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Xanthomonas albilineans (Ashby) Dowson: Study of Its Genetic Variability in Sugarcane Samples in Cuba

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Abstract: Four isolates of the phytopathogenic bacterium *Xanthomonas albilineans (Xa)* from samples taken in commercial fields of different Cuban provinces and two from the cultivar L55-5, artificially infected in the Camagüey and Matanzas Experimental Stations, were used for the extraction of total nucleic acids according to the CTAB protocol. They were amplified by PCR using four primer combinations: A: P1-M1, B: P1-M2, C: P2-M1 and D: P2-M2. Direct sequencing of the PCR products was performed on all samples and the nucleotide sequences were used to generate partial consensus of 16-23S rRNA, which were analyzed with the BLASTn program. In all the isolates evaluated, the presence of a common band at the height of 620 bp was visualized and the AFLP profile showed that there is homology when comparing the sequences corresponding to the 16-23S rDNA of *X. albilineans* of the different isolates; the phylogenetic analysis reflected a grouping of the isolates of the species, regardless of its geographical origin, different from others of the genus *Xanthomonas*. The comparison of the Cuban isolates with eight strains published in GenBank, showed marked similarity with them, with 100% nucleotide identity and percentage of coverage with a genome fragment of the GPE PC73 strain from Guadalupe, KO905 from the United States and a strain from Brazil. The predominance of the generalized circulation in Cuba of a single serovar is presumed.

Keywords: *Xanthomonas albilineans*, PCR, Sequence, Variability, Identity

1. Introduction

Leaf scald disease, caused by *Xanthomonas albilineans* (Ashby) Dowson, is a lethal disease of sugarcane. The bacterium colonizes stems and leaves, mobilizes systemically within plant tissues and manifests itself differently, with symptoms that frequently vary between cultivars, times of the year and localities, a condition that is attributed to environmental influences or pathogenic variations [12, 19, 27]. In Cuba, safely assessing genetic resistance within the management strategy is crucial for the control of the disease, since resistance to leaf scald within the breeding process is considered as a selection criterion.

The objective of this work lies in knowing the existence or not of different pathogenic variants of *Xa* and their influence on characterization of the resistance of cultivars as a measure to control the spread and effects of the disease.

2. Materials and Methods

2.1. Molecular Detection and Studies of Genetic Variability

The detection of *X. albilineans* in the samples to be studied was carried out by the immunochemical technique UMELISA-DAS, for which a set of AGDIA diagnostics was used and the confirmation by RT-PCR: four isolates obtained from samples from commercial areas of the Mayabeque, Matanzas, Camagüey and Las Tunas provinces and two of the cv. L55-5, which is used as inoculum in the resistance tests by artificial inoculation in the Experimental Stations of Jovellanos (Matanzas) and Florida (Camagüey), were used for the extraction of total nucleic acids according to the CTAB (Cetyl-Trimethyl Ammonium Bromide).

The PCR was performed with the primers FGPS1490 (5'-TGC GGC TGG ATC ACC TCC TT-3 ') and FGPS132 (5'-

CCG GGT TTC CCC ATT CGG-3'), which amplify a 620bp product, corresponding to the 16-23S rRNA region [13]. A PTC100 thermal cycler (MJ Research, USA) was used, with the following reaction program: one cycle at 94°C for 1 min; 40 cycles at 94°C for 30 s, a cycle at 65°C for 30 s and 72°C for 1 min and a final cycle at 72°C for 5 min.

The PCR reaction mixture was taken as a negative control and instead of adding DNA, sterile distilled water was used. As positive control, strain *Xa* DNA was used, which was extracted from isolates of naturally infected plants. For each sample, PCR was performed in triplicate. The amplified products were visualized by electrophoresis in 1% agarose gels, in a horizontal equipment (Biorad) at 100 volts for 30 min. They were then stained with ethidium bromide (Sambrook et al., 1989).

Characterized *X. albilineans* isolates were used for the analysis of the amplified fragment length polymorphism (AFLP) following of the Bachem procedures [3]; their genomic DNA was digested with the restriction enzymes PstI and MseI, according to the manufacturer's recommendations. Four primer combinations were used for PCR amplification: A: P1-M1, B: P1-M2, C: P2-M1 and D: P2-M2 (Table 1).

Table 1. *Primers used in AFLP reactions.*

Código	Cebador	Secuencia 5´-3´	Bases selectivas
P1	PstI-1	GACTGCGTACATGCA	AA
P2	PstI- 2	GACTGCGTACATGCA	AC
M1	MseI-1	ATGAGTCCTGAGATAG	ACC
M2	MseI-2	ATGAGTCCTGAGATAG	GGC

Amplification was performed in a PTC100 thermal cycler (MJ Research, USA) with the following reaction program: one cycle of 94°C (30 s), 65°C (30s); 12 cycles where the hybridization temperature of the primers decreases 0.7°C per cycle, from 94°C (30s), 65°C (30s) and 72°C (1 min) followed by 29 cycles of 94°C (30s), 56°C (30s) and 72°C (60s), with a final extension of 90°C (3 min). The reaction products were kept on ice until separated by 9.0% polyacrylamide gel electrophoresis. Bands were visualized by silver staining (PROMEGA).

2.2. 16-23S rRNA Sequencing

PCR products were sequenced on all samples in an automated sequencer (ABI PRISM® 377, Hitachi, Japan) with reagent sets (Perkin-Elmer ABI-PRISM DyeTerminato Cycle Sequencing Kit). The nucleotide sequences obtained in both directions with a selected isolate were used to generate partial consensus of the 16-23S rRNA, and were subsequently analyzed with the BLASTn program to reconfirm the molecular identity [1].

Sequences of the isolate from Camagüey (C1) and Jovellanos (C2) were aligned with the Clustal X program [25]. Its phylogeny was determined using the MEGA5 program [24], with the nearest neighbor method with a re-sampling analysis of 1,000 repetitions; 26 reference sequences corresponding to isolates of *Xanthomonas* sp. were included for the analysis.

3. Results

3.1. Molecular Detection and Studies of Genetic Variability

The migration in 1% agarose gel of the amplified product with the primers FGPS1450 and FGPS132 of the four isolates evaluated allowed to visualize the presence of a common band at the height of 620 bp, all corresponding to the expected size, while the control did not originate signals amplification, a result that confirms the identity of the isolates as *X. albilineans* in the sampled areas (Figure 1).

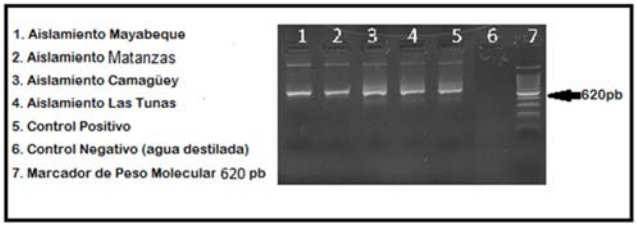

Figure 1. *Amplification products in 1% agarose gel.*

The AFLP analysis (Figure 2) made it possible to compare a small fragment of the genome of an isolate from Jovellanos (Matanzas) and another from Florida (Camagüey); the sequences corresponding to the 16-23S rDNA of *X. albilineans* showed that there is homology between both isolates. Some authors consider that AFLP could be a useful tool to demonstrate polymorphism at the level of the entire genome of the bacterium *X. albilineans* [18, 26].

Figure 2. *AFLP band pattern of X. albilineans isolates from Jovellanos (1) and Florida (2).*

The comparison of one of these Cuban isolates with eight

strains published in the International Gene Bank, demonstrated its marked similarity with them, with 100% nucleotide identity and percentage of coverage with a fragment of the genome of the GPE PC73 strain of Guadalupe (Table 2).

Table 2. *Comparison of the DNA segment of the intergenic region of the Cuban isolate of X. albilineans with sequences of strains published in the International Gene Bank. 16-23S rRNA sequencing.*

Descripción	Coverage (%)	e-value	Identity (%)
Xanthomonas albilineans GPE PC73 complete genome [17]	100%	0.0	100%
X. albilineans strain IBSBF 326 16S-23S ribosomal RNA intergenic spacer and 23S ribosomal RNA gene, partial sequence [6]	95%	0.0	99%
X. albilineans strain IBSBF 1374 16S-23S ribosomal RNA intergenic spacer and 23S ribosomal RNA gene, partial sequence [6]	94%	0.0	99%
X. albilineans clone K0905 16S-23S ribosomal RNA intergenic spacer and 23S ribosomal RNA gene, partial sequence [23]	85%	0.0	100%
X. albilineans strain ICMP196 16S-23S ribosomal RNA intergenic spacer, complete sequence [14]	78%	0.0	99%
X. albilineans intergenic spacer and 23S ribosomal RNA gene, partial sequence [7]	57%	0.0	100%
X. albilineans transfer RNA-Ala, intergenic spacer, and transfer RNA-Ile gene [8]	19%	2,00E-54	97%
X. albilineans transfer RNA-Ala, intergenic spacer, and transfer RNA-Ile gene [8]	19%	8,00E-53	96%

3.2. 16-23S rRNA sequencing

The sequences of the PCR products of the *X. albilineans* isolates from Jovellanos and Florida were homologous. The phylogenetic analysis, which included sequences from different geographic regions, as well as from other species of this genus available in GenBank (Figure 3), demonstrated that, without distinction of origin, the *X. albilineans* grouped together and separately from the other species.

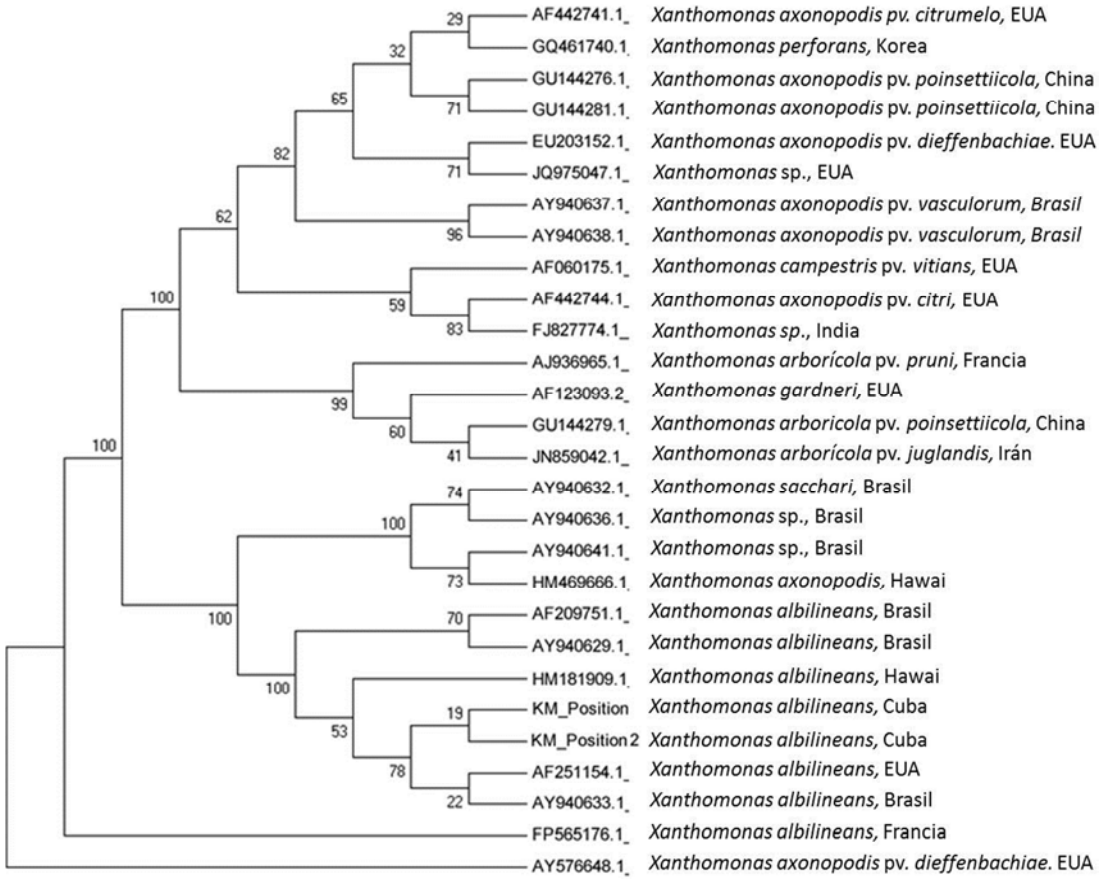

Figure 3. *Phylogenetic tree of Xanthomonas albilineans samples based on the 16-23S rRNA nucleotide sequence.*

4. Discussion

Although [20] described three serovars of *X. albilineans* based on immunological properties of a worldwide collection of the pathogen, some authors have published no correlation between genetic markers and those of pathogenicity of the bacterium [5]. In Guadalupe [4], among 75 isolates of the bacterium inoculated in one cultivar, they found variation of this character in 19 of them. However, in Venezuela [2] obtained band patterns for bacteria identified as *X.*

albilineans that are not similar to those described by other authors in other regions of the world.

Studies carried out with isolates obtained from sugarcane plants with symptoms of leaf scald disease from Gabon, indicate greater aggressiveness than that observed in the isolates from Guadalupe and the USA [13], while in Mexico [9], amplified a band of 288 bp of four strains of *X. albilineans* obtained in Veracruz and Chiapas, of which two were more aggressive, but all presented 98% nucleotide identity with an isolate from Brazil.

In France [21], found that the measurement of the *X. albilineans* genome is smaller than the rest of the sequenced related genera and the GPE PC73 variant reflects characteristics that distinguish it from the rest of the species; in China [10], verified on the basis of the concatenated sequence of genes, that 14 strains from this country had a 99.9-100% similarity to each other and to five strains from the Indies Westerners and the United States.

5. Conclusions

It was observed that there is nucleotide similarity between the isolates obtained in two localities where resistance studies are carried out in Cuba, which rules out the possibility that the inconsistency of the symptoms is caused by different serovars of the pathogen.

The comparison with eight strains published in the International Gene Bank showed 100% identity and coverage with a fragment of the genome of the GPE PC73 strain of Guadalupe and also, although for a smaller fragment, it showed similarity with the KO905 strains from United States and one from Brazil. The epidemiological characteristics of leaf scald in Cuba and the results of genetic and molecular studies reflect the possibility of the predominance of the circulation of a single serovar, which should be demonstrated with the performance of other studies.

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