Bacterial Diversity Analysis of Huanglongbing Pathogen-Infected Citrus using PhyloChips and 16S rDNA Clone Library Sequencing

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The bacterial diversity associated with citrus leaf midribs was characterized from citrus groves that contained the Huanglongbing (HLB) pathogen, which has yet to be cultivated *in vitro*. We employed a combination of high-density phylogenetic 16S rDNA microarray and 16S rDNA clone library sequencing to determine the microbial community composition of symptomatic and asymptomatic citrus midribs. Our results revealed that citrus leaf midribs can support a diversity of microbes. PhyloChip analysis indicated that 47 orders of bacteria from 15 phyla were present in the citrus leaf midribs while 20 orders from 8 phyla were observed with the cloning and sequencing method. PhyloChip arrays indicated that nine taxa were significantly more abundant in symptomatic midribs compared to asymptomatic midribs. *Candidatus* Liberibacter asiaticus (Las) was detected at a very low level in asymptomatic plants, but was over 200 times more abundant in symptomatic plants. The PhyloChip analysis was further verified by sequencing 16S rDNA clone libraries, which indicated the dominance of Las in symptomatic leaves. These data implicate Las as the pathogen responsible for HLB disease.

Citrus is the most important commercial fruit crop in Florida. In recent years, citrus Huanglongbing (HLB), also called citrus greening, has severely affected Florida's citrus production and hence has drawn an enormous amount of attention. HLB is one of the most devastating diseases of citrus (6,13), characterized by blotchy mottling with green islands on leaves, as well as stunting, fruit decline, and small, lopsided fruits with poor coloration. The disease tends to be associated with a phloem-limited fastidious α-proteobacterium given a provisional *Candidatus* status (*Candidatus* Liberobacter spp. later changed to *Candidatus* Liberibacter spp.) in nomenclature (18,25,34). Previous studies indicate that HLB infection

causes disorder in the phloem and severely impairs the translocation of assimilates in host plants (5,27,40). Tatineni and colleagues discovered that the HLB bacteria were unevenly distributed in phloem of bark tissue, vascular tissue of the leaf midrib, roots, and different floral and fruit parts (43).

Unsuccessful attempts in culturing the pathogen are notably hampering efforts to understand its biology and pathogenesis mechanism. Using a modified Koch's Postulates approach, Jagoueix and colleagues were able to re-infect periwinkle plants from a mixed microbial community harvested from HLB diseased plants (25). Emergence of the disease in otherwise healthy plants led to the conclusion that HLB was associated with *Candidatus* Liberibacter sp. based on its 16S rDNA sequence (18,25). Currently, three species of the pathogen are recognized from trees with HLB disease based on 16S rDNA sequence: *Ca.* Liberibacter asiaticus (Las), *Ca.* Liberibacter africanus (Laf), and *Ca.* Liberibacter americanus (Lam); Las is the most prevalent species among HLB diseased trees (5,12,18,25,44). Las is naturally transmitted to citrus by the psyllid, *Diaphorina citri* Kuwayama, and can be artificially transmitted by grafting from citrus to citrus and dodder (*Cuscuta campestris*) to periwinkle (*Catharanthus roseus*) or tobacco (*Nicotiana tabacum* Xanthi) (5). Based on current research regarding the associations of Liberibacter *in planta* there is not enough evidence to implicate Liberibacter as the definitive causal agent of HLB disease due to its resistance to cultivation *in vitro*. It is possible that HLB disease may be the result of complex etiology where Liberibacter interacts with other endophytic bacteria. However, there is not enough evidence regarding its association(s) *in planta* to make this conclusion, nor is it known whether associated microbial communities play a role in expression of pathogenic traits.

It has been noticed that certain trees (called escape plants) may survive in citrus groves heavily infected with HLB pathogen. Because these escape plants have the same genotype as susceptible plants and have developed under similar edaphic and climatic conditions, a possible explanation for the lack of HLB symptoms may lie in the nature of the microbial community associated with these plants. In a study of the endophytic bacteria associated with *Xylella fastidiosa* infected citrus branches, the endophyte *Curtobacterium flaccumfaciens* was found more frequently in asymptomatic citrus trees infected by *Xylella fastidiosa* (2). It was also reported that *C. flaccumfaciens* was able to reduce symptoms caused by *Xylella fastidiosa* when *Catharanthus roseus* (Madagascar periwinkle) was used as host plant (29). Microbial community analysis may lead to isolation and identification of novel bacteria with a potential of identifying biocontrol agent(s) against the HLB pathogen. Identification of biocontrol organisms from a niche similar to that of the pathogen would be particularly promising for effective disease control.

Microbial community analysis may help solve the puzzles regarding the causal agent of the HLB disease and symptom difference among citrus trees in infected groves. Little is known about the bacterial community composition associated with citrus except some studies on the citrus phyllosphere and *Xylella fastidiosa* infected citrus (2,51). The phloem microbiome can be characterized by either cultivation based or cultivation-independent methods. However, the portion of microbial diversity estimated through conventional culture techniques amounts to only 0.1 to 10% of the total diversity (46), indicating that techniques based on laboratory cultivation might be significantly biased. In fact, it has been observed that in many environmental samples the bacteria that are most dominant and abundant are not cultivable (28,37,41). Due to the limitations of cultivation based methods, in recent years molecular methods of community

analysis have been widely used. Multiple methods have been developed, and among them, 16S rDNA based methods are the most popular due to remarkably high conservation of this gene in all bacteria which enables a universal phylogeny (47). 16S rDNA based phylogenetic analysis has been commonly employed to characterize the microbial diversity in a variety of ecological niches such as plants (9, 42), soils (28), subsurface sediments and rocks (8). The high-density 16S ribosomal RNA gene oligonucleotide microarray, PhyloChip, has recently been developed and effectively applied to study bacterial population diversity and is more powerful and sensitive in identifying bacteria in the environment (7,14,39).

The main objective of the study was to test the hypothesis that other bacteria besides *Ca*. Liberibacter spp. are associated with citrus greening disease. The differences between the relative abundance, species richness and phylogenetic diversity of the microbial communities associated with the leaf midribs of HLB symptomatic and asymptomatic citrus trees were investigated using high-density 16S rDNA microarray PhyloChip and 16S rRNA gene clone library methods.

MATERIALS AND METHODS

Plant material collection and DNA extraction. Leaf samples were collected from citrus groves in Dover (Grove 1) and Lake Placid (Grove 2), FL. Asymptomatic leaves (8 leaves × 6 trees × 2 groves) and leaves showing HLB blotchy mottling (8 leaves × 6 trees × 2 groves) were randomly collected and brought to the lab in a cooler with ice in January and February with 10 days apart in 2008. The two citrus groves chosen in this study were confirmed to be HLB positive for more than 2 years previously. The two groves are separated by 110 Kilometers and both groves are planted to Valencia oranges (*Citrus sinensis*). The leaves were washed in tap water and surface sterilized in 35% bleach (2% active Cl^-) and 70% (v/v) ethanol for 2 min each

and rinsed three times with sterile water. Later, midribs of leaves were separated, frozen in liquid nitrogen, and stored at -80°C. All the midribs of the eight leaves from a single tree were pooled and DNA was extracted using the Wizard® Genomic DNA purification kit (Promega Corp., Madison, WI) following the protocol for isolating genomic DNA from plant tissue. The DNA was again purified once by phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v) and choloroform:isoamyl alcohol (24:1, v/v) following the standard protocol (38). The DNA was precipitated, washed with 70% (v/v) ethanol and resuspended in RNase and DNase free water. For cloning, bacterial plasmid DNA was isolated with the Wizard® miniprep DNA purification system (Promega).

PCR detection of Las. PCR using Las specific primers A2/J5 (23) was performed to confirm the presence of Las in the samples. All PCR reactions in this study were performed in a DNAEngine® Peltier thermal cycler (BioRad Laboratories, Hercules, CA). Amplification of the DNA was conducted in a 20 µL total volume using Speed Star™ HS polymerase (Takara Bio INC, Otsu, Shiga, Japan). The PCR conditions for Speed Star™ HS polymerase were 2 min of pre-denaturation at 94°C, followed by 35 cycles of 10 s of denaturation at 94°C, 10 s of annealing at 65°C, 1 min of extension at 72°C, then a single final extension of 4 min at 72°C.

PCR amplification and sample preparation for 16S rDNA PhyloChip analysis. For PhyloChip analysis, DNA was amplified separately from six asymptomatic and six symptomatic trees from each of the two groves sampled. Amplification of DNA was performed in 25 µL PCR reaction using 1.5 U of Ex *Taq* polymerase (Takara Bio INC, Otsu, Shiga, Japan). Gradient PCR was conducted with annealing at 48-58°C for 25 cycles (gradient = 48.0, 48.3, 48.9, 49.7, 50.8, 52.3, 54.0, 55.4, 56.5, 57.3, 57.8, 58.0 °C). Primers 27f (5'-AGAGTTTGATCMTGGCTCAG) and 1492r (5'-GGYTACCTTGTTACGACTT) were used to amplify the 16S rDNA regions of

bacteria (31). The PCR products of all 12 gradients belonging to one sample were pooled before electrophoresis. Amplified PCR products were electrophoresed on 1% agarose gel, and the desired ~1.5 kb bands were gel purified and shipped on ice for PhyloChip analysis.

PhyloChip analysis. PCR product was quantified using Egels (Invitrogen Corp. Carlsbad, CA), and 1000 ng of bacterial PCR product was fragmented with DNAse, biotin labeled and hybridized as previously described (7). The microbial community analysis was resolved as a subset of 8743 potentially detected taxa with corresponding hybridization scores reported as arbitrary units (au). Each taxon consists of a set of 25 to 30 perfect match-mismatch probe pairs. For a taxon to be reported in this analysis, 90% of probe pairs in its set (probe fraction (pf) > 0.92) must meet these conditions: (i) the perfect match has an intensity of at least 1.3 times higher than the mismatch and (ii) both perfect match and mismatch are 500-fold above background. Hybridization scores for a taxon are reported for all samples if at least one sample out of the twelve has pf > 0.92. Hybridization scores are an average of the difference between perfect match and mismatch fluorescent intensities of all probe pairs excluding the highest and lowest. Final hybridization scores were normalized to an average of 2500 au for each PhyloChip. In presentation of relative abundances of reported taxa, hybridization scores were converted to 16S copy number based on the empirically-determined log-linear relationship between copy number of applied 16S rDNA PCR product and hybridization score; for analysis of richness by group, presence or absence was determined based on a probe fraction cutoff of 0.9 for each taxon within that group (7).

Statistical Analysis. To estimate richness (S), we used a probe fraction value of 0.9 as a cutoff, below which the taxon was deemed absent. Previously probe fraction was found to correlate well with richness patterns displayed by clone library analysis (14).

All statistical analyses were performed using JMP (SAS Institute, Inc., Cary NC), PCOrd (McCune and Mefford), or R (R Team, 2005). Multi-response permutation procedure (MRPP) was used to test the null hypothesis that the ordination contained distinct subgroups that were statistically separate from one another. All statistical significance was evaluated at a *p*-value of 0.05, unless otherwise noted. Regression analysis of environmental variables against the ordination coordinates was performed as previously published (4). Student's t-tests were performed as unpaired, two-tailed tests evaluated to a significance level of 0.05.

PCR amplification, cloning, and sequencing of bacterial 16S rDNA. As the DNA extracted from citrus midribs contained a mixture of plant and bacterial DNA, it was necessary to use a PCR primer that is specific to bacterial 16S rDNA sequence. We used universal primers 799f (5'-AACMGGATTAGATACCCKG) (9) and 1492r (31) that was shown to amplify most bacterial species and to exclude plastid DNA. The PCR products from mitochondrial using the primer pair 799f/1492r were approximately 1.5 times larger than bacterial 16S rDNA product, which easily allowed the separation of the PCR products from those of bacteria. DNA extracted from six asymptomatic and six symptomatic trees from each grove (six asymptomatic, six symptomatic from two groves = 24 samples) were used as templates to amplify the bacteria-specific 16S rDNA region using 799f and 1492r primers. Primer 1492r amplifies most eubacteria (31). PCR conditions and number of cycles were exactly the same as mentioned in the sample preparation for 16S rDNA PhyloChip analysis section (see above). The PCR products of all gradients (twelve) belonging to one sample type (e.g., symptomatic leaves of tree 1 from grove 1) were pooled before electrophoresis. The PCR products were electrophoresed on 1% agarose gel and the bacteria-specific expected size DNA band (735 bp) was gel purified using Wizard® SV Gel and PCR Clean-Up System (Promega) and ligated into pGEM T-easy cloning vector

(Promega). The ligation mixture was transformed into chemically competent *Escherichia coli* (DH5α), and transformants were selected on LB agar + Ampicillin (50 µg/ml). The positive clones with desired plasmids were screened by blue-white screening using 40 µL of X-gal (2% w/v) and 7 µL of IPTG (20% w/v) per plate. The white colonies were picked and plasmids containing 16S rDNA inserts were sequenced using T7 universal primer. Sequencing was performed at the sequencing facility of the Interdisciplinary Center for Biotechnology Research at University of Florida.

Phylogenetic analysis of 16S rDNA clone library. The sequenced rDNA regions were compared to Ribosomal Database Project II (Release 10 Update 3) (http://rdp.cme.msu.edu/index.jsp) (10) using 'Naive Bayesian rRNA Classifier' Version 2.0 to identify the nearest phylogenetic neighbor (confidence level of 95%). Homologies of the sequences were further verified using the Basic Local Alignment Search Tool (BLAST) algorithm (1). Sequences with more than 98% similarity was considered to be of the same operational taxonomic unit (OTU).

RESULTS

PCR detection of Las in tested samples. All plant midribs used to identify the bacterial populations associated with citrus leaf midribs were screened for the presence of Las using PCR assays containing specific primers A2/J5 (23). Midribs were chosen since they are phloem rich and we intended to identify microbiomes in the same niches as Las since Las is known to be phloem limited. An expected 703 bp PCR product was amplified from all 12 symptomatic plants and 2 out of 12 asymptomatic plants in Grove 1 (Dover) and Grove 2 (Lake Placid) (Fig. 1). Negative controls showed no amplification (data not shown).

PhyloChip bacterial community analysis. The microbial communities detected in the vascular tissues of citrus leaves were comprised of 117 taxa from 15 different phyla of bacteria, spanning the diversity of the bacterial phylogenetic tree (Table S1, Table 1). There were 15 hits that were homologous to chloroplasts that were assumed to be plant-derived and excluded from further analysis. The α-Proteobacteria has the highest richness, accounting for 26.5% of all taxa detected. Other phyla that were well-represented included Acidobacteria (6.8%), delta-Proteobacteria (5.98%), gamma-Proteobacteria, and Firmicutes (5.13%) (Table S1, Table 1).

In looking at the differences in richness between symptomatic and asymptomatic plants from Grove 1 and Grove 2, it is evident that there are some populations that co-vary with evidence of pathogenesis and grove location (Table 1). There was an overall increase in the bacterial richness in the symptomatic plants from Grove 2. The increased richness seems to come mostly from bacteria in the phylum α-Proteobacteria, and from the orders Caulobacterales, Sphingomonadales, and Rhizobiales. Measures of richness do not indicate changes in absolute abundance, but hybridization scores do have a linear relationship to absolute abundance when examining single taxon between different treatments (7). From the 117 taxa detected as the leaf midrib microbial community, we separately examined the individual taxa that were significantly different ($P < 0.05$) between symptomatic and asymptomatic plants. Only nine taxa were significantly different, and all were more abundant in symptomatic compared to asymptomatic plants (Fig. 2). In general, the differences were modest and comprised a 50% increase to a doubling of relative abundance, with one notable exception. The taxa otu_7603, representing Las, was detected at a very low level in asymptomatic plants, but over 200 times more abundant in symptomatic plants.

There were no discernable differences between the overall microbial communities in symptomatic and asymptomatic plants, as determined by ordination (data not shown). This community analysis was based on relative abundance of individual taxa detected by PhyloChip. There were three symptomatic plants from Grove 2 that were separated from the rest of the trees based on analysis of the microbial community; these three samples (G2S3, G2S4, and G2S6) were consistently elevated in terms of bacterial richness compared to the rest of the samples, suggesting that either Grove 2 in general or these plants specifically harbored a more complex microbial community.

16 rDNA clone library sequencing and phylogenetic analysis. In order to verify the PhyloChip data and understand the relative abundance of different bacteria associated with HLB affected citrus, the 16S rDNA amplicons used for the PhyloChip analysis were employed to construct a 16S rDNA clone library. However, sequencing of 192 clones indicated that they were all from chloroplast. This was due to the dominance of citrus plant DNA, and that the primers 27f and 1492r could not differentiate chloroplast 16S rDNA from bacterial 16S rDNA. Chelius and Triplett (9) designed primer 799f in combination with 1492r (31) that successfully differentiated bacterial 16S rDNA from chloroplast DNA and mitochondrial products. Thus, clone libraries of 16S rDNA were constructed using the 16S rDNA PCR products amplified using primers 799f and 1492r.

In total, 2062 clones were generated from cloning the 16S rDNA regions amplified using the same set of the genomic DNA samples that were used to amplify rDNA regions for PhyloChip analysis. These sequence data have been submitted to the GenBank databases under accession numbers (FJ387589-FJ388874). All sequence matches that were homologous to chloroplasts or cyanobacteria were assumed to be plant-derived, likely from plastids, and

excluded from analysis. In the total population of sequenced clones, the database search placed clones into eight phyla: (i) Proteobacteria (47.1%), (ii) Bacteroidetes (14.1%), (iii) Dictyoglomi (0.4%), (iv) Actinobacteria (0.3%), (v) Chlamydiae (0.2%), (vi) Firmicutes (0.1%), and (vii) TM7 (0.05%), (viii) Verrucomicrobia (0.05%), and 37.6% of the clones originated from chloroplast (Table 2).

On average more than 86% of the clones from symptomatic trees belonged to phylum proteobacteria to which Las belongs (Fig. 3). Further analysis of individual sequences of the above clones showed 99% identity with the Asia strain "Sihui" (GenBank accession number EU644449) and Florida strain (GenBank accession number EU982421). Las was the only common bacterium found in all the 12 symptomatic trees from two citrus groves. Las was identified in five of six asymptomatic trees from Grove 1 while in three of six asymptomatic trees from Grove 2 (Fig. 3). Significantly, midribs from symptomatic leaves contained higher (G1S = 88% and G2S = 84%) percentage clones that matched with Las 16S rDNA, where as asymptomatic leaves have lower percentage (G1A = 55% and G2A = 10%).

Comparison of cloning with PhyloChip analysis. A comparison between clone library sequencing and PhyloChip analysis of the microbial community showed that PhyloChip detected broader richness of taxa compared to cloning: PhyloChip detected 15 phyla in the citrus leaf midribs whereas cloning detected eight phyla (Table 3). Otherwise the two methods are largely in accordance. The PhyloChip detected all of the phyla identified by cloning except Dictyoglomi. Both methods detect an overabundance of α-Proteobacteria generally and Las species specifically.

DISCUSSION

Molecular techniques based on PCR have made it possible to study the diversity of microorganisms in natural environments without culturing (50). These techniques are valuable in increasing our understanding of the microbial communities despite some amplification biases demonstrated due to primer selection, number of amplification cycles, and template concentration (36,48). A diverse assemblage of microorganisms was observed in the citrus leaf midribs from HLB positive citrus groves with both PhyloChip analysis and 16S rDNA clone library sequencing. That so many more orders of bacteria were detected by PhyloChip (47 orders from 15 phyla) compared to cloning and sequencing (20 orders from 8 phyla) indicates that PhyloChip is more comprehensive in identification of microorganisms from environmental samples than 16S rDNA clone library sequencing. This is consistent with previous reports that compared clone library data with PhyloChip community analysis from environmental samples (14, 14b). The PhyloChip used in this study contains 8741 taxa representing all 121 demarcated bacterial and archaeal orders (7). The size of the clone library might also contribute to the difference of the data. It has been suggested that 40,000 sequencing reactions are required to document 50% of the richness of certain environmental samples, which is laborious, costly, and time-consuming (15). Typical 16S rDNA clone libraries include fewer than 1000 sequences (17,26,33). Our total clones for the asymptomatic and symptomatic samples are 957 and 1105, respectively. The selection of different primers for PCR amplifications for PhyloChip analysis (27f and 1492r) and the construction of clone library (799f and 1492r) might contribute to the difference even though both sets of primers are universal primers of bacteria (9,49). In addition, there is also a possibility that PhyloChip array approach causes nonspecific hybridization leading to false positives, though this is most likely to hamper discrimination of taxa at the genus or family level (14, 14b). While this may inflate the number of species-level taxa detected per

family, this does not affect either the phylum-level richness of the community or the change in relative abundance of Las, which are the two main points we meant to address with PhyloChip analysis.

Our study indicated that *Ca*. Liberibacter spp. was the dominant bacterium that is always detected from citrus showing HLB symptoms. 16S rDNA cloning and sequencing showed that Las was the only common bacterium found in all the 12 symptomatic trees from two citrus groves. PhyloChip study indicated nine taxa were significantly different, and all were more abundant in symptomatic compared to asymptomatic plants. However, Las dominated in the symptomatic leaves while not in the asymptomatic leaves, and this observation of dominance of Las in the symptomatic leaves supports the association between HLB disease and Las in Florida (5). By PhyloChip the taxon otu_7603, representing Las, was detected at a very low level in asymptomatic plants, but over 200 times more abundant in symptomatic plants. Except Las, the eight taxa which were more abundant in symptomatic compared to asymptomatic plants included representatives from the groups Phyllobacter, Dehalicoccoides, Brevundimonas 6904 and 7359, Sphingobacterium, Verrucomicrobia, Caulobacter, and Syntrophobacter (Fig. 2), and these bacteria have not been reported to cause plant diseases so far. Their roles in the HLB symptom development remain to be investigated.

Some bacteria were detected at higher abundance from HLB asymptomatic samples over symptomatic ones. For example, Incertae sedis 5, Oxalobacteraceae, Alcaligenaceae, Hydrogenophilaceae, Rhodocylclaceae, Pseudomonadaceae, Nocardioidaceae, Propionibacteriaceae, Bacillaceae, Simkaniaceae, Verrucomicrobiaceae, and Saprospiraceae, some having biocontrol and plant growth promoting potential (3, 11,21,35), were only identified from asymptomatic samples based on the cloning. It is unknown whether they played significant

roles in suppressing the HLB symptom. The asymptomatic phenomenon might in some cases be due to the low titer of Las in the phloem, considering that previous results indicated a minimal Las population is required for the symptom development (N. Wang unpublished). Interestingly, clone library analysis and Las-specific PCR seemed to suggest that a few escape trees might exist (asymptomatic trees with heavy loads of the putative pathogen Las). Both methods found Las in the asymptomatic tree G1A4, while clone library also indicated the presence of Las at high titers in asymptomatic trees G1A1 and G2A5, and PCR showed Las in asymptomatic trees G1A4 and G2A3. It remains to confirm whether those trees can survive high population of Las without showing any disease symptoms or whether the endophytic microbial community play a role in the symptom suppression.

Citrus leaves can support a diversity of microbes either epiphytically or endophytically. PhyloChip analysis revealed the presence of 47 orders of bacteria in 15 phyla while 20 orders in eight phyla were observed with cloning and sequencing method from the citrus leaf midribs. Actinobacteria, Proteobacteria, and Firmicutes have been reported previously associated with plant leaves (22). The majority of the bacteria are insect transmitted or endosymbionts of insects. Las has been shown to be psyllid transmitted. Most of the clones from 16S rDNA library were closely related to bacteria reported as endosymbionts of various insects (16,19,20,45). Lacava et al. (29) have reported similarity between the endophytes of host plants and bacteria inhabiting head region of glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis*, an important vector of various strains of *Xylella fastidiosa*. Our study also indicated towards multipartite interactions between the host plant, insect vector and the associated microbial diversity. However, some bacteria such as Chlamydiae, AD3, Bacteroidetes, and mgA-2 have never been reported to be associated with plant leaves (3,7,24,32,52). This indicates that our understanding

of the extent of microbial diversity associated with plant leaves is still incomplete. It is not surprising that the bacterial population associated with citrus midribs seems to be quite different from and more diverse than the citrus phyllosphere (51). The majority of the bacterial population in our study are likely endophytic since surface sterilization was used. Surface sterilization was known to eliminate most microbes on the leaf surface but not all (9). The microbiome associated with citrus leaves from HLB pathogen infected groves in Florida is very different from that of *Xylella fastidiosa* infected citrus groves in Brazil (2). *Curtobacterium flaccumfaciens*, *Enterobacter cloacae*, *Methylobacterium* spp. *Nocardia* sp. and *Pantoea agglomerans* were reported from *Xylella fastidiosa* infected citrus branches in Brazil while not in our study (2). This might be due to the differences of the two geographic locations in the environment conditions in which the plants are grown (e.g., geographic areas and weather conditions), dominating pathogens associated with the plants, or sampled tissues (leaf midrib or branch).

This study represents a extensive molecular analysis of the bacterial composition in the citrus leaf midribs from HLB positive citrus groves. We have demonstrated that both symptomatic and asymptomatic leaves contain a diverse assemblage of bacteria. Some other bacteria have been identified from HLB diseased citrus besides *Ca*. Liberibacter. Las dominates in the symptomatic leaves compared to the asymptomatic leaves, implicating this organism as the causal agent of the HLB disease.

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Table 1. Richness of microbial communities by grove or evidence of symptoms from HLB diseased citrus groves based on PhyloChip analysis.

	Asymptomatic		Symptomatic		ANOVA		
Grove	1	2	1	2	Gr	Sy	X
Domain							
Bacteria	18.7 ± 2.2	22.17 ± 0.94	22.7 ± 2.5	51.2 ± 13.4	*	*	
Phylum (or Class, inset)							
Acidobacteria	0.17 ± 0.17	0.17 ± 0.17	0.33 ± 0.21	3.33 ±1.15		*	
Actinobacteria	0.67 ± 0.42	0.83 ± 0.17	0.83 ± 0.17	1.83 ± 0.6			
AD3	0.67 ± 0.21	0	0.83 ± 0.17	0.5 ± 0.22			
Bacteroidetes	0.83 ± 0.17	0	0.83 ± 0.17	2.33 ± 0.61	*		
BRC1	0.33 ± 0.21	0.83 ± 0.17	0.83 ± 0.17	0.83 ± 0.17			
Chlamydiae	0.17 ± 0.17	0.17 ± 0.17	0.5 ± 0.22	0.67 ± 0.21		*	
Chlorobi	0.17 ± 0.17	0.17 ± 0.17	0.33 ± 0.21	0.83 ± 0.17		*	
Chloroflexi	0	0.33 ± 0.21	0.33 ± 0.21	1.0 ± 0.52			
Firmicutes	0	0	0	0.67 ± 0.84			
Gemmatimonadetes	0	0	0	0.5 ± 0.34	*n.d.*		
Marine.group.A	0	0	0	0.5 ± 0.22	*n.d.*		
NC10	0	0	0	0.17 ± 0.17	*n.d.*		
Planctomycetes	1.33 ± 0.21	1.67 ± 0.21	1.33 ± 0.21	2.0 ± 0.0	*		
Proteobacteria	0.5 ± 0.34	0.5 ± 0.22	0.33 ± 0.33	14.0 ± 6.8			
a-proteobacteria	0.17 ± 0.17	0 ± 0	0.17 ± 0.17	19.0 ±9.1			*
β-proteobacteria	0	0.33 ± 0.21	0.33 ± 0.21	1.83 ± 1.25			
δ-proteobacteria	0.17 ± 0.17	0 ± 0	0 ± 0	4.5 ± 2.16			
ε-proteobacteria	0	0	0	0.33 ± 0.21			
γ-proteobacteria	0.17 ± 0.17	0	0.33 ± 0.21	3.17 ± 1.66	*		*
TM7	0.17 ± 0.17	0	0	0.5 ± 0.22	*n.d.*		
Unclassified	0.83 ± 0.17	1.0 ± 0	1.0 ± 0.26	2.0 ± 0.52			
Verrucomicrobia	0.5 ± 0.22	1.0 ± 0	1.17 ± 0.31	1.83 ± 0.6		*	

Numbers shown are the means plus or minus one standard error of the mean for 5 degrees of freedom. Statistical analysis was performed as ANOVAs for each phylum, where statistical significance is denoted for (*) $p < 0.05$. Statistical analysis was not performed on phyla containing fewer than 5 taxa total, denoted by '*n.d.*' Factors in the ANOVA analyses include Gr = Grove, Sy = symptomatic, X = cross-product.

Table 2. Relative abundance of clones from HLB asymptomatic or symptomatic citrus leaf midribs.

		Grove 1		Grove 2	
Plylum/Class[a]	Order/family	**Asymptomatic G1A**	**Symptomatic G1S**	**Asymptomatic G2A**	**Symptomatic G2S**
Proteobacteria					
Alphaproteobacteria					
	Rhizobiales				
	Phyllobacteriaceae	0.547	0.8845	0.1010	0.8443
	Bradyrhizobiaceae	0	0.0024	0.0106	0.0019
	Rhizobiaceae	0	0	0	0.0019
	Unclassified	0	0	0.0053	0
	Caulobacterales				
	Caulobacteraceae	0.0210	0.0049	0.0585	0.0019
	Sphingomonadales				
	Sphingomonadaceae	0	0.0049	0.0106	0
	Unclassified	0.0052	0.0073	0	0
Betaproteobacteria					
	Burkholderiales				
	Comamonadaceae	0.0052	0.0024	0.0106	0.0019
	Incertae sedis 5	0.0105	0	0.0212	0
	Oxalobacteraceae	0	0	0.0212	0
	Alcaligenaceae	0	0	0.0053	0
	Unclassified	0.0052	0	0	0
	Hydrogenophilales				
	Hydrogenophilaceae	0.0052	0	0	0
	Rhodocyclales				
	Rhodocyclaceae	0.0052	0	0	0
Gammaproteobacteria					
	Pseudomonadales				
	Moraxellaceae	0.0157	0	0	0.0019
	Pseudomonadaceae	0	0	0.0053	0
	Oceanospirillales				
	Halomonadaceae	0	0	0	0.0059
	Unclassified	0	0.0024	0	0
	Enterobacteriales				
	Enterobacteriaceae	0	0	0	0.0059
Deltaproteobacteria					
	Desulfuromonadales				
	Geobacteraceae	0	0	0	0.0019
	Unclassified	0	0.0024	0	0
Actinobacteria					
Actinobacteria					
	Actinomycetales				
	Actinomycetaceae	0	0.0049	0.0053	0.0019
	Nocardioidaceae	0	0	0.0053	0

		Grove 1		Grove 2	
Plylum/Class[a]	Order/family	**Asymptomatic G1A**	**Symptomatic G1S**	**Asymptomatic G2A**	**Symptomatic G2S**
	Propionibacteriaceae	0	0	0.0053	0
Firmicutes					
Bacilli					
	Bacillales				
	Bacillaceae	0	0	0.0106	0
	Lactobacillales				
	Carnobacteriaceae	0	0	0.0053	0
Chlamydiae					
Chlamydiae					
	Chlamydiales				
	Simkaniaceae	0	0	0.0212	0
Bacteroidetes					
Bacteroidetes					
	Bacteroidales				
	Bacteroidaceae	0	0.0024	0	0
Flavobacteria					
	Flavobacteriales				
	Flavobacteriaceae	0.3684	0.0614	0.6276	0.1157
	Unclassified	0	0.0024	0.0053	0.0019
	Unclassified	0	0	0	0.0099
Sphingobacteria					
	Sphingobacteriales				
	Flexibacteraceae	0	0.0122	0.0212	0.0019
	Saprospiraceae	0	0	0.0053	0
Verrucomicrobia					
	Verrucomicrobiales				
	Verrucomicrobiaceae	0.0052	0	0	0
Dictyoglomi					
Dictyoglomi					
	Unclassified	0.0052	0.0024	0.0372	0
TM-7		0	0.0024	0	0
Total		**1**	**1**	**1**	**1**

The numbers of clones in the libraries and data set were as follows: Grove 1 Asymptomatic, 190; Grove 1 Symptomatic, 407; Grove 2 Asymptomatic, 188; and Grove 2 symptomatic, 501. Totally there were 776 clones matching chloroplast or mitochondria products and were not included in this analysis.

Table 3. Phyla detected in different samples by high-density PhyloChip analysis or by cloning and sequencing

	PhyloChip				Coning and sequencing			
	Asymptomatic		**Symptomatic**		**Asymptomatic**		**Symptomatic**	
Plylum/Class	**Grove 1 (G1A)**	**Grove 2 (G2A)**	**Grove 1 (G1S)**	**Grove 2 (G2S)**	**Grove 1 (G1A)**	**Grove 2 (G2A)**	**Grove 1 (G1S)**	**Grove 2 (G2S)**
Proteobacteria	Y	Y	Y	Y	Y	Y	Y	Y
Alphaproteobacteria	Y	Y	Y	Y	Y	Y	Y	Y
Betaproteobacteria		Y	Y	Y	Y	Y	Y	Y
Deltaproteobacteria	Y			Y			Y	Y
Episilonproteobacteria				Y				
Gammaproteobacteria	Y		Y	Y	Y	Y		Y
Acidobacteria	Y	Y	Y	Y				
Actinobacteria	Y	Y	Y	Y		Y	Y	Y
AD3	Y		Y	Y				
Bacteroidetes	Y		Y	Y	Y	Y	Y	Y
BRC1	Y	Y	Y	Y				
Chlamydiae	Y	Y	Y	Y		Y		
Chlorobi	Y	Y	Y	Y				
Chloroflexi		Y	Y	Y				
Dictyoglomi					Y	Y	Y	
Firmicutes				Y		Y		
Gemmatimonadetes				Y				
NC10				Y				
Planctomycetes	Y	Y	Y	Y				
TM7	Y			Y		Y		
Unclassified Bacteria	Y		Y	Y	Y	Y	Y	Y
Verrucomicrobia	Y	Y	Y	Y	Y			

Y: positive; blank: negative.

Fig. legends

Fig. 1. Agarose gel electrophoresis of PCR products amplified using primers specific for Las. Primers target the 16S rDNA of Las using specific primers A2/J5 that result in amplicon of 703 bp (23). Total DNA extracted from symptomatic and asymptomatic leaf midribs of sweet orange trees were used as templates for PCR amplification. Symptomatic leaf midribs: Lanes 1-6; Asymptomatic leaf midribs: lanes 7-12; Upper panel: Grove1; Lower panel: Grove 2; M: DNA molecular weight size markers.

Fig. 2. Nine taxa were significantly different ($p < 0.05$) between the symptomatic and asymptomatic leaves in either grove of all the 117 taxa detected in the leaf midrib microbial community. These nine taxa are shown below as mean of hyb score, with error bars denoting standard error.

Fig. 3. Prevalence of *Ca*. Liberibacter asiaticus in clone libraries from asymptomatic and symptomatic trees in each of the two groves sampled.