Detection and Relative Titer of Candidatus Liberibacter asiaticus (Hemiptera: Psyllidae) Vector of Citrus Huanglongbing Disease

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ABSTRACT Candidatus Liberibacter asiaticus (CLas) bacterium has been strongly implicated as the causative agent of huanglongbing (HLB), or citrus greening, which is currently the most devastating citrus disease worldwide. HLB is transmitted by the Asian citrus psyllid, Diaphorina citri Kuwayama (Hemiptera: Psyllidae), in a persistent manner. We used quantitative-polymerase chain reaction (PCR) to detect CLas in dissected organs of individual D. citri adults infected with HLB in the laboratory or collected from field-infected citrus trees in South Florida. The proportion of infected (CLas-positive) dissected organs was 47–70% for the salivary glands, 72–80% for the alimentary canal, and 79–97.5% for the rest of the insect body. Statistical analysis indicated that, in both field- and laboratory-infected D. citri, the proportion of infected salivary glands was significantly lower than that of other parts in the insect body. With field-collected psyllids, the relative copy number of CLas genomes, compared with psyllid genomic DNA in each sample, was significantly higher in both the salivary gland and alimentary canal compared with that in the rest of the insect body for both males and females. These results provide the first PCR confirmation of CLas in the alimentary canal and salivary glands of D. citri and strongly suggest that the salivary glands constitute an important transmission barrier to CLas in the psyllid vector. Our results also suggest that CLas may replicate or accumulate in both the alimentary canal and salivary glands of D. citri.

KEY WORDS huanglongbing, citrus greening, vector relations, transmission barrier, salivary gland

The Asian citrus psyllid, Diaphorina citri Kuwayama (Hemiptera: Psyllidae), is an important pest of citrus (Citrus spp.) in the United States and other parts of the world because it is a vector of huanglongbing (HLB) or citrus greening disease, which is considered one of the world’s most serious diseases of citrus (Hung et al. 2004, Bové 2006, Gottwald 2010). HLB infects various citrus cultivars and causes substantial economic losses by promoting fruit drop, rendering fruit inedible and shortening the life span of infected trees (Miyakawa 1980). There are currently three closely related phloem-limited, nonculturable bacteria associated with this disease that have been provisionally categorized according to the International Code of Nomenclature of Bacteria and named as follows: Candidatus Liberibacter asiaticus (isolates from Asia and the Americas), Candidatus Liberibacter africanus (isolates from Africa), and Candidatus Liberibacter americanus (isolates from Brazil) (Gottwald 2010).

Limited information related to the acquisition and transmission of HLB-associated bacteria by D. citri has been published previously (Capoor et al. 1974, Xu et al. 1988, Xu et al. 2004, Bransky and Rogers 2007). D. citri developing on trees infected by HLB can acquire the pathogen most efficiently during the nymphal stage, and new adults from these nymphs are infected and may immediately be able to transmit the disease (Xu et al. 1988). Uninfected adult D. citri feeding on a diseased tree can acquire Ca. L. asiaticus (CLas) at a much lower percentage than nymphs, as determined by polymerase chain reaction (PCR) tests, but apparently cannot transmit, or transmit at very low frequency (Inoue et al. 2009). CLas persists and/or multiplies in the vector and consequently, after acquiring the pathogen, adults remain infective throughout their life (Xu et al. 1988, Hung et al. 2004). Normally, low HLB transmission rates by D. citri (1.3–12.2%) have been reported previously (Hung et al. 1984, Pelz-Stelinski et al. 2010). However, HLB often spreads quickly in a citrus planting, particularly if the planting is young (Xu et al. 1988). Thus, to understand the epidemiology of this disease, a better understanding of pathogen–vector interactions is essential.
With another phloem-limited bacterium that causes citrus stubborn disease, *Spiroplasma citri*, replication of the pathogen in the leafhopper vector requires crossing the midgut lining; multiplying to high titers in the hemolymph; and subsequently infecting other organs, including the salivary glands. Only after *S. citri* reaches and multiplies in cells of the salivary glands can the pathogen be inoculated by the vector into host plants (Fletcher et al. 1998, Bové et al. 2003). Yet, basic information is lacking on the localization of CLas or other HLB associated bacteria in various tissues of the psyllid vector and on the cellular and molecular interactions affecting vector competence. Although Inoue et al. (2009), using quantitative-PCR (Q-PCR), recently reported that CLas apparently multiplies in the nymphs but not in the adults of *D. citri*, it is not known whether this bacterium just circulates or also multiplies in specific organs and tissues of the vector. The alimentary canal and salivary glands are known as the most important barriers to the transmission of persistently and propagatively transmitted pathogens (Ammar 1994, Gray and Gildow 2003, Weintraub and Beanland 2006, Hogenhout et al. 2008). However, because CLas are nonculturable and thus antibodies to it are difficult to obtain, it has never been previously identified in the alimentary canal, the salivary glands, or other tissues of *D. citri*. Electron microscopic observations of bacteria-like structures were reported in the salivary glands and alimentary canal of *D. citri* and another citrus psyllid *Trioza erytreae* that were exposed to HLB-infected plants (Chen et al. 1973, Moll and Martin 1973, Xu et al. 1988), but no immunolabeling or molecular labeling methods were used to confirm the identity of such bacteria in these studies.

Thus, the purpose of the present investigation was to detect and identify CLas by using Q-PCR in the alimentary canal and salivary glands of *D. citri*, as a step toward a better understanding of vector interactions of CLas with its psyllid vector at the organ and cellular levels, particularly with regard to transmission barriers of this important pathogen.

**Materials and Methods**

**Collecting *D. citri* From Field-Infected Citrus Trees and From HLB-infected Laboratory Colony.** Live adults of the Asian citrus psyllid *D. citri* were collected from ‘Valencia’ orange [*Citrus sinensis* (L.) Osbeck] trees at the USDA citrus grove in Fort Pierce, in east central Florida. A majority of these trees were naturally infected with HLB/CLas, as evidenced by disease symptoms and previous PCR tests (not included here). Psyllid adults were collected by aspiration into glass vials from trees showing symptoms of HLB. Three psyllid samples were taken on 14 October, 16 November, and 14 December 2009 (tests 1–3, respectively; Table 1). In separate tests, we used adult *D. citri* from a laboratory colony that has been maintained for several generations on HLB-infected citrus plants (rough lemon, *Citrus junibara* Lush.). Healthy control *D. citri* adults were taken from a laboratory colony established during 2000 that has been maintained on healthy orange jasmine [*Murraya paniculata* (L.) Jack] trees, and more recently on healthy citrus trees (*Citrus macrophylla* Wester) in the greenhouse (as described by Hall et al. 2007). No wild psyllids are introduced into this colony, and individuals from the colony are PCR-assayed quarterly to ensure the colony remains free of CLas. Both healthy control and field- or laboratory-infected adults were starved (in glass vials) at room temperature for at least 3 h before dissection to help in clearing their gut contents. Subsequent to starvation, in some cases, they also were kept in the refrigerator (at 4°C) for 1–2 d before dissection. However, all adults were still alive when dissected.

**Dissecting *D. citri* Organs and Preparing Them for Q-PCR.** *D. citri* adults were dissected on clean glass slides under a stereomicroscope. The slides were setup by drawing three circles of liquid blocker (PAP Pen, Electron Microscopy Sciences, Warrington, PA) on a slide; each insect was dissected on a separate (circled) part of the slide. Two no. 2 insect pins, fine forceps, and a new razor blade were cleaned with 70% ethanol and used for dissections. A small drop of clear nail polish was placed in the center of the circle on the marked slide, and a live psyllid was placed on top of the wet nail polish dorsal side up. A clean razor blade was used to make an incision on the far right dorsal side of the psyllid, the integument was pulled apart by using two insect pins, and two drops of sterile double distilled water were then placed onto the exposed psyllid’s internal tissues.

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**Table 1. Proportion of CLas-infected (PCR-positive) organs of *D. citri* by using Q-PCR on dissected adults collected from HLB-infected citrus trees in Fort Pierce, FL, October–December 2009**

| Test no. | Date      | Alimentary canal | Salivary glands | Other body parts |
|---------|-----------|------------------|-----------------|-----------------|
|         |           | Male  | Female | All  | Male  | Female | All  | Male  | Female | All  |
| 1       | 14 Oct.   | 5/9   | 6/11   | 11/20 | 6/9   | 7/11   | 13/20 | 6/9   | 5/11   | 11/20 |
| 2       | 16 Nov.   | 9/14  | 14/16  | 23/30 | 4/14  | 5/16   | 9/30  | 13/14 | 14/16  | 27/30 |
| 3       | 14 Dec.   | 13/17 | 12/13  | 25/30 | 9/17  | 5/13   | 14/30 | 15/17 | 12/13  | 27/30 |
| Total   |           | 27/40 | 32/40  | 59/80 | 19/40 | 17/40  | 36/80 | 34/40 | 31/40  | 65/80 |

% infected

| Test no. | Date      | Alimentary canal | Salivary glands | Other body parts |
|---------|-----------|------------------|-----------------|-----------------|
|         |           | Male  | Female | All  | Male  | Female | All  | Male  | Female | All  |
| 1       | 14 Oct.   | 67.5  | 80.0   | 73.75 | 47.5  | 42.5   | 45.0  | 85.0  | 77.5   | 81.25 |
| 2       | 16 Nov.   | 9/14  | 14/16  | 23/30 | 4/14  | 5/16   | 9/30  | 13/14 | 14/16  | 27/30 |
| 3       | 14 Dec.   | 13/17 | 12/13  | 25/30 | 9/17  | 5/13   | 14/30 | 15/17 | 12/13  | 27/30 |
| Total   |           | 27/40 | 32/40  | 59/80 | 19/40 | 17/40  | 36/80 | 34/40 | 31/40  | 65/80 |

% infected

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*a* In each test, eight to 10 healthy control adults were similarly dissected and processed for Q-PCR, and none of their dissected organs proved to be CLas-positive.

*b* Number of CLas-positive organs/number tested.

*c* In test 1 the salivary glands were attached to the head when processed for Q-PCR, but in tests 2 and 3 the salivary glands were completely excised from the head and processed separately (two salivary glands per insect per tube).
The alimentary canal (Fig. 1) is located mainly at the anterior part of the abdomen and the posterior part of the thorax. The filter chamber, midgut loop, and Malpighian tubules (Fig. 1C and D) were carefully separated from the rest of the body and placed in a 1.5-ml tube containing 150 μl of lysis buffer from DNeasy kit (QIAGEN, Valencia, CA), making sure that no other tissues were attached to it. The salivary glands are located in the anterior part of the thorax immediately behind the head (Fig. 1A and B). In the first test (test 1, Table 1) both salivary glands, still attached to the head (for easier handling), were separated from the rest of the body and placed in the lysis buffer tube. But in later tests (tests 2 and 3, Table 1), the salivary glands were carefully and completely excised from the head and processed separately (two salivary glands per insect per tube with 150 μl of lysis buffer). The rest of the body, designated here as “other body parts,” containing mostly the abdomen, including the reproductive organs, along with part of the thorax, was placed into a third tube with 150 μl of lysis buffer. The sex of each dissected individual psyllid was recorded. In each test, organs from 20 to 40 field- or laboratory-infected D. citri adults and eight to 10 healthy control adults were dissected and processed for Q-PCR.

Fig. 1. Gross anatomy and location of the alimentary canal and salivary glands of D. citri, as observed in unstained preparations by stereomicroscopy (A and C) or by confocal laser scanning microscopy of organs stained with the nuclear stain propidium iodide (B and D). Abbreviations: amg, anterior (descending) midgut; asg, accessory salivary gland; cgm, compound ganglionic mass; es, esophagus; fc, filter chamber; h, head; mt, Malpighian tubule; ol, optic lobe; pmg, posterior (ascending) midgut; sg1 and sg2, principal salivary glands 1 and 2; seg, subesophageal ganglion. (Online figure in color.)
DNA Extraction and Q-PCR. We used DNeasy blood and tissue kit (catalog no. 69506, QIAGEN) for DNA extraction. The salivary glands, alimentary canal, and other body parts from a single psyllid were each placed in an impact-resistant 1.5-ml tube that contained enough glass beads to cover the bottom of the tube and 150 µl of lysis buffer. The insect tissues were homogenized using the Fastprep-24 at 4 m/s for 30 s, and then protease K (20 µl) was added to each tube. DNA extraction was carried out according to the manufacturer’s instructions, and the DNA was finally collected in 30 µl of nuclease-free water. The samples were stored at 80°C for further processing.

All samples were quantified by 260/280 spectrophotometry by using a NanoDrop ND-1000 (Thermo Fisher Scientific, Wilmington, DE). The Q-PCR reactions were run on DNA isolated from specified body parts of *D. citri* by using the APHIS-recommended TaqMan protocol and specified primers and probe (Li et al. 2006). Reactions were run in a 96-well format by using a 7500 Fast Instrument (Applied Biosystems, Foster City, CA), and every 96-well run included a known positive psyllid sample and three no template negative control samples. The PCR reaction contained 10 µl of TaqMan Universal PCR Mix (Applied Biosystems). The 16S rDNA forward primer HLB AS used was 5'-TCGAGCCGCTAGTCTCACAC-3', and the 16S rDNA reverse primer HLB R used was 5'-GGCGTTATGGGCTAGAAGAAGGTAAG-3'. The Q-PCR probe HLB P used was 5'-GAG/AGGTTGAAGCTACACCACCC/3' TAMRA. Each reaction contained 2 µl of the DNA. Two samples from each organ/body part per insect were analyzed with Q-PCR in two independent runs, and the average of the two readings was used to determine whether each organ was CLas-positive. With healthy control psyllid adults (*N* = 30), cycle threshold (Ct) values for the alimentary canals, salivary glands and the majority (83.3%) of the other body parts were “undetectable” (>40), but for 16.7% of the other body parts Ct values averaged 37.16 (±0.30). Thus, only Ct values <35 were considered CLas-positive, which has been also adopted previously by Bonani et al. (2010).

Relative Titers of *Ca. L. asiaticus* in Psyllid Organs. In field-collected adults, the relative titer of CLas genomes with respect to the psyllid genome copy number was determined by comparison to Q-PCR results for the *D. citri* Ribosomal S20 psyllid gene (GenBank accession DQ673424). A TaqMan protocol was developed using the following primers and probes: left primer was Dci-S20-L: 5'-GCCCAAGGGCCCAATCA-3', right primer was Dci-S20-R: 5'-GGACCTTACGGGTGGTTATTCTG-3', and FAM-labeled probe was 5'-AATGCCCACCAAAGTT-3'. Reaction conditions were the same as described for Las detection. Relative titer of CLas genomes to *D. citri* genomes was calculated by dividing the Ct value for the psyllid ribosomal S20 gene by the Ct value for the CLas 16S rRNA sequence. Because the Ct value is inversely proportionate to the abundance of the target sequence the ratio had to be inverted (expressed as psyllid Ct/CLas Ct and not CLas Ct/psyllid Ct) to show a ratio representative to relative abundance.

Statistical Analysis. Proportions of organs/body parts (alimentary canal, salivary gland, and other body parts) infected by CLas (PCR-positive) among males, females, and over both sexes were calculated for each of the three tests performed on three independent field samples (Table 1). The mean (±SEM) proportions were calculated over all three tests. Chi-square tests were conducted first on the raw data, and then an analysis of variance (ANOVA) was conducted on proportions of CLas-positive organs (weighted on the number of adults sampled in each test). Means and SEM were computed using PROC MEANS (SAS Institute 2008). ANOVAs were conducted using PROC GLM (SAS Institute 2008), and Tukey’s honestly significant difference (HSD) studentized range test was used for multiple comparisons (*P* = 0.05–0.001).

Confocal Laser Scanning Microscopy of the Salivary Glands and Other Organs. To show the structure and location of the salivary glands and other dissected parts of *D. citri* adults (Fig. 1), dissected parts were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, and 0.1% Triton X-100; washed in phosphate-buffered saline, pH 7.4; and 0.1% Triton X-100 (PBS-T); and stained for 5 min with 3 nM solution of the fluorescent nucleic acid stain propidium iodide (Invitrogen, Carlsbad, CA) before the final wash in PBS-T (Ammar and Hogenhout 2005). They were then mounted on glass slides and examined by an LSM 5 confocal laser scanning microscope (Carl Zeiss, Thornwood, NY).

Results

Location and Gross Morphology of the Alimentary Canal and Salivary Glands of *D. citri*. The gross morphology and location of the alimentary canal in *D. citri* (Fig. 1A, C, and D) are essentially as described by Cicero et al. (2009), but the four “ventricular appendages” described by these authors are termed Malpighian tubules here in accordance with the general anatomy of Hemiptera and other psyllids (Brittain 1923, Chapman 2003). The alimentary canals dissected out and used here for Q-PCR tests included the filter chamber, midgut loop (anterior and posterior midgut), Malpighian tubules, and parts of the esophagus and hindgut (Fig. 1C and D). The principal salivary glands (Fig. 1A and B) are paired, semiopaque, heart-shaped bodies located in the pro- and mesothoracic segments, dorsal to the compound ganglionic mass, whereas the accessory salivary glands are much smaller tubular structures attached to the principal salivary glands antero-laterally (Fig. 1B). The compound ganglionic mass, which represents an amalgamation of the abdominal and thoracic ganglia as described for Hemiptera and other psyllids (Brittain 1923, Ammar 1985, Chapman 2003), is more opaque and much larger than each of the principal salivary glands.

Q-PCR Detection of CLas in Dissected Organs of *D. citri* Collected From Field-Infected Citrus Trees. Q-PCR results of three tests conducted on dissected organs of *D. citri* adults, field-collected between 14...
October and 14 December 2009 are presented in Table 1. Preliminary statistical analyses using a chi-square test or ANOVA on the proportion of infected (CLas-positive) organs indicated no significant differences in this regard between male and female psyllids ($\chi^2$ test: $P < 0.204$–0.505) or between the three tests conducted on psyllid samples collected 14 October, 16 November, and 14 December 2009 (ANOVA: $F = 0.49$–0.58, $df = 2, P < 0.602$–0.645). With pooled data from both sexes ($N = 80$), the mean percentage $\pm S.E.$ of infected alimentary canals was 71.8 $\pm$ 6.6, that of infected salivary glands was 46.9 $\pm$ 6.7, and that of infected other body parts was 78.8 $\pm$ 7.8. ANOVA on the proportion of infected organs, weighted on the number of adults sampled in each test indicated that a significantly lower proportion of the salivary glands were CLas-infected compared with that of the alimentary canal or other body parts ($F = 8.45, df = 2, P < 0.001$), but no significant differences were found in this respect between the alimentary canal and other body parts.

**Q-PCR Detection of CLas in Dissected Organs of Laboratory-Infected D. citri.** In laboratory-infected psyllids, the percentages of infected salivary glands, alimentary canals and other body parts were 70, 80, and 97.5% respectively ($N = 40$). Chi-square analysis indicated that the proportion of infected salivary glands was significantly lower than that of other body parts ($\chi^2 = 11.11, df = 1, P < 0.001$) but not significantly lower than that of the alimentary canal ($\chi^2 = 1.07, df = 1, P < 0.302$). However, the proportion of infected alimentary canal was also significantly lower than that of other body parts ($\chi^2 = 5.999, df = 1, P < 0.014$).

**Relative Titer of CLas in Psyllid Organs.** The titer of detected CLas genomes relative to psyllid genomes in dissected organs/body parts of individual adults of *D. citri* collected from HLB-infected field trees was calculated by dividing Ct values for a ribosomal S20 protein gene present in the nuclear genome of *D. citri* over the Ct value for the CLas 16S rRNA sequence in each sample in tests 2 and 3 (Table 2), where the salivary glands were completely excised from the head before Q-PCR analysis. Pooled data from both tests indicated that the mean relative titer for CLas was 0.9286 in the alimentary canal, 0.9490 in the salivary glands and 0.7714 in the rest of the body ($N = 60$). ANOVA indicated that the relative titer of CLas was significantly higher in both the salivary glands and alimentary canal compared with that in the rest of the body ($F = 18.77, df = 2, P < 0.0001$), with no significant differences between the salivary gland and alimentary canal or between males and females (Table 2).

### Discussion

HLB is a destructive disease of citrus causing great losses in many countries of Asia, Africa, and North and South America (da Graça 1991, Bove 2006, Gottwald 2010). In the United States, China, and some other parts of the world, CLas has been shown to be associated with this disease in both plants and in the psyllid vector *D. citri*, mainly by using PCR methods (Bove 2006, Inoue et al. 2009). Although Koch postulates remain to be completed, Tyler et al. (2009) recently provided evidence, using phloem metagenomic DNA, that strongly suggested that CLas is the main, if not the only, phloem microbe present in plants with severe HLB symptoms in the United States.

Transmission characteristics of HLB in *D. citri* indicate a persistent pathogen–vector relationship (Capoor et al. 1974, Xu et al. 1988, Pelz-Stelinski et al. 2010), which implies that the HLB-causing bacterium circulates, infects and/or replicates, in various organs and tissues of the vector, including the alimentary canal and salivary glands. These two organs are recognized as the most important transmission barriers of persistently and propagatively transmitted plant pathogens, including viruses (Gray and Gildow 2003, Hogenhout et al. 2008, Ammar et al. 2009) and bacteria (Purcell and Nault 1991, Bove et al. 2003, Ammar and Hogenhout 2006, Weintraub and Beanland 2006). However, CLas has never been positively identified previously in the alimentary canal, the salivary glands or any specific tissues of *D. citri*. Furthermore, previous PCR investigations on HLB/CLas were either carried out on plant material (Bove 2006, Li et al. 2009) or on whole insects (nymphs, adults, or eggs) of *D. citri* (Hung et al. 2004, Inoue et al. 2009). Thus, ours is the first Q-PCR study on HLB using dissected organs or body parts of *D. citri* and the first PCR confirmation of CLas in the alimentary canal and salivary glands of this important insect vector.

Using PCR assays on whole insects, Inoue et al. (2009) reported the association between Q-PCR detection of CLas in whole psyllids and the ability of *D. citri* to transmit this bacterium to new plant hosts. Their work showed that the nymphal stage of *D. citri* is much more efficient in acquiring CLas compared with the adults. Rogers (2010) found that 60–100% of

### Table 2. Relative titer of CLas in psyllid organs based on Q-PCR of dissected adults of *D. citri*

| Sex     | No. tested | Alimentary canal | Salivary glands | Other body parts |
|---------|------------|------------------|----------------|-----------------|
|         |            | Mean  | SE        | Mean  | SE        | Mean  | SE        |
| Male    | 31         | 0.9289a | 0.0308 | 0.9630a | 0.0546 | 0.7896b | 0.0240 |
| Female  | 29         | 0.9253a | 0.0319 | 0.9314a | 0.0417 | 0.7518b | 0.0259 |
| Overall | 60         | 0.9256a | 0.0250 | 0.9490a | 0.0351 | 0.7714b | 0.0176 |

In each row, means followed by different letters are significantly different ($P < 0.001$).

* Ratio represented as Ct of *D. citri* S20 gene/Ct of CLas 16S rRNA gene.
D. citri nymphs acquired CLas from diseased plants, whereas acquisition by adults reached only 40% after 5 wk of feeding on infected plants. In our study, the majority of the alimentary canals and other body parts tested were CLas-positive (72–79%), which suggests that the field-collected adults must have been feeding on infected trees at the nymphal, rather than the adult, stage. The ratio of infected organs did not differ significantly between the three groups of psyllid adults collected in three sampling dates (Table 1). In our study using laboratory-infected D. citri that acquired CLas during the nymphal stage, a very high proportion of infected alimentary canals and other body parts (80–97.5%) also were obtained. Previous studies on persistently transmitted bacterial and viral pathogens indicate that the insect alimentary canal/midgut is normally the first site of infection in the vector, from which the pathogen may enter the hemocoel and reach the salivary glands through the hemolymph or other tissues (Purcell and Nault 1991, Fletcher et al. 1998, Bové et al. 2003). Thus, the most important transmission barriers reported (suggested) have been: midgut-infection, midgut-escape (or exit), salivary gland-infection, and salivary gland-escape barriers in the vector (Ammar 1994, Hogenhout et al. 2008). Our studies indicate that, in D. citri samples collected from field-infected citrus trees, the proportion of CLas-infected salivary glands was significantly lower than that of infected alimentary canals or other body parts. Using laboratory-infected D. citri confirmed that the proportion of infected salivary glands were significantly lower than that of other body parts, but the proportion of infected alimentary canals was also significantly lower than that of other body parts, although somewhat higher than that of the salivary glands. Together, these results suggest that both the salivary gland and the alimentary canal constitute barriers to CLas infection or transmission in the psyllid vector, as they do in other pathogen–vector systems (Hogenhout et al. 2008), but the psyllid salivary glands seem to be more important in this regard compared with the alimentary canal. Although our analysis included only Q-PCR–based detection of the bacterium, previous work showing a strong correlation between Q-PCR–based detection in whole psyllids and transmissibility of HLB disease (Inoue et al. 2009, Rogers 2010) indicates that even if we are detecting some free bacterial DNA, it is probably associated with a higher abundance of viable pathogenic bacteria.

Interestingly, even though the proportion of CLas-infected salivary glands was generally lower than the proportion of infected alimentary canals or other body parts, the relative titer of CLas was significantly higher in the salivary glands and alimentary canals than in the rest of the body. The similarly high relative titer of CLas in the alimentary canal and salivary gland is intriguing and indicates replication, accumulation, or both in the alimentary canal and salivary gland cells. However, further evidence is required before we can suggest replication rather than accumulation of CLas in these two organs of the psyllid vector.

Our results are consistent with previous reports in which the rate of HLB transmission to citrus plants by individual D. citri has been usually low (1.3–16%), even though much higher percentages of psyllids were infected when assayed by PCR (Huang et al. 1984, Pelz-Stelinski et al. 2010). This is also true for several other persistently transmitted pathogens, including bacteria and viruses, where the rate of transmission is much lower than that of infected vectors (Bové et al. 2003, Hogenhout et al. 2008). However, among HLB-infected D. citri, the proportion of individuals with infected salivary glands and their transmission rates could vary depending on the age structure of a population, environmental conditions, and other factors.

Specific interactions between pathogen surface proteins and receptors in the midgut, salivary glands, or other vector tissues have been reported in other pathogen–vector systems, e.g., rhabdoviruses and bunyaviruses (Ullman et al. 2006, Ammar et al. 2009), Spiroplasma and other bacteria (Fletcher et al. 1998, Yu et al. 2000, Bové et al. 2003, Weintraub and Beanland 2006, Killiny et al. 2006, Suzuki et al. 2006), and Plasmodium (Ghosh et al. 2001). These interactions are thought to be major determinants of the specificity with which such pathogens are transmitted only by certain species, races, or developmental stages of their insect vectors (Ammar 1994, Bové et al. 2003, Weintraub and Beanland 2006). One of the suggested and innovative ways to combat such diseases is to find and block such receptors in the midgut, salivary glands, or other tissues of the vectors (Ghosh et al. 2001, Hogenhout et al. 2008). Our results show that because there is a much lower rate of infection of the salivary glands with CLas compared with the rest of the body, there is a significant barrier that could be an excellent point of interdiction for blocking the circulative movement of the bacterium within the vector. Thus, further work is needed on the pathogen–vector interactions of HLB-causing bacteria at the cellular and molecular levels to find new ways to combat this serious and threatening disease in citrus around the world.

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