Measuring the Genetic Diversity of *Xanthomonas axonopodis* pv. *manihotis* Within Different Fields in Colombia

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ABSTRACT

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Cassava bacterial blight, caused by *Xanthomonas axonopodis* pv. *manihotis*, is a widespread disease that affects cassava (*Manihot esculenta*). We collected 238 *X. axonopodis* pv. *manihotis* strains by intensively sampling single fields in four edaphoclimatic zones (ECZs) in Colombia. DNA polymorphism of different *X. axonopodis* pv. *manihotis* populations was assessed by restriction fragment length polymorphism (RFLP) analyses, repetitive sequence-based polymerase chain reaction (rep-PCR), and amplified fragment length polymorphism (AFLP) assays. Genetic diversity, phenetic relationships among strains, and the coefficient of genetic dif-

ferentiation were determined. All strains were tested for aggressiveness on the susceptible cassava cv. MCOL 1522. Strains were also tested for virulence on cassava differentials adapted to the strains' respective ECZs. Our study showed that the Colombian *X. axonopodis* pv. *manihotis* population has a high degree of genetic diversity. The hierarchical analysis of diversity showed genotypic differentiation at all levels, among ECZs, among fields within ECZs, and among strains within fields planted to several cassava genotypes. New RFLP haplotypes were detected, leading to the characterization of a new pathotype. Dendrograms from AFLP were more robust than those from RFLP data. A close association between the strains' geographical origin and DNA polymorphism was obtained using RFLP and AFLP data. We suggest that the host played a role in causing pathogen differentiation.

Cassava bacterial blight (CBB), caused by *Xanthomonas axonopodis* pv. *manihotis*, is a major constraint to cassava (*Manihot esculenta* Crantz) cultivation worldwide (15). Cassava provides food and income for approximately 500 million people living in the tropics. Cassava is propagated by planting pieces of stem, permitting the pathogen to be disseminated through infected planting material. Root yield losses ranging from 12 to 100% were reported (15). Losses induced by CBB also affect the planting material. Incidence of the disease can be reduced by a combination of cultural practices, sanitation measures, and the use of varietal resistance (15). The use of varietal resistance is the most realistic and appropriate way to control the disease (15).

Genetics of resistance to CBB have not been extensively studied in cassava, and genes governing resistance have not yet been documented. At least two sources of resistance to CBB exist, one from *M. glaziovii* and the other from *M. esculenta* (7). Whether the two sources of resistance to CBB act in the same way or are controlled by the same alleles is still unknown. Resistance from both sources is thought to be polygenic and additively inherited, with narrow-sense heritability ranging from 25 to 65% (7). The difference between resistant and susceptible cultivars is expressed as a variation in the rate of *X. axonopodis* pv. *manihotis* colonization of vascular tissues. For this reason, resistance is usually considered as quantitative (10). No clear-cut gene relationship has been demonstrated, and hypersensitive responses have never been observed in cultivated varieties. Studies on the genetics or number of disease resistance loci involved in host plant resistance to CBB have not been conducted.

As was recently observed in Africa, changes in the pathogen's population structure may lead to the overcoming of the deployed re-

sistance. The dissemination of *X. axonopodis* pv. *manihotis* through infected cuttings has important implications for controlling the disease. Understanding pathogen migration and how it influences population genetic structure is, therefore, important.

Genetic diversity of *X. axonopodis* pv. *manihotis* was mainly characterized by restriction fragment length polymorphism (RFLP) analyses (22,27,28). RFLP analyses using a plasmid probe that harbors a pathogenicity gene (*pthB*) (26) are more discriminatory than are genomic probes or ribotyping (22,27). A 1996 study of distribution of pathogen diversity among edaphoclimatic zones (ECZs) and sites in Colombia revealed the genetic variability of *X. axonopodis* pv. *manihotis* in that country and population substructuring at the ECZ level (22).

Caution must be taken when inferring phylogeny based on data from one or a few molecular markers (19). To avoid potential bias in genetically characterizing a pathogen population, the population structure must be inferred from a variety of neutral markers (13). Recently, two techniques were shown to be very useful for studying plant pathogen population structures: repetitive sequence-based polymerase chain reaction (rep-PCR) and amplified fragment length polymorphism (AFLP). rep-PCR is simple and fast (14) and has been used for characterizing xanthomonads and pseudomonads (14,24). AFLP is a novel DNA fingerprinting method that can be used on any DNA, regardless of origin or complexity (29). AFLP analyses offer the possibility of a broader genome coverage. Their usefulness in characterizing bacterial populations was shown by Janssen et al. (8), and their applicability to the study of *X. axonopodis* pv. *manihotis* populations and their high discriminatory power were demonstrated by Restrepo et al. (21) when characterizing *X. axonopodis* pv. *manihotis* populations with low levels of genetic diversity. The AFLP technique allowed a very fine discrimination of pathogen populations, clearly and reliably differentiating bacterial strains (3). A high degree of discrimination among isolates can be achieved despite the monomorphic nature of a population or species (9,21).

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The objective of this study was to conduct a hierarchical analysis of the *X. axonopodis* pv. *manihotis* population structure in Colombia by examining the variation and microgeographical distribution of the pathogen in different fields located in four ECZs. Resulting information should be useful for guiding deployment strategies and designing epidemiology studies (16). A *X. axonopodis* pv. *manihotis* collection was sampled in 1997 from six experimental and two farmers' fields. In this study, all of the strains were analyzed by RFLP, AFLP assays, and pathogenicity on a susceptible cassava cultivar. A selected set of strains was further analyzed by rep-PCR and pathogenicity on a set of 14 cassava differentials. The molecular techniques were compared for their efficiency in measuring and describing genetic diversity.

MATERIALS AND METHODS

Bacterial strains and collection of infected samples. Collections were made from eight fields located in four ECZs: 1 = sub-humid tropics, 2 = acid-soil savannas, 5 = high-altitude tropics, and 2/5 = an ECZ sharing characteristics with ECZs 2 and 5 (Fig. 1). Fields were sampled intensively at random and along diagonals. Samples of infected stems or leaves were collected from various cassava plants within each field. Collections were made from one improved cultivar at site F, from two traditional cultivars at site A, and from various improved cultivars at the other sites (B, C, D, E, G, and P). An average of 31 samples from infected plants was collected per field. Of each sample, a single bacterial colony was isolated on YPGA medium (5 g of yeast extract, 5 g of glucose, 5 g of Bacto Peptone, and 15 g of agar per liter). We obtained 238 strains and preserved them in 60% glycerol at -80°C .

Following the equation $N = \log(1 - P)/\log(1 - F)$ (13), in which N = number of samples ($N = 238$) required to detect a given haplo-

type present at a frequency (F) at least once with a probability of 95%, the sampling strategy permitted us to detect variants present at a frequency of 1.2% in the population ($N = 238$) and, in average, 1.95% in ECZs ($N = 59$) and 2% in fields ($N = 31$).

RFLP and rep-PCR characterization. All of the strains were analyzed by RFLP analysis using the plasmid probe *pthB*. Probe *pthB* is a 5.6-kilobase (kb) *EcoRI* fragment that was cloned from an indigenous plasmid and harbors a pathogenicity gene related to the *avrBs3/pthA* gene family (26). Probe *pthB* has shown homology to sequences in other *Xanthomonas* spp. (25). All procedures were identical to that previously described (22) except that, before DNA extraction, the strains were grown for 12 h on YPGA solid medium and then collected from the petri dish and centrifuged twice at $6,000 \times g$ for 5 min in 1 M NaCl. Extracted DNA was digested with the restriction enzyme *EcoRI* at 37°C for 5 h, as recommended by the enzyme manufacturer (Gibco BRL, Gaithersburg, MD). Each strain was analyzed at least twice, and the RFLP banding patterns were compared in a single blot to confirm that each pattern was unique.

A set of 19 strains, representing the 19 *pthB* haplotypes detected, was further characterized by rep-PCR. The primers used for PCR amplification were ERIC1R 5'-ATGTAAGCTCCTGGGG-ATTAC-3'; ERIC2 5'-AAGTAAGTGACTGGGGTGAGCG-3') and REP (REP1R-I 5'-IIICGICGICATCIGGC-3'; REP2-I 5'-ICGICTTATCIGGCCTAC-3').

PCR protocols were as described by Vera Cruz et al. (24), except for the composition of the buffer (100 mM Tris-HCl, pH 8.3; 33.5 mM MgCl_2 ; and 500 mM KCl). PCR amplifications were performed in an automated thermal cycler and the program was as described by Vera Cruz et al. (24). A 10- μl portion of amplified PCR product was separated by electrophoresis on 1.5% agarose gels in 0.75 $\times$ Tris-acetate-EDTA buffer (40 mM Tris-HCl and 1 mM EDTA, adjusted to pH 7.6 with glacial acetic acid) at 45 V for 15 h, stained with ethidium bromide, and scanned with a video system (Eagleeyell; Stratagene Inc., La Jolla, CA). Amplifications were done at least twice for each strain.

AFLP assays. All the strains were characterized by AFLP analysis as described by Vos et al. (29) with modifications as previously described by Restrepo et al. (21). DNA was digested with one combination of enzymes (*EcoRI/MseI*) and then ligated to the respective adapters. The selective amplifications were performed using two combinations of primers, *EcoRI*+*T/MseI*+*T* and *EcoRI*+*T/MseI*+*A* (21). After completing the second amplification, products were separated on a 6% polyacrylamide denaturing gel as previously described (21). Reproducibility of AFLP was assessed by twice loading the same PCR product in either the same gel or different gels.

Pathogenicity tests. All strains were evaluated for virulence on the susceptible cassava cv. MCOL 1522 as previously described (22). Disease severity was rated on a scale of 0 to 5, in which 0 = healthy plant and 5 = complete wilting. According to the disease reaction, virulence of strains was classified into three categories: 0 to 2.49 (not aggressive), 2.5 to 3.49 (weakly aggressive), and 3.5 to 5.0 (very aggressive).

Strains corresponding to the new RFLP/*pthB* haplotypes detected in this study were inoculated by stem puncture on a set of 14 cassava differentials that were previously selected (S. Restrepo, M. C. Duque, and V. Verdier, unpublished data). Plants were arranged in the greenhouse according to a randomized complete block design in four replicates. Uninoculated plants were included in each experiment as a control. The experiment was conducted twice. Plants with a mean disease reaction of ≤ 3 were grouped as resistant, while those with a disease reaction of >3 were classified as susceptible (28).

Data analysis. Each unique banding pattern generated by the *pthB* probe, AFLP primer combinations, or rep-PCR primers was regarded as a haplotype. The presence of a band was coded as 1 and its absence as 0. We used NTSYS-pc (version 1.80; Exeter Software, Setaukat, NY), to calculate a similarity matrix using Jaccard's coefficient of similarity. Cluster analysis was done with the un-

Fig. 1. In each edaphoclimatic zone (ECZ), strains were collected in different sites. A = Cajibío, B = Mondomo, C = Matazul, D = Carimagua, E = Vilavencio, F = Caracoli, G = Pivijay, and P = Santander de Quilichao.

weighted pair-group method using arithmetic averages in the SAHN program of NTSYS-pc. A dendrogram was constructed with the TREE option. Correlation between the RFLP and AFLP assays was determined by the NTSYS-pc option MXCOMP, which compared similarity matrices generated for each technique, using Jaccard's coefficient of similarity with the same set of strains.

The robustness of each phenogram was evaluated by a bootstrap analysis of each data set (5) using the computer program WinBoot (32). Each phenogram was reconstructed 1,000 times by repeated sampling with replacement. The frequency with which a particular grouping was identified was taken to reflect the strength of the grouping.

The diversity of strains from each field, ECZ, and the entire country was estimated by Nei and Tajima's diversity index (H_T) (18). A genetic diversity of 1 means that the diversity is so high that any two haplotypes sampled from a population are different. A genetically uniform population will have a diversity of 0 because any two individuals sampled will be identical. A genetic differentiation coefficient (G_{ST}) was calculated as $G_{ST} = (H_T - H_s)/H_T$, in which H_T is the total genetic diversity of *X. axonopodis* pv. *manihotis* in Colombia and H_s is the average genetic diversity in each ECZ or field.

Analysis of molecular variance (AMOVA) was used to estimate variance components for RFLP haplotypes, partitioning the variation among haplotypes, within and among ECZs. Genetic distance calculated as a Euclidean metric distance was computed between all pairs of isolates. Significance levels for variance component estimates were computed by nonparametric permutation procedures. The analyses were undertaken with the ARLEQUIN program (Arlequin version 1.1; Genetics and Biometry Laboratory, University of Geneva, Switzerland).

The aggressiveness of strains was analyzed, using the SAS general linear models procedure PROC GLM to assess differences among strains collected in the same and different fields.

RESULTS

RFLP analysis. A total of 23 bands, ranging in size from 2.4 to 26 kb, was scored. With the *pthB* probe, we found 19 distinct haplotypes among the 238 Colombian strains collected in 1997 (Table 1; Fig. 2). Nine of the haplotypes (C4, C6, C8, C9, C12, C13, C15, C17, and C18) found in 167 strains had been recovered in 1995 to 1996 (21), and the remaining 10 (C27, C28, C29, C30, C31, C33, C34, C35, C36, and C37) were new (Table 1). Two haplotypes (C34 and C37) were represented by a single strain, whereas others were represented by as many as 41 strains (e.g., C4). The haplotypes most frequent in ECZs 1, 2, 2/5, and 5 were C18 (67% of the analyzed strains), C4 (54%), C35 (71%), and C8 (65%), respectively. Seven of the eight haplotypes described for ECZ 2 were detected at site E (Table 1). All of the haplotypes present in ECZs 1 and 5 could be detected in sites G and B, respectively (Table 1).

In Colombia, the total genetic diversity of the *X. axonopodis* pv. *manihotis* collection based on the RFLP/*pthB* analysis was 0.9 (Table 2). ECZ 2 had the highest level of haplotypic diversity (0.68), with eight haplotypes among 81 strains analyzed (Tables 1 and 2). The population at sites A and F appeared to be monomorphic. Pathogen diversity was high within the fields planted with several improved cultivars (Table 2). In contrast, the diversity index was 0 for sites A and F, in which only two and one cultivars, respectively, were cultivated (Table 2). The G_{ST} estimate was large at both the field and ECZ levels (0.5 and 0.4, respectively). For each ECZ considered separately, the G_{ST} estimate was larger in ECZs 1 and 5 ($G_{ST} = 0.67$ and 0.63 , respectively), in which subpopulations at sites F and A are highly homogeneous, with only one haplotype detected at each site (Table 2). From the Euclidean distance matrix between all pairs of isolates, AMOVA partitioning of variance components revealed highly significant ($P < 0.001$) differences at all levels. Of the total genetic variation, 49.6% was attributable to differences among ECZs, 18.8% to differences among fields within ECZs, and 31.6% to differences among strains within fields.

rep-PCR analyses. The ERIC primers yielded two haplotypes (Fig. 3A). Amplification with the REP primers yielded three haplotypes (Fig. 3B). The size of the amplification products ranged from 800 to 3,200 base pairs (bp). The haplotypes obtained were highly similar among the 19 strains representing the 19 *pthB* haplotypes described above, with differences limited to the presence or absence of one to four bands. The haplotypes of the strains were highly reproducible with each of the two primer sets.

AFLP assays. Reproducibility was estimated by running amplifications of the strain CIO 378 on the same and different gels. Band-

Fig. 2. Southern blot analysis of *EcoRI*-digested genomic DNA from *Xanthomonas axonopodis* pv. *manihotis* strains hybridized with the *pthB* probe. The 19 haplotypes detected are presented as follows: lane 1, strain CIO 521 (haplotype C4); lane 2, CIO 716 (C6); lane 3, CIO 726 (C8); lane 4, CIO 745 (C9); lane 5, CIO 657 (C12); lane 6, CIO 520 (C13); lane 7, CIO 713 (C15); lane 8, CIO 614 (C17); lane 9, CIO 691 (C18); lane 10, CIO 527 (C27); lane 11, CIO 645 (C28); lane 12, CIO 640 (C29); lane 13, CIO 571 (C30); lane 14, CIO 574 (C31); lane 15, CIO 704 (C33); lane 16, CIO 503 (C34); lane 17, CIO 556 (C35); lane 18, CIO 755 (C36); and lane 19, CIO 730 (C37). M = 1-kilobase ladder, molecular weight marker.

TABLE 1. *Xanthomonas axonopodis* pv. *manihotis* strains used to study the genetic diversity of this bacterium in Colombia

Site ^a	ECZ ^b	No. of strains	Strain	<i>pthB</i> haplotype	Aggregation class (%) ^c	Cluster ^d	
						RFLP	AFLP
A	5	24	CIO 378 to 402	C8	M (6.6), H (93.4)	1	1
B	5	35	CIO 726 to 761	C8, C9, C36, C37	M (5), H (95)	1, 2, 8, 9	4, 5, 6, 7, 9, 10, 11, 14
C	2	26	CIO 611 to 637	C4, C12, C17	M (6.3), H (95.7)	4, 5, 7	2
D	2	22	CIO 509 to 531	C4, C12, C13, C17, C27	H (100)	3, 4, 5, 6, 7	12
E	2	30	CIO 503 and 638 to 667	C4, C12, C17, C27, C28, C29, C34	M (6.6), H (93.4)	3, 4, 5, 7, 13, 16, 17	15
P	2/5	54	CIO 536 to 590	C30, C31, C35	L (1.8), M (5), H (93.2)	11	13, 14
F	1	14	CIO 676 to 690	C18	L (7.1), H (92.9)	10	3, 15, 16
G	1	33	CIO 691 to 724	C6, C15, C18, C33	M (9), H (91)	10, 12, 14, 15	3, 6, 7, 8, 11, 15, 16, 17, 18

^a Fields at A = Cajibío, B = Mondomo, C = Matazul, D = Carimagua, E = Villavicencio, P = Santander de Quilichao, F = Caracoli, and G = Pivijay.

^b Edaphoclimatic zones: 1 = subhumid tropics, 2 = acid-soil savannas, 5 = high-altitude tropics, and 2/5 = ECZ with characteristics of ECZs 2 and 5.

^c H = very aggressive, M = weakly aggressive, and L = not aggressive. Percentages (in parentheses) represent the proportions of strains corresponding to each category.

^d Amplified fragment length polymorphism (AFLP) and restriction fragment length polymorphism (RFLP) clusters, numbered 1 to 18, are indicated per group of strains.

ing patterns, when loaded in the same gel, were identical, and when loaded in different gels, showed differences of less than 1%. With the two primer combinations selected, 130 bands were evaluated, 75 for primer combination *EcoRI*+*T/MseI*+*T* and 55 for primer combination *EcoRI*+*T/MseI*+*A*. The total number of bands obtained with the two AFLP combinations is four times more than that obtained with the RFLP/*pthB* technique. Strains belonging to *pthB* haplotype C8 were differentiated into 20 different AFLP haplotypes. The percentage of polymorphic bands was 90% for both combinations, showing the high level of polymorphism detected by this technique. With only one exception, all of the strains sharing the same AFLP haplotype were collected in the same field. Strains CIO 689 and CIO 707 showed the same AFLP and RFLP haplotypes, but were collected in fields F and G, respectively, both located in ECZ 1. Polymorphisms were detected among AFLP fingerprints, using the primer combination *EcoRI*+*T/MseI*+*T* for strains collected in fields B and G (Fig. 4).

Cluster analysis. To study the genetic relationships among strains, rep-PCR, RFLP/*pthB*, and AFLP haplotypes were analyzed separately by cluster analysis. The correlation coefficient between the AFLP and RFLP similarity matrix was low ($r = 0.48$). *X. axonopodis* pv. *manihotis* strains clustered into 17 groups at 70% similarity when considering the RFLP data (Fig. 5). Strains were clustered into 18 groups at 70% of similarity, using AFLP data (Fig. 6). Genetic similarities occurred between 0 and 1 for dendrograms derived from the RFLP data and between 0.5 and 1 for the AFLP dendrograms.

Clusters obtained through the RFLP technique using the *pthB* probe grouped strains belonging to the same *pthB* haplotypes, except for the three haplotypes (C35, C31, and C30) detected in field P and found in cluster 11 (Table 1; Fig. 5). This may suggest that the strains collected in field P are highly similar and that some haplotypes probably arose through evolution from other strains present in the same field or that they share a common ancestor. Bootstrap values shown in the RFLP dendrogram (Fig. 5) are very low, particularly for branches formed between similarity levels 0 and 0.5. Bootstrap values are higher for the AFLP dendrogram (Fig. 6), showing that clusters obtained by AFLP are more robust and more reliable for phenetic studies.

Using the AFLP technique, most strains collected from field F clustered with the strains collected from field G (Table 1; Fig. 6). The correlation between the geographical origin of the strains at field level and DNA polymorphism is higher for AFLP than for RFLP data. For example, using RFLP, strains collected from fields C, D, and E located in ECZ 2 were frequently grouped together (clusters 4, 5, and 7). According to AFLP data, these strains were grouped in three distinct clusters (Table 1; Fig. 6).

Combining the REP and ERIC data, strains were grouped in four clusters (data not shown) at 96% similarity. rep-PCR clusters were not correlated to AFLP or RFLP clusters.

Pathogenicity tests. Approximately 92% of *X. axonopodis* pv. *manihotis* strains were very aggressive (disease index >3.5) when evaluated on the susceptible cassava cv. MCOL 1522. No significant differences were observed in aggressiveness among strains collected at different sites (Table 1). All of the strains collected from field D were very aggressive. At site P, 15% of the strains were either weakly aggressive or not aggressive. No correlation was found between the aggressiveness of the strains and AFLP or RFLP haplotypes.

When the strains representing the 10 new RFLP haplotypes were inoculated onto cassava cultivars adapted to the respective strains' ECZ, five pathotypes were characterized, three previously detected (pathotypes 1-2, 2-3, and 5-1) and one new (pathotype 2-9) (Table 3). Another new pathotype (2-5-1) was defined for strains collected at site P (ECZ 2/5) and inoculated on two cultivars adapted to ECZs 2 and 2/5. The cassava differentials used had been selected to characterize strains collected in ECZs 1, 2, and 5; therefore, another set of differentials has to be selected for characterizing ECZ 2/5 strains, because we do not yet know which cultivars present the better adaptation to this ECZ.

DISCUSSION

Our study showed that the Colombian *X. axonopodis* pv. *manihotis* population has a high degree of genetic diversity. The hierarchical analysis of diversity showed genotypic differentiation at all levels, among ECZs, among fields within ECZs, and among strains within fields. The geographical differentiation of the *X. axonopodis* pv. *manihotis* population in ECZs confirmed our previous results, showing that RFLP haplotypes are specific to each ECZ (22). Migration of haplotypes among fields located in the same ECZ is thought to be important in maintaining the high levels of genetic variability within an ECZ (22). Within an ECZ, the same haplotype can be found in different fields. In ECZ 2, haplotype C4 was found at the three sites visited and was always collected from the same cultivar (M CUB 74), indicating that the strain probably migrated through exchanges of contaminated planting materials. The current study also indicated that the *pthB*/RFLP variation was distributed within fields. In experimental fields, the genetic variation in *X. axonopodis* pv. *manihotis* was distributed in a fine-scaled pattern on a microgeographical scale ($H_s = 0.68$ in field E). This microgeographic distribution was previously shown with other pathogens (16,17,30). This high within-field component of diversity was largely due to the large number of genotypes cultivated in fields B, C, D, E, and G.

Host selection is a critical determinant of the ultimate genetic composition of a pathogen population in an agroecosystem (13, 17). To assess the role of host diversity in structuring pathogen population, we related pathogen diversity to the number of improved

TABLE 2. Haplotypic diversity (H_s) of *Xanthomonas axonopodis* pv. *manihotis* population within each field, each edaphoclimatic zone (ECZ), and for the entire Colombian *X. axonopodis* pv. *manihotis* collection (H_T)^a

Field	Site	ECZ ^b	Cassava cultivars		Genetic diversity index ^c		
			Type	Number	H_s (field)	H_s (ECZ)	G_{ST} (ECZ) ^d
A	Cajibío	5	Traditional	2	0	0.53	0.63
B	Mondomo	5	Improved	33	0.66	0.53	0.63
C	Matazul	2	Improved	30	0.62	0.68	0.30
D	Carimagua	2	Improved	30	0.64	0.68	0.30
E	Villavicencio	2	Improved	30	0.68	0.68	0.30
P	Santander de Quilichao	2/5	Improved	16	0.46	0.46	0.48
F	Caracoli	1	Improved	1	0	0.52	0.67
G	Pivijay	1	Improved	30	0.61	0.52	0.67

^a H_T (Colombia) = 0.9, G_{ST} (ECZ) = 0.4, and G_{ST} (field) = 0.5.

^b Zones: 5 = high-altitude tropics, 2 = acid-soil savannas, 2/5 = ECZ with characteristics of ECZs 2 and 5, and 1 = subhumid tropics.

^c Diversity index, based on the restriction fragment length polymorphism (RFLP) haplotypes was calculated as described by Nei and Tajima (18).

^d The coefficient of genetic differentiation (G_{ST}) was calculated for both field and ECZ levels based on the RFLP haplotypes.

cultivars planted. Pathogen populations isolated from fields cultivated with one or two cultivars (whether traditional or improved) were less diverse than were populations derived from fields (e.g., sites E, G, and B) where a large number of improved cultivars were planted. In a previous study, genetic diversity of a large group of cassava cultivars representing the genetic diversity available in cassava germ plasm was assessed using AFLP. A 79% level of polymorphism was found showing the high level of diversity within a collection of various improved cultivars (23). All of the fields planted to many cultivars were representative of the pathogen diversity found in the respective ECZ, suggesting that sampling can be done in one field per ECZ.

We have developed robust and efficient genetic markers, useful for studying *X. axonopodis* pv. *manihotis* populations. Depending on the purposes of the study, either the RFLP or AFLP techniques may be suitable for use. The RFLP/*pthB* technique is useful for estimating genetic diversity, because the distribution of the haplotypes reflects the adaptation of both host and pathogen to the different ecosystems. Diversity at loci involved in host-pathogen interactions seems to be affected by differences in host population structure, and this diversity is detected by RFLP/*pthB*. The DNA sequence of the *pthB* probe is highly homologous to the *Xanthomonas* *avrBs3* gene family (26). Plasmid DNA sequences homologous to aviru-

lence genes used as probes (such as *avrXa10* from *X. oryzae* pv. *oryzae*) are useful in population studies because of their role in plant-pathogen interactions (1,19). They provided a unique opportunity to evaluate the acquisition of virulence in the bacteria populations under the influence of host resistance and to correlate those changes with race specificity and symptom expression (1,11,19). These markers also represent convenient tools for tracking strains in epidemiological studies (6).

However, the use of RFLP/*pthB* data led to an ecological and physiological picture of the *X. axonopodis* pv. *manihotis* population, not a genetic and phenetic classification reflecting the overall similarity of strains. A striking feature of the *pthB* gene is the nearly perfect direct repeats flanking the gene (26). The presence of such repeats suggests that the *pthB* gene may transpose and be moved around by horizontal gene exchange. However, it is difficult to know whether our results on the *pthB* haplotypic structure, like the detection of new haplotypes, reflect the horizontal transfer of plasmid genes among strains of different lineages or convergence in the pattern of arrangement of plasmid DNA (20). Mechanisms for evolution of genes involved in host-pathogen interactions have been studied in a number of systems and could have occurred through recombination between related avirulence genes, insertional inactivation, or mutation of one or a few bases. Selection forces also cause the genes to rearrange in the case of the *avrBs3* gene family (12,31).

The development of a technique for establishing chromosomal genetic relatedness, like AFLP, provides a basis for an evolutionary analysis of the *X. axonopodis* pv. *manihotis* pathotypes. Neutral markers could also be very useful in the design and interpretation of studies of the ecological and physiological characteristics of the bacteria (4). Most strains collected in the same field clustered together when using AFLP technique, showing that field populations of *X. axonopodis* pv. *manihotis* each represent a different phenetic group. However, at field B (ECZ 5), different phenetic groups exist, suggesting that different founder effects are occurring, probably

Fig. 3. Agarose gel showing repetitive sequence-based polymerase chain reaction (rep-PCR) patterns of strains representing each *pthB* haplotype of *Xanthomonas axonopodis* pv. *manihotis*. The patterns were generated by rep-PCR with **A**, ERIC and **B**, REP primers. Lane 1, strain CIO 521 (haplotype C4); lane 2, CIO 716 (C6); lane 3, CIO 726 (C8); lane 4, CIO 728 (C9); lane 5, CIO 657 (C12); lane 6, CIO 520 (C13); lane 7, CIO 713 (C15); lane 8, CIO 522 (C17); lane 9, CIO 691 (C18); lane 10, CIO 656 (C27); lane 11, CIO 645 (C28); lane 12, CIO 643 (C29); lane 13, CIO 553 (C30); lane 14, CIO 541 (C31); lane 15, CIO 704 (C33); lane 16, CIO 503 (C34); lane 17, CIO 536 (C35); lane 18, CIO 736 (C36); and lane 19, CIO 730 (C37). M = 1-kilobase ladder, molecular weight marker. Arrows indicate polymorphic bands and the characteristic pattern obtained with each primer (REP or ERIC) is indicated by an asterisk.

Fig. 4. Banding pattern after amplified fragment length polymorphism analysis of *Xanthomonas axonopodis* pv. *manihotis* strains collected at two sites. The primer combination *EcoRI*+*T/MseI*+*T* was used. **B** = Mondomo and **G** = Pivijay. Lane 1, strain CIO 736; lane 2, CIO 735; lane 3, CIO 734; lane 4, CIO 733; lane 5, CIO 732; lane 6, CIO 731; lane 7, CIO 730; lane 8, CIO 729; lane 9, CIO 728; lane 10, CIO 727; lane 11, CIO 726; lane 12, CIO 724; lane 13, CIO 723; lane 14, CIO 722; lane 15, CIO 721; lane 16, CIO 720; lane 17, CIO 719; lane 18, CIO 718; lane 19, CIO 717; lane 20, CIO 716; lane 21, CIO 715; lane 22, CIO 714; lane 23, CIO 713; and lane 24, CIO 712.

Fig. 5. Similarities among restriction fragment length polymorphism/*pthB* haplotypes of *Xanthomonas axonopodis* pv. *manihotis*. The dendrogram of relationships among haplotypes was derived by cluster analysis of similarities between haplotypes, using the unweighted pair-group method using arithmetic averages. Sites of collection are indicated (A = Cajibío, B = Mondomo, C = Matázul, D = Carimagua, E = Villavicencio, F = Caracolí, G = Pivijay, and P = Santander de Quilichao). The number at the branches show the percentage of times the cluster consisting of the strains (indicated at the right of that branch) occurred.

because new cultivars have been recently introduced. The inconsistencies within some clusters (e.g., strains from fields B and G, ECZs 1 and 5, respectively, had clustered together) may reflect exchanges that have occurred between phenetic groups or also could be explained by the presence of a common ancestor among strains originating from different ECZs.

The utility of DNA-based approaches has been enhanced tremendously by the application of PCR. Using a PCR-based technique, a large number of strains can be analyzed. At the intrapathovar level, rep-PCR is capable of discerning whether pathovars represent a single or several evolutionary lines (14). In the case of *X. axonopodis* pv. *manihotis*, rep-PCR was not useful in discriminating RFLP haplotypes. AFLP and rep-PCR techniques likewise showed greatly different discriminatory power. In the current study, AFLP and rep-PCR results were not correlated. However, more strains need to be analyzed by rep-PCR to determine the existence or lack of correlation between both techniques.

Molecular analysis used in combination with virulence analysis could provide evidence on the genetic relationship of population changes and race changes. Since 1983, the entire cassava breeding

strategy has been based on improving cultivars adapted to specific ECZs (2). In a previous study, the geographical differentiation of pathogenic behavior at the ECZ level was shown (S. Restrepo, M. C. Duque, and V. Verdier, *unpublished data*). Representative strains of the new *pthB* haplotypes were, thus, inoculated onto cassava cultivars adapted to the respective strains' ECZ. A new pathotype was detected among the strains of ECZ 2 representing the new haplotypes. We suggest that the genetic variation detected with RFLP/*pthB* is evolving more rapidly than the virulence phenotypes. However, without analyzing more strains from each *pthB* haplotype, or AFLP cluster on all the cassava differentials, we cannot make any conclusion about the correlation between genomic type and pathotype.

To learn more about population dynamics within *X. axonopodis* pv. *manihotis*, the next step is to study how the genetic structure relates to the prevalence and distribution of virulence characteristics. Additionally, knowledge of the population dynamics in experimental fields over time will enable us to determine whether resistance breakdown is associated with corresponding changes in the genetic structure or virulence phenotypes of the pathogen's population.

TABLE 3. Pathotypes of *Xanthomonas axonopodis* pv. *manihotis* defined after analysis of reactions between strains and cassava cultivars adapted to the same edapho-climatic zone (ECZ), Colombia

ECZ ^a	Patho- type	Strain	Cultivar															
			MBRA 685	M COL 22	M VEN 25	M COL 1505	MBRA 12	M NGA 2	CM 5286-3	CM 2177-2	MBRA 902	CM 6438-14	CM 523-7	CG 402-11	M COL 1522	M COL 2261	CM 2177-2	CM 523-7
1	1-2 ^b	CIO 704	R ^c	S	S	S	S	R										
2	2-3 ^b	CIO 503, 640, 645							S	S	S	S	S					
2	2-9	CIO 527							S	R	R	R	R					
5	5-1 ^b	CIO 730, 755												S	S	S		
2-5	2-5-1	CIO 571, 574, 556															S	S

^a Zones: 1 = subhumid tropics, 2 = acid-soil savannas, 5 = high-altitude tropics, and 2/5 = ECZ with characteristics of ECZs 2 and 5.

^b Pathotypes as previously described (S. Restrepo, M. C. Duque, and V. Verdier, unpublished data).

^c Reactions: R = resistant reaction with a mean disease rating of ≤ 3 , and S = susceptible reaction with a mean disease rating >3 .

Fig. 6. Simplified dendrograms obtained by cluster analysis, using the un-weighted pair-group method using arithmetic averages among amplified fragment length polymorphism haplotypes of *Xanthomonas axonopodis* pv. *manihotis*. The number at the branches show the percentage of times the cluster (indicated at the right of that branch) occurred. Shaded areas represent different sampling sites. Clusters were delineated at an arbitrary level of similarity = 0.70.

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LITERATURE CITED

- Adhikari, T. B., Vera Cruz, C. M., Zhang, Q., Nelson, R. J., Skinner, D. Z., Mew, T. W., and Leach, J. E. 1995. Genetic diversity of *Xanthomonas oryzae* pv. *oryzae* in Asia. Appl. Environ. Microbiol. 61:966-971.
- Anonymous. 1987. Annual Report, CIAT (Centro Internacional de Agricultura Tropical). 1987. CIAT, Cali, Colombia.
- Clerc, A., Manceau, C., and Nesme, X. 1998. Comparison of randomly amplified polymorphic DNA with amplified fragment length polymorphism to assess genetic diversity and genetic relatedness within genospecies III of *Pseudomonas syringae*. Appl. Environ. Microbiol. 64:1180-1187.
- Eardly, B. D., Materon, L. A., Smith, N. H., Johnson, D. A., Rumbaugh, M. D., and Selander, R. K. 1990. Genetic structure of natural populations of the nitrogen-fixing bacterium *Rhizobium meliloti*. Appl. Environ. Microbiol. 56:187-194.
- Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39:366-369.
- Gagnevin, L., Leach, J. E., and Pruvost, O. 1997. Genomic variability of the *Xanthomonas* pathovar *mangiferaeindicae*, agent of mango bacterial black spot. Appl. Environ. Microbiol. 63:246-253.
- Hahn, S. K., Terry, E. R., Leuschner, E. K., Akobundu, I. O., Okali, C., and Lal, R. 1979. Cassava improvement in Africa. Field Crop Res. 2:193-226.
- Janssen, P., Coopman, R., Huys, B., Swings, J., Bleeker, M., Vos, P., Zabeau, M., and Kersters, K. 1996. Evaluation of the DNA fingerprinting method AFLP as a new tool to bacterial taxonomy. Microbiology 142:1881-1893.
- Keim, P., Kalif, A., Schupp, J., Hill, K., Travis, S. E., Richmond, K., Adair, D. M., Hugh-Jones, M., Kuske, C. R., and Jackson, P. 1997. Molecular evolution and diversity in *Bacillus anthracis* as detected by amplified fragment length polymorphisms markers. J. Bacteriol. 179:818-824.
- Kpémoua, K., Boher, B., Nicole, M., Calatayud, P., and Geiger, J. P. 1996. Cytochemistry of defense responses in cassava infected by *Xanthomonas campestris* pv. *manihotis*. Can. J. Microbiol. 42:1131-1143.
- Leach, J. E., Rhoads, M. L., Vera Cruz, C. M., White, F. F., Mew, T. W., and Leung, H. 1992. Assessment of genetic diversity and population structure of *Xanthomonas oryzae* pv. *oryzae* with a repetitive DNA element. Appl. Environ. Microbiol. 58:2188-2195.
- Leach, J. E., and White, F. F. 1996. Bacterial avirulence genes. Annu. Rev. Phytopathol. 34:153-179.
- Leung, H., Nelson, R. J., and Leach, J. E. 1993. Population structure of plant pathogenic fungi and bacteria. Adv. Plant Pathol. 10:157-205.
- Louws, F. J., Fulbright, D. W., Stephens, C. T., and de Bruijn, F. J. 1995. Differentiation of genomic structure by rep-PCR fingerprinting to rapidly classify *Xanthomonas campestris* pv. *vesicatoria*. Phytopathology 85:528-536.
- Lozano, J. C. 1986. Cassava bacterial blight: A manageable disease. Plant Dis. 70:1089-1093.
- McDonald, B. A., and Martinez, J. P. 1990. DNA restriction fragment length polymorphisms among *Mycosphaerella graminicola* (anamorph *Septoria tritici*) isolated from a single wheat field. Phytopathology 80:1368-1373.
- McDonald, B. A., McDermott, J. M., Allard, R. W., and Webster, R. K. 1989. Coevolution of host and pathogen populations in the *Hordeum vulgare*-*Rhynchosporium secalis* pathosystem. Proc. Natl. Acad. Sci. U.S.A. 86:3924-3927.
- Nei, M., and Tajima, F. 1981. DNA polymorphism detectable by restriction endonucleases. Genetics 97:145-163.

19. Nelson, R. J., Baraoidan, M. R., Vera Cruz, C. M., Yap, I. V., Leach, J. E., Mew, T. W., and Leung, H. 1994. Relationship between phylogeny and pathotype for the bacterial blight pathogen of rice. *Appl. Environ. Microbiol.* 60:3275-3283.
20. Pinero, D., Martinez, E., and Selander, R. K. 1988. Genetic diversity and relationships among isolates of *Rhizobium leguminosarum* biovar *phaseoli*. *Appl. Environ. Microbiol.* 54:2825-2832.
21. Restrepo, S., Duque, M. C., Tohme, J., and Verdier, V. 1999. AFLP fingerprinting: An efficient technique for detecting genetic variation of *Xanthomonas axonopodis* pv. *manihotis*. *Microbiology* 145:107-114.
22. Restrepo, S., and Verdier, V. 1997. Geographical differentiation of the population of *Xanthomonas axonopodis* pv. *manihotis* in Colombia. *Appl. Environ. Microbiol.* 63:4427-4434.
23. Sánchez, G., Restrepo, S., Duque, M. C., Fregene, M., Bonierbale, M., and Verdier, V. 1999. AFLP assessment of genetic variability in cassava accessions (*Manihot esculenta*) resistant and susceptible to the cassava bacterial blight (CBB). *Genome* 42:163-172.
24. Vera Cruz, C. M., Ardales, E. Y., Skinner, D. Z., Talag, J., Nelson, R. J., Louws, F. J., Leung, H., Mew, T. W., and Leach, J. E. 1996. Measurement of haplotypic variation in *Xanthomonas oryzae* pv. *oryzae* within a single field by rep-PCR and RFLP analyses. *Phytopathology* 86:1352-1359.
25. Verdier, V., Boher, B., Maraite, H., and Geiger, J. P. 1994. Pathological and molecular characterization of *Xanthomonas campestris* strains causing diseases of cassava (*Manihot esculenta*). *Appl. Environ. Microbiol.* 60:4478-4486.
26. Verdier, V., Cuny, G., Assigbetsé, K., Geiger, J. P., and Boucher, C. 1996. Characterization of pathogenicity gene *pthB* in *Xanthomonas campestris* pv. *manihotis*. Abstr. G-71 in: Abstr. Book 8th Int. Congr. Plant Microbe Interact. G. Stacey, B. Mullin, and P. Gresshoff, eds. University of Tennessee, Knoxville.
27. Verdier, V., Dongo, P., and Boher, B. 1993. Assessment of genetic diversity among strains of *Xanthomonas campestris* pv. *manihotis*. *J. Gen. Microbiol.* 139:2591-2601.
28. Verdier, V., Restrepo, S., Mosquera, G., Duque, M. C., Gerstl, A., and Laberry, R. 1998. Genetic and pathogenic variation of *Xanthomonas axonopodis* pv. *manihotis* in Venezuela. *Plant Pathol.* 47:601-608.
29. Vos, P., Hogers, R., Bleeker, M., Reijmans, M., Van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kluper, M., and Zabeau, M. 1995. AFLP: A new technique for DNA fingerprinting. *Nucleic Acids Res.* 23:4407-4414.
30. Xia, J. Q., Correll, J. C., Lee, F. N., Marchetti, M. A., and Rhoads, D. D. 1993. DNA fingerprinting to examine microgeographic variation in the *Magnaporthe grisea* (*Pyricularia grisea*) population in two rice fields in Arkansas. *Phytopathology* 83:1029-1035.
31. Yang, Y., and Gabriel, D. W. 1995. Intragenic recombination of a single plant pathogen gene provides mechanism for the evolution of new host specificities. *J. Bacteriol.* 177:4963-4968.
32. Yap, I., and Nelson, R. J. 1996. Winboot: A program for performing bootstrap analysis of binary data to determine the confidence limits of UPGMA-based dendrograms. IRRI Discussion Paper Series No. 14. International Rice Research Institute, Manila, Philippines.