Assessment of genetic diversity among strains of *Xanthomonas campestris* pv. *manihotis*

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Three-hundred and twenty-six strains of *Xanthomonas campestris* pv. *manihotis* from 22 countries were studied to detect and assess genetic and evolutionary relationships within the pathovar. A range of techniques was used for this study including restriction fragment length polymorphism (RFLP) analysis. The probes used for the RFLP analysis were 16+23S rRNA genes from *Escherichia coli* and three restriction fragments from the chromosomal and plasmid DNA of *X. campestris* pv. *manihotis*. Analysis of the rRNA probe data showed five RFLP groups whilst the other three probes were used to further sub-divide these groupings. Genetic variability of *X. campestris* pv. *manihotis* was pronounced in strains from South America where the host plant originated but was limited in strains from other regions. The results obtained confirm the hypothesis that the pathogen has been introduced only recently to Africa and suggests that African strains have not as yet diversified significantly at the chromosomal level. Our results indicate that rRNA and DNA probes are useful tools for epidemiological studies and in following the genetic evolution of strains.

Introduction

Xanthomonas campestris pv. *manihotis* is the causal agent of cassava bacterial blight. Symptoms are characterized by an angular leaf spot and a systemic infection of the stem which leads to wilting and die-back. This disease causes severe yield losses in cassava growing areas (Lozano & Sequeira, 1974a). Cassava bacterial blight was first reported in Brazil in 1912 (Bondar, 1912). It was observed in Colombia and Venezuela in 1971 (Lozano & Sequeira, 1974b) and has occurred in most African and Asian countries since 1972 (Williams *et al.*, 1973; Maraite & Meyer, 1975; Booth & Lozano, 1978).

Several reports (Lozano & Sequeira, 1974a; Maraite *et al.*, 1981; Grousson *et al.*, 1990) have demonstrated differences among strains of *X. campestris* pv. *manihotis* based on pathogenicity, physiological and biochemical characteristics. Plasmid profiles have also been reported

to be variable among strains of *X. campestris* pv. *manihotis* (Verdier, 1988). In contrast, studies based on protein electrophoretic analysis (Vauterin *et al.*, 1991) and DNA homology, (Van den Mooter *et al.*, 1987) have indicated that *X. campestris* pv. *manihotis* constitutes a genetically and phenotypically homogeneous group of bacteria.

RFLP analysis has been used to differentiate plant-pathogenic bacteria at the pathovar level (Lazo & Gabriel, 1987; Denny *et al.*, 1988; Leach *et al.*, 1990; Qhobela *et al.*, 1990) as well as to assess relatedness among bacterial strains (Gabriel *et al.*, 1988; Cook *et al.*, 1989, 1991; Hartung & Civerolo, 1989; Leach *et al.*, 1992; Pruvost *et al.*, 1992; Qhobela *et al.*, 1991; Qhobela & Clafin, 1992; Xu & Gonzales, 1991). The conserved nature of rRNA genes allowed the use of a 16+23S rRNA probe from *Escherichia coli* for the identification of bacteria and for taxonomic studies (Grimont *et al.*, 1989). This probe was used for phylogenetic studies of human pathogenic bacteria (Scieux *et al.*, 1992) and recently of phytopathogenic bacteria (Berthier *et al.*, 1993). It has been established previously on a small number of strains, that the *Escherichia coli* 16+23S rRNA probe was a useful probe to detect restriction polymorphisms in the *X. campestris* pv. *manihotis* genome (Berthier *et al.*, 1993). This study has been

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Abbreviation: RFLP, restriction fragment length polymorphism.

Table 1. *Strains of X. campestris pv. manihotis*

Strain no. and reference in other collections*	Origin	Year of isolation	Isolated by
LMG 776 (NCPBB 2443, HMB 72, CFBP 2603)	Colombia	1970	J. Lozano (CIAT)
ORST 1 (CIAT 1060, CFBP 1849)	Colombia	1970	
CIAT 1058	Colombia	1970	
NCPBB 2444	Colombia	1970	
CFBP 1851 (CIAT 1111)	Colombia	1974	
CIAT 1199	Colombia	1981	
CIAT 1222	Colombia	1984	
ORST 211	Colombia	1992	V. Verdier (This laboratory)
ORST 2 (CIAT 1061, CFBP 1850)	Venezuela	1971	J. Lozano (CIAT)
CIAT 1129	Venezuela	1974	
ATCC 23380 (HMB 68, NCPBB 1159)	Brazil	1941	W. H. Burkholder (New York, USA)
HMB 70 (NCPBB 1160, LMG 5273)	Brazil	1941	O. A. Drummond, O. Hipolito (LMG)
NCPBB 348	Brazil	1954	M. P. Starr (Davis, USA)
HMB 55a (NCPBB 1834†, LMG 784)	Brazil	1965	C. Robbs (Rio de Janeiro, Brazil)
CIAT 1065	Brazil	1971	J. Lozano (CIAT)
NCPBB 2550	Brazil	1973	A. L. G. Pereira (NCPBB)
ORST 7 (CFBP 1854)	Brazil	1973	J. R. Neto (CFBP)
HMB 23 (LMG 770)	Brazil	1973	A. L. G. Pereira (NCPBB)
CIAT 1117	Brazil	1974	J. Lozano (CIAT)
ORST 3 (CIAT 1120, CFBP 1852)	Brazil	1974	
ORST 5 (CFBP 1855)	Brazil	1974	J. R. Neto (CFBP)
ORST 6 (CFBP 1856)	Brazil	1976	
HMB 79 (LMG 778)	Brazil	1978	A. Takatsu (Brasilia, Brazil)
LMG 777 (HMB 78)	Brazil	1978	
LMG 779 (HMB 80)	Brazil	1978	
UnB 64	Brazil	1978	
UnB 9	Brazil	1980	
UnB 49	Brazil	1980	
UnB 129	Brazil	1981	
UnB 184	Brazil	1981	
UnB 185	Brazil	1982	
UnB 215	Brazil	1982	
UnB 216	Brazil	1982	
UnB 289	Brazil	1982	
UnB 291	Brazil	1983	
UnB 351	Brazil	1983	
UnB 589	Brazil	1985	
UnB 638	Brazil	1986	
UnB 724	Brazil	1988	
UnB 833	Brazil	1991	
CIAT 1162	Mexico	1976	J. Lozano (CIAT)
HMB 25 (NCPBB 3060, LMG 771)	Nigeria	1976	H. Maraite (Louvain, Belgium)
ORST 42	Nigeria	1978	J. F. Daniel (This laboratory)
ORST 43	Nigeria	1978	
CFBP 1857 (ORST A202.1)	Nigeria	1978	
CFBP 1858 (ORST A203.1)	Nigeria	1978	
CFBP 1859 (ORST A205.1)	Nigeria	1978	
CFBP 1860 (ORST A207)	Nigeria	1978	
ORST 34	Benin	1982	
ORST 35	Benin	1982	M. Ride (Angers, France)
ORST 36	Benin	1982	
ORST 37	Benin	1982	
ORST 38	Benin	1982	
CFBP 1944	Ivory Coast	1979	
LMG 5249 (HMB 203)	Ivory Coast	1981	H. Maraite (Louvain, Belgium)
ORST 55	Ivory Coast	1984	J. F. Daniel (This laboratory)
ORST 56	Ivory Coast	1984	
198 strains from ORST	Congo	1977-1992	B. Boher, J. F. Daniel, V. Verdier (This laboratory)

Table 1 (cont.)

Strain no. and reference in other collections*	Origin	Year of isolation	Isolated by
29 strains from ORST	Togo	1987-1991	B. Boher, V. Verdier (This laboratory)
HMB 6 (LMG 767)	Zaire	1973	H. Maraite (Louvain, Belgium)
HMB 9 (LMG 768, NCPBB 3058)	Zaire	1973	
LMG 769 (NCPBB 3059, HMB 10)	Zaire	1973	
LMG 766 (HMB 3)	Zaire	1973	
ORST 44	Zaire	1979	J. F. Daniel (This laboratory)
ORST 45	Zaire	1979	
ORST 46	Zaire	1979	
ORST 47	Zaire	1979	
ORST 48	Zaire	1979	
ORST 49	Zaire	1979	
ORST 50	Zaire	1979	
ORST 51	Zaire	1979	
ORST 52	Zaire	1979	
ORST 53	Zaire	1979	
ORST 54	Zaire	1979	B. Boher (This laboratory)
ORST 186	Zaire	1987	
ORST 187	Zaire	1987	J. F. Daniel (This laboratory)
ORST 39	Central Africa	1977	
ORST 40	Central Africa	1977	
ORST 41	Central Africa	1977	G. Persley (LMG)
LMG 5287 (NCPBB 3161)	Cameroon	1976	
HMB 27 (LMG 629)	Cameroon	1977	H. Maraite (Louvain, Belgium)
ORST 212	Cameroon	1992	V. Verdier (This laboratory)
ORST 213	Cameroon	1992	
ORST 214	Cameroon	1992	
ORST 215	Cameroon	1992	
ORST 216	Cameroon	1992	
ORST 217	Cameroon	1992	
LMG 780 (HMB 81)	Uganda	1979	H. Maraite (Louvain, Belgium)
LMG 782 (HMB 93)	Uganda	1979	
LMG 783 (HMB 148)	Kenya	1979	
LMG 5288 (NCPBB 3194)	Niger	1978	J. Bradbury (Kew, UK)
CIAT 1160	Malaysia	1976	J. Lozano (CIAT)
LMG 765	Malaysia	1980	G. Lan (LMG)
CIAT 1135	Taiwan	1975	J. Lozano (CIAT)
LMG 774 (HMB 60)	Taiwan	1978	H. Maraite (Louvain, Belgium)
CIAT 1163	Sumatra	1976	J. Lozano (CIAT)
CIAT 1181	Philippines	1977	
CIAT 1203	Dominican Rep.	1981	
HMB 71 (NCPBB 1161, LMG 775)	Mauritius	1946	G. Orian (LMG)
CFBP 2624	Reunion Island	1986	O. Pruvost (CIRAD, La Réunion)
CFBP 2635	Reunion Island	1987	J. C. Girard (CIRAD, La Réunion)

* Abbreviations: ATCC, American Type Culture Collection, Rockville, MD, USA; CFBP, Collection Française de Bactéries, Phytopathogènes, Angers, France; CIAT, *Xanthomonas* collection, Centro Internacional de Agricultura Tropical, Cali, Colombia; HMB, Collection of H. Maraite, Louvain La Neuve, Belgium; LMG, Laboratorium voor Microbiologie Gent Culture Collection, Universiteit Gent, Belgium; NCPBB, National Collection of Plant Pathogenic Bacteria, Harpenden, UK; ORST, Collection du Laboratoire de Phytopathologie, ORSTOM, Brazzaville, Congo; UnB, Collection of A. Takatsu, Universidade Nacional de Brasília, Brazil.

† A pathovar reference strain.

extended here to a total of 326 strains of *X. campestris* pv. *manihotis* from worldwide geographical locations, to assess genetic diversity and evolutionary relationships with special emphasis on the African population of the

pathogen. Different techniques were used to distinguish strains of *X. campestris* pv. *manihotis* including biochemical characteristics, pathogenicity tests on host plants and restriction fragment length polymorphism

(RFLP) analyses of genomic DNA. Probes used for RFLP studies reported here were evaluated for their utility for genetic characterization and in epidemiological studies of *X. campestris* pv. *manihotis*.

Methods

Bacterial strains. Three-hundred and twenty-six strains of *X. campestris* pv. *manihotis* isolated from *Manihot esculenta* in 22 countries were used in this study. The sources and geographical origin of the bacterial strains are listed in Table 1. The 29 strains from Togo and 198 strains from the Congo were isolated from samples collected in different ecological zones and at different times. Control strains of *X. campestris* pv. *cassavae* (HMB 29), *X. campestris* pv. *phaseoli* (CIAT 214), *X. campestris* pv. *malvacearum* (NCPBP 633) and *X. campestris* pv. *undulosa* (UPB 670) were included in the RFLP study. All strains were stored as frozen glycerol stocks and were maintained on YPGA medium (l⁻¹: 5 g yeast extract, 5 g Bactopeptone, 5 g glucose, 15 g agar, pH adjusted to 7.2).

Phenotypic studies. Starch hydrolysis was carried out using a medium containing 0.5% (w/v) yeast extract, 0.5% (w/v) Bactopeptone, 0.2% (w/v) soluble starch and 1.5% (w/v) agar in distilled water. Tests were inoculated with a drop of a suspension of approximately 10⁹ cells (ml distilled water)⁻¹. After 5 d incubation at 30 °C, Lugol iodine solution was poured on the plates. Starch hydrolysis was indicated by the presence of clear zones in the black-stained medium around the colonies. The *in vitro* susceptibility towards 20 antibiotics was determined by the technique described by Grousson *et al.* (1990), using sensitivity bio-discs (bioMerieux). The following antibiotics were tested (per disk): 10 µg ampicillin, 30 µg cefuroxime, 25 µg amoxicillin, 30 µg cefotaxime, 30 µg kanamycin, 10 U penicillin G, 10 µg streptomycin, 300 U polymyxin B, 30 µg chloramphenicol, 100 µg carbenicillin, 30 µg cefalotin, 30 µg cefoxitin, 15 µg erythromycin, 30 µg neomycin, 30 µg nalidixic acid, 30 µg rifampicin, 250 µg sulfamide, 30 µg tetracycline, 75 µg ticarcillin and 15 µg virginiamycin. Inhibition zones were recorded after 48 h according to the manufacturer's recommendations (bioMerieux). Utilization of carbon sources was tested using the basal medium of Hayward (1964). The following carbon sources were sterilized separately by filtration and were tested at the recommended concentration of 0.2% (w/v) (Grousson *et al.*, 1990): amidon, D-galactose, ribose, glycerol, xylose, cellobiose, L-rhamnose, arabinose, maltose, lactose, trehalose, mannose, raffinose, esculin, D-fructose, saccharose, D-glucose, salicin and erythritol.

Phytopathogenicity tests. Pathogenicity of all strains of *X. campestris* pv. *manihotis* was tested on the cassava cultivar Mpembé from the Congo. This was propagated from cuttings. Inoculations were done according to methods described by Maraite *et al.* (1981). The development of symptoms was monitored over 1 month, and the observed reactions rated according to a scale described by Grousson *et al.* (1990). Three plants were inoculated with a given strain.

RFLP analysis, restriction digests, electrophoresis and blotting. Bacteria were cultured in a liquid medium containing peptone (10 g l⁻¹), casamino acids (1 g l⁻¹) and yeast extract (1 g l⁻¹) (pH adjusted to 7.2). Total genomic DNA was extracted from overnight cultures by the method of Boucher *et al.* (1985). DNA (5 µg) was digested with the restriction endonuclease *EcoRI* according to the manufacturer's specifications (Eurogentec). Electrophoresis of DNA was carried out in 0.7% agarose gels in TBE buffer at 3 V cm⁻¹ for 12 h. Capillary transfers were performed either to Hybond N⁺ following manu-

facturer's specifications (Amersham) or to nitrocellulose membrane as described by Southern (1975).

Screening for probes. A genetic library was constructed by ligation of *EcoRI* fragments of strain *X. campestris* pv. *manihotis* CFBP 1851 into the vector Bluescript M13 (Maniatis *et al.*, 1982). Two DNA clones, pBS6 and pBS8, which have a 6 and 8 kb *EcoRI* insert, respectively, were selected from the genomic library of strain *X. campestris* pv. *manihotis* CFBP 1851. These clones allowed the detection of polymorphism in the genome of strains of *X. campestris* pv. *manihotis*.

A CsCl-purified plasmid DNA (44 kb) from the same strain was also digested with *HindIII* and fragments were separated using agarose gel electrophoresis. They were visualized with ultraviolet light (320 nm) after staining gels for 15 min in 1 mg ethidium bromide l⁻¹. A 13 kb *HindIII* fragment was excised from the agarose gel and eluted with glassmilk (Bio 101) before ligation into vector Bluescript M13. It has been shown that this DNA fragment (pBSF2) contains pathogenicity genes of *X. campestris* pv. *manihotis* (V. Verdier, unpublished data).

Competent *E. coli* cells (strain DH5α) were transformed with the different ligation mixtures and plated on Luria broth medium supplemented with ampicillin at 100 µg ml⁻¹ and with Xgal at 30 µg ml⁻¹. White colonies were screened for inserts using the boiling mini-prep method (Maniatis *et al.*, 1982). Large-scale preparations of recombinant DNA (insert with vector) were made by the alkaline lysis method and purified by precipitation with polyethylene glycol (Maniatis *et al.*, 1982). Concentrations of recombinant DNA were calculated by measurement of A₂₆₀.

Hybridization procedure. The recombinant Bluescript plasmids were labelled with [³²P]deoxycytidine triphosphate by random priming (Multiprime, Amersham). Prehybridization and hybridization (2 h) of Southern blots were performed at 65 °C in conditions described by the manufacturer (Amersham). Two post-hybridization stringency washes were done with 0.1% SSC (1 × SSC = 0.15 M-sodium chloride and 0.015 M-sodium citrate, pH 7.0), 0.1% SDS at 65 °C for 20 min each. Autoradiography was done at -80 °C with X-Ray film (Hyperfilm-MP, Amersham) with intensifying screens (Amersham). All the experiments were done at least twice for each strain.

Hybridization with acetyl amino fluorene (AAF)-labelled ribosomal 16+23S rRNA genes from *E. coli* and AAF-labelled pBR322 DNA, which hybridizes to the molecular mass marker RaouII, was performed according to the manufacturer's recommendations (Eurogentec). The presence or absence of specific restriction fragments was observed on autoradiograms and on the nitrocellulose membrane, and the size was estimated from the reference marker (RaouII).

Hierarchical cluster analysis. A computer program (Miller, 1992) was used for calculating genetic distances between the strains based on fragment data. Fragment data were entered as fragment sizes in kb. The Nei coefficient (Nei, 1987) was calculated for each strain and cluster analysis was done by the unweighted pair group method (UPGMA) using the Restsite program (Miller, 1992).

Results

RFLP patterns

Using the 16+23S rRNA probe, five different patterns were obtained from the 326 strains tested. Two of the hybridizing fragments (9 and 1.5 kb) were common to all the 326 strains. The other seven fragments (10, 7.4, 7, 3.6, 2.3, 2, 1.9 kb) allowed the distinction of the five patterns (RNA groups 1, 2, 3, 4, 5) (Fig. 1).

Fig. 1. Southern hybridization of *Eco*RI-digested genomic DNA of *X. campestris* pv. *manihotis* probed with AAF-labelled 16+23S rRNA genes from *E. coli* and AAF-labelled pBr322 DNA. All 326 strains used exhibited one of these five representative patterns each corresponding to an RNA group. Lanes: A, strain CFBP 1851 (Colombia), RNA group 3; B, strain ATCC 23380 (Brazil), RNA group 4; C, strain LMG 777 (Brazil), RNA group 2; D, strain NCPPB 2444 (Colombia), RNA group 5; E, strain CIAT 1163 (Sumatra), RNA group 1; R, molecular mass standard Raoult.

The cluster analysis resulted in five groups, each group represented strains with identical RFLP patterns (Fig. 2). The rRNA probe did not show any polymorphism among the 271 African strains (Table 2). A representative strain from each African country was subsequently used to construct the dendrogram (Fig. 2). Strains belonging to RNA groups 2, 3 and 4 were located on the same branch of the phylogenetic tree and may be closely related. Strains in RNA groups 1 and 5 were more distantly related. In contrast to the homogeneous African strains (RNA1), those from South America were heterogeneous and gave the five different patterns (RNA groups 1, 2, 3, 4, 5). *X. campestris* pv. *phaseoli*, pv. *malvacearum*, pv. *cassavae* and pv. *undulosa* groups were clearly distinct from the *X. campestris* pv. *manihotis* groups.

The three DNA probes, pBS8, pBS6 and pBSF2 allowed the distinction of different subgroups within the five RNA groups defined above (Table 2).

Hybridization profiles with the pBS8 probe revealed different patterns. Two or three distinct bands were observed for strains representing the BS8 groups 1, 2 and 3, whereas multiple bands were observed for the strains

belonging to the BS8 groups 4 and 5 (Fig. 3), suggesting the presence of a repetitive sequence in different regions of the genome. The cluster analysis resulted in two main groups (data not shown). The first group (which included strains belonging to RNA groups 1 and 5) is divided in three subgroups (BS8 1, 2 and 3). Within the second group (which included RNA groups 2, 3 and 4) six subgroups occurred (BS8 groups 4, 5, 6, 7, 8, 9). Considerable polymorphism was observed among South American strains which were found in all nine subgroups, whilst all the African strains were clustered in the same subgroup (Table 2). Similar results were obtained with the pBS6 probe. Cluster analysis (data not shown) of data obtained with this probe, allowed the distinction of the strains into 17 RFLP groups (Table 2). All strains contained the repetitive cloned sequence in pBS6 in high copy number (Fig. 4).

The plasmid probe pBSF2 was used to detect polymorphism among strains of *X. campestris* pv. *manihotis* (Fig. 5) and produced 26 different hybridization patterns. A high level of polymorphism was observed among RNA group 2 which included 13 Brazilian strains, one Venezuelan strain and one Mauritian strain (Table 2). Using the pBSF2 and pBS8 probes, no hybridization was observed with *X. campestris* pv. *undulosa* (strain UPB 670) and *X. campestris* pv. *cassavae* (strain HMB 29) genomic DNA. Hybridization with *X. campestris* pv. *malvacearum* (strain NCPPB 633) and *X. campestris* pv. *phaseoli* (strain CIAT 214) genomic DNA gave different patterns with pBSF2 and pBS8, respectively (data not shown), clearly distinct from those of *X. campestris* pv. *manihotis* defined above. pBS6 was not tested on these four strains. pBluescript vector DNA did not hybridize with *X. campestris* pv. *manihotis* genomic DNA.

Pathogenic characteristics

All the *X. campestris* pv. *manihotis* strains, except four strains (one Brazilian, one Ivory Coast and two Congolese strains), caused characteristic pathogenic symptoms on cassava plants. Variation in virulence was observed but was not related to the geographical origin of strains or to the RFLP groups defined above.

Phenotypic features

Starch hydrolysis was demonstrated for all the strains of *X. campestris* pv. *manihotis* tested. All African strains showed a limited ability to hydrolyse starch when compared to strains from South America. Strains originating from South America and Asia showed a strong amylase activity (Table 3).

Table 2. Placement of strains of *X. campestris* pv. *manihotis* in RFLP groups based on four different DNA probes

Origin	Strain no.	No. of RFLP groups with probe:*			
		rRNA	pBS8	pBS6	pBSF2
Colombia	LMG 776, NCPPB 2444	5	2	3	2
	ORST 1	1	3	6	11
	CIAT 1058, CIAT 1199	5	1	1	1
	CIAT 1222	5	2	2	3
	CFBP 1851	3	8	14	24
	ORST 211	1	2	4	6
Venezuela	ORST 2	2	4	10	19
	CIAT 1129	1	2	4	4
Brazil	HMB 055, UnB 64, UnB 216, UnB 289, UnB 291, UnB 589, ORST 3, HMB 79, UnB 215	1	2	4	5,7,8,9,10
	CIAT 1065, UnB 724	1	2	5	6
	ORST 6	1	3	6	12
	NCPPB 2550, UnB 49, UnB 129, UnB 9, UnB 184	2	4	9	15,16,17
	UnB 638	2	4	13	17
	HMB 023, LMG 779	2	4	10	20
	ORST 5	2	4	10	19
	UnB 833	2	4	7	23
	LMG 777	2	5	12	21
	ORST 7	2	6	11	22
	UnB 351	2	7	9	18
	HMB 70	3	8	14	24
	ATCC 23380, NCPPB 348	4	9	15	24
	UnB 185	4	9	16	25
	CIAT 1117	5	1	1	1
	CIAT 1162	5	2	2	2
	CIAT 1203	5	2	2	2
	CIAT 1160	5	1	1	1
	LMG 765	1	2	5	5
	CIAT 1135, LMG 774	4	9	17	26
Taiwan	CIAT 1163	1	2	5	5
Philippines	CIAT 1181	1	2	5	6
Mauritius	HMB 71	2	4	10	20
Reunion Island	CFBP 2624, CFBP 2635	1	3	6	14
Nigeria†	HMB 25, ORST 42, ORST 43, CFBP 1858, CFBP 1859, CFBP 1860	1	3	6	11
Benin†	ORST 34, ORST 35, ORST 36, ORST 37, ORST 38	1	3	6	11
Ivory Coast†	CFBP 1944, LMG 5249, ORST 55, ORST 56	1	3	6	11
Central Africa†	ORST 39, ORST 40, ORST 41	1	3	6	11
Cameroun†	LMG 5287, HMB 27, ORST 212, ORST 213, ORST 214, ORST 215, ORST 216, ORST 217	1	3	6	11
Uganda†	LMG 780, LMG 782	1	3	6	13
Kenya†	LMG 783	1	3	6	11
Niger†	LMG 5288	1	3	6	11
Zaire†	HMB 6, HMB9, LMG 769, LMG 766, ORST 44, ORST 45, ORST 46, ORST 47, ORST 48, ORST 49, ORST 50, ORST 51, ORST 52, ORST 53, ORST 54, ORST 186, ORST 187	1	3	6	11
Congo†	198 strains ORST	1	3	6	11,12
Togo†	29 strains ORST	1	3	6	11,12

* Each number represents one RFLP group (strains with identical RFLP patterns form a group). Numbers are not comparable across rows.

† African countries.

Fig. 2. Dendrogram of genetic distance using the computer program Restsite (Miller, 1992), showing the relationships among strains of *X. campestris* pv. *manihotis* based on RFLP analysis using AAF-labelled rRNA 16+23S as a probe. Sixty-two representative strains of *X. campestris* pv. *manihotis* (out of the 326 used) were used in the construction of the dendrogram. The number of strains studied is indicated within parentheses. Four strains from others pathovars were also included. *, Unclustered strains.

Fig. 3. Southern hybridization of *Eco*RI-digested genomic DNA of six strains of *X. campestris* pv. *manihotis* probed with 32 P-labelled clone pBS8. Lanes: A, strain LMG 777 (Brazil), BS8 group 5; B, strain UnB 833 (Brazil), BS8 group 4; C, strain LMG 5287 (Cameroon), BS8 group 3; D, strain CIAT 1065 (Brazil), BS8 group 2; E, strain CIAT 1117 (Brazil), BS8 group 1; F, strain NCPPB 2444 (Colombia), BS8 group 2; R, molecular mass standard Raoult.

Only minor differences for susceptibility towards antibiotics *in vitro* were noted. All the strains were resistant to three antibiotics (per disk: 300 U polymyxin B, 250 μ g sulfamide, 10 μ g ampicillin), and sensitive to (per disk) 10 μ g streptomycin, 30 μ g kanamycin, 15 μ g erythromycin, 30 μ g chloramphenicol, 30 μ g rifampicin and 30 μ g tetracycline. A variable response was observed with the other antibiotics, which was not related to the geographical origin of strains.

Growth on different carbon sources was similar for all strains tested: growth was observed on D-glucose, D-mannose, trehalose, cellobiose, fructose, saccharose, xylose and galactose. Poor or no growth was observed in basal medium supplemented with rhamnose, amidon, arabinose, erythritol, esculin, glycerol, lactose, maltose, raffinose, ribose and salicin.

Discussion

Berthier *et al.* (1993) reported that RFLP analysis with the rRNA probe could distinguish the pathovar *manihotis* from other pathovars, as well as from other xanthomonads. In this initial study involving 42 strains of *X. campestris* pv. *manihotis* it was established that there were three distinct RFLP patterns with the rRNA probe. All the strains from Africa gave the same pattern in contrast with the six strains from South America which gave three patterns (Berthier *et al.*, 1993). This study has been extended here to a total of 326 strains of

Fig. 4. Southern hybridization of *Eco*RI-digested genomic DNA of 11 strains of *X. campestris* pv. *manihotis* probed with 32 P-labelled clone pBS6. Lanes: A, strain CIAT 1135 (Taiwan), BS6 group 17; B, strain UnB 185 (Brazil), BS6 group 16; C, strain CFBP 1851 (Colombia), BS6 group 14; D, strain LMG 777 (Brazil), BS6 group 12; E, strain LMG 779 (Brazil), BS6 group 10; F, strain NCPPB 2550 (Brazil), BS6 group 9; G, strain UnB 638 (Brazil), BS6 group 13; H, strain UnB 833 (Brazil), BS6 group 7; I, strain CFBP 2624 (Reunion Island), BS6 group 6; J, strain CIAT 1117 (Brazil), BS6 group 1; K, strain NCPPB 2444 (Colombia), BS6 group 3; R, molecular mass standard Raoult.

Fig. 5. Southern hybridization of *Eco*RI-digested genomic DNA of 14 strains of *X. campestris* pv. *manihotis* probed with ³²P-labelled clone pBSF2. Lanes: A, strain LMG 777 (Brazil), BSF2 group 21; B, strain ORST 7 (Brazil), BSF2 group 22; C, strain HMB 023 (Brazil), BSF2 group 20; D, strain LMG 779 (Brazil), BSF2 group 20; E, strain HMB 71 (Brazil), BSF2 group 20; F, strain ORST 5 (Brazil), BSF2 group 19; G, strain ORST 2 (Venezuela), BSF2 group 19; H, strain LMG 5288 (Niger), BSF2 group 11; I, strain LMG 783 (Kenya), BSF2 group 11; J, strain LMG 780 (Uganda), BSF2 group 13; K, strain ORST 203 (Congo), BSF2 group 12; L, strain ORST 64 (Congo), BSF2 group 11; M, strain ORST 133F (Congo), BSF2 group 11; N, strain CFBP 2624 (Reunion Island), BSF2 group 14; R, molecular mass standard Raoult.

Table 3. Distribution of amylase activity among strains of *X. campestris* pv. *manihotis*

Amylase activity was assessed by clarity of zones surrounding the colony (0–1.5 cm, low amylase activity; 1.5–3 cm, strong amylase activity).

Origin	RFLP groups with rRNA probe	Number of strains:		
		Tested	With strong amylase activity	With low amylase activity
Africa				
Nigeria	1	7	0	7
Benin	1	5	0	5
Ivory Coast	1	4	0	4
Congo	1	198	0	198
Togo	1	29	0	29
Zaire	1	17	0	17
Central Africa	1	3	0	3
Cameroon	1	8	0	8
Uganda	1	2	0	2
Kenya	1	1	0	1
Niger	1	1	0	1
Mauritius	1	1	0	1
Reunion Island	1	2	0	2
Asia				
Taiwan	4	1	1	0
Malaysia	1,5	1	0	1
South America				
Columbia	1,3,5	3	2	1
Venezuela	1,2	1	1	0
Brazil	1,2,3,4,5	10	7	3

X. campestris pv. *manihotis* from various geographical areas including 284 additional strains of African, Asian and Latin American origin. Using RFLP analysis, our results demonstrate that *X. campestris* pv. *manihotis* is more genetically diverse in South America than in Africa. A high level of polymorphism could be detected among South American strains with all four probes tested.

On the basis of the present data we have shown that the rRNA gene restriction pattern (ribotypes) allowed the distinction of geographical groups of *X. campestris* pv. *manihotis*. The RFLP patterns with the rRNA probe were as determined previously (Berthier *et al.*, 1993), however two additional RFLP groups that corresponded to strains isolated in South America and Asia were characterized. Moreover, hybridization with the other cloned DNA probes revealed more genomic polymorphism than the rRNA probe. The probe pBS6 contains a repetitive DNA sequence present in many copies in DNA from all strains of *X. campestris* pv. *manihotis* tested. With the probe pBS8, the African strains were distinguished from Asian and South American strains in that DNA from African strains contained fewer copies of the repetitive element pBS8. Our results are very similar to those of Leach *et al.* (1992) who have used a repetitive element to measure the genetic diversity of the *X. oryzae* populations.

The DNA probes used in this study allowed the assessment of relatedness of different strains. Some strains were clustered in the same RFLP group with the plasmid (pBSF2) or genomic probes (pBS6, pBS8) used: Brazilian, Colombian and Malaysian strains; Mexican, Dominican and Colombian strains; Sumatran, Philippine, Malaysian, Brazilian and Colombian strains; Brazilian, Colombian and African strains. The fact that all the 274 African strains studied were clustered in the same RFLP group with South American strains is consistent with the hypothesis of the recent introduction of this pathogen from South America to African countries. Lack of polymorphism observed among African strains isolated in different geographical locations (West, Central and East Africa), demonstrate a clonal population structure for the pathogen. The results suggest that these strains have not as yet diversified at the chromosomal level. RFLP analysis of the 196 strains

from Congo showed no polymorphism amongst the strains. This is in spite of the fact that the strains were collected from different ecological zones, over a period of 15 years in the Congo (1976–1991) at different seasons (both rainy and dry seasons) and even from different cultivars of the host plant. This shows that none of these different cultivars offered sufficient selection pressure to result in any detectable genetic changes. Nevertheless, the probe from a DNA plasmid fragment (pBSF2) revealed some DNA polymorphisms in African strains. This is not surprising since some plasmids can be conjugatively transferred to facilitate genetic exchange in bacterial strains (Coplin, 1989; Eberhard, 1990). Plasmid DNA RFLP profiles were also a more sensitive measure of diversity in bacterial strains as shown in *X. campestris* pv. *citri* (Pruvost *et al.*, 1992).

The cloned plasmid DNA fragment (pBSF2) hybridized to total genomic DNA of some pathovars of *X. campestris* with pathovar-specific patterns and allowed distinction of polymorphism within strains of these pathovars (V. Verdier, unpublished data). The pBSF2 clone could possibly be used to identify some strains from other pathovars of *X. campestris*.

The high level of diversity observed in the genome of South American strains could be a consequence of the host genotype diversity, confirming an accepted concept in the host–pathogen coevolution and in biotype selection (Leonard & Czochor, 1980). A few reports have shown that genetic diversity among cassava plants was extensive in the South American areas, whereas it was more restricted in African countries (Lefevre, 1988). This is probably because cassava is a crop native to South America and was introduced into Africa only in the 16th and 18th centuries by the Portuguese traders. Recent introduction of cassava plants has been also accomplished by different 'International Centers' from Colombia (CIAT) to Nigeria (IITA) via seeds, cuttings and *in vitro* plantlets.

RFLP analysis is more precise and more discriminative to assess genetic diversity, than other methods such as serology, phenotypic analysis and fatty acid profiling. On the basis of SDS-PAGE analyses of cellular protein, Vauterin *et al.* (1991) could not find any obvious differences among 14 strains of *X. campestris* pv. *manihotis* from different geographical origins. These strains were included in the present study and the RFLP analysis enabled the detection of genetic groups within these strains.

Biochemical and physiological variations within the pathovar have been reported but no correlation was observed between the characteristics studied and virulence or geographic origin (Maraité *et al.*, 1981; Van den Mooter *et al.*, 1987; Verdier, 1988; Grousson *et al.*, 1990). Our results on the phenotypic characteristics agree

with these previous reports and have shown that only minor differences were evident in the biochemical features tested. The only biochemical characteristic that allowed a clear distinction between geographical groups was the ability to hydrolyse starch. Such results have been described for *X. campestris* pv. *manihotis* (Maraité *et al.*, 1981) and also for *X. campestris* pv. *vasculorum* in which differentiation of Eastern and Southern African strains was possibly using starch hydrolysis (Qhobela & Calflin, 1992).

We have evaluated the potential use of the RFLP technique to describe the pathogen population structure over time and space, in a worldwide collection of *X. campestris* pv. *manihotis*, as well as in an African population (Congo). The different probes used in this study are extremely useful tools for following the genetic evolution of strains. RFLP data suggests that all African strains and Asian strains have a common centre of origin in the South American cassava areas. It is likely that dissemination of this pathogen has been brought about by humans, either with infected cuttings as reported by Kwage (1982) in Sudan or with infected seeds (Elango & Lozano, 1980).

Presently, control of the disease is based essentially on the use of cassava-resistant germ plasma. Information on genetic variability of the pathogen and consequently its potential for adaptation to new cassava cultivars will give the crop breeders a broader knowledge in the development of new, more resistant cultivars.

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