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Complete Genome Sequence of Citrus Huanglongbing Bacterium, '*Candidatus* Liberibacter asiaticus' Obtained Through Metagenomics

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Citrus huanglongbing is the most destructive disease of citrus worldwide. It is spread by citrus psyllids and is associated with a low-titer, phloem-limited infection by any of three uncultured species of α-Proteobacteria, '*Candidatus* Liberibacter asiaticus', '*Ca.* L. americanus', and '*Ca.* L. africanus'. A complete circular '*Ca.* L. asiaticus' genome has been obtained by metagenomics, using the DNA extracted from a single '*Ca.* L. asiaticus'–infected psyllid. The 1.23-Mb genome has an average 36.5% GC content. Annotation revealed a high percentage of genes involved in both cell motility (4.5%) and active transport in general (8.0%), which may contribute to its virulence. '*Ca.* L. asiaticus' appears to have a limited ability for aerobic respiration and is likely auxotrophic for at least five amino acids. Consistent with its intracellular nature, '*Ca.* L. asiaticus' lacks type III and type IV secretion systems as well as typical free-living or plant-colonizing extracellular degradative enzymes. '*Ca.* L. asiaticus' appears to have all type I secretion system genes needed for both multidrug efflux and toxin effector secretion. Multiprotein phylogenetic analysis confirmed '*Ca.* L. asiaticus' as an early-branching and highly divergent member of the family Rhizobiaceae. This is the first genome sequence of an uncultured α-proteobacteria that is both an intracellular plant pathogen and insect symbiont.

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Nucleotide sequence data for the Whole Genome Shotgun project has been deposited in the DDBJ/EMBL/GenBank database under project accession number ABQW00000000. The version described in this paper is the first version, ABQW01000000.

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Citrus huanglongbing (HLB), popularly known as citrus greening disease, was first noted in China in the early 20th century (Zhao 1981). The disease is associated with three species of phloem-restricted α-Proteobacteria, '*Candidatus* Liberibacter asiaticus' in Asia, '*Ca.* L. africanus' in Africa (Jagoueix et al. 1994), and '*Ca.* L. americanus' in Brazil, South America (Teixeira 2005). '*Ca.* L. asiaticus' has a wide host range and can infect, although not necessarily cause disease, on most rutaceous species and some solanaceous species (Halbert and Manjunath 2004). HLB is transmitted by two phloem-feeding insect vectors, the Asian citrus psyllid *Diaphorina citri* and the African citrus psyllid *Trioza erytreae*, and is considered the most destructive disease of citrus in the world (Bove 2006; Brlansky and Rogers 2007; Callaway 2008; Gottwald et al. 2007; Stokstad 2006). Management of the disease is not only difficult but also expensive, and no cure is currently available for infected trees. Approximately 100 million infected citrus trees have been destroyed by the disease throughout Asia, with an additional one million trees eliminated in Brazil since the first report of the disease in São Paulo in 2004 (Gottwald et al. 2007). In the U.S., HLB was first discovered in August 2005 in South Florida, seven years after the introduction of *D. citri* into the state (Sutton et al. 2005). Since that time, HLB has spread to all of Florida's citrus-growing counties and has recently been reported in the state of Louisiana (Southern Plant Diagnostic Network 2008). Of even greater concern is the fact that the vector *D. citri* can now be found in other citrus-producing states, including Texas (Da Graça and Korsten 2004), Hawaii, and California (Citrus Research Board 2008).

In addition to the Liberibacter sp. that is affecting the citrus industry, a new Liberibacter species, '*Ca.* L. solanacearum', was recently associated with the emerging 'zebra chip' disease of potatoes in the U.S. and tomatoes in New Zealand (Liefting et al. 2008). '*Ca.* L. solanacearum' is closely related to '*Ca.* L. asiaticus', although '*Ca.* L. solanacearum' is not associated with citrus HLB nor has it been found in Asian citrus psyllids (Li et al. 2008b).

Attempts to obtain the entire Liberibacter genome using HLB-affected plants have been unsuccessful, largely due to the fact that the '*Ca.* L. asiaticus' bacterium has not been cultured and is present in very low titers in its hosts (the copy ratio of '*Ca.* L. asiaticus' DNA to host plant genomic DNA is 1:1,000) (Li et al. 2006). To date, only 24,477 nonredundant base pairs

(bp) from five contigs and two singlets (covering 12 full-length genes, two partial genes, one pseudogene, and several intergenic regions of the bacterium) have been obtained from HLB-affected plant DNA extracts by various methods (Hocquellet et al. 1999; Jagoueix et al. 1994, 1997; Lin et al. 2008; Okuda et al. 2005; Villechanoux et al. 1992). Another possible source of '*Ca.* L. asiaticus' DNA is the insect vector. Despite the fact that most infected psyllids collected from HLB-affected citrus trees carry low titers of '*Ca.* L. asiaticus', some heavily infected adult psyllids harboring up to 10^{10} '*Ca.* L. asiaticus' cells per head (Li et al. 2008a) can be found. Whole-genome amplification methods, such as multiple displacement amplification (MDA) (Dean et al. 2002), have been successfully applied to obtain whole insect genomes from a small amount of initial DNA extract such as that found in first instar larval mosquitoes or single adult legs (Gorrochotegui-Escalante and Black 2003). In this study, we report the first completed genome of the uncultured '*Ca.* L. asiaticus' bacterium obtained by metagenomics using MDA and pyrosequencing of the DNA extracted from a single psyllid carrying a high titer of '*Ca.* L. asiaticus' bacterium.

RESULTS AND DISCUSSION

Concentration of '*Ca.* L. asiaticus' bacterial DNA from a single psyllid.

Because of the low titer, phloem restriction, and uneven distribution of '*Ca.* L. asiaticus' in all tested host plants, we chose to obtain bacterial DNA from *D. citri*. In a *D. citri* population from Florida, approximately 3% of the infected population carried a '*Ca.* L. asiaticus' population of approximately 10^8 bacterial cells per head, which was estimated to yield a 1:1 copy ratio of '*Ca.* L. asiaticus' DNA to *D. citri* DNA (Li et al. 2008a). This ratio was approximately 1,000-fold higher than that of '*Ca.* L. asiaticus' DNA to plant DNA obtained from symptomatic tissues of HLB-affected citrus plants. Based on the linear regression of the absolute standard curve for '*Ca.* L. asiaticus' using real-time polymerase chain reaction (PCR) and the fact that there are three copies of template 16S rDNA per '*Ca.* L. asiaticus' genome, it was calculated that Psyllid #62 (Psy62) contained 1.29×10^{10} copies of the '*Ca.* L. asiaticus' genome per microliter of DNA. Using multiplex PCR, the DNA copy ratio of several possible sources of DNA were determined (Fig. 1). The copy ratios of the main targets (*D. citri*, '*Ca.* L. asiaticus', and a Wolbachia sp.) were 1, 576, and 28, respectively. Although the '*Ca.* L. asiaticus' bacterium contains three copies of 16S rDNA and the *Wolbachia* sp. contains only one copy, the predominance of the '*Ca.* L. asiaticus' DNA found in the Psy62 sample would provide an adequate source for obtaining the '*Ca.* L. asiaticus' genome sequence.

Differentiation of '*Ca.* L. asiaticus' genomic DNA in the Psy62 sample.

After MDA amplification of Psy62 DNA and 454 pyrosequencing, a total of 419,571 shotgun reads (average read length of 216 bp) were generated, containing 90,813,125 bp. These shotgun reads were assembled into 1,475 contigs covering 2,386,844 bp. Each contig ranged in size from 500 to 186,240 bp. A total of 320 PCR confirmation reactions (covering approximately 551 kb of Psy62 DNA) were run against both HLB-affected and unaffected samples obtained from citrus, periwinkle, and psyllids (Fig. 2), to determine which of the 1,475 contigs were associated with '*Ca.* L. asiaticus' and which were from other sources of DNA such as *Wolbachia* spp. or *D. citri*. Ultimately, 38 contigs covering 1,225,539 bp were confirmed by PCR to have originated from '*Ca.* L. asiaticus'. These '*Ca.* L. asiaticus' contigs were assembled from 91,875 reads and covered 19,885,425 bp, providing an estimated 16-fold coverage of the '*Ca.* L. asiaticus' genome. Extensive PCR reactions that allowed the joining of HLB-associated contigs resulted in closure of the '*Ca.* L. asiaticus' chromosome. The gaps between these contigs were 853 bp on average, ranging from 24 to 2,885 bp. Of the 551 kb of confirmation PCR DNA, 122,400 bp were resequenced and only 24 bp were inconsistent with the original 454 assembly. Only three misassemblies were discovered and Sanger sequencing was performed to correct these misassembly errors. These indicated that the Psy62 '*Ca.* L. asiaticus' genome obtained by MDA and 454 pyrosequencing had an accuracy ≥99.9% and that the genomes among the population of '*Ca.* L. asiaticus' cells in psyllid Psy62 were quite uniform.

General features of the Psy62 '*Ca.* L. asiaticus' draft genome.

Recently, pulsed-field gel electrophoresis revealed the main chromosome of the related species '*Ca.* L. americanus' to be circular and approximately 1.26 Mb in size (Wulff et al. 2008). Consistent with these results, our '*Ca.* L. asiaticus' genome appears to be a single circular chromosome containing 1,227,204 bp, with an average GC content of 36.5% (Table 1). Upon annotation, three operons for rRNA genes and a total of 44 tRNA genes were found within the genome. The replication origin of the chromosome was predicted at position 436,457, where the GC skew reached the global minimum. In the vicinity,

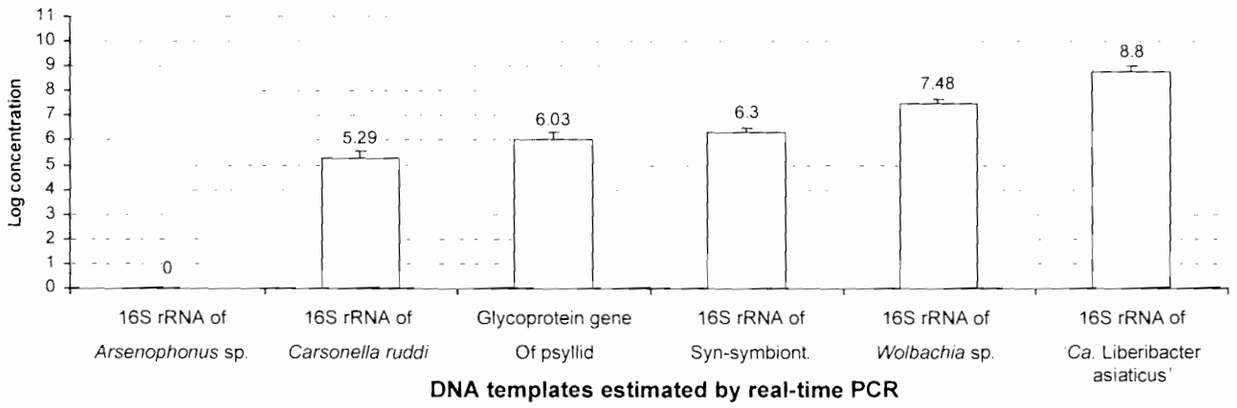

Fig. 1. Multiplex real-time polymerase chain reaction–based estimation of 16S rDNA copy numbers of the five major endosymbiotic bacteria from a single Asian citrus psyllid (*Diaphorina citri*), namely, *Arsenophonus* sp., *Carsonella ruddi*, *Wolbachia* sp., Syn-symbiont, and '*Candidatus* Liberibacter asiaticus'. Note the logarithmic scale.

three DnaA boxes were arranged in inverted orientations. Approximately 74% of the 1,136 predicted coding sequences have homologs with known and putative functions, while the other 26% represent hypothetical conserved open reading frames, which are conserved in other microorganisms but with unknown function. A total of 32 genes were determined to be pseudogenes. Table 2 contains a summary of the COG (Clusters of Orthologous Groups database) assignments and their functional categories. As with most reduced genomes, there were a relatively low number of genes involved in the biosynthesis of compounds readily taken up from the host and regulatory elements, including σ factors, probably reflecting the reduced environmental challenges associated with an intracellular lifestyle (Konstantinidis and Tiedje 2004). By contrast, there were a high number of genes, particularly for a small genome, involved in cell motility (4.5% in '*Ca.* L. asiaticus' as compared with 0.1% in the *Wolbachia* sp. and 1.2% in α-Proteobacteria [Table 2]), including type IV pili and flagellar genes. There were 92 genes involved in active transport, including 40 ATP-binding cassette (ABC) transport genes. In contrast to the *Wolbachia* sp., no transposons or insertion elements were identified in the '*Ca.* L. asiaticus' genome (Wu et al. 2004). However, 12 phage-related genes were found, indicating that a phage or prophage may be present.

Phylogenetic analysis and comparative genomics.

The '*Ca.* L. asiaticus' genome from Pys62 was incorporated into a robust species tree for α-Proteobacteria, based on 104 conserved genes that show no evidence of horizontal transfer (Williams et al. 2007). Two of these 104 genes were not identified in '*Ca.* L. asiaticus', and to ensure that only vertically transmitted genes were used, an additional eight genes were rejected that scored poorly in a hidden Markov model (HMM) for the family. Bayesian phylogenetic analysis of the concatenation of the trimmed alignments for the remaining 94 proteins produced a tree with identical topology and similar lengths to that obtained previously, with 100% bootstrap support for every branch (Fig. 3). The current analyses using robust genome-wide, gene-based phylogeny places '*Ca.* L. asiaticus' within the order *Rhizobiales* in agreement with the previous 16S and other gene-based analyses (Doddapaneni et al. 2008; Williams et al. 2007). Furthermore, for the first time and using large samples of both bacteria and genes, we are able to specifically show that within the order *Rhizobiales*, '*Ca.* L. asiaticus' is closely associated with members of the *Rhizobiaceae* family. Based on its position in the tree, which received 100% Bayesian support, '*Ca.* L. asiaticus' can be considered an early-branching member of the *Rhizobiaceae* family. It is interesting to note that the distance from the root is far longer for '*Ca.* L. asiaticus' than for any other tested member of the order *Rhizobiales* (indeed, exceeded among all α-Proteobacteria only by *Neorickettsia sennetsu*), suggesting that it has evolved much more rapidly. Such rapid genome evolution is typical of host-restricted symbionts or pathogens, usually explained by elevated genetic drift resulting both from population bottlenecks and relaxed selection on many genes (Moran et al. 2008).

Analysis of the putative protein coding gene sequences of Psy62 '*Ca.* L. asiaticus' (1,136 proteins) with the genomes of other related bacteria supports phylogenetic positioning of this bacterium rather than positioning by a niche-based genome

Table 1. General features of '*Candidatus* Liberibacter asiaticus' genome

Feature	Corresponding no.
Size (bp)	1,227,204
GC%	36.5
rRNA operons	3
tRNA	44
Total number of predicted genes	1186
Protein coding genes	1136
Genes assigned to the COG (Clusters of Orthologous Groups) database	836
Hypothetical proteins	362
Pseudogenes	32

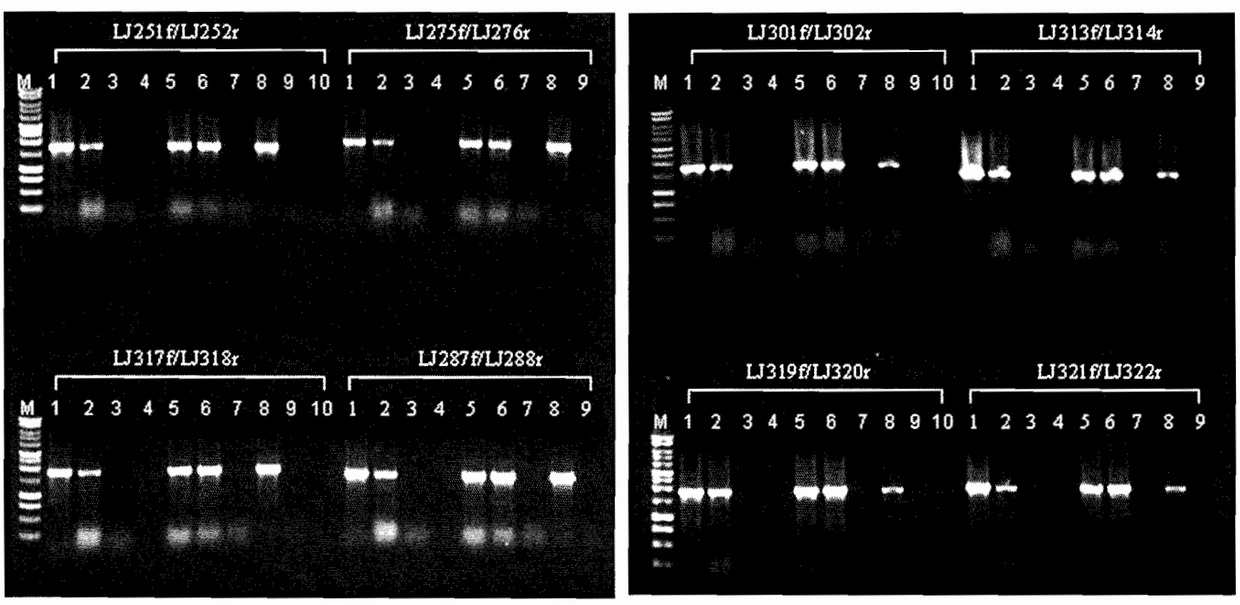

Fig. 2. Polymerase chain reaction (PCR) confirmation of the '*Candidatus* Liberibacter asiaticus' genome sequences using infected vs. noninfected citrus, periwinkle, and psyllids. Lane M, 1-kb DNA ladder; lane 1, Citrus huanglongbing (HLB)-affected citrus from field; lane 2,HLB-affected citrus from green house; lane 3, HLB-free citrus from field; lane 4, HLB-free citrus from green house; lanes 5 and 6, HLB-affected periwinkles from greenhouse; lane 7, HLB-free periwinkle from green house; lane 8, pooled DNA from eight individual '*Ca.* Liberibacter asiaticus'–infected psyllids; lane 9, pooled DNA from 10 individual '*Ca.* Liberibacter asiaticus'–free psyllids; and lane 10, no DNA negative control. Numbers above PCR reactions beginning with LJ correspond to the primer pairs used for each PCR reaction.

adaptation. A four-way comparison of the '*Ca.* L. asiaticus' genome was performed with the genomes of *Sinorhizobium meliloti* (3,341 proteins), *Candidatus* Phytoplasma asteris (754 proteins), and *Wolbachia pipientis* (1,208 proteins) (Fig. 4). A total of 174 proteins were present in all four genomes, with a majority of these proteins playing a role in the core metabolic pathways of the cell. There were 7 to 255 proteins present in three of the four genomes compared, although no common proteins were found among the other three genomes if *S. meliloti* was excluded. In this analysis, one protein (Na+/H+-dicarboxylate symporter) was found to be common only between *W. pipientis* and '*Ca.* L. asiaticus' (both are insect symbionts) but none of the other genomes, while no common proteins existed only between '*Ca.* P. asteris' and '*Ca.* L. asiaticus' (the two phloem pathogens and insect endosymbionts). On the other hand, 279 proteins (24.8%) present in '*Ca.* L. asiaticus' are also present only in *S. meliloti* (two closely related members of the *Rhizobiaceae* family).

Metabolic pathways.

In order to provide a framework from which the metabolic needs of the organism could be deduced, all major metabolic pathways and enzyme reactions predicted from the '*Ca.* L. asiaticus' genome sequence were mapped onto established metabolic pathways and are summarized here. Briefly, '*Ca.* L. asiaticus' contains all 14 genes that typically encode NADH dehydrogenase subunits [A-N], a major component of the respiratory electron transport chain. However, no homologs corresponding to the terminal stages of oxidative phosphorylation were identified. This includes the absence of two key enzymes, polyphosphate kinase (EC 2.7.4.1) and inorganic diphosphatase (EC 3.6.1.1), that metabolize pyrophosphate to Pi and ADP. Likewise, the '*Ca.* L. asiaticus' genome does not appear to have homologs for the cytochrome bc1 complex (EC 1.10.2.2), cytochrome c oxidase, the cbb3-type (EC 1.9.3.1), or the cytochrome bd complex although all four cytochrome O ubiquinol oxidase subunits [I to IV] have been identified. This is interesting because α-Proteobacteria (the class containing '*Ca.* L. asiaticus') typically employ cytochrome c oxidases as terminal oxidases, whereas γ-Proteobacteria (the class containing *E. coli* and *Xylella fastidiosa*) employ quinol oxidases (Foster et al. 2005). Unlike *E. coli*, which has two terminal oxidases (Anraku and Gennis 1987), the respiratory complex of '*Ca.* L. asiaticus' resembles that of the citrus pathogen *X. fastidiosa* (Bhattacharyya et al. 2002), which has one terminal oxidase encoded for by the cyo operon and is active only under oxygen-rich conditions (Cotter et al. 1990; Rice and Hempfling 1978). Therefore, due to the lack of key enzymes involved in oxidative phosphorylation as well as absence of diverse terminal oxidases, it can be concluded that '*Ca.* L. asiaticus' has a limited capacity for aerobic respiration.

Because of the limited aerobic capabilities, pathways necessary for anaerobic respiration were also investigated. The '*Ca.* L. asiaticus' genome did not contain any genes involved in sulfur metabolism; however, several enzymes involved in nitrogen metabolism, such as a NAD+ synthase (EC 6.3.1.5), gluta-

Table 2. Summary of COG (Clusters of Orthologous Groups) database assignments by functional category and correlation with the total number of open reading frames

Functional class and categories		'*Candidatus* Liberibacter asiaticus' genome		Correlation[a]	% in *Wolbachia* AE017321	% in α-proteobacteria[b]
		No. of genes	% of total			
Information storage and processing						
J	Translation, ribosomal structure & biogenesis	123	10.83	−	13.91	4.31
K	Transcription	29	2.55	+	2.07	5.69
L	DNA replication, recombination, repair	75	6.60	−	6.21	4.36
Cellular processes						
D	Cell division and chromosome partitioning	13	1.14	−	1.03	0.71
V	Defense mechanisms	5	0.44	No	0.23	0.97
O	Postranslational modification	47	4.14	No	5.86	3.24
M	Cell envelope biogenesis, outer membrane	57	5.02	No	3.79	4.34
P	Inorganic ion transport and metabolism	25	2.20	No	4.02	4.79
U	Intracellular trafficking and secretion	7	0.62	No	3.33	1.78
N	Cell motility	51	4.49	+	0.11	1.18
T	Signal transduction	18	1.59	+	1.15	3.70
Metabolism						
F	Nucleotide transport and metabolism	44	3.87	−	4.25	1.61
G	Carbohydrate transport and metabolism	23	2.02	No	2.76	4.90
E	Amino acid transport and metabolism	48	4.23	No	4.37	8.18
H	Coenzyme metabolism	53	4.67	No	3.79	2.97
I	Lipid metabolism	33	2.90	No	2.99	3.54
C	Energy production and conversion	62	5.46	+	7.82	5.15
Q	Secondary metabolite transport and metabolism	8	0.71	+	1.26	2.80
Poorly characterized						
R	General function prediction only	69	6.07	No	7.24	10.62
S	Function unknown	46	4.05	No	3.56	6.06
	Not in COG	300	26.4		20.23	19.03
		1,136	100.00		100.00	100.00

[a] The expected positive (+), negative (−), or no correlation (No) of genome size with the number of genes in each COG assignment category (Konstantinidis and Tiedje 2004).

[b] Representative small (940 genes encoded by 1.08 Mb) α-Proteobacteria genome.

mine synthetase (EC 6.3.1.2), and glutaminase (EC 3.5.1.2), along with those enzymes involved in the glutamate metabolism were identified, suggesting a dependence of '*Ca.* L. asiaticus' on nitrogen metabolism. The above results imply that '*Ca.* L. asiaticus' can not reduce sulfate and possibly depends heavily upon nitrogen utilization to generate energy through anaerobic respiration.

Key enzymes such as PfkA, which encodes for 6-phosphofructokinase (EC 2.7.1.11) in bacteria (Koonin and Galperin 2003), and Pgm, which encodes a phosphoglucomutase (EC 5.3.1.9), were present in the genome, while no enzymes for the Entner-Doudroff pathway were uncovered, indicating that glycolysis is the major pathway for the catabolism of monosaccharides. Based on the enzymes that are present, '*Ca.* L. asiaticus' has the ability to metabolize sugars such as glucose, fructose, and xylulose but not mannose, galactose, rhamnose, or cellulose. How these sugars are transported into the cell remains unclear, since the only phosphotransferase system (PTS) protein identified within the genome was a SgaT homolog, a component of a PTS permease. Considering the multiple ABC transporter proteins in the '*Ca.* L. asiaticus' genome, it is highly possible that these may be involved in sugar transport. Several obligate intercellular parasites, such as *Chlamydia trachomatis* and *Rickettsia prowazeki*, have developed a unique system for obtaining energy. These bacteria can act as energy parasites and scavenge ATP from their host through the use of an ATP/ADP translocase (Hatch et al. 1982; Winkler 1976). Interestingly, '*Ca.* L. asiaticus' encodes for an ATP/ADP translocase in addition to its ATP synthase, allowing it to both synthesize ATP as well as uptake this energy source directly from its surroundings. Additionally, genes for the tricarboxylic acid (TCA) cycle, including a gene encoding for citrate synthase (*gltA*), the principal port of entry of acetyl-

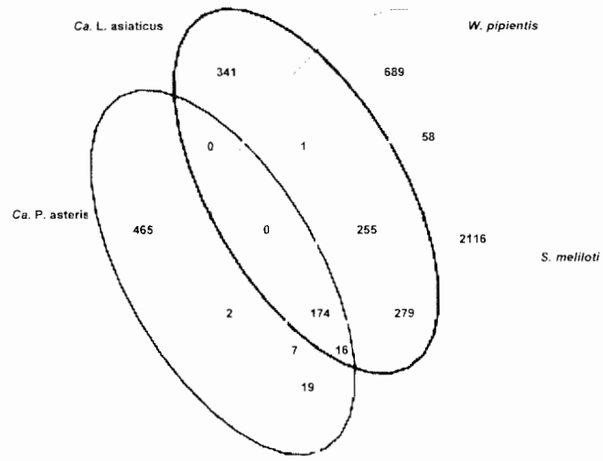

Fig. 4. Venn diagram comparing homologous and unique proteins among '*Candidatus* L. asiaticus', *Wolbachia pipientis* (an insect symbiont), *Sinorhizobium meliloti* (a member of the *Rhizobiaceae* family), and '*Ca.* P. asteris' (a phloem pathogen and insect symbiont).

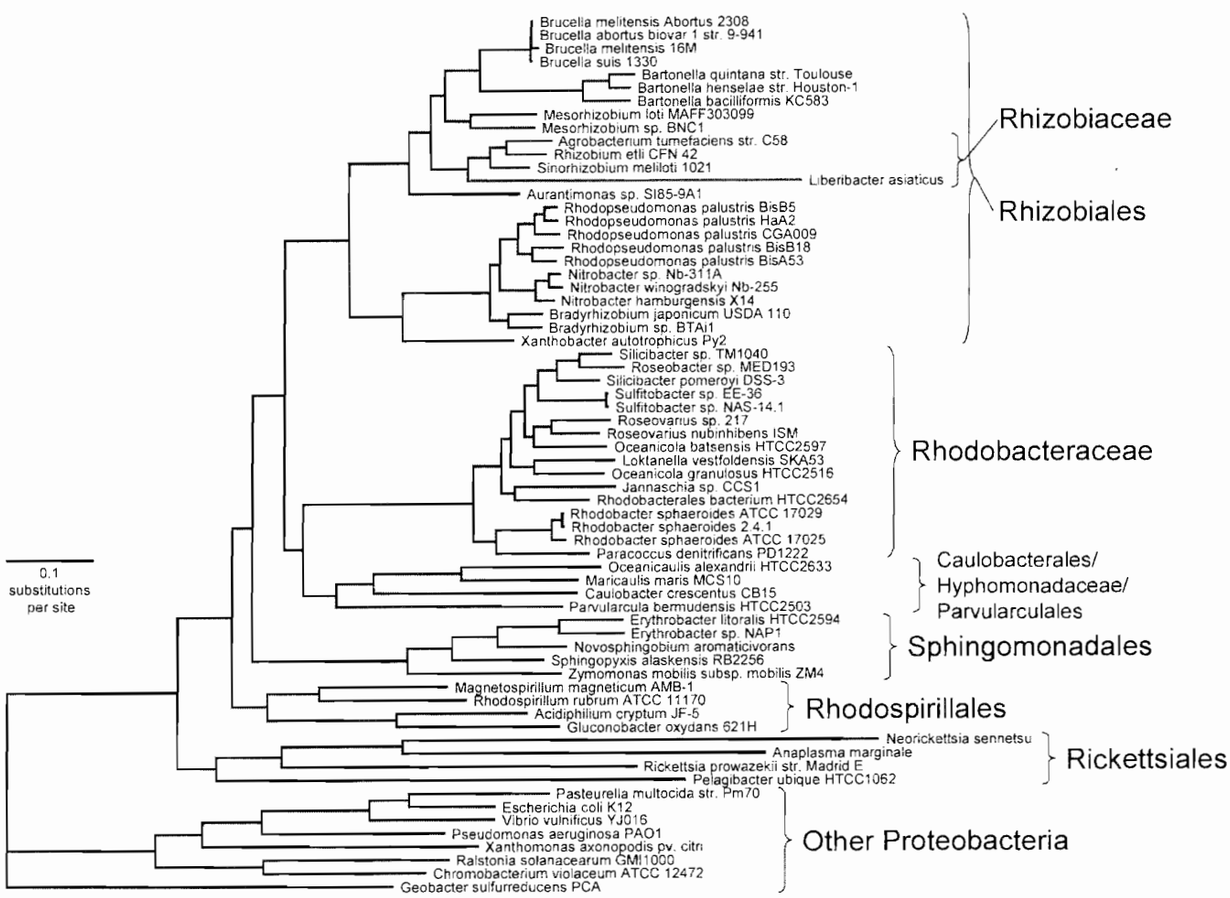

Fig. 3. Consensus tree from Bayesian phylogenetic analysis based on 94 proteins. Branches for the 46 strains most distant to '*Candidatus* L. asiaticus' have been removed for clarity. All nodes had 100% support.

CoA into the TCA cycle that is often used as an indicator of the presence of a functional TCA cycle, were identified in '*Ca.* L. asiaticus'. Given the presence of a functional TCA cycle, '*Ca.* L. asiaticus' may utilize a range of amino acids as energy sources. These include glutamate, alanine, aspartate, glycine, serine, threonine, methionine, cysteine, arginine, proline, histidine, tyrosine, phenylalanine, and tryptophan. With the exception of proline and tryptophan, all other amino acids listed above have been identified as components of the phloem sap in other studies (Ohshima et al. 1990), and thus, sap may act as the primary source of these amino acids for '*Ca.* L. asiaticus'.

Enzymes involved in the conversion steps from metabolic intermediates to the synthesis of amino acids have been identified in '*Ca.* L. asiaticus' for only two candidates, phenylpyruvate to phenylalanine and asparate to lysine. Those involved in the synthesis of tryptophan, tyrosine, leucine, isoleucine, and valine from metabolic intermediates are absent. Three of the above amino acids are known to be present in phloem, which is probably the direct source of these amino acids, while the source of the other two amino acids, tryptophan and valine, has yet to be determined.

One of the enzymes in the pentose phosphate pathway (PPP), known as transaldolase (EC 2.2.1.2), is absent in '*Ca.* L. asiaticus'. However, '*Ca.* L. asiaticus' has other enzymes of the PPP and the full pathways for both purine and pyramidine biosynthesis, suggesting the existence of a functional but divergent form of this enzyme in '*Ca.* L. asiaticus'. Previous studies have shown that transaldoase exists as several uncharacterized isoforms in bacterial genomes (Kube et al. 2008), thus coinciding with our hypothesis.

Transport proteins and types of secretion systems.

Similarity searches of the 1,136 '*Ca.* L. asiaticus' proteins classified 137 as transporter proteins. These 137 proteins fall into five of the nine known classification categories. Of these proteins, nine proteins belong to the channels/pores class of transporters, 24 proteins belong to the electrochemical potential-driven transporters, 92 are classified under primary active transporters, one belongs to the group translocators class, and the remaining 11 proteins can be classified under the incompletely characterized transport systems class of transporters. Of the 92 primary active transporters, 40 are ABC transporters, which is typical of α-Proteobacteria with a wide host range but in stark contrast to intracellular bacteria of a similar size, in which the average is 15 ABC transporters (Davidson et al. 2008). It is quite possible that some of these transporters affect virulence, host range, or symptom elicitation, alone or in combination. For example, the phosphate transport system found in '*Ca.* L. asiaticus' utilizes an ABC-type transporter that is known to mediate the uptake of phosphate into the cell and has been reported in several studies to be associated with the virulence of the bacteria (Daigle et al. 1995; Mantis and Winans 1993; von Kruger et al. 1999). Another example of an ABC transporter required for virulence in other organisms is the zinc transport system (*znuABC*) (Garrido et al. 2003). The presence of *znuABC* genes in '*Ca.* L. asiaticus' may allow the bacteria to uptake this micronutrient from the phloem, resulting in a local zinc deficiency and, thus, the mimicry of the symptoms between HLB-affected and zinc-deficient plants.

Secretion systems.

Not surprisingly for an intracellular bacterial pathogen whose route of infection involves direct injection by the psyllid into host cells, no type III or IV secretion systems or their effectors, including avirulence genes, were found. Type III and IV systems can play key offensive roles in allowing extracellular pathogens to attack both plant and animal hosts (Fauvart and Michiels 2008; Felix et al. 2008; Munkvold et al. 2008). Also not found in the genome were plant cell-wall degradation enzymes such as cellulases, pectinases, xylanases, or endoglucanases, which require type II secretion. In addition, genes typically involved in the external secretion of extracellular enzymes as part of the main terminal branch (MTB) of the type II secretion system (Johnson et al. 2006) were not present.

However, all proteins required for the first step of the type II secretion system, the general secretory pathway (TC 3.A.5.), which is responsible for the export of proteins to the periplasm (Pugsley 1993), were found in the '*Ca.* L. asiaticus' genome from Psy62. In addition, 10 putative proteins required for pilin secretion and assembly as part of the MTB (TC 3.A.15) were found, indicating the potential for type IV pilus secretion and assembly. Four putative pilin subunit (COG3847) proteins were also found. Although pilus formation by '*Ca.* L. asiaticus' needs to be experimentally verified, if present, the type IV pili could be used for autoaggregation and biofilm formation, as in *Xylella* spp., in which it is responsible for the obstruction of xylem flow (De La Fuente et al. 2008).

A nearly complete set of 30 genes involved in flagella biosynthesis were also found in the '*Ca.* L. asiaticus' genome (Supplementary Table S1). However, despite extensive published electron microscopy of '*Ca.* L. asiaticus' in both citrus and psyllid hosts (Bove 2006), there is no evidence to our knowledge that '*Ca.* L. asiaticus' cells are flagellated. One gene encoding the flagella motor switch protein (*fliN*) and three genes encoding the flagella motor protein and chemotaxis motility protein (*motB*, *motC*, and *motD*) appear to be pseudogenes (Supplementary Table S2). This, perhaps, may be the reason for the lack of an observable structure.

Several complete type I secretion systems were present. Two primary functions for the type I secretion machinery have been elucidated. The first one is defensive, involving multidrug efflux, protecting the bacterium against toxic environmental chemicals, antibiotics produced by other bacteria, and phytoalexins produced by hosts. Multidrug efflux has been demonstrated as an important mechanism for bacterial survival in members of genera *Erwinia*, *Rhizobium*, *Agrobacterium*, *Bradyrhizobium*, and *Xanthomonas* (Reddy et al. 2007). The other function is offensive, allowing the secretion of a variety of degradative enzymes and offensive effectors, some of which are antibiotics and others involved in plant or animal pathogenicity. Offensive enzymes and effectors known to be secreted via the type I system include a limited number of hydrolases (proteases, phosphatases, esterases, nucleases, and glucanases) and a relatively large number of protein toxins, including RTX hemolysins and bacteriocins (Delepelaire 2004; Koronakis et al. 2004). In gram-negative bacteria, type I secretion systems are typically composed of three protein components, two of which are localized in the inner membrane and one, TolC, that traverses both the periplasm and outer membrane (Koronakis et al. 2004). Although most phytopathogenic bacteria possess multiple copies of *tolC*, only one copy was found in the '*Ca.* L. asiaticus' genome. Interestingly, only one copy of *tolC* is also found in each of the *Xylella* genomes (Reddy et al. 2007). Two type I defensive system gene fusions were found in '*Ca.* L. asiaticus', both in COG1132: i) ABC-type multidrug exporter family fused ATPase and ii) inner membrane subunits. In addition, one complete type I offensive system was found, with both genes in close proximity, i.e., COG4618 (ATPase) and COG0845 (membrane fusion protein). A putative type I effector, a hemolysin (COG1253), was found close to the two type I offensive system genes. Since knockouts of the single *tolC* gene in *Xylella* spp. are completely nonpathogenic and highly sensitive to phytoalexins (Reddy et al. 2007), this raises

the possibility of a chemical- or gene-engineered approach to attack the single '*Ca.* L. asiaticus' target.

Successful plant pathogens have long been thought to have the ability to avoid eliciting plant defense responses, to actively suppress plant defenses, or both, mainly through effectors secreted by a type III system (Abramovitch and Martin 2004). Given the relatively reduced genome size of '*Ca.* L. asiaticus' and the lack of a type III secretion system, '*Ca.* L. asiaticus' may primarily rely upon an avoidance strategy. The lack of type II plant cell-wall degradative enzymes avoids the problem of eliciting defense responses based on autodegradation products of the plant cell wall (oligogalacturonides) (Orozco-Cardenas and Ryan 1999). On the other hand, it is more difficult to see how '*Ca.* L. asiaticus' can avoid eliciting plant recognition of nonself, pathogen-associated molecular patterns (PAMPs), particularly of lipopolysaccharide (LPS) and LPS fragments (Braun et al. 2005) and possibly also of flagellin and flagellin fragments (Gomez-Gomez et al. 1999; Marutani et al. 2005). '*Ca.* L. asiaticus' encodes 57 genes in COG functional category M (cell envelope biogenesis and outer membrane, including LPS), which is 5% of the total and approximately what would be expected for a genome of this size (Table 2). Therefore LPS fragments may elicit a PAMP response, even if flagellin or flagellin fragments are not produced. This raises the intriguing possibility that '*Ca.* L. asiaticus' secrets a non–type III suppressor of host defense. Indeed, spiroplasmas and phytoplasmas, which do not have type III systems, are also injected into the cytoplasm of phloem cells by their insect vectors and secrete proteins into the phloem cell cytoplasm, allowing movement of these proteins to other plant cells via plasmodesmata (Hogenhout and Loria 2008). One such protein is a plant gene-regulating effector, SAP11, which contains a nuclear localization signal that is functional in plant cells (Hogenhout et al. 2008).

HLB symptoms and '*Ca.* L. asiaticus' bacterial titer vary among infected plants. Some disease symptoms, such as yellow shoots, chlorosis, and vein corking are nondescript and difficult to distinguish from nutrient deficiencies. There is no indication that '*Ca.* L. asiaticus' actively conditions pathogenicity, since analysis of the genome revealed no toxins, enzymes, or specialized secretion systems. Instead, the intracellular lifestyle of '*Ca.* L. asiaticus' is better described as parasitic rather than pathogenic, with disease symptoms arising primarily as a result of host metabolic imbalances caused by '*Ca.* L. asiaticus' nutrient depletion or interference of transportation. Infection by '*Ca.* L. asiaticus' leads to the plugging of sieve pores, primarily by callose deposition. The phloem blockage or damage then leads to massive accumulation of starch in leaves and nutrient deficiencies in sink organs (Kim et al. 2009). How '*Ca.* L. asiaticus' is able to infect such a wide variety of citrus plants and their relatives is currently under investigation.

CONCLUSION

Because of the rapid spread and threat of HLB to the citrus industries in the world, efforts are being made to decipher the genetic information of the HLB bacterium '*Ca.* L. asiaticus'. Due to its fastidious nature, however, this bacterium remains unculturable, representing a major obstacle towards the advancement of the field over the past century. In spite of this limitation, we have obtained and annotated the entire genome of '*Ca.* L. asiaticus', using MDA and 454 pyrosequencing technologies on DNA extracted from a single '*Ca.* L. asiaticus'–infected Asian citrus psyllid (*D. citri*). This is the first genome sequence of an uncultured α-Proteobacteria that acts as both an intracellular plant pathogen and an insect symbiont. It is important to note that '*Ca.* L. asiaticus' contains genetic features distinctive to obligate intracellular bacteria (Moran 2002), such as having a small genome size (1.23 Mb for '*Ca.* L. asiaticus'), a low GC content (36.5% for '*Ca.* L. asiaticus'), and a significant genome reduction compared to other members of the *Rhizobiaceae* family. The information revealed by the completed genome may make it possible to identify those conditions necessary for its growth as well as aid in our understanding of how this pathogen becomes established in both its vector and plant hosts. This genome may also provide information relevant to two other genomes of HLB bacteria, '*Ca.* L. americanus' and '*Ca.* L. africanus', thus lending insight into measures to control this devastating disease and sustain the citrus industry.

MATERIALS AND METHODS

Psyllid treatment.

All experiments using live *D. citri* were performed in the insect-proofed greenhouse in the United States Horticulture Research Laboratory, United States Department of Agriculture (USDA)-Agricultural Research Service (Fort Pierce, FL, U.S.A.). Clean adult psyllids maintained in cages were transferred to a cage with an HLB-affected lemon plant (*Citrus limon*) with a high titer of '*Ca.* L. asiaticus' bacterium. After feeding for 45 days on the infected plant, 15 psyllids were collected, using an aspirator, and were stored in 95% ethanol for further use.

DNA extraction from psyllids.

DNA from individual psyllids was extracted as described (Hung et al. 2004). Briefly, individual psyllids were air-dried for 10 min., were homogenized in a 1.5-ml tube containing 300 μl of extraction buffer (100 mM Tris-HCl, pH 8.0, 50 mM EDTA, 500 mM NaCl, 1% *N*-lauroylsarcosinel), and were incubated at 55°C for 1 h. An equal volume of phenol/chloro form/isoamyl alcohol (25:24:1) was then added to each tube, and the tube was vortexed and centrifuged at 12, 000 × *g*, 4°C for 10 min. The supernatant (approximately 200 μl) was transferred to a new 1.5-ml tube containing 500 μl of 100% ethanol, which was gently inverted several times and was centrifuged at 14,000 × *g*, 4°C for 10 min. The resulting DNA pellet was washed once with 70% ethanol. The pellet was resuspended in 15 μl of water and was stored at –20°C. All extractions were performed in a laminar flow hood to avoid contamination.

Real-time PCR.

Quantitative TaqMan real-time PCR amplifications were performed with the specific primers and probes (Supplementary Table S3), using a SmartCycler II (Cepheid, Sunnyvale, CA, U.S.A.) in a 25-μl reaction volume, according to the standardized conditions and program (Li et al. 2006). Reactions used 1μl of the original DNA extracts obtained directly from infected psyllids or 2 μl of the MDA-generated DNA obtained as described above. To estimate DNA template concentrations, absolute standard curves were established using the plasmid DNA cloned with PCR amplicons from each template, as described previously (Li et al. 2008c). The data were analyzed using SmartCycler software version 2.0D.

MDA.

DNA extracted from Psy62 was used as a template for MDA, using the REPLI-g whole genome amplification kit (Qiagen, Germantown, MD, U.S.A.). Briefly, 40 ng of DNA was subjected to whole genome amplification in a 50-μl reaction by random priming and strand-displacement synthesis at 30°C, according to the manufacturer's recommendations. After an overnight incubation, the reaction was terminated by incu-

bating at 65°C for 3 min. Approximately 8.5 μg of DNA was obtained from a MDA reaction as estimated by a NanoDrop 1000 spectrophotometer (Wilmington, DE, U.S.A.). DNA was stored at –20°C.

Sequencing and assembly.

Initially, 454 pyrosequencing and sequence assemblies were conducted by the Interdisciplinary Center for Biotechnology Research, University of Florida, Gainesville. DNA sequencing was performed as described by Margulies and associates (2005), with slight modifications as specified by 454 Life Sciences (Roche, Branchburg, NJ, U.S.A.). Briefly, high molecular–weight DNA was sheared by nebulization to a size range of 300 to 800 bp. DNA fragment ends were repaired and phosphorylated using T4 DNA polymerase and T4 polynucleotide kinase. Adaptor oligonucleotides 'A' and 'B' supplied with the 454 Life Sciences sequencing reagent kit were ligated to the DNA fragments using T4 DNA ligase. Purified DNA fragments were hybridized to DNA capture beads and were clonally amplified by emulsion PCR. DNA capture beads containing amplified DNA were deposited in individual regions of a 70 × 75 mm PicoTiter plate, and DNA sequences were determined using the GS-FLX instrument. DNA sequence information from the initial and supplementary runs was combined in a single assembly using Newbler sequence assembly software (version 1.1.03.24).

Sequence validation and annotation.

To confirm that the assembled contigs were correct, 320 sets of confirmation primers (Supplementary Table S4) were designed based on the contig sequences predicted to be from '*Ca.* L. asiaticus', each set yielding an average PCR product of 1,900 bp. DNA from HLB-affected and HLB-free citrus, periwinkle, and psyllid samples was used to validate the target DNAs and assembled contigs. PCR products of the expected size (at least one amplified product from each contig) were combined from all positive samples and were purified and resequenced to confirm their sequence identity. After confirmation, the '*Ca.* L. asiaticus' sequences were annotated using the National Center for Biotechnology Information (NCBI) annotation pipeline with GeneMarkS (Besemer et al. 2001) program and the FGENESB software (SoftBerry Inc., Mount Kisco, NY, U.S.A.). Briefly, protein coding (mRNA) genes were identified using Markov chain models with self-trained parameters. Their functions were assigned by similarity searches against the NCBI COG database (Tatusov et al. 1997) and Conserved Domain Database (Marchler-Bauer et al. 2002). Potential rRNA genes were identified by BLAST against bacterial rRNA databases. tRNAs were predicted using tRNAscan-SE (Lowe and Eddy 1997). Ribosome binding sites, promoters, and terminators prediction information was used as evidences to identify potential operons. Automatic annotations were stored in the myCAP system, a web application with database backend, for review and manual curation (available online).

Comparative genomics and contig gap closure.

A BLASTP search of predicted '*Ca.* L. asiaticus' proteins was followed by mapping the contigs representing predicted orthologs using the genomes of two phylogenetically related organisms, *Sinorhizobium meliloti* and *Bartonella quintana*, as potential scaffolds using Circos software (available online). After mapping the contigs, end primers were designed and used to amplify Psy62 DNA. Amplified DNA was then sequenced and was used to close gaps of the chromosome.

Bayesian phylogenetic analysis. Orthologs of '*Ca.* L. asiaticus' were identified for 102 of the 104 conserved proteins (all except CcmC and MnmA) that were used in a previous phylogenetic tree constructed for 72 α-Proteobacteria and eight non–α-Proteobacteria strains (Williams et al. 2007). Each '*Ca.* L. asiaticus' ortholog was ranked relative to those for the other 80 species using the HMM score, with a median ranking of 12th worst, and eight protein families (UvrD, UbiA, UvrA, YaeL, LigA, RpsH, RpsO, and AlaS) for which the '*Ca.* L. asiaticus' proteins scored no better than third worst were rejected. Sequences for the remaining 94 protein families were aligned, and ambiguous portions of the alignments were removed to prepare a concatenated protein sequence with 23,219 amino acids. Three single-chain 800,000-generation MrBayes (available online) runs were performed, and all trees from the latter 400,000 generations showed identical topology and were used to prepare a consensus tree.

In silico analysis of proteins involved in metabolism and transport.

Reconstruction of predicted '*Ca.* L. asiaticus' metabolic pathways was carried out using IdentiCS software (Sun and Zeng 2004). Annotated enzyme models were used to map the '*Ca.* L. asiaticus' data onto the KEGG (Kyoto Encyclopedia of Genes and Genomes) metabolic pathways (Kanehisa and Goto 2000; Kanehisa et al. 2006, 2008). A comparison between '*Ca.* L. asiaticus' proteins and proteins in the Transporter Classification database of the Saier Lab Bioinformatics Group (available online) was made to classify and group transporter proteins in the newly annotated genome according to the widely accepted Transporter Classification system.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

University of Florida Biotechnology Vision server myCAP system: vision.biotech.ufl.edu/mycap
Genome Sciences Centre's Circos software: mkweb.bcgsc.ca/circos
MrBayes software: mrbayes.csit.fsu.edu
Saier Lab Bioinformatics Group's Transporter Classification database: www.tcdb.org/index.php