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Internal Extracellular Bacteria of *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae), the Asian Citrus Psyllid

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Abstract The Asian Citrus Psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae), is an invasive insect pest that transmits *Candidatus Liberibacter* spp. This insect/pathogen system was first identified in North America in the early 2000's and has become the top threat to the citrus industry. Limited options for management of this problem exist; therefore, innovative pest management strategies are being developed. In this study, we describe the first step toward a paratransgenic approach (also referred to symbiotic control) for control of the insect vector or the pathogen. Culturable bacteria from the gut of Asian Citrus Psyllids were identified using standard culture techniques followed by sequencing of the cultured microorganisms. Further, 454 pyrosequencing of the gut was performed to audit bacterial presence in order to begin to identify any relationship between psyllid symbionts and *C. Liberibacter* spp.

Introduction

Diaphorina citri Kuwayama (Hemiptera: Psyllidae), the Asian Citrus Psyllid, is a vector of the phloem-limited bacterium *Candidatus Liberibacter* asiaticus (Ca. Las). Ca. Las is considered to be the etiological agent of Huanglongbing (HLB), a devastating disease of citrus. *D. citri* have been shown to transmit Ca. Las in both nymphal and adult stages [18, 29] yet microorganisms are suspected to grow primarily in the adult stage [4, 42] especially in the salivary glands and alimentary canal [1]. Growth of Ca. Las in the adult stage, thus, increases the vector capacity of *D. citri* and concurrently, the transmission efficiency of *Ca.* Las.

Some aspects of the transmission efficiency of *D. citri* remain undefined. For example, when a microorganism is acquired by *D. citri* through feeding, such as Ca. Las, do indigenous microbiota affect the acceptance and/or growth of the newly acquired microorganism? Some evidence exists to support this question [14] with a strong positive correlation determined between *Wolbachia* and Ca. Las, for example. Several reports also exist on other host responses to the introduction of allochthonous microorganisms for both invertebrate and vertebrate organisms (i.e., [17, 38, 40]). The responses are variable and often complex (i.e., [31]); however, in some cases, they can be predicted by knowing the composition of the indigenous microbiota [34, 37]. This type of information would be useful for understanding the role of *D. citri* for Ca. Las transmission (i.e., [26]), and ultimately, HLB management.

The aim of the work was twofold: (1) to acquire culturable bacteria that can be used in studies to begin to understand more about the microbial ecology of *D. citri* particularly as it relates to *Ca. Las* establishment and transmission, and (2) to acquire potential candidate bacteria that can be used for paratransgenic control of *Ca.* Las and management of HLB disease. While numerous reports exist on bacteria that inhabit Ca. Las (i.e., [16, 24, 33, 35]), herein we report on a survey of *D. citri* for the presence of

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aerobic and anaerobic bacteria using both culture and culture-independent approaches. Our intent is to add to what is currently known about the internal microbial inhabitants of *D. citri* paying close attention to any variability that may exist with the gut community. While the work is basic in nature, our work directly relates to finding suitable microorganisms that could be applied in a para-transgenic strategy to control HLB.

Materials and Methods

Psyllids

Dead *D. citri* adults ($N = 227$) were shipped on ice, overnight from the USDA-ARS Horticultural Laboratory in Fort Pierce, Florida to California State University where they were processed the day of receipt, or kept at 0 °C until processed, generally within 24 h. *D. citri* were surface sterilized using a three-step chemical soak described in Lauzon et al. [20]. Whole abdomens or individual gut samples, fore-, mid-, and hindgut, were surveyed for bacterial inhabitants.

Culture Techniques

Abdomens and individual gut samples were individually and aseptically placed into pre-sterilized Reasoner's 2 (R2) broth (Difco Laboratories, Detroit, MI) and incubated at 24 °C for 24 h or when turbidity was observed. Individual cultures were streaked for isolation and, isolated, pure colonies of aerobic bacteria were identified using standard biochemical and morphological tests (Bergey's 2002) in companion with API analytical strips (BioMerieux, Durham, NC). Samples were also streaked directly onto pre-reduced anaerobically sterilized media (Hardy Diagnostics, Santa Maria, CA) and incubated in an anaerobe chamber held at 24°–26° for 48 h or when visible growth presented. Pure cultures were subsequently streaked onto R2 agar and incubated overnight under air to verify anaerobic requirement. All isolates were also identified using biochemical and molecular techniques, the latter described below.

Culture-Independent Techniques for Identification of Isolated Bacteria

Bacterial DNA was isolated from 18 h-old pure cultures from abdomens and individual gut samples using the DNeasy kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. DNA was quantified using a UV-spectrophotometer (Genesys 8, Thermo Scientific, Rochester, NY) and amplified using polymerase chain reaction under the following conditions:

Two sets of primers were used to amplify regions specific for almost all bacterial 16S sequences. A region of approximately 1,500 bp from the 16S rRNA gene was amplified using the primers B27F (5′AGAGTTTGA TCMGGC TCAG-3′) and 1492R (5′-GGTTACCTTGT-TACGACTT-3′) [19]. Each 25-μL PCR mixture contained 10 ng DNA, 2X PCR Master Mix (Promega, Madison,WI), and primers. The amplification cycle consisted of an initial denaturation step of 2 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at 55 °C and 1 min at 72 °C, and a final extension step of 7 min at 72 °C. Template DNA was omitted from the reaction mixture to serve as a negative control.

Five μL of each suspension was electrophoresed on 1 % agarose gels in 1X TBE buffer, which were then stained with ethidium bromide and examined under UV light for visualization of PCR products. Five μL of PCR products were subjected to an ExoSAP-IT kit (Affymetrix, Inc) at 37 °C for 15 min followed by 80 °C for 15 min, to cleave excess primers and inactivate free nucleotides. Cleaned PCR products were subsequently used as the template for sequencing.

DNA from all samples was subjected to a BigDye V3.1 Terminator Kit (Applied Biosystems, Foster City, CA), and the reactions were run in a PTC-200 Thermal Cycler (MJ Research, Waltham, MA). Conditions were initial denaturation at 96 °C for 1 min, followed by 25 cycles at 96 °C for 10 s, 50 °C for 5 s, 60 °C for 4 min. X-Terminator Mix (Applied Biosystems, CA, USA) was added, and DNA was sequenced using a 3130 Genetic Analyzer (Applied Biosystems, CA, USA).

Peak Scanner™ Software v1.0 (Applied Biosystem, CA, USA) was used to view the sequence peaks, and each sequence was run in a BLAST search limited to a bacterial database. Bacterial DNA from pure cultures was identified by examining the top-scoring sequences from the BLAST search results.

Culture-Independent Techniques for Determination of Bacterial Communities Using 454-Pyrosequencing

Bacterial DNA was recovered from *D. citri* and *Bactericera cockerelli* (another psyllid that is known to transmit *C. Liberibacter* spp.) using the Qiagen DNeasy Blood and Tissue Kit (Qiagen). *B. cockerelli* was used as an out-group in this study to determine the relative effect of *C. Liberibacter* spp. presence on the bacterial community within a host insect. This insect was included to see whether the presence of *Ca.* Liberibacter could have an effect on

indigenous microbiota of more than just ACP. Insect DNA was particularly screened for *C. Liberibacter* spp. using conventional PCR protocol modified from Hung et al. [18]. This focus aimed to determine if any noticeable changes in the bacterial presence existed between *C. Liberibacter* spp.+ and *C. Liberibacter* spp.− pysllids. Twenty-five μL reactions included 12.5 μL Ampli Taq Gold 360 master mix (Applied Biosystems, Foster City, CA), 1.0 μL GC enhancer (Applied Biosystems, Foster City, CA), 1 μL of each Las-specific primer, 2 μL of extracted DNA, and 7.5 μL of nuclease-free water. PCR products were verified by agarose electrophoresis. Six samples of each species were chosen, three positive for *C. Liberibacter* spp. and three negative. These samples were subjected to sequencing of the bacterial 16 s small ribosomal subunit using Roche 454 amplicon pyrosequencing as described in Dowd et al. [11–13]. and Hail et al. [15]. Sequencing was performed at the Research and Testing Laboratories in Lubbock, Texas.

Bacterial sequencing data were analyzed using the QIIME Microbiome Pipeline [9]. Raw reads were screened for chimeric sequences and they were removed; remaining sequences were split by sample and the barcodes and primer sequences were removed. Operational taxonomic units (OTUs) were then chosen using the Uclust method, grouping 97 % similar sequences as one reprehensive sequence. Taxonomy was then assigned to OTUs using the ribosomal database project (RDP) 16 s database [41], and taxonomy summary plots were generated. Multiple rarefactions were used to calculate alpha diversity using the Cho1 Test, and a random sample size of 4,000 sequences was chosen. Beta diversity and Jackknifed Beta diversity were calculated from which weighted (copy number of OTU taken into account) and unweighted (copy number of OTU not taken into account) bootstrapped phylogenetic trees were generated [3, 27].

Results

Two hundred and twenty seven ACP reared previously on citrus were sampled on two different dates. Gram-positive bacteria isolated included members of Bacillales, *Lysinibacillus* sp., *Paenibacillus* sp., and *Bacillus cereus*, *Staphylococcus saprophyticus,* and, *Streptomyces* sp., a filamentous bacterium in the Actinomycetales. Gram-negative bacteria were identified as Enterobacteriales, *Enterobacter* spp., and *Pantoea agglomerans*, and Pseudomonales, *Pseudomonas putida, Chryseomonas luteola*, *Alcaligenes xylosoxidans denitrificans*, and *Acromobacter* sp. (Tables 1 and 2). The identities of pure isolates of these bacteria were confirmed using standard morphological and biochemical tests and culture-independent methods. No anaerobic bacteria were isolated from *D. citri.*

Table 1 Culturable bacteria isolated from *D. citri* reared on citrus ($N = 227$)

Gram-negative species	Gram-positive species
Alcaligenes xylosoxidans	*Bacillus cereus*
Chryseomonas luteola	*Paenibacillus* sp.
Enterobacter aerogenes	*Lysinibacillus* sp.
Enterobacter cloacae	*Staphylococcus saprophyticus*
Enterobacter gergoviae	*Streptomyces* sp.
Pantoea agglomerans	
Pseudomonas putida	

Table 2 Culturable bacteria from *D. citri* on two different sampling dates

Trial 1 $N = 152$	Trial 2 $N = 45$
Alcaligenes xylosoxidans	*Alcaligenes xylosoxidans*
Bacillus cereus	
	Chryseomonas luteola
Enterobacter cloacae	*Enterobacter cloacae*
	Enterobacter aerogenes
Lysinibacillus sp.	
Paenibacillus sp.	
Pantoea agglomerans	*Pantoea agglomerans*
Pseudomonas putida	*Pseudomonas putida*
	Staphylococcus saprophyticus
Streptomyces sp.	

Sequencing using the 454 Pyrosequencing platform revealed the presence of the most populous bacterial genera in all ACP and *B. cockerelli* samples, respectively, as *Wolbachia*, *Ralstonia*, and members in the family Tremblayaceae, all of which are α-proteobacteria and β-proteobacteria. A *Staphylococcus* sp. was found in ACP in low numbers. Actinomycetales were not observed. Pseudomonales' one OTU was called to the *Pseudomonas* genus and was present in ACP. One member of the Enterobacteriales was found to be present in small numbers in ACP. In both weighted and unweighted phytogenetic analysis, insects that contained *C. Liberibacter* spp. had a marked impact on bacterial community analysis. More diverse microbial inhabitants were found in *D. citri* in the presence of *Ca.* Las. The amount of *Wolbachia* spp. in both *D. citri* and *B. cockerelli* is greater in insects infected with *Ca.* Las (Fig. 1).

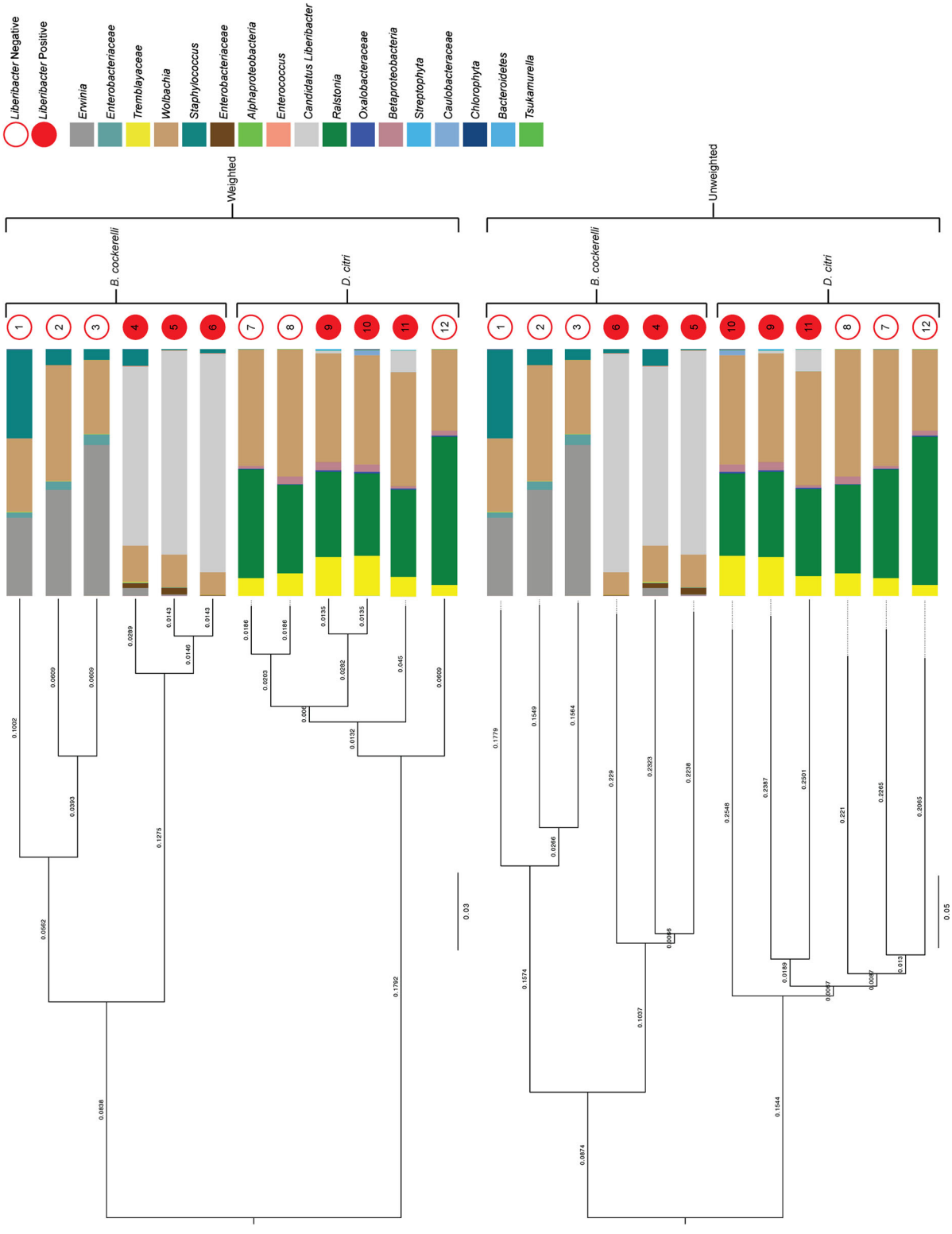

Fig. 1 Weighted and unweighted phylogenetic trees based on bacterial diversity of *Candidatus Liberibacter* infected and non-infected potato psyllid (*Bactericera cockerelli*) and Asian citrus psyllid (*Diaphorina citri*). *Jackknifed trees* are shown with branch distance values. Percentage of overall community composition in each sample is shown in histograms. *Each color* reflects an OTU, classifications are as specific as is available. *Solid dots* denote *Liberibacter* positive insects, and *empty dots* negative samples. *Numbers* in the *circles* denote the sample identity

Discussion

Diaphorina citri reared on citrus-harbored bacteria typically described as citrus endophytes [2, 5] and insect symbionts, such as *P. agglomerans* [10, 21, 22] and *A. xylosoxidans denitrificans* [6]. Our findings were similar to those reported by others [24, 30, 36]; however, our findings were biased due to the focus on obtaining culturable bacteria, and the limitations of basic DNA extraction, amplification, and sequencing based on 16 s rRNA. To get a more complete description of the microbiota of *D. citri*, Sagaram et al. [30], for example, found that PhyloChip analysis was more comprehensive in terms of microbial identifications than 16S rRNA gene clone library sequencing.

We focused our survey on culturable bacteria due to our parallel interest in finding suitable candidates for paratransgenic control of HLB. Two bacterial species, *Alcaligenes xylosoxidans* [6] and *P. agglomerans* [28, 39] have already been considered as candidates for paratransgenesis. Other microbial surveys of *D. citri* reported bacterial species, including intracellular [14, 24] and anaerobic bacteria [15], both groups often refractory to culture or use in the field. Our survey did not yield any anaerobic cultures which can be explained by the typical physiological state of many bacteria when removed from their habitats that make them difficult to culture [25, 32]. This may be true for other bacterial species, including aerobes and facultative anaerobes residing within *D. citri.* For this reason, paired studies (culture-based surveys and culture-independent surveys) are key in fully exploring bacterial communities.

As researchers begin to unravel the epidemiology of HLB disease, microbe–microbe interactions are also beginning to emerge as important areas of study and may provide opportunities for exploitation. Trivedi et al. [36] found that *Ca. Las* influences microbial communities in citrus and our findings are in line. Both *D. citri* and *B. cockerelli* displayed shifts in their gut microbiota in the presence of *Ca.* Las. For our purposes, shifts should be examined to assess the establishment and/or efficiency of an introduced bacterium (PTG agent) in the presence and absence of *Ca.* Las. In turn, microbes in *D. citri* may influence *Ca. Las* establishment and/or transmission. This notion is supported by the findings of Boissière et al. [7] whose findings suggested that mosquitoes harboring members of the Enterobacteriaceae are more likely to be infected by *Plasmodium falciparum.* Often, the focus of insect-symbiotic associations is on how the insect selects its symbionts; however, microorganisms also have a "choice" on their host [23] and influence each other in terms of growth and expression of virulence factors.

Host plant endophytic composition may be the first important factor in influencing *Ca. Las* transmission efficiency by indirectly seeding *D. citri* with bacteria that affect *Ca. Las* establishment and transmission. While speculative in nature, evidence does exist that support this notion (i.e., [7, 8, 30, 36]). If the case, the vector capacity and transmission efficiency for *Ca. Las* may be able to be manipulated artificially either using host plant endophytes or insect symbionts. An endophyte that cycles between and among *D. citri* and plants, such as that exhibited by *Homalodisca coagulata*, the glassy winged sharpshooter and *A. xylosoxidans denitrificans* [6], and that is associated with inhibition of *Ca. Las* would be ideal.

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Conflict of interest The authors declare that they have no conflict of interest.

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