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Genomic Variability of the *Xanthomonas* Pathovar mangiferaeindicae, Agent of Mango Bacterial Black Spot†

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The genetic diversity of 138 strains of the *Xanthomonas* pathovar mangiferaeindicae, which were isolated from three different hosts (mango, ambarella, and pepper tree) in 14 different countries, was assessed with restriction fragment length polymorphism markers. An analysis of patterns obtained by hybridization with an *hrp* cluster probe from *Xanthomonas oryzae* pv. oryzae separated 11 of the strains from all of the other strains, which suggested that these 11 strains may not be *Xanthomonas* pv. mangiferaeindicae strains. Hybridization with an avirulence gene from *X. oryzae* pv. oryzae and a repetitive DNA fragment from *Xanthomonas* pv. mangiferaeindicae separated the remaining 127 strains into four groups that were consistent with both geographic and host origins. The group with the greatest diversity consisted of strains from Southeast Asia, where mango originated. Other groups and subgroups contained strains that were either from widely separated countries, which suggested that wide dissemination from a single site occurred, or from localized areas, which suggested that evolution of separate lineages of strains occurred. One group of strains contained only strains isolated from pepper trees in Réunion, indicating that pepper tree may not be an alternate host for *Xanthomonas* pv. mangiferaeindicae strains.

Mango bacterial black spot, an important disease of mango (*Mangifera indica* L.), is endemic in the major mango-producing regions of the world (Asia, southern and eastern Africa, western Oceania, and the Indian Ocean). The only known occurrence of the disease in the New World is in Brazil, where it was reported for the first time in 1954 (23). The disease results in reductions in market value and fruit yield. Lesions on leaves are angular, raised, black, and necrotic, whereas lesions on fruits are star shaped and erumpent with an infectious gummy exudate. Occasionally, twig cankers can develop (25). Bacteria enter the plant through wounds, stomata, or lenticels; systemic infection is unknown. Bacterial cells survive mostly in lesions and on aerial organs as epiphytes (25, 27). The pathogen is believed to spread between continents or countries through transport of contaminated plant material and, on a smaller scale, by cultural practices and by wind-driven rains (e.g., hurricanes) (15).

The pathogenic agent, first identified in the 1940s, was classified in 1980 as *Xanthomonas campestris* pv. mangiferaeindicae (6, 19, 20). Recently, Vauterin et al. (37) did not include the pathovar mangiferaeindicae in their extensive reclassification of the genus *Xanthomonas*. Most strains belonging to the pathovar produce white colonies (16), but the colonies of a few strains from Brazil, South Africa, and Réunion Island are yellow (25). Most characterized strains have been isolated from mango. However, strains isolated from other members of the mango family (Anacardiaceae), including ambarella (*Spondias cytherea* Sonnerat) (29) and pepper tree (*Schinus terebenthifolius* Radii) (22), were pathogenic when they were inoculated onto mango leaves (22, 26). Because until very recently the taxonomy of the genus *Xanthomonas* was ruled by the pathovar concept established by Dye et al. (6), all of the strains mentioned above were provisionally classified as members of pathovar mangiferaeindicae.

Control of the disease by chemicals in regions where the disease is endemic is limited. Current research is oriented toward integrated pest management, principally using resistant cultivars of mango. To facilitate the screening of cultivars in breeding programs (5, 39), we initiated an analysis of the pathogen's variability and population structure.

Recent studies in which physiological and biochemical tests, sensitivity to antibiotics, heavy metals, and bacteriophages, serological grouping, plasmid profiles, and multilocus isozyme analysis were used showed that strains of pathovar mangiferaeindicae display intrapathovar diversity (21, 24, 35). Although useful for grouping strains for genus and species classification, these techniques are limited in their ability to group strains at the pathovar level. Thus, to clarify the relationships between strains belonging to pathovar mangiferaeindicae, more discriminating techniques are required.

Techniques that assess variation in genomic DNA provide additional reliable tools to evaluate strain variability. In general, genomic variability is independent of external factors, and the techniques involved are adaptable to studies involving large numbers of strains. Furthermore, because these techniques allow for the measurement of many markers, they usually result in more comprehensive information than other methods. One molecular technique commonly used for comparisons of genome structure is restriction fragment length polymorphism (RFLP) analysis. Several types of DNA probes have been used for RFLP analysis of bacterial species. Avirulence (*avr*) genes (1, 18), *hrp* genes (4, 13, 33, 36), and repetitive sequences that either are of unknown nature (7, 8, 10, 13) or are transposable elements (1, 3, 12, 18) have been useful for studies on the population structure within pathovars, for comparing different pathovars, and for differentiating nonpathogenic and pathogenic *Xanthomonas* strains. Depending on the probe used, variability was observed at different levels. For instance, in *Xanthomonas oryzae* pv. oryzae, repeated sequences and *avr* genes allow differentiation between strains of

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a single pathovar (18), whereas *hrp* gene organization is usually conserved within a pathovar and allows differentiation of and comparison between pathovars (33, 36).

In this study, a repetitive element that was cloned from *Xanthomonas* pv. mangiferaeindicae, an avirulence gene from *X. oryzae* pv. oryzae (9), and an *hrp* cluster from *X. oryzae* pv. oryzae (17) were used as probes to differentiate several groups of pathovar mangiferaeindicae strains and to assess the relationships among these groups. Our purpose was to establish the relationships among pathovar mangiferaeindicae strains isolated from different hosts and in different countries to increase our understanding of the diversity of xanthomonads associated with members of the Anacardiaceae.

MATERIALS AND METHODS

Bacterial strains and plasmids. The geographic origins and other relevant characteristics of the 138 *Xanthomonas* pv. mangiferaeindicae strains used in this study are described in Table 1. Prior to the study, all strains were tested for pathogenicity for mango by using the leaf inoculation protocol of Pruvost and Luisetti (26) with the bacterial suspension concentrations adjusted to 10^6 and 10^8 CFU/ml. All strains were pathogenic for mango; i.e., they produced angular black necrotic lesions and therefore were considered members of pathovar mangiferaeindicae. No significant differences were found among the white-colony-producing strains. However, the yellow-colony-forming strains were less aggressive than the other strains; they consistently produced fewer lesions on mango leaves (7a). A few strains of *Xanthomonas* pv. mangiferaeindicae were available from Brazil (five strains) and the French West Indies (two strains); the disease has been reported only once from each of these countries, and the strains included in this study are the only ones maintained in collections. For preservation, cells were adsorbed on beads with a cryoprotectant by using a Microbank kit (Pro-Lab Diagnostics) and stored at −80°C. For routine use, cultures were grown on YPGA medium (7 g of yeast extract per liter, 7 g of peptone per liter, 7 g of glucose per liter, 16 g of agar per liter; pH 7.2) at 28°C.

Plasmid pBSavrXa10 contains avirulence gene *avrXa10* from *X. oryzae* pv. oryzae and was described previously (9). Plasmid p23-44 consists of a 23-kb insert containing part of the *hrp* cluster from *X. oryzae* pv. oryzae; the fragment is inserted in pHM1 and was isolated by homology with other *hrp* clusters (17).

DNA extraction. DNA was extracted from *Xanthomonas* pv. mangiferaeindicae cells grown overnight at 28°C in 30 ml of yeast extract-peptone broth (7 g of yeast extract per liter, 7 g of peptone per liter) by the hexadecyltrimethylammonium bromide method (2). Plasmids were extracted from *Escherichia coli* by the alkaline lysis procedure (31) and were purified with a Prep-A-Gene plasmid purification kit (Bio-Rad, Hercules, Calif.).

Construction of a partial genomic library. DNA of *Xanthomonas* pv. mangiferaeindicae strain 1717 was partially digested with *Sau*3AI, and the fragments were separated in a 0.7% agarose gel. The area of the gel corresponding to 2- to 5-kb fragments was excised and dialyzed in Tris-borate-EDTA buffer (31). After phenol-chloroform extraction, the DNA fragments were ligated to *Bam*HI-digested pBluescript II KS+ (Stratagene, La Jolla, Calif.) by using T4 DNA ligase (Promega, Madison, Wis.) and standard techniques (2). Recombinant plasmids were electroporated into *E. coli* DH5-α MCR (Gibco BRL, Gaithersburg, Md.) by using settings of 550 V and 5 ms with a model T-100 electroporator (Biotechnical and Experimental Research, Inc., San Diego, Calif.).

Electrotransformed cells were incubated in Luria-Bertani broth (31) with shaking for 45 min at 37°C and then plated onto Luria-Bertani agar medium containing 100 mg of carbenicillin per ml and 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside per ml. White colonies were transferred by colony blotting to a nylon membrane (14). DNA was cross-linked with UV light and subsequently probed with biotinylated (see below) total DNA from *Xanthomonas* pv. mangiferaeindicae strain 1717. Colonies which hybridized strongly were selected, and plasmid DNA was extracted. The plasmid DNA was labeled with biotin and used to probe blots containing *Bam*HI-digested genomic DNAs from *Xanthomonas* pv. mangiferaeindicae and other xanthomonads.

Digestion, electrophoresis, and blotting. Bacterial DNA (2 to 3 μg) was digested to completion with *Bam*HI or *Eco*RI as described by the manufacturer (Boehringer Mannheim, Indianapolis, Ind.). DNA fragments were separated in a 0.7% agarose gel by electrophoresis at 1.5 V/cm for 16 h in TBE buffer and were transferred to a Photogene nylon membrane (Gibco BRL) by using a Bio-Rad vacuum blotter according to the manufacturer's instructions. A 1-kb ladder (Gibco BRL) was included as a size standard in each gel.

Biotin labeling, hybridization, and detection. Plasmid preparations (pBSavrXa10, p23-44, and pLGX1) and total DNA were labeled with biotin-14-dCTP by random priming by using a BioPrime labeling kit (Gibco BRL). The pHM1 and pBluescript vectors do not hybridize with *Xanthomonas* DNA (data not shown); therefore, the entire plasmid was labeled.

Blots were prehybridized in a rotary hybridization oven (Appligene, Illkirch, France) for 2 h at 65°C. After hybridization overnight at 65°C, blots were washed under high-stringency conditions (11). Detection of biotinylated, hybridized probe with streptavidin-alkaline phosphatase conjugate and chemiluminescent substrate was performed by using a PhotoGene detection kit (Gibco BRL) according to the manufacturer's instructions. Blots were exposed to X-Omat AR film (Kodak, Rochester, N.Y.) for 15 min to 4 h, depending on the signal intensity.

Analysis of band patterns. The presence or absence of bands on the blots was converted into binary data; i.e., the presence of a band was coded as 1, and the absence of a band was coded as 0. In cases where two bands were not clearly distinguishable, neither band was considered in the analysis. The Jaccard similarity coefficients (J) (34) between strains ($J = N_{ab}/[N_{ab} + N_a + N_b]$, where N_{ab} is the number of bands that strains a and b have in common and N_a and N_b are the numbers of bands exclusive to strains a and b, respectively) were calculated by using the SIMQUAL procedure in the NTSYS program, version 1.80 (28), and then transformed into dissimilarity coefficients ($1 - J$). The program *abcd* (version 2, 1993; Centre National de la Recherche Scientifique, Marseille, France) was used to construct a tree with the neighbor-joining method of Saitou and Nei (30). Correction for the lengths of tree branches according to the least-square values was done with the same program. Clusters were determined by using Ward's minimum variance option of the PROC CLUSTER procedure of SAS Institute, Inc., Cary, N.C. The values for the cubic clustering criterion, pseudo F, and pseudo t^2 were used as estimators of the most reliable number of clusters as described by Adhikari et al. (1).

RESULTS

Isolation of a repetitive element from *Xanthomonas* pv. mangiferaeindicae. A plasmid containing a 1.8-kb insert was selected from 200 clones obtained from a partial *Xanthomonas* pv. mangiferaeindicae strain 1717 genomic library by colony blotting electroporated *E. coli* strains and probing them with total digested labeled DNA from strain 1717. Five clones strongly hybridized to the probe and were expected to contain sequences that are frequently found in the genome of *Xanthomonas* pv. mangiferaeindicae. When labeled plasmids from these strains were used as probes with *Bam*HI-digested DNA from *Xanthomonas* pv. mangiferaeindicae, at least 30 fragments hybridized. The five independent clones gave exactly the same hybridization patterns, indicating that they contain a fragment which has multiple copies in the genome. One plasmid was randomly chosen and named pLGX1.

When hybridized under low-stringency conditions with DNAs from 30 other *Xanthomonas* pathovars, pLGX1 showed homology with 11 pathovars, although it never hybridized with more than seven fragments (7b).

Polymorphism of the restriction fragments hybridizing with p23-44, pBSavrXa10, and pLGX1. *Eco*RI-digested DNA from *Xanthomonas* pv. mangiferaeindicae was hybridized with p23-44, a plasmid containing a 23-kb *hrp* cluster from *X. oryzae* pv. oryzae. Each unique genotype (i.e., each unique RFLP pattern) is referred to below as a haplotype. Twenty-one band positions were visually recorded, and there were three to six bands per haplotype. The distribution of the different haplotypes (designated haplotypes hrp1 to hrp8) obtained with this probe is shown in Table 2.

A single pattern was found for most of the *Xanthomonas* pv. mangiferaeindicae strains. This group of strains, which is referred to below as group I, consisted of 127 white-colony-producing strains which were isolated from mangoes and pepper trees from all countries investigated except Brazil. The absence of polymorphism made the *hrp* probe useless for analyzing diversity among the group I strains. Seven unique *hrp* patterns were identified among the 11 other strains (group II).

The hybridization profiles for 12 group I strains obtained with probes pLGX1 and pBSavrXa10 are shown in Fig. 1. For each of the 127 group I strains, at least 30 *Bam*HI fragments hybridized with pLGX1. Only bands that ranged in size from 1.6 to 6.2 kb were analyzed. A total of 31 bands were scored, and there were 13 to 18 bands per strain, resulting in 43 pLGX1-derived haplotypes. Eighteen bands were scored for probe pBSavrXa10, and there were 6 to 10 bands per strain; 40

TABLE 1. Strains of *Xanthomonas* pv. mangiferaeindicae used in this study

Geographic origin	Strain(s)[a]	Haplotype[b]	Group or subgroup[c]	Host (colony color)[d]
Australia	2921	8	C3	Mango
	2935, JF28-5	10	C3	Mango
	JF28-4	11	C3	Mango
	JF28-7, JF28-8, JF28-9, JF28-10, JF28-11, JF28-12	12	C3	Mango
	JF28-13	13	C3	Mango
	JJ238-46, JJ238-47	14	C3	Mango
Brazil	2912, 2913, 2914			Mango
	2923, 2924			Mango (yellow)
Comoro Islands	2931, JF955	1	A	Mango
French West Indies	2547, 2623			Ambarella
India	1716^T	15	C2	Mango
	2916	16	B	Mango
	2917	17	C2	Mango
	JP682	18	B	Mango
	JP683	19	B	Mango
Japan	JN570, JN571, JN572, JN574, JN576, JN577, JN578, JN582, JN583	20	B	Mango
	JN573, JN579	21	B	Mango
	JN575, JN581	22	B	Mango
Mauritius Island	2930	23	C1	Mango
	JF29-5	9	A	Mango
	2936	24	A	Mango
	JF29-2	25	C1	Mango
	JG98-3	26	A	Mango
New Caledonia	JM23-2	1	A	Mango
	JM22-1, JM22-2, JM22-3, JM23-1	2	A	Mango
	JJ234-4, JJ234-5, JJ234-6, JM24-2	9	A	Mango
	JJ234-1	27	A	Mango
	JJ234-3	28	A	Mango
	JM22-4	29	A	Mango
	JM23-3	30	A	Mango
	JM24-4	31	A	Mango
Philippines	JK147-1	32	B	Mango
	JK147-2	33	B	Mango
Réunion Island	2925, 2932, N236-4, N236-8, A5-1, A8, JP501-1, JP501-2, JP501-3, JP501-4, JP501-5, JP501-6	2	A	Mango
	A23, B17	9	A	Mango
	1717	34	C1	Mango
	2927	35	A	Mango
	2933	37	C1	Mango
	A10	40	A	Mango
	A11-1	41	C1	Mango
	A11-2	42	C1	Mango
	A13	43	C1	Mango
	A14	44	A	Mango
	A17	45	C1	Mango
	A24-1	46	C1	Mango
	A26-1	47	A	Mango
	A30	48	C1	Mango
	A36	49	C1	Mango
	A5-4	50	C1	Mango
	A6-2	51	C1	Mango
	A6-1	52	C1	Mango
	B16	53	C1	Mango
	JE545-2	54	C1	Mango
	JF30-1, JF30-2, JF30-3, JF30-6	55	A	Mango
	JP517	56	C1	Mango
	JP709	57	C1	Mango
	JP737	58	A	Mango
	2928, 2939	36	D	Pepper tree
	2938	38	D	Pepper tree
	2940	39	D	Pepper tree
	JP738, JP739, JP743	59	D	Pepper tree
	JP740, JP741	60	D	Pepper tree
	JP742	61	D	Pepper tree
	JP757, JP758, JP759, JP760	62	D	Pepper tree
	2918, 2919, 2920			Mango (yellow)

Continued on following page

TABLE 1—*Continued*

Geographic origin	Strain(s)[a]	Haplotype[b]	Group or subgroup[c]	Host (colony color)[d]
Rodrigues Island	JP782-1	63	A	Mango
	JP782-2	2	A	Mango
South Africa	2915	1	A	Mango
	2926, 2934, JF953, JG725, JG726, JG727	2	A	Mango
	JF950, JF951, JF952	3	A	Mango
	JG730, JG729	4	A	Mango
	JG731	5	A	Mango
	JG732	6	A	Mango
	JK119-2	7	A	Mango
	JK119-1			Mango (yellow)
Taiwan	2929	64	B	Mango
Thailand	JM20	65	B	Mango
	JM21	66	B	Mango

[a] Strains 2921, 2935, 2912, 2913, 2914, 2923, 2924, 2931, 2547, 2623, 1716^T (T = type strain), 2916, 2917, 2930, 2936, 2925, 2932, 1717, 2927, 2933, 2928, 2939, 2938, 2940, 2918, 2919, 2920, 2915, 2926, 2934, and 2929 were obtained from the Collection Française de Bactéries Phytopathogènes, Institut National de la Recherche Agronomique, Angers, France. Other designations for these strains can be obtained from us.
[b] Haplotypes were determined from combined pBSavrXa10 and pLGX1 data.
[c] Groups and subgroups were determined by Ward's method.
[d] If no colony color is indicated, the colonies are white.

different haplotypes were differentiated. When combined, the RFLP data obtained with the two probes differentiated 66 haplotypes (Table 1). Most of the 11 group II strains did not hybridize with pLGX1 or pBSavrXa10; the only exception was one yellow-colony-producing strain, strain JK119-1 from South Africa, which had three fragments homologous to pLGX1. Because of this, group II strains were assumed to be members of a different pathovar and were excluded from the statistical analysis of the overall population diversity. The diversity of the group I strains was assessed by using only data obtained with the pLGX1 and pBSavrXa10 probes.

Statistical analysis of RFLP data for group I strains. When the data were analyzed separately and compared, the major groups revealed by the two probes were consistent. Most of the differences found were differences in the ability to separate subgroups within a main group. For example, strains isolated from pepper trees on Réunion Island could not be differentiated from a group of strains isolated from mangoes with probe pLGX1. Probe pBSavrXa10, however, clearly separated these isolates from the other groups. Therefore, the data were combined into a single 66-by-49 matrix to maximize the ability to differentiate (66 haplotypes by 31 and 18 band positions revealed by probes pLGX1 and pBSavrXa10, respectively).

Figure 2 shows the resulting neighbor-joining tree with the clusters obtained by Ward's method superimposed. A principal-coordinate analysis also was performed with the dissimilarity coefficients, and the results were similar to those obtained by Ward clustering (data not shown). The neighbor-joining tree is shown because it provides a better representation of the distances between strains. Ward clustering is more suitable for forming objective groups. To obtain the advantages of both representations, we superimposed the Ward clusters onto the neighbor-joining tree in Fig. 2.

The 127 group I strains were clustered into four groups (designated groups A to D) with Ward's clustering method. Four was found to be the most reliable number of groups for this set of data according to the following three statistical tests recommended by SAS (32): the cubic clustering criterion, pseudo F, and pseudo t^2 values (local maximum for the cubic clustering criterion and pseudo F, local minimum for pseudo t^2). The groups were consistent with the neighbor-joining tree branches. When other methods of clustering were used, similar groups were formed; only the details of the minor branches of clusters varied (data not shown).

To determine the diversity within groups and between groups, the average dissimilarity coefficient ($1 - J$) was calculated (Table 3). Group A showed little genetic diversity, although it contained strains from widespread geographic areas (South Africa, Mascarene Islands, Comoro Islands, and New Caledonia; a total of 59 strains from six countries). Group B, which was the most diverse group, contained strains that originated from Southeast Asia (21 strains from five countries). Group C consisted of strains from two of the Mascarene Islands (Réunion and Mauritius) (18 strains), India (2 strains),

TABLE 2. Different haplotypes obtained by hybridization of total *Eco*RI-digested DNAs with probe p23-44 containing an *hrp* gene cluster[a]

hrp group	*hrp* haplotype	No. of strains	Country(ies)	Host(s)	Colony color
I	hrp1	127	All countries except Brazil	Mango, pepper tree	White
II	hrp2	2	French West Indies	Ambarella	White
	hrp3	3	Brazil	Mango	White
	hrp4	2	Brazil	Mango	Yellow
	hrp5	1	Réunion	Mango	Yellow
	hrp6	1	Réunion	Mango	Yellow
	hrp7	1	Réunion	Mango	Yellow
	hrp8	1	South Africa	Mango	Yellow

[a] Strains that produced the hrp1 pattern were placed in group 1, while the other haplotypes were placed in group II.

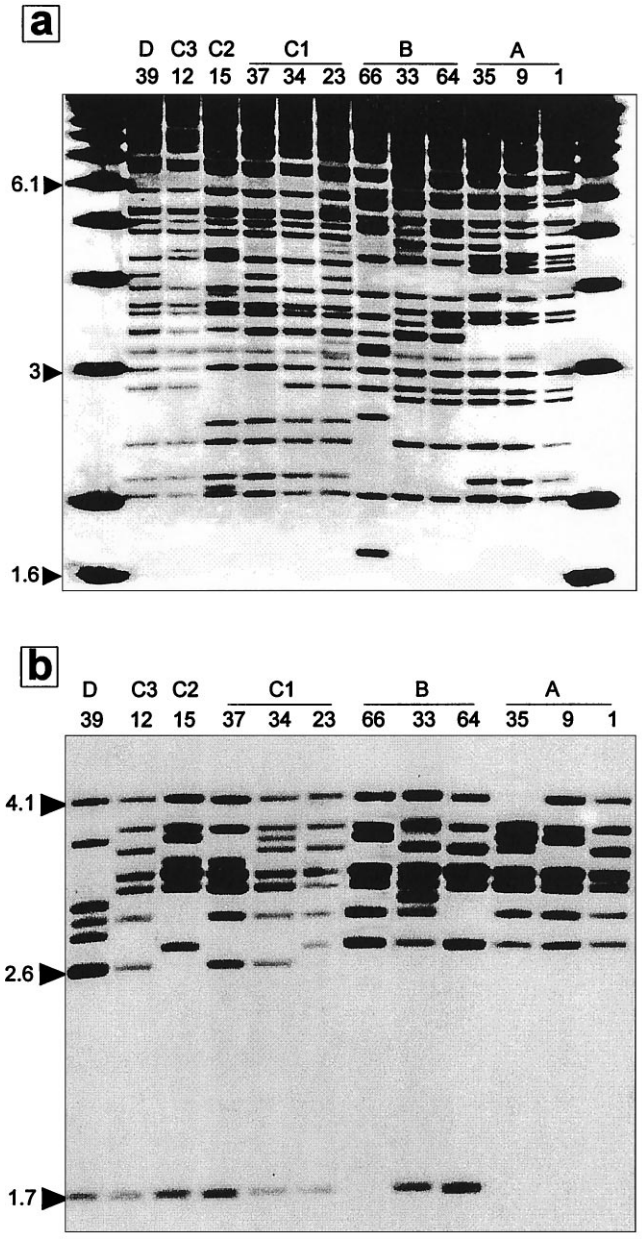

FIG. 1. Southern blot of digested DNAs from *Xanthomonas* pv. mangiferaeindicae group I strains obtained with probes pLGX1 (a) and pBSavrXa10 (b). The strains represent the groups (indicated by capital letters) obtained by Ward clustering. Lane 1, haplotype 1 (strain 2915); lane 9, haplotype 9 (strain JJ234-4); lane 35, haplotype 35 (strain 2927); lane 64, haplotype 64 (strain 2929); lane 33, haplotype 33 (strain JK147-2); lane 66, haplotype 66 (strain JM21); lane 23, haplotype 23 (strain 2930); lane 34, haplotype 34 (strain 1717); lane 37, haplotype 37 (strain 2933); lane 15, haplotype 15 (strain 1716); lane 12, haplotype 12 (strain JF28-7); lane 39, haplotype 39 (strain 2940). Sizes (in kilobases) are indicated on the left. Southern blots were scanned by using a Hewlett-Packard model ScanJet IIcx scanner and the Aldus PhotoStyler 2.0 program.

and Australia (13 strains). When these strains were analyzed separately from the rest of the population by the Ward clustering method, the following three subgroups of group C were distinguished: subgroup C1, which contained only the Mascarene Islands strains; subgroup C2, which contained the Indian strains; and subgroup C3, which contained the Australian strains. Finally, group D contained 14 strains, all of which were isolated in Réunion from pepper trees.

DISCUSSION

Using RFLP analysis, we found that strains previously identified as pathovar mangiferaeindicae strains based on pathogenicity and host origin are genetically heterogeneous. Two groups were differentiated. Group I, which includes most of the strains, contains the typical strains of *Xanthomonas* pv. mangiferaeindicae (i.e., white-colony-forming strains isolated from mangoes or pepper trees). Group II contains strains that produce yellow-pigmented colonies or were isolated either from ambarella in the French West Indies or from mango in Brazil.

The three different probes that were utilized for this study revealed different aspects of the variability in pathovar mangiferaeindicae. When the *hrp* cluster was used as a probe, a single band pattern was observed for the 127 group I strains, which are typical of pathovar mangiferaeindicae. Very different hybridization profiles were obtained for the 11 group II strains, and most of these strains had no common band. The results obtained with the repetitive element contained in pLGX1 and with the *avr* gene were consistent with one another in terms of strain grouping and revealed polymorphism among the group I strains. Therefore, while the *hrp* probe was useful for distinguishing group I from group II, *avr* and pLGX1 probes were useful for establishing relationships between strains within group I.

The distribution of strains in the different *hrp* RFLP haplotypes was comparable to the groups obtained by other workers with biochemical and serological data, with minor exceptions. Pruvost et al. described eight groups based on biochemical comparisons of a subset of our collection of strains (24). The *hrp* RFLP group I strains are distributed in four of these biochemical clusters, and the group II strains constitute the other four groups. Serovar 1, as described by Pruvost (21), for the most part included our group I strains, but also included the white-colony-producing strains belonging to our group II isolated from mangoes in Brazil. Serovar 2 contained all of the yellow-colony-producing strains and the strains isolated from ambarella and was comparable to *hrp* RFLP group II. More recently, Somé and Samson (35) used isozyme profiles obtained with esterase, phosphoglucomutase, and superoxide dismutase and differentiated four groups; isozyme group 1 was similar to our *hrp* RFLP group I, while groups 2, 3, and 4 included strains belonging to our group II. Thus, as shown by all types of analyses, *hrp* RFLP group I is fairly homogeneous, whereas group II is heterogeneous and is composed of strains that are not related to each other or to any other group (i.e., the cluster is forced).

Based on the work of Stall and Minsavage (36) on *Xanthomonas* pathovars and the work of Scholtz et al. (33) and Legard et al. (13) on *Pseudomonas syringae*, the structure and position of *hrp* clusters in the genomes of plant-pathogenic xanthomonads and pseudomonads are conserved within a pathovar. Thus, the strains in group I are probably members of the same pathovar (pathovar mangiferaeindicae), and the 11 strains in group II, which have diverse *hrp* patterns, are probably members of pathovars other than pathovar mangiferaeindicae. When inoculated onto mango, the group II strains caused symptoms similar to black spot. However, many of these strains, (e.g., the yellow-colony-forming mango strains) are weakly aggressive on mango (21). Such strains are very rare in Réunion, where regular, intensive sampling has been undertaken. Strains isolated from ambarella were highly aggressive on ambarella and devastated an attempt to cultivate ambarella in the French West Indies. These strains were pathogenic on mango when they were artificially inoculated, but the bacterial

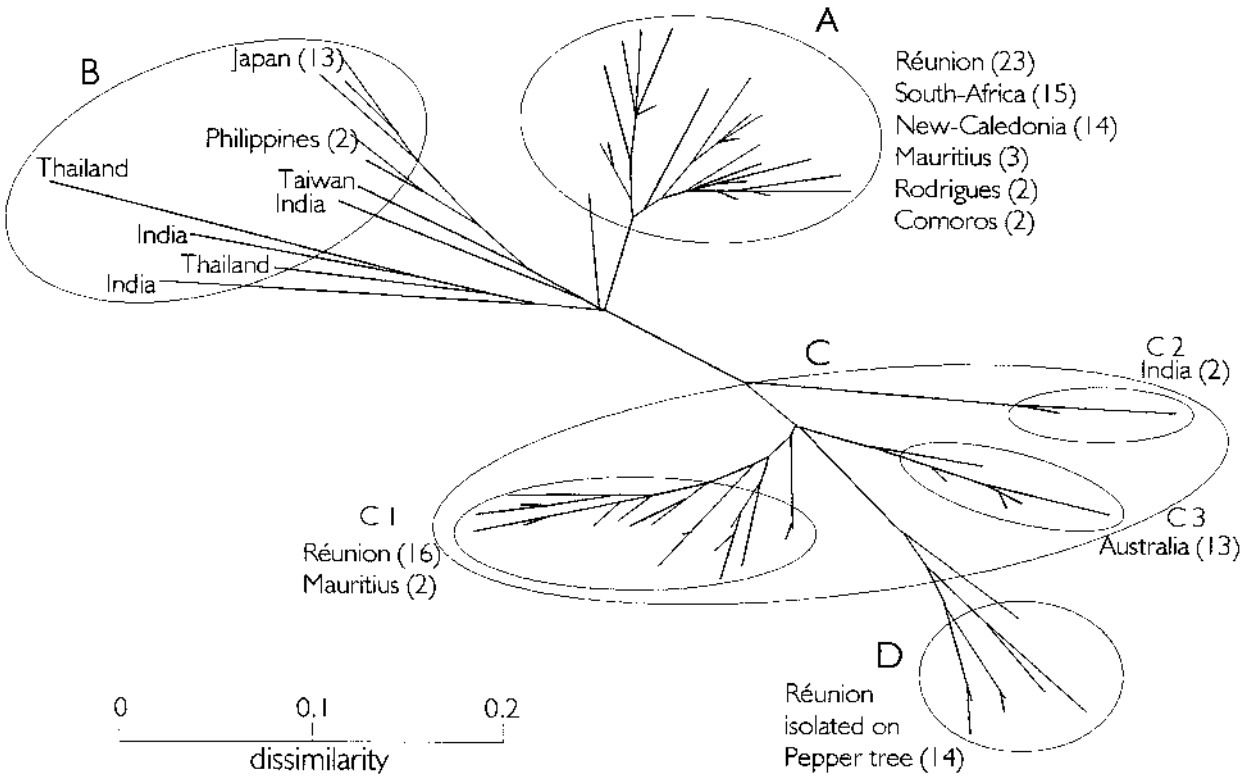

FIG. 2. Neighbor-joining tree showing the relationships among the 127 group I strains as determined by hybridization profiles with probes pBSavrXa10 and pLGX1. Groups A, B, C, and D and subgroups C1, C2, and C3 obtained by the Ward clustering method are superimposed. The countries of origin and the number of strains (in parentheses) for each country are indicated. The distance $(1 - J)$ from one strain to another is represented on the neighbor-joining tree by the sum of the lengths of the branches that join them.

black spot disease has not been reported in the French West Indies, although there are mangoes there (26, 29). Since group II strains are rarely or never found in naturally infected mangoes, their role in mango black spot is questionable compared to other strains, and their classification with pathovar mangiferaeindicae strains should be reevaluated by using a polyphasic approach.

Although analysis with the *hrp* probe suggested that all group I strains belong to the same pathovar, analysis with the *avr* and pLGX1 probes revealed distinct lineages within which strains are relatively similar (Fig. 1). This is in agreement with the fact that the genus *Xanthomonas* is predominantly composed of clonal populations (7, 10); that is, xanthomonads are thought to evolve mostly by mutation or by insertion-deletion of mobile DNA elements, and major changes are not seen over relatively large periods of time. Some haplotypes are shared by strains from countries that are very distant from one another. This suggests that populations of *Xanthomonas* pv. mangiferaeindicae can be spread over very large distances, probably through exchanges of contaminated propagative material. Conversely, the presence of strains in the same location that belong to different haplotypes or different clusters could indicate that several distinct introduction events occurred.

Four groups were apparent according to Ward clustering at a level of similarity greater than 65%. When the geographic origins of the strains in each cluster are considered, several interesting observations emerge. Group B, which exhibits the greatest diversity (almost as great as the diversity observed in the entire collection), contains only strains from Asia. Asia is the area where mangoes originated and therefore is probably the area where the disease originated. If this is true, the pathogen would have been present for a longer time in Asia than in other areas and would be predicted to be more diverse there than in other parts of the world. Analysis of more strains from Asia will be necessary to confirm this hypothesis. The group A strains include 59 isolates that are from different geographic areas but are similar (except for one strain from Réunion). Many strains belonging to this cluster could not be differentiated from one another (one haplotype was shared by 22 strains from three countries). The high degree of similarity among strains from six countries could be explained by a recent, but widespread, introduction of the disease into these countries (for instance, by large-scale introduction of diseased plant material from a single place or from very few places). This hypothesis is difficult to test since information concerning the origin of the mango cultivars used in these regions is not available. Group C contains three subclusters that are geo-

TABLE 3. Average dissimilarity coefficients $(1 - J)$ for strains within clusters and for strains belonging to different group I clusters[a]

Cluster	Distances within and between clusters			
	A	B	C	D
A	0.16	0.35	0.40	0.51
B		0.34	0.43	0.52
C			0.21	0.35
D				0.20

[a] The average dissimilarity coefficient for the entire collection of Group I strains was 0.35.

graphically well-separated and have high degrees of internal similarity (Fig. 2). Each subcluster could correspond to a discrete introduction of strains that evolved in the region separately from the other populations. This is feasible, since the geographic origins of strains in subgroups C1 and C3 are islands that are relatively isolated from the rest of the world. The two strains from India found in subgroup C2 are another example of the fact that Asian strains have a diversity which approaches that of the total population.

Finally, group D includes strains from Réunion Island that were collected from black spot lesions on pepper tree (Brazilian pepper), a weed tree belonging to the family Anacardiaceae which is sometimes used as windbreak around mango orchards. These strains were considered members of pathovar mangiferaeindicae because they are pathogenic when they are inoculated onto mango and cannot be differentiated in phenotypic studies (22, 24). DNAs from group D strains hybridize with the three probes and yield patterns similar to the patterns obtained with strains belonging to groups A, B, and C. However, an analysis of pBSavrXa10 and pLGX1 patterns indicated that group D strains clearly constitute a separate group. Strains collected over several years from pepper trees many kilometers apart always belonged to group D, whereas all of the mango strains collected in Réunion, even strains collected from trees that were very close to a pepper tree hedgerow, clustered either in group A or in group C. Our information does not support the hypothesis of Pruvost et al. that pepper trees constitute an inoculum reservoir in areas where they are present together with mango trees (22). Further studies will address why strains from pepper trees do not cause the disease on mango. One hypothesis is that a lineage of strains from mango diverged to become pathogenic on pepper tree and over time lost the ability to naturally infect mango. One approach to understanding the relationship between mango and pepper tree strains would be to determine if the disease occurs on pepper tree in Brazil, where pepper tree originated, and to compare the genetic relationship of bacteria obtained from this tree to other groups of *Xanthomonas* pv. mangiferaeindicae strains. The small number of strains collected from mango in Brazil that have been tested are distantly related to the other mango and pepper tree strains (they belong to group II), suggesting that the pepper tree strains found in Réunion did not originate in Brazil.

Often, repetitive elements that previously were used to assess genetic diversity appear to be mobile elements that can move within the genome of an organism (40). Although the repetitive DNA fragment contained in pLGX1 has not been characterized, the occurrence of repeated sequences raises a question concerning the reliability of measurements of variability made with mobile DNA elements. Previous studies showed that different mobile elements gave comparable results that were consistent with results obtained with other probes, indicating that they are good markers of genetic diversity (18, 38). This could be because either they are one cause of genetic diversification (by inserting in the genome they create mutations) or they are indicators of the time elapsed since two strains diverged from a common ancestor.

When hybridized to DNAs from *Xanthomonas* pathovars, pLGX1 homologs were found in high copy numbers in the genomes of typical strains of *Xanthomonas* pv. mangiferaeindicae, but not in the genomes of other pathovars. This could mean that the repetitive element contains sequence that might be present only in the genome of that *Xanthomonas* pathovar. Therefore, the element may be useful for detection and identification based on DNA-DNA specificity.

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