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Molecular Epidemiology of *Xanthomonas campestris* pv. *musacearum*, the Causal Agent of Xanthomonas Wilt of Banana and Enset

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Abstract

Xanthomonas wilt of enset and banana was first described in Ethiopia in 1968 and is now a serious disease that is spreading in East and Central Africa. Earlier studies identified *Xanthomonas campestris* pv. *musacearum* as the causal agent. This paper describes the characterisation of strains of the bacterium that were isolated in four countries between 1968 and 2006. Phylogenetic analysis of partial nucleotide (nt) sequences of the gyrase B gene and internal transcribed spacer (ITS) region, and genomic amplicon fingerprints using repetitive sequence PCR and fatty acid methyl esters, showed that all strains of the pathogen belonged to the same genotype. Twenty strains originating from Ethiopia, Uganda, the Democratic Republic of Congo and Rwanda had nt sequence identities above 98%. When compared to other species of bacteria, the pathogen was not found to be related to *X. campestris*, but to *X. vasicola*, a species that contains *X. vasicola* pv. *holcicola*, a sorghum pathogen. Further, a group of sugarcane and maize pathogens that had been classified as *X. axonopodis* pv. *vasculorum*, but which were atypical of the species and had been proposed for reclassification as *X. vasicola* pv. *vasculorum*, were found to be most similar to the banana and enset strains. The results support the reclassification of *X. campestris* pv. *musacearum* as *X. vasicola* pv. *musacearum*. In addition, the data reveal that the recent occurrence of Xanthomonas wilt in Uganda and other East African countries is a consequence of the spread of the pathovar first recognised on enset in Ethiopia. Possible hypotheses to explain evolutionary pathways of the three strains of Xanthomonas that affect enset, banana, sorghum, sugarcane and maize are proposed.

INTRODUCTION

Xanthomonas wilt, caused by *Xanthomonas campestris* pv. *musacearum*, is emerging as a devastating disease of banana (*Musa* spp.) and enset (*Ensete ventricosum*) in the Great Lake Region of East Africa. On affected plants, *X. campestris* pv. *musacearum* typically causes progressive yellowing and wilting of leaves, with fruits ripening prematurely and unevenly with internal brown discolourations. When stems are cut, pockets of pale yellow bacterial ooze appear within 5 to 15 minutes (Yirgou and Bradbury, 1974; Tushemereirwe et al., 2004). Yellow or brown streaks occur in the vascular tissues of infected plants. Other symptoms of the floral parts include wilted bracts, shrivelled and rotted male buds, and yellow-brown flower stalks (Yirgou and Bradbury, 1968; Tushemereirwe et al., 2004). *Xanthomonas campestris* pv. *musacearum*

was first described in Ethiopia in 1968 on enset (Yirgou and Bradbury, 1968) and in 1974 on banana (Yirgou and Bradbury, 1974). In 2001, the disease was detected on banana in Mukono and Kayunga districts in Uganda (Tushemereirwe et al., 2004). Since then, the disease has spread widely, affecting different banana genotypes in the major banana-growing areas of Uganda (Tushemereirwe et al., 2004). Further outbreaks have been reported in the Democratic Republic of Congo (Ndungo et al., 2006), Rwanda (Reeder et al., 2007), Tanzania (Mgenzi et al., 2006) and most recently in Teso and Busia districts of Kenya (Anon., 2008).

In a recent analysis using sequences of the gene encoding the gyrase B protein (*gyrB*), fatty acid methyl esters (FAME) and genomic amplicon fingerprints using Repetitive sequence PCR (rep-PCR) profiling, all strains of *X. campestris* pv. *musacearum* were similar to *X. vasicola*, which comprises a sorghum pathogen, *X. vasicola* pv. *holcicola*, and a sugarcane and maize pathogen, *X. vasicola* pv. *vasculorum* (Dookun et al., 2000; Vauterin et al., 1995). In addition, pathogenicity studies revealed that *X. campestris* pv. *musacearum* is able to induce hypersensitive response in maize plants. Based on these results, *X. campestris* pv. *musacearum* has been reclassified as *X. vasicola* pv. *musacearum* (Aritua et al., 2007a,b). Whereas the above report provided the first genetic information of *X. campestris* pv. *musacearum*, no data exist on the molecular epidemiology of *X. campestris* pv. *musacearum* to date. Therefore, in order to gain more genetic as well as provide evolutionary information, we evaluated phylogenetic relationships among 20 isolates of the bacterium collected between 1968 and 2005.

MATERIALS AND METHODS

Sources, Culturing and DNA Isolation

The strains of the pathogen studied in this work included the first recorded isolates from enset and banana in Ethiopia (Yirgou and Bradbury, 1968; 1974) as well as strains of the pathogen from recent outbreaks in Uganda, Rwanda and the Democratic Republic of Congo (Aritua et al., 2007b). The strains were obtained from CAB International (UK), the National Collection of Plant Pathogenic Bacteria (NCPPB) maintained at the Central Science Labouratory, York (UK) and the National Agricultural Research Organisation (Uganda). DNA was extracted from 48-h old cultures grown on YPGA at 28°C and purified using the Wizard® Genomic DNA Purification Kit (Promega). The DNA extracts were standardised to approximately 25-50 ng DNA μl^{-1}.

Generation of Ribosomal Internal Transcribed Spacer (ITS) Region

Generation of partial sequences of the ITS region was performed in PCR reactions with 27F (5'-AGTGTTTGATCCTGGCTCAG-3', forward) and FGPS-132 (5'-CCGGGTTTCCCCATTCGC-3', reverse) primers (Goncalves and Rosato, 2002). The amplification volume of 25 µl reaction contained, in addition to H_2O, 2.0 µl DNA, 2.5 µl of 10x buffer (HotMaster Taq Buffer), 2.5 mM $MgCl_2$, 1.25 mM dNTPs, 0.3 pMol each primer and 0.75 U Taq (Eppendorf). The following thermal cycling parameters were used: initial denaturation at 94°C for 2 min followed by 30 cycles of denaturation at 94°C for 30 sec, primer annealing at 58°C for 30 sec and DNA extension at 72°C for 50 s. The final extension step was at 72°C for 5 min.

Generation of DNA *gyrB*

The partial sequence of gyrase sub unit B gene (*gyrB*) was amplified by PCR using primers X.Gyr.fsp1 (5'-CAGGGCAAGAGCGAGCTGTA-3', forward) and X.Gyr.rsp1 (5'-CAAGGTGCTGAAGAT CTG GTC-3', reverse) (Aritua et al., 2007b). Approximately 50 ng of DNA was added to a solution containing 5 µl of 10x buffer (HotMaster Taq Buffer), 2.5 mM $MgCl_2$, 1.25 mM dNTPs, 0.3 pMol each primer and 1.25 U Taq (Eppendorf) in a final volume of 50 µl. The samples were heated at 94°C for 2 min and processed through 35 cycles of 30 sec denaturation at 94°C, 1 min annealing at 55°C and 1 min primer extension at 72°C. A final step was performed at 72°C for 5 min.

Fatty Acid Methyl Esters Analysis and Library Generation

FAMEs were analysed as described by Stead (1992). FAMEs were separated using a Hewlett-Packard Model 6890 gas chromatograph equipped with a 25-m fused silica capillary column using hydrogen as carrier gas and identified with known standards using a Microbial Identification System Software Package (Microbial Identification System, MIDI). The fatty acid profiles generated were compared with the commercial library TSBA40 of a wide range of aerobic bacteria and an in-house Central Science Laboratory library (NCPPB3) of many plant-pathogenic bacteria.

Phylogenetic Analysis

In both PCRs, products of the predicted sizes were purified with a Wizard® SV Gel and PCR Clean-up Systems (Promega) and sequenced directly (in both directions) at MWG-Biotech. Multiple pairwise sequence alignments and percentage identity estimates were generated using the Clustal V software within the MEGALIGN package (DNAstar). Sequence distances were estimated using the algorithm of Jukes and Canter (1969), taking into account all positions and all omissions and deletions. Before phylogeny construction, tree topology was inferred using the neighbour-joining algorithm, and confidence values for the grouping from the multiple alignments were derived using 1000 bootstrapped data sets (Van der Peer and De Wachter, 1993). Rooted clustering trees were drawn and visualised by using TREECON version 1.3b.

RESULTS

Analysis of ITS Region

Multiple alignments of the partial nucleotide (nt) sequences approximating to 500 bp revealed a difference of <2% over all nucleotides examined for all isolates from banana. The sequence of a single enset isolate was also identical to the banana isolates. Comparison of the ITS sequences to those for known xanthomonads in the DNA DataBank of Japan (DDBJ) (http://www.ddbj.nig.ac.jp/), the European Molecular Biology Laboratory (EMBL) (http://www.ebi.ac.uk/) and GenBank at National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) showed that the *X. campestris* pv. *musacearum* sequences were over 98% identical to *X. vasicola* strains from maize and sugarcane (Vauterin et al., 1995). The sequences were also nearly identical to the ITS sequences of the type strain of *X. vasicola* pv. *holcicola*, the causal agent of Xanthomonas wilt of sorghum. However, *X. campestris* pv. *musacearum* isolates shared a relatively low identity (>10% nt divergence) with strains of *X. campestris* with which it is currently classified. Phylogenetic analysis grouped all the isolates of *X. campestris* pv. *musacearum* together with those of *X. vasicola* pv. *holcicola* without coincident separation (Fig. 1).

Analysis of *gyrB* Sequences

Details of the results have already been presented in Aritua et al. (2007b). Congruent with the ITS sequences, multiple sequence alignment identified inter-pathovar sequence similarity between *X. campestris* pv. *musacearum* and *X. vasicola* from sugarcane at 100%, whereas *X. vasicola* pv. *holcicola* possessed small sequence variation (Genbank accession no. DQ676938-53 and DQ667683). In comparison with other *Xanthomonas* species, *X. campestris* pv. *musacearum* showed much lower levels of sequence identity: *X. campestris* pv. *campestris* showed 84.9% identity to type strain NCPPB528, *X. axonopodis* pv. *citri* 93.0%, *X. oryzae* pv. *oryzae* 94.9%, *X. campestris* pv. *vesicatoria* 93.2% and *X. arboricola* pv. *celebensis*, also a pathogen of banana, 89.1%. A bootstrapped phylogenetic analysis on the sequences from the above studies together with the available Genbank sequences is presented in Fig. 2. No differences were evident that related isolate to geography or time.

Fatty Acid Methyl Esters Analysis

The FAME profiles for enset and banana isolates were all typical of the genus *Xanthomonas*, and agreed with those for the *gyrB* and ITS sequences. Cluster analysis of the FAME profiles using Unweighted Pair Group Method with Arithmetic averages (UPGMA) clustered all the enset and banana isolates in a single homogeneous group together with those from sugarcane (data not shown). They clustered very close to *X. vasicola* pv. *holcicola*, but were distant to other *Xanthomonas* species, including the type strain of *X. campestris* (Aritua et al., 2007b).

DISCUSSION AND CONCLUSIONS

To develop control measures against Xanthomonas wilt, it will be necessary to understand the disease's epidemiology and identity of *X. campestris* pv. *musacearum*. In this study, sequence analyses of the ITS loci and the protein-coding gene (*gyrB*) revealed a limited nucleotide divergence among 20 isolates collected between 1968 and 2005 from Ethiopia, Uganda, the Democratic Republic of Congo, Tanzania and Rwanda. Little genetic variation was observed in the pathogen over this time span (38 years). This was consistent with FAME and rep-PCR profiling, that also indicated that the current populations of *X. campestris* pv. *musacearum* are homogenous (Aritua et al., 2007a,b). Absence of notable differences with respect to time of isolation, geographic origins or host (banana and enset) suggests that the recent outbreak of Xanthomonas wilt across the Great Lake region of East Africa resulted from *X. campestris* pv. *musacearum* originating in Ethiopia.

Speculation as to why a pathogen that has existed for 40 years remains homogenous is not known. Presumably, it has not experienced significant selection pressure during that time for diversification to occur. One also wonders why the pathogen remained in Ethiopia without further spread for almost four decades.

The phylogenetic analyses based on rep-PCR and FAME profiles (Aritua et al., 2007b) and the sequences of the gyrase B and ITS loci found that the banana isolates (this study) were most similar to strains of *X. vasicola* from sorghum, maize and sugarcane originating in Africa. Thus, these bacteria may have evolved to be pathogenic on these crops in the same location. Measurement of genetic diversity among isolates of other pathovars of *X. vasicola* could provide vital evidence regarding their origin, although an African origin is possible given the African origins of sorghum and enset. The

musacearum pathovar may have developed on enset in Ethiopia as a result of a close association with *X. vasicola*-infected sorghum. Spread to banana may have come later followed by dissemination from Ethiopia as a result of the movement of banana planting material. The very high similarity among all banana strains and the enset strain indicates that pathovar evolution has occurred relatively recently. In conclusion, results of this work have demonstrated genetic homogeneity within *X. campestris* pv. *musacearum*, its relatedness to *X. vasicola* strains and a possible origin from sorghum.

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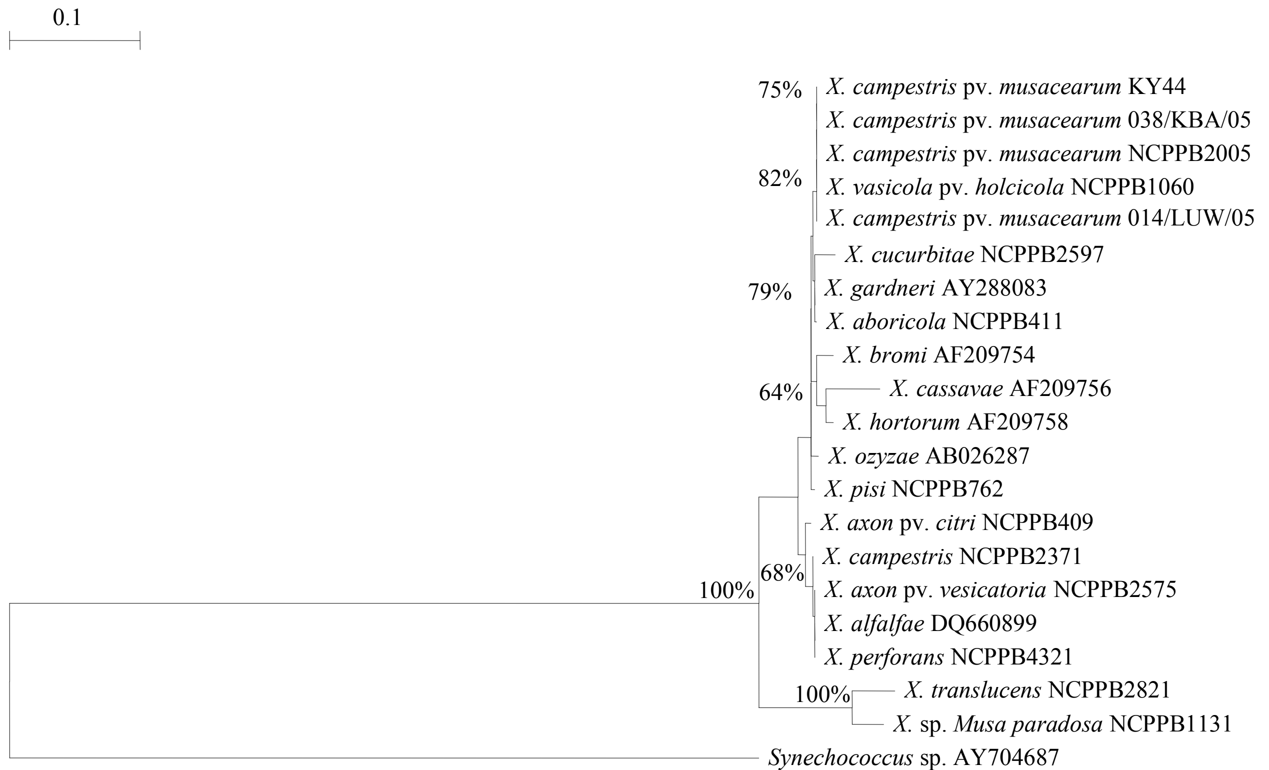

Fig. 1. Phylogram based on partial ITS sequences. Note identity between *Xanthomonas campestris* pv. *musacearum* and sorghum strain of *X. vasicola* pv. *holcicola*. Scale bar is substitutions/nucleotide. Percentages at nodes are bootstrap values from 1000 samplings. Access number refers to the Genbank accession number from where these sequences were obtained. NCPPB, National Collection of Plant Pathogenic Bacteria (UK). IMI, International Mycological Institute. The sequence of *Synechococcus* sp. (Access no. AY704687) was used as the outgroup.

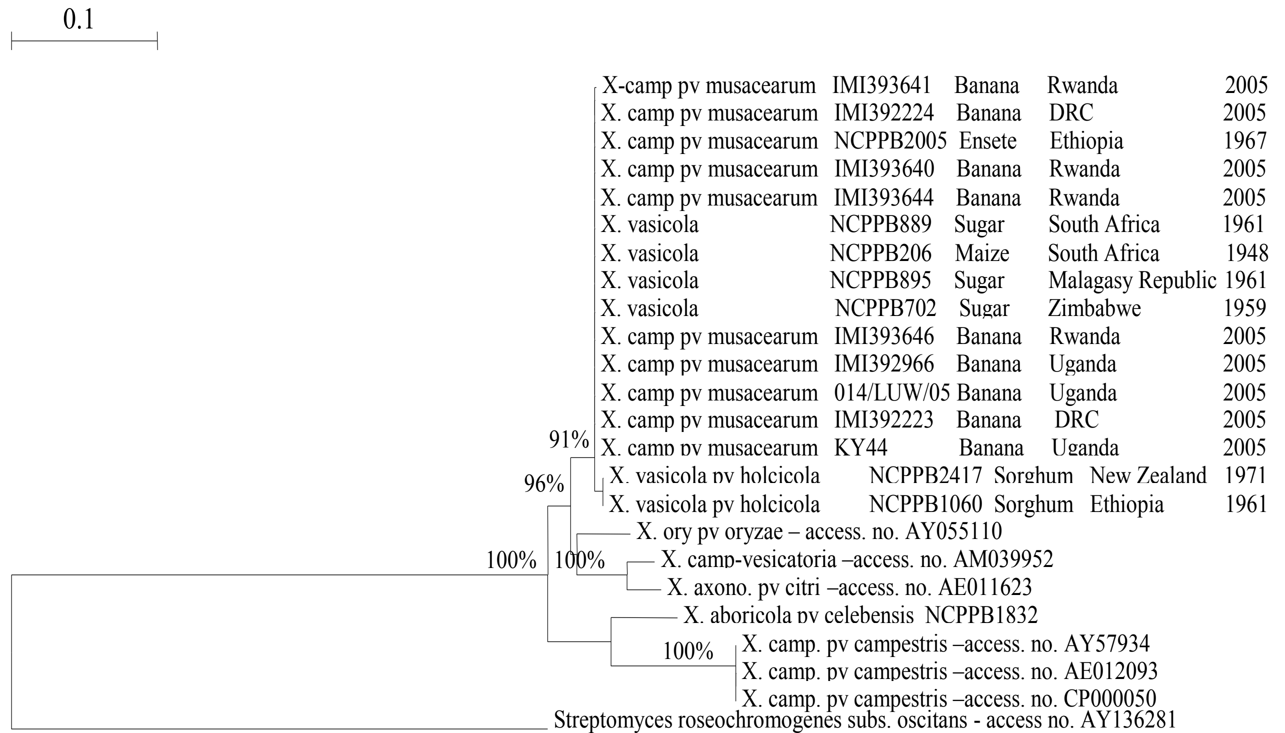

Fig. 2. Phylogram based on partial *GyrB* sequences. Note identity between *Xanthomonas campestris* pv. *musacearum* and sugarcane strains of *X. vasicola*, and their close similarity to *X. vasicola* pv. holcicola. Scale bar is substitutions/nucleotide. Percentages at nodes are bootstrap values from 1000 samplings. The sequence of *Streptomyces roseochromogenes* subsp. *oscitans* (AY136281) is used as the outgroup. Published with permission (Aritua et al., 2007b).