Citrus greening bacterium is now available in culture — so what's next?

By Nabil Killiny

A dynamic research collaboration between several labs at Washington State University (Gang, Beyenal and Omsland labs), University of Arizona (Brown lab) and University of Florida (Killiny lab) recently reported an important step in the long-sought culture of the bacterium [*Candidatus* Liberibacter asiaticus (*C*Las)] associated with huanglongbing (HLB). The work, published in the journal Biofilm, describes a new approach (a membrane biofilm reactor) developed for growing the bacterial culture from diseased extracts of Hamlin sweet orange leaves and stems. It also outlines a modified formula of the medium used in the biofilm reactor for growing the bacteria. These modifications included adding vitamins and other essential nutrients, such as salts and trace minerals. So far, the lab-grown bacterial biofilm culture has been sustained and sub-cultured for more than two years in the labs.

PREVIOUS ATTEMPTS

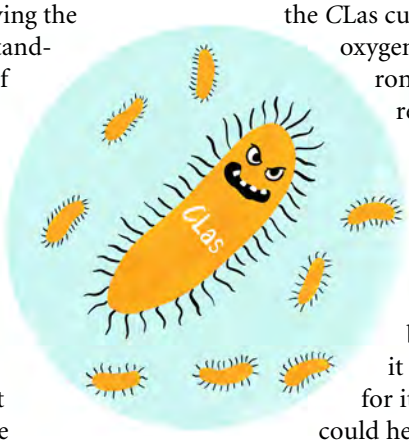
Scientists worldwide have thus far been unable to cultivate the bacterium (*C*Las) associated with citrus greening disease in a pure or sustained culture. Attempts in the United States included co-culturing with *Actinobacterium* and other organisms, using citrus vein extract and a growth factor, and adding citrus juice to the growth medium. Recently, a Japanese group successfully co-cultured the Ishi-1 strain of *C*Las with *Comamonadaceae, Flavobacteriaceae*, *Microbacteriaceae* and *Pseudomonadaceae* in a modified medium. These attempts only resulted in short-term viability and limited growth *in vitro*. These failed attempts at culturing provided unique insights into the world of *C*Las.

In the current host-free biofilm

culture, obtaining the genome of *C*Las and determining the chemical composition of psyllid haemolymph (blood) and citrus phloem sap (the two sites where the bacterium multiplies) helped identify key nutrients required for growing the bacterium in a continuous biofilm culture. However, the biofilm contains other bacteria which are believed to support *C*Las growth.

WHY IS THIS IMPORTANT?

Having *C*Las in culture opens up new avenues for studying the pathogen and understanding the mechanisms of HLB. For instance, until now, the idea that *C*Las causes citrus greening disease had been "putative," meaning it was *suspected* to be the causal pathogen but had not been proven.



Therefore, the first critical study using the biofilm-cultured *C*Las should attempt to fulfill the last two of Koch's postulates. To prove a certain organism is the cause of a disease, Koch's postulates requires it: 1) be isolated from diseased organisms and not healthy ones, and able to be grown in disease active culture, 2) must cause disease when introduced to healthy organisms and 3) be re-isolated from the newly diseased host.

So, in the case of *C*Las, scientists need to introduce the biofilm culture into healthy citrus trees and wait for symptoms to develop. Then the two culture organisms (the one used as the inoculum and the one recovered from the newly infected trees) should be compared genetically to be sure they are identical. If so, the postulates have been fulfilled and *C*Las can be confirmed as the causal agent of HLB.

Confirming that *C*Las is indeed the cause of HLB will allow the bacterial culture to be used for extensive studies in bacterial lifestyle, physiology and pathogenicity mechanisms. Having the citrus greening bacteria available will help researchers understand the chemical and physical requirements for growth, cellular communication and metabolism, and identify any weak points that could be exploited to eradicate the disease.

Until now, lack of a lab culture has hampered in-depth study of *C*Las. For example, it is believed that *C*Las can only survive in the presence of other bacteria that help provide some of its nutrients and communication signals. These mutualistic relationships can be confirmed by exclusion studies, e.g., selectively removing cohabiting bacteria from the culture until *C*Las stops growing.

The Biofilm article reported that the *C*Las culture preferred a low-oxygen, neutral pH environment, providing researchers clues about its metabolism. In addition, scientists can now investigate which physiological processes in *C*Las are responsible for biofilm formation, as it seems to be critical for its multiplication and could help provide new strategies for defeating it.

CONCLUSION

Recently, the Japanese group who developed a co-culture of *C*Las showed that altering the host microbial community with antibiotics could be a new approach to help mitigate citrus greening, but much more research is still needed. Given the limited time to find a solution, and the fact that *C*Las is in a microbial community inside its host plant and insect vector, scientists are encouraged by the availability of the biofilm culture for use as a tool to screen antimicrobial compounds.

Many antibiotics can be tested, rapidly and simultaneously, for their ability to kill the culture *in vitro*, before being tested in trees. This saves both time and money on field experiments, which until now have been the only viable method of testing. Identifying the most effective antibiotics and antimicrobials in the lab will hasten their approval for safety and efficacy in citrus trees.

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