Citrus Huanglongbing: a newly relevant disease presents unprecedented challenges

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ABSTRACT

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 N_2 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 *rrs* and *rpl* genes and in the *omp* and *rpo*B loci of HLB-associated Liberibacters, is well documented (15, 38, 53, 57). Bastianel et al. (15) used an *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 *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 *rpo*B 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 *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 *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 *ser*A-*trm*U-*tufB*-*sec*E-*nus*G-*rpl*KAJL-*rpo*B 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 *nus*-*rplL* operon in 65 *Ca.* L. asiaticus isolates and reported that Japanese *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 *Ca.* L. asiaticus psy 62 strain. A dendogram analysis of diversity within these 27 SSR loci among *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 *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 phosphotranferase 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 *Spiroplasma citri* and *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 *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 *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 *Ca.* L. asiaticus even though the oxygen level in the phloem is lower than atmospheric levels.

CA. L. ASIATICUS TRANSMISSION

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 *Ca.* L. asiaticus. Tremendous efforts have been made in recent years to understand the mechanism of the psyllid transmission of *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 nymphal 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

40% after 5 weeks of feeding on *Ca.* L. asiaticus-infected plants (114). Inoue et al. (74) suggested that multiplication within psyllids is required for efficient transmission, and it is difficult for adults to transmit the pathogen unless they acquire the pathogen as nymphs.

The transmission of *Ca.* L. asiaticus from parent to offspring (transovarial) occurs at a rate of 2-6% (114). *Ca.* L. asiaticus has been detected in *Ca.* L. asiaticus-negative female genitalia and later in their offspring after mating with a *Ca.* L. asiaticus-infected male (98). This finding is consistent with the occasional detection of *Ca.* L. asiaticus in psyllid ovaries (8). However, it has also been reported that transovarial passage of *Ca.* L. asiaticus by *D. citri* does not occur (144,153).

Seed transmission

Although *Ca.* L. asiaticus is located in the seed coat (127), it appears not to be seed-transmitted (3, 68, 123). Most data suggest that seedlings do not develop symptoms typical of HLB from HLB-infected seeds and that *Ca.* L. asiaticus is not present in the seedlings germinated from HLB-affected seeds (3, 68, 123). Hilf (68) reported the presence of *Ca.* L. asiaticus in 10% of 'Sanguenelli' sweet orange seedlings but not in 'Conners' grapefruit seedlings generated from infected seeds. Additionally, *Ca.* L. asiaticus was not detected in 'Ridge Pineapple' tissue at 3 months post-grafting onto the abovementioned 'Sanguenelli' seedlings. Thus, it does not appear that seed transmission occurs or plays a significant role in *Ca.* L. asiaticus transmission.

VIRULENCE MECHANISM

Understanding the citrus and *Ca.* L. asiaticus interaction and the virulence mechanism of 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

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

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

536 Sucrose is the primary photoassimilate in phloem transported from mature leaves to sink
537 organs (159). Sucrose accumulation in *Ca.* L. asiaticus-infected leaves suggests that
538 photoassimilate translocation is impaired by *Ca.* L. asiaticus infection, most likely due to phloem
539 blockage (47,48,81,82). Koh et al. (82) carried out CO_2 pulse-labeling experiments and
540 determined that *Ca.* L. asiaticus infection interferes with photoassimilate export from source
541 leaves. In healthy leaves, 81% of ^{14}C (measured at time 0) disappeared within 24 h, while only

542 46% of radioactivity was released from *Ca.* L. asiaticus infected leaves. The delayed export of
543 fixed ^{14}C from the *Ca.* L. asiaticus infected leaves suggests that the starch buildup in the
544 chloroplasts of *Ca.* L. asiaticus infected leaves may have resulted from the delayed translocation
545 of photosynthates. This reduced photoassimilate transportation might contribute to the small,
546 misshapen, and poorly colored fruit containing aborted or partially developed seeds. Sucrose
547 deficiency has been associated with fruit growth arrest (59). Importantly, the flavedo from *Ca*. L.
548 asiaticus-infected trees has been reported to have a lower carbohydrate content (116).
549 Additionally, Fan et al. (48) compared the phloem transport activity in the midribs of source
550 leaves of tolerant rough lemon (*C. jambhiri*) and susceptible sweet orange (*C. sinensis*) in
551 response to *Ca*. L. asiaticus infection. Their study indicated that although microscopic changes
552 e.g., callose deposition in sieve elements and phloem cell collapse, were found in both infected
553 species, the phloem transport activity of rough lemon was much less affected by HLB than in
554 sweet orange.

555 Starch accumulation has also been reported to be increased in infected aerial tissues but
556 depleted in roots. Interestingly, it has been observed that many genes involved in photosynthesis
557 are repressed, most likely due to increased sucrose/glucose levels, as photosynthesis/chlorophyll-
558 associated genes, such as those encoding photosystem-II 5-kDa protein, photosystem-I subunit O
559 and a chlorophyll A-B binding family protein, were down-regulated by *Ca.* L. asiaticus infection
560 (4,47,81). However, bark samples and symptomless leaves also contain higher levels of starch
561 than healthy controls without visible phloem blockage (45). This seems to suggest that other
562 mechanisms in addition to phloem blockage might also be involved in HLB disease
563 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.*

610 L. asiaticus genome encodes a set of general L-amino acid permease proteins that are able to
611 transport a variety of amino acids into the cell. In addition, a gene encoding branched chain
612 proton-glutamate transporter that is able to import both glutamate and aspartate is present in the
613 genome. Also, *Ca*. L. asiaticus possess a thiamine ABC transporter not found in *L. crescens*,
614 presumably to compensate for the inability to synthesize thiamine (89).

615 *Ca.* L. asiaticus encodes 137 transporter proteins with 92 genes that are involved in active
616 transport, including 40 ABC transport genes. Recently, Li et al. (90) analyzed all of the ABC
617 transporter-related proteins in *Ca*. L asiaticus and identified 14 ABC transporter systems and 7
618 non-transporting ABC proteins. The study showed that the bacterium could use these ABC
619 transporters to import metabolites (amino acid and phosphates) and enzyme cofactors (choline,
620 thiamine, iron, manganese, and zinc); resist organic solvent, heavy metal, and lipid-like drugs;
621 maintain the composition of the outer membrane; and secrete virulence factors. The large
622 number of transporter proteins might play a critical role in providing *Ca.* L. asiaticus with
623 necessary nutrients and cause a metabolic imbalance in citrus. Interestingly, *Ca.* L. asiaticus
624 encodes one zinc transport system (*znuABC*) (41). Vahling-Armstrong et al. (143) demonstrated
625 that the *znuABC* system of *Ca*. L. asiaticus is functional and is responsible for high-affinity zinc
626 uptake. Therefore, this system might contribute to the zinc deficiency associated with HLB-
627 affected trees. A comparison of the *znuABC* homologues of *S. meliloti* and *Ca*. L. asiaticus also
628 revealed the existence of distinct modes of regulation between the zinc import systems, despite
629 the intracellular-plant niche that is common to both bacteria (143). Although zinc ABC
630 transporters are also present in *L. crescens*, they show very low sequence similarity with *Ca*. L.
631 asiaticus (89). This variation in zinc ABC transport proteins may contribute to the differences in
632 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 bust 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. crescens* (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).

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 predicated peroxidases that might defend *Ca.* L. asiaticus against ROS, including superoxide radicals, hydrogen peroxide, and hydroxyl radicals. SC1 and SC2 also encode two predicated 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.

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 crecentus* (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).

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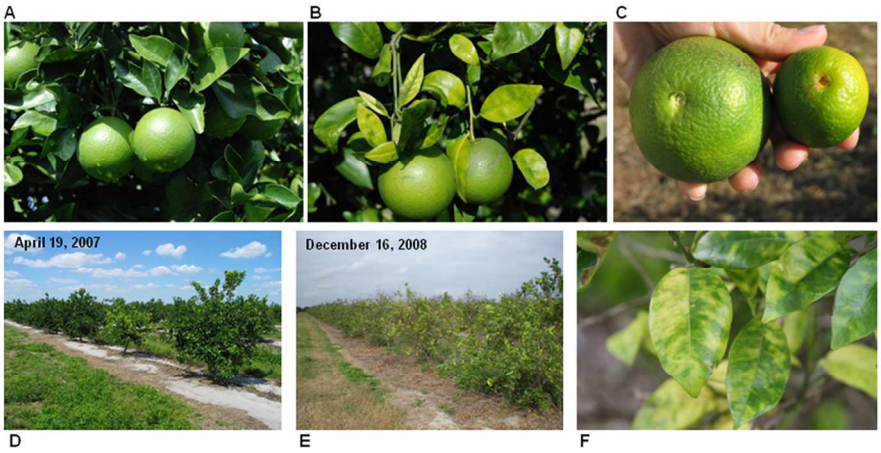

Fig. 1

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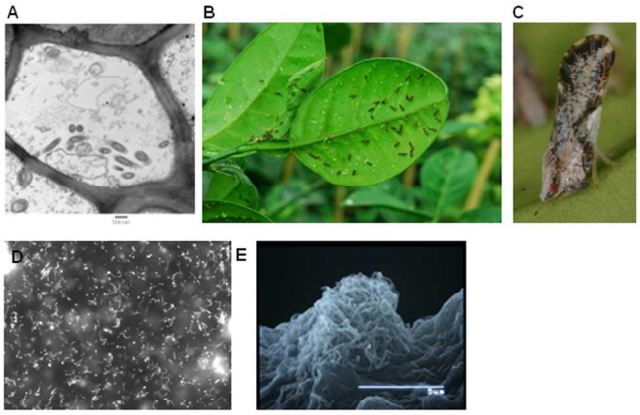

Fig. 2

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