Controlled replication of ‘Candidatus Liberibacter asiaticus’ DNA in citrus leaf discs

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Summary

‘Candidatus Liberibacter asiaticus’ is a fastidious bacterium and a putative agent of citrus greening disease (a.k.a., huanglongbing, HLB), a significant agricultural disease that affects citrus fruit quality and tree health. In citrus, ‘Ca. L. asiaticus’ is phloem limited. Lack of culture tools to study ‘Ca. L. asiaticus’ complicates analysis of this important organism. To improve understanding of ‘Ca. L. asiaticus’–host interactions including parameters that affect ‘Ca. L. asiaticus’ replication, methods suitable for screening pathogen responses to physicochemical and nutritional variables are needed. We describe a leaf disc-based culture assay that allows highly selective measurement of changes in ‘Ca. L. asiaticus’ DNA within plant tissue incubated under specific physicochemical and nutritional conditions. qPCR analysis targeting the hypothetical gene CD16-00155 (strain A4) allowed selective quantification of ‘Ca. L. asiaticus’ DNA content within infected tissue. ‘Ca. L. asiaticus’ DNA replication was observed in response to glucose exclusively under microaerobic conditions, and the antibiotic amikacin further enhanced ‘Ca. L. asiaticus’ DNA replication. Metabolite profiling revealed a moderate impact of ‘Ca. L. asiaticus’ on the ability of leaf tissue to metabolize and respond to glucose.

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Introduction

‘Candidatus Liberibacter asiaticus’ is a fastidious bacterium and a putative causative agent of huanglongbing (HLB), a highly destructive disease of citrus worldwide (Bové, 2006). ‘Ca. L. asiaticus’ is transmitted between trees by the Asian citrus psyllid (Diaphorina citri) (McClean and Oberholzer, 1965; Capoor et al., 1967; Martinez and Wallace, 1967). Within plants, ‘Ca. L. asiaticus’ is detected in phloem sieve tubes (Ding et al., 2015). Qualitative maintenance of ‘Ca. L. asiaticus’ in co-culture with other bacteria has been reported (Davis et al., 2008; Parker et al., 2013), and moderate replication in nutrient broth of the atypical Ishi-1 strain of ‘Ca. L. asiaticus’ was recently demonstrated (Fujwara et al., 2018). The ability to activate ‘Ca. L. asiaticus’ DNA replication in the context of natural tissue can be exploited in the analysis of host–pathogen interactions.

Several studies have identified alterations in primary metabolite content in ‘Ca. L. asiaticus’-infected plant tissues (Fan et al., 2010; Slisz et al., 2012; Albrecht et al., 2016; Killiny and Nehela, 2017). Because defence responses in plants can be energy dependent, changes in plant central carbon metabolism can affect initiation of defence responses after encounter with a pathogen (Berger et al., 2007). Moreover, pathogens could take advantage of the resulting re-arrangements in plant physiology upon infection in order to improve survival. Therefore, an understanding of whether ‘Ca. L. asiaticus’ infection affects host responses to specific nutrients can reveal pathogen-induced manipulation of host metabolic status and capacity.

The ‘Ca. L. asiaticus’ genome has been derived via metagenomics-based assembly (Duan et al., 2009), and metabolic pathway reconstruction based on the genome sequence has been used to predict major metabolic features of ‘Ca. L. asiaticus’ (Duan et al., 2009). Similar to other bacterial obligate intracellular parasites including species of the genus Rickettsia (Driscol et al., 2017) and phytoplasmas (Oshima et al., 2013), the ‘Ca. L. asiaticus’ genome has undergone genome reduction (Duan et al., 2010).
suggesting that the bacterium relies on the host to obtain essential metabolites in order to replicate. ‘Ca. L. asiaticus’ appears to be adapted to the lower oxygen tension of phloem sap (~7%) (van Dongen et al., 2003), and the genome encodes some components necessary for aerobic respiration (Duan et al., 2009). However, genes for cytochrome bd (cydAB), a terminal oxidase associated with bacteria specifically adapted to microaerobic environments (Borisov et al., 2011), do not appear to be encoded by the ‘Ca. L. asiaticus’ genome (Duan et al., 2009). Moreover, ‘Ca. L asiaticus’ appears to encode a partial glycolytic pathway in which the gene pgi, encoding glucose 6-phosphate isomerase, is missing. This apparent defect would likely severely reduce the efficiency of ‘Ca. L. asiaticus’ glucose metabolism.

In an effort to establish a method to more effectively screen variables that impact the potential for ‘Ca. L. asiaticus’ DNA replication, we developed a leaf disc assay that allows quantification of the absolute load of ‘Ca. L. asiaticus’ DNA within leaf discs incubated under different physicochemical and nutritional conditions. We hypothesize that conditions that produce an increase in relative DNA content represent conditions likely to trigger and/or support ‘Ca. L. asiaticus’ replication within leaf tissue. We test the effects of glucose and oxygen availability on ‘Ca. L. asiaticus’ replication of DNA in situ.

Results

Primary metabolite profiles derived from healthy or Ca. L. asiaticus ‘infected leaves in response to glucose

This study is based on citrus trees maintained under semi-field conditions with exposure to natural light. Figure 1A summarizes seasonal characteristics in citrus tree development and physiology and reported changes in ‘Ca. L. asiaticus’ loads in trees. The main leaf flush occurs in the early spring and minor flushes follow in the summer and early fall. Flushing is generally synchronized with higher populations of ‘Ca. L. asiaticus’ in the leaves. Upon measuring pathogen titre in March, June and September of 2018 (Fig. 1B), seasonal variability in ‘Ca. L. asiaticus’ titre with non-statistically significant changes in bacterial loads was observed between seasons, in agreement with previous results from Florida (Hijaz and Killiny, 2014).

Glucose is a critical carbon source in most organisms, including citrus, where glucose is found in phloem sap (Hijaz and Killiny, 2014). To test the effect of ‘Ca. L. asiaticus’ infection on the ability of citrus leaf tissue to respond to glucose, we used gas chromatography–mass spectrometry (GC-MS) to assess primary metabolite profiles of healthy or ‘Ca. L. asiaticus’-infected leaves in the presence or absence of glucose (Figs 2 and 3). The analysis was performed with samples harvested during months correlating with critical stages in ‘Ca. L. asiaticus’ and citrus biology (e.g. Fig. 1). While early spring correlates with tree emergence from winter dormancy, the summer represents onset of higher temperatures and extended daylight (geographical region: Lake Alfred, FL, USA) with direct effects on tree metabolism and photosynthetic activity. Leaf discs were cut from the midribs of surface decontaminated leaves that had also been pre-screened for colonization by ‘Ca. L. asiaticus’ by the measurement of genome equivalents (GE) by quantitative PCR, thus avoiding performing metabolite analysis on tissue that is not directly colonized by the pathogen (Fig. 1B, March and June). Leaf discs were then incubated for 3 days in either plain PBS or PBS supplemented with 10 mM glucose. Incubations were performed in the dark to prevent photosynthetic activity, a variable that could mask responses and a process not consistent with the end goal of this study (i.e. screening of variables that facilitate ‘Ca. L. asiaticus’ DNA replication). Figures 2 and 3 show heatmaps for corresponding metabolite profiles measured in March and June 2018 respectively. Consistent clustering between samples replicates was not observed in March (Fig. 2A), suggesting ‘Ca. L. asiaticus’ only moderately affects primary metabolite profiles or the ability of young/flowering leaf tissue to metabolize glucose. When signal intensities for
individual metabolites from all replicates in each treatment group were averaged (Fig. 2B), clear separation between treatment groups was observed, but the overall change in metabolite abundances was minimal. In June, ‘Ca. L. asiaticus’ infection resulted in a shift in metabolite profile consistent with differential ability of infected tissue to respond to glucose (Fig. 3A). Compared to healthy tissue, ‘Ca. L. asiaticus’-infected material generally showed reduced levels of glucose and immediate metabolites of glucose (glucose 1-phosphate, glucose 6-phosphate and fructose 6-phosphate). Additionally, several amino acids (including lysine, tyrosine, isoleucine, glycine, valine, alanine, methionine, proline, aspartic acid and serine) showed a trend towards reduced levels in infected tissue. Healthy tissue incubated with glucose displayed an overall different metabolite profile with elevated levels of most detected metabolites. Pathogen-infected tissue incubated without glucose generally grouped with healthy tissue incubated without glucose. As for the month of March, averaging signal intensities for individual metabolites from all replicates in each treatment group (Fig. 3B) revealed moderate differences in the abundances of specific metabolites. However, averaging also highlighted the effect of ‘Ca. L. asiaticus’ infection in reducing pools of critical metabolites (including citric acid, glucose-1-P and the amino acids serine, phenylalanine, lysine, tyrosine, proline and glycine) in both the absence and presence of glucose. Figures S1 and S2 show quantitative comparisons of select saccharides (Fig. S1) or TCA cycle (Fig. S2) intermediates in leaf discs incubated in the absence or presence of glucose. In March, but not in June, infected leaf discs showed elevated levels of glycolytic intermediates under control conditions (absence of glucose). During incubation with glucose, tissue analysed in March showed reduced levels of glycolytic intermediates, while tissue analysed in June showed elevated levels of fructose-6-P, glucose-6-P and sucrose in non-infected tissue incubated with glucose. TCA cycle intermediates exhibited extensive variability between treatment groups. Quantitative differences in relative abundance of selected sugars were variable and within two- to threefold, thus considered moderate in nature. Overall, the observed shifts in metabolite profile are consistent with ‘Ca. L. asiaticus’ infection affecting leaf metabolism and responses to glucose.

**Design of a leaf disc assay to test the effect of physicochemical and nutritional conditions on ‘Ca. L. asiaticus’ DNA replication**

We developed a leaf disc-based assay, exploiting a natural niche for ‘Ca. L. asiaticus’, to identify physicochemical and nutritional requirements for ‘Ca. L. asiaticus’ replication (Fig. 4). Although leaf tissue naturally contains all the nutrients required by ‘Ca. L. asiaticus’ for replication, supplementation of limiting nutrients is expected to stimulate replication thus allowing detection of pathogen responses without detailed knowledge of ‘Ca. L. asiaticus’ metabolic requirements and capabilities.

Measurement of GE is a widely used method to identify gross increases in DNA replication (although not necessarily cell division). We designed an oligonucleotide primer pair specific to the conserved ‘Ca. L. asiaticus’ hypothetical gene CD16-00155 (strain A4) as a basis for quantification of ‘Ca. L. asiaticus’ DNA. Nucleotide sequence BLAST with CD16-00155 did not result in detection of similar sequences in other bacteria (including ‘Ca. L. americanus’, ‘Ca. L. solanacearum’ and L. crescens), consistent with the utility of using detection of CD16-00155 for highly specific detection and quantification of ‘Ca. L. asiaticus’. To validate the specificity and selectivity of the primers designed for detection of CD16-00155, PCR amplification was performed on 50 ng of total DNA (tDNA) isolated from healthy or ‘Ca. L. asiaticus’-infected leaves, tDNA isolated from L. crescens and the unrelated (animal) pathogen Coxiella burnetii (Fig. S3A). Amplification of CD16-00155 allowed detection of ‘Ca. L. asiaticus’ DNA with expected specificity and selectivity in that amplification was only observed in ‘Ca. L. asiaticus’-infected plant tissue. To assess reaction sensitivity, amplification was tested over a concentration gradient of template tDNA. Relative quantification of ‘Ca. L. asiaticus’ based on CD16-00155 was as sensitive as that observed when using 16S rDNA as target (Fig. S3B and C). Sequencing of the PCR and qPCR reaction products from amplification of CD16-00155 confirmed amplification of the target sequence (data not shown). To facilitate absolute quantification of ‘Ca. L. asiaticus’ DNA, CD16-00155 was cloned into a plasmid vector from which a standard curve was generated (Fig. S4).

‘Ca. L. asiaticus’ infection of citrus is characterized by extensive variability in pathogen titre in shoots throughout the canopy (Tatineni et al., 2008; Louzada et al., 2016). To establish the leaf disc assay platform with the lowest possible variability between replicate samples and independent experiments, the ‘Ca. L. asiaticus’ load in leaves was characterized. Initially, we quantified the absolute bacterial load in different parts of the leaf including the midrib, leaf blade, petiole and stem based on quantitative detection of CD16-00155. In agreement with other reports (Tatineni et al., 2008; Fujikawa et al., 2013), ‘Ca. L. asiaticus’ was most and consistently abundant in the midrib (data not shown). To determine the variability of pathogen colonization among leaves, multiple leaves from different branches were randomly collected and surface sterilized, and then, two leaf discs
were punched from the midrib of each leaf and tDNA extracted for GE analysis (Fig. 5A). Out of twelve tested leaves, four had ‘Ca. L. asiaticus’ loads equal to or higher than $1 \times 10^4$ GE/200 ng DNA, while the rest of the leaves had ‘Ca. L. asiaticus’ load lower than $1 \times 10^2$ GE/200 ng DNA. In one leaf, the ‘Ca. L. asiaticus’ load was below the detection limit. Additionally, upon quantifying the absolute ‘Ca. L. asiaticus’ load along the midrib in segments equal to two leaf discs (5 mm diameter), we observed that while some sections show a ‘Ca. L. asiaticus’ load higher than $1 \times 10^3$ GE/200 ng DNA, other parts show very low or even undetectable bacterial loads (Fig. 5B). To distribute the pathogen evenly between culture samples, leaf discs prepared from several leaves were pooled and then divided evenly between cultures to assure detectable and similar levels of ‘Ca. L. asiaticus’ for any given experiment regardless of time of tissue harvest, greatly reducing overall assay variability (Fig. 4).

**Stimulation of Ca. L. asiaticus’ DNA replication within leaf discs**

As exemplified by host cell-free replication of the bacterial obligate intracellular parasite *C. burnetii*, bacteria can exhibit highly specific physicochemical requirements for...
replication (Omsland et al., 2008; Esquerra et al., 2017). ‘Ca. L. asiaticus’ is adapted to citrus phloem sap, a microaerobic environment (van Dongen et al., 2003; Geigenberger, 2003). Therefore, the dependency of ‘Ca. L. asiaticus’ DNA replication on specific O₂ availability was tested (Fig. 6). While no significant changes in GE were observed after incubation under normoxic (air/~20% O₂) conditions, a 4.6-fold increase in bacterial load was observed between day 0 (d0, 1.9 × 10³ ± 1.9 × 10² GE) and day 3 (d3, 8.7 × 10³ ± 1.4 × 10³ GE) when leaf discs were incubated in the presence of glucose, and the level of O₂ was reduced to 10% (Fig. 6A and B), suggesting that ‘Ca. L. asiaticus’ is indeed a microaerophile. Reducing available O₂ to 2.5% did not positively affect replication (Fig. S5). ‘Ca. L. asiaticus’ appears optimally adapted to an environment where the oxygen level is approximately 10%.

‘Ca. L. asiaticus’ may be able to utilize glucose directly or benefit from a product of glucose metabolism (e.g. ATP) following oxidation by the leaf tissue. Incubation of leaf discs from ‘Ca. L. asiaticus’-infected plants in PBS containing different concentrations of glucose revealed dose-dependent increases in GE (Fig. 6C). Relative to d0 (3 × 10³ ± 5.8 × 10² GE), leaf discs incubated for...
three days with 10 mM glucose (9.5 × 10^3 ± 1.4 × 10^3 GE) had significantly higher GE counts, equivalent to a 3.2-fold increases in DNA. Relative to d0, incubation with 0.5 mM glucose (d3, 4.7 × 10^3 ± 1.3 × 10^3 GE) showed a non-significant 1.6-fold increase in GE counts, consistent with a dose-dependent effect of glucose on ‘Ca. L. asiaticus’ DNA replication. Incubation of autoclaved leaf discs did not produce any increases in GE over the 3-day incubation. ‘Ca. L. asiaticus’ may utilize glucose directly by importing glucose into the cytoplasm via a glucose/galactose transporter (e.g. CD16-00155, A4 strain; Zheng et al., 2014). However, the possibility that leaf tissue actively converts glucose into a downstream metabolite used by ‘Ca. L. asiaticus’ cannot be excluded.

Genome sequence analysis has revealed that ‘Ca. L. asiaticus’ encodes a nearly complete glycolytic pathway, but is missing glucose 6-phosphate isomerase (pgi, EC 5.3.1.9; Duan et al., 2009; Fagen et al., 2014b). Based on mutational analysis in Escherichia coli (Charusanti et al., 2010; Long et al., 2018), loss of pgi can have significant negative implications for utilization of glucose with corresponding re-arrangements of metabolic flux. Because the genome(s) of ‘Ca. L. asiaticus’ has been obtained via metagenomics sequencing and pathogen isolates may differ in genetic makeup, we used PCR of several genes to validate expected presence or absence of genes, including pgi, between tDNA isolated from healthy or ‘Ca. L. asiaticus’-infected leaf tissue and gDNA isolated from L. crescens (Fig. 6D). While pgi (using primers internal to the L. crescens orthologue), 16S-rDNA, and the gene encoding chorismate synthase were detected in L. crescens, only 16S-rDNA and the hypothetical sequence CD16-00155 were detected in tissue containing ‘Ca. L. asiaticus’. Among Liberibacter species, only L. crescens appears to encode chorismate synthase (Fagen et al., 2014b). Although based on lack of detection, given the positive controls (16S-rDNA and hypothetical gene CD16-00155) included in this experiment and the pattern of positive amplification between species and sample types, our results strongly support the absence of pgi from the ‘Ca. L. asiaticus’ strain used in this study.

‘Ca. L. asiaticus’ does not appear to be sensitive to the antibiotic amikacin (Zhang et al., 2014). In addition, antibiotics have been used to suppress the growth of
specific bacteria and thus reduce the complexity of microbial communities in citrus (Zhang et al., 2013). Therefore, we tested whether the response of ‘Ca. L. asiaticus’ DNA replication was positively affected by the presence of amikacin during incubation (Fig. 7); leaves were pre-screened for the presence of pathogen DNA to further reduce assay variability. Incubation of leaf discs from ‘Ca. L. asiaticus’-infected plants in PBS containing 10 mM glucose in the absence or presence of amikacin showed that incubation with amikacin resulted in a 3.03-fold potentiation in ‘Ca. L. asiaticus’ DNA content (d3 glucose, 3.89 × 10^4 GE; glucose and amikacin, 1.15 × 10^5 ± 2.8 × 10^4 GE; Fig. 7) after three days of incubation. Compared to the starting material, an overall 11.1-fold increase in GE was observed (d0, 1.03 × 10^4 ± 3.3 × 10^3 GE; d3 glucose and amikacin, 1.15 × 10^5 ± 2.8 × 10^4 GE).

Discussion

We have established an assay based on incubating citrus leaf discs in solution to enable screening of parameters that affect replication of ‘Ca. L. asiaticus’ DNA in situ. An increase in ‘Ca. L. asiaticus’ DNA within leaf discs was observed under reduced oxygen availability (10% O_2), but not under normoxic (air) conditions. Moreover, glucose stimulated ‘Ca. L. asiaticus’ replication in a dose-dependent manner in situ. Incubation with the antibiotic amikacin further stimulated ‘Ca. L. asiaticus’ DNA replication, suggesting improved ‘Ca. L. asiaticus’ activity upon potential changes in the microbial community structure in response to amikacin. A comparison between the metabolite profiles derived from healthy versus ‘Ca. L. asiaticus’-infected leaf discs following incubation with glucose revealed a trend consistent with a moderate alteration of metabolism in

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infected tissue. Collectively, these findings are consistent with a model in which ‘Ca. L. asiaticus’ replicates optimally under microaerobic conditions and produces moderate changes in the metabolite makeup of its replicative environment, possibly as a means to increase availability of nutrients that promote pathogen replication and viability.

Several metabolomics studies have revealed changes in the metabolite profiles of citrus leaves and fruit juice after infection with ‘Ca. L. asiaticus’ (Slisz et al., 2012; Hijaz et al., 2013; Killiny, 2017). For example, concentrations of sugars, organic acids, amino acids and lipids can be altered in response to infection. Additional studies have demonstrated altered carbohydrate (e.g. glucose and fructose) content in ‘Ca. L. asiaticus’-infected leaves (Fan et al., 2010; Albrecht et al., 2016). Levels of glucose and fructose have been shown to vary depending on the area of the infected leaves and time after infection (Albrecht et al., 2016), suggesting that seasonal changes may additionally be affected by spatiotemporal activity within individual leaves. Our results show that the levels of primary metabolites in ‘Ca. L. asiaticus’-infected leaves from trees exhibit some yet inconsistent variability when exposed to glucose (Figs 2 and 3). Overall, these data show that ‘Ca. L. asiaticus’ infection can affect the metabolite profile of infected tissue and the metabolic response of leaf tissue to specific nutrients, in this case illustrated by the response to incubation with glucose. As revealed upon averaging signal intensities from metabolites detected in March or June, the abundances of several metabolites (including quinic acid, myo-inositol, sucrose, glucose 6-phosphate and fructose 6-phosphate) shifted in opposite directions in the two months shown, indicating that responses to glucose treatment may be season-dependent.

‘Ca. L. asiaticus’ has been described to have limited capacity for aerobic respiration (Duan et al., 2009). Despite the lack of cytochrome bd (cydAB), a terminal oxidase typically associated with microaerobic metabolism, ‘Ca. L. asiaticus’ was able to undergo DNA replication, but only under microaerobic condition (Fig. 6). ‘Ca. L. asiaticus’ DNA replication under microaerobic conditions despite the absence of a terminal oxidase indicative of a microaerophilic lifestyle could be related to organism sensitivity to oxidative stress. Importantly, the cytochrome o ubiquinol oxidase encoded by ‘Ca. L. asiaticus’ could also function under microaerobic conditions (Tseng et al., 1996).

‘Ca. L. asiaticus’ DNA replication was observed upon incubation of leaf discs with glucose. ‘Ca. L. asiaticus’ is either able to metabolize glucose, predicted from metabolic pathway reconstruction (Fagen et al., 2014b), and analysis of E. coli mutants with defects in pgI (Charusanti et al., 2010; Long et al., 2018), or responds to a glucose-dependent alteration in leaf physiology, such as synthesis of ATP. Ability of ‘Ca. L. asiaticus’ to utilize glucose is in agreement with gene expression profiling of ‘Ca. L. asiaticus’ (Yan et al., 2013) demonstrating that genes encoding enzymes involved in glycolysis are expressed in planta. Similar to E. coli (Charusanti et al., 2010) and as predicted for ‘Ca. L. asiaticus’ (Fagen et al., 2014b), the pathogen may adapt to loss of pgI by rerouting metabolic flux through the pentose phosphate pathway (PPP). In short, ‘Ca. L. asiaticus’ may bypass the early conversions in glycolysis to generate glycer-aldehyde-3-phosphate via the PPP (Fagen et al., 2014b), allowing ‘Ca. L. asiaticus’ to produce pyruvate from glucose. The apparent absence of the PPP enzyme transaldolase (E. C. 2.2.1.2) in ‘Ca. L. asiaticus’ (Fagen et al., 2014b) may compromise generation of glyceraldehyde-3-phosphate via PPP activity. Moreover, the absence in ‘Ca. L. asiaticus’ of genes shown to have significance for detoxification of methylglyoxal (MG; Jain et al., 2017) may predispose ‘Ca. L. asiaticus’ to MG sensitivity and thus make metabolism of glucose a suboptimal carbon source for this pathogen. Regardless, conservation of a nearly complete glycolytic pathway, including the enzyme that facilitated entry of glucose into the pathway, is consistent with oxidation of glucose by ‘Ca. L. asiaticus’. Genome sequence analysis based on metagenomics assembly showed that ‘Ca. L. asiaticus’ is similar to ‘Ca. L. solanacearum’ (Lin et al., 2011) in that it does not encode a phosphotransferase system (PTS), a common bacterial machinery for transporting carbohydrates (Kotb et al., 2001). However, ‘Ca. L. asiaticus’ does encode a single glucose/galactose...
transporter (CD16-03615, strain A4; Zheng et al., 2014), suggesting that ‘Ca. L. asiaticus’ can take up glucose. Recent analysis of broth-based culture of the Ishi-1 strain of ‘Ca. L. asiaticus’ (Fujiwara et al., 2018) lends support to the finding that carbohydrates, including glucose, are important for optimal growth of ‘Ca. L. asiaticus’. Because ‘Ca. L. asiaticus’ Ishi-1 does not harbour a pro-phage that appears to have a major impact on pathogen culturability (Fleites et al., 2014; Fujiwara et al., 2018), the hypothesis that glucose can be used directly by the ‘Ca. L. asiaticus’ strain used in this study cannot be tested until a chemically defined medium that supports axenic growth of a wider variety of ‘Ca. L. asiaticus’ strains becomes available.

The ‘Ca. L. asiaticus’ genome encodes an apparently intact ATP/ADP transporter (nttA; Duan et al., 2009; Vahling et al., 2010; Jain et al., 2017), suggesting the pathogen acts like an ‘energy parasite’ by importing ATP directly from the host akin to the obligate intracellular bacteria *Rickettsia prowazekii* (Piano and Winkler, 1991; Driscoll et al., 2017) and *Chlamydia trachomatis* (Hille-Lee and McClarty, 1999). It is possible that leaf tissue converts glucose to ATP and therefore that the increase in ‘Ca. L. asiaticus’ GE within leaf discs as observed in this study is an indirect response to glucose.

Documented seasonal variability in ‘Ca. L. asiaticus’ loads in infected trees (Lopez-Buenfil et al., 2017) may affect the utility of leaf discs prepared from citrus in screening physicochemical and nutritional conditions that affect *in situ* replication. Indeed, we observed optimal assay responses between March and September. This limitation could be based on the natural biology of the interaction between ‘Ca. L. asiaticus’ and citrus trees, including increased pathogen activity in the spring and early summer when the flush develops and trees are at their highest level of activity. Use of greenhouse- or growth-chamber cultivated plants that are subjected to less seasonal variability may allow assay responses that are consistent throughout the year.

In conclusion, we have developed a strategy to assess ‘Ca. L. asiaticus’ responses to physicochemical and nutritional variables in the context of leaf tissue. Because responses in ‘Ca. L. asiaticus’ DNA replication were observed within three days, the methods presented herein are suitable for medium-throughput screening of conditions that influence pathogen DNA replication *in situ*. ‘Ca. L. asiaticus’ responses to different conditions were determined by measuring bacterial GE by qPCR targeting a single-copy hypothetical gene that appears unique to ‘Ca. L. asiaticus’, thus reducing the likelihood of detecting DNA related to organisms other than ‘Ca. L. asiaticus’. Unlike published methods (e.g. Zhang et al., 2014) to assess ‘Ca. L. asiaticus’ responses to chemical stimuli (e.g. antibiotics) that yield a qualitative output (e.g. disease transmission), the assay described herein allows such analysis under conditions where pathogen replication is activated and the level of activation is quantitated at the ‘Ca. L. asiaticus’ cellular level. Methods other than qPCR would have to be used to correlate DNA replication with potential cell division. Because ‘Ca. L. asiaticus’ DNA synthesis is measured in the context of host tissue, it is not possible to conclude whether test conditions affect the pathogen directly or indirectly via altered host physiology. Moreover, ‘Ca. L. asiaticus’ may benefit from the ability of another microbe to utilize glucose in the production of one or more secreted metabolite(s) subsequently acquired and used by ‘Ca. L. asiaticus’. Regardless, this study establishes a method for controlled activation of ‘Ca. L. asiaticus’ DNA replication within natural tissue.

**Experimental procedures**

*Bacterial strains and culture condition*

*Liberibacter crescens* BT-1 (ATCC® BAA-2481™) was cultured in liquid BM7 medium at 28°C and 20% O2 tension (Fagen et al., 2014a). One shot™ TOP 10 chemically competent *Escherichia coli* (Invitrogen, Carlsbad, CA, USA) was cultured in Luria-Bertani (LB) liquid medium supplemented with 50 mg ml⁻¹ ampicillin at 37°C with agitation at 250 r.p.m.

*Establishment and maintenance of citrus trees*

Leaves from *Citrus sinensis* (L.) Osbeck (Hamlin) trees were maintained at the Citrus Research and Education Center, Lake Alfred, FL, USA. Trees were kept in outdoor cages (semi-field conditions) to allow seasonal responses to temperature and light in a facility approved by the United States Department of Agriculture-Animal and Plant Health Inspection Service. Specifically, citrus trees were housed in large outdoor cages, 6' wide × 12' long × 6' high, (183 cm × 366 cm × 183 cm) constructed with amber-coloured 400 mesh Lumite screen (#1412B Bioquip, Rancho Domingo, CA). The screen enclosure allows trees to receive natural sunlight, rainfall and humidity, while protecting the trees from frost damage and most insects. In periods of extremely cold weather (< 5°C), the cages are covered with 4 mm plastic sheeting until the outside temperature rises above 5°C. The trees were inoculated by grafting with infected material and tissue harvesting initiated 9 months later when the trees started to show symptoms consistent with HLB. Trees were trimmed regularly (every 3 months) to stimulate new shoots. Plants were irrigated twice weekly (three times per week during hot weather) and fertilized once every week using 20-10-20 NPK.
fertilizer (Peter’s Fertilizer, Allentown, PA, USA). Plant material was harvested in the morning and shipped overnight from Florida to Washington, refrigerated upon arrival and used for experimentation within seven days.

Leaf disc assay
‘Ca. L. asiaticus’-infected leaves were surface sterilized as follows: Leaves were soaked in 70% ethanol for 15 min, rinsed with autoclaved water 2–3 times transferred to a sterile container containing 10% bleach with 0.01% Tween-20 for 15 min and then washed four times with sterile deionized water to remove the bleach from the leaf surface. Surface-sterilized leaves were punched with sterile and disposable 5 mm leaf punches (Integra Miltex, PA, USA) and then carefully mixed in order to assure that the average bacterial load among groups of five leaf discs was equivalent. Subsequently, groups of five leaf discs were transferred into individual wells of 12-well plates containing 1.5 ml per well of different test media and incubated for 3 days. The basal PBS consisted of 8.1 mM Na2HPO4, 1.47 mM KH2PO4, 2.7 mM KCl, 136.8 mM NaCl, 0.9 mM CaCl2 and 0.5 mM MgCl2. All incubations were performed in the dark to prevent photosynthetic activity in the leaf discs from affecting ‘Ca. L. asiaticus’ DNA replication. All incubations were done in regularly calibrated tri-gas incubators (Panasonic Healthcare Corporation, Wood Dale, IL, USA) adjusted to 28°C; for microaerobic incubations, oxygen was displaced by nitrogen gas. Maintenance of natural leaf colour over 3 days of incubation is consistent with maintenance of general tissue integrity and viability for at least 3 days (Fig. S6). The response of ‘Ca. L. asiaticus’ to different conditions was measured by increases in gross ‘Ca. L. asiaticus’ DNA content by qPCR.

Extraction of total DNA
To extract total DNA from citrus leaf discs, two to five (depending on type of experiment) ‘Ca. L. asiaticus’-infected leaf discs, stored at −20°C before extraction, were placed into screw cap Lysing Matrix H tubes (MP Biomedicals, Irvine, CA, USA) and then homogenized using a Fastprep-24 System (MP Biomedicals) for 60 s at 6 m s⁻¹. Leaf discs were homogenized dry. Following homogenization, either 600 or 200 µl extraction buffer was added to samples containing the equivalent of 5 or 2 leaf discs respectively. Subsequent extraction of DNA was performed using the Wizard Genomic DNA Purification Kit (Promega Corp., Madison, WI, USA) according to the manufacturer’s protocol. Genomic DNA of L. crescens and C. burnetii was extracted using the Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research Corp., Irvine, CA, USA). The concentration of sample DNA was determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Quantitative and conventional PCR
Primers used in this study are listed in Table S1. All quantitative PCR (qPCR) reactions were performed using a CFX318 real-time PCR Detection System (Bio-Rad, Hercules, CA, USA). Briefly, 10 µl qPCR reactions contained 5 µl 2 × SYBR green qPCR master mix (IQ™ SYBER® GREEN supermix, Bio-Rad), 0.2 µM of each primer (qPCR-CD16-00155 F and qPCR-CD16-00155 R) and 0.2 µg template DNA. The amplification conditions for 16S rDNA followed published protocols (Jagoueix et al., 1996; Orce et al., 2015). All reactions were performed in triplicate with a positive, autoclaved infected leaf discs as a negative and ‘no template’ controls.

Absolute quantification of ‘Ca. L. asiaticus’ was based on qPCR of the hypothetical gene CD16-00155 (‘Ca. L. asiaticus’, strain A4). The CD16-00155 sequence was amplified from total DNA extracted from the midrib of ‘Ca. L. asiaticus’-infected leaves by conventional PCR using 0.2 µg of DNA template, 0.2 µM primer, 0.25 mM dNTP, 1× buffer and 0.125 µl of Phusion high-fidelity DNA polymerase (Thermo Fisher Scientific). The amplification product was cloned into the pCR™ 4-TOPO® vector (Invitrogen) and then transformed into E. coli TOP10 cells (Invitrogen; Fig. S4). Transformants were selected using LB medium supplemented with 50 µg ml⁻¹ ampicillin at 37°C and extracted plasmid sequenced to validate the insertion. The plasmid was extracted from E. coli using the PureLink kit (Invitrogen) according to the manufacturer’s instructions. Plasmid concentration was determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific), and plasmid copy numbers were calculated based on the molecular weight of the plasmid. A standard curve was generated by serial dilution of the plasmid, and the absolute quantification of ‘Ca. L. asiaticus’ extrapolated from the standard curve and presented as genome equivalents (GE).

Primary metabolite derivatization and gas chromatography time-of-flight mass spectrometry analysis
Extraction was carried out using a slight modification of an established procedure (Lee and Fiehn, 2008). To assure metabolite profiles represented that of infected leaf tissue, leaves subjected to metabolite profiling were pre-screened for the presence of ‘Ca. L. asiaticus’ DNA; only leaves with a GE load above 100 per 200 ng DNA were used in the analysis. After incubation, a defined amount of powdered freeze-dried citrus leaves (ca. 5–14 mg) was suspended in 500 µl of extraction solvent.
Statistical analyses

Statistical analyses were performed using Prism software (GraphPad Software, San Diego, CA, USA). Unless otherwise noted, data were collected from at least three independent biological replicates each consisting of at least three technical replicates. t-test, one-way ANOVA and Tukey HSD post hoc tests were performed for relevant data sets. Depicted data illustrate the mean ± SEM.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

E. A. and A. B. designed and performed experiments and analysed data. D. R. G., N. K. and A. O. designed and directed experiments, and analysed data. E. A., A. B. and A. O. drafted the manuscript. E. A., A. B., D. R. G., N. K., H. B. and A. O. revised the manuscript. H. B., N. K., D. R. G. and A. O. designed the study.

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Candidatus Liberibacter asiaticus DNA replication

**Supporting information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Fig. S1.** Effect of glucose treatment on abundance of selected saccharides.

**Fig. S2.** Effect of glucose treatment on abundance of selected TCA cycle intermediates.

**Fig. S3.** Selective measurement of ‘Ca. L. asiaticus’ GE by qPCR.

**Fig. S4.** Map of plasmid standard used for absolute quantification of ‘Ca. L. asiaticus’ based on gene CD16-00155.

**Fig. S5.** Assessment of the ability of ‘Ca. L. asiaticus’ to replicate in citrus leaves under different oxygen conditions.

**Fig. S6.** Maintenance of tissue integrity and natural leaf color over three days of incubation in the absence of light.

**Table S1.** Primers used in this study.