Techniques for the Specific Detection of the Two Huanglongbing (Greening) Liberobacter Species: DNA/DNA Hybridization and DNA Amplification by PCR

S. Jagoueix, J. M. Bové, and M. Garnier

ABSTRACT. Based on the 16S rDNA sequences, as well as the sequence of the *rpl*KAJL-*rpo*BC operons (β operon) leading to probes In 2.6 and AS 1.7, two liberobacter species causing citrus Huanglongbing (greening) have been recognized. They are Liberobacter asiaticum and Liberobacter africanum, respectively. The two liberobacter species can be detected specifically in infected citrus trees by DNA-DNA hybridization using probe In 2.6 for L. asiaticum and probe AS 1.7 for L. africanum. A PCR detection method has also been developed with primers derived from the 16S rDNA sequences. These primers allow amplification of the 16S rDNA of the two liberobacter species, and do not amplify DNA from other citrus pathogenic bacteria or bacteria which might occasionally be present on citrus samples. When digested by *Xba*I, the DNA amplified by PCR from a Huanglongbing-infected tree yields a restriction profile of DNA specific for L. asiaticum or L. africanum.

The bacterium associated with citrus Huanglongbing (HLB) (Greening) has recently been taxonomically characterized by studying the nucleotide sequence of its 16S ribosomal RNA gene (rDNA) (5). This work showed that the HLB bacterium belongs to the alpha subdivision of the proteobacteria where it represents a new "*Candidatus*" genus to which we have given the name Liberobacter. In addition, we have described two "*Candidatus*" species: Liberobacter asiaticum, present in Asia and transmitted by the Asian psyllid *Diaphorina citri* Kuwayama and Liberobacter africanum, present in Africa and transmitted by the African psyllid *Trioza erytreae* (Del Guercio) (3, 5). Both Liberobacter species as well as both psyllid vectors are present in Reunion and Mauritius islands (4).

In 1992, a DNA probe, In 2.6, containing genes of the well known bacterial β operon was selected for the detection of L. asiaticum (2, 10) and, in 1995, a similar probe, AS 1.7, was obtained for the detection of L. africanum, by PCR amplification of the corresponding genes in the β operon of L. africanum (8). From the 16S rDNA sequences of the two liberobacter species, we have defined specific primers for PCR amplification of the DNA of the two species (6). Detection of the HLB liberobacters by DNA/DNA hybridization with probe In 2.6 and AS 1.7 and by PCR are presented.

Fig. 1 shows the results of dot-blot hybridization at high stringency of probes AS 1.7 (Panel I) and In 2.6 (Panel II) with the DNA extracted from healthy (A1, A2, B 1 to 7) or Liberobacter-infected citrus midribs from South Africa (A3 and C1 to 7), India (A4), China (A5), Taiwan (A6), and the Philippines (A7). These results show that each probe is able to recognize the homologous liberobacter species. However, despite the high stringency conditions, a faint hybridization signal is observed with the heterologous species when large amounts of DNA (from 1 to 10 µg) are deposited onto the membrane. This is not surprising as the two probes share 74% nucleotide homology (5, 8). No hybridization signals were observed with DNA extracted from healthy citrus even when large amounts of DNA were blotted on the membrane (A1, A2 and B1 to 7).

Fig. 2 shows the results of DNA amplification by PCR using primers

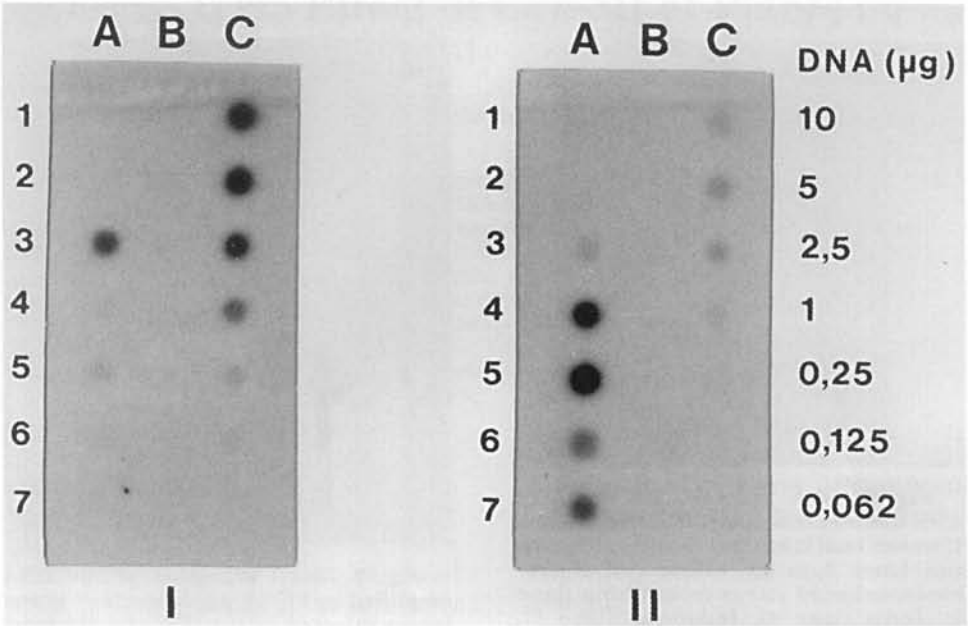

Fig. 1. Dot blot hybridization at high stringency of probes AS 1.7 (panel I) and In 2.6 (panel II) with the DNA extracted from healthy citrus plant (A1, A2, and B1 to 7) and from liberobacter-infected citrus plant from South Africa, (A3 and C1 to 7); India (A4), China (A5), Taiwan (A6), and the Philippines (A7).

defined on the sequence of the 16S rDNA. No amplification is obtained when water or DNA extracted from healthy citrus is present in the PCR mixture (lanes 1 and 2). Amplified DNA of the expected size (i.e. 1,160 bp, and corresponding to the DNA length between the two primers) is observed not only with citrus infected by L. asiaticum from China (lane 3), India (lane 4), Indonesia (lane 5), Nepal (lane 6), the Philippines (lane 7), Taiwan (lane 8) but also with L. africanum from South Africa (lane 9) and Zimbabwe (lane 10).

The specificity of the amplification reaction for the HLB liberobacters has been studied by using as a PCR template a mixture of DNA extracted from the following bacteria: *Xanthomonas campestris* pv. *citri,* the agent of citrus canker, *Xylella fastidiosa*, responsible for citrus variegated chlorosis, *Spiroplasma citri,* the agent of citrus stubborn disease, Phytoplasma aurantifolia (witches' broom disease of lime), *Acinetobacter lwoffii*, sometimes found associated with citrus, and *Agrobacterium tumefaciens*, a plant pathogenic bacterium belonging to the same phylogenetic group as Liberobacter spp. As expected, amplification of a 1,500 bp DNA band occurred when universal primers for the amplification of procaryotic 16S rDNA were used, but no amplification was observed with the liberobacter-specific primers that we have defined (6).

PCR is a very rapid procedure, but it gives rather poor results when performed directly on crude plant homogenates, as these contain inhibitors of the Taq DNA polymerase used during the PCR reaction. This is why DNA is generally purified from the plant to be tested before performing PCR. Extraction of DNA is a lengthy procedure, and, therefore, we have developed a quick method to prepare plant extracts suitable for DNA amplification by

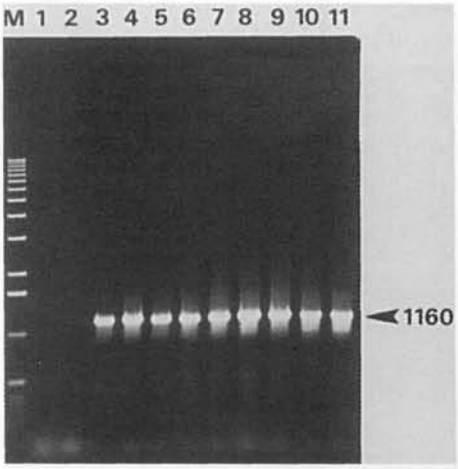

Fig. 2. DNA amplification by PCR with liberobacter specific primers. Lane 1: water; lane 2: extract from healthy citrus; lanes 3 to 10: extracts of liberobacter-infected citrus from China (lane 3), India (lane 4), Indonesia (lane 5), Nepal (lane 6), the Philippines (lane 7), Taiwan (lane 8) and extracts of L. africanum from South Africa (lane 9) and Zimbabwe (lane 10). M: 1 kb ladder (Gibco).

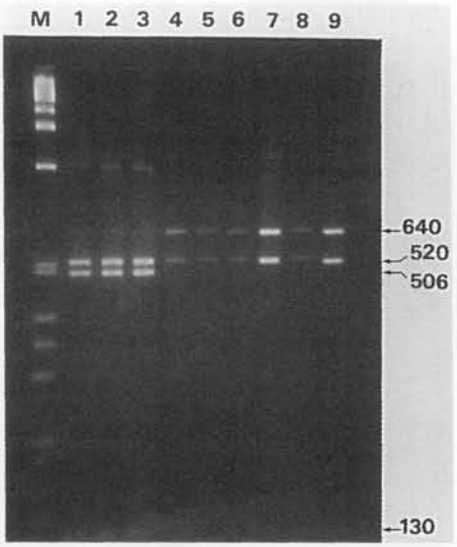

Fig. 3. Xba I digestion of the DNA amplified by PCR from extracts of citrus leaves infected by the liberobacter from South Africa (lanes 1, 2), Zimbabwe (lane 3), China (lane 4), India (lane 5), Indonesia (lane 6), Nepal (lane 7), the Philippines (lane 8), Taiwan (lane 9), M: 1 kb ladder (Gibco).

PCR. The method is based on proteinase K and SDS treatment of citrus midribs, followed by trapping of the extracted DNA on a silica resin (Promega) from which it is recovered by elution with hot water. Using this procedure, results consistent with those of hybridization and/or EM have been obtained for the detection of the two HLB liberobacters species.

The sensitivity of the PCR reaction for the detection of the liberobacters has been assessed with samples containing different amounts of healthy and Liberobacter-infected midribs, collected on citrus seedlings from the greenhouse. A DNA band was still observed when one midrib (about 3 cm long) from an infected leaf and 10 midribs (each about 10 cm long) from healthy leaves were combined for quick extraction of DNA (as described above), before performing PCR.

Even though the rDNA amplified from the two liberobacter species has the same size (1,160 bp), we were able to distinguish between L. asiaticum and L. africanum, knowing that, as shown by DNA sequencing, the DNA amplified from L. asiaticum has only one *Xba* I restriction site, yielding two *Xba* I fragments of 520 bp and 640 bp (Fig. 3, lanes 4 to 9); whereas the DNA amplified from L. africanum has an additional *Xba* I restriction site, yielding three fragments of 520 bp, 506 bp and 130 bp (Fig. 3, lanes 1, 2, 3). With DNA amplified from a mixture of L. asiaticum and L. africanum, the *Xba* I restriction pattern shows that, as expected, four bands (640 bp, 520 bp, 506 bp and 130 bp) appeared (4).

CONCLUSION

The two liberobacter species, L. asiaticum and L. africanum, can be detected by DNA/DNA hybridization with specific probes, In 2.6 and AS 1.7 respectively, as well as by DNA

amplification by PCR. Restriction of the amplified DNA with restriction enzyme *Xba* I allows identification of the liberobacter species present. We have applied these techniques to the detection of the liberobacter species present in orchard trees in various countries of Asia and Africa (1, 4, 7, 9).

LITERATURE CITED

1. Bové, J. M., Nguyen Minh Chau, Ha Minh Trung, J. Bourdeaut, and M. Garnier
1996. Huanglongbing (greening) in Vietnam: detection of Liberobacter asiaticum by DNA-hybridization with probe In 2.6 and PCR-amplification of 16S ribosomal DNA, p. 258-266. *In*: Proc. 13th Conf. IOCV., IOCV, Riverside
2. Bové, J. M., M. Garnier, Y. S. Ahlawat, N. K. Chakraborty, and A. Varma
1993. Detection of the Asian strains of the greening BLO by DNA-DNA hybridization in Indian orchard trees and *Diaphorina citri* psyllids, p. 258-263. *In*: Proc. 12th Conf. IOCV. IOCV, Riverside.
3. Garnier, M. and J. M. Bové
1996. Distribution of the Huanglongbing (greening) Liberobacter species in fifteen African and Asian countries, p. 388-391. *In*: Proc. 13th Conf. IOCV., IOCV, Riverside.
4. Garnier, M., S. Jagoueix, P. Toorawa, M. Grisoni, R. Mallessard, A. Dookun, S. Saumtally, J. C. Autrey, and J. M. Bové
1996. Both Huanglongbing (greening) Liberobacter species are present in Mauritius and Reunion, p. 392-394. *In*: Proc. 13th Conf. IOCV., IOCV, Riverside.
5. Jagoueix, S., J. M. Bové, and M. Garnier
1994. The phloem-limited bacterium of greening disease of citrus is a member of the α subdivision of the proteobacteria. J. Intern. Syst. Bacteriol. 44: 397-386.
6. Jagoueix, S., J. M. Bové, and M. Garnier
1996. PCR detection of the two liberobacter species associated with greening disease of citrus. Mol. Cell. Probes 10: 43-50.
7. Korsten, L., S. Jagoueix, J. M. Bové, and M. Garnier
1996. Huanglongbing (greening) detection in South Africa, p. 395-398. *In*: Proc. 13th Conf. IOCV., IOCV, Riverside.
8. Planet, P., S. Jagoueix, J. M. Bové, and M. Garnier
1995. Detection and characterization of the African citrus greening liberobacter by amplification, cloning and sequencing of the *rpl*KAJL-*rpo*BC operon. Curr. Microbiol. 30: 137-141.
9. Regmi, C., M. Garnier, and J. M. Bové
1996. Detection of the Huanglongbing (greening) liberobacter in Nepal by DNA-DNA hybridization, p. 267-270. *In*: Proc. 13th Conf. IOCV., IOCV, Riverside.
10. Villechanoux, S., M. Garnier, J. Renaudin, and J. M. Bové
1992. Detection of several strains of the bacterium-like organism of citrus greening disease by DNA probes. Curr. Microbiol. 24: 89-95.