreover, A^w is yet another introduction of the A genotype. This confirms that at least three introductions of A type strains into Florida have occurred since the 1980s. Finally, the MI genotype belongs to main groups defined by BOX and ERIC PCRs. The origin of this genotype is uncertain because of its widespread distribution, which includes almost all the citrus areas affected by CBC.

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