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Detection and Characterization of the African Citrus Greening Liberobacter by Amplification, Cloning, and Sequencing of the *rpl*KAJL-*rpo*BC Operon

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Abstract. Greening disease of citrus is caused by a phloem-restricted, uncultured bacterium, recently characterized and named *Liberobacter*. As shown previously, a probe encoding ribosomal protein genes (*rpl*KAJL-*rpo*BC operon) from an Asian liberobacter could detect all Asian liberobacter strains tested, but not African strains. Using the sequence of the *rpl*KAJL-*rpo*BC operon of the Asian liberobacter strain from Poona (India), we have defined primers for PCR amplification of the equivalent genes of an African liberobacter strain. The amplified fragment was cloned in pUC18 and successfully used as a probe to detect African liberobacter strains by Southern and dot hybridizations. Sequence comparisons of the African and Asian liberobacter operons indicate that they represent two different species in the proposed genus *Liberobacter*.

Greening is a severe and widely distributed disease of citrus [3]. For many years, two forms of the disease have been recognized: an Asian form, relatively heat tolerant, because symptoms of the disease occur at temperatures well above 30°C, and an African form, heat sensitive, with no symptoms above 30°C [1]. A bacterial-like organism (BLO), which could not be cultured so far, is associated with the disease [4, 5]. For phylogenetic purposes, we have recently cloned and sequenced the 16S rDNA of an Asian and an African strain of the BLO [8]. The two organisms have been found to belong to a new phylum in the α-proteobacteria, and the name *Liberobacter* has been proposed for these organisms [8]. This is the first time that uncultured plant pathogenic bacteria have been phylogenetically characterized, but criteria for defining species in this new phylum are lacking.

In 1992, using total DNA from greening-infected periwinkle midribs, we were able to clone a 2.6-kbp liberobacter DNA fragment representing the *rpl*KAJL-*rpo*BC operon and coding in particular for four ribosomal proteins [14, 15]. The liberobacter strain used in this work was an Asian strain from India (Poona strain). When the 2.6-kbp fragment (In-2.6) was used as a probe in Southern or dot hybridizations, hybridization signals were observed at high stringency with all Asian strains, but not with the African strain tested. Only when moderate hybridization stringencies were used did hybridization also occur with the African strain, but a restriction fragment length polymorphism was revealed [15]. Because the *rpl*KAJL-*rpo*BC operon codes for conserved proteins (ribosomal proteins), it might provide taxonomic information for organisms with close phylogenetic relationships such as the Asian and African liberobacters. Also, in order to develop a probe for the detection of African strains, we have now PCR amplified, cloned, and sequenced a 1676-bp fragment of the *rpl*KAJL-*rpo*BC operon of an African strain (Nelspruit strain) of the liberobacter.

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Materials and Methods

Plant material. Healthy periwinkle (*Catharantus roseus*) and sweet orange (*Citrus sinensis*) seedlings as well as such plants infected with a liberobacter strain from South Africa (Nelspruit strain) were grown in a greenhouse at 25°C during the day and 20°C at night. Those infected with heat-tolerant Asian strains were grown at 30°C in the day and 25°C at night. We have described previously the origin of the strains [14] and their experimental transmission to periwinkle plants [2].

Bacterial strain and cloning vector. *Escherichia coli* TG1 [12] was used as the host for cloning. Plasmid pUC18 was the cloning vector.

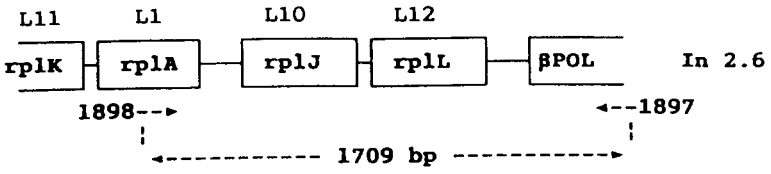

Fig. 1. Schematic representation of gene arrangement in In-2.6. Location of the PCR primers is indicated by arrows.

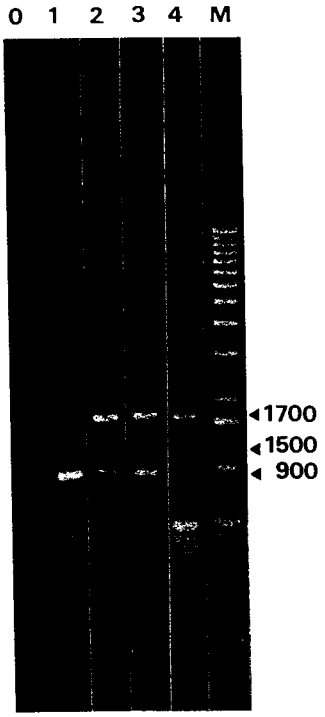

Fig. 2. Agarose gel electrophoresis of PCR products amplified with primers 1898–1897 from water (track 0), or from DNAs extracted from healthy periwinkle midribs (track 1), periwinkle midribs infected with the liberobacters from Nelspruit (track 2), Poona (track 3), and from recombinant M13-replicative form containing In-2.6 (track 4). M: 1 kb ladder (Gibco BRL).

Extraction of DNA from healthy and greening-infected plants. DNA was extracted from leaf midribs by the CTAB method of Murray and Thompson [10].

PCR amplification of a fragment of the *rpl*KAJL-*rpo*BC operon of the Nelspruit liberobacter. Two primers defined from the sequence of In-2.6 of the Poona liberobacter (Indian strain) [15] were used. They have the following sequences: Forward primer fp 1898: 5′-GTC ATC GCA TAT GGG ATG TGG-3′ (positions 848–868); reverse primers rp 1897: 5′-CGT CTT TAG TCA TAA GAG G-3′ (positions 2557–2539). When these primers are used with Poona liberobacter DNA, the theoretical size of the amplified DNA is 1709 bp. The location of the primers is indicated in Fig. 1. PCR was done according to [11] in a DNA thermal cycler (Perkin-Elmer Cetus) with 2.5 units of Taq polymerase (Gibco BRL) for 50 μl of reaction mixture containing 1 μl of DNA. Amplification was for 30 cycles, each composed of 60 s at 92°C, 30 s at 47°C, and 90 s at 72°C. The last cycle was followed by chain extension for 10 min at 72°C.

Cloning of the amplified DNA. The 50 μl containing the amplified DNA were adjusted to 200 μl with sterile water, phenol extracted, and the DNA precipitated with ethanol. The precipitate was resuspended in 10 μl of sterile water and phosphorylated with T4 polynucleotide kinase (Pharmacia) according to the manufacturer's instructions. Blunt ends were obtained with the Klenow fragment of DNA polymerase I. The fragments were cloned in approximately 1 μg of pUC18 plasmid DNA, restricted with *Sma*1, and the ligation mixture was used to transform competent *E. coli* TG1 cells as described in [7, 8].

Sequencing. DNA was sequenced by the method of Sanger [13] with the T7 sequencing kit (Pharmacia). The sequence was aligned with that of the Poona liberobacter by use of Nucaln software. The deduced protein sequences from the Nelspruit liberobacter were compared with those from the Poona liberobacter with Sequaid software. The sequence of AS-1.7 of the African liberobacter has been deposited in GeneBank data base (accession number U09675).

Southern and dot hybridizations. Conditions for Southern and dot hybridizations at high or intermediate stringencies have been described elsewhere [15].

Results

Amplification and cloning of a fragment of the *rpl*KAJL-*rpo*BC operon of the Nelspruit liberobacter. Results of PCR amplifications with primer pair 1898–1897 are shown in Fig. 2. As expected, a clear DNA band of about 1700 nucleotides was obtained when amplification was carried out with the recombinant M13 replicative form containing In-2.6 from the Poona liberobacter [14] (track 4). A similar band was obtained when DNAs from periwinkle plants infected with either the Poona or the Nelspruit liberobacters (tracks 3 and 2 respectively) were used as templates. However, an additional band of about 950 bp was also present. This band was similarly observed in track 1, where amplification was performed with DNA from healthy periwinkle plants. Therefore, this 950-bp band probably results from the amplification of the equivalent ribosomal protein genes from the plant operon. Finally, an additional faint band of about 1500 bp was present in the amplified DNA from Nelspruit liberobacter-infected periwinkle. It probably corresponds to hybridization of the primer(s) at another position on the DNA.

The total amplified DNA, of which an aliquot was used for Fig. 2, track 2, was cloned in pUC 18. Seventeen clones were shown to contain inserts of either one of the three sizes revealed in Fig. 2, track 2.

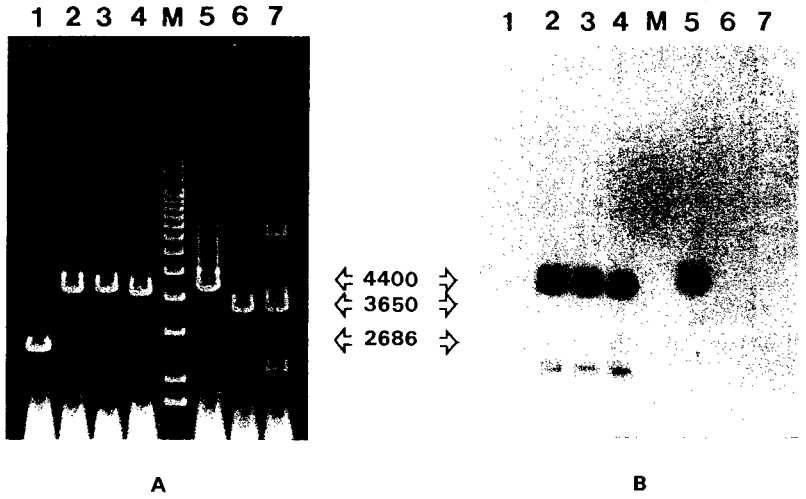

Fig. 3. Panel A. Agarose gel electrophoresis of various *Eco*RI restricted plasmids. Panel B. Southern hybridization between In-2.6 as probe and the plasmids of panel A. Native pUC 18 (track 1); recombinant pUC18 plasmids with inserts of 1700 bp (tracks 2, 3, 5), 1500 bp (track 4), and 950 bp (tracks 6, 7). M: 1 kb ladder.

This is illustrated on Fig. 3A for six of the clones. Plasmids of tracks 2, 3, and 5 contain a 1700-bp insert, plasmid of track 4 a 1500-bp insert, and plasmids of tracks 6 and 7 a 950-bp insert. After Southern hybridizations at moderate stringency with In-2.6 as the probe (Fig. 3B), hybridization signals were obtained with the DNAs from tracks 2, 3, 4, and 5, but not with those of tracks 6 and 7. This indicates that the 1700 and 1500 bp inserts are probably DNA fragments from the Nelspruit liberobacter, while that of 950 bp is of plant origin.

Sequence of the 1700-bp fragment. For confirmation that the 1700-bp fragment is part of the *rpl*KAJL-*rpo*BC operon of the Nelspruit liberobacter, its sequence was determined. The total number of nucleotides is 1676, and this fragment will be called AS-1.7. Two complete and two incomplete potential open reading frames (ORFs) were observed. Their comparison with the ORFs of In-2.6 from the Poona liberobacter revealed that they correspond to the 3′ end of ribosomal protein L1, to the complete genes for ribosomal proteins L10 and L12, and to the 5′ end of RNA polymerase β subunit gene, as expected from the primers that were used for PCR (Fig. 1). The percentage of G + C in AS-1.7 is 36.6, while that in In-2.6 is 39.1. The putative Shine Dalgarno ribosomal binding sequences identified previously in In-2.6 [15] were also found in AS-1.7 and are 100% homologous. The overall nucleotide homology between the two fragments is 74.2%. This relatively low homology for similar organisms explains why no hybridization was observed when In-2.6 was hybridized at high stringency to DNA from Nelspruit liberobacter-infected plants. As shown in Table 1, DNA homology is much lower in the intergenic regions than in the ORFs.

Table 1. Percentage of homology between nucleotide and protein sequences in AS-1.7 and In-2.6

Genes	Percentage of nucleotide homology between the genes in AS 1.7 and In 2.6	Percentage of amino acid homology between proteins in AS 1.7 and In 2.6
3′end of *rpl*A (L1)	93	
Intergenic region L1/L10	55	
*rpl*J (L10)	80.8	84.8
Intergenic region L10/L12	57.1	
*rpl*L (L12)	78.1	90.1
Intergenic region	66.7	
5′end of RNA polymerase β subunit	89.6	94.1

Indeed, the intergenic regions L1/L10, L10/L12, and L12/β show respectively 55%, 57.1%, and 66.7% homology, while the two complete ORFs *rpl*J (L10) and *rpl*L (L12) have respectively 80.8% and 78.1% homology. In addition, the intergenic region L1/L10 in AS-1.7 has a 32-nucleotide deletion and that between L12 and β is 22 nucleotides shorter than that of the Poona liberobacter. When the amino acid sequences of the two complete ORFs, *rpl*J (L10) and *rpl*L (L12) of AS-1.7 as deduced from the nucleotide sequence, were compared with those of In-2.6, amino acid homology was 84.8% for protein L10 and 90.1% for protein L12.

Hybridization of AS-1.7 with African and Asian liberobacter strains at high and moderate stringencies. When AS-1.7 was used as the probe in Southern hybridizations (Fig. 4) with *Hin*dIII-restricted DNA from healthy periwinkle (track 1) or citrus (track 4) plants, no hybridization signals were obtained whether

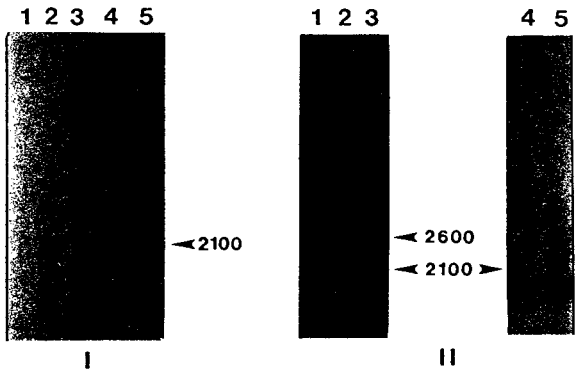

Fig. 4. Southern hybridizations at high (panel I) or moderate (panel II) stringencies between AS-1.7 as the probe and *Hin*dIII-restricted DNAs extracted from healthy periwinkle plants (track 1), Nelspruit (track 2)-, or Poona (track 3)-infected periwinkle plants, healthy citrus plants (track 4), Nelspruit-infected citrus plants (track 5).

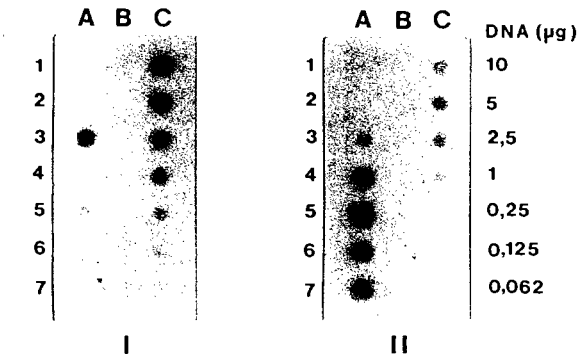

Fig. 5. Dot blot hybridizations at high stringency between probes AS-1.7 (panel I) or In-2.6 (panel II) and DNA extracted from healthy periwinkle plants (A, 1 and 2 and B, 1 to 7) or periwinkle plants infected with liberobacter strains from Nelspruit (A, 3), Poona (A, 4), Fujian, China (A, 5), Thaïland (A, 6) and the Philippines (A, 7). The amount of DNA deposited on tracks B and C of both panel I and II is indicated; 5 µg of DNA was deposited in track A.

hybridization was carried out at high (panel I) or intermediate (panel II) stringencies. At high stringency, a DNA band of 2.1 kbp was observed with DNA from periwinkle or citrus plants infected with the African Nelspruit strain (panel I, tracks 2 and 5); a faint DNA band of about 3 kbp was also present. No hybridization occurred with DNA from periwinkle or citrus plants infected with the Asian Poona strain (panel I, track 3). At intermediate stringency (panel II), the same 2.1-kbp and 3-kbp bands were revealed with DNA from periwinkle and citrus plants infected with the African Nelspruit strain (tracks 2 and 5), and a 2.6-kbp fragment was now present on track 3, where DNA from Poona-infected periwinkle plants was used.

Dot hybridizations at high stringency between In-2.6 or AS-1.7 and DNA from periwinkle plants infected with Asian or African strains of the greening liberobacter are illustrated in Fig. 5. It shows that AS-1.7 hybridized with the DNA of the African Nelspruit strain (Panel I, A3 and C1 to C6). A strong positive signal was still observed with 1 µg of plant DNA spotted onto the membrane (Panel 1, C4). On the contrary, no hybridization was observed with DNA from healthy (A1, A2, and B1 to B7) or periwinkle plants infected with Asian liberobacter strains from India (A4), China (A5), Thailand (A6), and the Philippines (A7). As expected from previous work [14], opposite results were obtained when In-2.6 was used as the probe; In-2.6 hybridized with DNA from periwinkle plants infected with all Asian strains tested (Panel II, A4 to A7), but it gave no or only a faint signal with DNA from periwinkle plants infected with the African Nelspruit strain (Panel II, A3 and C1 to C7). Probe AS-1.7 was recently used with midvein DNA from various citrus species collected in orchards in Zimbabwe, South Africa, Reunion Island, and Mauritius Island. The probe gave strong hybridization signals with several of these species, showing that African liberobacter strains are present in these regions and that African strains other than the Nelspruit strain are successfully detected with probe AS-1.7 (unpublished results).

Conclusion

With PCR it has been possible to amplify part of the *rpl*KAJL-*rpo*BC operon of an African liberobacter strain (Nelspruit strain) and to compare it with its counterpart from the Asian liberobacter Poona (India) strain. The percentage of homology between the oligonucleotide sequences of the Asian and African liberobacter operons is 74.2%. Because this operon codes in particular for highly conserved proteins (ribosomal proteins), it is likely that the percentage of homology of total DNA of the two strains will be below 70%, suggesting that African and Asian liberobacters are two different species [9] in the new liberobacter phylum that we have identified in the α-proteobacteria. At high stringency, the Asian and African probes when used together should detect all liberobacter strains and when used singly should be able to distinguish between African and Asian strains. In this way, we have recently demonstrated that both African and Asian liberobacter strains were present on Mauritius and Reunion Islands (unpublished results). The presence of the two *Liberobacter* species in

Reunion and Mauritius Islands had never been demonstrated before but was suspected, because both the Asian and the African psyllid vectors are present in these islands.

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