rRNA operons and genome size of '*Candidatus* Liberibacter americanus', a bacterium associated with citrus huanglongbing in Brazil

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Huanglongbing is one of the most severe diseases of citrus worldwide and is associated with '*Candidatus* (*Ca.*) Liberibacter africanus' in Africa, '*Ca.* Liberibacter asiaticus' in Asia and the Americas (Brazil, USA and Cuba) and '*Ca.* Liberibacter americanus' (Lam) in Brazil. In the absence of axenic cultures, genetic information on liberibacters is scarce. The sequences of the entire 23S rRNA and 5S rRNA genes from Lam have now been obtained, using a consensus primer designed on known $tRNA^{Met}$ sequences of rhizobia. The size of the Lam genome was determined by PFGE, using Lam-infected periwinkle plants for bacterial enrichment, and was found to be close to 1.31 Mbp. In order to determine the number of ribosomal operons on the Lam genome, probes designed to detect the 16S rRNA gene and the 3′ end of the 23S rRNA gene were developed and used for Southern hybridization with I-*Ceu*I-treated genomic DNA. Our results suggest that there are three ribosomal operons in a circular genome. Lam is the first liberibacter species for which such data are available.

INTRODUCTION

Huanglongbing (HLB) is one of the worst diseases of citrus and endangers the very existence of citriculture (Bové, 2006). Known in China since the 1870s and South Africa since 1928, HLB emerged in 2004, 2005 and 2006, respectively, in São Paulo state (Brazil), Florida (USA) and Cuba (Bové, 2006; Llauger *et al.*, 2008). Three '*Candidatus* Liberibacter' species are associated with HLB. In Africa, only '*Candidatus* Liberibacter africanus' (Laf) has been detected and, in Asia, '*Candidatus* Liberibacter asiaticus' (Las) has been the only species to be found (Jagoueix *et al.*, 1994, 1997; Bové, 2006). São Paulo state harbours, in addition to Las (Coletta-Filho *et al.*, 2004; Teixeira *et al.*, 2005a), a third liberibacter species, '*Candidatus* Liberibacter americanus' (Lam) (Teixeira *et al.*, 2005a, b, c). Citrus trees in Florida and Cuba may be infected only with Las. Liberibacters are not available in axenic culture. They are phloem sieve tube-restricted members of a novel subgroup of the *Alphaproteobacteria*, with members of the alpha-2 subgroup as their closest relatives (Jagoueix *et al.*, 1994, 1997). Two psyllid insect vectors, in which the liberibacters circulate and multiply, are responsible for the rapid spread of liberibacters between citrus species, as well as citrus relatives such as *Murraya paniculata* (jasmine orange) (Lopes *et al.*, 2005). The African citrus psyllid *Trioza erytreae* transmits Laf in Africa, while the Asian citrus psyllid *Diaphorina citri* vectors Las and/or Lam in Asia and the Americas (Bové, 2006; Yamamoto *et al.*, 2006). Each of the three liberibacters can be transmitted by dodder (*Cuscuta campestris*) to periwinkle plants (*Catharanthus roseus*), in which they reach higher titres than in citrus and induce severe symptoms (Garnier & Bové, 1983; Teixeira *et al.*, 2008a).

Because the liberibacters have not been available in culture, infected periwinkle plants have been one of the major experimental sources of liberibacters. However, as it is difficult to get liberibacter DNA that is uncontaminated with plant DNA, only a few liberibacter genes have been characterized, as follows: the genes for the rRNA (*rrn*) operon, the *rplKAJL–rpoBC* gene cluster or *β*-operon and a few additional genes (*nusG*, *pgm*, *omp*, hypothetical protein gene) isolated by the RAPD method (Hocquellet *et al.*, 1999). The 16S rRNA gene and the 16S–23S intergenic region were obtained by PCR amplification with universal primers for prokaryotic rRNA genes, using DNA from liberibacter-infected periwinkle plants as target DNA (Jagoueix *et al.*, 1994, 1997). The rRNA gene region has recently been extended (Lin *et al.*, 2008). Part of the *β*-operon was obtained in the early 1990s as a 2.6 kbp DNA

Abbreviations: HLB, huanglongbing; Laf, '*Candidatus* Liberibacter africanus'; Lam, '*Candidatus* Liberibacter americanus'; Las, '*Candidatus* Liberibacter asiaticus'.

The GenBank/EMBL/DDBJ accession numbers for the *rrs* and *rrl*/*rrf* gene sequences of Lam isolate São Paulo are FJ036892 and FJ036893, respectively.

fragment (In-2.6) by random cloning of DNA from periwinkle plants infected with the Poona (India) strain of Las (Villechanoux *et al.*, 1993). Next, DNA from periwinkle plants infected with the South African Nelspruit strain of Laf was amplified with a primer pair designed from In-2.6 and yielded, after cloning and sequencing, a 1.7 kbp DNA fragment (AS-1.7) of the *β*-operon of Laf (Planet *et al.*, 1995). Comparison of the sequences of In-2.6 and AS-1.7 confirmed that the Asian and African liberibacters represented two distinct species (Planet *et al.*, 1995). When, in 2005, the third liberibacter species, Lam, was described (Teixeira *et al.*, 2005a, b, c), its *β*-operon was not available. The *β*-operon of the American liberibacter, as well as three upstream genes (*tufB*, *secE* and *nusG*), have now been obtained by the technique of chromosome walking and characterized (Teixeira *et al.*, 2008a). Furthermore, the *β*-operon sequence of Laf, only partially known previously, was completed, making it possible to compare the *β*-operon sequences of African, Asian and American liberibacter strains over a length of ~3000 bp (Teixeira *et al.*, 2008a). The *β*-operon gene cluster of Las, as well as the *omp* locus (Hocquellet *et al.*, 1999; Bastianel *et al.*, 2005), have recently been extended by an improved genomic walking technique (Lin *et al.*, 2008).

Evidence for the presence of more than one *rrn* operon in liberibacters was discovered while comparing the 16S–23S intergenic regions of Las and Laf (Jagoueix *et al.*, 1997). Here, we show that Lam probably has three *rrn* operons on a circular genome, the size of which is 1.29–1.34 Mbp, based on data from PFGE and hybridization assays. The sequences of the 23S and 5S rRNA genes of Lam have also been obtained.

METHODS

Plant material and DNA extraction. Seedlings of periwinkle (*Catharanthus roseus* 'Peppermint Cooler') were grown in the Bordeaux laboratory greenhouse at ~25 °C during the day and ~20 °C at night. The initial periwinkle plant infected with Lam was obtained by dodder transmission from a symptomatic sweet orange seedling that had been graft-inoculated with the São Paulo strain of Lam. To generate large numbers of Lam-infected periwinkle plants, pieces of shoot from the initial symptomatic periwinkle plant were top-grafted onto healthy periwinkle seedlings. The plants were used when they showed generalized symptoms. Symptomatic periwinkle leaves infected with Lam were used as the source material for DNA extraction and the preparation of high-molecular-mass liberibacter DNA. Uninfected periwinkle leaves were used as controls. Whenever symptomatic leaves were used, they were always checked by PCR to confirm infection with Lam (Teixeira *et al.*, 2005b).

DNA preparations from symptomless, uninoculated periwinkle plants and symptomatic plants infected with Lam were obtained from leaf midribs by the CTAB procedure (Murray & Thompson, 1980). The DNA preparations were treated with RNase A and proteins were removed by phenol treatment before quantification of DNA from UV absorption at 260 nm.

Amplifying, cloning and sequencing of ribosomal genes. The sequences of the 16S rRNA gene (*rrs*) (GenBank accession number AY859542.1) and the 16S–23S intergenic region (AY742824.1) of Lam were from Teixeira *et al.* (2005c).

The DNA upstream of *rrs* was obtained by PCR amplification with degenerate forward primer rrs_UpDeg (5′-AGAAAGRGARACG-TGGRCGGC), based on consensus sequences upstream of the 16S rRNA gene from selected rhizobia (see below), and reverse primer GB3 (Teixeira *et al.*, 2005b) (Fig. 1). PCR was carried out in a 50 µl reaction mixture containing 200 ng DNA, 1× PCR buffer (Invitrogen), 2.0 mM $MgCl_2$, 0.2 mM of each dNTP, 500 nM of each primer and 1.5 U *Taq* DNA polymerase. PCR conditions were as follows: initial denaturation at 95 °C for 2 min, 35 cycles of 92 °C for 30 s, 67 °C for 30 s and 72 °C for 90 s and a final extension of 72 °C for 4 min. The amplicon from Lam-infected periwinkle DNA was cloned into the pGEMT-Easy vector (Promega). One microlitre of the ligation mixture was then used to transform competent *Escherichia coli* DH10B by electroporation (Bio-Rad). The cloned DNA was sequenced using Genome Express facilities (http://www.gexbyweb.com/gexbyweb) with T7 promoter primer and SP6 primer.

The *rrl* gene, together with the *rrf* gene, was obtained by PCR amplification with forward primer GB4 (5′-TTACCGACGTTAG-ATAACCGGACG), designed from the 16S–23S intergenic region (Teixeira *et al.*, 2005c), and reverse primer GB11 (5′-CTACCGGGC-TGCTCCACCCC), designed to anneal at the $tRNA^{Met}$ sequence located at the end of the *rrn* operon. The sequence of GB11 was based on the consensus sequences downstream of *rrf* from selected rhizobia (see below). PCR was carried out as described above, except that the annealing temperature was 64 °C. *E. coli* transformation and sequencing were as described, except that the following primers were also used for sequencing: GB5 (5′-GCTACCTTAGGACCGTTATA-GTTACG), GB6 (5′-GTAACTATAACGGTCCTAAGGTAGCG), GB9 (5′-CTTTAAGCAGGAACCCTTGG) and GB13 (5′-GGCTGGATGT-GGAAGCTGGGTA) (Fig. 1).

Sequence analysis. Sequences of the *rrs*, *rrl* and *rrf* genes and 1 kbp sequences upstream and downstream of the *rrn* genes were retrieved from the NCBI Complete Microbial Genomes database (http://www.ncbi.nlm.nih.gov/genomes/MICROBES/microbial_taxtree.html) for the following strains: *Agrobacterium tumefaciens* C58 (GI:159185562), *Rhizobium leguminosarum* bv. viciae 3841 (GI:116249766), *Sinorhizobium meliloti* 1021 (GI:15963753), *Brucella melitensis* $16M^T$ (GI:17988344), *Bartonella henselae* Houston-1^T (GI:49474831), *Nitrobacter winogradskyi* Nb-255 (GI:75674199) and *Bradyrhizobium japonicum* USDA 110 (GI:27375111). The sequence of the 16S rRNA gene and part of the 23S rRNA gene from Las isolate GuangXi-GL-1 (GenBank accession no. DQ778016; Lin *et al.*, 2008) was also selected. Multiple sequence alignments were performed using CLUSTAL W software (Thompson *et al.*, 1994). Searches for identities between sequences were carried out using the BLAST algorithm (Altschul *et al.*, 1997). tRNA searches were conducted with tRNAscan-RE (Lowe & Eddy, 1997).

Sequences obtained from cloned PCR fragments were trimmed to remove plasmid sequences and amplification primers and consensus sequences were generated from multiple overlapping sequences with CodonCode Aligner (CodonCodeCorporation).

Preparation of DNA probes

Probe for ribosomal protein genes. A DNA fragment of 878 bp encompassing *rplA* and *rplJ* of Lam was PCR-amplified in the presence of DIG–11-dUTP (Roche) as described previously (Teixeira *et al.*, 2008a), yielding probe rplAJ.

Probes for rRNA genes. An *rrs* amplicon was obtained with universal bacterial primers fD1/rD1 (Weisburg *et al.*, 1991) from a DNA preparation of Lam-infected periwinkle leaves (Teixeira *et al.*, 2005b)

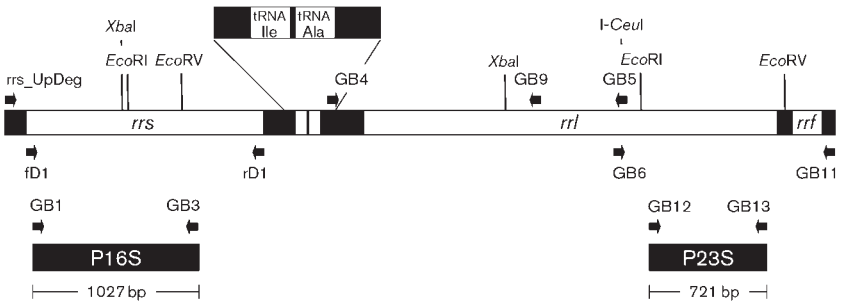

Fig. 1. Schematic representation of the *rrn* operon in Lam. *rrs*, 16S rRNA gene; *rrl*, 23S rRNA gene; *rrf*, 5S rRNA gene. The 16S–23S intergenic spacer region contains tRNA genes for Ile and Ala. Positions of restriction sites are indicated by vertical lines. The positions of primers on the *rrn* operon are given by arrows. Sequences corresponding to probes P16S and P23S are depicted.

and used as template in a second PCR with primers GB1 and GB3 in the presence of DIG–11-dUTP (Roche), yielding an *rrs* probe of 1027 bp named P16S (Fig. 1).

Probe P23S (721 bp) was generated by amplification with forward primer GB12 (5′-GGTAGGCATTGAAGCAGAGGCG) and reverse primer GB13 (5′-GGCTGGATGTGGAAGCTGGGTA) in the presence of DIG–11-dUTP (Roche), in a nested reaction with the 23S rRNA gene as template (see above) (Fig. 1).

Preparation of Lam genomic DNA High-molecular-mass genomic DNA was prepared as described previously (Neimark & Kirkpatrick, 1993; Padovan *et al.*, 2000) with minor modifications. Briefly, 1.5 g periwinkle leaf midrib was ground in 5 ml ice-cold extraction buffer [100 mM Na_2HPO_4, 30 mM NaH_2PO_4, pH 7.2, 2 % PVP 40 000, 10 mM EDTA, pH 8.0, 0.15 % BSA, 1 mM isoascorbic acid, 10 % sucrose and 1 % macerozyme (Yakult)], using a Homex model 6 homogenizer (Bioreba). The resulting suspension from multiple samples was transferred into centrifuge tubes (6 × 29 ml), incubated for 1 h in a water bath at 30 °C with occasional gentle stirring and centrifuged at 1500 ***g*** for 5 min and the supernatant was filtered through two layers of cheesecloth. Centrifugation and filtration were repeated. The filtered, low-speed supernatants were pooled and centrifuged at 18 000 ***g*** for 30 min at 4 °C. The resulting green pellet was gently resuspended in 20 ml suspension buffer (20 mM Tris/HCl, 50 mM EDTA, pH 8.0, 10 % sucrose). The suspension was again centrifuged at 1500 ***g*** for 5 min, filtered and centrifuged at 18 000 ***g***. Each pellet was resuspended in 150 µl suspension buffer; the pellets were pooled and centrifuged at 18 000 ***g***. The final pellet was gently resuspended in 150 µl suspension buffer, brought to 40 °C for 3 min and mixed with an equal volume of 2 % molten, low-melting-point agarose (Bio-Rad) dissolved in 2 × TSE (0.2 M Tris/HCl, 20 mM EDTA, pH 8.0, 0.2 M NaCl) maintained at 50 °C. The agarose suspension was moulded into blocks by pipetting 80 µl of the suspension into plastic moulds and allowed to set at 4 °C for 15 min. Agarose blocks were expelled into lysozyme buffer (10 mM Tris/HCl, pH 7.5, 50 mM NaCl, 100 mM EDTA, 0.5 % sarcosine). Lysozyme was freshly added to a final concentration of 1 mg ml^{-1}, and the blocks were left in the lysozyme solution for 36 h at 37 °C, replacing the lysozyme solution with fresh solution every 12 h. The last lysozyme solution was replaced with lysis buffer (100 mM EDTA, 0.5 % SDS). Proteinase (Roche) was added to a final concentration of 1 mg ml^{-1} and left at 50 °C for 5 days, the proteinase solution being replaced every 12 h. At the end of the proteinase treatment, blocks were washed three times with TE (10 mM Tris/HCl, 1 mM EDTA, pH 8.0) and twice with 1 mM PMSF in TE buffer to inactivate the proteinase. The blocks were stored at 4 °C in 20 mM Tris/HCl, 50 mM EDTA (pH 8.0) before treatment with or without restriction endonucleases and used for PFGE.

Restriction endonuclease digestion and PFGE. Agarose blocks were incubated for 1 h in 1 × restriction enzyme buffer supplied with the enzyme, and digested in 80 µl enzyme buffer containing BSA (final concentration 100 µg ml^{-1}) and 50 U enzyme at the recommended temperature for 18 h. Enzymes used were *Sma*I, *Bam*HI, I-*Ceu*I, *Apa*I, *Sac*II, *Sal*I, *Not*I, *Pau*I, *Sfi*I, *Smi*I and *Pme*I.

PFGE was performed by the contour-clamped homogeneous electric field technique using the CHEF-DR III system (Bio-Rad) with 1 % PFGE agarose (Bio-Rad). Electrophoresis was performed at 6 V cm^{-1} at an included angle of 120° in 0.5 × TBE buffer (45 mM Tris/borate, 1 mM EDTA; Bio-Rad) maintained at 14 °C with varying ramped pulse times. Pulse conditions were selected according to the size ranges of the DNA fragments under study. Molecular masses were estimated by comparisons with a lambda ladder (catalogue no. N0340S; New England Biolabs) and chromosome standards of *Saccharomyces cerevisiae* and *Hansenula wingei* (Bio-Rad catalogue numbers 170-3605 and 170-3667).

For resolution of low-molecular-mass DNA fragments (<30 kbp), DNA prepared by the CTAB protocol was used in large amounts (150 µg per lane). Restriction endonucleases *Eco*RI, *Xba*I, *Eco*RV and *Bgl*II were used according to the manufacturer's instructions (New England Biolabs and Promega) and electrophoresis was performed in the CHEF-DR III system.

Southern hybridization. For Southern hybridization, PFGE gels were stained with ethidium bromide followed by extensive destaining before image capture under UV light, using FluorS with software QuantityOne (Bio-Rad). A long destaining period was critical for optimal visualization of digested fragments. Gels were treated for three successive 15 min periods in solution A (0.25 M HCl) and then in solution B (0.5 M NaOH, 1.5 M NaCl) and finally in solution C (0.25 M Tris/HCl, pH 7.5, 1.5 M NaCl). Gels were blotted overnight onto nylon membranes (NitranSuperCharge; Schleicher & Schuell) in 10 × SSC. The membranes were washed in 2 × SSC for 5 min and the DNA was fixed by baking the membranes at 80 °C for 2 h (Charles & Ishikawa, 1999). Pre-hybridization and hybridization were performed at 42 °C. Four low-stringency washings were carried out for 15 min each in 2 × SSC containing 0.5 % SDS at room temperature, followed by two washings for 30 min each in 0.1 × SSC containing 0.1 % SDS at 65 °C. Detection of DIG-labelled probes was done according to the manufacturer's recommendations (Roche).

RESULTS AND DISCUSSION

Sequences of Lam *rrl* and *rrf*

Sequence alignments from *rrn* regions of selected genomes were used to design primers in highly conserved regions. Downstream of *rrs* there are three highly conserved regions: (i) an I-*Ceu*I site, located in the *rrl* gene (Liu *et al.*, 1993); (ii) the *rrf* gene, with high sequence identity to that in species of the *Rhizobiales* and (iii) a 78 bp stretch

overlapping the $tRNA^{Met}$ region. Primer GB11, designed to this region, and primer GB4, designed to the *rrs*/*rrl* intergenic region, were used to amplify *rrl* and *rrf* and the *rrl*/*rrf* intergenic region as a 3142 bp fragment that was cloned and sequenced. Upstream of *rrs*, a conserved DNA stretch of 21 nucleotides was identified and used to design degenerate primer rrs_UpDeg. Using primer rrs_UpDeg in combination with primer GB3, the 5′ part of *rrs* and the upstream DNA were amplified. The amplified DNA spanned the fD1 recognition site (Weisburg *et al.*, 1991) up to the rrs_UpDeg-binding site, adding 158 bp to the previous *rrs* sequence (Teixeira *et al.*, 2005c).

Based on the results above, the *rrl* and *rrf* genes span 2803 and 119 bp, respectively, and they are separated by a 41 bp intergenic region that contains no tRNAs. The *rrl* gene from Lam had the highest sequence identity (95 %) to the *rrl* gene from Las strain Sihui (GenBank accession no. EU644449), followed by the sequences of *Shinella zoogloeoides* ATCC 19623^T (X88894) and *A. tumefaciens* C58 (AE007870.2), both with 89 % identity. The *rrf* gene from Lam again had the highest sequence identity (95 %) to the *rrf* gene from Las strain Sihui (GenBank accession no. EU644449), while the *rrf* genes from both *Sinorhizobium meliloti* 1021 (AL591688) and *Sinorhizobium medicae* WSM419 (CP000738) shared 88 % identity with the Lam sequence. All these bacteria belong to the *Rhizobiaceae*.

The complete *rrs* gene from Lam strain São Paulo (this work and Teixeira *et al.*, 2005c) has 1495 bp and the closest match (95 % sequence identity) is the complete *rrs* sequence from Las strain GuangXi-GL-1 (GenBank accession no. DQ778016.1), followed by (i) the partial *rrs* sequences from liberibacters associated with members of the Solanaceae, '*Ca.* Liberibacter' sp. NZ082226 (EU834130.1) and '*Ca.* Liberibacter psyllaurous' Tx15 (EU812556.1), and Laf (L22533), with 94 % sequence identity, and (ii) the complete sequences from *R. leguminosarum* 3841 (AM236080) and *Rhizobium etli* CFN 42^T (CP000133.1), with 90 % identity. The sequence of the *rrs*/*rrl* intergenic region is also known (Teixeira *et al.*, 2005c). In total, the Lam *rrn* represents a stretch of 5187 bp between primers rrs_UpDeg and GB11 (Fig. 1).

Analysis of Lam DNA by PFGE and Southern hybridization: estimation of genome size

After mild homogenization of midribs from Lam-infected periwinkle leaves, preparations enriched in liberibacter cells were obtained by alternating low- and high-speed centrifugations. The final pellet was resuspended and immobilized in agarose blocks before DNA extraction by lysozyme and proteinase treatments. Similar blocks were obtained from midribs of uninoculated periwinkle plants. Carrying out the treatments for DNA preparation on material embedded in agarose blocks is thought to minimize mechanical shearing of DNA strands. In the experiments shown in Fig. 2, the blocks were submitted to PFGE without any restriction endonuclease treatment, and the gels were stained with ethidium bromide. PFGE has been used widely to obtain high-molecular-mass DNA from non-cultured bacteria such as phytoplasmas and to determine the size of their genomes (Marcone *et al.*, 1999). In the PFGE gels (Fig. 2a, b), a faint band (arrows) is present in lanes containing DNA from plants infected with the liberibacter (lanes L), but not in lanes containing DNA from uninfected plants (lanes PW). We conclude that this band represents liberibacter DNA for the following reasons. (i) It can be stained with ethidium bromide. (ii) After transfer of the gel shown in Fig. 2(b) to a nylon membrane and Southern hybridization with probe rplAJ, specific to Lam, a hybridization signal was observed in lane L, but not in lane PW of Fig. 2(c), and the position of the signal corresponded very precisely to the position of the band on the PFGE gel of Fig. 2(b). (iii) As indicated above, in the results shown in Fig. 2(a, b), the DNA blocks were not treated with restriction endonuclease prior to PFGE. However, when such treatments were applied, in particular with endonucleases such as I-*Ceu*I and *Sal*I, which cut liberibacter genomic DNA, no bands were observed in the lanes L and, after transfer to a nylon membrane and Southern analysis, no hybridization signal was detected in the region corresponding to the signal in Fig. 2(c) (data not shown).

From comparisons with DNA size markers, the DNA band in Fig. 2 had an estimated size of 1.29 Mbp. In addition to the hybridization signal given by the 1290 kbp DNA band,

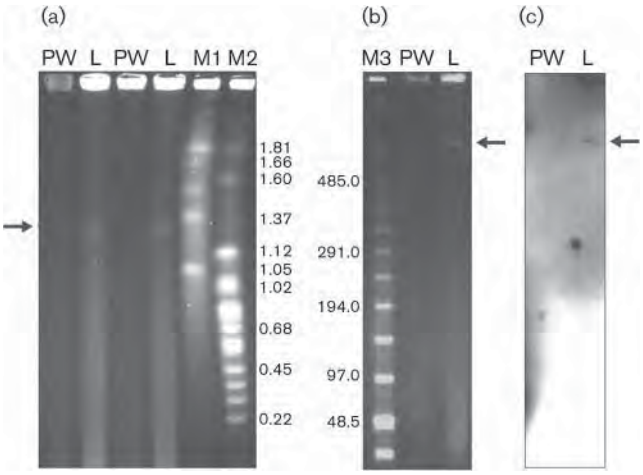

Fig. 2. PFGE of chromosomal DNA of Lam isolate São Paulo. (a, b) Ethidium bromide-stained gels of undigested PFGE of chromosomal DNA from healthy periwinkle plants (PW) and plants infected with Lam (L). PFGE parameters were 1–12 s for 6 h and 60–120 s for 16 h (a) or 2–40 s for 19 h (b) at 6 V cm^{-1}. (c) Southern blot hybridization of membrane from the gel shown in (b) with probe rplAJ. Arrows point to chromosomal DNA of Lam. DNA size markers (kbp) are as follows: M1, DNA from *Hansenula wingei*; M2, DNA from *Saccharomyces cerevisiae*; M3, λ DNA ladder.

Fig. 2(c) shows a rather strong hybridization signal in the well of lane L, indicating that an appreciable amount of liberibacter DNA did not move out of the well during PFGE. As circular genomic DNA does not migrate as effectively as linearized DNA (Marcone *et al.*, 1999; Neimark & Kirkpatrick, 1993), some of the DNA in the well might correspond to the circular form of genomic DNA, and the 1.29 Mbp DNA band to the linearized form. In general, the DNA blocks are submitted to gamma irradiation before PFGE in order to introduce single, random cuts into the circular double-stranded DNA genome molecules and increase the number of linearized genomes moving into the PFGE gel. In the experiments reported here, gamma irradiation was not used, and this may explain why the band of linearized genomic DNA was faint. As expected, there was no hybridization band in the PW lanes or in the PW well.

Next, the DNA in the agarose blocks from liberibacter-infected leaves and from uninfected leaves was submitted to restriction enzyme treatments (Fig. 3). The I-*Ceu*I endonuclease seemed particularly interesting to use, since the unique location of its restriction site is within the *rrl* gene of most bacteria (Liu *et al.*, 1993), and the enzyme is frequently used to determine the number of *rrn* operons during PFGE mapping. In Lam, the I-*Ceu*I site is towards the 3′ end of the *rrl* gene (Fig. 1). In experiments with endonucleases, the DNA to be digested is not only the putative linearized DNA of 1.29 Mbp, but also the more abundant, putative circular undigested DNA remaining in the well during PFGE experiments such as those of Fig. 2(b, c). Under these conditions, digestion with I-*Ceu*I resulted in three bands of high-molecular-mass DNA (C1, C2 and C3, in descending order of mass) when blocks with DNA from liberibacter-infected leaf midribs were used (Fig. 3; PFGE gel, I-*Ceu*I, lane L), but no bands were seen with the blocks with DNA from uninfected control leaves (Fig. 3; PFGE gel, I-*Ceu*I, lane PW). Probe P16S hybridized with C1 and C3 (Fig. 3; P16S, I-*Ceu*I, lane L) and probe P23S hybridized with C2 and C3 (Fig. 3; P23S, I-*Ceu*I, lane L). Based on the DNA markers, the mean $\pm$ SD sizes of fragments C1, C2 and C3 generated upon digestion with I-*Ceu*I, estimated from three independent experiments, were found to be respectively 494 ± 4, 447.7 ± 3 and 399.7 ± 3 kbp. The sum of the sizes of fragments C1, C2 and C3 amounted to 1.341 Mbp, a value close to that of 1.29 Mbp found for the DNA band of Fig. 2. The size of the Lam genome would thus be in the range 1.29–1.34 Mbp.

Of the restriction enzymes used, *Apa*I, *Not*I, *Pau*I, *Sfi*I and *Pme*I did not cut the Lam genomic DNA. I-*Ceu*I cut the chromosome in three fragments (Fig. 3). Restriction enzymes *Sma*I, *Bam*HI, *Sac*II, *Sal*I and *Smi*I gave different restriction patterns (not shown).

A genome size in the range of 1.29–1.34 Mbp places Lam in the lower range of genome sizes among alphaproteobacteria, with *Neorickettsia sennetsu* Miyayama^T (GenBank accession no. NC_007798) having the smallest sequenced alphaproteobacterial genome, namely 859 kbp. Of all bacteria capable of self-replication in artificial media, the mollicute *Mycoplasma genitalium* G-37^T (GenBank accession no. NC_000908) has the smallest genome, at 580 kbp. Although the liberibacters have not yet been cultured axenically *in vitro*, the genome of Lam appears to be about twice as large as that of the culturable *M. genitalium*. Phytoplasmas also belong to the class *Mollicutes* and, like the liberibacters, are (i) associated with plant diseases, (ii) restricted to the plant sieve tubes in the phloem, (iii) have sieve tube-feeding insects as vectors and (iv) are not available in culture. Their known genome sizes vary from 530 kbp for the bermudagrass white leaf phytoplasma to 1350 kbp for the stolbur phytoplasma (Marcone *et al.*, 1999). As pointed out previously (Marcone *et al.*, 1999;

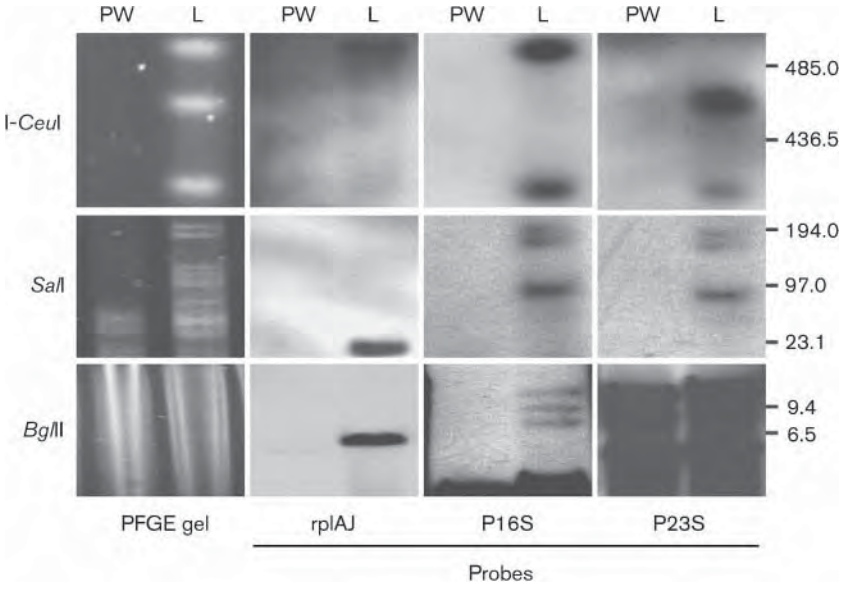

Fig. 3. PFGE of DNA of Lam isolate São Paulo and hybridization with probes rplAJ, P16S and P23S after Southern blot. Plugs containing chromosomal DNA of healthy periwinkle (PW) or Lam-infected periwinkle (L) digested with I-*Ceu*I or *Sal*I were electrophoresed at 2–40 s for 40 h, 6 V cm^{-1} at 120° included angle (I-*Ceu*I) or 1–12 s for 4 h and 60–120 s for 16 h at 6 V cm^{-1} (*Sal*I). DNA of healthy periwinkle (PW) or Lam-infected periwinkle (L) digested with *Bgl*II was electrophoresed at 0.5–12 s for 12 h, 6 V cm^{-1}. DNA size markers (kbp) are *Saccharomyces cerevisiae* DNA and a λ DNA ladder.

Neimark & Kirkpatrick, 1993), the genome sizes of many phytoplasmas are larger than those of many culturable human and animal mycoplasmas and, therefore, small genome size alone does not explain the fact that phytoplasmas or liberibacters have not yet been obtained in axenic culture.

Number of *rrn* operons on a circular liberibacter genome

As I-*Ceu*I cuts only within the *rrl* gene (Liu *et al.*, 1993) and I-*Ceu*I digestion of the liberibacter genome yields three DNA fragments (C1, C2 and C3), the genome is either circular with three ribosomal *rrn* operons or linear with two ribosomal *rrn* operons. The following results suggest that the genome is circular with three *rrn* operons.

After digestion with I-*Ceu*I, the hybridization signal with probe rplAJ showed that the *rplKAJL–rpoB* operon is located in the C1 fragment (Fig. 3). Probe P16S, binding to an extended portion of the *rrs* gene, hybridized with fragments C1 and C3, and the hybridization signal with C1 was stronger than with C3 (Fig. 3), suggesting that C1 carries two *rrs* genes and C3 only one. Probe P23S, binding to the 3′ portion of the *rrl* gene, hybridized with C2 and C3, the hybridization signal with C2 being stronger than with C3, suggesting that C2 carries two *rrl* genes. Bands C1, C2 and C3 were obtained reproducibly, with the hybridization signals to C1 and C2 being stronger than that of C3 in independent experiments. However, in some Southern blot experiments, high-molecular-mass DNA not cut with restriction enzymes failed to be detected, probably because of small amounts of liberibacter DNA in the infected leaves, especially in the winter months when growing conditions for plants were less favourable than at other times of the year.

After genomic DNA digestion with *Sal*I, hybridization with probe P23S revealed three bands with sizes of 199, 181 and 110 kbp. The two larger bands were close together but clearly distinct (Fig. 3). The same hybridization pattern was obtained with probe P16S, even though the two upper bands were less distinct. Interestingly, the known sequences of the Lam *rrn* operon have no *Sal*I site (this work and Teixeira *et al.*, 2005c). However, *Sal*I digestion does produce restriction fragments (Fig. 3; PFGE gel, *Sal*I, L), and these fragments must result from restriction sites located between the *rrn* operons. The fact that the same three fragments hybridized with P16S and P23S is further evidence for the occurrence of three *rrn* operons in Lam.

Additional data concerning the number of *rrn* operons were obtained after digestion with frequently cutting enzymes. In this case, restriction enzyme treatments were carried out on DNA in solution, without immobilization in blocks. Digestion with *Bgl*II and hybridization with probe P16S resulted in three hybridizing bands with DNA from liberibacter-infected periwinkle only (Fig. 3; P16S, L). Hybridization with probe P23S was hindered by plant DNA and could not be evaluated (Fig. 3; P23S, PW and L). As there are no *Bgl*II sites in the sequences of the known Lam *rrn* operon (this work and Teixeira *et al.*, 2005c), the *Bgl*II sites must be downstream and upstream of each *rrn* operon, accounting for three hybridization signals with probe P16S and further supporting the occurrence of three *rrn* operons in Lam.

Digestion of genomic DNA with I-*Ceu*I produces three fragments (C1, C2 and C3) and the results of Southern blot hybridizations with probes P16S and P23S are consistent with the presence of three *rrn* operons (Fig. 3). A genome with three *rrn* operons and three DNA fragments upon digestion with I-*Ceu*I should have a circular configuration. If the genome is linear with three *rrn* operons, four DNA fragments would be generated upon digestion with I-*Ceu*I. A linear genome generating three DNA fragments upon I-*Ceu*I digestion should have only two *rrn* operons. Hybridization with probes P16S and P23S for fragments C1, C2 and C3 accounts for a circular genome with three *rrn* operons, and not for a linear genome with two or four *rrn* operons. Besides, Southern hybridization results indicate that two *rrn* operons are in the same orientation and the third is in the opposite orientation (Fig. 4): (i) fragment C1 displayed hybridization only for P16S, which indicates that the two *rrn* operons located at the ends of C1 (*rrnA* and *rrnB*) are in opposite orientations; (ii) fragment C2 hybridized only with P23S, which also indicates that it harbours two *rrn* operons in opposite orientations; and (iii) as C3 had hybridization signals with both P16S and P23S, it must harbour two *rrn* operons in the same orientation. Fig. 4 summarizes the position and orientation of Lam *rrn* operons based on our hybridization results.

Among bacterial genomes, the number of *rrn* operons varies considerably, from one to 15 (Klappenbach *et al.*, 2000; Charles & Ishikawa, 1999). In *E. coli* K-12, the

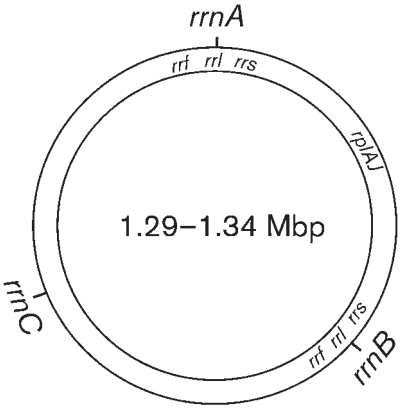

Fig. 4. Ribosomal operon location in the Lam genome. Locus *rplAJ* is between the *rrnA* and *rrnB* operons (fragment C1 of 445 kbp), with *rrnA* orientated anticlockwise and *rrnB* clockwise. Genome size is derived from PFGE data. Tick marks indicate I-*Ceu*I restriction sites.

number of *rrn* operons was estimated to be seven by restriction endonuclease digestion and hybridization assays (Kiss *et al.*, 1977). In alphaproteobacteria, the *rrn* copy number is between one and five (Klappenbach *et al.*, 2000). We present evidence for three *rrn* operons in the Lam genome; the *rrn* operon number in the other liberibacter species is unknown, although previously our group had indicated that liberibacters harbour at least two 16S rRNA genes (Jagoueix *et al.*, 1997; Garnier *et al.*, 2000).

Finally, probe rplAJ hybridized to a single *Bgl*II restriction fragment (Fig. 3; rplAJ, *Bgl*II, L). Similar results were obtained with *Eco*RI, *Xba*I and *Eco*RV (not shown). These data suggest that the *rplKAJL–rpoB* or β-operon is present in Lam as a single copy, as in all other proteobacteria, and is well-suited for real-time, quantitative PCR (Teixeira *et al.*, 2008b).

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