



# Update on culturing of the citrus greening bacterium

By Nabil Killiny, Anders Omsland, Haluk Beyenal and David R. Gang

**T**he suspected causative agent of citrus greening disease, *Candidatus Liberibacter asiaticus* (CLAs), is one of many plant pathogens that has not been isolated and grown in pure culture. One major reason for this is its small genome size; it is missing the genes for several metabolic pathways critical for independent living and growth.

Instead, CLAs parasitizes its hosts (psyllids and citrus plants) for essential nutrients. CLAs also relies on the metabolites or other growth factors produced by neighboring bacteria found within the citrus host phloem sap and psyllid host gut.

So far, growing CLAs in pure culture has proved challenging. However, scientists found a way to grow it in biofilm culture using necessary microorganisms and nutrients. Diseased extracts of Hamlin sweet orange leaves

and stems and infected psyllids were used to grow CLAs in a biofilm by laboratories at Washington State University (Gang, Beyenal and Omsland labs), University of Arizona (Brown lab) and University of Florida (Killiny lab).

CLAs has been sustained and sub-cultured for more than two years. This multi-expertise/multi-university collaboration used new approaches (biofilm engineering) for growing the bacterial culture. The team's research outlines the engineered modified medium for growing the bacteria in a biofilm reactor that included salts and trace minerals, vitamins and other essential nutrients.

## TESTING ANTIBIOTICS

In a recent study, the research team used infected Asian citrus psyllids (ACP) as a source of CLAs to test different antibiotics against

the various bacterial populations in a biofilm-grown culture. It was expected that the antibiotics would alter other microbes so that CLAs would grow faster or could be eliminated in the mixed culture. The researchers hypothesized that the presence of selected antibiotics could reduce the presence of CLAs by targeting it directly (just as amoxicillin targets strep throat) or other antibiotics could enhance or reduce the presence of CLAs by manipulating the composition of the mixed microbial culture.

Some of the antibiotics targeted the bacteria associated with CLAs rather than CLAs itself. Treatments with low concentrations of vancomycin or streptomycin showed more CLAs compared to untreated mixed microbial cultures, while treatments with higher doses of vancomycin and streptomycin were associated with reduced growth. Conversely, polymyxin B was

more effective with lower doses than higher ones.

The growth of CLAs varied with the microbial community composition. The presence of bacteria from the *Pseudomonadaceae* family correlated with the enhanced growth of CLAs, while in cultures with high abundances of *Bacillus cereus*, CLAs levels were very low. This means that *B. cereus* reduced CLAs, which could be a potential therapy if the mechanism is understood.

Some antibiotics may be more effective against fast-growing bacteria that compete directly with CLAs for nutrients. Alternately, some antibiotics may interfere with bacteria that CLAs depends on, thus reducing the nutrient pool for CLAs multiplication. Low levels of antibiotics may act as signaling molecules instead of antimicrobials, resulting in paradoxical effects. More studies are needed to understand these complex mechanisms. However, manipulating the microbial community surrounding CLAs could be a new strategy for controlling CLAs populations in either psyllids or citrus trees.

## LEARNING FROM BIOFILM CULTURES

Without a pure culture, researchers cannot directly test the nutrition requirements and metabolism of CLAs, what treatments are effective against the bacterium or whether it is truly the agent responsible for HLB symptoms in citrus. However, the possibility of culturing CLAs in biofilm form does allow researchers to home in on its microenvironment (pH and oxygen requirements), its apparently mutualistic lifestyle (it may require other bacteria for survival) and its pathogenicity in dual hosts.

It should be noted that researchers still have not proven that CLAs is the organism that causes citrus greening disease. To prove an organism causes a disease, it must be isolated from the diseased host, cause the disease when introduced into a healthy host and then be reisolated from that formerly healthy (now diseased) host. It can be confirmed as the causative agent if the bacteria match genetically. This confirmation will aid in further investigations of the CLAs pathosystem.

Researchers have tested some of the biofilm cultures' infectivity by feeding

the nymphs cultures with a technique developed in the Killiny lab. In this technique, droplets of the biofilm culture were placed on the mouthparts of ACP nymphs. The nymphs acquired the droplets within a minute and were transferred to healthy citrus plants to feed on them. Six months later, CLAs symptoms were observed and CLAs was detected growing in citrus plants. This partially confirms that CLAs (with its microbial community) is the causal agent of citrus greening.

## LOOKING AHEAD

Better therapies can be developed if researchers have a purer CLAs-containing culture. Work is now being done to optimize culture conditions and antibiotic levels, with the goal of increasing CLAs titers even higher and changing the biofilm community composition. The hope is that better conditions will allow for substantial improvement in the purity of biofilm cultures, ultimately leading to pure cultures, if possible.

Understanding that CLAs is part of

a microbial community and may not be viable without specific community members gives researchers new avenues for study. This includes the roles and relationships between CLAs and other bacterial species in the community, the dual lifestyle of CLAs in two hosts (plant and vector insect) and cellular communication between species.

Most importantly, the availability of the biofilm culture will allow for rapid screening of a wide array of antimicrobial peptides and other compounds against CLAs and its companion bacteria in the laboratory. Competition assays using the biofilm reactors will be an invaluable tool to understanding CLAs metabolism and growth requirements. 🍊

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The advertisement features the Carden logo, which consists of a stylized green and brown shape above the word "carden" in lowercase. To the right, the slogan "We keep you growing..." is written in a white, cursive font. The background is a photograph of several bright orange oranges hanging from a tree with green leaves. At the bottom, a green banner contains the text "SPECIALIZED RISK MANAGEMENT FOR THE AGRICULTURE INDUSTRY" in white, followed by "Carden & Associates, Inc.", the phone number "888.296.7533", and the website "www.cardeninsurance.com".