APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 2005, p. 6473–6478 Vol. 71, No. 11
0099-2240/05/$08.00+0 doi:10.1128/AEM.71.11.6473–6478.2005

Diversity of "*Candidatus* Liberibacter asiaticus," Based on the *omp* Gene Sequence

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Received 17 December 2004/Accepted 6 June 2005

Huanglongbing (yellow dragon disease) is a destructive disease of citrus. The etiological agent is a noncultured, phloem-restricted alpha-proteobacterium, "*Candidatus* Liberibacter africanus" in Africa and "*Candidatus* Liberibacter asiaticus" in Asia. In this study, we used an *omp*-based PCR-restriction fragment length polymorphism (RFLP) approach to analyze the genetic variability of "*Ca*. Liberibacter asiaticus" isolates. By using five different enzymes, each the 10 isolates tested could be associated with a specific combination of restriction profiles. The results indicate that the species "*Ca*. Liberibacter asiaticus," even within a given region, may comprise several different variants. Thus, *omp*-based PCR-RFLP analysis is a simple method for detecting and differentiating "*Ca*. Liberibacter asiaticus" isolates.

Huanglongbing (HLB), previously called greening disease, is one of the most severe diseases of citrus in Asia, Africa, the Arabian Peninsula, and the islands of Mauritius, Reunion, and Madagascar (11, 12). The causal agent of HLB is a noncultured, sieve tube-restricted alpha-proteobacterium, "*Candidatus* Liberibacter africanus" in Africa and "*Candidatus* Liberibacter asiaticus" in Asia (19). The two "*Ca*. Liberibacter" species are transmitted by the psyllid vectors *Trioza erytreae* (Del Guercio) in Africa (25) and *Diaphorina citri* (Kuwayama) in Asia (3, 24). Monoclonal antibodies directed against "*Ca*. Liberibacter" isolates from different geographical areas have been shown to react with one or several isolates, but none of the antibodies reacted with all isolates. In these studies, 11 isolates of the two "*Ca*. Liberibacter" species were classified in seven distinct serotypes, suggesting that there is genomic variation between isolates (9, 10, 14). However, sequence analysis of the *rplKAJL-rpoBC* operon, the 16S rRNA gene, and the intergenic 16S/23S rRNA gene spacer region did not reveal any differences between the various isolates of "*Ca*. Liberibacter asiaticus" (13, 19, 21, 27, 33, 34).

To further characterize the liberibacters at the molecular level, additional DNA fragments have been isolated by the randomly amplified polymorphic DNA method (18). Sequence analyses of six such fragments showed that they were part of the "*Ca*. Liberibacter" genome and led to identification of the *nusG*, *pgm*, and *omp* genes, as well as a gene encoding a conserved hypothetical protein. The *omp* gene, encoding an outer membrane protein (Omp), was thought to be the most promising candidate for studying inter- and intraspecies variability. In bacteria, Omps are involved in various functions. They participate in exchanges with the external environment, and in some cases they may be involved in pathogenicity (23). Although the three-dimensional structures of Omps are conserved, their amino acid sequences are not. The *omp* nucleotide sequences show considerable variation between species and even between strains of the same species, and they have been shown to be helpful for studying bacterial biodiversity (4, 7, 29, 36). For example, *omp* has been used to differentiate *Brucella* species, which, like the liberibacters, belong to the α subdivision of the *Proteobacteria* (5).

In this study, we determined the nucleotide sequences of the *omp* genes of "*Ca*. Liberibacter africanus" and "*Ca*. Liberibacter asiaticus" isolates, and we found that each isolate can be characterized by a specific PCR-restriction fragment length polymorphism (RFLP) profile.

MATERIALS AND METHODS

Plant material. Periwinkle and *Citrus* seedlings infected with various geographical isolates of "*Ca*. Liberibacter asiaticus" and "*Ca*. Liberibacter africanus" were maintained by grafting in a greenhouse. The culture conditions for maintaining healthy and infected plants in the greenhouse have been described previously (35).

DNA isolation. Total DNA of a *Citrus* plant was extracted from 0.5 g of leaf midribs using a Wizard DNA purification kit (Promega Biosciences Inc., San Luis Obispo, Calif.) by the method of Jagoueix et al. (20). Total DNA of periwinkle plants was extracted from 2 g of leaf midribs by the hexadecyltrimethylammonium bromide method of Murray and Thompson (26). Plant DNA preparations were treated with DNase-free RNase prior to further analyses.

Plasmids were prepared from *Escherichia coli* XL1-Blue transformants with the Wizard Plus Miniprep DNA purification system (Promega Biosciences Inc., San Luis Obispo, Calif.).

PCR amplification and RFLP analyses. Amplification was carried out in a 25-μl reaction mixture containing 2 μl of "wizard extract", 0.1 μg of "hexadecyltrimethylammonium bromide-extracted" DNA, or 1 ng of purified plasmid as the target DNA and 2 U of *Taq* DNA polymerase (Promega Biosciences Inc., San Luis Obispo, Calif.) with the buffer recommended by the supplier. Amplification was carried out for 40 cycles, each consisting of 40 s at 92°C, 40 s at 55°C, and 3 min at 72°C. The annealing temperature was determined based on the melting temperatures of the primers used for amplification. Primer sequences are listed in Table 1. The positions of primers are shown in Fig. 1. PCR detection of "*Ca*. Liberibacter" in the plants was carried out with primers OA1 and OI2c or primers OI1 and OI2c as described previously (19, 20). For RFLP analyses, the entire *omp* gene of "*Ca*. Liberibacter" was amplified with primers HP1asinv and Lp1c. PCR products were digested with TacI, ApoI, SwaI, SacI, MssI, and SspI, and the DNA fragments were separated by 2% agarose gel electrophoresis using standard procedures.

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TABLE 1. Primers used in this study

Primer	Nucleotide sequence	Position	Target	Accession no.
OMP1	5′ GGGGAAATAAGTGTATAGAAA 3′	860–880	*omp* gene from "*Ca*. Liberibacter africanus"	AY642158
HP1inv	5′ GGGTCACGGGTTTTATGAATTTGTTG 3′	602–627	Gene homologous to *yaeL* from "*Ca*. Liberibacter africanus"	AY642158
OMP8	5′ GTGATTCGTCGTGAGCTTGATTTTAG 3′	2010–2035	*omp* gene from "*Ca*. Liberibacter africanus"	AY642158
OMP8inv	5′ CTAAAATCAAGCTCACGACGAATCAC 3′	2035–2010	*omp* gene from "*Ca*. Liberibacter africanus"	AY642158
Lp3c	5′ ACCCGAACATATAACAGCCA 3′	3325–3306	Gene homologous to *lpxD* from "*Ca*. Liberibacter asiaticus"	AY642159
HP1asinv	5′ GATGATAGGTGCATAAAAGTACAGAAG 3′	506–532	*omp* gene from "*Ca*. Liberibacter asiaticus"	AY642159
Lp1c	5′ AATACCCTTATGGGATACAAAAA 3′	2926–2904	Gene homologous to *lpxD* from "*Ca*. Liberibacter asiaticus"	AY642159
OMP1asmc	5′ CATTGATTCGAATTCGATCTGGTAATC 3′	1424–1450	*omp* gene from "*Ca*. Liberibacter asiaticus"	AY642159
OMP2asmc	5′ CGGATAGAAATTGAGGGGAATGATCAA 3′	1618–1644	*omp* gene from "*Ca*. Liberibacter asiaticus"	AY642159
OMP9	5′ TCGGATTATGGCTACGCCTTATTTTT 3′	2081–2106	*omp* gene from "*Ca*. Liberibacter africanus"	AY642158
OMP2	5′ TGGACTGCCGATATCAGAA 3′	1616678–1616660	*omp* gene from *Sinorhizobium meliloti*	NC_003047
OA1	5′ GCGCGTATGCAATACGAGCGGCA 3′		16S rRNA gene from "*Ca*. Liberibacter africanus"	L22533
OI1	5′ GCGCGTATTTTATACGAGCGGCA 3′		16S rRNA gene from "*Ca*. Liberibacter asiaticus"	L22532
OI2c	5′ GCCTCGCGACTTCGCAACCCAT 3′		16S rRNA gene from "*Ca*. Liberibacter asiaticus"	L22532
AP1*	5′ GTAATACGACTCACTATAGGGC 3′		Adaptor for chromosome walking	
AP2*	5′ ACTATAGGGCACGCGTGGT 3′		Adaptor for chromosome walking	

FIG. 1. Gene organization of the *omp* gene regions of "*Ca*. Liberibacter africanus" (isolate "SouthAfrica-Nelspruit") and "*Ca*. Liberibacter asiaticus" (isolate "India-Poona"). The arrows indicate the positions of primers. The arrowheads indicate the directions of transcription.

Cloning and sequencing. PCR-amplified DNA was subjected to electrophoresis on a 0.8% agarose gel, purified from the gel band according to the QIAquick kit (QIAGEN S.A., Courtaboeuf, France) manual instructions, and ligated to the pGEM-T easy vector (Promega Biosciences Inc., San Luis Obispo, Calif.). Two microliters of the ligation mixture was used to transform *E. coli* XL1-Blue competent cells by electroporation (8). Cloned DNA was sequenced using Genome Express facilities (Genome Express, Meylan, France).

Southern blot hybridization. Total DNA (10 μg) extracted from periwinkle plants infected either with "*Ca*. Liberibacter africanus" (isolate "SouthAfrica-Nelspruit") or with "*Ca*. Liberibacter asiaticus" (isolates "India-Poona," "Thailand-NakhomPathom," and "Philippines-Lipacity") was digested with EcoRI. Restricted DNA was fractionated by 1% agarose gel electrophoresis, blotted onto positively charged nylon membranes by the alkali procedure, and hybridized with a digoxigenin-dUTP-labeled probe specific for the "*Ca*. Liberibacter africanus" *omp* gene. The 1,290-bp probe was obtained by PCR amplification with primers OMP1 and OMP2 in the presence of digoxigenin-dUTP (17). Hybridization signals were detected with anti-digoxigenin-alkaline phosphatase conjugate and 2-hydroxy-3-naphthoic acid-2′-phenylanilide phosphate as the substrate by following the supplier's instructions. Fluorescent signals were analyzed with a Fluor-S Multimager phosphorimager and the Quantity-One software (Bio-Rad Laboratories Inc., Hercules, Calif.).

Chromosome walking. Chromosome walking was performed using a Universal Genome Walker kit (BD Biosciences Clontech, Palo Alto, Calif.) by following the manufacturer's instructions. DNA from periwinkle plants infected with "*Ca*. Liberibacter asiaticus" (isolate "India-Poona") was digested with restriction enzymes Bst1101, Eco105I, KspAI, SmaI, and StuI. For "*Ca*. Liberibacter africanus" (isolate "SouthAfrica-Nelspruit"), DNA was digested with restriction enzymes EcoRV, PvuII, ScaI, and StuI.

To construct genomic DNA libraries, each batch of digested DNA was ligated to an adapter. For "*Ca*. Liberibacter asiaticus," a first PCR amplification (seven cycles, each consisting of 25 s at 94°C and 3 min at 72°C; 32 cycles, each consisting of 25 s at 94°C and 3 min at 67°C; and a 7-min final extension at 67°C) using primers AP1 and OMP1asmc was followed by a nested PCR (five cycles, each consisting of 25 s at 94°C and 3 min at 72°C; 20 cycles, each consisting of 25 s at 94°C and 3 min at 67°C; and a 7-min final extension at 67°C) using internal primers AP2 and OMP2ascm. Primers AP1 and AP2 were designed from the adaptor sequence. In the case of "*Ca*. Liberibacter africanus," the primers used were primers OMP8 and AP1 for the PCR and primers OMP9 and AP2 for the nested PCR. After amplification, the larger PCR products were gel purified, cloned, and sequenced.

Sequence analyses. DNA and protein sequence analyses were performed using the programs proposed by Infobiogen (http://www.infobiogen.fr/index.html). Searches for homologies in general databases (http://www.ncbi.nlm.nih.gov/BLAST) were carried out using the BLAST program (1). Multiple-sequence alignment was performed using the MULTALIN (6; http://www.toulouse.inra.fr/Igc/multalin/multalin.html) or CLUSTALW (32; http://www.infobiogen.fr/services/analyseq/cgi-bin/clustalw_in.pl) software. Phylogenetic trees were con-

structed by using the MEGA2 program (22; http://evolution.genetics.washington.edu/phylip.html). Six "*Ca*. Liberibacter" *omp* sequences were used in this work; one was from "*Ca*. Liberibacter africanus" isolate "SouthAfrica-Nelspruit" (accession no. AY642158), and five were from "*Ca*. Liberibacter asiaticus" isolates "India-Poona" (accession no. AY642159), "China-Behai" (accession no. AY842429), "Thailand-NakhomPathom" (accession no. AY842432), "Nepal-Pokhara" (accession no. AY842430), and "Philippines-Lipacity" (accession no. AY842431).

In addition to the "*Ca*. Liberibacter" sequences, the *omp* sequences of the following bacteria were included in this study: *Sinorhizobium meliloti* (accession no. NP 385608; coding for a putative outer membrane protein), *Agrobacterium tumefaciens* (accession no. NP 354388; coding for AGR_C_2554p, an Omp1 precursor), *Brucella suis* (accession no. NP 698159; coding for a bacterial surface antigen), *Brucella melitensis* (accession no. NP 539747; coding for an outer membrane protein), *Bartonella henselae* (accession no. AAL 66374; coding for Omp89), *Mesorhizobium loti* (accession no. NP_102404; coding for an outer membrane protein), *Rhodospirillum rubrum* (accession no. ZP_00015130; coding for an outer membrane protein), *Bradyrhizobium japonicum* (accession no. NP_770352; coding for a probable outer membrane protein), and *E. coli* (accession no. NP_285871; *yaeT* gene coding for a hypothetical protein).

RESULTS

Determining the *omp* nucleotide sequences of "*Ca*. Liberibacter africanus" and "*Ca*. Liberibacter asiaticus." While the expected size was around 2.3 kbp, only 519 nucleotides of the *omp* sequence were known at the time that we started this study. In order to determine the complete nucleotide sequence, the *omp* gene of "*Ca*. Liberibacter africanus" (isolate "SouthAfrica-Nelspruit") was PCR amplified with primers OMP1 and OMP2, which was designed from the 3′ end of the *omp* gene of *S. meliloti*. Since "*Ca*. Liberibacter" species have never been cultured, we used total DNA extracted from periwinkle plants infected with this "*Ca*. Liberibacter africanus" isolate as the target DNA. Unexpectedly, amplification yielded a 1,285-bp product instead of the expected 2,300-bp product. Sequence analysis showed that this fragment corresponded to the 5′ moiety of the "*Ca*. Liberibacter africanus" *omp* gene. Attempts to amplify the 3′ part of the gene by chromosome walking failed.

Similar experiments were carried out with "*Ca*. Liberibacter asiaticus" isolate "India-Poona", which was also shown to possess *omp* sequences by Southern blot hybridization with the *omp* probe (data not shown).

To determine the entire sequence of the "*Ca*. Liberibacter asiaticus" isolate "India-Poona" *omp* gene, the 5′ part was first amplified with primers HP1 and OMP8inv, designed from the sequence of "*Ca*. Liberibacter africanus" (Table 1 and Fig. 1), and the nucleotide sequence of the 1.2-kbp fragment was determined. Then the nucleotide sequence of the 3′ part of the gene was determined by the chromosome walking technique.

The complete sequence of the "*Ca*. Liberibacter africanus" *omp* gene was obtained by combining the nucleotide sequences of the 1,285-bp OMP1-OMP2 and the 1,500-bp OMP8-Lp3c fragments (primer Lp3c was designed from the "*Ca*. Liberibacter asiaticus" sequence). Additional sequences flanking the *omp* gene were determined by the chromosome walking method.

The sequenced regions of "*Ca*. Liberibacter africanus" and "*Ca*. Liberibacter asiaticus" were 3,694 and 3,701 bp long, respectively, and were found to have the same gene organization (Fig. 1). The *omp* region comprises three putative coding sequences, the 3′ end of an incomplete open reading frame (ORF), a short 71-bp ("*Ca*. Liberibacter africanus") or 66-bp ("*Ca*. Liberibacter asiaticus") intergenic region, the *omp* gene followed by a short 47-bp ("*Ca*. Liberibacter africanus") or 29-bp ("*Ca*. Liberibacter asiaticus") intergenic region, and the 5′ end of an incomplete ORF. The incomplete ORFs flanking *omp* were found to be highly conserved (more than 57% identity for the ORF upstream of *omp* and 68% identity for the ORF downstream of *omp*) in the two "*Ca*. Liberibacter" species. The ORF upstream of *omp* putatively encodes a conserved hypothetical polypeptide exhibiting 50% similarity with a hypothetical zinc metallopeptidase of *A. tumefaciens* str. C58 (best hit for "*Ca*. Liberibacter africanus") and 51% similarity with a putative integral membrane protein of *Neisseria meningitidis* Z2491 (best hit for "*Ca*. Liberibacter asiaticus"). The incomplete ORF downstream of *omp* encodes a putative 146-amino-acid polypeptide in the case of "*Ca*. Liberibacter africanus" and a putative 271-amino-acid polypeptide in the case of "*Ca*. Liberibacter asiaticus," which exhibit 63% and 70% similarity, respectively, with a probable UDP-3-*O*-3-hydroxymyristoyl glucosamine *N*-acyltransferase of *S. meliloti* that is encoded by the *lpxD* gene and is involved in lipopolysaccharide biosynthesis (28). The gene organization of the *omp* region of "*Ca*. Liberibacter" species was similar to the organization found in the α-proteobacteria *S. meliloti*, *A. tumefaciens*, and *B. henselae*, as well as in the γ-proteobacteria *E. coli* and *Salmonella enterica* serovar Typhi (15). In these two organisms, however, an additional gene encoding a hypothetical protein was located between the Omp-encoding gene *yaeT* and *lpxD*.

Sequence analyses of "*Ca*. Liberibacter africanus" and "*Ca*. Liberibacter asiaticus" *omp* genes. The *omp* genes of "*Ca*. Liberibacter africanus" (isolate "SouthAfrica-Nelspruit") and "*Ca*. Liberibacter asiaticus" (isolate "India-Poona") were 2,340 and 2,346 bp long, respectively, beginning with a GTG start codon preceded by a ribosome binding site and ending with a TAG stop codon. The nucleotide sequences exhibited 72.2% identity, whereas the encoded polypeptides had 58% identical and 86.5% similar amino acids. Protein alignment also showed that blocks of identical amino acids were distributed all along the protein sequence with the exception of two short regions, one at the N-terminal end (positions 1 to 39) and the other in the central part of the protein (positions 508 to 565).

A BLAST search for homologies revealed that the Omp of "*Ca*. Liberibacter africanus" exhibited 37% identity and 55% similarity over 743 amino acids with the group 1 outer membrane protein precursor Omp1 of *A. tumefaciens* (accession no. NP_354388.1) and 37% identity and 56% similarity with a putative outer membrane protein of *S. meliloti*, whereas the Omp of "*Ca*. Liberibacter asiaticus" exhibited 40% identity and 62% similarity over 744 amino acids and 38% identity and 61% similarity over 743 amino acids with the outer membrane proteins of *S. meliloti* and *A. tumefaciens*, respectively. A search for conserved domains showed that the "*Ca*. Liberibacter" Omp protein belongs to the COG4775 family, which includes the outer membrane protective antigen Oma87 from *Pasteurella multocida* (23) and Omp1 of *A. tumefaciens* (16). Interestingly, structure predictions revealed that Omp of "*Ca*. Liberibacter" had an organization similar to that of *A. tumefaciens*, *S. meliloti*, *B. melitensis*, and *B. henselae*. In particular, it possessed a single hydrophobic transmembrane segment (po-

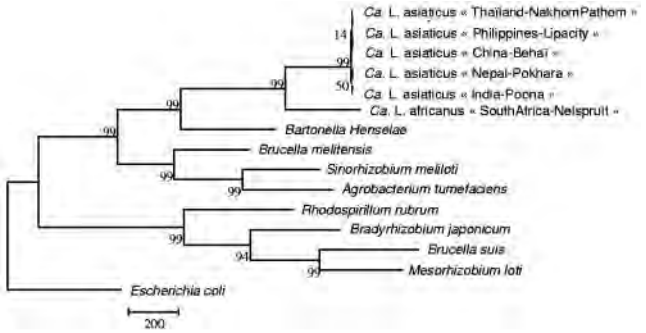

FIG. 2. Maximum-parsimony phylogenetic tree generated from alignment of *omp* sequences from "*Ca*. Liberibacter" and other α-proteobacteria. *E. coli* was added as an outgroup. Bootstrap values are shown at the nodes.

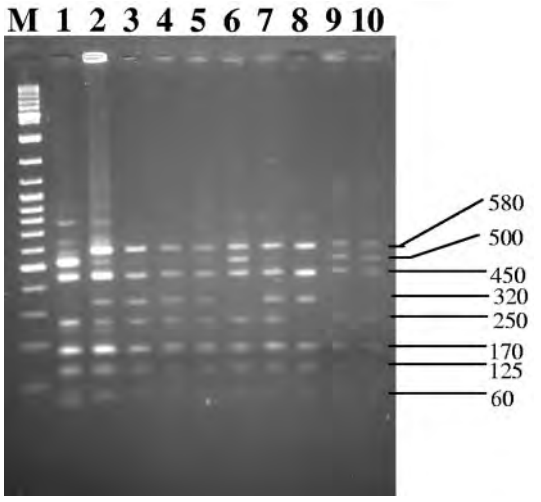

FIG. 3. TaqI restriction profile analyses of PCR-amplified DNAs from *Citrus* infected with various isolates of "*Ca*. Liberibacter asiaticus." Amplification was performed with primers HP1asinv and Lp1c. Lane M, 1-kb DNA ladder; lane 1, "India-Poona"; lane 2, "Vietnam-Bentre"; lane 3, "Thailand-NakhomPathom"; lane 4, "Philippines-Lipacity"; lane 5, "China-Behaï"; lane 6, "Nepal-Pokhara"; lane 7, "Taiwan-Taiwan12"; lane 8, "China-Yunnan"; lane 9, "India-Chetally"; lane 10, "India-Kodali."

sitions 19 to 41), while the major part of the polypeptide was amphiphilic, suggesting that it is exposed at the cell surface. Most of the Omps are known to have a phenylalanine (F) residue as the carboxyl-terminal amino acid (30). In the "*Ca*. Liberibacter" species, however, the carboxyl-terminal amino acids were arginine (R) for "*Ca*. Liberibacter africanus" and methionine (M) for "*Ca*. Liberibacter asiaticus." A similar situation was found in *E. coli*, in which the *yaeT*-encoded polypeptide ends with a tryptophan (W) residue (accession no. NP_414719) (2).

Phylogenetic analyses. To further characterize the "*Ca*. Liberibacter" Omp, the nucleotide sequences of the *omp* genes from five isolates of "*Ca*. Liberibacter asiaticus" (isolates "India-Poona," "Thailand-NakhomPathom," "Philippines-Lipacity," "China-Behaï," and "Nepal-Pokhara") were determined. These sequences were aligned with the *omp* sequences from "*Ca*. Liberibacter africanus" isolate "SouthAfrica-Nelspruit" and nine other bacteria, including eight α-proteobacteria and the γ-proteobacterium *E. coli*. A phylogenetic tree was constructed using the maximum-parsimony method (Fig. 2). The finding that all "*Ca*. Liberibacter" isolates grouped together was consistent with phylogenetic studies based on the 16S rRNA gene sequences showing that "*Ca*. Liberibacter" species are members of a new lineage in the α subdivision of the *Proteobacteria* (19). Interestingly, the group was divided into two subgroups corresponding to the two "*Ca*. Liberibacter" species, one containing the "*Ca*. Liberibacter africanus" "SouthAfrica-Nelspruit" isolate and the other comprising all five "*Ca*. Liberibacter asiaticus" isolates. Whereas the levels of identity between the Omp amino acid sequences of "*Ca*. Liberibacter africanus" isolate "SouthAfrica-Nelspruit" and "*Ca*. Liberibacter asiaticus" ranged from 58.5 to 59%, the levels of identity were 99.5 to 99.9% for the various isolates of "*Ca*. Liberibacter asiaticus." As shown by the *omp* phylogenetic tree, the liberibacters are more closely related to proteobacteria belonging to the α-2 subgroup.

***omp*-based RFLP analyses of "*Ca*. Liberibacter" isolates.** The *omp* sequences of the two "*Ca*. Liberibacter" species exhibited significant variability, including differences in restriction sites. RFLP assays based on the *omp* sequences were carried out in order to differentiate various isolates of "*Ca*. Liberibacter asiaticus" from India ("India-Poona," "India-Chetally," "India-Kodali"), Vietnam ("Vietnam-Bentre"), Thailand ("Thailand-NakhomPathom"), Taiwan ("Taiwan-Taiwan12"), The Philippines ("Philippines-Lipacity"), China ("China-Behaï" and "China-Yunnan"), and Nepal ("Nepal-Pokhara"). Primers HP1asinv and Lp1c (Fig. 1 and Table 1) were used to amplify the *omp* gene from these 10 isolates. The PCR products (approximate size, 2.4 kbp) were restricted with five distinct enzymes, SspI, ApoI, TaqI, SacI, and MssI. As illustrated in Fig. 3, restriction with TaqI yielded four distinct profiles (profiles T1 to T4). Profile T1, representative of the "India-Poona" isolate (lane 1), was characterized by four major signals at around 500, 450, 250 and 170 bp. Profile T2, which possessed two additional signals at around 320 and 580 bp, was obtained for isolates "Vietnam-Bentre" (lane 2), "Thailand-NakhomPathom" (lane 3), "Philippines-Lipacity" (lane 4), "China-Behaï" (lane 5), and "Taiwan-Taiwan12" (lane 7). Compared to profile T1, profile T3 was characterized by the presence of a signal at around 580 bp and the absence of the 320-bp signal. Profile T3 was shared by isolates "Nepal-Pokhara" (lane 6), "India-Chetally" (lane 9), and "India-Kodali" (lane 10). Compared to profile T2, profile T4, from isolate "China-Yunnan" (lane 8), lacked the 250-bp signal. Similarly, four distinct restriction patterns (patterns M1 to M4) were obtained with MssI, two distinct restriction patterns (patterns A1 to A2) were obtained with ApoI, two distinct restriction patterns (patterns S1 to S2) were obtained with SspI, and two distinct restriction patterns (patterns Sa1 to Sa2) were obtained with SacI (Table 2). On the basis of their restriction patterns, the various isolates could be classified in nine groups. Isolate "Philippines-Lipacity" was differentiated from the other isolates by its distinctive ScaI restriction pattern. Similarly, isolate "China-Yunnan" yielded a unique SspI profile. Restriction with ApoI revealed two groups, one comprising isolates "India-Poona," "Nepal-Pokhara," "India-Chetally,"

TABLE 2. RFLP profiles of isolates of "*Ca*. Liberibacter asiaticus"

Group	Isolate	Profile				
		SspI	ApoI	TaqI	SacI	MssI
1	India-Poona	S1	A1	T1	Sa1	M1
4	Nepal-Pokhara	S1	A1	T3	Sa1	M3
3	India-Chetally	S1	A1	T3	Sa1	M4
3	India-Kodali	S1	A1	T3	Sa1	M4
7	Vietnam-Bentre	S1	A2	T2	Sa1	M1
5	Thailand-Nakhom Pathom	S1	A2	T2	Sa1	M2
6	Taiwan-Taiwan12	S1	A2	T2	Sa1	M3
8	China-Behaï	S1	A2	T2	Sa1	M4
2	Philippines-Lipacity	S1	A2	T2	Sa2	M3
9	China-Yunnan	S2	A2	T4	Sa1	M4

and "India-Kodali" and one comprising isolates "Vietnam-Bentre," "Thailand-NakhomPathom," "Taiwan-Taiwan12," "China-Behaï," "Philippines-Lipacity," and "China-Yunnan," whereas restriction with TaqI and restriction with MssI each identified four different groups (Table 2). These results indicate that, based on the variability of the *omp* sequence, all but two "*Ca*. Liberibacter asiaticus" isolates ("India-Chetally" and "India-Kodali") could be discriminated.

DISCUSSION

Two distinct bacterial species, "*Ca*. Liberibacter africanus" and "*Ca*. Liberibacter asiaticus," are responsible for HLB in Africa and Asia, respectively. Detection and identification of the two "*Ca*. Liberibacter" species were achieved by 16S rRNA gene-based PCR-RFLP analysis (20), as well as PCR amplification of ribosomal protein genes (18). While these tools allowed specific detection of the two HLB "*Ca*. Liberibacter" species, they were not useful for studying the variability of "*Ca*. Liberibacter" isolates from various geographical areas. Monoclonal antibodies, alone or in combination, were too specific to detect all isolates, but they made it possible to classify "*Ca*. Liberibacter" isolates in seven serogroups (9). The antigenic diversity of "*Ca*. Liberibacter" isolates has not been characterized at the molecular level.

In this work, we studied the genetic variability of "*Ca*. Liberibacter" isolates by looking at the putative sequence variations of the *omp* gene. The *omp* regions of "*Ca*. Liberibacter africanus" and "*Ca*. Liberibacter asiaticus" were found to have the same gene organization as the *omp* regions of other α-proteobacteria, and the genes upstream and downstream of *omp* were homologous to *E. coli yaeL* and *lpxD*, respectively. Although the Omps are conserved among gram-negative bacteria, they exhibit sequence variations, which have been used to study biodiversity in various pathogenic bacteria (4, 5, 7). In "*Ca*. Liberibacter," the levels of amino acid identity ranged from 58.5% between "*Ca*. Liberibacter africanus" isolate "SouthAfrica-Nelspruit" and "*Ca*. Liberibacter asiaticus" isolate "India-Poona" to 99.9% between isolates "Philippines-Lipacity" and "Thailand-NakhomPathom." The phylogenetic tree constructed from *omp* DNA sequence alignments matched exactly the tree based on the 16S rRNA gene sequences, suggesting that the *omp* gene is part of the core genome and was not acquired by horizontal transfer from unrelated bacteria. As expected, all "*Ca*. Liberibacter asiaticus" isolates clustered in a branch distinct from the branch containing "*Ca*. Liberibacter africanus." The phylogenetic tree also showed that "*Ca*. Liberibacter asiaticus" isolates were distributed in two groups depending on their geographical origins; one group contained isolates from India and Nepal, and the other contained isolates from Thailand, The Philippines, and China. However, due to the high levels of identity, resulting in low bootstrap values, and the relatively low numbers of isolates available, the true occurrence of these two groups should be investigated further. Interestingly, however, based on the *omp* nucleotide sequence, each "*Ca*. Liberibacter asiaticus" isolate could be identified by a specific PCR-RFLP pattern, in spite of the high levels of identity. In particular, isolates from similar geographic areas, such as "China-Yunnan" and "China-Behaï" or "India-Poona" and "India-Kodali," did not have similar RFLP patterns. Isolates "India-Kodali" and "India-Chetally," which had identical RFLP profiles, probably represent a single isolate, as they were collected at the same time from the same citrus orchard (J. M. Bové and M. Garnier, unpublished data). The genetic diversity of "*Ca*. Liberibacter asiaticus" isolates as revealed by *omp*-based PCR-RFLP analysis is in good agreement with serological studies showing the occurrence of seven distinct serogroups (9). However, in contrast to RFLP analysis, serological methods did not necessarily differentiate isolates from different countries (9). Thus, RFLP analysis seems to be more discriminating. As a whole, these results indicate that "*Ca*. Liberibacter asiaticus," even within a given region, includes several different variants, which can be distinguished by a PCR-RFLP method based on the *omp* gene sequence. This technique might also be useful for studying "*Ca*. Liberibacter" diversity on the basis of phenotypic traits, such as virulence and insect transmissibility. Huanglongbing has recently been reported from São Paulo State, Brazil, and a new "*Ca*. Liberibacter" species, "*Ca*. Liberibacter americanus," was found to be associated with the disease (31).

ACKNOWLEDGMENTS

This work was funded by INRA, University Victor Segalen Bordeaux 2, and CIRAD-FLHOR.

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