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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 (Fujiwara *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* (Driscoll *et al*., 2017) and phytoplasmas (Oshima *et al*., 2013), the '*Ca. L. asiaticus*' genome has undergone genome reduction (Duan *et al*.,

2009) 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*.

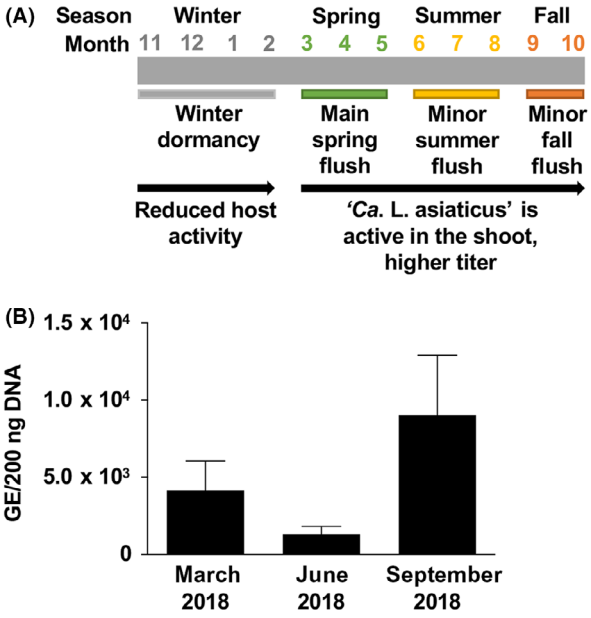

Fig. 1. Seasonal development of citrus and bacterial loads during critical stages of the growing season. A. Schematic of citrus tree annual cycle in Florida, USA. Observed seasonal variability of '*Ca. L. asiaticus*' in the shoot matches the elevated photosynthetic activity during main flush development in the spring during blooming and minor flush development during the summer and early fall. B. Average '*Ca. L. asiaticus*' DNA content in leaf tissue harvested in the spring, summer or fall.

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 (Hu *et al.*, 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

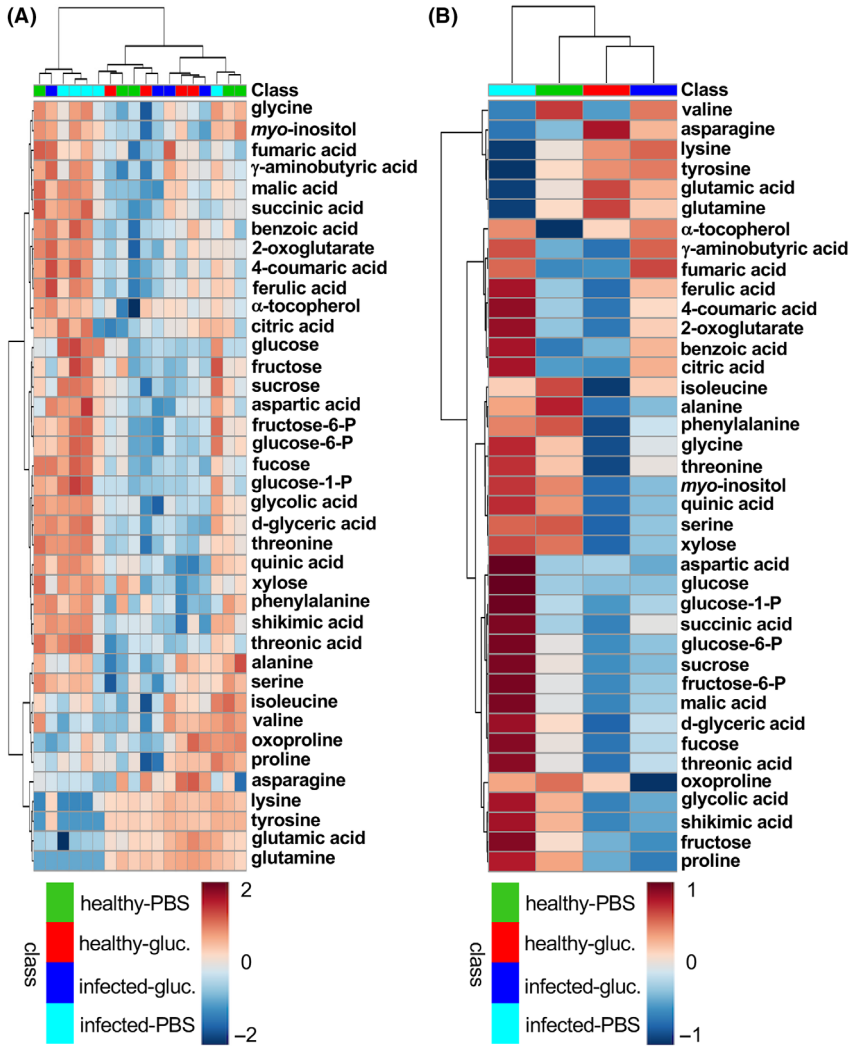

Fig. 2. Primary metabolite profiles in leaves from healthy or '*Ca. L. asiaticus*'-infected plants following incubation with glucose (March). Heatmaps show differences between healthy or '*Ca. L. asiaticus*'-infected citrus leaves harvested in March after three days of incubation in the presence or absence of glucose. Sterilized leaf discs were prepared, and primary metabolites extracted and analysed as described in Experimental procedures.

A. Autoscaled relative abundances of a set of reliably identified metabolites are coloured blue (lower abundance) to red (higher abundance). Heatmap rows correspond to the analytes (n = 4–5).

B. Averages of autoscaled signal intensities from each treatment group. Hierarchical clustering with Euclidean distance as similarity measure and Ward's linkage as clustering algorithm was performed using METABOANALYST 4.0

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 1×10^4 GE/200 ng DNA, while the rest of the leaves had '*Ca. L. asiaticus*' load lower than 1×10^2 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 1×10^3 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

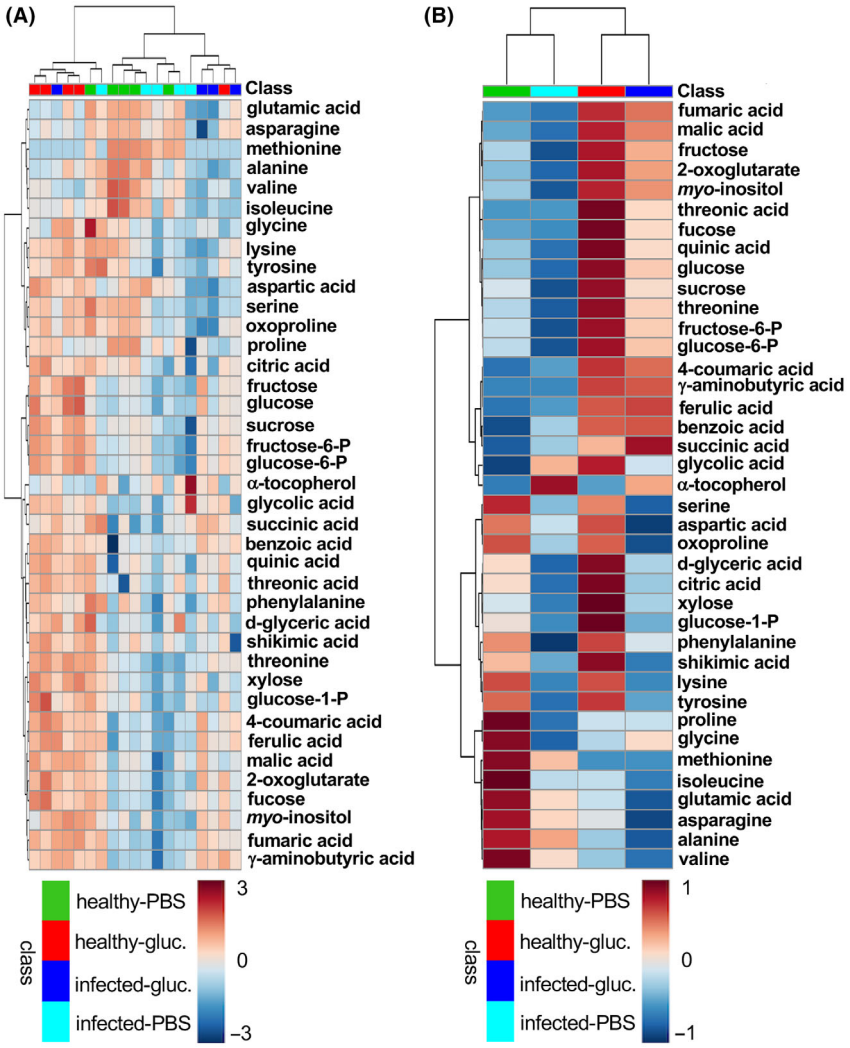

Fig. 3. Primary metabolite profiles in leaves from healthy or '*Ca. L. asiaticus*'-infected plants following incubation with glucose (June). Heatmaps show differences between healthy or '*Ca. L. asiaticus*'-infected citrus leaves harvested in June after 3 days of incubation in the presence or absence of glucose. Sterilized leaf discs were prepared, and primary metabolites extracted and analysed as described in Experimental procedures.

A. Autoscaled relative abundances of a set of reliably identified metabolites are coloured blue (lower abundance) to red (higher abundance). Heatmap rows correspond to the analytes (n = 4–5).

B. Averages of autoscaled signal intensities from each treatment group. Hierarchical clustering with Euclidean distance as similarity measure and Ward's linkage as clustering algorithm was performed using METABOANALYST 4.0.

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 O_2 availability was tested (Fig. 6). While no significant changes in GE were observed after incubation under normoxic (air/~20% O_2) conditions, a 4.6-fold increase in bacterial load was observed between day 0 (d0, $1.9 \times 10^3 \pm 1.9 \times 10^2$ GE) and day 3 (d3, $8.7 \times 10^3 \pm 1.4 \times 10^3$ GE) when leaf discs were incubated in the presence of glucose, and the level of O_2 was reduced to 10% (Fig. 6A and B), suggesting that '*Ca. L. asiaticus*' is indeed a microaerophile. Reducing available O_2 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 ($3 \times 10^3 \pm 5.8 \times 10^2$ GE), leaf discs incubated for

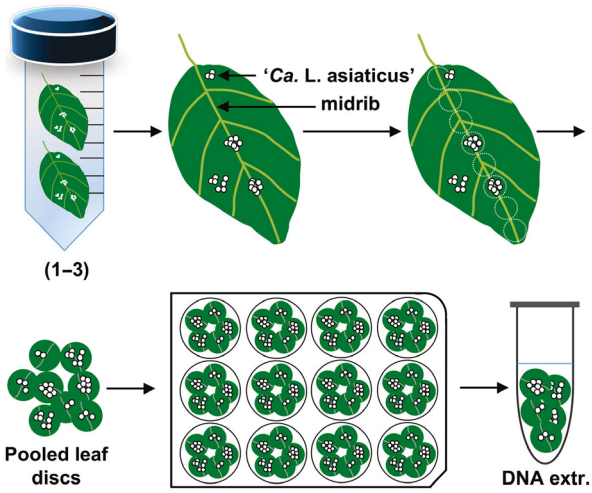

Fig. 4. Schematic of experimental design for analysing '*Ca. L. asiaticus*' DNA replication in leaf discs. Infected leaves were collected from several branches to reduce overall sample variability. Leaves were surface sterilized and rinsed using a 3-step protocol (1 – 70% ethanol; 2 – 10% bleach + 0.01% Tween-20; and 3 – sterile water). After preparation, leaf discs were pooled and divided into groups of five discs to normalize the number of bacteria per sample, necessary to reduce sample variability originating from uneven distribution of '*Ca. L. asiaticus*' in infected tissue. After incubation, samples were processed and analysed as described. White dots indicate uneven distribution of '*Ca. L. asiaticus*' within the leaf.

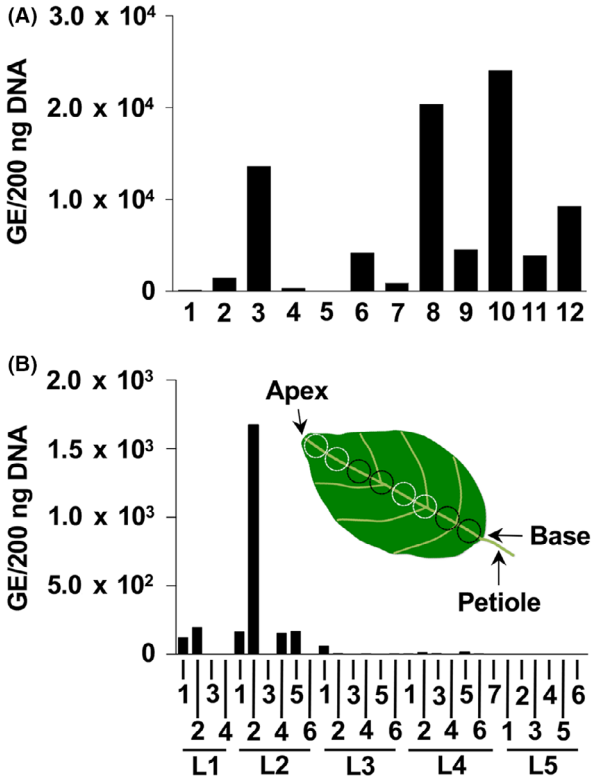

Fig. 5. Distribution of '*Ca. L. asiaticus*' in the shoot and within citrus leaves.
A. Distribution of '*Ca. L. asiaticus*' among leaves collected from different branches. Two leaf discs were collected along the midribs of twelve leaves from different branches and GE measured by qPCR. Data from a representative experiment are shown.
B. Distribution of '*Ca. L. asiaticus*' along the midrib of infected leaves. Five infected leaves (L1–L5) from different branches were tested for distribution of '*Ca. L. asiaticus*' along the midrib. Two leaf discs were prepared by punching discs along the midrib for each section of the leaf from the apex of the leaf to the base (insert). Data from a representative experiment are shown.

three days with 10 mM glucose ($9.5 \times 10^3 \pm 1.4 \times 10^3$ 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, $4.7 \times 10^3 \pm 1.3 \times 10^3$ 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. CD16-03615, 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

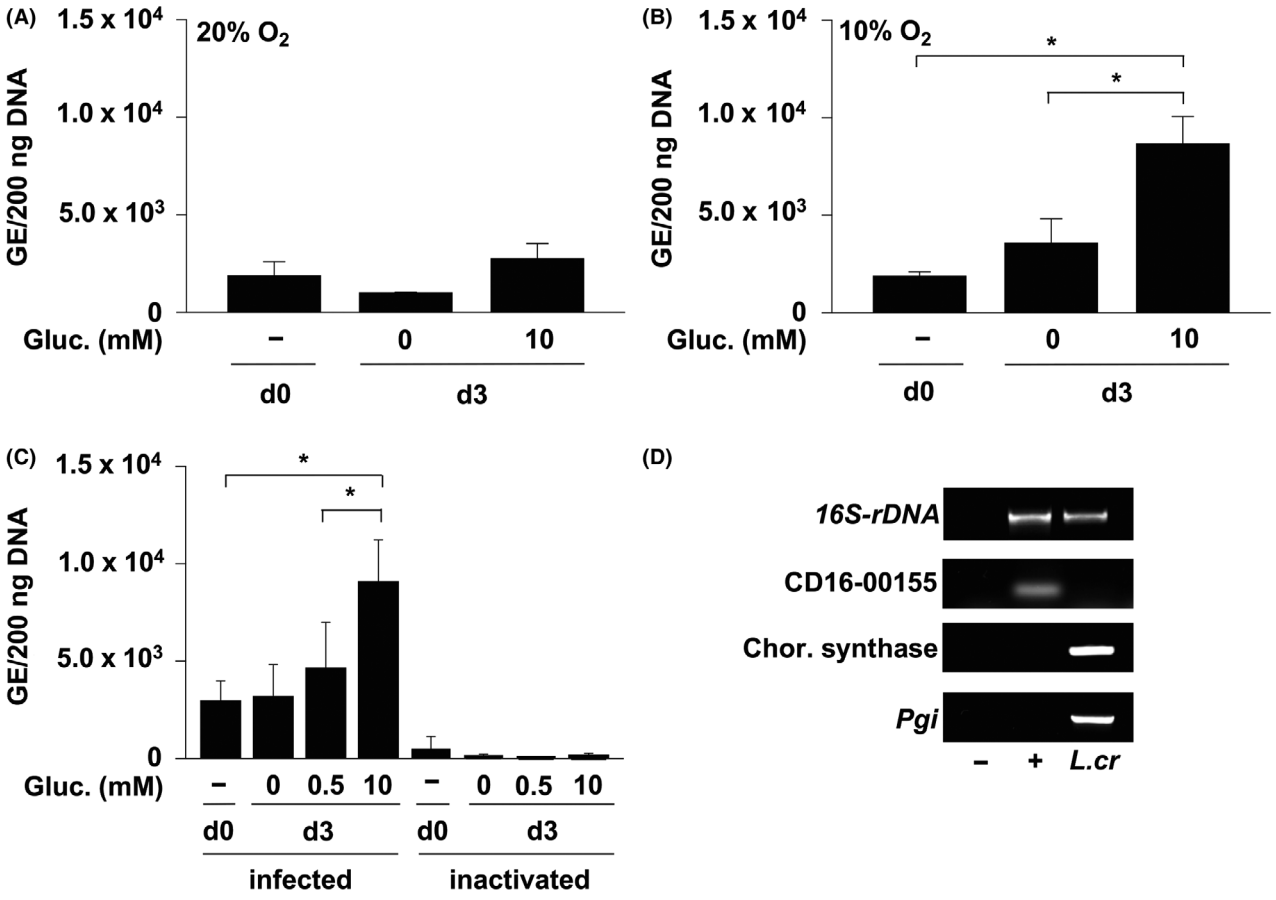

Fig. 6. '*Ca. L. asiaticus*' DNA replication in citrus leaf discs is activated by glucose availability under microaerobic conditions. To determine the capacity of '*Ca. L. asiaticus*' to undergo DNA replication *in situ*, '*Ca. L. asiaticus*'-infected leaf discs were incubated for three days in plain PBS (control) or PBS supplemented with 10 mM glucose under (A) 20% or (B) 10% O_2 and GE measured. (C) The ability of '*Ca. L. asiaticus*' to undergo DNA replication was measured using a dose–response assay. Viable or autoclaved ('inactivated') '*Ca. L. asiaticus*'-infected leaf discs were incubated for three days in plain PBS (control), or PBS supplemented with 0.5 or 10 mM glucose, and GE measured. Autoclaved leaves were included as a negative control. Asterisk denotes a significant difference (one-way ANOVA, Tukey HSD post hoc test) between indicated samples ($P < 0.05$). (D) Confirmation of the absence of *pgi* in '*Ca. L. asiaticus*' by conventional PCR using DNA template extracted from healthy or '*Ca. L. asiaticus*'-infected leaves, and DNA extracted from *L. crescens*.

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, $3.8 \times 10^4 \pm 1.07 \times 10^4$ GE; glucose and amikacin, $1.15 \times 10^5 \pm 2.8 \times 10^4$ GE; Fig. 7) after three days of incubation. Compared to the starting material, an overall 11.1-fold increase in GE was observed (d0, $1.03 \times 10^4 \pm 3.3 \times 10^3$ GE; d3 glucose and amikacin, $1.15 \times 10^5 \pm 2.8 \times 10^4$ 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

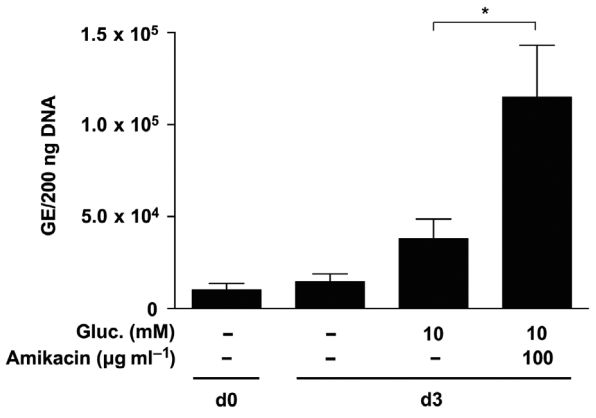

Fig. 7. The effect of amikacin on '*Ca. L. asiaticus*' DNA replication in leaf discs incubated with glucose. To assess the effect of amikacin on '*Ca. L. asiaticus*' DNA replication *in situ*, '*Ca. L. asiaticus*'-infected leaf discs were incubated for three days in plain PBS (control) or PBS supplemented with 10 mM glucose in the absence or presence of 100 µg ml^{-1} amikacin. Data were collected from three independent experiments done with three technical replicates. Asterisk denotes a significant difference (one-way ANOVA test, Tukey HSD post hoc test) among the indicated test groups ($P < 0.05$).

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 glyceraldehyde-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 (Kotrba *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* (Plano and Winkler, 1991; Driscoll *et al*., 2017) and *Chlamydia trachomatis* (Iliffe-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. asiaticis' 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% O_2 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 sinesis* (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 Na_2HPO_4, 1.47 mM KH_2PO_4, 2.7 mM KCl, 136.8 mM NaCl, 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$. 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

(methanol:2-propanol:water, 5:2:2 v:v:v). After adding 1.0 μg of the internal standard salicylic acid-d6 (C/D/N Isotopes, Canada), the material was extracted by shaking at room temperature for 10 min (Vortex) and sonication at room temperature for 10 min (Branson 5510 sonication bath, Branson Ultrasonics Corp, CT, USA). The extracts were then centrifuged for 10 min at 21 000 × *g*, and the supernatants transferred into new vials. The extracts were dried under vacuum. Dry residues were suspended in 500 μl of 50% aqueous acetonitrile and re-extracted as above by sequential vortexing and sonication. The debris was again removed by centrifugation, and the supernatants dried under vacuum. The dry residues were suspended in 10 μl *O*-methoxylamine hydrochloride (30 mg ml^{-1} in pyridine, Sigma, St. Louis, MO, USA) and incubated for 90 min at 30°C and 1000 r.p.m. Subsequently, samples were derivatized with 90 μl of *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) with 1% trimethylchlorosilane (TMCS; Thermo Fisher Scientific) for 30 min at 37°C and 1000 r.p.m. Samples were spiked with a mixture of linear alkanes for the calculation of retention indices. Gas chromatography–mass spectroscopy analysis was performed using a Pegasus 4D time-of-flight mass spectrometer (LECO, MI, USA) equipped with a Gerstel MPS2 autosampler and an Agilent 7890A GC oven. The derivatization products were separated on a 30 m, 0.25 mm i.d., 0.25 μm df Rxi-5Sil® column (Restek, Bellafonte, PA, USA) with an IntegraGuard® pre-column using ultrapure He at a constant flow of 0.9 ml min^{-1} as carrier gas. The linear thermal gradient started with a one minute hold at 70°C, followed by a ramp to 300°C at 10°C min^{-1}. The final temperature was held for 5 min prior to returning to initial conditions. Mass spectra were collected at 70 eV and 17 spectra s^{-1}. The injection port was held at 240°C, and 2 μl of the sample was injected at an appropriate split ratio. After deconvolution and peak alignment, a typical experiment yielded over 800 distinct chemical features/analytes. Peak alignment and spectrum comparisons were carried out using the Statistical Compare feature of the ChromaTOF® software (LECO, MI, USA). Peak identification was conducted using the Fiehn primary metabolite library (Kind *et al*., 2009) with an identity score cut-off of 600. Based on comparison to reference mass spectra, over 200 analytes could be assigned a probable identity, with confirmation of specific compounds accomplished by comparison to an in-house custom library of ~120 authentic standards. Approximately 40 primary metabolites were consistently and reliably detected in all samples and thus included in the final analysis. The internal standard and the initial tissue weight were used for normalization. Statistical analyses with selected metabolites were carried out using METABOANALYST 4.0 (Chong *et al*., 2018).

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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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.

