Citrus Huanglongbing: a newly relevant disease presents unprecedented challenges

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Running Title: Citrus Huanglongbing

Key words: Candidatus Liberibacter asiaticus, citrus greening, evolution, genetic diversity, host range, genome analysis, virulence mechanism, agroecosystem.

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Citrus huanglongbing (HLB) is one of the oldest citrus diseases and has been known for over a century. HLB is caused by *Candidatus* Liberibacter spp. that are phloem-limited fastidious α-proteobacteria and infect hosts in different Kingdoms (i.e., Animalia and Plantae). When compared to well-characterized, cultivatable plant pathogenic Gram-negative bacteria, the interactions of uncultured insect-vectored plant pathogenic bacteria, including *Ca.* Liberibacter, with their hosts remain poorly understood. *Ca.* Liberibacter spp. have been known to cause HLB, which has been rapidly spreading worldwide, resulting in dramatic economic losses. HLB presents an unprecedented challenge to citrus production. In this review, we focus on the most recent research on citrus, *Ca.* L. asiaticus, and psyllid interactions, specifically considering the following topics: evolutionary relationships among *Ca.* Liberibacter spp., genetic diversity, host range, genome analysis, transmission, virulence mechanisms, and the ecological importance of HLB. Currently, no efficient management strategy is available to control HLB, although some promising progress has been made. Further studies are needed to understand citrus, *Ca.* L. asiaticus, and psyllid interactions to design innovative management strategies. While HLB has been problematic for over a century, we can only win the battle against HLB with a coordinated and deliberate effort by the citrus industry, citrus growers, researchers, legislatures, and governments.
INTRODUCTION

Citrus huanglongbing (HLB) is one of the oldest diseases in citrus and has been known in East Asia for over a century (reviewed by 21, 30, 61). However, this disease was largely ignored until its recent introduction to the Americas. HLB poses an unprecedented challenge in newly infected citrus production areas.

HLB is characterized by blotchy mottling with green islands on leaves. Infected shoots are stunted, and the branches gradually die as the disease progresses. Fruit from diseased trees may be small and lopsided, with poor coloration (Fig. 1). HLB greatly damages the citrus industry by shortening the trees’ lifespan and reducing fruit yield and quality characteristics, such as total soluble solids (TSS) content, acidity, and the TSS/acidity ratio (Fig. 1) (14, 31, 137). HLB can debilitate the productive capacity of citrus trees, with reported losses of 30–100% (10). It has also been observed that HLB-diseased trees are more adversely affected by extremes of temperature and moisture than are healthy trees. Consequently, symptoms of stress, e.g., excessive leaf loss and premature fruit drop, occur in HLB diseased trees. This stress intolerance is thought to result partially from a loss of fibrous root function. Recently, Graham and colleagues surveyed the root status of HLB-affected trees. HLB-diseased, 4-year-old Valencia orange trees showed a 30% and 37% reduction in fibrous root mass density for presymptomatic and symptomatic trees, respectively, compared to healthy trees (76).

All commercial citrus species and scion cultivars are susceptible to HLB infection regardless of rootstock (21). However, a recent analysis of 30 different genotypes of citrus to Florida isolates of Ca. L. asiaticus indicated that there are differences in host response to HLB, e.g., sensitive, moderately tolerant, and tolerant. The sensitive genotypes include C. halimii, Nules clementine mandarin, Valencia sweet orange, Madam Vinous sweet orange, Duncan
grapefruit, Ruby red grapefruit, and Minneola tangelo whereas the most tolerant genotypes are Eureka lemon, Persian lime, Carrizo citrange, and Severinia buxifolia (52).

HLB is widespread in most citrus areas of Asia, Africa, and the Americas. Importantly, HLB and the Asian citrus psyllid (ACP, *Diaphorina citri*) (vector of *Ca. L. asiaticus*) are expanding to new citrus production areas (Fig. 2). In the past 14 years, the ACP has been found in Florida, Texas, California, Arizona, Hawaii, Louisiana, Georgia, and Alabama in the U.S.A., as well as in parts of South and Central America, Mexico, and the Caribbean. Meanwhile, HLB has been identified in Florida (2005), Louisiana (2008), South Carolina (2009), Louisiana (2008), Georgia (2009), and most recently in Texas and California (2012) of the USA; it has also been discovered in Cuba, Belize, Jamaica, Mexico, and other countries in the Caribbean.

HLB is associated with a phloem-limited fastidious α-proteobacterium given provisional *Candidatus* status (*Candidatus* Liberobacter spp. later changed to *Candidatus* Liberibacter spp.) (Fig. 2) in its nomenclature (57, 75). Currently, three species of *Ca. Liberibacter* are recognized in trees with HLB disease based on 16S rDNA sequence: *Ca. L. asiaticus*, *Ca. L. africanus*, and *Ca. L. americanus*. Circumstantial evidence indicates that HLB is caused by *Ca. Liberibacter* spp., although Koch’s postulates have not been fulfilled due to the difficulty in culturing the bacterium, as reported previously (21). Two recent studies of bacterial diversity associated with HLB disease further support that *Ca. L. asiaticus* is the sole pathogen responsible for HLB in Florida (117,141). In a study by Sagaram et al. (117), *Ca. L. asiaticus* was detected at a very low level in asymptomatic plants but was over 200 times more abundant in symptomatic plants based on PhyloChip analysis. The PhyloChip analysis results were further verified by sequencing of the 16S rRNA gene clone libraries, which indicated the dominance of *Ca. L. asiaticus* in symptomatic leaves. *Ca. L. asiaticus* is absent or present in small populations in asymptomatic
plants. In a study by Tyler et al. (141), three next-generation high-throughput sequencing platforms, 454, Solexa, and SOLiD, were used to obtain metagenomic DNA sequences from the phloem tissue of HLB-diseased citrus trees. Only Ca. L. asiaticus was identified from the phloem tissue. This phloem metagenomic DNA provided further evidence to verify the presence of Ca. L. asiaticus in infected tissues, and no other disease agents were present in the phloem.

Phytoplasma has been found in trees showing HLB-like symptoms in Brazil and China (27,128). However, phytoplasma has not been identified in HLB-diseased trees in Florida (117,141). In addition, no phytoplasma has been reported in psyllids collected from Indonesia and Florida (103,126). Based on these current studies, the research community agrees that HLB is caused by Ca. Liberibacter, which distinguishes HLB from the disease caused by phytoplasma.

**EVOLUTIONARY RELATIONSHIPS BETWEEN CA. LIBERIBACTER SPP. AND RELATED BACTERIA**

All Ca. Liberibacter spp. belong to the Gram-negative α-proteobacteria in the family Rhizobiaceae. The taxonomy of the Ca. Liberibacter spp. is based on the 16S rRNA gene sequence rather than traditional methods such as morphology, growth, enzymatic activity, metabolism and DNA-DNA hybridization (84) due to the difficulty of culturing the bacteria. Phylogenetic analysis has shown that Ca. L. asiaticus is an “early branching member” of Rhizobiaceae, and the long branch of Ca. L. asiaticus in the phylogenetic tree suggests rapid evolution of this pathogen (41). The recent discoveries of Ca. L. europaeus and Ca. L. solanacearum suggest that Ca. Liberibacter spp. may be widespread in psyllids and their host plants. A bacterial isolate initially isolated from the bunchy top diseased hybrid mountain papaya (Carica stipulate x C. pubescens) is recently characterized as the first cultured member of genus
Liberibacter and is named as *Liberibacter crescens* (89). Further studies are needed to investigate whether *Ca.* Liberibacter spp. occur in other psyllid species and their host plants.

Interestingly, all *Ca.* Liberibacter spp. are phloem-restricted and transmitted by psyllids except that *L. crescens* is reported to be present in the periphery of phloem and the association of the bacterium with insects has not yet been determined (89). It is most likely that *Ca.* Liberibacter spp. evolved from the same ancestor in the Rhizobiaceae family through adaptive, diversifying, and reductive evolutionary processes that occur during host adaptation (129). This evolution is possible due to the intimate relationship between members of the Rhizobiaceae family and plant roots (54). The intimate associations of *Ca.* Liberibacter spp. with plants as endophytes predispose them to frequent encounters with herbivorous insects, providing *Ca.* Liberibacter spp. with ample opportunities to colonize and eventually evolve alternative associations with insects (110). The genome sizes of the bacteria closely related to *Ca.* Liberibacter spp. range between 3.4 Mb (*Agrobacterium* sp. H13-3), 5.7 Mb (*Agrobacterium tumefaciens* C58), 6.3 Mb (*Agrobacterium vitis* S4), 6.5 Mb (*Rhizobium etli* CFN 42), 6.7 Mb (*Sinorhizobium meliloti* 1021), and 7.3 Mb (*Agrobacterium radiobacter* K84); in contrast, the much reduced genome size of *Ca.* Liberibacter spp. ranges from 1.23 Mb for *Ca.* *L. asiaticus*, 1.26 Mb for *Ca.* *L. solanacearum*, to 1.5 Mb for *L. crescens* (89). The reduced genome size and low GC content of *Ca.* *L. asiaticus* and *Ca.* *L. solanacearum* are hypothesized to be the result of stable and nutrient-rich environments and attenuated purifying selection due to small population sizes and strong bottleneck effects (105,107,150). Hartung et al. (67) compared the genome of *Ca.* *L. asiaticus* with other members of *Rhizobiales*, including *S. meliloti*, *Bradyrhizobium japonicum* (both N₂ fixing endosymbionts), *A. tumefaciens* (plant pathogen), and *Bartonella japonicum* (an intracellular mammalian pathogen). Whole-genome comparisons have identified
at least 50 clusters of conserved microsyntenous orthologous genes (MOG) found on the chromosomes of all five metabolically diverse species (67). The existence of so many MOGs in these inter-specific genomic comparisons reflects the underlying evolutionary relationships among these species. Because *S. meliloti* is a close phylogenetic relative of *Ca. L. asiaticus*, it is likely that the two bacteria deploy a similar repertoire of mechanisms for avoiding defenses elicited in host plant cells by their invasion, or, in the case of beneficial root nodule bacteria, recruitment or “welcome entry” (87). Approximately 182 *pSymA* (megaplasmid of *S. meliloti*) carrying nonessential ‘accessory’ genes involved in maintaining intimate intracellular plant interactions with host alfalfa) encoded proteins have sequence similarity (≤E-10) with *Ca. L. asiaticus* proteins (87). These proteins are involved in amino acid uptake, the cell surface structure, chaperonins, electron transport, the export of bioactive molecules, cellular homeostasis, the regulation of gene expression, signal transduction and the synthesis of amino acids and metabolic cofactors. The presence of multiple orthologs is consistent with the hypothesis that these proteins may be of particular importance in the host/microbe interactions, and their duplication likely facilitates their ongoing evolution (87).

The transition between hosts subjects *Ca. L. asiaticus* to a dramatic change in habitat, even though the sugar concentrations in the vector hemolymph and plant phloem are comparable (106). The phloem seems to be a more suitable environment for *Ca. L. asiaticus* compared to the psyllid 's hemolymph. Recently, we used quantitative reverse transcription PCR to compare the gene expression of *Ca. L. asiaticus* in planta and in psyllid. Of the 381 genes that were analyzed, 182 were up-regulated in planta compared with in psyllid (*p* < 0.05), 16 genes were up-regulated in psyllid (*p* < 0.05), and 183 genes showed no significant difference (*p* = 0.05) between expression in planta and expression in psyllid. Our study indicated that the expression
of *Ca. L. asiaticus* genes involved in transcriptional regulation, the transport system, the
secretion system, flagellar assembly, the metabolic pathway, and stress resistance was
significantly changed in a host-specific manner to adapt to the distinct environments of plant and
insect (154). The biased gene induction of *Ca. L. asiaticus* *in planta* compared to in psyllid
suggests that it is more active *in planta* compared to a passive and idle status in psyllid. In
addition, it has been suggested that *Ca. L. asiaticus* forms a biofilm in the psyllid (Fig. 2),
whereas biofilm formation has not been reported for *Ca. L. asiaticus* *in planta*. It is possible that
the biofilm formation of *Ca. L. asiaticus* in the psyllid is either stress induced, as reported for
other bacteria such as *Pseudomonas aeruginosa* (60), or adjusts its physical status to be suitable
for psyllid transmission. Together, these pieces of evidence suggest the vector role of psyllids
for *Ca. L. asiaticus* to its ultimate plant host. It remains to be determined how *Ca. L. asiaticus*
interacts with psyllids in the short lifespan of the vector.

Interestingly, *Ca. L. asiaticus* lacks a complete restriction-modification system (RM) (41,
92). Thus, *Ca. L. asiaticus* is vulnerable to prophage integration, as evidenced by the presence of
several phage-derived gene sequences within its genome. This could result in an enhanced rate
of evolution in *Ca. L. asiaticus* through phage-mediated recombination events (92). In addition,
*Ca. L. asiaticus* lacks three proteins involved in DNA replication and repair that are present in
*Ca. L. solanacearum*: LexA, DnaE, and RadC. Consequently, it has been suggested that *Ca. L.
asiaticus* (41) rapidly evolves, which is typical of host-restricted symbionts and pathogens, due to
the elevated genetic drift resulting both from population bottlenecks and from relaxed selection
on many genes (41,105). A geographic range of *Ca. L. asiaticus* variants based on phylogenetic
analysis, have been reported, although no differences in phenotype have been reported (15,36).

**GENETIC DIVERSITY**
The detection of genetic diversity within pathogen populations is fundamental for ecological and epidemiological studies of a disease. The genetic structure within a given pathogen is an indispensable prerequisite for determining sources of infection and risk management for diseases. In previous studies, monoclonal antibodies directed against Ca. L. asiaticus isolates from different geographical locations have been shown to react with one or several isolates, but none of the antibodies react with all of the isolates (55,58). Gao et al. (55) classified 11 Ca. L. asiaticus isolates from different geographical locations into six different serotypes, suggesting that there is significant genomic variation among isolates. In further studies, molecular techniques provided useful complementary tools for the identification and genetic characterization of Ca. L. asiaticus. The genetic diversity, primarily at several loci in the \textit{rrs} and \textit{rpl} genes and in the \textit{omp} and \textit{rpoB} loci of HLB-associated Liberibacters, is well documented (15, 38, 53, 57). Bastianel et al. (15) used an \textit{omp}-based PCR-restriction fragment length polymorphism (RFLP) to analyze the genetic variability of Ca. L. asiaticus isolates and showed that, even within a given region, several different variants exist. The \textit{omp} gene was further assayed by various restriction endonucleases to investigate the genetic diversity of 23 Ca. L. asiaticus isolates with different symptoms from seven provinces in China (69). The study revealed that different isolates were distributed in three subgroups depending on their geographical origins, and no genetic evidence for host determination was observed. The alignments in a 1.5-Kb region of the \textit{rpoB} of the Ca. L. asiaticus and Ca. L. africanus strains revealed that the strain from China differed by two single-nucleotide polymorphisms (SNPs) from the Japan, Florida and Brazil strains, which were identical at this locus (38). In many Japanese and several South Asian isolates, including those from Taiwan, Indonesia, the Philippines, Vietnam, and Thailand, the 16S rDNA genes are identical (126,130). However,
numerous SNPs have been reported in many Chinese isolates and two Indian isolates collected
from Southwest India (2). Phylogenetic analysis with 16S rDNA sequences and SNPs of the \textit{omp}
gene region revealed that the northeastern Indian isolates were genetically closer to common
Asian isolates from Japan, Taiwan, and Vietnam than to the Indian isolates reported previously
from western parts of India (104). This result showed that the Asian-common strains of \textit{Ca. L.}
asiaticus, as well as the other diverse atypical strains, are distributed in India. On the basis of the
11 nucleotide substitutions in the 11,168-nucleotide sequence of the \textit{serA-trmU-tufB-secE-nusG-rplKAJL-rpoB} gene cluster and its flanking region, Furuya et al. (53) showed that one unique
genetic group is dominant around the Okinawa Main Island of Japan, whereas several different
isolates were found to be frequently distributed around islands near Taiwan. Tomimura et al.
(130) applied duplex PCR that can simultaneously amplify the DNA pol and \textit{nus-rplL} operon in
65 \textit{Ca. L. asiaticus} isolates and reported that Japanese \textit{Ca. L. asiaticus} isolates contain at least
two distinct genotypes, and the genotype that had the DNA pol is highly homogeneous. Katoh et
al. (79, 80) identified 27 simple single repeats (SSRs) with 4-63 nucleotides per unit in the
genome of the \textit{Ca. L. asiaticus psy 62} strain. A dendogram analysis of diversity within these 27
SSR loci among \textit{Ca. L. asiaticus} isolates from India, East Timor, Papua New Guinea and Florida
showed that the clusters were mostly consistent with the geographical origin of the isolates (79,
80). Furthermore, the differences in the nucleotide sequences were not associated with the
differences in the citrus host from which the isolates were originally derived. Recently, a
genomic region (CLIBASIA\_05640 to CLIBASIA\_05650) of \textit{Ca. L. asiaticus} showing hyper
variability was identified and investigated using 262 bacterial strains (188 from China and 74
from Florida) (149). Based on the characteristic electrophoretic profiles of the PCR amplicons
generated by a specific primer set, eight electrophoretic types (E-types) were identified; in
contrast, strains from China predominantly consisted of E-types A and B, whereas E-type G was predominant in Florida.

Chen et al. (26) identified the bacteriophage repressor protein C1 as a genetic marker containing small tandem repeats in the genome of Ca. L. asiaticus and comprehensively analyzed the tandem repeat numbers (TRNs) in Ca. L. asiaticus populations from Guangdong, China and Florida. An analysis of TRNs showed that the bacterial population in Guangdong consisted predominantly of strains with a TRN of 7 and was different from that in Florida, where most of the isolates had a TRN of 5. Moreover, two TRN subgroups, one widely distributed throughout Florida and the other limited to central Florida, were identified. Zhou et al. (158) described the genetic diversity of Ca. L. asiaticus by using hypervariable prophage genes with intragenic tandem repeats. Sequence conservation within the individual repeats but an extensive variation in the repeat numbers, rearrangement, and the sequence flanking the repeat region indicated the diversity and plasticity of the Ca. L. asiaticus bacterial populations in the world. These differences were found not only in samples of distinct geographical origins but also in samples from a single origin and even from a single Ca. L asiaticus-infected sample. An analysis of a prophage terminase gene revealed genetic variations in the populations of two citrus growing provinces in China (94). Differences between the two sets of populations were postulated to be the result of evolutionary genetic drift due to their geographical separation over an estimated period of 30 to 40 years.

**HOST RANGE**

When discussing hosts in HLB, two types of plants are of concern: the plant that supports the psyllid vectors and the plant in which the bacterial pathogen can multiply. Research shows that the two types of plants have different significance in HLB management. Compared to the
wide physiological host range of the bacterial pathogens, the psyllid vectors have a relatively
narrow host range. Considering the low vector-pathogen specificity, this may have potential
implications for the disease epidemiology (61, 65).

**Host of vector**

Halbert and Manjunath (65) have provided lists of plant species that are hosts to *D. citri* and *Ca. Liberibacter* spp.. Because many of the hosts on the two lists were included based
on field surveys (i.e., observations of plant symptoms or psyllid behavior) and only a few have
been verified by PCR tests, the host status of various plants has not been experimentally
established. Psyllids can feed on many citrus species and close relatives of citrus, but the
preferred hosts are *Murraya paniculata* (Orange jasmine, mock orange) (11) and *Citrus
aurantifolia* (65). Tsai and Liu (139) found that the grapefruit was the best host of *D. citri* out of
the four plants studied: *Murraya paniculata* (L.) Jack (orange jasmine), *Citrus
jambhiri* Lushington (Rough lemon), *C. aurantium* L. (sour orange), and *C. x paradisi* Macfad.
(grapefruit); there was no significant difference among the other three hosts. Continuous shoot
growth of *M. paniculata* plays an important role in maintaining ACP populations when citrus
flush is not available (140). Based on greenhouse studies, Halbert and Manjunath (65) suggested
that the two Florida native *Zanthoxylum* plants, *Z. clavahercules* L. and *Z. fagara* (L.) Sarg.,
and *Casimiroa edulis* Llave & Lex. may be non-hosts (or very poor hosts, as in the case of *Z.
fagara*) of the ACP. In line with these greenhouse observations, the authors also reported that
no ACP were found on *Z. fagara* plants growing next to an infested lime grove in South Florida
(65).

**Citrus hosts**
Due to the difficulty of detecting the HLB-associated bacteria with certainty, the available information on the host range of the liberibacters is based primarily on symptoms (65). Most citrus cultivars, especially commercial ones, are susceptible to some degree, regardless of their rootstock (21, 65). However, one characteristic of HLB is that different degrees of disease and symptoms are induced in different types of citrus. Furthermore, different isolates of Ca. L. asiaticus can cause varying degrees of disease in citrus cultivars (137). The most severe symptoms are found on sweet orange, mandarin, tangelo, and grapefruit, followed by lemon, rough lemon, and sour orange (21, 65, 83, 94, 138). Small-fruited acid lime trees (C. aurantifolia) are only slightly affected, but clear-cut blotchy mottle symptoms can be observed on leaves.

There is no real resistance to HLB in citrus species, but some species and cultivars have some tolerance. Several extensive field surveys have demonstrated that some cultivars were more susceptible to decline than others (83). For example, grapefruit was more tolerant than most of the sweet orange cultivars. Some citrus species (C. indica Tan. and C. macroptera Montr.) remained symptom-free under heavy inoculum pressure (18), which may indicate a certain degree of resistance. In Taiwan, severe leaf yellowing was first noticed in Ponkan mandarin, Tankan tangor, and Liucheng sweet orange but not in Wentan pummelo in the field in 1951 (101). The pummelo cultivar that was formerly resistant to HLB eventually became infected and displayed HLB symptoms approximately 30 years after HLB first appeared (70,125). The kumquat (Fortunella margarita (Lour.) Swingle), which was formerly resistant to HLB, recently became infected and displayed yellow mottling symptoms in 2006 (138). It was assumed that the change in host range was due to the evolution of HLB strains in pathogenicity. Most of the information on different citrus genotype reactions to HLB has been accumulated.
from observations of field trees made under different conditions, at different geographic
locations, and at different times.

Various studies have also reported that several citrus relatives, such as Severinia
buxifolia (Poiret) Ten. (34,72,73,115), Limonia acidissima L. (73,83), Clausena lansium (35,37),
and Toddalia lanceolata Lam (85), could harbor HLB-associated bacteria.

**Alternative hosts**

Field observation and laboratory studies have confirmed that *M. paniculata* is a preferred
ACP host; however, its alternative host status for HLB-associated bacteria is not yet clear
(73,96,148). Hung et al. (73) used a graft inoculation technique to demonstrate that *Ca. L.
asiaticus* can replicate in the Chinese box orange (*Severinia buxifolia*) and the wood apple
(*Limonia acidissima*) but not in the common jasmine orange (*M. paniculata*) or the curry leaf
(*M. euchrestifolia*). On the contrary, Halbert and Manjunath (65) have found consistent
symptoms in inoculated *M. paniculata* plants. *M. paniculata* shows leaf yellowing, defoliation
and dieback on branches when infected with *Ca. L. asiaticus* or *Ca. L. americanus* in Brazil (95,
96). Zhou et al. (157) also found *M. paniculata* to be naturally infected with *Ca. L. asiaticus* in
Florida. Zhou et al. (157) concluded that *M. paniculata* can serve as an infection source for *Ca.
L. asiaticus* because it can host *Ca. L. asiaticus* for at least 2 months, and *Ca. L. asiaticus* can be
transmitted to the sweet orange during this time. Controlled inoculation experiments with two
isolates of *Ca. L. asiaticus* using *D. citri* as vector showed that *M. paniculata* is variable as a
reservoir host of the HLB associated pathogen (33). Because the bacterial population in *M.
paniculata* becomes extremely low after 5 months, *M. paniculata* (as well as another
*Murraya* species, *M. exotica*) could only serve as a bridging host if citrus are present during that
period of time. Field surveys conducted in Florida and Brazil found an extremely low incidence
of *Ca. L. asiaticus* in ornamental *M. paniculata* and associated psyllids (*D. citri*) (96,148).

However, the importance of *M. paniculata* in HLB disease epidemics should not be underestimated, as it is a preferred host of ACP and is not being subjected to any strict tree-eradication programs or insect control measures. In the Western Cape Province of South Africa, *Calodendron capense*, an ornamental rutaceous tree (Cape chestnut tree), showed blotchy mottle leaves and was found to be infected with a liberibacter. The new liberibacter was characterized as subspecies “capensis” of *Ca. L. africanus* (57).

**Non-Rutaceous hosts**

Some hosts outside the Rutaceae family can be experimentally inoculated with *Ca.* Liberibacter spp., and they are used in various HLB studies. It has been demonstrated that all three citrus liberibacters can be transmitted to periwinkle plants by dodder (*Cuscuta* spp., in Cuscutaceae family) (56). Dodder can be effectively colonized by *Ca. L. asiaticus* and *Ca. L. americanus*, and the bacteria can multiply internally to a high level. The bacteria are unevenly distributed in dodder as in citrus (66). Dodder can be used to transmit HLB-associated pathogens to citrus (154,156), non-Rutaceous plants such as periwinkle (*Catharanthus roseus* L. G. Don, in Apocynaceae family) (56,66) and several solanaceous plants such as tomato (40) and tobacco (*Nicotiana tobacum* L. cv. ‘Xanthii’) (56), which indicates that *Ca. L. asiaticus* has a wide physiological host range. Fan et al. (46) reported that the non-Rutaceae plants *Pithecellobium lucidum* Benth showed yellow shoots, mimicking the symptom of HLB, in a citrus orchard in Fujian, China, where citrus plants were severely infected by HLB. The results of a low *Ca. L. asiaticus* bacterial titer and the lack of psyllid propagation in this host plant indicated that the new host is an opportunistic host of HLB.

**GENOME ANALYSIS**
Despite the difficulty in acquiring pure genomic DNA, *Ca. L. asiaticus* has been sequenced successfully, which provides a basis for the assessment of the metabolic and functional capabilities of the pathogens. Genomic analysis of *Ca. L. asiaticus* has provided useful insights into the biology and pathogenicity of the HLB pathogen (41). Here, we emphasize two main aspects of the metabolic capacity related to the central carbohydrate metabolism and respiration of *Ca. L. asiaticus* and offer a perspective that is slightly different than a previous analysis (41).

**Central carbohydrate metabolism**

*Ca. L. asiaticus* is able to metabolize a very limited set of sugars, including glucose, as a carbon and energy source. The genome sequence provides evidence for a near complete set of glycolytic enzymes, with the possible exception of glucose-6-phosphate isomerase, though this gene may be an example of non-orthologous gene displacement. *Ca. L. asiaticus* lacks a glucose phosphotransferase system (PTS) system. Glucose is most likely imported into the cell via a glucose/galactose transporter, which is present in *Ca. L. asiaticus*. Thus, *Ca. L. asiaticus* is likely able to utilize glucose as a carbon and energy source.

The *Ca. L. asiaticus* genome encodes a full inventory of enzymes necessary for the tricarboxylic acid (TCA) cycle. The conversion of glucose and TCA intermediates to pyruvate provides the majority of pyruvate in the cell because enzymes for the direct formation of pyruvate, such as serine dehydratase, alanine racemase and alanine dehydrogenase, are not present in the genome. The lack of a glyoxylate bypass indicates that isocitrate lyase and malate synthase are also absent from the genome, suggesting that the bacterium is incapable of growth on acetate and/or fatty acids. This information indicates that *Ca. L. asiaticus* uses exogeneous fumarate, malate, succinate and L-aspartate as carbon substrates for the TCA cycle and pyruvate
generation as energy sources. This conclusion is supported by the fact that a C4 dicarboxylate transport protein has been identified in the *Ca. L. asiaticus* genome. The import of L-aspartate is facilitated by the existence of an ABC-type L-amino acid transport cassette comprising substrate-binding, permease and ATP-binding components.

**Respiratory chain**

*Ca. L. asiaticus* has a respiratory chain capable of transferring electrons from reduced substrates to oxygen under microaerophilic growth conditions. It appears that malate, fumarate, succinate, aspartate and glutamate can be used as carbon sources by this organism, as enzymes that utilize these compounds are encoded in the genome. A malate dehydrogenase that would allow the oxidation of malate to oxaloacetate and thus feeds into the TCA cycle is present. The reducing equivalents generated are transferred down to an exogeneously derived quinone pool.

An important component of the *Ca. L. asiaticus* aerobic respiratory chain identified in the genome is the NADH dehydrogenase complex. It appears that the reduced genome of this phytopathogen is devoid of genes for the biosynthesis of menaquinone and ubiquinone. Thus, for a functional respiratory chain, exogenous quinone needs to be used.

The absence of nitrate, sulfate, fumarate and trimethylamine reductase systems suggests that *Ca. L. asiaticus* does not have an anaerobic respiratory scheme. Duan et al. (41) suggested that anaerobic respiration by *Ca. L. asiaticus* occurs, based on the observation of enzymes involved in nitrogen metabolism such as NAD⁺ synthase, glutamine synthetase, and glutaminase. However, there is a clear distinction between the enzymes involved in nitrogen metabolism and electron acceptors for an anaerobic respiratory chain. The functions of the enzymes identified in nitrogen metabolism (e.g., NAD⁺ synthase, glutamine synthetase, and glutaminase) do not define a respiratory chain. We did not find evidence of any electron acceptors for anaerobic respiration.
using nitrogen, specifically nitrate or nitrite reductases. In the absence of these acceptors, it is difficult to have a respiratory chain coupled to the reduction of nitrogen compounds. In addition, both \textit{Spiroplasma citri} and \textit{Serratia marcescens} could infect phloem, and both are facultative anaerobe bacteria that make ATP by aerobic respiration when oxygen is present (1,151). This is further supported by the culture condition of \textit{L. crescens} at the presence of oxygen (89). It is important to note that oxygen is present in the phloem. In a previous study of \textit{Ricinus communis}, oxygen levels in phloem were shown to range from 21\% (v/v) at the surface to 7\% (v/v) in the vascular region and 15\% (v/v) toward the hollow center of the stem, compared with 21\% (v/v) oxygen in air (145). Thus, phloem can support aerobic respiration of \textit{Ca. L. asiaticus} even though the oxygen level in the phloem is lower than atmospheric levels.

\textbf{\textit{Ca. L. ASIATICUS TRANSMISSION}}

\textit{Ca. L. asiaticus} may spread locally and regionally via citrus psyllids and can be disseminated by the propagation of contaminated scion budwood by grafting (66). Grafting is a common practice in citrus production that maintain the horticultural characteristics of a scion. Preventing HLB transmission via grafting has been taken into consideration in management and regulation and is easily achievable. Grafting transmission was recently reviewed by Halbert and Manjunath (65), and the reader is referred to that excellent review. Psyllid transmission is the dominant factor in the epidemiology of HLB, and stopping psyllid transmission has been the major focus of the citrus industry despite the extreme difficulty of preventing psyllid transmission of \textit{Ca. L. asiaticus}. Tremendous efforts have been made in recent years to understand the mechanism of the psyllid transmission of \textit{Ca. L. asiaticus}, with the aim of designing innovative management strategies to combat HLB. In addition, seed transmission has been a concern because the
rootstocks used to produce trees are grown locally from seeds. Therefore, we will mainly discuss psyllid transmission and will briefly discuss seed transmission of *Ca. L. asiaticus*.

**Psyllid transmission of *Ca. L. asiaticus***

*Ca. Liberibacter spp.* is naturally transmitted by two vectors: the ACP *Diaphorina citri* (Kuwayama (Hemiptera: Sternorrhyncha: Psyllidae)) and the African psyllid *Trioza erytreae* (del Guercio) (Hemiptera: Sternorrhyncha: Triozidae). *D. citri* is responsible for the transmission of *Ca. L. asiaticus* in Asia and the Americas and *Ca. L. americanus* in Brazil. *T. erytreae* is responsible for the transmission of *Ca. L. africanus* in the Middle East, Mauritius, Reunion, and Africa (9,65). It was demonstrated that *T. erytreae* is able to transmit *Ca. L. asiaticus* under experimental conditions (100).

A psyllid can acquire the pathogen during the nymphaal and adult stages (23, 74, 114, 153). Acquisition by nymphs ranged from 60 to 100%, whereas acquisition by adults reached 40% after 5 weeks of feeding on *Ca. L. asiaticus*-infected plants under laboratory conditions (114). Similar results were observed under field conditions (114). It was reported that the ACP can acquire *Ca. L. asiaticus* in a minimum of 15 min to 24 h (22,23). However, Pelz-Stelinski et al. (114) indicated that adult psyllids were unable to acquire *Ca. L. asiaticus* in the first week of pathogen exposure. The acquisition rate of *Ca. L. asiaticus* by the adult ACP was positively affected by prolonged feeding (23,114). The latent period required for *Ca. L. asiaticus* to incubate inside the psyllid following acquisition before it can be transmitted can vary from one (153) to eight days post-acquisition (24).

*Ca. L. asiaticus* has been reported to be transmitted by ACP in a persistent manner (23,74,153). Psyllids were reported to maintain *Ca. L. asiaticus* for 12 weeks (71), which covers most of the approximately 90-day lifespan of psyllids (93). It has also been shown that an
infected adult retains its infectivity throughout the adult stage (153). In addition, _Ca._ L. asiaticus has been reported to invade various psyllid organs and tissues. A transmission electron microscopy study indicated that _Ca._ L. asiaticus could invade cells of the salivary gland, the filtration chamber of the foregut, and the cells of the midgut and hindgut (153). This observation was further validated by quantitative real time PCR (QPCR) and fluorescence in situ hybridization (FISH) analyses (7,8). QPCR indicated that _Ca._ L. asiaticus was present in the salivary glands, the alimentary canal, and the rest of the insect body. FISH analysis indicated that _Ca._ L. asiaticus was detected in the filter chamber, midgut, Malpighian tubules, haemolymph, salivary glands, ovaries and in the muscle and fat tissues of psyllids.

Multiple studies have suggested that _Ca._ L. asiaticus is propagative in psyllids. Based on QPCR analysis, the mean concentration of _Ca._ L. asiaticus increased over time in psyllid after acquisition feeding by fifth instars (74). Ammar et al. (6,7) also reported that in both field- and laboratory-infected _D. citri_, the proportion of infected salivary glands was significantly lower than the alimentary canal and the rest of the insect body. However, the relative copy number of the _Ca._ L. asiaticus genome relative to psyllid genomic DNA was significantly higher in both the salivary gland and alimentary canal compared with the rest of the insect body for both male and female psyllids. The distribution pattern of _Ca._ L. asiaticus is similar to other propagative plant pathogenic bacteria that are known to multiply in their hemipteran insect hosts. e.g., phytoplasmas, _Spiroplasma kunkelii_ and _Spiroplasma citri_ (6,20,49). Collectively, previous studies seem to suggest that _Ca._ L. asiaticus replicates in psyllids. However, it has also been reported that the retention of _Ca._ L. asiaticus in adult psyllids that acquired the pathogen as nymphs decreased over time, which suggests that _Ca._ L. asiaticus does not persist in _D. citri_ (114). Considering that most experiments conducted thus far are not comprehensive, as they rely
on either symptoms, PCR, or FISH analysis, the combining of multiple approaches to conduct a
more comprehensive study of this subject is desirable.

Overall, two different models exist regarding the psyllid transmission of Ca. L. asiaticus
to plants. One model is based mainly on studies by Capoor et al. (23) and Xu et al. (153). In
both studies, the transmission assays were conducted using indicator citrus plants. Their model
suggests that the fourth to fifth instar nymphs and adults can acquire Ca. L. asiaticus and
transmit the pathogen to the plant. The emerged adults that fed on infected plants as nymphs
could transmit the pathogen in a shorter latent period than could psyllids that fed on infected
plants only as adults.

Another model is mainly based on the study by Inoue et al. (74) and Pelz-Stelinski et al.
(114). Inoue et al. (74) suggested that the multiplication of Ca. L. asiaticus in psyllids is
essential for efficient transmission and that it is difficult for adults to transmit the pathogen
unless they acquire Ca. L. asiaticus as nymphs. Pelz-Stelinski et al. (114) showed that acquisition
by only adult psyllids did not result in Ca. L. asiaticus-infected plants after more than 1 year of
incubation after inoculation. Both models suggest that psyllids could acquire Ca. L. asiaticus as
nymphs and adults, but they disagree on the role of acquisition of Ca. L. asiaticus by only adults
in Ca. L. asiaticus transmission. Inoue et al. (74) reported that when psyllids fed on infected
plants as adults, the percentage of Ca. L. asiaticus-positive psyllids declined continuously after
an acquisition access period of 24 h, and the concentration of Ca. L. asiaticus did not increase
significantly over time in Ca. L. asiaticus. Furthermore, Ca. L. asiaticus was not transmitted to
plants and did not cause HLB disease. However, the concentration of Ca. L. asiaticus
significantly increased over time after acquisition feeding by fifth instars. It was also reported
that acquisition by nymphs ranged from 60 to 100%, whereas acquisition by adults only reached
474 40% after 5 weeks of feeding on *Ca. L. asiaticus*-infected plants (114). Inoue et al. (74)
475 suggested that multiplication within psyllids is required for efficient transmission, and it is
476 difficult for adults to transmit the pathogen unless they acquire the pathogen as nymphs.
477
478 The transmission of *Ca. L. asiaticus* from parent to offspring (transovarial) occurs at a
479 rate of 2-6% (114). *Ca. L. asiaticus* has been detected in *Ca. L. asiaticus*-negative female
480 genitalia and later in their offspring after mating with a *Ca. L. asiaticus*-infected male (98). This
481 finding is consistent with the occasional detection of *Ca. L. asiaticus* in psyllid ovaries (8).
482 However, it has also been reported that transovarial passage of *Ca. L. asiaticus* by *D. citri* does
483 not occur (144,153).
484
485 **Seed transmission**
486
487 Although *Ca. L. asiaticus* is located in the seed coat (127), it appears not to be seed-
488 transmitted (3, 68, 123). Most data suggest that seedlings do not develop symptoms typical of
489 HLB from HLB-infected seeds and that *Ca. L. asiaticus* is not present in the seedlings
490 germinated from HLB-affected seeds (3, 68, 123). Hilf (68) reported the presence of *Ca. L.
491 asiaticus* in 10% of ‘Sanguenelli’ sweet orange seedlings but not in ‘Conners’ grapefruit
492 seedlings generated from infected seeds. Additionally, *Ca. L. asiaticus* was not detected in
493 ‘Ridge Pineapple’ tissue at 3 months post-grafting onto the abovementioned ‘Sanguenelli’
494 seedlings. Thus, it does not appear that seed transmission occurs or plays a significant role in
495 *Ca. L. asiaticus* transmission.
496
497 **VIRULENCE MECHANISM**
498
499 Understanding the citrus and *Ca. L. asiaticus* interaction and the virulence mechanism of
500 the pathogen is critical to designing innovative management strategies to control HLB. However,
due to the difficulty in culturing *Ca. L. asiaticus*, our understanding of its virulence mechanism is very limited, despite some promising progress.

**Phloem blockage and aberrations**

Phloem blockage has been suggested to be a major reason for HLB disease symptom development (81). HLB-associated phloem blockage results from plugged sieve pores rather than HLB bacterial aggregates because *Ca. L. asiaticus* does not form aggregates in citrus (81).

Given the size of *Ca. L. asiaticus*, approximately 2 µm in length and 0.1 to 0.2 µm in diameter (21) or 0.33 to 0.66 µm in diameter and 2.6-6.3 µm in length (66), it is unlikely that a single HLB bacterium could plug the sieve pores, which range from less than 1 µm to approximately 14 µm (44). Phloem blockage is partially due to the deposits of large amounts of callose as confirmed by staining with aniline blue. Phloem proteins might also be involved in phloem blockage since the PP2 gene was induced in HLB diseased citrus compared to healthy control (81). However, PP2 has been suggested to be a defense response of the host to restrict further spread of the pathogen within the sieve tubes. Analysis of recovered apple from apple proliferation disease has indicated that callose accumulation and phloem-protein deposition in the sieve elements might contribute to the recovery of the infected plant by forming physical barriers, preventing the movement of *Ca. Phytoplasma mali* from the roots and re-colonization of the crown (109).

Considering that PP2 genes are not induced in the early stage of infection at 5-9 weeks after graft inoculation (4), the phloem protein does not appear to play a critical role in plant defense against *Ca. L. asiaticus*. Instead, the plugging of the sieve elements might block phloem transportation, leading to nutrient depletion of neighboring cells.

Callose deposition in the sieve plates has also been observed by Koh et al. (82).

Additionally, Koh and colleagues observed callose accumulation around plasmodesmata pore...
units (PPUs) connecting companion cells and sieve elements. It was suggested that callose accumulated around PPUs before starch began to accumulate in the chloroplasts. This suggestion was based on the observation that PPUs in the *Ca. L. asiaticus* infected asymptomatic leaves were stained for callose at levels similar to that of PPUs in the symptomatic leaves. Transmission electron microscopy also indicated that PPUs with abnormally large callose deposits were more abundant in the *Ca. L. asiaticus* infected samples than the healthy leaves. Callose formation around PPUs in *Ca. L. asiaticus* infected leaves inhibited the symplastic flow of solutes from companion cells into sieve tubes, thereby reducing the phloem loading efficiency based on the monitoring of a symplast fluorescent tracer carboxyfluorescein diacetate (CFDA). In healthy leaves, CFDA is imported into the veins. In contrast, the fluorescence in minor veins is often dimmer than it is in the surrounding non-vascular tissue in *Ca. L. asiaticus* infected leaf samples, indicating that CFDA remains in the non-vascular tissue.

This blockage harms not only plant cells but also *Ca. L. asiaticus*. Therefore, *Ca. L. asiaticus* might eventually become nonviable in completely blocked sieve elements (135). Interestingly, large numbers of *Ca. L. asiaticus* cells were found in phloem sieve tubes in tissue samples from pre-symptomatic young flushes, but they were not found in highly symptomatic leaf samples (51).

Sucrose is the primary photoassimilate in phloem transported from mature leaves to sink organs (159). Sucrose accumulation in *Ca. L. asiaticus*-infected leaves suggests that photoassimilate translocation is impaired by *Ca. L. asiaticus* infection, most likely due to phloem blockage (47,48,81,82). Koh et al. (82) carried out CO$_2$ pulse-labeling experiments and determined that *Ca. L. asiaticus* infection interferes with photoassimilate export from source leaves. In healthy leaves, 81% of $^{14}$C (measured at time 0) disappeared within 24 h, while only
46% of radioactivity was released from Ca. L. asiaticus infected leaves. The delayed export of fixed $^{14}$C from the Ca. L. asiaticus infected leaves suggests that the starch buildup in the chloroplasts of Ca. L. asiaticus infected leaves may have resulted from the delayed translocation of photosynthates. This reduced photoassimilate transportation might contribute to the small, misshapen, and poorly colored fruit containing aborted or partially developed seeds. Sucrose deficiency has been associated with fruit growth arrest (59). Importantly, the flavedo from Ca. L. asiaticus-infected trees has been reported to have a lower carbohydrate content (116). Additionally, Fan et al. (48) compared the phloem transport activity in the midribs of source leaves of tolerant rough lemon (C. jambhiri) and susceptible sweet orange (C. sinensis) in response to Ca. L. asiaticus infection. Their study indicated that although microscopic changes e.g., callose deposition in sieve elements and phloem cell collapse, were found in both infected species, the phloem transport activity of rough lemon was much less affected by HLB than in sweet orange.

Starch accumulation has also been reported to be increased in infected aerial tissues but depleted in roots. Interestingly, it has been observed that many genes involved in photosynthesis are repressed, most likely due to increased sucrose/glucose levels, as photosynthesis/chlorophyll-associated genes, such as those encoding photosystem-II 5-kDa protein, photosystem-I subunit O and a chlorophyll A-B binding family protein, were down-regulated by Ca. L. asiaticus infection (4,47,81). However, bark samples and symptomless leaves also contain higher levels of starch than healthy controls without visible phloem blockage (45). This seems to suggest that other mechanisms in addition to phloem blockage might also be involved in HLB disease development.
Other microscopic aberrations have been observed in the *Ca. L. asiaticus* infected Madam Vinous sweet orange seedlings (51), including swelling of the middle lamella between cell walls surrounding the sieve elements. The development of HLB symptoms correlated with an increasing degree of microscopic aberrations. Interestingly, large numbers of *Ca. L. asiaticus* cells were observed in tissue samples from asymptomatic young flushes but not in highly symptomatic leaf samples (51). In addition, microscopic studies of leaf samples from symptomatic sweet orange field trees demonstrated necrosis in the phloem, massive accumulation of starch in the plastids, aberrations in cambial activity, and excessive phloem formation and phloem collapse (81,119). It was suggested that extensive phloem necrosis contributes to the blockage of the phloem transportation, which leads to other anatomical changes. Consequently, these changes are responsible for the blotchy mottle, yellowing, leatheriness, and vein clearing on the leaves of infected trees (119).

**Metabolic imbalances by nutrient depletion**

Duan et al. (41) suggested that *Ca. L. asiaticus* is parasitic rather than pathogenic, causing host metabolic imbalances by nutrient depletion or interference with transportation, which results in HLB symptoms.

Knowledge of the carbon source and sugar metabolism of the *Ca. L. asiaticus* facilitates understanding of its pathogenicity. *Ca. L. asiaticus* may disrupt host cellular metabolic functions by importing multiple host-cell metabolites for growth and development, ultimately leading to disease expression. *Ca. L. asiaticus* has the ability to metabolize sugars such as glucose, fructose, and xylulose but not mannose, galactose, rhamnose, or cellulose (41). The concentrations of fructose and glucose are very low in the phloem sap (28,50); therefore, consumption of fructose by *Ca. L. asiaticus* during infection may initiate a shift in the host metabolite distribution. Fan et
al. (47) observed a remarkable accumulation of glucose but not fructose and suggested that Ca. L. asiaticus might preferentially utilize fructose, similar to *Spiroplasma citri*. Thus, Ca. L. asiaticus infection will result in reduced fructose concentrations and the accumulation of glucose in the infected host tissues. Glucose accumulation will subsequently favor the repression of enzymes involved in photosynthesis and contribute to HLB symptom development. Interestingly, the consumption of fructose by *Spiroplasma citri* has been implicated in affecting phloem loading of sucrose, sugar accumulation in source leaves, and causing disease symptoms, including yellowing. Sugar and starch accumulations have been observed previously in citrus trees infected by Ca. Liberibacter (81,119). It is possible that Ca. L. asiaticus could affect the phloem loading of sucrose in citrus and result in starch accumulation. Such mechanisms of pathogenicity are based not on specific genes, such as genes for toxins, but on deviations in sugar metabolism. However, Ca. L. asiaticus encodes only one sugar transporter for glucose/galactose (41). It is unknown how Ca. L. asiaticus imports fructose from its host. Thus, this hypothesis needs further validation.

*Ca. L. asiaticus* encodes a relatively low number of genes involved in the biosynthesis of compounds, which are readily taken up from the host. Analysis of the *de novo* amino acid biosynthetic pathways of Ca. L. asiaticus has revealed that they are capable of producing serine, glycine, cysteine, aspartate, lysine, threonine, glutamate and arginine and incapable of making histidine, tyrosine, thiamine, phenylalanine, tryptophan, asparagine, isoleucine, methionine, alanine, valine, leucine and proline. Interestingly the culturable nature of *L. crescens* is postulated to be in part due to the presence of genes involved in the synthesis of essential amino acids phenylalanine and tyrosine (89). The deficiencies in amino acid biosynthesis can be countered by the bacterium through the import of exogeneous amino acids. Accordingly, the *Ca.
L. asiaticus genome encodes a set of general L-amino acid permease proteins that are able to transport a variety of amino acids into the cell. In addition, a gene encoding branched chain proton-glutamate transporter that is able to import both glutamate and aspartate is present in the genome. Also, Ca. L. asiaticus possess a thiamine ABC transporter not found in L. crescens, presumably to compensate for the inability to synthesize thiamine (89).

**Ca. L. asiaticus** encodes 137 transporter proteins with 92 genes that are involved in active transport, including 40 ABC transport genes. Recently, Li et al. (90) analyzed all of the ABC transporter-related proteins in Ca. L asiaticus and identified 14 ABC transporter systems and 7 non-transporting ABC proteins. The study showed that the bacterium could use these ABC transporters to import metabolites (amino acid and phosphates) and enzyme cofactors (choline, thiamine, iron, manganese, and zinc); resist organic solvent, heavy metal, and lipid-like drugs; maintain the composition of the outer membrane; and secrete virulence factors. The large number of transporter proteins might play a critical role in providing Ca. L. asiaticus with necessary nutrients and cause a metabolic imbalance in citrus. Interestingly, Ca. L. asiaticus encodes one zinc transport system (*znuABC*) (41). Vahling-Armstrong et al. (143) demonstrated that the *znuABC* system of Ca. L. asiaticus is functional and is responsible for high-affinity zinc uptake. Therefore, this system might contribute to the zinc deficiency associated with HLB-affected trees. A comparison of the *znuABC* homologues of *S. meliloti* and Ca. L. asiaticus also revealed the existence of distinct modes of regulation between the zinc import systems, despite the intracellular-plant niche that is common to both bacteria (143). Although zinc ABC transporters are also present in *L. crescens*, they show very low sequence similarity with Ca. L. asiaticus (89). This variation in zinc ABC transport proteins may contribute to the differences in the virulence of Liberibacter genus. A twin arginine translocation (Tat) protein export pathway
and an additional iron ABC transporter are present in *L. crescens* but not in *Ca. L. asiaticus* (89).

The significance of these two transporters is not currently known, but their existence may explain why *L. crescens* is less fastidious than *Ca. L. asiaticus*.

*Ca. L. asiaticus* also encodes an ATP/ADP translocase in addition to its ATP synthase so that it can utilize the energy source directly from its host, as do other obligate intercellular parasites, such as *Rickettsia prowazeki* (41,142,152).

**Hormone**

Phytohormones have been known to influence citrus fruit set, productivity, and plant response to plant pathogen infection (116). Rosales and Burns (116) compared the phytohormones in symptomatic fruit (S), asymptomatic fruit (AS) from symptomatic trees, and healthy fruit (H) from asymptomatic trees harvested from ‘Valencia’ sweet orange trees (*Citrus sinensis* (L.) Osbeck). It was shown that S and AS harvested 7 and 12 months after full bloom produced significantly less ethylene than H. The indole-3-acetic acid (IAA) and abscisic acid (ABA) contents in flavedo from the stylar end, middle section or stem end of fruit were higher in S flavedo than in AS and H. Although ethylene promotes abscission, the ethylene-IAA balance is known to play a regulating role in controlling fruit abscission (121). The four-fold lower IAA content in the stem end of S is suggested to accelerate abscission, although ethylene production in the whole fruit is lower. The IAA content was higher in the misshapen region compared to the normal-growing areas of S of the fruit. The hypodermal cell size was also increased in the corresponding regions. Therefore, IAA has been suggested to play a role in the development of misshapen fruit areas (116).

**Suppression or avoidance of plant defense**
Unsuccessful attempts to culture *Ca. L. asiaticus* have slowed the dissection of molecular mechanisms of pathogenesis and the avoidance or suppression of plant innate immunity. It has been suggested that *Ca. L. asiaticus* elicits a delayed defense response (81). How *Ca. L. asiaticus* manipulates the plant defense response is critical to its survival *in planta*.

The reduced genome of *Ca. L. asiaticus* and transmission by psyllids might allow it to avoid PAMP-triggered immunity. Plants use pattern recognition receptors (PRRs), which are typically localized in the plant cell membrane, to respond to microbial- or pathogen-associated molecular patterns (MAMPs or PAMPs, respectively) (77,78). Plants recognize a wide range of bacterial PAMPs, most of which are derived from structural components of the bacterial cell (112). PAMPs induce rapid and transient production of reactive oxygen species in an oxidative burst following the recognition of a variety of pathogens (12, 39, 63, 112). In addition, *Ca. L. asiaticus* lacks type II plant cell-wall degrading enzymes, which have been known to elicit defense responses based on autodegradation products of the plant cell wall (oligogalacturonides) (113). However, *Ca. L. asiaticus* still contains 57 genes in cell envelope biogenesis, the outer membrane, including lipopolysaccharides (LPS), and most flagellar genes (41), which might function as PAMPs. It has been shown that *Ca. L. asiaticus* contains a functional *fla* gene encoding a flagellin and hook-associated protein of 452 amino acids that contains the conserved flg22 (160). The *fla* gene could partially complement the corresponding *Sinorhizobium meliloti fla* mutant. Transient expression *in planta* indicated that Fla_{Las} induced cell death and callose deposition in *Nicotiana benthamiana* and that the transcription of *BAK1* and *SGT1*, which are associated with plant innate immunity, was upregulated. The synthetic Flg22_{Las} peptide could not induce plant cell death but retained the ability to induce callose deposition (160). The influence of flagellin and Flg22_{Las} on the induction of cell death and callose deposition is similar to that of
other known flagellin and Flg22 (111). Thus, it has been suggested that *Ca. L. asiaticus* flagellin may act as a PAMP and trigger host plant resistance to the HLB bacteria (160). However, flagella have not been observed for *Ca. L. asiaticus*, even though most flagellar genes are present in the genome (41). In addition, FLAGELLIN-SENSING2 (FLS2) is a transmembrane receptor kinase that binds to bacterial flagellin or flg22 through a physical interaction within the FLS2 extracellular domain (5,42). It is unknown how *Ca. L. asiaticus* perceives the flagellin or flg22 and other PAMPs because *Ca. L. asiaticus* resides in the phloem, an intracellular environment rather than an extracellular environment. Interestingly, several components of a fimbrial low-molecular-weight protein (flp) pilus system encoded by Tad family proteins and involved in tight adherence of the bacteria were present in pathogenic and uncultured *Ca. L. asiaticus* but not in non-pathogenic and culturable *L. crescent* (89). Diversity of the flp pilus operon is predicted to contribute to variation in virulence among pathogenic species and further studies are warranted to deduce its role in the pathogenicity of *Ca. L. asiaticus*.

It is noteworthy that the dissemination of *Ca. L. asiaticus* relies on its psyllid vector. Therefore, it bypasses the preformed and certain induced plant defenses, such as stomata closure (64,102), that are encountered by free-living bacteria such as *Pseudomonas* and *Xanthomonas*.

Plants also utilize polymorphic nucleotide binding (NB) leucine-rich repeat (LRR) protein products encoded by most R genes to recognize pathogens inside the cell (77). This process is mediated through the direct or indirect reorganization of effectors by NB-LRR, resulting in effector-triggered immunity. *Ca. L. asiaticus* does not encode type III or IV secretion systems or their effectors (41). *Ca. L. asiaticus* might encode other unidentified effectors that are recognized by the host plant once it is inside the phloem. It has been known that cytoplasmic proteins are able to recognize pathogens. In resistant tomato plants, the
cytoplasmic protein kinase Pto in plants carrying the nucleotide-binding site-LRR gene Prf recognizes AvrPto and AvrPtoB and leads to effector-triggered immunity (108). However, it is unlikely that the plant defense against Ca. L. asiaticus is enough to suppress the HLB pathogen. Microarray analysis has been used to understand the molecular mechanisms underlying HLB disease development (4, 81, 47, 48, 91, 99). It has been suggested that the infection of citrus with Ca. L. asiaticus does not lead to a significant induction of defense-related genes in the early stages, approximately 5-9 weeks after inoculation. The citrus host is unable to suppress the pathogen, resulting in the compatibility of the interaction (4, 81).

In addition, Ca. L. asiaticus could further suppress the plant defense. Our preliminary data indicate that Ca. L. asiaticus contains CLIBASIA_00255, which encodes a salicylate hydroxylase that in turn converts salicylic acid (SA) into catechol, a product that does not induce resistance (146). CLIBASIA_00255 has been shown to be highly induced in planta compared with in psyllid. SA has been reported to play a central role in plant defenses by mediating defense responses against pathogens in a number of plant species (122). SA is important for basal defense, the hypersensitive response, and systemic acquired resistance (SAR) (43).

Expressing salicylate hydroxylase in plants has been shown to abolish plant defenses by degrading SA. For example, Arabidopsis plants carrying the nahG gene, which encodes a salicylate hydroxylase, are defective in non-host resistance to Pseudomonas syringae pv. phaseolicola strain 3121 (147). Our preliminary analysis indicates that SA hydroxylase is able to degrade SA using the crude extract of E. coli expressing SA hydroxylase. Our data suggest that the modulation of SA production could be one of the mechanisms deployed by Ca. L. asiaticus to evade plant defense responses (131,132). This is consistent with the previous finding that a large
number of defense-related genes were down-regulated or expressed at very low levels in Ca. L. asiaticus infected citrus (4,81).

725 Prophages SC1 and SC2

It has been reported that Ca. L. asiaticus carries an excision plasmid prophage, SC2, and a chromosomally integrated prophage, SC1, that becomes lytic in citrus (156). SC1 and SC2 have been suggested to contribute to the pathogenicity of Ca. L. asiaticus. SC1 carries suspected lytic cycle genes, and phage particles associated with Ca. L. asiaticus have been observed in the phloem of infected periwinkle using transmission electron microscopy, although phage particles are not observed in citrus. A lytic burst of Ca. L. asiaticus inside a living phloem cell might trigger a cell death or apoptosis cascade, resulting in the subsequent death of the citrus phloem cell. This seems to explain the difficulty of observing Ca. L. asiaticus in symptomatic citrus leaf midribs (52, 81). However, Ca. L. asiaticus has been be observed in young asymptomatic tissues (51,52). SC1 and SC2 also encode multiple virulence factors that might contribute to the pathogenicity of Ca. L. asiaticus (156). SC1 and SC2 encode two predicted peroxidases that might defend Ca. L. asiaticus against ROS, including superoxide radicals, hydrogen peroxide, and hydroxyl radicals. SC1 and SC2 also encode two predicted adhesins, which might be useful in transmission by psyllids (156). However, an analysis of multiple isolates of Ca. L. asiaticus from different geographical locations has indicated that SC1 and SC2 are not universally present (Bill Schneider, personal communication). Leonard et al. (89) have reported that the culturable L. crescens contains two prophages (LC1 and LC2) which are not homologous to each other or to the tandem prophage region in Ca. L. asiaticus. The involvement of SC1 and SC2 in the pathogenicity of Ca. L. asiaticus needs further characterization.

745 Serralysin and hemolysin
Ca. L. asiaticus encodes multiple putative virulence genes, including genes encoding serralysin and hemolysin. Serralysin, a putative T1SS effector that is encoded by CLIBASIA_01345 and is located next to the T1SS locus in the genome, was identified using a computational analysis of Ca. L. asiaticus (29, 41). In our recent study, we found that the expression of CLIBASIA_01345 was up-regulated in planta compared with in psyllid (154). Serralysin is a secreted metalloprotease produced by a wide range of microorganisms, including plant and human pathogenic bacteria such as S. marcescens, P. aeruginosa, E. chrysanthemi, Proteus mirabilis and Caulobacter crescentus (32, 97). It has been shown that serralysin inactivates diverse antimicrobial proteins and peptides (118). For example, serralysin produced by P. mirabilis was reported to degrade host immunoglobulins and cleave antimicrobial peptides, including human β-defensin and LL-37 (17). The production of antimicrobial proteins and peptides is one of the major defense strategies utilized by a plant in response to infection by pathogenic organisms (25). The up-regulation of the serralysin biosynthesis gene in planta indicates that Ca. L. asiaticus may also utilize serralysin to modify the plant defenses, possibly by degrading host antimicrobial peptides. It has also been suggested that serralysin might aid in the acquisition of carbon and nitrogen for bacterial growth and metabolism through the proteolysis of host proteins and nutrient uptake (16, 17). Serralysin may further help Ca. L. asiaticus survive in its hosts. In addition, the introduction of exogenous antimicrobial peptides to citrus plants, by various transgenic approaches, is being used to control HLB. The presence of serralysin poses a potential challenge in the selection of efficient antimicrobial peptides against Ca. L. asiaticus. Thus, the serralysin of Ca. L.asiaticus could be a potential target for screening antimicrobial compounds to control HLB.
Hemolysin produced by animal and insect pathogens is believed to induce cell lysis, necrosis, and apoptosis (89); increase the availability of iron to the pathogen (124); and cause the leakage of ions, water, and low molecular weight molecules out of and into the host cell (62).

Hemolysin is present in other plant pathogenic bacteria, including phytoplasmas and Xylella fastidiosa (13,19), and is postulated to play an important role in degrading proteins produced by host cells in the defense reaction or by degrading host proteins for the uptake of essential nutrients (86). Like serralysin, the production of hemolysin by Ca. L. asiaticus may play an important role in facilitating survival of Ca. L. asiaticus inside the phloem by contributing to nutrient acquisition, ion transfer, and phloem necrosis.

ECOLOGICAL IMPORTANCE OF HLB

HLB not only directly affects plant production, but it also affects the agro-ecosystem. It has been reasonably postulated that the disruption of multi-trophic interactions in a stable ecosystem under the influence of a phytopathogen will cause community reorganization and changes in local feedback interactions. However, there is a paucity of knowledge on the extent to which such community shifts may occur, the dynamics of the changes involved and the putative effects on the functioning of ecosystems. Few studies have used HLB and citrus as disease-host models to evaluate fluctuations in the diversity, composition, structure and functional potential of plant-associated microbial communities in response to disease infection (117,133,134,136).

The profiling of bacterial diversity using various molecular- and culture-based methods has shown that HLB infection has a profound effect on the structure and composition of the bacterial community associated with citrus leaves (117), roots (133,136), and rhizospheres (134). Unique phylotypes and genotypes of bacteria have been found to be associated with HLB-infection, but apparently not in healthy citrus (133,136,141). Both culture- and molecular-based
assessments of bacterial diversity associated with citrus roots showed that the isolation frequency of bacterial isolates possessing various plant beneficial properties was significantly higher in HLB asymptomatic samples. The majority of bacterial types in the roots of healthy citrus were similar to known plant-growth promoting bacteria, including *Bacillus, Burkholderia, Caulobacter, Lysobacter, Paenibacillus, Pantoea* and *Pseudomonas,* while *in planta* levels of most of these types of bacteria were reduced in HLB-infected samples (133,136).

Representatives of the phylum *Actinobacteria,* particularly *Curtobacterium* species, were detected only in healthy samples (133,136). Taxon specific QPCR analysis has also revealed that the bacterial community changes not only qualitatively but also quantitatively (133,134).

Overall, various reports have shown that the infection of citrus by HLB has a profound effect on the structure and composition of the citrus-associated bacterial community.

Trivedi et al. (134) used QPCR and functional microarray ‘GeoChip 3.0’ to evaluate the effect of HLB on the functional diversity of the bacterial community associated with the citrus rhizosphere. Both analyses revealed that HLB has a significant negative effect on the functional diversity of rhizosphere microflora. Many of the genes involved in key ecological processes such as nitrogen, carbon, phosphorus, and sulfur cycling; metal homeostasis and resistance; and xenobiotic contaminant degradation were absent in the rhizosphere of HLB infected trees.

Carbon cycle gene distributions in the rhizosphere of citrus were significantly affected by HLB. The shift in the patterns of rhizodeposition and changes in the carbon utilization and fixation potential of microbial communities in response to HLB can have long-term effects on carbon storage and sequestering. Both GeoChip 3.0 and QPCR analyses revealed that HLB infection leads to a decreased abundance of various genes involved in N cycling, independent of their taxonomic origin. Shifts in the microbial community of these specialist bacteria can have a
strong impact on agro-ecosystem sustainability. According to the insurance hypothesis, species richness has a positive effect on ecosystem productivity through a buffering effect against disturbances. As shown by several studies (117,133,134,136,141), HLB infection can drastically influence the structure and function of citrus-associated bacterial communities, which could potentially have severe consequences on the stability and productivity of ecosystems.

**CONCLUDING REMARKS**

With the citrus industry of Florida and possibly that of the entire USA at stake, the need to control HLB and the challenges involved in doing so are unprecedented. However, no “silver bullet” has been identified to control HLB and stop it from spreading to new citrus production areas, although some promising progress has been made. Further studies are needed to understand the interactions among citrus, *Ca. L. asiaticus*, and psyllids to design innovative management strategies to control HLB. We also presume the availability of *L.crescens* in culture will greatly speed the hunt for effective treatments against HLB. While HLB has been a problem for over a century, the battle against HLB can only be resolved with a coordinated and deliberate effort from by the citrus industry, growers, researchers, legislatures, and governments.

**FIGURE LEGENDS**

Fig. 1. HLB causes dramatic symptoms in citrus. A. Healthy Valencia sweet orange (Citrus *sinensis*); B. Valencia with HLB disease; C. Typical fruit from healthy trees (left) and from severely diseased HLB trees (right); D. Citrus grove before HLB; E. Citrus grove after HLB
infection; F. Typical blotchy mottling with green islands on leaves from HLB trees (C, D, E, & F: courtesy of Mike Irey, U.S. Sugar Corp.).

Fig. 2. The Ca. L. asiaticus life-cycle involves the replication of the microbe in plants and psyllids. A. TEM picture of Ca. L. asiaticus in the phloem of citrus (Courtesy of Dr. Svetlana Y. Folimonova and Diann Achor, Citrus Research and Education Center (CREC), University of Florida (UF)); B & C. Asian citrus psyllid (D. citri) feeding on citrus plants (C courtesy of Dr. Michael Rogers, Citrus Research and Education Center, University of Florida); D. A scanning electron micrograph of Ca. L. asiaticus on the exterior surface of the psyllid midgut (courtesy of Dr. Michael Davis, CREC, UF); E. Ca. L. asiaticus acquired from psyllids stained with a DNA-binding fluorochrome SYTO 13 (courtesy of Dr. Michael Davis).

ACKNOWLEDGMENTS

We thank Dr. Hao Hu for his valuable suggestions regarding the host range of Ca. L. asiaticus and its psyllid vector.

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Fig. 1

177x133mm (300 x 300 DPI)