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ON HUANGLONGBING



**Session 4:**  
**Pathogen Genome  
and Sequencing**

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#### 4.1 Evaluation of potential pathogenicity genes identified by genomic sequencing of *Ca. Liberibacter asiaticus*.

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Huanglongbing (HLB), also known as citrus “greening” is a lethal disease of citrus that is now found in every county where citrus is grown in Florida. Based primarily on 16S rDNA sequence analysis, HLB is associated with at least three different species of *Candidatus Liberibacter*. None of the candidate species have been cultured; consequently Koch’s postulates have not been completed. The titer of these phloem-limited bacteria is so low that the genome size has not been estimated and < 25 kb of nonredundant genomic DNA has been publically available. Nearly all PCR tests to confirm HLB are based on two genomic regions, and phylogenetic information relevant to strain identity has not been assessed.

**Curation.** Using dodder transmission, we have continuously transmitted HLB from a single infected Florida citrus tree to citrus and periwinkle for two years; by PCR, this HLB “strain”, UF506, is associated with *Ca. L. asiaticus* (Las). We developed a DNA extraction protocol that enriched for Las and greatly reduced chloroplast and mitochondrial DNA contamination (as determined by PCR) and used multiple displacement amplification (MDA) to obtain sufficient DNA for shotgun library sequencing. To date, 14,000 sequencing reactions from this library have resulted in the identification of > 68 kb of new Las DNA sequence in the 15 largest contigs. These contigs have been validated by PCR primers against at least two plant sources (citrus and periwinkle). One of the contigs, currently 7.2 kb in size, completely encompasses and extends the existing 988 bp singlet for DNA polymerase from Las (GenBank M94320). Twenty new primer sets have been developed and validated against Florida Las samples; some primers revealed potential phylogenetic differences between the Florida and one Brazilian Las samples tested.

**New Las genomic sequence.** A total of 1,127 contigs of size > 1 kb was obtained from the 14,000 shotgun library reads. The largest 100 contigs, ranging in size from 1.2 to 17 kb, totaling 224 kb of DNA, were analyzed by comparisons against the GenBank nonredundant database. These contigs fell into the following categories:

"Las"	Other bacterial	Plant nuclear	Chloro	Mito	Other	Mis-assembly	Total (kb)
82 kb	23 kb	71 kb	26 kb	11 kb	3 kb	7 kb	217 kb
38%	11%	33%	12%	5%	1%	3%	100%

Those contigs that appeared to be plant nuclear DNA, chloroplast, mitochondrial or bacterial with >50% GC content were eliminated from further consideration. Of the 82 kb of presumptive “Las” DNA, 68 kb was confirmed to be authentic UF506 DNA by PCR. Additional primers were subsequently designed in different regions of several larger contigs, and large PCR products were confirmed to be authentic Las assemblies by hybridization against both Las-infected and uninfected citrus and periwinkle. The % GC content of these contigs varied from 35 - 44%.

Preliminary examination of presumptive ORFs from the largest assembled contigs were identified by BLASTX. The majority of ORFs from these larger contigs appeared to be either phage related or hypothetical unknowns, indicating a bias in our metagenomic library, and possibly the existence of a prophage or plasmid associated with Las. However, one of these

showed similarity to an RTX (repeats in toxin) family effector. As a result of our work with *Xylella fastidiosa* (Xf), we knew that RTX proteins can be involved in host-specific symptom elicitation as a result of Type I secretion (Gabriel, 2008). Type I secretion systems are used for defense against plant antimicrobial compounds (eg., in *Erwinia chrysanthemi* (Barabote et al. 2003), *E. amylovora* (Burse et al. 2004), *Agrobacterium tumefaciens* (Palumbo et al. 1998; Peng and Nester 2001), *Rhizobium etli* (Gonzales-Pasayo and Martinez-Romero, 2000), *Bradyrhizobium japonicum* (Krummenacher and Narberhaus 2000) and *X. fastidiosa* (Reddy et al., 2007). More importantly, Type I secretion systems are also used for pathogen offensive purposes, being capable of directly secreting toxins and enzymes directly from the bacterial cytoplasm to the external medium. Since Las is an intracellular pathogen, this type of system could provide the basis for HLB symptom elicitation, and possibly, for Las growth in citrus. RTX proteins can be host range determining or contributing factors in plant pathogenic bacteria. Primers were designed to amplify this RTX protein coding region for use as a probe against a UF506 fosmid library. These fragments were sequenced and confirmed the SeqWright shotgun assembly in these regions.

**Identification of Las fosmids carrying a putative Las Type I gene.** Because of the sheer number of sequencing reactions (ca. 200,000) that would have to be made to begin to assemble even a small (1.5 Mb) genome, combined with the apparent overrepresentation of prophage or plasmid DNA in our largest contigs, we reasoned that the shorter contigs, once validated, could be used as probes to identify rare, but long contiguous UF506 DNA regions in a fosmid library. A fosmid library was therefore constructed consisting of 1,400 colonies. A random sampling of 18 of these were examined by restriction digestion and the average insert size was 41 kb. A 1.4 kb amplified region was labeled with  $^{32}\text{P}$  and used to probe the entire fosmid library on nylon membranes. Identified fosmids are currently being used for primer walking, adding an additional 11 kb of new Las sequence to our assembly. While this process was being carried out, the Las strain psy62 genome, obtained from psyllids, was made available to us (Duan, Y.P. et al., 2008).

**Use of the Duan et al (2008) psy62 database.** The Duan et al (2008) partial genomic DNA database, containing > 1.2 Mb of Las genomic sequence, was much more complete than our own Las genomic sequence data, and this database confirmed the presence of a Type I system, including CLIBASIA\_04987 (*tolC*), an essential outer membrane component of Type I secretion, and CHRN\_01308, an ATP-binding cassette (ABC) transporter. The Duan et al (2008) genomic sequence allowed identification of at least two likely Type I effectors: a partial serralyisin (CLIBASIA\_05478), which is an RTX protease, and a full length hemolysin (CLIBASIA\_02850), which is an RTX toxin.

PCR fragments of both the partial psy62 serralyisin (CLIBASIA\_05478) and the hemolysin (CLIBASIA\_02850) were found in UF506, and these and other potential RTX effectors in UF506 are currently being investigated. If confirmed, the protein coding regions of any full length RTX encoding genes will be re-cloned into the transient expression vector pYD40.1 (Duan et al., 1999). We plan to determine if transient expression of any full length RTX genes in plants using an *Agrobacterium tumefaciens* delivery system will indicate a potential effector function of these genes in citrus.

## Citations

- Barabote RD, et al. 2003. *Erwinia chrysanthemi* tolC is involved in resistance to antimicrobial plant chemicals and is essential to pathogenesis. *J. Bacteriol.* 185:5772-5778.
- Burse A, Weignart H, Ullrich MS. 2004. The Phytoalexin-inducible multidrug efflux pump AcrAB contributes to virulence in the fire blight pathogen, *Erwinia amylovora*. *Mol. Plant-Microbe Interact.* 17:43-54.
- Duan YP, Castaneda A, Zhao G, Erdos GW, Gabriel DW. 1999. Expression of a single, host-specific gene in citrus cells elicits division, enlargement and cell death. *Molec. Plant-Microbe Interact.* 12:556-560.
- Duan YP, Zhou LJ, Hall D, Li WB, Liu L, Gottwald TR. 2008. GenBank direct submission.
- Gabriel DW 2008. Role of Type I secretion in Pierce's Disease. 2008 Pierce's Disease Research Symposium Proceedings, in press.
- Gonzales-Pasayo R, Martinez-Romera E. 2000. Multiresistance genes of *Rhizobium etli* CFN42. *Mol. Plant-Microbe Interact.* 13:572-577.
- Krummenacher P, Narberhaus F. 2000. Two genes encoding a putative multidrug efflux pump RND/MFP family are cotranscribed with an *rpoH* gene in *Bradyrhizobium japonicum*. *Gene* 241:247-254.
- Palumbo JD, Kado CI, Phillips DA. 1998. An isoflavonoid-inducible efflux pump in *Agrobacterium tumefaciens* is involved in competitive colonization of roots. *J. Bacteriol.* 180:3107-3113.
- Peng WT,, Nester EW. 2001. Characterization of a putative RND-type efflux system in *Agrobacterium tumefaciens*. *Gene* 270:245-252.
- Reddy JD, Reddy SL, Hopkins DL, Gabriel DW. 2007. TolC is required for pathogenicity of *Xylella fastidiosa* in *Vitis vinifera* grapevines. *Molec.Plant-Microbe Interact.* 20:403-410.

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