Understanding the Physiology of *Liberibacter asiaticus*: An Overview of the Demonstrated Molecular Mechanisms

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Abstract

Citrus greening disease, or huanglongbing, may entirely eradicate all varieties of citrus cultivars worldwide in the near future. This disease is caused by non-cultivable bacteria of the genus *Liberibacter*; among them, the more pathogenic being *Liberibacter asiaticus*. The complexity of the host–pathogen relationship, associated with the impossibility of performing research using axenic cultures, has severely hindered the basic research on microbiology. Since its genome sequence was published in 2009, most of the scientific publications in the field were dedicated to in silico analysis and selection of targets to design early detection methods. The knowledge gained with these approaches felt short to articulate effective methods to control the disease progression. There is a critical need to understand the basic biology of bacteria to design effective strategies to inactivate central mechanisms of pathogenesis. In this review, we summarize the scientific progress made by studying *L. asiaticus*' biology through direct experimentation. The evidence collected thus far is not enough to understand *L.*

asiaticus' fundamental biology. It is imperiously necessary to increase the basic research to identify relevant biological clues to control citrus greening. The gained knowledge may also help to prevent potential catastrophic diseases in other crops of significant importance caused by other unculturable *Liberibacter* species.

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Introduction

Huanglongbing (HLB), also known as citrus greening, is a pernicious disease affecting the citrus industry in most of the top citrus-producing countries worldwide, with no control method available currently. This disease causes characteristic symptoms in the citrus host, including blotchy mottled leaves, yellow shoots, and small, deformed fruit [Bové, 2006]. The trees suffer from shorter lifespans, premature fruit drop, and a reduction in fruit quality [Bassanezi et al., 2009; Bové, 2006]. The resulting reduction in marketable fruit has had a drastic economic effect on the citrus industry. HLB has already devastated the citrus industry in many areas of Asia, Africa, and America. In the United States, this pathogen has rapidly spread to Florida, California, Louisiana, South Carolina,

Georgia, and Texas [da Graça et al., 2015, 2016; Halbert and Manjunath, 2004; Kumagai et al., 2012]. In Florida alone, HLB has cost over USD 7.80 billion in lost revenues and more than 7,000 jobs in a 7-year span, from the 2006 to 2007 harvest to the 2013–2014 harvest [Hodges et al., 2015]. In 2017, the net oranges production in Florida fell behind California, with almost double acreage in production. These numbers clearly demonstrate the immediate and critical need to combat and eradicate citrus greening, to preserve the economy of international citrus producers.

Three species of phloem-limited Alphaproteobacteria in the order Rhizobiales have been associated with HLB: *Liberibacter asiaticus* (Las), *Liberibacter africanus* (Laf), and *Liberibacter americanus* (Lam) [Bové, 2006]. These bacteria are transmitted into the citrus host via a psyllid vector, *Diaphorina citri* or *Trioza erytreae* [Bové, 2006; Gottwald, 2010]. *L. asiaticus* is the most pathogenic of these [Bové, 2006; Lopes et al., 2009], and is also the species associated with HLB in the United States. Identifying potential treatment targets of *L. asiaticus* has been particularly challenging, as there is currently no method for maintaining *L. asiaticus* in culture in a laboratory setting. This means that all knowledge pertaining to its physiology has been derived from predictions based on the information encoded in its genome.

Much of the research completed to date concentrates on detection, transmission, and phylogenetics of *L. asiaticus* or on interactions between *L. asiaticus* and the plant or between *L. asiaticus* and the psyllid vector, with the focus on the host rather than on the bacteria. We will omit these areas of investigation from the discussion enclosed herein, as there are several excellent reviews already available [Arredondo Valdés et al., 2016; Bové, 2006; Gottwald, 2010; da Graça et al., 2016; Grafton-Cardwell et al., 2013; Halbert and Manjunath, 2004; Wang et al., 2017; Wang and Trivedi, 2013].

In this review, we will focus on the advances in describing the physiology of *L. asiaticus*, investigated through the use of molecular techniques, as complement to in silico approaches. Although valuable information can be gathered through these large-scale data-rich methods, they fell short in predicting precise strategies directed to minimize citrus greening disease. Thoroughly mining the vast amount of data currently available is an important step in identifying potential targets for the development of therapeutics. Here, we will summarize relevant in silico assessments but we will mainly focus on studies that undertake the functional characterization of *L. asiaticus* proteins through direct molecular assays (Table 1).

Omics Approaches

The approaches covered by the umbrella term – omics – are aimed primarily at studying a specific sample in a non-targeted and non-biased manner [Horgan and Kenny, 2011]. These studies, contrary to traditional experimentation, are utilized to generate hypotheses to understand a complex system when little or nothing is known about the physiological process being screened. These techniques provide an excellent foundation for future, more targeted experimentation, and allow for the development of data-driven hypotheses. In this section, we will discuss the application of genomics, transcriptomics, and proteomics to *L. asiaticus* as a means of identifying genes/proteins for further investigation.

Metagenomics

The first complete genome of *L. asiaticus* was sequenced from the DNA contained within a single psyllid, Psyllid #62 [Duan et al., 2009]. The DNA was concentrated by multiple displacement amplification (MDA), sequenced by 454 pyrosequencing, and finally PCR verified to belong to *L. asiaticus* rather than alternate DNA sources, such as *D. citri* or other endosymbionts [Duan et al., 2009]. The genome was found to be small, roughly 1.23 Mb in size, with a high GC content. Automatic genomic annotation predicted 1,136 genes encoding proteins; 36.5% of them were classified as uncharacterized or hypothetical. There were a low number of genes associated with the biosynthesis of compounds already transported from the host, like amino acids, and regulatory transcriptional proteins, including σ factors, a high number of genes pertaining to cell motility or active transport, and 12 phage-related genes. The genome sequence suggests that *L. asiaticus* has a restricted capability for aerobic respiration, cannot reduce sulfate but may utilize nitrogen for anaerobic respiration, and is unable to synthesize at least 5 amino acids. *L. asiaticus* is predicted to encode ABC transporters, proteins required for the general secretory pathway, type I secretion systems, and flagella biosynthesis proteins; however, it does not encode type III or IV secretion systems [Duan et al., 2009].

Comparative Genomics

The genome of *L. asiaticus* was compared with that of *L. solanacearum* and *L. crescens* BT-1, the closest cultured relative, to acquire knowledge regarding the current inability to culture most *Liberibacter* species [Fagen et al., 2014]. Compared to *L. crescens*, *L. asiaticus* was found to lack the following: pathways for synthesis of 6 amino ac-

Table 1. Summary of *Liberibacter asiaticus* proteins characterized via traditional molecular and genetic methods

Protein name ^a	Locus tag	Gene annotation ^b	Function prediction	Importance/significance	Source
PrbP	CLIBASIA_01510	CarD family transcriptional regulator	Activator of gene expression; regulates some ribosomal genes	<ul style="list-style-type: none"> – Interacts with promoter DNA and RNA polymerase; hypothesized role in promoting open complex formation – Other members of the family involved in pathogenesis, persistence, cell viability, and resistance to antibiotics and stress – In <i>M. tuberculosis</i>, PrbP mutations cause reduced viability and increased sensitivity to antibiotics and oxidative stress – Tested chemical resulted in the recovery of HLB symptoms and inhibited <i>L. asiaticus</i> in infected citrus; evidence for treatment methods targeting PrbP 	Gardner et al., 2016; Pan et al., 2017
LdtR	CLIBASIA_01180	MarR family transcriptional regulator	Global activator/repressor of gene expression	<ul style="list-style-type: none"> – 5-fold upregulation in planta vs. psyllid – Genome-wide activator/repressor of gene expression allowing <i>L. asiaticus</i> to sense/adapt to environment – Involved in osmotic stress tolerance, important in phloem – Regulated processes include cell motility, CW biosynthesis, energy production and conversion, growth and cell division, and transcription – Characterization of ligand binding pocket permits structure-based strategies for rational drug design and virtual inhibitor screening 	Pagliai et al., 2014, 2015, 2017
NttA	CLIBASIA_01040	ATP/ADP translocase	Nucleotide transporter; imports ATP from host to bacterial cytoplasm	<ul style="list-style-type: none"> – Maintained through genome reduction and conserved in <i>L. solanacearum</i> – Alteration of host intracellular ATP levels could lead to increased starch accumulation as seen in HLB-infected citrus – Implication in targeted drug design and improved culturability of <i>L. asiaticus</i> 	Vahling et al., 2010
Znu #1		Zn transport system; ABC transporter	Facilitates metal ion translocation across the bacterial IM	<ul style="list-style-type: none"> – Zn²⁺ is essential in many structural elements and as enzymatic cofactor for various important biological processes – Zinc uptake from host may cause localized zinc deficiency in citrus leading to HLB symptoms – Znu mutants in other pathogens result in diminished virulence 	Vahling-Armstrong et al., 2012
ZnuA1 ZnuB1 ZnuC1	CLIBASIA_03020 CLIBASIA_03030 CLIBASIA_03025	Periplasmic SBP Membrane spanning permease ATPase	Metallochaperone; binds metal ions in the periplasm Structural subunit; transfer metal ions across IM ATP-driven molecular motor driving translocation		
Znu #2		Zn/Mn/Fe transport system; ABC transporter	Facilitates metal ion translocation across the bacterial IM	<ul style="list-style-type: none"> – ZnuA2: Unique mechanism of metal binding/release with low binding affinity; may have evolved to prevent Zn toxicity – ZnuA2: Reversibly binds both Mn²⁺ and Zn²⁺, allowing transport of both ions – ZnuA2: Characterization of this unusual SBP improves understanding of a metal ion transport system important for cell viability 	Vahling-Armstrong et al., 2012; Sharma et al., 2015, 2016

Table 1. (continued)

Protein name ^a	Locus tag	Gene annotation ^b	Function prediction	Importance/significance	Source
ZnuA2 ZnuB2 ZnuB2-2 ZnuC2	CLIBASIA_02120 CLIBASIA_02130 CLIBASIA_02135 CLIBASIA_02125	Periplasmic SBP Membrane spanning permease ATPase	Metallochaperone; binds metal ions in the periplasm Structural subunit; transfer metal ions across IM ATP-driven molecular motor driving translocation		
LasA _I LasA _{II}	Hyv _I Hyv _{II}	Hypothetical protein	Trimeric autotransporter (T5cSS or AT-2) self-transporting protein system; secreted virulence factors; target plant mitochondria	<ul style="list-style-type: none"> – May affect host mitochondria and chloroplast function and manipulate energy production during <i>L. asiaticus</i> infection (connected to ATP-ADP translocase) – Play a role in adherence and aggregation 	Hao et al., 2013
SecA	CLIBASIA_01060	Pre-protein translocase subunit of the Sec protein translocase complex	ATP hydrolysis provides energy for translocation of pre-proteins across the IM from cytoplasm to periplasm	<ul style="list-style-type: none"> – Essential component of Sec machinery, one of the few secretion systems encoded by <i>L. asiaticus</i> genome – Role in secretion of effector proteins – important pathogenicity determinants involved in virulence – Crystal structure enables structure-based drug design strategies to develop highly specific antimicrobial compounds and virtual inhibitor screening 	Akula et al., 2011, 2012; Hu et al., 2016
LepB	CLIBASIA_04190	Type I signal peptidase	Cleaves N-terminal SP from pre-proteins; allows release and proper folding of mature protein from the bacterial IM	<ul style="list-style-type: none"> – See SecA for importance of Sec system – Essential to cell viability – When inhibited, accumulation of secretory pre-proteins in IM leads to cell death – Good antimicrobial target due to periplasmic catalytic domain; drugs do not have to penetrate the IM 	Prasad et al., 2016
NA	SC2_gp095	Hypothetical protein; putative phage-related peroxidase	Detoxifies ROS; alters plant defense	<ul style="list-style-type: none"> – Proposed role in evading early detection of <i>L. asiaticus</i> in phloem by suppressing host defenses through detoxification of H₂O₂ – May cause observed long incubation period of HLB by attenuating IIR until significant titer level reached and oxidative damage occurs 	Jain et al., 2015
BCP	CLIBASIA_00485	Bacterioferritin comigratory protein; thioredoxin-dependent thiol peroxidase	1-Cys Peroxiredoxin (Prxs) family protein; detoxifies ROS; alters plant defense	<ul style="list-style-type: none"> – Suggested in defensive role, protecting cells from H₂O₂-induced damage promoting <i>L. asiaticus</i> infection – Putative DNA-binding protein; prevents oxidative damage to DNA – Potential target for antibacterial treatment 	Singh et al., 2016
SahA	CLIBASIA_00255	Salicylate hydroxylase	Flavoprotein monooxygenase; naphthalene degradation pathway (proximal step); degrades SA; alters plant defense	<ul style="list-style-type: none"> – 3.5-fold upregulation in planta vs. psyllid – SA is an endogenous defense signal important for activating plant IIR – SA hydroxylases metabolize SA, limiting plant defenses – Critical virulence factor for infectivity and persistence in citrus – Not essential for bacterial growth, reducing the possibility of resistance development 	Li et al., 2017
NA	SC1_gp110	Hypothetical protein; possible holin	Forms holes in bacterial IM upon aggregation	<ul style="list-style-type: none"> – Heterologous expression of holin and endolysin resulted in cell lysis and stopped bacterial growth – Understanding mechanisms of induction of lytic cycle for SC1/2, leading to expression of late genes, is a possible method for HLB management – May also suggest necessary host factors for free-living culture resulting in improved culturability of <i>L. asiaticus</i>. 	Fleites et al., 2014

Table 1. (continued)

Protein name ^a	Locus tag	Gene annotation ^b	Function prediction	Importance/significance	Source
NA	SC1_gp035	Hypothetical protein; possible endolysin	Degrades bacterial CW	– See SC1_gp110	Fleites et al., 2014
Fla	CLIBASIA_02090	Flagellin domain-containing protein	Forms the filament of flagellum; contains flg22-like domain (strong PAMP)	– Important PAMP in plant IIR – <i>L. asiaticus</i> flg22 is weaker PAMP than other pathogens, possibly to reduce plant IIR – Understanding the mechanism and regulation of flagellum production may be critical for describing the HLB pathosystem	Zou et al., 2012
FabI	CLIBASIA_01735	Enoyl-acyl carrier protein (ACP) reductase I	Catalyzes last step in fatty acid synthesis; fatty acid chain elongation	– Fatty acids are vital for bacterial growth and survival; FabI is essential in the elongation of fatty acids – Crystal structure permits structure-based strategies for rational drug design and virtual inhibitor screening	Jiang et al., 2014
LotP	CLIBASIA_03135	Uncharacterized protein; peptidase S16 lon domain-containing protein	Uncharacterized family of proteins; GroEL-interaction protein; ATPase activity in vitro; may have a central role in HLB-citrus pathosystem	– Sequence highly conserved among <i>Rhizobiaceae</i> family (56–86% identity) – >7-fold induction in planta vs. psyllid – May be involved in response to temperature and osmotic stresses and in decreased plant IIR to <i>L. asiaticus</i> – Understanding role of GroEL-LotP may shed light on virulence and infectivity	Loto et al., 2017

^a Gene annotation based on annotations in NCBI, EggNOG, and KEGG databases.

^b NA, protein name not assigned.

SBP, substrate-binding protein; IM, inner membrane; CW, cell wall; ROS, reactive oxygen species; SP, signal peptide; SA, salicylic acid; PAMP, pathogen-associated molecular pattern; HLB, huanglongbing; IIR, innate immune response; dpi, days post inoculation.

ids, the shikimic acid pathway, the (R)-pantothenate biosynthetic pathway or transporter, a functional folate production pathway, polyamine synthesis pathway, glucose-6-phosphate isomerase necessary in Embden–Meyerhof glycolytic pathway, a cytochrome d type ubiquinol oxidase for aerobic respiration, 5 ABC transporters (*niaP*, *ftsEX*, *fhuBCD*, *oppABCD*, and *raxB*), the twin arginine transport system, 4 two-component signal transduction systems, multi-copper oxidase, zinc uptake regulation (ZUR) protein, the stringent response, LpxL protein involved in lipid A biosynthesis, several peptidoglycan recycling proteins, indole-3-acetamide production pathway. Alternatively, *L. asiaticus* encodes 107 potentially secreted proteins, 47 more than *L. crescens*, which may act as potential effectors as well as an ATP/ADP transporter [Fagen et al., 2014].

To verify the involvement of these genes in the culturability of *L. crescens*, Tn5 transposon mutagenesis was utilized to produce an insertion library. The genes that could not foster a Tn5 insertion were considered to be essential for growth in culture, which accounted for 314 protein-coding genes [Lai et al., 2016]. The results of the mutagenesis are consistent with those of Fagen et al.

[2014], and the authors suggest targeting ABC transporters and lipopolysaccharide biosynthesis genes for the development of antimicrobial treatments [Lai et al., 2016].

Transcriptomics

The gene expression pattern of *L. asiaticus* in both of its hosts, the citrus plant and the psyllid vector, was compared via qRT-PCR to identify genes essential for adaptation within the different hosts [Yan et al., 2013]. Of the 381 genes analyzed, 182 were upregulated in planta versus in psyllid, 16 genes were upregulated in psyllid versus in planta, and 183 were not statistically different. Based on these findings, gene expression related to “transcriptional regulation, transport system, secretion system, flagella assembly, metabolic pathway, and stress resistance” were found to change relative to the host [Yan et al., 2013]. There were also a large number of genes encoding hypothetical proteins that were significantly altered with the host.

Proteomics

To ascertain some information regarding the mechanisms of pathogenesis of *L. asiaticus*, every predicted protein was analyzed by computational methods fol-

lowed by manual curation of subcellular localization, spatial structure, and function [Cong et al., 2012]. All of the data from these analyses were compiled as a website (http://prodata.swmed.edu/liberibacter_asiaticus/) with a page for each protein. The following information is listed for each protein: basic information from existing annotations; prediction of local sequence properties such as secondary structure, signal peptides, transmembrane helices, etc.; close homologs and phylogenetic distribution; homologous protein families and conserved domains; homologous structures or homology modeling [Cong et al., 2012].

Transcriptional Regulators

As a result of significant genome reduction in *L. asiaticus*, only 2% of the genome (11 genes) encodes the transcription factors that regulate all gene expressions [Duan et al., 2009; Gardner et al., 2016; Moran, 2002; Pagliai et al., 2014]. Consequently, the identification of high affinity chemical inhibitors targeting a single transcription factor could result in pleiotropic effects, greatly hindering bacterial adaptation and survival [Pagliai et al., 2014]. *L. asiaticus* acts as an intracellular plant pathogen as well as an insect symbiont and, therefore, must have the ability to significantly alter gene expression in a host-specific manner to adapt to two very distinct environments [Duan et al., 2009; Yan et al., 2013]. As the bacterium senses environmental changes during the transition from psyllid to citrus host, regulation is altered to activate genes involved in survival and pathogenicity. Among the affected genes, 6 transcription factors are upregulated in citrus, including the 2 transcription factors that have been functionally characterized via biochemical techniques, LdtR and PrbP [Gardner et al., 2016; Pagliai et al., 2014, 2015, 2017; Yan et al., 2013].

PrbP belongs to the CarD_CdnL_TRCF superfamily; it is a transcriptional activator that has a suggested role in stabilizing the RNA polymerase (RNAP)/open promoter complex formation at some rRNA gene promoters [Gardner et al., 2016; Srivastava et al., 2013; Stallings et al., 2009; Stallings and Glickman, 2011]. This is accomplished through direct interactions between PrbP and the β -subunit of RNAP and a recognition sequence in the promoter region DNA [Gardner et al., 2016]. The transcription of rRNA is a rate-limiting step in bacterial growth, and the formation of the RNAP/open promoter complex is tightly regulated [Bartlett et al., 2000;

Häkkinen et al., 2013; Jin et al., 2012; Zhou and Jin, 1998]. Small molecules targeting PrbP disrupt the open complex formation, decreasing the transcription and reducing viability of *L. asiaticus* in the citrus plant [Gardner et al., 2016]. A functional in vitro screening identified tolfenamic acid (TA) as a PrbP ligand that disrupted PrbP-RNAP and PrbP-DNA interactions. PrbP binding pocket was extensively characterized and the amino acids in the active pocket were precisely described after site-directed mutagenesis analysis [Pan et al., 2017]. A direct consequence of PrbP inhibition was the arrest of *L. crescens* growth. Furthermore, TA inhibited *L. asiaticus* in infected citrus seedlings [Gardner et al., 2016]. TA was applied as a root soak and foliar spray to HLB-infected seedlings 2 times over a 2-week period, and the condition of the plants was monitored over an 11-month period. The results of this treatment study were very promising with rapid recovery of the root system, slower but significant recovery of the canopy, and an 80–95% drop in *L. asiaticus* titer level in 75% of plants. In field application of TA is currently under evaluation. The authors suggest that a combined application method and/or combined treatment with TA and heat may be complementary treatments to treat highly infected plants with exceptional vascular damage [Gardner et al., 2016].

The MarR family transcriptional regulator, LdtR, was shown to mediate osmotic stress, such as that encountered in the phloem [Pagliai et al., 2014]. Insertional mutation of the homologous gene in *Sinorhizobium meliloti* resulted in morphological changes and increased sensitivity to osmotic stress [Pagliai et al., 2014]. Small molecules that interact with LdtR were identified that caused a phenotype in *S. meliloti* and *L. crescens* similar to the insertional mutants [Pagliai et al., 2014]. These small molecules were then tested in a citrus shoot assay and shown to decrease the expression of LdtR and a gene regulated by LdtR potentially involved in cell wall biosynthesis. The binding pocket for one of these small molecules, benzobromarone, was in silico identified. The prediction was verified by site-directed mutagenesis, electrophoretic mobility shift assay, thermal denaturation, isothermal titration calorimetry, and in vivo assays [Pagliai et al., 2015]. LdtR DNA binding sequence was precisely determined; it was found that LdtR controls the expression of nearly 180 genes. This detailed information regarding the binding mechanism will allow for the development of antimicrobials for LdtR and other MarR family members [Pagliai et al., 2017].

Transport Proteins

L. asiaticus genome encodes 137 proteins classified as transporter proteins, an exceptionally high number compared to other intracellular bacteria with a similar genome size [Duan et al., 2009]. These transporters may be involved in pathogenicity, host range, and symptom elicitation [Duan et al., 2009]. The following transport proteins from *L. asiaticus* have been functionally characterized: NttA, ATP/ADP translocase; ZnuABC, zinc uptake system.

ATP Translocase (NttA)

NttA was predicted to contain 12 transmembrane helices and have a molecular mass and pI consistent with other known ATP/ADP translocases. The expression of NttA in *Escherichia coli* confirmed the function of this protein, exhibiting the uptake of ATP from the medium over time [Vahling et al., 2010]. This translocase is specific for ATP/ADP, however, high concentrations of other nucleotides may be transported or block ATP import. A protein with high homology to NttA was found in *L. solanacearum* based on protein sequence [Vahling et al., 2010]. A recent publication enhances the importance of this translocase as a potential pathogenicity factor. Citrus greening-infected trees accumulate high concentration of ATP in the leaf as a consequence of an unusual upregulation of ATP synthases [Pitino et al., 2017].

Znu System

The zinc uptake (Znu) system imports zinc for use as a cofactor or structural element of many proteins. The ZnuA protein binds zinc in the periplasm and ZnuBC transports it into the cell. In *L. asiaticus*, there are 2 gene clusters homologous to ZnuABC; however, only one of the clusters is capable of complementing the function of a partially inactivated Znu system in *E. coli* or *S. meliloti* [Vahling-Armstrong et al., 2012]. The regulator of the Znu system is the Zur protein in *E. coli* and *S. meliloti*, but a gene encoding homologous to this protein does not exist in *L. asiaticus* indicating that an alternative mode of regulation may exist [Vahling-Armstrong et al., 2012].

ZnuA1 is clearly indicated in the uptake of zinc, due to the presence of conserved coordinating residues, but this protein only shares 22% sequence identity with ZnuA2 [Sharma et al., 2015]. The highest identity, from sequence analysis of ZnuA2, is with cluster A-I family Mn/Fe-specific solute binding proteins (SBPs); however, the crystal structure depicting the mechanism of metal binding more closely resembles the cluster A-I family Zn-specific SBPs

[Sharma et al., 2015]. Biophysical characterization of ZnuA2 through surface plasmon resonance and circular dichroism revealed that this protein exhibits a low metal-binding affinity [Sharma et al., 2016]. The crystal structure of ZnuA2 bound to zinc was determined. From both these structures, it was shown that ZnuA2 binds Zn^{2+} and Mn^{2+} pentavalently with square pyramidal geometry, unlike the usual tetrahedral geometry seen elsewhere [Sharma et al., 2016]. It was hypothesized that this protein evolved to bind Mn^{2+} and reversibly bind Zn^{2+} , avoiding zinc toxicity but also allowing for the transport of Mn^{2+} [Sharma et al., 2016].

Secretion Systems and Effector Proteins

Effector proteins are virulence determinants secreted by the bacteria to alter host processes and thereby improve survival/pathogenicity. Due to the absence of most secretion systems in *L. asiaticus*, it is believed that the Sec secretion pathway, or Sec-translocon, is responsible for the export of these effector proteins outside the cytoplasm [Prasad et al., 2016], and another system should be necessary to cross the outer membrane. Prediction software was employed to analyze *L. asiaticus* strain psy62 proteins for the presence of a signal peptide, resulting in the identification of 166 proteins. In total, 151 of these proteins were shared among the 3 strains of *L. asiaticus*. Confirmation of functional signal peptides was analyzed by alkaline phosphatase assays in *E. coli*, for which in-frame gene fusions of *phoA* lacking a signal peptide linked to the *L. asiaticus* gene of interest were produced and phosphatase activity observed chromogenically. Of the 166 proteins initially identified, 86 proteins tested positive for a signal peptide in the phosphatase assay [Prasad et al., 2016].

Autotransporters (LasA_I/LasA_{II})

Autotransporters are large, multi-domain proteins consisting of a Sec-dependent signal peptide, a secreted passenger domain, and a translocator domain. The signal peptide directs the pre-protein to the periplasm via the Sec pathway, the translocator domain forms a pore in the outer membrane, and the passenger domain is cleaved and exported through the pore [Hao et al., 2013]. LasA_I and LasA_{II} from *L. asiaticus* were predicted autotransporters due to sequence analysis of architectural features, despite the lack of a signal peptide and no sequence homology at the amino acid level [Hao et al., 2013]. GFP-fusions with LasA_I C-terminus or full-length LasA_{II} revealed polar localization. LasA_I was found to localize to

the cell surface where the passenger domain is cleaved and transported out of the cell [Hao et al., 2013]. Tobacco transiently expressing a LasA_I translocator domain-GFP fusion was shown to target the leaf mitochondria, and leaves infiltrated with LasA_I or LasA_{II} showed altered morphology of the mitochondria and chloroplasts. This suggests that these autotransporters may affect the mitochondria and chloroplast function in *L. asiaticus*-infected plants [Hao et al., 2013].

ATPase (SecA)

The general secretory (Sec) pathway translocates unfolded proteins across the cytoplasmic membrane, leading to either a secreted protein or a protein integrated into the membrane [Akula et al., 2011, 2012; Hu et al., 2016; Manting and Driessen, 2000]. The SecA protein, which exhibits ATPase activity, is the subunit of this system that drives the protein translocation [Van den Berg et al., 2004; Economou and Wickner, 1994]. Blocking the activity of SecA, the driving force for this pathway, would inhibit protein translocation and have a significant effect on bacterial survival. The X-ray crystal structure of SecA from *E. coli* was used for initial homology modeling, and a virtual screening of chemicals was completed by molecular docking to identify 20 structures for biological assays [Akula et al., 2011, 2012; Papanikolaou et al., 2007]. The effectiveness of these chemicals as inhibitors of SecA activity was assessed by measuring the ATPase activity of purified SecA protein from *L. asiaticus* with the addition of each chemical. The 5 most effective compounds from the in vitro assay were tested for antimicrobial activity against *Agrobacterium tumefaciens* and were determined to be suitable candidates [Akula et al., 2012]. From these five compounds, a new similarity search was conducted to find 11 compounds for testing [Dean, 1994; Hu et al., 2016; Willett et al., 1998]. Since these compounds have poor aqueous solubility, a micro-emulsion was developed for application [Hu et al., 2016]. The minimum inhibitory concentration and minimum bactericidal concentration of the inhibitors was assessed with 8 bacteria (including *L. crescens*, *A. tumefaciens*, and *Bradyrhizobium japonicum*). The results obtained were similar to those described for streptomycin. The result of this study was the identification of potent SecA inhibitors as well as a microemulsion formula for improved application [Hu et al., 2016].

Signal Peptidase I (LepB)

The type I signal peptidase from *L. asiaticus*, CLIBASIA_04190 or LepB, cleaves the signal peptide from proteins transported via the Sec translocon. Despite only 34%

identity with the homolog in *E. coli*, the *lepB* gene from *L. asiaticus* partially complemented the *lepB* mutant of *E. coli* [Prasad et al., 2016].

Plant Immune Response Modulators (Antioxidants)

Peroxidase (SC2_gp095)

Ectopic expression of SC2_gp095 in *L. crescens* BT-1 resulted in smaller zones of inhibition around disks soaked with H₂O₂ compared to a control, illustrating enhanced peroxide degradation activity [Jain et al., 2015]. This hydrogen peroxide degradation activity was confirmed via a spectrophotometric activity assay. This activity could not be observed in transformed *E. coli*. The SC2_gp095 protein was demonstrated to be secreted by transformed *L. crescens* but not *E. coli*, suggesting that the non-classical secretion mechanism may be species-specific [Jain et al., 2015]. Lastly, expression of SC2_gp095 enhanced the growth of *L. crescens* in liquid culture and transient expression in tobacco detoxified H₂O₂ [Jain et al., 2015]. This protein may function in the detoxification of H₂O₂ during the initial stages of *L. asiaticus* infection, playing a critical role in colonization.

Peroxiredoxin (BCP)

Peroxiredoxins (Prxs) are thiol-specific antioxidants produced by bacteria to detoxify reactive oxygen species (ROS). The Prxs family can be subdivided into 1-Cys or 2-Cys depending on the presence of only the peroxidatic cysteine residues or both the peroxidatic and resolving cysteine residues [Wood et al., 2003]. *L. asiaticus* encodes a bacterioferritin comigratory protein (BCP), which is a 1-Cys Prxs lacking the resolving Cys [Singh et al., 2017]. Purified BCP from *L. asiaticus* was shown to catalyze peroxide-dependent DTT oxidation with various substrates, protect cells from H₂O₂-mediated apoptosis, and act as an antioxidant by scavenging ROS [Singh et al., 2017].

Salicylic Acid Hydroxylase (SahA)

A recently characterized effector protein in *L. asiaticus*, known as CLIBASIA_00255 or SahA, is a salicylic acid (SA) hydroxylase that degrades SA produced by the plant's immune response and thus abolishes plant defenses [Li et al., 2017]. The *SahA* gene was shown to be up-regulated in citrus compared to the psyllid host. The functional activity of SahA was confirmed by measuring the rate of NADH oxidation as well as degradation of SA by recombinantly purified SahA [Li et al., 2017]. Further,

the effect of SahA was studied during the infection of tobacco with the non-host pathogen *Xanthomonas citri* subsp. *citri* A306 (p53hrpX). The overexpression of SahA in tobacco suppresses the plant hypersensitive response and abolishes SA accumulation. Also, in citrus plants, the infection of *L. asiaticus* attenuates the plant responses to exogenous SA compared with the response in healthy plants [Li et al., 2017]. Future studies must be completed to identify effector proteins in *L. asiaticus*, as these are likely involved in virulence of this pathogen.

Prophage Late Gene Products

L. asiaticus carries 2 similar prophages, named SC1 and SC2 in strain UF506, with little evidence of a lytic phase in infected citrus and no evidence in psyllids [Fleites et al., 2014; Zhang et al., 2011]. SC2 does not appear to encode lytic cycle genes and appears to encode lysogenic conversion genes that may encode virulence factors that can be exploited by *L. asiaticus* to increase pathogenicity [Fleites et al., 2014; Zhang et al., 2011]. Conversely, SC1 does encode lytic cycle genes, and phage particles have been observed in *L. asiaticus*-infected periwinkle. Further investigation into the mechanisms involved in the induction of the lytic cycle is necessary to determine the feasibility of using this as a management strategy in infected citrus [Fleites et al., 2014].

The SC1 prophage encodes a holin (SC1_gp110) and an endolysin (SC1_gp035) that are expressed upon lytic cycle induction to allow for mature phage release [Fleites et al., 2014]. Holins aggregate in the bacterial inner membrane to form holes through which the endolysin accesses the bacterial cell wall and degrades the murein layer [Fleites et al., 2014]. Expression of these proteins in *E. coli* was toxic, confirming the annotated functions.

Transcription at the holin gene promoter appears to be regulated by an activator in *L. asiaticus* and *L. crescens*. Potentially, a repressor present in psyllids and citrus cell-free extracts may negatively regulate the expression of this gene [Fleites et al., 2014]. This is supported by the lack of holin promoter-driven reporter gene expression in *E. coli* versus constitutive promoter activity in *L. crescens*. This was further supported by a strong inhibition of holin promoter-driven reporter gene expression in *L. crescens* treated with crude psyllid or plant extracts [Fleites et al., 2014]. The expression of this holin in *L. asiaticus* upon separation from the host may be a contributing factor behind the inability to culture this bacterium.

Other Proteins

Flagellin (Fla)

The genome of *L. asiaticus* encodes a complete set, 30 genes, of flagella biosynthesis genes. The *fla* gene encodes a flagellin domain-containing protein that possesses a 22 amino acid highly conserved flagellin domain, flg22, at the N-terminus, and a C-terminal helical region [Zou et al., 2012]. Callose deposition and obliteration of vascular sieves was described as a central characteristic in HLB-infected plants [Kim et al., 2009]. The *fla* gen from *L. asiaticus* was shown to partially complement a *S. meliloti* mutant lacking *fla* genes [Koh et al., 2012]. The transient expression of FlaLas in tobacco induced cell death, callose deposition, a defense reaction which was measured via the upregulation of plant innate immunity proteins, BAK1/SGT1 [Zou et al., 2012]. In contrast, expression of a synthetic flg22 peptide in tobacco induces callose deposition [Zou et al., 2012] but does not result in cell death as it does when produced by other pathogens like *Pseudomonas* and *X. citri* subsp. *citri* [Felix et al. 1999; Chinchilla et al., 2007]. An understanding of the formation and regulation of flagellum may be essential for fully dissecting the host-pathogen interaction in HLB.

Enoyl-Acyl Carrier Protein Reductase I (FabI)

Fatty acids are essential for bacterial viability. Bacterial fatty acid biosynthesis is catalyzed by a type II fatty acid synthase and the enoyl-acyl carrier protein (ACP) reductase I, or FabI. These enzymes are critical in the catalysis of the final step of this pathway [Jiang et al., 2014]. The crystal structure of FabI from *L. asiaticus* was determined in both the apo form and in complex with NAD⁺. The main differences between other FabI structures and the FabI of *L. asiaticus* is the oligomeric state (tetramer vs. hexamer, respectively) and the substrate-binding loop (disordered vs. ordered, respectively) [Jiang et al., 2014]. From this investigation, isoniazid was identified as a competitive inhibitor with NADH. This work provides the framework for the development of inhibitors for FabI [Jiang et al., 2014].

LotP

Analysis of the transcriptome data generated by Yan et al. [2013] revealed that a large number of genes that were significantly induced in planta encoded hypothetical proteins, including the 3 genes displaying the largest induction (>7-fold) [Loto et al., 2017; Yan et al., 2013]. One of these genes, encoding CLIBASIA_03135 (re-named LotP), was recently biochemically characterized

to determine its biological significance. In silico analysis of LotP revealed that the sequence is highly conserved among the Rhizobiaceae family (56–86% identity), and it is vastly distributed in α -proteobacteria (35% plant-associated bacteria, 1/2 of these are plant pathogens) [Loto et al., 2017]. This protein was previously misannotated as an LA1 aminopeptidase, but structural analysis suggested that LotP actually belongs to a different, uncharacterized family of proteins. Co-immunoprecipitation assays identified GroEL as the main interacting protein, and a physical interaction between GroEL and LotP was confirmed via two-hybrid assay [Loto et al., 2017]. LotP is predicted to be critical to the pathogenesis in planta through this GroEL interaction and may have a role in masking GroEL from the plant immune defense system. GroEL is very important in many insect-vector pathosystems, and it is hypothesized that LotP participates in GroEL moonlighting activity to enhance the pathogenicity of *L. asiaticus*; however, further investigation is necessary to unveil the precise role of LotP in the HLB-citrus pathosystem [Loto et al., 2017].

Conclusions

The first case of citrus greening disease in Florida was officially recognized in 2005 (Miami Dade County), by the end of 2008 the disease was fully disseminated in the whole peninsula. Immediately after this massive *L. asiaticus* irruption, significant amounts of economic and human resources were redirected to minimize the potential agricultural, economic, and social consequences of this disease. Most of the studies employing molecular techniques thus far completed have focused on the citrus host or psyllid vector rather than the causative pathogen. This review summarized the available literature describing the biology of *L. asiaticus* that was generated through the use of classical molecular and genetic assay methods. Overall, the results obtained after a decade of research offer no answers to the citrus industry. The future of the Florida citrus industry, and probably the fate of citrus production worldwide, largely depend on a prompt delivery of new and effective biotechnological strategies to control *L. asiaticus* epidemic.

A detailed analysis of the relevant scientific literature indicates that the fundamental research was mainly committed to sequence *L. asiaticus* genome, propose early detection methods, and perform in silico analysis of *L. asiaticus* genome. Still, since the *L. asiaticus* genome was published, 36% of the encoding genes remains uncharac-

terized, and 26% of them are not classified in the COG database. These approaches, although important, largely relegates the scientific efforts in the field of functional biology. Two important factors contributed to the low volume of scientific publications directed to understand the basic biology of *L. asiaticus*: the inability to obtain axenic *L. asiaticus* cultures associated with the high complexity of the bacteria–host interaction. These elementary methodological difficulties have made the scientific advance tediously slow. Thus, there is an exceptional void in the knowledge base, reflecting demonstrated molecular mechanisms of pathogenesis from the perspective of the *L. asiaticus* bacterium that must be filled to develop targeted antimicrobial treatments. The citrus industry is on the precipice of obliteration. A successful, sustainable treatment method for HLB remains elusive; yet, new *Liberibacter* species and subspecies continues to emerge [Morris et al., 2017; Roberts et al., 2015; Roberts and Pietersen, 2017; Wu et al., 2015; Zheng et al., 2017]. A variety of important crops are infected by this bacteria genus, the *Solanaceae* family is probably the most representative and economically significant [Hawkes, 1999]. All pathogenic *Liberibacter* species are not cultivable, the innocuous *L. crescens* is the only member of the group used as reference. It has become critical that we employ the most effective means available for the determination of efficient therapeutics and for understanding the modes of persistence of *Liberibacter*, to protect significant food resources affected by species of this genus.

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Disclosure Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

J.F.C., C.F.G., and G.L.L. conceived, wrote, and coordinated the review article. All authors revised and approved the final version of the manuscript.

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