Proc. Fla. State Hort. Soc. 120:136–137. 2007.

Culturing Fastidious Prokaryotes—Points to Consider when Working with Citrus Huanglongbing or Greening

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Additional index words. citrus greening, *Candidatus* Liberibacter asiaticus, phloem-limited bacteria, xylem-limited bacteria, media formulation

The first of the fastidious prokaryotic plant pathogens to be grown in axenic culture was the phloem-limited *Spiroplasma citri,* which causes stubborn disease of citrus. Other fastidious prokaryotic plant pathogens followed and include *Spiroplasma kunkelii* (corn stunt) and *Spiroplasma phoeniceum* (periwinkle yellows). The vast majority of spiroplasmas are associated with arthropods and do not cause plant diseases. The axenic culture of fastidious xylem-limited bacteria followed the work on these phloem-limited fastidious bacteria. The gram-negative bacterium causing Pierce's disease of grapevines and now known as a pathogenic variant of *Xylella fastidiosa* was the first isolated in culture in 1978. The gram-positive coryneform bacterium causing ratoon stunting disease of sugarcane, *Clavibacter xyli* subsp. *xyli* (*Liefsonia xyli* subsp. *Xyli*) was the second isolated in culture. Media formulations for culturing these bacteria have served as models for the development of other media for the culture of fastidious plant-associated microbes. Knowledge gained in such efforts may lead to the culture of the citrus greening bacterium. The importance of culturing the causal agent as a tool for the management of citrus greening is discussed.

Fastidious prokaryotic plant pathogens are called fastidious because they have specific nutritional requirements, so much so that some have not been grown in culture. Others that have been grown in culture have very exacting requirements. The first fastidious prokaryotes were discovered in the late 1960s (Doi et al., 1967; Ishie et al., 1967). These were wall-free prokaryotes called mycoplasma-like-organisms for a while, but now known as phytoplasmas. None of the phytoplasmas have been grown in culture. Shortly after phytoplasmas were discovered, helical wall-free prokaryotes called spiroplasmas were also discovered in association with plant disease. In the early 1970s, the spiroplasma causing citrus stubborn disease, *Spiroplasma citri*, was the first of the fastidious prokaryotes isolated in axenic culture (Fudl-Allah et al., 1972; Saglio et al., 1971). To date, only three spiroplasma species have been shown to cause plant disease, namely *S. citri*, *S. kunkelii* (corn stunt) and *S. phoeniceum* (periwinkle yellows), but many more have been isolated from arthropods.

The plant pathogenic phytoplasmas and spiroplasmas both inhabit the phloem of plants, but there are other groups of fastidious prokaryotic plant pathogens—the xylem-limited bacteria and the phloem-inhabiting bacteria. These bacteria differed from the phytoplasmas and spiroplasmas by being bound by a cell wall in addition to a cytoplasmic membrane. In 1978, the gram-negative, xylem-inhabiting fastidious prokaryote causing Pierce's disease of grapevines, and now known as a pathogenic variant of *Xylella fastidious*, was the first of the fastidious xylem-inhabiting group to be isolated in axenic culture (Davis et al., 1978). The gram-positive, xylem-limited bacterium causing ratoon stunting disease of sugarcane now known as *Clavibacter xyli* subsp. *xyli* (synonym *Liefsonia xyli* subsp. *xyli*) was next (Davis et al., 1980). Since then all known fastidious, xylem-limited, prokaryotic plant pathogens have been cultured, but the phloem-inhabiting pathogens have not. Among the fastidious phloem-inhabiting prokaryotes that remain uncultured is the one associated with citrus huanglongbing (HLB) disease, also known as greening disease. This bacterium is presently characterized as *Candidatus* Liberibacter spp. (Jagoueix et al., 1994; Teixeira et al., 2005). The following is a discussion of points to consider when attempting culturing the HLB bacterium.

Direct and Indirect Advantages of Culturing *Candidatus* Liberibacter spp.

Candidatus Liberibacter spp. are thought to cause HLB because they are the only entity that has been constantly associated with the disease. This was initially accomplished by microscopy, but polymerase chain reaction (PCR) using specific primers is presently the preferred method. Their actual role as the pathogen has not been proven by completion of Koch's postulates, which is a series of tests requiring isolation of the pathogen in pure culture, inoculation of the host with the pathogen from culture, production of typical symptoms in the inoculated host, and re-isolation of the pathogen in culture. Obviously, being able to culture the suspected pathogen is the key to completion of Koch's postulates. In addition to confirming the pathogenicity of the HLB bacterium, culturing of the HLB bacterium will facilitate the development of methodologies for better detection and identification of the bacterium and hence the disease. The development of further detection methods will facilitate studies on the epidemiology of HLB, which will, in turn, assist in developing better management strategies for the disease. We can learn the times of the year when the bacterium is at the highest concentration in the tree, the exact location of the bacterium, and when this population coincides with the increase in psyllid vector populations. Certain cultivars may be better

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hosts for the pathogen than others. This could facilitate studies to develop resistant cultivars for the future. Once the bacterium is in culture, genomic studies can proceed with the sequencing of its DNA leading the way to molecular biology studies of the host/pathogen/vector relationships that might identify genes of interest for disease management.

Approaches, Alternatives, and Limitations to Culturing *Candidatus* Liberibacter spp.

Since the HLB bacterium has not been grown in culture, using the knowledge gained while culturing other fastidious prokaryotic plant pathogens to develop a method to culture the HLB pathogen would seem to be a valid approach. We used the knowledge and medium developed while culturing the Pierce's disease pathotype of *Xylella fastidiosa* to isolate other xylella pathotypes (Davis et al., 1981: Davis et al., 1983;), *Clavibacter xyli* (Davis et al., 1980), and an unidentified λ-proteobacterium from California buckeye, *Aesculus californica* (Davis, unpublished). Subsequently, the medium for the buckeye bacterium was optimized and used in attempts to isolate the bacterium associated with papaya bunchy top disease (Davis et al., 1996). Although the papaya bunchy top bacterium was not isolated, attempts at the same time to isolate a bacterium from mountain papaya or babaco, *Vasconcella* ×*heilbornii*, yielded an isolate that proved to be interesting. The DNA sequence of the 16s rRNA gene of the babaco bacterium has a 92% homology to that of the South American species of the HLB bacterium, *Candidatus* L. americanus, making it possibly the closest relative of the liberibacters that can be grown in culture. Consequently, we have been have been using the growth of the babaco bacterium to help devise different media in attempts to isolate the HLB bacterium. Interestingly, some media developed for the babaco bacterium also support luxuriant growth of the corn stunt spiroplasma, *Spiroplasma kunkelii*, one of the few phloem-inhabiting, fastidious prokaryotes to have been grown in culture.

Numerous limitations exist to obtaining fastidious prokaryotic plant pathogens in culture in addition to the composition of the culture medium. As with the isolation of most plant pathogenic bacteria, the presence of contaminating bacteria that may be epi- or endophytic always exists. However, with the use of enriched media, the possibility of contaminates is even greater. Another limitation is providing at least the critical concentration of the pathogen in the inoculum to permit growth. The HLB bacterium appears to be present in low concentrations and unevenly distributed in plant phloem. Studies are presently under way to determine the optimum time and location within plants to harvest tissue for inoculum preparation. The psyllid vector may harbor higher concentrations of the bacterium, but determining which individual vectors are infected and decontaminating those presents a problem. Other factors affecting growth include incubation temperature, pH, osmolarity, and oxygen tension.

The possibility exists that the HLB bacterium lacks vital gene functions that would allow it to grow in axenic culture. The intimate association or co-evolution of the HLB bacterium with its hosts may have permitted such gene functions to be provided by the host. In this case, it may be necessary to grow the HLB bacterium in co-culture with another organism that can provide the missing metabolites. Candidates for co-culture include other microorganisms and insect and animal cell cultures.

What if the HLB Bacterium Is Cultured?

If the HLB bacterium is grown in culture, then Koch's postulates as proof of pathogenicity must be completed. This will entail optimizing the culture medium to permit consistent isolation from infected plants, which, in turn, will allow the establishment of a consistent association of the bacterium with the disease. Pathogenicity tests will need to be completed, which will entail devising methods to reintroduce the pathogen into the plant. If mechanical means of inoculation fail, then injecting psyllids with the bacterium and using the infected psyllids to transmit the bacterium into the phloem of plants may be an alternate approach. Following plant inoculation, it will need to be determined if symptoms characteristic of HLB are produced and if the bacterium can be re-isolated from the symptomatic plant but not non-inoculated plants. If this is all completed successfully, then we can be confident that the bacterium causing HLB has been isolated. The study can then be extended to test other strains of the HLB bacterium to determine if they respond in the same manner to efforts to culture them. If not, then it will become necessary to optimize culturing for these strains.

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