Isolation of "*Candidatus* Liberibacter" Genes by RAPD and New PCR Detection Technique

A. Hocquellet, J. M. Bové, and M. Garnier

ABSTRACT. The RAPD technique has been extensively applied to reveal polymorphism between closely related genomes. We have used it to isolate genes of the uncultured phloem-restricted bacterium "*Candidatus* Liberibacter" sp. of citrus huanglongbing (HLB). Using 102 random 10-mer primers, eight DNA bands were amplified only from HLB-infected plant DNA. These bands were cloned and analyzed. Six of them corresponded to fragments of the liberibacter genome as shown by sequence and hybridization experiments, indicating that the RAPD technique is appropriate to isolate genes from uncultured phloem-restricted plant bacteria. Four genes, *nus*G, pgm, omp, and an hypothetical protein gene, were identified in the DNA fragments; they will be used for further characterization of the bacterium. A PCR detection method based on the amplification of part of the ß operon of the two liberibacter species has been developed. Compared to the previously described PCR detection method in which the 16S ribosomal RNA gene was amplified and which required restriction of the amplified DNA to distinguish between "*Candidatus* Liberibacter asiaticus" and "*Candidatus* Liberibacter africanus", the new PCR approach allows direct identification of the two species.

In order to characterize the bacterium associated with citrus huanglongbing (HLB), the following genes of the bacterium have been isolated: (i) the highly conserved 16S ribosomal RNA (rRNA) gene obtained by PCR using universal primers (3); (ii) the more variable intergenic 16S/23S region which was also amplified and cloned (5); (iii) three other-HLB DNA fragments were isolated after random cloning of total DNA from infected plants (7). One of these fragments encoded several genes of the well-known ß operon; they corresponded to four ribosomal proteins (L1, L10, L11, and L12) and to the 5' end of the β subunit of the RNA polymerase gene (8).

Sequence analyses of the 16S rDNA and the ß operon revealed that the HLB bacterium was a new bacterial genus that we named "*Candidatus* Liberibacter" and in which two species could be defined "*Candidatus* Liberibacter asiaticus" and "*Candidatus* Liberibacter africanus". This work also resulted in the development of specific and sensitive detection techniques for the liberibacters by DNA/DNA hybridization and PCR (2, 4, 7). However, in order to better characterize the liberibacters, isolation of additional genes is needed.

For that purpose, the Random Amplified Polymorphic DNA (RAPD) technique (10) has been carried out on healthy and HLB-infected plant DNA. This method is able to reveal polymorphism in genomic DNA without any prior knowledge on the genome, as this PCR-based technique uses single arbitrary 10-mer primers.

Amplification of 16S-rDNA by PCR, allows detection of both liberibacter species. However, in order to distinguish between the two species, digestion of the amplified DNA with restriction enzyme *Xba*I must be carried out. This step is time consuming and difficult to achieve when little DNA is amplified in a given sample. This is why an alternative PCR method was developed. It is based on the amplification of part of the ß operon leading to one size product for "*Candidatus* L. asiaticus" and another for "*Candidatus* L. africanus".

"*Candidatus* Liberibacter" sp. gene isolation using RAPD as well as PCR detection of the two liberibacters with primers A2/J5 selected

in ribosomal protein genes *rpl*A and *rpl*J are presented in this paper.

RAPD amplification of DNA from healthy and "*Candidatus* Liberibacter"—infected plants. Out of 102 RAPD primers used to amplify DNA extracted from healthy or HLB-infected citrus or periwinkle plants, eight primers revealed additional PCR products on the profiles obtained when DNA from infected plants was used as compared to those obtained with DNA from healthy plants. Figure 1A shows the RAPD profiles obtained with one of these primers (P3).

Four bands (P3-Af, P3-As, A17-Af and C13-Af) evidenced with three primers (P3, A17, and C13) were further studied. They were specific of the profiles corresponding to infected plant DNA. Their size were approximately 700 bp, 340 bp, 550 bp and 1 kbp, respectively. Three of them were obtained from plants infected with "*Candidatus* L. africanus" and one from plants infected with "*Candidatus* L. asiaticus".

Analysis of the selected RAPD products. The selected RAPD fragments were reamplified, radiolabeled and used as probes in dot-blot hybridization against DNA extracted from healthy and infected citrus plants in order to verify that they corresponded to "*Candidatus* Liberibacter" DNA. In parallel, sequences of the different cloned fragments were determined and compared with those present in data banks.

When used as probe under high stringency conditions, fragment P3-Af hybridized strongly with DNA from "*Candidatus* L. africanus"-infected citrus plants but not with DNA extracted from healthy or "*Candidatus* L. asiaticus"-infected plants (Fig. 1B). This indicates that this PCR product is, indeed, DNA of "*Candidatus* L. africanus". The sequence of P3 AF (662 bp) had no homology with any of the sequences in the GenBank database.

Similarly, P3-As hybridized with DNA from citrus infected with different strains of "*Candidatus* L. asiaticus" Poona (India), Lipa City (Philippines), Nakom Pathom (Thailand), Taipei (Taiwan) and Fuzhou

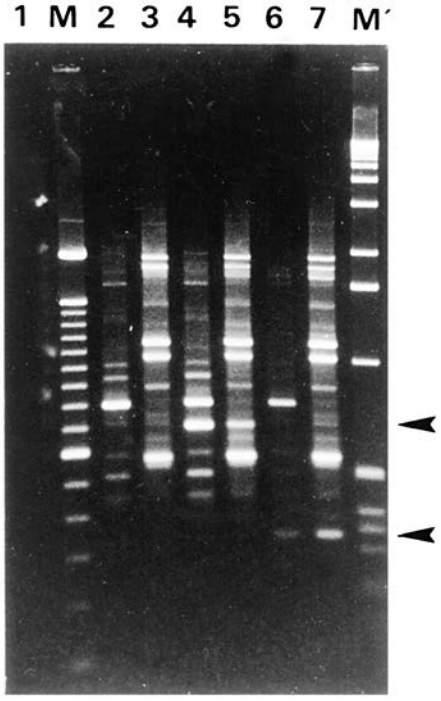
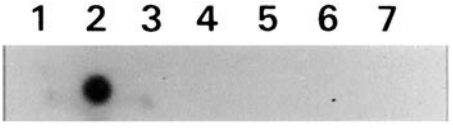


Fig. 1. A. RAPD profile obtained with primer P3 (A). Amplifications were performed from water (lane 1), DNA from healthy periwinkle (lane 2), and citrus (lane 3); DNA from "*Candidatus* L. africanus"-infected periwinkle (lane 4) and citrus (lane 5); DNA from "*Candidatus* L. asiaticus"-infected periwinkle (lane 6) and citrus (lane 7). M: size marker.



B. Dot blot hybridization of probe P3Af with DNA extracted from healthy citrus (1), citrus infected with "*Candidatus* L. africanus" (2), citrus infected with "*Candidatus* L. asiaticus" from India (3), the Philippines (4), Thailand (5), Taiwan (6) and China (7).

(People's Republic of China). The strongest signal was observed with the homologous strain, Poona, the faintest with the Thai strain. Whether this is due to polymorphism or to low concentration of liberibacter in citrus infected with the Thai strain is not known. P3-As also hybridized very faintly with DNA extracted from "*Candidatus* L. africanus"-infected citrus plants. No hybridization was obtained with healthy plant DNA. This result suggested that this RAPD product was a DNA fragment of the liberibacter genome. This was confirmed by sequence analysis. Indeed, one putative ORF encoding 94 amino acids exhibited homology with part of the "*Candidatus* L. asiaticus" *nus*G gene that we have partially cloned and sequenced previously (GenBank accession number: M94319) (8). The P3-As sequence extends the previously *nus*G gene sequence of "*Candidatus* L. asiaticus" at the 5'end.

Probe A17-Af generated a strong hybridization signal with DNA extracted from "*Candidatus* L. africanus"-infected citrus plant DNA and a very weak one with other DNAs. Sequence analysis, revealed a truncated putative ORF encoding a peptide of 173 amino acids with strong homology (79%) with a phosphoglucomutase (PGM) of *Agrobacterium tumefaciens*, a bacterium belonging to the α proteobacteria phylogenetic group. This enzyme is also a plant enzyme which explains the faint hybridization with healthy plant DNA.

Finally, C13-Af, hybridized strongly only with DNA from "*Candidatus* L. africanus"—infected plants. This clone was 1,114 bp in length and contained two putative ORFs. The first, located at the 5' moiety encoded a 159 putative amino acid peptide with 50% homology with hypothetical proteins of unknown function sequenced from *Haemophilus influenza* and *Escherichia coli*. The second putative ORF, encoded a peptide of 173 amino acids sharing 57% homology with the N-terminal part of a type 1 outer membrane protein (OMP) of *Brucella abortus*, another α–Proteobacterium.

Thus, RAPD is an efficient method to obtain DNA from uncultured bacteria. The bands revealed with the five other primers will be analyzed in the same way and it is likely that more liberibacter genes will be isolated.

PCR detection of "*Candidatus* Liberibacter" sp. by amplification of genes in the ß operon with primer A2/J5. Comparison of the *rpl*KAJL-*rpo*BC operon (ß operon) sequences of the two liberibacter species contained in the cloned fragment In 2.6 of "*Candidatus* L. asiaticus", strain Poona (8) GenBank accession number: M94319 and AS 1.7 of "*Candidatus* L. africanus", strain Nelspruit (6) GenBank accession number: U09675 has shown that they are quite different especially within the intergenic regions which have not only different sequences, but also different lengths. This provides a way to distinguish the two liberibacter species after PCR amplification by the size of the PCR-amplified DNA. However, genes for ribosomal proteins are also part of the plant genome and considerable care must be taken to design primers which do not amplify host genes, or those of other bacteria. Several primer combinations were tested and many were found inadequate in that they give multiple bands or amplifications with certain healthy citrus species. Finally, two primers located in genes *rpl*A and *rpl*J could be selected. Primer A2 5'-TATAAAGGTTGACCTTTCGAGTTT-3' is at the 3'-end of the *rpl*A gene (position 869-892 on the In 2.6 sequence, and in position 22-45 on the AS 1.7 sequence). Primer J5 5'-ACAAAAGCAGAAATAGCACGAACAA-3', is at the 3'-end of the *rpl*J gene (position

1547-1571 on the In 2.6 sequence and position 666-690 on the AS 1.7 sequence).

The primers were used in a PCR reaction containing in 50 µl, 1 µM of each primer, 200 µM of each of the four dNTPs, 78 mM Tris-HCl pH 8.8, 17 mM $(NH4)_2SO_4$, 10 mM b-mercaptoethanol, 2 mM $MgCl_2$, 0.05% W1 detergent (Gibco BRL), 200 µg. ml-1 BSA and 2.5 units of Taq polymerase (Gibco BRL). Two microliters of "wizard extract" (4) or various amounts of plant DNA were used as template in the reaction. Amplification was carried out in a thermocycler (Thermojet, Eurogentec) with the following program: 35 cycles each at 92°C for 20 s, 62°C for 20 s and 72°C for 45 s. As controls, primers OA1, OI1 and OI2c were used to amplify the "*Candidatus* Liberibacter" 16S rDNA as described previously (4).

Eight µl of each PCR reaction mixture were analyzed by electrophoresis on 2% agarose gel for *rpl*A-*rpl*J amplification or on 0.7% agarose gels for 16S rDNA amplification in 1× Tris-Acetate-EDTA buffer and visualized by ethidium bromide staining.

Figure 2 shows the result of PCR with the primer pair A2/J5 on "wizard extracts" obtained from healthy sweet orange plants or from sweet orange plants infected with different geographical strains of the two "*Candidatus* Liberibacter" species, maintained in the greenhouse. A band of about 650 bp was obtained with extracts from "*Candidatus* L. africanus", strain Nelspruit, South Africa (lane 3) and strain Mauritius 23 (lane 8)-infected plants. A band around 700 bp was amplified from plants infected with different strains of "*Candidatus* L. asiaticus": Poona (India) (lane 4), Fuzhou (People's Republic of China) (lane 5), Lipa City (Philippines) (lane 6), Sarawak (Malaisia) (lane 7), Mauritius strain 79 (lane 9), Nepal (lane 10), Reunion (lane 11), Taipei (Taiwan) (lane 12), Nakhom Pathom (Thailand) (lane 13) and Vietnam (lane 14). No amplification was observed when water (lane 1) or extract of healthy sweet orange (lane 2) were used. The sizes of the PCR products obtained with primers A2/J5 were in agreement with the sizes calculated from the ß operon sequences of the two "*Candidatus* Liberibacter" species: 669 bp for "*Candidatus* L. africanus" strain Nelspruit and 703 bp for "*Candidatus* L. asiaticus" strain Poona.

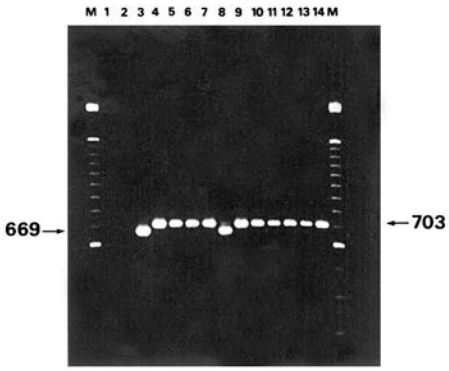


Fig. 2: Agarose gel electrophoresis of the DNA amplified with primers A2 and J5 from: Water (lane 1), healthy citrus extracts (lane 2), extracts from citrus infected with "*Candidatus* L. africanus", strain Nelspruit South Africa (lane 3) and strain Mauritius 23 (lane 8), or "*Candidatus* L. asiaticus", strains Poona, India (lane 4), Fuzhou, China (lane 5), Lipa city, the Philippines (lane 6), Sarawak, Malaysia (lane 7) Mauritius (lane 9), Nepal (lane 10), Réunion (lane 11), Taiwan (lane 12), Nakhom Pathom, Thailand (lane 13), Vietnam (lane 14). M: 100bp marker (Gibco, BRL).

Specificity of the amplification for "*Candidatus* Liberibacter". The specificity of the amplification with primer A2/J5 for the HLB liberibacters was evaluated using DNA extracted from other citrus-associated bacteria such as *Xylella fastidiosa*, *Xanthomonas axonopodis* pv. citri, *Acinetobacter lwoofi*, *Spiroplasma citri*; or "*Candidatus* Phytoplasma aurantifolia". PCR was also performed on

DNA extracted from *Agrobacterium tumefaciens*, a phytopathogenic bacterium belonging to the same phylogenetic cluster as "*Candidatus* Liberibacter", and with *Escherichia coli* or the stolbur phytoplasma. Amplification products of the expected size were obtained only with extracts from "*Candidatus* L. africanus" or "*Candidatus* L. asiaticus"-infected plants. No amplification was observed from water, or from extracts of the other bacteria tested (result not shown). In order to verify that no PCR inhibitors were present in the extracts from which no amplification was obtained, a PCR reaction in which 16S rDNA bacterial universal primers fd1/rp1 (9) was carried out. In all cases, a 1,500 bp band was obtained (data not shown).

Sensitivity of the PCR test for "*Candidatus* Liberibacter" detection. Various amounts of DNA (10 ng to 0.001 ng) purified from "*Candidatus* L. africanus" or "*Candidatus* L. asiaticus"-infected plants were used for amplification with primers A2/J5. In both cases, a band was still visible when 0.01 ng of DNA was used as template. No amplification was observed with water or with up to 10 ng of healthy citrus DNA. When the "16S rDNA PCR" test (4) was performed on the same DNA samples, a PCR product of 1,160 bp was obtained with 0.1 ng of "*Candidatus* L. africanus" DNA or "*Candidatus* L. asiaticus" DNA but not with 0.01ng. These results indicate that the PCR test based on amplification of the *rpl*A-*rpl*J genes seems slightly more sensitive than the test based on the amplification of the 16S rDNA.

Amplification from samples infected with both "*Candidatus* Liberibacter" species. When "wizard extracts" prepared from an equal amount of midribs containing either one or the other of the two liberibacter species, were used for amplification with primers A2/J5, a band corresponding to each species was obtained (Fig. 3, lane 6): the upper band corresponding to "*Candidatus* L. asiaticus" (lane 9); and the lower band corresponding to "*Candidatus* L. africanus" (lane 3). When different proportion of midribs (1/10, 1/50) were mixed together, the intensity of the PCR band corresponding to the species in lower amount decreased proportionally: lanes 5 and 7 for 1:10; and lanes 4 and 8 for 1:50 mixtures, respectively.

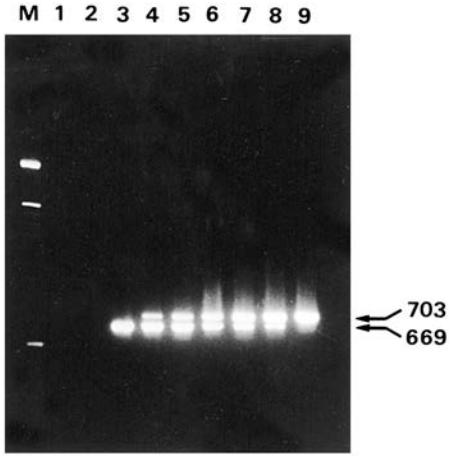


Fig. 3: Agarose gel electrophoresis of the DNA amplified with primers A2 and J5 from water (lane 1), healthy citrus extracts (lane 2) mixed-infected extracts with "*Candidatus* L. africanus" and "*Candidatus* L. asiaticus" in the following proportions 100/0 (lane 3) 50/1 (lane 4), 10/1 (lane 5) 50/50 (lane 6) 1/10 (lane 7) 1/50 (lane 8) 0/100 (lane 9).

Primers A2/J5 have been tested in comparison with primers OI1/OI2c/OA1 on field sample extracts from several different Asian and African countries (2). The sensitivity is equivalent or even slightly higher with primers A2/J5. In addition, because the amplified product is smaller than with the ribosomal primers, the PCR is somehow less affected by DNA degradation in the sample.

Note added in proof: Results from this paper are described in more details in publications 1 and 2.

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