Progress and Obstacles in Culturing ‘*Candidatus Liberibacter asiaticus*’, the Bacterium Associated with Huanglongbing

Marcus V. Merfà,¹ Edel Pérez-López,¹ Eber Naranjo,¹ Mukesh Jain,² Dean W. Gabriel,² and Leonardo De La Fuente^{1,†}

¹ Department of Entomology and Plant Pathology, Auburn University, Auburn, AL 36849, U.S.A.

² Department of Plant Pathology, University of Florida, Gainesville, FL 32611, U.S.A.

Accepted for publication 12 April 2019.

ABSTRACT

In recent decades, ‘*Candidatus Liberibacter* spp.’ have emerged as a versatile group of psyllid-vectored plant pathogens and endophytes capable of infecting a wide range of economically important plant hosts. The most notable example is ‘*Candidatus Liberibacter asiaticus*’ (CLAs) associated with Huanglongbing (HLB) in several major citrus-producing areas of the world. CLAs is a phloem-limited α -proteobacterium that is primarily vectored and transmitted among citrus species by the Asian citrus psyllid (ACP) *Diaphorina citri*. HLB was first detected in North America in Florida (USA) in 2005, following introduction of the ACP to the State in 1998. HLB rapidly spread to all citrus growing regions of Florida within three years, with severe economic consequences to growers and considerable expense to taxpayers of the state and nation. Inability to establish CLAs in culture (except transiently) remains a significant scientific challenge toward effective HLB management. Lack of axenic cultures has restricted functional genomic analyses, transfer of CLAs to either insect or plant hosts for fulfillment of Koch’s postulates, characterization of host-pathogen interactions and effective screening of antibacterial compounds. In the last decade, substantial progress has been made toward CLAs culturing: (i) three reports of transient CLAs cultures were published, (ii) a new species of *Liberibacter* was identified and axenically cultured from diseased mountain papaya (*Liberibacter crescens* strain BT-1), (iii) psyllid hemolymph and citrus phloem sap were biochemically characterized, (iv) CLAs phages were identified and lytic genes possibly affecting CLAs growth were described, and (v) genomic sequences of 15 CLAs strains were made available. In addition, development of *L. crescens* as a surrogate host for functional analyses of CLAs genes, has provided valuable insights into CLAs pathogenesis and its physiological dependence on the host cell. In this review we summarize the conclusions from these important studies.

Keywords: Asian citrus psyllid, bacteriology, ‘*Candidatus Liberibacter asiaticus*’, citrus greening, huanglongbing, *Liberibacter crescens*, plant pathogen, unculturable bacteria

‘*Candidatus Liberibacter asiaticus*’ (CLAs), ‘*Ca. Liberibacter americanus*’ (CLAm), and ‘*Ca. Liberibacter africanus*’ (CLAf) are Gram-negative bacteria in the α subdivision of Proteobacteria (Jagoueix et al. 1994). These three ‘*Ca. Liberibacter* spp.’ have been associated with Citrus Huanglongbing (HLB), a devastating disease considered one of the most damaging plant diseases worldwide, causing substantial economic losses in Asia,

Africa, Oceania, and the Americas (Bové 2014). CLAs is widespread in all continents affected by HLB, including the Americas with reports in United States, Mexico, Brazil, and Cuba; Africa with reports in Ethiopia, and Reunion Island; and Oceania (Wang et al. 2017).

The term ‘*Candidatus*’ is used to record putative prokaryotic taxa, where the identifying information is obtained from genomic sequencing of natural samples and supported by observation of cells. However, traditional taxonomic description of such organisms in pure culture is still missing (Murray and Stackebrandt 1995). Besides the taxonomical problem, the lack of critical molecular and physiological information of ‘*Candidatus*’ organisms hamper the understanding of the biological interactions, and subsequent development of control strategies for pathogens like CLAs (Stewart 2012).

Despite very limited success toward growing CLAs in vitro (Davis et al. 2008; Parker et al. 2014; Sechler et al. 2009), not one of the pathogenic *Liberibacter*s has been maintained in continuous axenic culture to date. The aim of this review is to critically summarize the advances in CLAs culturing and integrate recent molecular and

[†]Corresponding author: L. De La Fuente; lzd0005@auburn.edu

Current address of Edel Pérez-López: Department of Biology, University of Saskatchewan, Saskatoon SK S7N 5E2, Canada

Funding: Financial support was provided by Agriculture and Food Research Initiative competitive grant from the USDA National Institute of Food and Agriculture, Citrus Disease Research and Extension (2016-70016-24844 and 2015-70016-23010); and HATCH AAES (Alabama Agricultural Experiment Station) program provided to L. De La Fuente.

The author(s) declare no conflict of interest.

Modified: 19 Sep 2019.

© 2019 The American Phytopathological Society

chemical information that provides insight into the requirements for free-living growth of Liberibacters in culture. We have compared and consolidated information from completely sequenced genomes of CLAs (Duan et al. 2009; Katoh et al. 2014), CLam (Wulff et al. 2014), CLaf (Lin et al. 2015), and the cultured but not evidently pathogenic *Liberibacter crescens* genome (Leonard et al. 2012). Also included are plant host and insect vector metabolomic analyses (Killiny 2016, 2017; Killiny et al. 2017b), and recent discoveries using *L. crescens* as a surrogate (Jain et al. 2017a, b) to identify traits that may be hampering CLAs culturing. The ability of culturing CLAs (and other 'Ca. Liberibacter spp.') will be fundamental help to (i) fulfill Koch's postulates; (ii) efficiently assess virulence across different CLAs genotypes; (iii) screen efficiently antibacterial compounds; (iv) conduct functional studies to understand the basis of CLAs pathogenicity and insect transmission; and (v) use this information to allow development of novel control methods based on the molecular pathogen–plant–insect interactions.

VASCULATURE-RESTRICTED CITRUS PATHOGENS SUCCESSFULLY CULTURED

Several fastidious bacteria besides CLAs have been associated with citrus diseases (Davis and Brlansky 2007). The phloem-inhabiting *Spiroplasma citri*, which is a citrus plant pathogen associated with citrus stubborn disease, was the first phloem-limited fastidious prokaryote isolated in axenic culture (Saglio et al. 1971). The second fastidious prokaryote isolated in axenic culture was the xylem-limited *Xylella fastidiosa*, which is the causal agent of Pierce's disease of grapevines (Davis et al. 1978). *X. fastidiosa* subsp. *pauca*, which infects citrus was cultured several years later (Chang et al. 1993). Successful culturing of these vasculature-limited,

fastidious plant pathogens relied on a nutritionally complex culture medium containing undefined components (Davis et al. 1978). The highly restricted nutritive value of xylem sap and significantly larger genome size of *X. fastidiosa* (2.5 Mb) (Table 1) may be contrasted to the nutritional environment of CLAs, given that phloem is physiologically and nutritionally more complex than xylem. Pathogenic Liberibacters in general have undergone reductive genome evolution with an average genome size of 1.2 Mb, but *S. citri* has a small genome (1.6 Mb) as well (Table 1), and nevertheless it has been cultured in vitro.

CLas TRANSIENT CULTURES

CLas has not yet been established in culture except transiently. In the past decade, three main studies (Davis et al. 2008; Parker et al. 2014; Sechler et al. 2009) have contributed to the progress toward the goal of culturing CLAs. Davis et al. (2008) reported that cocultivation of CLAs was possible in the presence of another accidentally contaminant bacterium, *Propionibacterium acnes*. Although CLAs/*P. acnes* survived multiple passages in vitro, CLAs was not able to grow in pure culture without *P. acnes*. However, confirmation of CLAs growth in vitro outside its plant or vector hosts remains a seminal contribution. The authors also suggested that through a mutualistic relationship with another bacterium, CLAs could obtain requisite nutrients and/or chemical signals required for its growth. A few years later, Parker et al. (2014) reported viable CLAs cultures in vitro for several weeks in a medium containing commercial grapefruit juice, also in the presence of other microflora found in grapefruit seeds. Moreover CLAs was found to remain viable for several months inside biofilms formed by other bacteria (Parker et al. 2014). However, in the absence of continuous CLAs

TABLE 1
Genome size and GC content of several vascular bacterial pathogens and insect symbionts

Strain ^a	Genome size (bp)	GC content (%)	GenBank Accession number
' <i>Candidatus</i> Liberibacter asiaticus'			
Psy62	1,227,204	36.5	NC_012985.3
Ishi-1	1,190,853	36.32	AP014595
'Ca. Liberibacter solanacearum'			
CLso-ZC1	1,258,278	35.24	NC_014774
LsoNZ1	1,312,416	35.32	JMTK01000000
'Ca. Liberibacter americanus'			
PW_SP	1,176,071	31	AOFG00000000
Sao Paulo	1,195,201	31.1	NC_022793.1
'Ca. Liberibacter africanus'			
PTSAPSY	1,192,232	34.5	NZ_CP004021.1
<i>Liberibacter crescens</i>			
BT-1	1,504,659	35.36	NC_019907.1
<i>Xylella fastidiosa</i>			
Temecula 1	2,519,802	51.8	NC_004556
<i>Spiroplasma citri</i>			
R8-A2	1,599,709	25.1	NZ_CP013197.1
' <i>Candidatus</i> Carsonella ruddii'			
NHV	159,923	15.6	NC_018418.1
Thao2000	162,504	14.2	NC_018415.1

^a Fastidious bacteria *Xylella fastidiosa* and *Spiroplasma citri* were initially identified as unculturable plant pathogens. '*Candidatus* Carsonella ruddii' is a primary *Diaphorina citri* symbiont. Whenever multiple genomes of the same species were available, only a representative genome in terms of size and GC content was included here.

growth, no bona fide independent “cultivation” was claimed. These results also contributed to the belief that CLAs are able to survive independently of either plant or insect hosts. The CLAs growth pattern oscillated over time, resembling cryptic growth and indicating depletion of specific nutritional or signaling components from the growth medium, causing partial loss of bacterial population, followed by revival of the persisting population using the content of dead cells as growth stimulators (Parker et al. 2014). In 2009, Sechler et al. (2009) reported that CLAs and CLam were grown in pure culture in vitro to the point where these strains “grown on Liber A medium were pathogenic on citrus”, thus “partially fulfilling Koch’s postulates”. A cursory reading of Sechler et al. (2009) would suggest that sustainable culturing had been achieved, but no such claim was made. The claim of partial fulfillment of Koch’s postulates was greeted with skepticism and is now regarded by consensus as unreproducible. No follow-up publications using this approach to culture CLAs are available. Very recently, Fujiwara et al. (2018) reported a coculture of CLAs strain Ishi-1 (that has no phages according to genomic data) in vitro associated with phloem-associated microbiota. The authors followed the population of CLAs by DNA quantification, and observed increase in DNA amounts over time, therefore suggesting growth; but no direct quantification of viability was provided. In agar plates, the authors could not see distinctive colonies, but a few cells were found after microscopic observations. In their manuscript the authors conclude that CLAs need other microflora for effective growth, based on suppression of certain “helper” bacteria by antibiotics. The authors conclude that CLAs are resistant to oxytetracycline and many other antibiotics, which contradicts previous reports (Zhang et al. 2014). Reproducibility of these findings by other research groups is needed.

AVAILABLE NUTRIENTS IN CLAs HOSTS

The CLAs genome encodes a large repertoire of transporter proteins (137 transporter proteins, including 40 ABC-type), enabling uptake and assimilation of all requisite nutrients from its extracellular environment, either from the host or culture medium (Wang and Trivedi 2013). In plants, CLAs are strictly intracellular and phloem-limited. Following insect acquisition via feeding, however, CLAs become extracellular, then enter, traverse, and exit the midgut epithelial cell layer and the basement membrane, emerging into the insect host hemolymph (Ghanim et al. 2017). CLAs colonize the psyllid host as a systemic, circulative and propagative endosymbiont, both inter- and intracellularly, across several organs *en route* to the salivary glands, which it penetrates to again become intracellular, prior to emergence into the salivary gland lumen, from which it can be secreted into plant hosts (Ammar et al. 2011). The capacity to cross multiple membrane barriers and survive both intra- and extracellularly suggests regulatory and mechanistic complexity and capacity to cope with multiple microenvironments. Nevertheless, CLAs have a highly reduced genome, lost multiple enzymes and entire biosynthetic pathways, as well as secretion systems from its genome (Duan et al. 2009). CLAs therefore apparently have a stringent metabolic dependence on phloem sieve tube, psyllid cell, and hemolymph contents to support its physiological and reproductive requirements (Killiny et al. 2017b).

In recent years, the chemical composition of psyllid hemolymph and the phloem sap of fourteen citrus varieties and periwinkle (*Catharanthus roseus*, an alternate laboratory host of CLAs) have been characterized in detail (Hijaz and Killiny 2014; Killiny 2016, 2017; Killiny et al. 2017b). Table 2 summarizes these results in comparison with Medium G, used for transient cultures of CLAs (Parker et al. 2014), since it is the only CLAs medium for which chemical characterization is available. A critical appraisal of these data are fundamental and crucial for identification of metabolic requirements for maintaining CLAs viability and sustained free

living growth in vitro. The major compounds found in these environments are discussed below (Fig. 1). Hypotheses on the ability of CLAs to utilize certain compounds is based mainly in genomic annotation, since in all cases functional data demonstrating ability to assimilate these compounds has not been tested, due to the limitation of nonavailable CLAs axenic cultures. Information was included below whenever functionality of certain genes was demonstrated in surrogate bacterial systems.

Sugars. Sugars are the most abundant energy-rich compounds found in the phloem sap of different citrus varieties and periwinkle, as well as in the hemolymph of Asian citrus psyllid (ACP). The main sugars present in these environments are glucose, fructose and sucrose (Hijaz and Killiny 2014; Killiny 2016, 2017; Killiny et al. 2017b). Bioinformatic analyses of the CLAs genome indicated that sugars such as glucose and fructose could be internalized via an ABC transporter (glucose/galactose MSF transporter) and utilized through glycolysis and downstream tricarboxylic acid (TCA) cycle (Duan et al. 2009; Wang and Trivedi 2013). It was hypothesized that in the absence of pyruvate biosynthetic enzymes, the majority of pyruvate production in CLAs could possibly be via glycolytic breakdown of glucose (Wang and Trivedi 2013). Glycolytic utilization of sugars in CLAs for production of pyruvate was, however, ruled out owing to the absence of phosphoglucose isomerase (PGI), the key rate limiting enzyme of glycolysis (Jain et al. 2017b). Further supporting the lack of glycolysis is the fact that CLAs, unlike *L. crescens*, lack a functional glyoxalase system, required for detoxifying methylglyoxal, a highly cytotoxic, nonenzymatic byproduct of glycolysis (Jain et al. 2017b). Pyruvate and other organic acids are abundantly available in the phloem sap and hemolymph environments; therefore, it is likely that CLAs can readily absorb and assimilate pyruvic acid and other organic acids as primary carbon source. CLAs were also found to be deficient in a sucrose transporter gene (*sut*), which was functionally characterized to be present only in the culturable *L. crescens* (Jain et al. 2017b).

Some sugar derivatives are also abundantly present in both CLAs host environments assessed, including sugar alcohols such as myo-inositol, scyllo-inositol, and chiro-inositol (Hijaz and Killiny 2014; Killiny 2016, 2017; Killiny et al. 2017b). Killiny and coworkers hypothesized that the high sugar alcohol content and multiple isomeric forms of these compounds in phloem sap and hemolymph is indicative of an important role in the survival and/or pathogenicity of CLAs. Nevertheless, there is still no evidence that these sugar alcohols can be used by CLAs as a carbon source, and there is no direct correlation between the sugar alcohol levels and relative susceptibility of citrus varieties toward HLB (Killiny 2017).

Organic acids. Organic acids are abundant in citrus and periwinkle phloem sap as well as in ACP hemolymph, represented by mono-, di- and tricarboxylic acids. The main organic acids present in both these environments are lactic, glycolic, pyruvic, fumaric, succinic, malic, citric, isocitric and 2-ketoglutaric acids (Killiny 2016, 2017; Killiny et al. 2017b). *L. crescens* can utilize 2-ketoglutarate as the primary carbon source (Fagen et al. 2014a, b). CLAs genome encodes the necessary enzymes to use malate, fumarate and succinate (Wang and Trivedi 2013). Many of these organic acids are intermediates in the TCA cycle and could be directly used by CLAs to produce energy (Fernie et al. 2004). The presence of a malate hydrogenase in the genome of CLAs should allow the oxidation of malate to oxaloacetate, thus feeding the TCA cycle. CLAs lack isocitrate lyase and malate synthase enzymes in its genome, indicating that CLAs may acquire these compounds exogenously from its extracellular environment. The presence of a C4 dicarboxylate transport protein in the CLAs genome also supports this prediction (Wang and Trivedi 2013).

As mentioned above, CLAs seem to lack glycolysis and could possibly import and utilize extracellular pyruvic acid as the entry point into an intact TCA cycle, since all the TCA cycle enzymes are

present in all *Liberibacters*, providing the reducing equivalents for subsequent ATP synthesis. Jain et al. (2017b), however, argued that noncyclic or shunted parts of the TCA pathway are more likely to function in CLAs, consistent with the abundant availability of ATP (Pitino et al. 2017) and TCA cycle intermediates in its extracellular environment. The cyclic flux of TCA cycle intermediates is

dependent upon high energy demand and several bacteria operate noncyclic variations in the TCA cycle in order to generate biosynthetic precursors for lipids and amino acids under anaerobic or microaerophilic growth conditions (Tian et al. 2005).

Amino acids. CLAs is able to use many proteinogenic amino acids as carbon and nitrogen sources, including alanine, glutamate,

TABLE 2
Main common compounds found in citrus and periwinkle phloem sap, psyllid hemolymph, and G medium (grapefruit juice), based on studies referenced in the main text^a

Compounds ^b	Periwinkle	Psyllid	Citrus	G medium ^c
Sugars^d				
Glucose	X	X	X	X
Fructose	X	X	X	X
Sucrose	X	X	X	X
Myo-inositol	X	X	X	X
Scyllo-inositol	X	X	X	NF
Chiro-inositol	X	X	X	NF
Organic acids				
2-Ketoglutaric acid	X	X	X	NF
Lactic acid	X	X	X	NF
Glycolic acid	X	X	X	NF
Fumaric acid	X	X	X	NF
Succinic acid	X	X	X	X
Malic acid	X	X	X	X
Citric acid	X	X	X	X
Iso-citric acid	X	X	X	X
GABA	X	X	X	X
Amino acids				
Leucine	X	X	X	X
Tryptophan	X	X	X	X
Isoleucine	X	X	X	X
Tyrosine	X	X	X	X
Valine	X	X	X	X
Histidine	X	X	X	NF
Asparagine	X	X	X	NF
Methionine	X	X	X	X
Alanine	X	X	X	X
Proline	X	X	X	X
Energy compounds				
ATP	NA	X	X	NA
ADP	NA	X	X	NA
AMP	NA	X	X	NA
GTP	NA	X	X	NA
GDP	NA	X	X	NA
NAD	NA	X	X	NA
NADP	NA	X	X	NA
FAD	NA	X	X	NA

^a X, compound identified; NF, not found; and NA, not analyzed.

^b These compounds were selected based on the characterizations made by Killiny (2016, 2017); Hijaz and Killiny (2014); Killiny et al. (2017a, b); Parker et al. (2014).

^c Medium G refers to grapefruit juice used and characterized by Parker et al. (2014).

^d Although these sugars are present in the environments where '*Candidatus Liberibacter asiaticus*' lives, it probably does not use them as carbon and energy sources, since it lacks key genes of the glycolysis pathway, a sucrose transporter, and a methylglyoxal detoxifying system.

aspartate, glycine, serine, methionine, cysteine, arginine, proline, histidine, threonine, tyrosine, phenylalanine, and tryptophan (Duan et al. 2009). CLAs has the ability to internalize amino acids through ABC-type transporters. It also encodes a number of general L-amino acid permease proteins to import a variety of amino acids, and also a branched-chain proton-glutamate transporter, which can import both glutamate and aspartate from the environment (Duan et al. 2009; Li et al. 2012; Wang and Trivedi 2013). CLAs is able to synthesize de novo only six of the 20 proteinogenic amino acids, namely, lysine, serine, glycine, glutamine, threonine, and arginine. Therefore, CLAs must overcome the lack of the remaining de novo amino acid biosynthesis by acquiring these from the surrounding environment. The phloem sap of sweet orange and the hemolymph of ACP have all the amino acids necessary to meet the growth and metabolic requirements of CLAs (Hijaz and Killiny 2014; Killiny et al. 2017b). Notably, a high proline-to-glycine ratio was positively correlated with high CLAs infectivity in plant hosts (Steamou et al. 2017). These authors also noted a positive association of arginine, gamma-aminobutyric acid (GABA) and proline with CLAs susceptibility in various hosts. Although the phloem sap of periwinkle appears to lack leucine, tryptophan, tyrosine, histidine and methionine, CLAs grows well in periwinkle (Killiny 2016). Since these amino acids cannot be synthesized de novo by CLAs, the source of these amino acids for CLAs growth in periwinkle is puzzling.

Some nonproteinogenic amino acids, specifically GABA, were present in all host environments supporting CLAs growth (Hijaz and

Killiny 2014; Killiny 2016, 2017; Killiny et al. 2017b). GABA is an important neurotransmitter in mammals, insects, and other organisms, and it also accumulates in plants in response to biotic and abiotic stresses (Ham et al. 2012). It has been proposed that CLAs indirectly upregulates the TCA cycle of the plant host via the GABA shunt to satisfy the increased demand for TCA cycle intermediates for supporting CLAs growth (Nehela and Killiny 2018). GABA was demonstrated to function environmentally as a cell-to-cell signal mediator by inhibiting the action of acyl-homoserine lactones (quorum sensing molecule) on *Agrobacterium tumefaciens* (Chevrot et al. 2006). If quorum sensing is important for CLAs growth and multiplication, as suggested by Yan et al. (2013), it may be important to avoid use of GABA in culture media.

Choline is an essential nutrient derived from serine and is required for biosynthesis of the osmoprotectant glycinebetaine in plants. Extracellular choline is abundant both in xylem as well as phloem exudates (Gout et al. 1990), is critical for phloem development and conductivity (Dettmer et al. 2014), and is important for some plant-pathogen infections (Chen et al. 2013). A phospholipid derivative, phosphatidylcholine (PC) represents almost one half of the total lipid content present in eukaryotic cell membranes. However, PC is present only in bacteria that live in close association with eukaryotic hosts, such as *A. tumefaciens*, *Sinorhizobium meliloti*, and *Pseudomonas aeruginosa* (Aktas et al. 2010). PC strongly affects the physicochemical properties of bacterial membranes, including fluidity, permeability and membrane potentials. PC-deficient phenotypes of these bacteria are

Microenvironments and conditions in nature

FIGURE 1

Summary of conditions for growth of 'Candidatus Liberibacter asiaticus' (CLAs) in nature in plant host and insect vectors. The environmental conditions in the phloem sieve tubes of plant hosts and insect vectors hemolymph are discussed in the manuscript, and should be taken into consideration for development of culture medium supporting axenic growth of CLAs. SUT, sucrose uptake transporter; NttA, ATP/ADP translocase; *gloA*, glyoxalase; *pgi*, phosphoglucosyltransferase; *lpxXL*, lipid A C28 acyltransferase; and *acpXL*, acyl carrier protein.

marked by temperature and sodium dodecyl sulfate (SDS) sensitivity, attenuated virulence, loss of Type 4 secretion, reduced growth, and motility and increased biofilm formation (Aktas et al. 2010). There are two pathways for PC biosynthesis. The enzyme phospholipid N-methyltransferase catalyzes de novo PC biosynthesis via three-fold sequential N-methylations of phosphatidylethanolamine using the methyl donor S-adenosylmethionine. Alternatively, nutritional choline can also be incorporated into the membrane via the single step enzymatic action of phosphatidylcholine synthase. While proteins for both these pathways exist in (cultured) *L. crescens* (WP_015272535.1 and WP_015272978.1, respectively), CLas lacks the ability to synthesize PC de novo. However, CLas encodes a predicted ABC transporter import system with high substrate specificity for choline (CLIBASIA_RS01090) (Li et al. 2012). CLas also encodes a phosphatidylcholine synthase (CLIBASIA_RS03145), indicating that CLas is capable of utilizing extracellular choline, present in both plant and insect hosts. Choline is likely to be a necessary component in CLas culture media.

Fatty acids. Fatty acids are also detected in low amounts in the phloem sap of periwinkle and citrus plants and in high amounts in the hemolymph of ACP (Hijaz and Killiny 2014; Killiny 2016, 2017; Killiny et al. 2017b). Analysis of the genome of CLas showed that this bacterium is not likely able to utilize fatty acids as a carbon source (Wang and Trivedi 2013). However, the recent successful axenic culture of the insect endosymbiont *Spiroplasma paulsonii* required lipid supplementation and was based on observations that the pathogen rapidly depleted specific host lipids (Masson et al. 2018), indicating that lipid supplementation of CLas culture media may require further investigation.

Nucleotides and cofactors. Nucleotides are also found in abundance in the phloem sap of sweet orange and hemolymph of ACP, and include all three adenine nucleotides (ATP, ADP, and AMP), two guanine nucleotides (GTP and GDP), two cytosine nucleotides (CDP and CTP), two uridine nucleotides (UDP and UTP), two nicotinamide nucleotides (NAD and NADP), and flavin nucleotide (FAD) (Hijaz et al. 2016; Killiny et al. 2017b). Because CLas encodes many ABC transport proteins in its genome (Li et al. 2012; Wang and Trivedi 2013), these exogenous sources of nucleotides are likely exploited by CLas as energy sources and as a source of cofactors (Hijaz et al. 2016). CLas lacks enzymes needed for the metabolism of purines and pyrimidines, which must therefore be acquired from the host (Hartung et al. 2011). However, genome analysis shows that CLas is presumably able to salvage nucleotides from the respective nucleoside diphosphates.

One significant and unusual feature found in the genome of CLas (and other pathogenic Liberibacters) is the presence of *nttA*, which has been demonstrated to encode an ATP/ADP translocase (Jain et al. 2017b; Vahling et al. 2010). Since CLas also encodes ATP synthase (Duan et al. 2009), it is likely capable of synthesizing its own ATP, as well as uptake ATP from its extracellular environment. ATP/ADP translocases are present in all plastids (Möhlmann et al. 1998) and are also encoded by only three other prokaryotes, all obligate and intracellular pathogens: *Chlamydia psittaci*, *Rickettsia prowazekii*, and *Lawsonia intracellularis*. ATP/ADP translocases catalyze the highly specific uptake of host ATP in exchange for the bacterial ADP (energy parasitism) (Schmitz-Esser et al. 2008).

CLas possibly has a limited ability to perform aerobic respiration, since it lacks homologs corresponding to the final stages of oxidative phosphorylation and many diverse terminal oxidases (Duan et al. 2009). In addition to the provision of nucleotides and cofactors, an exogenous supply of ATP in any axenic culture media is likely also important, since increased ATP levels are observed in both insect and plant hosts after CLas infection (Killiny et al. 2017a; Pitino et al. 2017). It has been suggested that some bacteria, including *Escherichia coli* and *Salmonella* spp., excrete ATP into their surroundings if their respiratory rates and ATP generation exceed their physiological requirements (Mempin et al. 2013).

Given that CLas has only been maintained transiently in cocultures with other bacteria (Davis et al. 2008; Fujiwara et al. 2018; Parker et al. 2014), it is possible that the cocultured bacteria may be sustaining the ATP requirement for CLas.

BEYOND NUTRIENTS: CONDITIONS TO CONSIDER FOR CLas CULTURE

pH. The significant levels of organic acids found in the phloem sap of both citrus and periwinkle plants renders these two environments slightly acidic (approximately pH 6.0 for citrus phloem sap and pH 5.85 for periwinkle phloem sap) (Killiny 2016; Hijaz and Killiny 2014). CLas has also been shown to grow inside acidic citrus fruits (Li et al. 2009), indicating that CLas growth may be supported in an acidic (or slightly acidic) environment. Medium G, which supports transient growth of CLas, is composed of 100% commercial grapefruit juice, and was used by Parker et al. (2014) to study the viability of CLas in vitro. It contained several sugars, organic acids and amino acids required for CLas growth and at an extremely acidic pH (close to 3.0).

Oxygen levels. CLas possibly has a limited ability to perform aerobic respiration due to the absence of the terminal oxidase complex (cytochrome bd), but possesses many enzymes with a role in nitrogen metabolism, including NAD⁺ synthase, glutamine synthetase, and glutaminase, indicating that CLas could rely on nitrogen to generate energy through anaerobic respiration (Duan et al. 2009). However, in a review that included new analyses of the genomes of CLas, Wang and Trivedi (2013) suggested that CLas does not perform anaerobic respiration because it lacks nitrate, sulfate, fumarate, and trimethylamine reductase systems. The authors argued that the absence of electron acceptors for an anaerobic respiratory chain, such as nitrate or nitrite reductase, prevents this type of respiration in CLas, considering that the enzymes found for nitrogen metabolism by Duan et al. (2009) are insufficient to define a respiratory chain (Wang and Trivedi 2013). This conclusion was supported by the fact that oxygen is present in the phloem of plants, even though at lower concentration than the surrounding atmosphere (Drew 1997). Taken together, the available information indicates that CLas is able to perform aerobic respiration, even under microaerophilic growth conditions. However, because it lacks genes for the biosynthesis of menaquinone and ubiquinone, CLas will need to acquire these compounds exogenously for a functional respiratory chain (Wang and Trivedi 2013).

GENETIC COMPONENTS

Comparative genomics and metabolic pathway analyses have revealed a trend for the reduction or complete absence of several biosynthetic pathways, metabolic enzymes, and secretion systems in the genomes of uncultured pathogenic Liberibacters. Genomic comparisons between cultured but likely nonpathogenic *L. crescens* (1.5 Mb) and CLas (1.2 Mb) have provided valuable clues about the genes missing from CLas that would cause growth problems, which may not be readily alleviated by simple media adjustments. As noted above, a functional glyoxalase pathway is absent in all pathogenic (and uncultured) Liberibacters, and present in *L. crescens* (Jain et al. 2017b). The two-enzyme glyoxalase system, consisting of glyoxalase I (GloA; lactoylglutathione lyase) and glyoxalase II (GloB; hydroxyacylglutathione hydrolase), provides the primary cellular defense against methylglyoxal-induced carbonyl stress and proteome glycation in both prokaryotes and eukaryotes. Methylglyoxal is a highly cytotoxic byproduct of glycolysis and is also produced via protein and lipid degradation. The CLas genome lacks *gloA*, and CLas circumvents a toxic buildup of cellular methylglyoxal pool by preventing sugar uptake and glycolytic utilization and instead scavenges ATP directly from the

host cell cytoplasm while infecting either plant or psyllid host (Jain et al. 2017b). An efficient mitigation of carbonyl stress would still be needed for methylglyoxal resulting from cellular metabolic processes in axenically cultured CLAs. This could be done by addition of specific methylglyoxal-binding compounds to the culture medium, to mitigate the effects of the methylglyoxal-induced carbonyl stress (Lv et al. 2011). An alternative strategy would be adding the *gloA* gene from *L. crescens* into CLAs. However, genetic modification protocols of CLAs have not been established to date.

As another example, biochemical analyses revealed the presence of a very long chain fatty acid (VLCFA)-modified lipid A in *L. crescens* LPS (Complex Carbohydrate Research Center, Athens, GA, unpublished data), a unique feature of several bacteria that form chronic intracellular infections within legumes and mammalian hosts. Homologs for both genes coding proteins involved in the biosynthesis of VLCFA-modified lipid A, *LpxXL* and *AcpXL*, are present in *L. crescens* (WP_015273388.1 and WP_015273393.1), but both are absent in all pathogenic (and uncultured) Liberibacters, including CLAs. Knockout mutations of various *L. crescens* genes have been readily obtained in the past (Jain et al. 2017a, b), but mutations affecting formation of VLCFA-modified lipid A in *L. crescens* have not been successful (M. Jain and D. Gabriel, unpublished data), leading to the speculation that VLCFA-modified lipid A may be essential for axenic growth of pathogenic Liberibacters. Introduction of *lpxXL* and *acpXL* from *L. crescens* into CLAs would aid in achieving this goal, if transformation protocols for CLAs are developed.

BACTERIOPHAGE GENES IN CLAs

CLAs strain UF506 carries an excision plasmid prophage, SC2, and a chromosomally integrated prophage, SC1 (Fleites et al. 2014; Zhang et al. 2011). SC1 becomes replicative in planta with expression of lytic genes (Fleites et al. 2014) and forms readily observed bacteriophage particles in periwinkle (Zhang et al. 2011) and citrus (Fu et al. 2015). By contrast, the replicative excision plasmid SC2 lacks lytic genes. SC1 and SC2 encode multiple virulence factors suggested to contribute to the fitness of CLAs in its hosts (Jain et al. 2015; Sudhan et al. 2018; Zhang et al. 2011). Fifteen different CLAs strains from various geographical locations have been sequenced to date with annotated genome information available in NCBI. Although CLAs strains have shown distinguishable genetic features, the genome structure and most of the genes previously identified as essential are conserved in the American and Asian strains (Lai et al. 2016; Yan et al. 2013). However, considerable heterogeneity has been observed in the resident prophage composition, with some strains lacking either or both prophages. For example, the Japanese strain Ishi-1 lacks both prophages (Katoh et al. 2014), and the Chinese strains A4 (Zheng et al. 2014a), YCpsy (Wu et al. 2015), and the American strains HHCA (Zheng et al. 2014b) and FL17 (Zheng et al. 2015) have only a single prophage. South Asian as well as American CLAs strains can be differentiated based on prophage gene content (Tomimura et al. 2009; Zheng et al. 2017) and display high variability in the prophage region (Duan et al. 2009; Katoh et al. 2014; Zhang et al. 2011; Zheng et al. 2016, 2017). The suspected role of some of the CLAs prophage genes (for example, SC2 peroxidase; Jain et al. 2015) in modulation of host defense responses might be the driving force behind the high level of genetic variability (Zheng et al. 2016, 2017).

Expression of the SC1-encoded lytic cycle gene encoding holin in *E. coli* inhibited bacterial growth (Fleites et al. 2014), and its expression was demonstrated to be transcriptionally suppressed by a small membrane permeable protein expressed by *Wolbachia* within the psyllid host (Jain et al. 2017a). Since phage particles have

been observed only in planta and never in the psyllid host, it has been suggested that activation of the phage lytic cycle may limit CLAs growth in vitro in the absence of the *Wolbachia*-expressed repressor protein. Wang and Trivedi (2013) suggested that a lytic burst of CLAs inside citrus phloem might trigger apoptotic death of citrus phloem cells, thus explaining the lack of CLAs in microscopic observations of citrus leaf midribs during advanced stages of HLB (Folimonova et al. 2009). Based on the studies mentioned above, CLAs strains lacking prophages such as Ishi-1 could be ideal for an attempt at culturing in axenic medium ex situ (Fujiwara et al. 2018). However, the lack of success culturing Ishi-1 (Fujiwara et al. 2018) may suggest that prophages are not the culprit for the lack of axenic culture. It is difficult to draw conclusions since the attempts to culture different CLAs (Davis et al. 2008; Parker et al. 2014; Sechler et al. 2009) have used very different, therefore noncomparable, culture conditions.

CELL CULTURE INFECTION MODELS AND BACTERIAL COCULTURES AS VALUABLE TOOLS

Chlamydia, *Rickettsia*, and *Mycobacterium leprae* are obligate intracellular bacteria requiring a host cell for replication and can only be studied in vitro using a cell culture system. Host cell culture strategies for such pathogens have been utilized with some degree of success (Hanson and Tan 2016). The systematic evaluation of *Coxiella burnetii* metabolic requirements inside the host cell culture system using expression microarrays, genomic reconstruction, and metabolite typing allowed successful isolation of the pathogen in axenic media (Omsland et al. 2013). A similar strategy to culture CLAs in host cell lines in vitro has not been published, despite the fact that establishment of primary cell culture lines of ACP has been reported (Marutani-Hert et al. 2009).

Bacterial coculture is another valuable approach for isolation of bacteria considered recalcitrant to axenic culture methods (Stewart 2012). Niche-specific bacterial communities coexist in complex crosstalk and metabolic relationships that can affect isolation of fastidious unculturable bacteria (Pham and Kim 2012). For this reason several coculture methods have been used for isolation of several fastidious and previous unculturable bacteria from diverse habitats (Ding et al. 2017; Tanaka and Benno 2015; Vartoukian et al. 2016a). Fujiwara et al. (2018) have recently reported that viability of CLAs phage-less strain Ishi-1 in vitro was dependent on the presence of several cocultured bacteria belonging to *Comamonadaceae*, *Flavobacteriaceae*, *Microbacteriaceae*, and *Pseudomonadaceae*, which are members of the core bacterial community in CLAs-infected citrus leaves. Davis et al. (2008) also demonstrated that CLAs could be maintained in vitro for several weeks in coculture with accidental contaminant *P. acnes*, indicating a mutually beneficial interrelationship between the two bacteria. Interestingly, *P. acnes*, *P. reudenreichii*, and *P. jensenii* have recently been used as a helper strains to grow nearly a dozen previously unculturable bacteria (Vartoukian et al. 2016a, b). Notably, *P. acnes* has also been shown to increase the viability of other bacteria such as *Staphylococcus aureus* in coculture (Tyner and Patel 2016).

Liberibacter crescens, A MODEL SYSTEM

L. crescens (Leonard et al. 2012) was isolated in axenic medium from the sap of diseased Babaco mountain papaya (*Carica stipulata* × *C. pubescens*) and has no known plant or insect hosts. *L. crescens* appears to be the most basal Liberibacter lineage, diverging from the other pathogenic 'Ca. Liberibacter spp.' early during reductive evolution of the genus (Nakabachi et al. 2013). Despite the differences among CLAs strains and *L. crescens* based on their genomes (Fagen et al. 2014b), engineering *L. crescens* for functional genomic studies of CLAs genes has helped in understanding CLAs physiology (Fleites et al. 2014; Jain et al. 2017a,

2018), and informs CLas culturing efforts. Several quite different bacterial strains have been utilized as surrogate hosts for functional characterization of CLas genes. For example, CLas transcriptional activator LdtR was functionally annotated using *Sinorhizobium meliloti* as a surrogate (Pagliai et al. 2014). However, with a genome size of ~6.7 Mb, *Sinorhizobium meliloti* not only possessed a larger repertoire of regulatory proteins as compared with CLas (Pagliai et al. 2014), but also provided a significantly different regulatory context than CLas. The genome content of *L. crescens* is highly similar and syntenic with the pathogenic ‘*Ca. Liberibacter* spp.’ (Nakabachi et al. 2013), and *L. crescens* may therefore be more suitable as a surrogate host for functional genomic studies of CLas genes, particularly in studies involving plant or psyllid inoculation assays. Interestingly, our recent study demonstrated that *L. crescens* can form biofilms in vitro under modified culture condition, allowing the use of this system to understand the process of biofilm formation in CLas, that has been described only in insect vectors so far (Naranjo et al. 2019). Clearly, either transforming CLas to enable its culturing, or causing *L. crescens* to be pathogenic by addition of CLas genes are two complimentary approaches to the same end. Although *L. crescens* is readily amenable to genetic transformation, to date CLas has not been transformed. In the absence of ability to transform CLas, axenic growth of this bacterium may be difficult or impossible. Clearly, development of CLas transformation protocols, even if utilizing transient cultures, remains a high priority for culturing efforts.

CONCLUDING REMARKS: WHAT HAVE WE LEARNED?

Despite the fact that more than a century has elapsed since its first reports, CLas remains enigmatic, and its pathogenicity and transmission functions are in many ways unknown. New reports appear regularly in the literature that reveal potential new mechanisms of CLas pathogenicity, details of the interaction between CLas and its citrus host or its psyllid vector host, or CLas associated with HLB affecting new countries or new citrus varieties. Reports of ‘*Ca. Liberibacter* spp.’ infecting other host species, such as tomato, potato and carrots (Nelson et al. 2013), pears (Thompson et al. 2013), or Australian eggplant psyllid (Morris et al. 2017), highlight a deserved focus on this genus as a causal agent of emerging diseases of multiple crops. A global multiomics and interdisciplinary vision is likely to find the key elements to be taken into account and accomplish the sought-after goal of culturing pathogenic ‘*Ca. Liberibacter* spp.’ Novel approaches are needed to find a successful method for CLas culturing, since simply testing mixtures of nutrients has proven to only advance the topic to a limited extent. Chemical signals found in the hosts may be missing in these transient cultures, and may be needed to achieve the culturing goal. Time is of the essence, CLas has already reduced citrus production in Florida by over 70% at the time of this writing.

LITERATURE CITED

- Aktas, M., Wessel, M., Hacker, S., Klüsener, S., Gleichenhagen, J., and Narberhaus, F. 2010. Phosphatidylcholine biosynthesis and its significance in bacteria interacting with eukaryotic cells. *Eur. J. Cell Biol.* 89:888-894.
- Ammar, E. D., Shatters, R. G., and Hall, D. G. 2011. Localization of *Candidatus Liberibacter asiaticus*, associated with citrus huanglongbing disease, in its psyllid vector using fluorescence *in situ* hybridization. *J. Phytopathol.* 159:726-734.
- Bové, J. M. 2014. Huanglongbing or yellow shoot, a disease of Gondwanan origin: Will it destroy citrus worldwide? *Phytoparasitica* 42:579-583.
- Chang, C., Garnier, M., Zreik, L., Rossetti, V., and Bové, J. 1993. Culture and serological detection of the xylem-limited bacterium causing citrus variegated chlorosis and its identification as a strain of *Xylella fastidiosa*. *Curr. Microbiol.* 27:137-142.
- Chen, C., Li, S., McKeever, D. R., and Beattie, G. A. 2013. The widespread plant-colonizing bacterial species *Pseudomonas syringae* detects and exploits an extracellular pool of choline in hosts. *Plant J.* 75:891-902.
- Chevrot, R., Rosen, R., Haudecoeur, E., Cirou, A., Shelp, B. J., Ron, E., and Faure, D. 2006. GABA controls the level of quorum-sensing signal in *Agrobacterium tumefaciens*. *Proc. Natl. Acad. Sci. USA* 103:7460-7464.
- Davis, M. J., and Brlansky, R. 2007. Culturing fastidious prokaryotes—Points to consider when working with citrus huanglongbing or greening. *Proc. Fla. State Hortic. Soc.* 120:136-137.
- Davis, M. J., Mondal, S. N., Chen, H., Rogers, M. E., and Brlansky, R. H. 2008. Co-cultivation of ‘*Candidatus Liberibacter asiaticus*’ with Actinobacteria from citrus with huanglongbing. *Plant Dis.* 92:1547-1550.
- Davis, M. J., Purcell, A. H., and Thomson, S. V. 1978. Pierce’s disease of grapevines: isolation of the causal bacterium. *Science* 199:75-77.
- Dettmer, J., Ursache, R., Campilho, A., Miyashima, S., Belevich, I., O’Regan, S., Mullendore, D. L., Yadav, S. R., Lanz, C., Beverina, L., Papagni, A., Schneeberger, K., Weigel, D., Stierhof, Y. D., Moritz, T., Knoblauch, M., Jokitalo, E., and Helariutta, Y. 2014. CHOLINE TRANSPORTER-LIKE1 is required for sieve plate development to mediate long-distance cell-to-cell communication. *Nat. Commun.* 5:4276.
- Ding, Z. W., Lu, Y. Z., Fu, L., Ding, J., and Zeng, R. J. 2017. Simultaneous enrichment of denitrifying anaerobic methane-oxidizing microorganisms and anammox bacteria in a hollow-fiber membrane biofilm reactor. *Appl. Microbiol. Biotechnol.* 101:437-446.
- Drew, M. M. C. 1997. Oxygen deficiency and root metabolism: Injury and acclimation under hypoxia and anoxia. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 48:223-250.
- Duan, Y., Zhou, L., Hall, D. G., Li, W., Doddapaneni, H., Lin, H., Liu, L., Vahling, C. M., Gabriel, D. W., Williams, K. P., Dickerman, A., Sun, Y., and Gottwald, T. 2009. Complete genome sequence of citrus huanglongbing bacterium, ‘*Candidatus Liberibacter asiaticus*’ obtained through metagenomics. *Mol. Plant-Microbe Interact.* 22:1011-1020.
- Fagen, J. R., Leonard, M. T., Coyle, J. F., McCullough, C. M., Davis-Richardson, A. G., Davis, M. J., and Triplett, E. W. 2014a. *Liberibacter crescens* gen. nov., sp. nov., the first cultured member of the genus *Liberibacter*. *Int. J. Syst. Evol. Microbiol.* 64:2461-2466.
- Fagen, J. R., Leonard, M. T., McCullough, C. M., Edirisinghe, J. N., Henry, C. S., Davis, M. J., and Triplett, E. W. 2014b. Comparative genomics of cultured and uncultured strains suggests genes essential for free-living growth of *Liberibacter*. *PLoS One* 9:e84469.
- Fernie, A. R., Carrari, F., and Sweetlove, L. J. 2004. Respiratory metabolism: Glycolysis, the TCA cycle and mitochondrial electron transport. *Curr. Opin. Plant Biol.* 7:254-261.
- Fleites, L. A., Jain, M., Zhang, S., and Gabriel, D. W. 2014. ‘*Candidatus Liberibacter asiaticus*’ prophage late genes may limit host range and culturability. *Appl. Environ. Microbiol.* 80:6023-6030.
- Folimonova, S. Y., Robertson, C. J., Garnsey, S. M., Gowda, S., and Dawson, W. O. 2009. Examination of the responses of different genotypes of citrus to huanglongbing (citrus greening) under different conditions. *Phytopathology* 99:1346-1354.
- Fu, S. M., Hartung, J., Zhou, C. Y., Su, H. N., Tan, J., and Li, Z. A. 2015. Ultrastructural changes and putative phage particles observed in sweet orange leaves infected with ‘*Candidatus Liberibacter asiaticus*’. *Plant Dis.* 99:320-324.
- Fujiwara, K., Iwanami, T., and Fujikawa, T. 2018. Alterations of *Candidatus Liberibacter asiaticus*-associated microbiota decrease survival of *Ca. L. asiaticus* in *in vitro* assays. *Front. Microbiol.* 9:3089.
- Ghanim, M., Achor, D., Ghosh, S., Kontsedalov, S., Lebedev, G., and Levy, A. 2017. ‘*Candidatus Liberibacter asiaticus*’ accumulates inside endoplasmic reticulum associated vacuoles in the gut cells of *Diaphorina citri*. *Sci. Rep.* 7:16945.
- Gout, E., Bligny, R., Roby, C., and Douce, R. 1990. Transport of phosphocholine in higher plant cells: ³¹P nuclear magnetic resonance studies. *Proc. Natl. Acad. Sci. USA* 87:4280-4283.
- Ham, T., Chu, S., Han, S., and Ryu, S. 2012. c-Aminobutyric acid metabolism in plant under environment stresses. *Korean J. Crop Sci.* 57:144-150.
- Hanson, B. R., and Tan, M. 2016. Intra-ChIP: Studying gene regulation in an intracellular pathogen. *Curr. Genet.* 62:547-551.
- Hartung, J. S., Shao, J., and Kuykendall, L. D. 2011. Comparison of the ‘*Ca. Liberibacter asiaticus*’ genome adapted for an intracellular lifestyle with other members of the rhizobiales. *PLoS One* 6:e23289.
- Hijaz, F., and Killiny, N. 2014. Collection and chemical composition of phloem sap from *Citrus sinensis* L. Osbeck (sweet orange). *PLoS One* 9:e101830.
- Hijaz, F., Manthey, J. A., Van der Merwe, D., and Killiny, N. 2016. Nucleotides, micro- and macro-nutrients, limonoids, flavonoids, and hydroxycinnamates composition in the phloem sap of sweet orange. *Plant Signal. Behav.* 11:e1183084.
- Jagueix, S., Bové, J. M., and Garnier, M. 1994. The phloem-limited bacterium of greening disease of citrus is a member of the subdivision of the Proteobacteria. *Int. J. Syst. Bacteriol.* 44:379-386.

- Jain, M., Fleites, L. A., and Gabriel, D. W. 2015. Prophage-encoded peroxidase in '*Candidatus Liberibacter asiaticus*' is a secreted effector that suppresses plant defenses. *Mol. Plant-Microbe Interact.* 28:1330-1337.
- Jain, M., Fleites, L. A., and Gabriel, D. W. 2017a. A small *Wolbachia* protein directly represses phage lytic cycle genes in "*Candidatus Liberibacter asiaticus*" within psyllids. *MSphere* 2:e00171-e17.
- Jain, M., Munoz-Bodnar, A., and Gabriel, D. W. 2017b. Concomitant loss of the glyoxalase system and glycolysis makes the uncultured pathogen "*Candidatus Liberibacter asiaticus*" an energy scavenger. *Appl. Environ. Microbiol.* 83:e01670-17.
- Jain, M., Munoz-Bodnar, A., Zhang, S., and Gabriel, D. W. 2018. A secreted '*Candidatus Liberibacter asiaticus*' peroxiredoxin simultaneously suppresses both localized and systemic innate immune responses in planta. *Mol. Plant-Microbe Interact.* 31:1312-1322.
- Katoh, H., Miyata, S. I., Inoue, H., and Iwanami, T. 2014. Unique features of a Japanese "*Candidatus Liberibacter asiaticus*" strain revealed by whole genome sequencing. *PLoS One* 9:e106109.
- Killiny, N. 2016. Generous hosts: What makes Madagascar periwinkle (*Catharanthus roseus*) the perfect experimental host plant for fastidious bacteria? *Plant Physiol. Biochem.* 109:28-35.
- Killiny, N. 2017. Metabolite signature of the phloem sap of fourteen citrus varieties with different degrees of tolerance to *Candidatus Liberibacter asiaticus*. *Physiol. Mol. Plant Pathol.* 97:20-29.
- Killiny, N., Hijaz, F., Ebert, T. A., Rogers, M. E., Alfred, L., and Harold Drake, E. L. 2017a. A plant bacterial pathogen manipulates its insect vector's energy metabolism. *Appl. Environ. Microbiol.* 83:e03005-e03016.
- Killiny, N., Hijaz, F., El-Shesheny, I., Alfaress, S., Jones, S. E., and Rogers, M. E. 2017b. Metabolomic analyses of the haemolymph of the Asian citrus psyllid *Diaphorina citri*, the vector of huanglongbing. *Physiol. Entomol.* 42:134-145.
- Lai, K. K., Davis-Richardson, A. G., Dias, R., and Triplett, E. W. 2016. Identification of the genes required for the culture of *Liberibacter crescens*, the closest cultured relative of the *Liberibacter* plant pathogens. *Front. Microbiol.* 7:547.
- Leonard, M. T., Fagen, J. R., Davis-Richardson, A. G., Davis, M. J., and Triplett, E. W. 2012. Complete genome sequence of *Liberibacter crescens* BT-1. *Stand. Genomic Sci.* 7:271-283.
- Li, W., Cong, Q., Pei, J., Kinch, L. N., and Grishin, N. V. 2012. The ABC transporters in *Candidatus Liberibacter asiaticus*. *Proteins* 80:2614-2628.
- Li, W., Levy, L., and Hartung, J. S. 2009. Quantitative distribution of '*Candidatus Liberibacter asiaticus*' in citrus plants with citrus huanglongbing. *Phytopathology* 99:139-144.
- Lin, H., Pietersen, G., Han, C., Read, D. A., Lou, B., Gupta, G., and Civerolo, E. L. 2015. Complete genome sequence of "*Candidatus Liberibacter africanus*", a bacterium associated with citrus huanglongbing. *Genome Announc.* 3:e00733-e15.
- Lv, L., Shao, X., Chen, H., Ho, C. T., and Sang, S. 2011. Genistein inhibits advanced glycation end product formation by trapping methylglyoxal. *Chem. Res. Toxicol.* 24:579-586.
- Marutani-Hert, M., Hunter, W. B., and Hall, D. G. 2009. Establishment of Asian citrus psyllid (*Diaphorina citri*) primary cultures. *In Vitro Cell. Dev. Biol. Anim.* 45:317-320.
- Masson, F., Copete, S. C., Schüpfer, F., Garcia-Arreaez, G., and Lemaitre, B. 2018. In vitro culture of the insect endosymbiont *Spiroplasma poulsonii* highlights bacterial genes involved in host-symbiont interaction. *MBio* 9:e00024-e18.
- Mempin, R., Tran, H., Chen, C., Gong, H., Kim Ho, K., and Lu, S. 2013. Release of extracellular ATP by bacteria during growth. *BMC Microbiol.* 13:301.
- Möhlmann, T., Tjaden, J., Schwöppe, C., Winkler, H. H., Kampfenkel, K., and Neuhaus, H. E. 1998. Occurrence of two plastidic ATP/ADP transporters in *Arabidopsis thaliana* L.—Molecular characterisation and comparative structural analysis of similar ATP/ADP translocators from plastids and *Rickettsia prowazekii*. *Eur. J. Biochem.* 252:353-359.
- Morris, J., Shiller, J., Mann, R., Smith, G., Yen, A., and Rodoni, B. 2017. Novel '*Candidatus Liberibacter*' species identified in the Australian eggplant psyllid, *Acizzia solanicola*. *Microbiol. Biotechnol.* 10:833-844.
- Murray, R. G. E., and Stackebrandt, E. 1995. Taxonomic note: Implementation of the provisional status *Candidatus* for incompletely described prokaryotes. *Int. J. Syst. Bacteriol.* 45:186-187.
- Nakabachi, A., Nikoh, N., Oshima, K., Inoue, H., Ohkuma, M., Hongoh, Y., Miyagishima, S. Y., Hattori, M., and Fukatsu, T. 2013. Horizontal gene acquisition of *Liberibacter* plant pathogens from a bacteriome-confined endosymbiont of their psyllid vector. *PLoS One* 8:e82612.
- Naranjo, E., Merfa, M. V., Ferreira, V., Jain, M., Davis, M. J., Bahar, O., Gabriel, D. W., and De La Fuente, L. 2019. *Liberibacter crescens* biofilm formation in vitro: Establishment of a model system for pathogenic '*Candidatus Liberibacter* spp.'. *Sci. Rep.* 9:5150.
- Nehela, Y., and Killiny, N. 2018. '*Candidatus Liberibacter asiaticus*' and its vector, *Diaphorina citri*, augment the tricarboxylic acid cycle of their host via the γ -aminobutyric acid shunt and polyamines pathway. *Mol. Plant-Microbe Interact.* 4:413-427.
- Nelson, W. R., Sengoda, V. G., Alfaro-Fernandez, A. O., Font, M. I., Crosslin, J. M., and Munyaneza, J. E. 2013. A new haplotype of "*Candidatus Liberibacter solanacearum*" identified in the Mediterranean region. *Eur. J. Plant Pathol.* 135:633-639.
- Omsland, A., Hackstadt, T., and Heinzen, R. A. 2013. Bringing culture to the uncultured: *Coxiella burnetii* and lessons for obligate intracellular bacterial pathogens. *PLoS Pathog.* 9:e1003540.
- Pagliai, F. A., Gardner, C. L., Bojilova, L., Sarnegrim, A., Tamayo, C., Potts, A. H., Teplitski, M., Gonzalez, C. F., Folimonova, S. Y., Lorca, G. L., and Gonzalez, C. F. 2014. The transcriptional activator LdtR from '*Candidatus Liberibacter asiaticus*' mediates osmotic stress tolerance. *PLoS Pathog.* 10:e1004101.
- Parker, J. K., Wisotsky, S. R., Johnson, E. G., Hijaz, F. M., Killiny, N., Hilf, M. E., and De La Fuente, L. 2014. Viability of '*Candidatus Liberibacter asiaticus*' prolonged by addition of citrus juice to culture medium. *Phytopathology* 104:15-26.
- Pham, V. H. T., and Kim, J. 2012. Cultivation of unculturable soil bacteria. *Trends Biotechnol.* 30:475-484.
- Pitino, M., Armstrong, C. M., and Duan, Y. 2017. Molecular mechanisms behind the accumulation of ATP and H₂O₂ in citrus plants in response to '*Candidatus Liberibacter asiaticus*' infection. *Hortic. Res.* 4:17040.
- Saglio, P., Laflèche, D., Bonisol, D. C., and Bové, J. M. 1971. Isolement et culture in vitro des mycoplasmes associés au stubborn des agrumes et leur observation au microscope électronique. *C. R. Acad. Sci. D* 272:1387-1390.
- Schmitz-Esser, S., Haferkamp, I., Knab, S., Penz, T., Ast, M., Kohl, C., Wagner, M., and Horn, M. 2008. *Lawsonia intracellularis* contains a gene encoding a functional Rickettsia-like ATP/ADP translocase for host exploitation. *J. Bacteriol.* 190:5746-5752.
- Sechler, A., Schuenzel, E. L., Cooke, P., Donnua, S., Thaveechai, N., Postnikova, E., Stone, A. L., Schneider, W. L., Damsteegt, V. D., and Schaad, N. W. 2009. Cultivation of '*Candidatus Liberibacter asiaticus*', '*Ca. L. africanus*', and '*Ca. L. americanus*' associated with huanglongbing. *Phytopathology* 99:480-486.
- Steamou, M., Olufemi, J. A., Simpson, C. R., and Jiftion, J. L. 2017. Contrasting amino acid profiles among permissive and non-permissive hosts of *Candidatus Liberibacter asiaticus*, putative causal agent of huanglongbing. *PLoS One* 12:e0187921.
- Stewart, E. J. 2012. Growing unculturable bacteria. *J. Bacteriol.* 194:4151-4160.
- Sudhan, D., Puttamuk, T., Vuttipongchaikij, S., and Chuawong, P. 2018. Cloning, overexpression, and purification of a gene of unknown function of prophage loci from '*Candidatus Liberibacter asiaticus*', the destructive bacterial pathogen of huanglongbing disease in citrus plants. *Protein Expr. Purif.* 150:72-80.
- Tanaka, Y., and Benno, Y. 2015. Application of a single-colony coculture technique to the isolation of hitherto unculturable gut bacteria. *Microbiol. Immunol.* 59:63-70.
- Thompson, S., Fletcher, J. D., Ziebell, H., Beard, S., Panda, P., Jorgensen, N., Fowler, S. V., Liefting, L. W., Berry, N., and Pitman, A. R. 2013. First report of '*Candidatus Liberibacter europaeus*' associated with psyllid infested Scotch broom. *New Dis. Rep.* 27:6.
- Tian, J., Bryk, R., Itoh, M., Suematsu, M., and Nathan, C. 2005. Variant tricarboxylic acid cycle in *Mycobacterium tuberculosis*: Identification of α -ketoglutarate decarboxylase. *Proc. Natl. Acad. Sci. USA* 102:10670-10675.
- Tomimura, K., Miyata, S. I., Furuya, N., Kubota, K., Okuda, M., Subandiyah, S., Hung, T. H., Su, H. J., and Iwanami, T. 2009. Evaluation of genetic diversity among '*Candidatus Liberibacter asiaticus*' isolates collected in Southeast Asia. *Phytopathology* 99:1062-1069.
- Tyner, H., and Patel, R. 2016. *Propionibacterium acnes* biofilm—A sanctuary for *Staphylococcus aureus*? *Anaerobe* 40:63-67.
- Vahling, C. M., Duan, Y., and Lin, H. 2010. Characterization of an ATP translocase identified in the destructive plant pathogen '*Candidatus Liberibacter asiaticus*'. *J. Bacteriol.* 192:834-840.
- Vartoukian, S. R., Adamowska, A., Lawlor, M., Moazzez, R., Dewhirst, F. E., and Wade, W. G. 2016a. In vitro cultivation of "unculturable" oral bacteria, facilitated by community culture and media supplementation with siderophores. *PLoS One* 11:e0146926.
- Vartoukian, S. R., Moazzez, R. V., Paster, B. J., Dewhirst, F. E., and Wade, W. G. 2016b. First cultivation of health-associated *Tannerella* sp. HOT-286 (BU063). *J. Dent. Res.* 95:1308-1313.
- Wang, N., Pierson, E. A., Setubal, J. C., Xu, J., Levy, J. G., Zhang, Y., Li, J., Rangel, L. T., and Martins, J. 2017. The *Candidatus Liberibacter*-host interface: Insights into pathogenesis mechanisms and disease control. *Annu. Rev. Phytopathol.* 55:451-482.

- Wang, N., and Trivedi, P. 2013. Citrus huanglongbing: A newly relevant disease presents unprecedented challenges. *Phytopathology* 103:652-665.
- Wu, F., Zheng, Z., Deng, X., Cen, Y., Liang, G., and Chen, J. 2015. Draft genome sequence of '*Candidatus Liberibacter asiaticus*' from *Diaphorina citri* in Guangdong, China. *Genome Announc.* 3:e01316-e15.
- Wulff, N. A., Zhang, S., Setubal, J. C., Almeida, N. F., Martins, E. C., Harakava, R., Kumar, D., Rangel, L. T., Foissac, X., Bové, J. M., and Gabriel, D. W. 2014. The complete genome sequence of '*Candidatus Liberibacter americanus*', associated with citrus huanglongbing. *Mol. Plant-Microbe Interact.* 27:163-176.
- Yan, Q., Sreedharan, A., Wei, S., Wang, J., Pelz-Stelinski, K., Folimonova, S., and Wang, N. 2013. Global gene expression changes in '*Candidatus Liberibacter asiaticus*' during the transmission in distinct hosts between plant and insect. *Mol. Plant Pathol.* 14:391-404.
- Zhang, M., Guo, Y., Powell, C. A., Doud, M. S., Yang, C., and Duan, Y. 2014. Effective antibiotics against '*Candidatus Liberibacter asiaticus*' in HLB-affected citrus plants identified via the graft-based evaluation. *PLoS One* 9:e111032.
- Zhang, S., Flores-Cruz, Z., Zhou, L., Kang, B. H., Fleites, L. A., Gooch, M. D., Wulff, N. A., Davis, M. J., Duan, Y. P., and Gabriel, D. W. 2011. '*Ca. Liberibacter asiaticus*' carries an excision plasmid prophage and a chromosomally integrated prophage that becomes lytic in plant infections. *Mol. Plant-Microbe Interact.* 24:458-468.
- Zheng, Z., Bao, M., Wu, F., Chen, J., and Deng, X. 2016. Predominance of single prophage carrying a CRISPR/cas system in '*Candidatus Liberibacter asiaticus*' strains in southern China. *PLoS One* 11:e0146422.
- Zheng, Z., Deng, X., and Chen, J. 2014a. Whole-genome sequence of '*Candidatus Liberibacter asiaticus*' from Guangdong, China. *Genome Announc.* 2:e00273-e14.
- Zheng, Z., Deng, X., and Chen, J. 2014b. Draft genome sequence of '*Candidatus Liberibacter asiaticus*' from California. *Genome Announc.* 2:e00999-e14.
- Zheng, Z., Sun, X., Deng, X., and Chen, J. 2015. Whole-genome sequence of '*Candidatus Liberibacter asiaticus*' from a huanglongbing-affected citrus tree in central Florida. *Genome Announc.* 3:e00169-e15.
- Zheng, Z., Wu, F., Kumagai, L., Polek, M., Deng, X., and Chen, J. 2017. Two '*Candidatus Liberibacter asiaticus*' strains recently found in California harbor different prophages. *Phytopathology* 107:662-668.

This article was modified on 19 Sep 2019.

ERRATUM / Volume 109, Number 7, 2019 / [PHYTO-02-19-0051-RVW](#)

In the article “Progress and Obstacles in Culturing ‘*Candidatus* Liberibacter asiaticus’, the Bacterium Associated with Huanglongbing” by M. V. Merfa, E. Pérez-López, E. Naranjo, M. Jain, D. W. Gabriel, and L. De La Fuente, the article mistakenly named Australia as having infections with ‘*Ca. Liberibacter* spp.’ associated with Huanglongbing. The article has been corrected.