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Short communication

Differentiation of *Xanthomonas* species pathogenic to sugarcane by PCR-RFLP analysis

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Abstract

The PCR-RFLP of the 16S-23S rDNA spacer region was used to differentiate *Xanthomonas* species pathogenic to sugarcane. Strains of *X. albilineans*, *X. campestris* pv. *vasculorum* Types A and B, *X. sacchari* and *Xanthomonas* sp. from Trinidad, South Africa and India were examined. The amplification products were digested with *Alu* I, *Hae* III, *Hpa* II and *Mbo* I and the results showed that the different groups of bacterial strains exhibited distinct RFLP patterns for each tested endonuclease, except *X. albilineans* and *X. sacchari* which could only be differentiated from each other by the digestion with *Hpa* II. The results also allowed the separation of *X.c.* pv. *vasculorum* Type A from *X.c.* pv. *vasculorum* Type B and strongly suggested that the analyzed *Xanthomonas* sp. strains belong to *X. sacchari*. Nine *X. campestris* (pv. not determined) strains included in this study showed identical profiles to *X.c.* pv. *vasculorum* Type A group and DNA–DNA hybridization experiments confirmed these results. PCR-RFLP of the 16S-23S rDNA spacer region could be applied as a reliable method for differentiating the xanthomonads pathogenic to sugarcane.

Sugarcane is one of the most important crops growing in the tropical and subtropical regions of the world. However, many factors affect yield and cause economical losses. Of these factors, the increased incidence of phytopathogens, mainly bacteria, represents a serious threat to sugarcane cultivation. The phytopathogenic bacteria include *Acidovorax avenae* subsp. *avenae*, *Herbaspirillum rubrisubalbicans*, *Leifsonia xyli* subsp. *xyli*, *Pantoea ananas*, *Pseudomonas syringae* pv. *syringae, Xanthomonas albilineans*, *X. campestris* pv. *vasculorum* Types A and B and *X. sacchari* (Bradbury, 1986; Rahimian, 1995; Baldani et al., 1996; Young et al., 1996; Evtushenko et al., 2000).

The three major bacterial diseases in sugarcane are caused by *L. xyli* subsp. *xyli*, *X. albilineans* and *X.c.* pv. *vasculorum*. *L. xyli* subsp. *xyli* is a fastidious organism, which causes the ratoon stunting disease. This disease causes severe losses in sugarcane crops and is considered one of the most important in the world because it does not show specific external symptoms apart from a nonspecific stunting of affected plants. *X. albilineans* is another vascular pathogen of worldwide distribution, which causes leaf scald disease. The bacteria may cause severe losses by death of entire stools of susceptible varieties and by affecting juice quality. Indirect losses can also occur by additional costs with the replacement of the destroyed stools and the restriction of the use of susceptible varieties. Latent infection is very common and apparently healthy infected stalks are undoubtedly the most important way of diseases dissemination. *X.c.* pv. *vasculorum*, the causal agent of gumming disease, is a third important sugarcane bacterial pathogen which causes serious losses including reduction of the crop production and difficulties in the processing of the mature stalks due to the presence of gum. The visible symptoms, mainly gum production, may be affected by the sugarcane varieties involved and/or environmental conditions such as humidity and temperature (Thomson, 1958; Hughes, 1961; Ricaud, 1969).

X. albilineans and *X.c.* pv. *vasculorum* may cause similar external symptoms and field diagnosis can be confusing (Hughes, 1961; Hayward, 1993). Consequently, correct identification of the pathogen is crucial to the control measures, development of resistant varieties and exchange of plant material and/or quarantine.

Molecular approaches are being applied increasingly as a tool to clarify the taxonomic positions of several groups of microorganism. The 16S rDNA gene, highly conserved among prokaryotic organisms, has been used extensively for elucidating phylogenetic relationships (Woese, 1987). However, 16S rDNA/16S rRNA sequences may be insufficient to differentiate strains within a species (Hauben et al., 1997). In contrast, nucleotide sequences of 16S-23S rDNA spacer regions represent more variable genetic markers for assessing the phylogenetic relationships between closely-related organisms (Gürtler and Stanisich, 1996). The 16S-23S intergenic region has been used as a target for PCR-based identification and the typing of many bacteria by fragment length profiling, restriction fragment length profiling or DNA sequencing (Seal et al., 1990; Honeycut et al., 1995; Gürtler and Stanisich, 1996; Maes et al., 1996; Manceau and Horvais, 1997). The utilization of molecular tools has already been applied for the diagnosis, study of variation and race differentiation of some sugarcane xanthomonads (Saumtally and Autrey, 1990; Qhobela and Claflin, 1992; Jaufeerally-Fakim et al., 2000; Lopes et al., 2001). In this study, PCR-RFLP of the 16S-23S rDNA spacer region was used to quickly differentiate the xanthomonads pathogenic to sugarcane. Twenty-six strains of *Xanthomonas* spp. were analyzed (Table 1). Other type and/or reference strains of *Xanthomonas* were also included for comparative purposes: *X. axonopodis* pv. *axonopodis* IBSBF 1444^{T}; *X. campestris* pv. *campestris* IBSBF 1163^{T}; *X. oryzae* pv. *oryzae* IBSBF 1392^{T} and *X. translucens* pv. *translucens* IBSBF 644. Nine Brazilian isolates identified as *X. campestris* (pv. not determined) (Carvalho, 1991) were also examined (Table 1). These isolates showed no differences in biochemical and cultural tests with *X.c.* pv. *vasculorum* but their identification was not confirmed due to the failure to detect gum production, a typical symptom of the gumming disease. All the strains were grown on NA (Levine, 1954) at 28 °C for 48 h, except *X. albilineans*, which was cultivated on YS (Dye, 1962) without NaCl plus 0.5% glucose.

Genomic DNA was extracted (Pitcher et al., 1989) and PCR amplification of the 16S-23S rDNA spacer region was performed (Destéfano and Rodrigues Neto, 2002). The amplification yielded a band of approximately 1.1 kilobase (kb) for all the strains tested. The restriction fragments were separated by electrophoresis in 3% agarose gels in 1X TAE buffer (Maniatis et al., 1982). The gels were stained with 0.1 μg per ml of EtBr and photographed under ultraviolet transillumination. The molecular weights of the fragments were determined by comparison with a 100 base pairs (bp) DNA ladder (Amersham Biosciences). PCR products (5 μl) were digested, individually, with each of the following restriction endonucleases *Dde* I, *Hae* III, *Hpa* II and *Mbo* I under conditions specified by the manufacturer (Amersham Biosciences). The results showed distinct RFLP patterns among the strains (Figure 1 and Table 1). The digestions with *Dde* I and *Hae* III generated fragments from 100 to 300 bp and from 100 to 700 bp, respectively. These endonucleases distinguished *X.c.* pv. *vasculorum* Types A and B from each other and from *X. albilineans* and *X. sacchari* (Figure 1A,B). On the other hand, the digestion with *Hpa* II revealed slightly different profiles among the strains, except for *X. albilineans*, which showed a very distinct profile, allowing its clear separation from all tested strains (Figure 1C). In the *Mbo* I digestions, which generated fragments ranging from 180 to 450 bp, the differentiation of *X.c.* pv. *vasculorum* Types A and B from *X. albilineans* and *X. sacchari* was also possible (Figure 1D). The nine Brazilian isolates previously classified as *X. campestris* (pv. not determined) showed identical banding profiles to those of the pathovar reference strain of *X.c.* pv. *vasculorum* Type A (IBSBF 695^{P}). The *Xanthomonas* sp. strains isolated from Trinidad, South Africa and India showed the same pattern as *X. sacchari* IBSBF 1313^{T} and IBSBF 1312 (Figure 1A–D).

The DNA profiles of the 16S-23S rDNA spacer regions were analyzed in a binary form (presence/absence) and similarity matrices were constructed using the Jaccard coefficient (S_J). Relationships among strains were calculated with the algorithm UPGMA (Unweighted Pair Group Method with Arithmetic mean) using the NTSYS-PC program (Rolf, 1989). Cluster analysis was performed on the data generated by the PCR-RFLP analysis. The data obtained from a total of 32 different bands ranging from 100 to 800 bp were scored and four distinct groups were identified (Figure 2). In Group I, strains of *X.c.* pv. *vasculorum* Type A and *X. campestris* (pv. not determined) showed 100% similarity each other and approx. 83% similarity to the type strain of *X.a.* pv. *axonopodis*. In Group II,

Table 1. *Xanthomonas* spp. strains used in this study and restriction patterns of the amplified 16S-23S rDNA spacer region digested with *Dde* I, *Hae* III, *Hpa* II and *Mbo* I

Strain	Accession number		Host	Place of isolation	Digestion groups			
	IBSBF	Other collections			*Dde* I	*Hae* III	*Hpa* II	*Mbo* I
X. albilineans	1374[T]	LMG 494[T]	*Saccharum officinarum*	Mauritius	B*	B	D	B
X. albilineans	326		*Saccharum officinarum*	Brazil	B	B	D	B
X. albilineans	654		*Saccharum* sp.	Brazil	B	B	D	B
X. campestris pv. *vasculorum* –	695[P]	LMG 901[P]	*Saccharum officinarum*	Mauritius	A	A	A	A
Type A	667	LMG 8709	*Roystonea regia*	Mauritius	A	A	A	A
	668	LMG 8713	*Thysanolaena maxima*	Mauritius	A	A	A	A
	696	LMG 8716	*Saccharum officinarum*	Reunion	A	A	A	A
	1319	LMG 903	*Thysanolaena maxima*	Mauritius	A	A	A	A
	1285	XBR-7[a]	*Saccharum* sp.	Unknown	A	A	A	A
X. sacchari	1313[T]	LMG 471[T]	*Saccharum officinarum*	Guadeloupe	B	B	B	B
	1312	LMG 476	*Saccharum officinarum*	Guadeloupe	B	B	B	B
X. campestris pv. *vasculorum* –	666	LMG 8718	*Saccharum officinarum*	Zimbabwe	C	C	C	A
Type B	1326	LMG 8284	*Zea mays*	South Africa	C	C	E	A
X. campestris (pv. not determined)	656		*Saccharum* sp. (hybrid)	Brazil	A	A	A	A
	660		*Saccharum* sp. (hybrid)	Brazil	A	A	A	A
	661		*Saccharum* sp. (hybrid)	Brazil	A	A	A	A
	665		*Saccharum* sp. (hybrid)	Brazil	A	A	A	A
	728		*Saccharum* sp. (hybrid)	Brazil	A	A	A	A
	730		*Saccharum* sp. (hybrid)	Brazil	A	A	A	A
	1615		*Saccharum* sp. (hybrid)	Brazil	A	A	A	A
	1616		*Saccharum* sp. (hybrid)	Brazil	A	A	A	A
	1617		*Saccharum* sp. (hybrid)	Brazil	A	A	A	A
Xanthomonas sp.	670	NCPPB 916	*Saccharum officinarum*	Trinidad	B	B	B	B
	671	NCPPB 888	*Saccharum officinarum*	South Africa	B	B	B	B
	867	NCPPB 917	*Saccharum officinarum*	Trinidad	B	B	B	B
	904	NCPPB1046	*Saccharum officinarum*	India	B	B	B	B

[T]Type strain. [P]Pathovar reference strain. [a]P. Rott, Center Cooperation Internacionale en Recherche Agronomique pour le Développement, BP 5032 Montpellier, Cedex 1, France. IBSBF: Culture Collection of Laboratório de Bacteriologia Vegetal, Instituto Biológico, Campinas, SP, Brazil. ICMP: International Collection of Micro-organisms from Plants, Auckland, NZ. LMG: Laboratorium voor Microbiologie, Gent, Belgium. NCPPB: The National Collection of Plant Pathogenic Bacteria, York, UK. *A–E: Different letters in each column represent distinct profiles obtained after digestion with endonucleases.

X.c. pv. *vasculorum* Type B strains IBSBF 666 and 1326 exhibited approx. 93% and approx. 75% similarity to type strains of *X.c.* pv. *campestris* and *X. oryzae* pv. *oryzae*, respectively. *X.c.* pv. *campestris* and *X. oryzae* pv. *oryzae* showed no clear differentiation between them. Group III was defined only by *X. translucens* pv. *translucens*, whereas the Group IV contained strains of *X. sacchari* and the four strains of *Xanthomonas* sp. (Hayward, 1962), which showed 100% similarity to each other and approx. 76% similarity to *X. albilineans.*

DNA–DNA hybridization experiments were performed to confirm the taxonomic position of the *X. campestris* (pv. not determined) strains herein allocated as *X.c.* pv. *vasculorum* Type A by PCR-RFLP of the 16S-23S rDNA spacer region. The genomic DNA from the strains of *Xanthomonas* were denatured and transferred to nylon membranes (Amersham Biosciences) using a Hybri slot-blot manifold as described by the manufacturer (Bio-Rad). The pathotype strain of *X.c.* pv. *vasculorum* Type A (IBSBF 695[P]) was labeled using a DIG DNA labeling kit, and the bands were detected with the chemiluminescent substrate CSPD as recommended by the manufacturer (Boehinger Mannheim). The DNA hybridization experiments were performed in triplicate and the amount of DNA immobilized onto the membranes normalized by a previous hybridization with a 16S rDNA

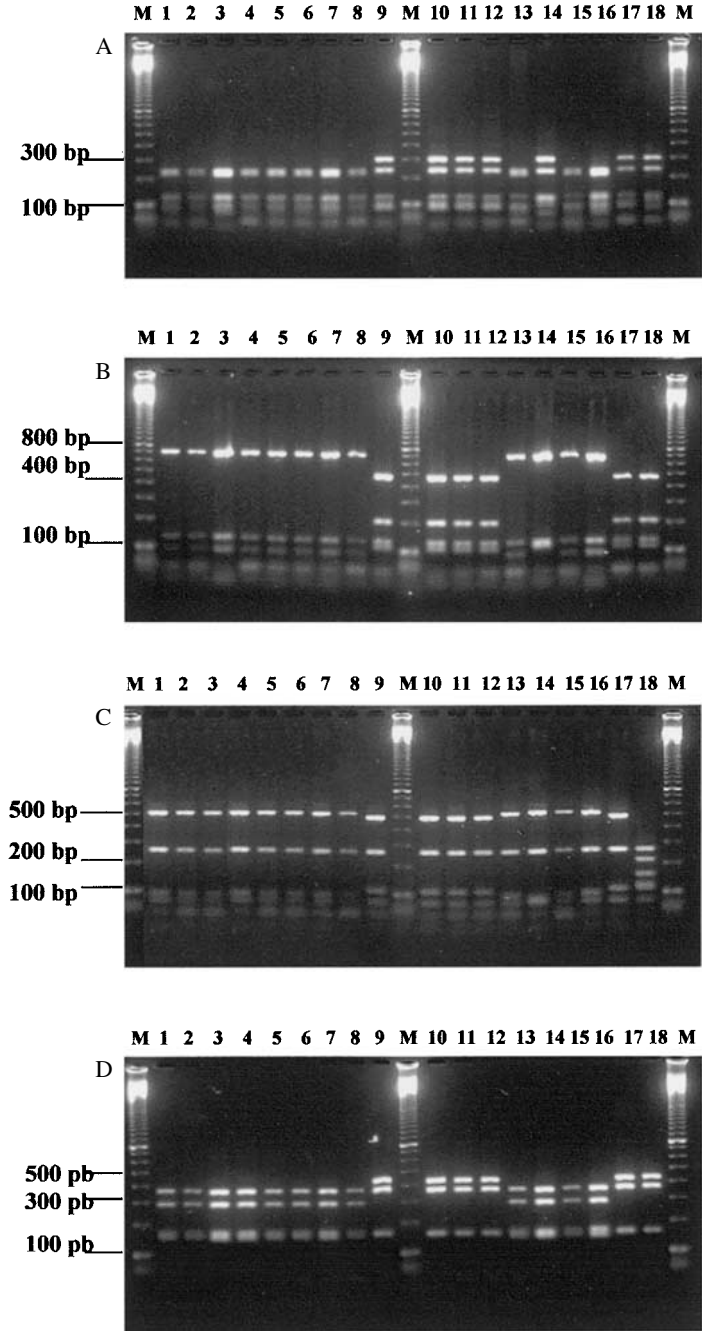

Figure 1. PCR-RFLP of the 16S-23S rDNA spacer regions digested with (A) *Dde* I; (B) *Hae* III; (C) *Hpa* II; and (D) *Mbo* I. M: 100 bp Marker (Amersham Biosciences). *X. campestris* (pv. not determined): lanes (1) 1615; (2) 1616; (3) 0665; (4) 0656; (5) 0660; (6) 0661; (7) 0728; (8) 0730; *Xanthomonas* sp.: lanes (9) 0670; (10) 0867; (11) 0671; (12) 0904; *X. campestris* pv. *vasculorum* Type A: lanes (13) 0695^{P}; (15) 0667; (16) 0668; *X.c.* pv. *vasculorum* Type B: lane (14) 0666; *X. sacchari*: lane (17) 1313^{T}; *X. albilineans*: lane (18) 1374^{T}.

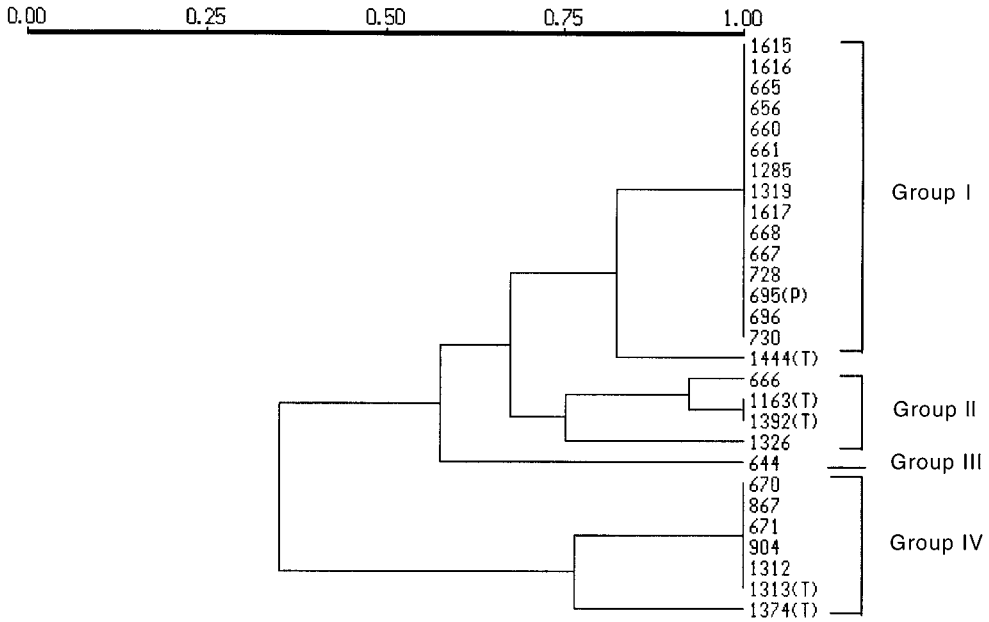

Figure 2. S_J-UPGMA cluster analysis of the RFLP patterns of the 16S-23S rDNA spacer region based on the combined restriction pattern (*Dde* I, *Hae* III, *Hpa* II and *Mbo* I). Scale bar indicates similarity.

probe amplified from *X.c.* pv. *vasculorum* Type A as described by Gonçalves and Rosato (2000). The signal, detected by exposure to X-ray film (Kodak), was measured using an Ultrascan XL-enhancer laser densitometer (LKB Bromma). *X.c.* pv. *vasculorum* Type A showed DNA homologies of 81–100% with *X. campestris* (pv. not determined) strains, confirming the results obtained by PCR-RFLP of the 16S-23S rDNA spacer region (data not shown).

The two strains of *X.c.* pv. *vasculorum* Type B showed homologies values of approx. 37% with *X.c.* pv. *vasculorum* Type A. Our data corroborate those of Vauterin et al. (1995), who found approx. 39% DNA homology values between these genomic species, proposing the reclassification of the former *X.c.* pv. *vasculorum* Types A and B as two new species: *X.a.* pv. *vasculorum* and *X. vasicola* pv. *vasculorum*, respectively. In addition, *X.c.* pv. *vasculorum* Type A showed approx. 83% similarity to the type strain of *X.a.* pv. *axonopodis* by PCR-RFLP and approx. 80% similarity in the DNA–DNA hybridization experiments, giving support to Vauterin's proposal.

The four *Xanthomonas* sp. strains (IBSBF 670, 671, 867 and 904) showed identical profiles to those of *X. sacchari* strains (IBSBF 1313^T and 1312) by PCR-RFLP of the 16S-23S rDNA spacer region. *X. sacchari* was proposed as a new genomic species by Vauterin et al. (1995) to allocate the two cited strains previously identified as *X. albilineans*. Hauben et al. (1997) also showed that these strains represent a separate group within the genus *Xanthomonas* by 16S rDNA analysis. However, Schaad et al. (2000) suggested that additional data and strains should be included in this species before it can be accepted as a new one. Our data strongly suggest that the *Xanthomonas* sp. strains herein belong to *X. sacchari*, but additional data based on DNA–DNA hybridization need to be obtained to confirm this proposal.

Our results indicate that the PCR-RFLP of the 16S-23S spacer region can be used to clearly differentiate the xanthomonads pathogenic to sugarcane such as *X.c.* pv. *vasculorum* Types A and B and *X. sacchari* from *X. albilineans*. This approach was shown to be a simple, rapid and reliable method for the diagnosis of the strains associated with several diseases of sugarcane plants.

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