6.6 Endosymbiotic microbiota of Asian Citrus Psyllid (*Diaphorina citri*)

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Huanglongbing (HLB), known in the U.S. as citrus greening disease, is one of the most severe diseases of citrus across the U.S., South America, Asia and Africa. The disease is thought to be associated with infection by an unculturable, gram-negative, phloem-limited bacteria, *Candidatus* Liberibacter spp., which belongs to the α-subdivision of the *Proteobacteria*. Huanglongbing is a threat to the world citrus industry. The disease is transmitted to healthy plants during feeding by the Asian citrus psyllid, *Diaphorina citri* (Hemiptera). Efforts to reduce *D. citri* include insecticides, biological control agents, and more recently efforts to develop RNAi based strategies against the host of bacteria associated with the psyllid and disease. Insects such as psyllids within the Hemiptera, feed from the phloem of plants ingesting a diet which includes rich carbohydrates but which is deficient in essential amino acids. Like most Hemipterans, these insects support maternally inherited bacterial mutualists, referred to as endosymbionts. The endosymbionts live within specialized host cells (bacteriocytes) and are generally thought to supplement their host's diet. It was reported *Buchnera aphidicola* synthesizes the vitamin riboflavin, which is essential to the aphid host's growth. In *D. citri*, five types of nucleotide sequences related to endosymbionts were determined, however some of secondary endosymbiont may not occur in all individuals. Knowledge of the bacteria community in *D. citri* will provide information for the production of novel strategies to manage or reduce *D. citri* by manipulation of its endosymbionts. In this study we have extended these studies by an analysis of rDNA from *D. citri*. In order to investigate the endosymbiotic microbiota of *D. citri*, we analyzed eubacterial 16S-23S rDNA amplified from *D. citri* feeding from citrus. We have used DNA sequence characterization to explore the microbial communities in psyllids. As many as eight endosymbiotic or gut fauna bacteria were identified.

Materials and Methods

DNA extraction, PCR and cloning rDNA. Whole DNA of psyllids was extracted by AquaPure Genomic DNA kits (BioRad, Hercules, CA, USA). Eubacterial 16S rDNA was amplified by PCR using primers 16SF and 16SR as described by Munson et al (Munson et al, 1991). A Eubacterial 16S rDNA and a partial 23S rDNA were amplified with 10F and 480R from Hansen et al (2007). These primers were selected because they do not amplify other psyllids (*G. brimblecombei*) primary endosymbiont, *Candidatus* Carsonella ruddii, an obligate endosymbiont that is reported to occur in all psyllid individuals (Hansen et al., 2007). PCR was conducted with Platinum PCR® SuperMix (Invitrogen,Carlsbad, CA, USA). The PCR product was cloned TA-cloning vector (Invitrogen, Carlsbad, CA, USA) and *E. coli* using ampicillin and X-gal blue-white selection system. White colonies were subjected to plasmid extraction and sequenced.

Results and Discussion

Previously, the genetic sequences were examined from adult psyllids collected from citrus trees. In these sequences, we successfully identified the psyllid primary endosymbiont, *Candidatus*

Carsonella ruddii. To avoid amplifying this primary endosymbiont bacteria, two pair of primers, 16S and a pair of 10F, 480R were chosen to detect novel endosymbiont bacterial sequences (Munson et al, 1991, Hansen et al., 2007). Almost the entire length of 16s rDNA was successfully amplified from the total psyllid DNA. A total of 47 cloned 1.5 kb DNA fragments were sequenced and subjected to homology searches in the DNA databases (NCBI). Sequence homology results found 40 of the 47 were 100% homologous to a *Syncytium* endosymbiont of *D. citri* (accession number EF433792) and seven were 100% homologous to *Wolbachia* endosymbiont of *D. citri* (accession number EF433793). The 2.5 kb of PCR product obtained with primer pair 10F, 480R contained six major types of sequences (A-E). These sequences were subjected to homology search in DNA databases (Table 1). The A-type and E-type sequences were members of the β-Proteobacteria, closely related to *Janthinobacterium* IC161 (99 %) and *Oxalobacteraceae* (97- 98 %). The B-type sequence was a member of the γ-Proteobacteria, closely related to *Escherichia* (96 %) and *Shigella* (95 %). The C-type sequence was a member of the γ-Proteobacteria, closely related to *Alkanindiges illinoisensis* (97 %). The D-type sequence was a member of the γ-Proteobacteria, closely related to *Acinetobacter* sp. (95 %) and *Alvinella pompejana* symbiont APG130A (97 %). *Acinetobacter* sp. are widespread in nature and can be obtained from water, soil and living organism. *Acinetobacter* are known to be involved in biodegradation of a number of different compounds (Abdel-El-Haleem, 2003). The F-type sequence was a member of the β-Proteobacteria, closely related to *Nitrosospira multiformis* (91 %). Biological functions of the endosymbionts of psyllids have not been investigated. Here we provide a first report of functional bacterial homologies from *D. citri* microbial data: such as Carbazole degrading bacteria, *Janthinobacterium* sp. IC161 (99%), ammonia-oxidizing bacterium, *Nitrosospira multiformis* ATCC25196 (CP000103) (91%), multidrug resistant bacteria, *Acinetobacter baumannii* ACICU(CP000863) (95%). These data suggest psyllids are supported by many endosymbiotic bacteria and various types of interactions between the *Ca.* Liberibacter species and other endosymbionts which occur in psyllids.

In summary, citrus greening is one of the most severe diseases of oranges in Asia and Africa, caused by an unculturable, gram-negative, phloem-limited bacteria, *Ca.* Liberibacter spp., belonging to the α-subdivision of the *Proteobacteria*. The disease, called citrus greening disease in the U.S. and known as Huanglongbing world-wide, was recently discovered in Florida, 2005. The disease is transmitted from infected to healthy plants by insects known as the Asian citrus psyllid, *Diaphorina citri* (Hemiptera) which are found throughout citrus in Florida. One strategy to manage citrus greening is aimed at suppression of psyllid populations. Insects such as psyllids within the Hemiptera, feed from the phloem of plants ingesting a diet which includes rich carbohydrates but is deficient in essential amino acids. These insects support maternally inherited bacterial mutualists, referred to as endosymbionts. The endosymbionts live within specialized host cells (bacteriocytes) and are generally thought to supplement their host's diet. In order to investigate the endosymbiotic microbiota of psyllids, we analyzed eubacterial 16S-23S rDNA amplified from *D. citri* and identified transcripts from a cDNA psyllid library made from field-collected Asian citrus psyllids feeding from citrus. These data suggest psyllids are supported by several endosymbiotic bacteria and live with a rich bacterial fauna of various types all of which may have important interactions between each other including Liberibacter asiaticus when it occurs in psyllids.

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Table 1. Endosymbiotic microbiota of Asian Citrus Psyllid (*Diaphorina citri*)

	Group	Function	Homology
Syncytium endosymbiont of *D. citri* (EF433792)			100%
Wolbachia endosymbiont of *D. citri* (EF433793)	Alphaproteobacteria		100%
Janthinobacterium sp. IC161	Betaproteobacteria	Carbazole degrading bacteria	99%
Oxalobacteraceae	Betaproteobacteria		97- 98 %
Escherichia	Gammaproteobacteria		96 %
Shigella	Gammaproteobacteria		95 %
Alkanindiges illinoisensis	Gammaproteobacteria	alkane-degrading strain	97%
Acinetobacter sp.	Gammaproteobacteria	multidrug resistant	95 %
Nitrosospira multiformis	Betaproteobacteria	ammonia-oxidizing bacterium	91%