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REVIEW

Why we should care about culturing the Huanglongbing associated bacterium '*Candidatus* Liberibacter asiaticus': the importance of terms and interpretations

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ABSTRACT

Having bacteria grown in pure culture has been the foundation of bacteriology, by allowing a wide range of microbiological studies to determine the functionality of a specific bacterium. However, most bacteria have not been axenically cultured to date, thus hindering the understanding of their role in the context of their host or environment. Among these uncultured bacteria are the recently emergent plant pathogens '*Candidatus* Liberibacter spp.'. This group is comprised of dynamic psyllid-vectored, phloem-limited plant pathogens and endophytes that harm a wide range of economically important crops worldwide. '*Candidatus* Liberibacter asiaticus' (CLas) is associated with Huanglongbing (HLB) in most of the main citrus-producing areas globally, a disease causing severe economic damages. Although the establishment of axenic cultures of CLas remains a major scientific challenge, many research groups have devoted efforts to culture this bacterium to aid in elucidating its virulence mechanisms to develop effective HLB management. This has led to the development of innovative systems to culture and grow CLas, however different authors have approached the concepts of bacterial culture and axenic culture in different manners, leading to confusion in the terminology used. In this review, we provide the scientific definitions of important terms in bacteriology, while critically reviewing the contribution of each of these important CLas culturing studies.

Keywords: plant pathogen, unculturable, axenic culture, HLB, CLas

Introduction

The establishment of pure cultures of microorganisms in laboratory conditions during the late nineteenth century has been a cornerstone of bacteriology. The ability to artificially grow bacteria enabled the development of studies to assess their physiology, taxonomy, ecology and pathology, as well as allowing studies of their morphology, virulence, antibiotic susceptibility and genome sequence, among other features (Austin 2017; Lagier et al. 2015a). However, not all bacterial species have been successfully grown in vitro. Currently, around 20,000 species have been described through culturing, while it is estimated that the total bacterial diversity ranges from 10^7 to 10^9 species (Curtis et al. 2002; Parte et al. 2020). The difference between microscopic and

culture counts is referred to as the "great plate count anomaly" (Staley and Konopka 1985). Although microbiologists have been working to replicate the distinct natural environments of bacteria to reduce this difference between bacterial diversity and culturability, the discrepancy is still high (Lagier et al. 2015b).

Depending on the features of each bacterium, strategies to mimic their habitats and determine specific growth conditions to allow their culturability may include analysis of required nutrients, temperature, oxygen (aerophilic, microaerophilic and anaerobic organisms), incubation time, use of reducing agents, addition of signal compounds and co-cultivation with one or more different species, since some bacteria depend on the metabolic interactions with their community to grow (Lagier et al. 2015a; Overmann et al. 2017). In addition, the use of new technologies of the omics era, specially metagenomics, has played a pivotal role in determining unknown metabolic features of unculturable bacteria and in performing culture-independent physiological and ecological analyses of these organisms (Overmann et al. 2017). However, although some authors may argue that metagenomics has the potential to replace bacterial culture (Austin 2017; Lagier et al. 2015b), culturing still plays a key role in modern functional microbiology.

The persistent case of '*Candidatus* Liberibacter asiaticus' unculturability and its implications

An important group of hitherto unculturable plant pathogenic bacteria is comprised by '*Candidatus* Liberibacter spp.', which are phloem-limited, fastidious Gram-negative bacteria of the α subdivision of Proteobacteria (Jagoueix et al. 1994; Wang and Trivedi 2013). Species of '*Ca.* Liberibacter' are a diverse group of plant pathogens and endophytes that cause diseases in numerous plant hosts (Merfa et al. 2019), including citrus, potato, tomato, carrot and pear (Bové 2014; Nelson et al. 2012; Thompson et al. 2013). However, in this review, we will focus on the subgroup causing the citrus Huanglongbing (HLB) disease.

HLB is the most single devastating disease of citrus worldwide (Gottwald 2010), causing meaningful economic losses in the Americas, Asia and Africa (Bové 2014). This disease has been associated with three '*Ca.* Liberibacter spp.': '*Ca.* Liberibacter africanus' (CLaf), '*Ca.* Liberibacter americanus' (CLam) and '*Ca.* Liberibacter asiaticus' (CLas), which is the focus species of this review due its prevalence worldwide and greater number of published studies (Bové

2014). The Asian Citrus Psyllid (ACP) *Diaphorina citri* is the insect vector for both CLas and CLam, while both CLas and ACP are believed to be native to Asia (Bové 2006; Nelson et al. 2013).

Because of the difficulty in culturing these bacteria, they are given the provisional *Candidatus* status (Merfa et al. 2019). Lack of CLas culturability impairs: (i) functional genomic analyses, which limits hypothesis testing; (ii) taxonomic identification and species name validation; (iii) fulfillment of Koch's postulates by transferring it to either insect or plant hosts; (iv) assessment of host-pathogen interactions; (v) screening of antimicrobial compounds; (vi) determination of virulence among different CLas genotypes; (vii) strain submission to microbial collections for sharing among laboratories; and, more importantly, (viii) development of novel management approaches to control this incurable disease (Bové 2006; Merfa et al. 2019; Pinevich et al. 2018; Wang and Trivedi 2013). Understanding the strategies by which a pathogen causes disease and overcomes plant defenses may allow the development of control measures for newly emerging plant diseases (Wang and Trivedi 2013). This may be possible by interfering with key elements of the pathogen's life cycle, infection process and pathogenicity determinants. Therefore, culturing CLas emerges as a priority, because it should enable functional studies and the development of management approaches to control HLB.

Although a reliable and reproducible method to culture CLas is yet to be developed, substantial progress in culturing this bacterium has been made by using different approaches. These include mimicking the natural environments where CLas lives, co-culture with one or more bacterial species and use of CLas-infected plant explants, all of which will be briefly detailed below in this review (Attaran et al. 2020; Davis et al. 2008; Fujiwara et al. 2018; Ha et al. 2019; Mandadi et al. 2017; Parker et al. 2014; Sechler et al. 2009). These studies show the need of CLas to grow in conditions close to its natural environments, and that it may obtain additional nutrients or chemical signals through a mutualistic relationship with other bacteria. However, there is great inconsistency in how these studies present the concept of an axenic bacterial culture and how this will contribute to control HLB. This is especially confusing for a lay audience, which includes citrus growers that have great interest in solving the HLB problem, as well as fund a considerable part of the ongoing research to solve this issue. Recently, we reviewed and provided insights into the requirements for CLas culturability by mainly analyzing its genome and the chemical composition of the environments where it lives (Merfa et al. 2019). In this review, we would like to provide guidelines on how to accurately use the technical terms comprising bacterial culture. In

addition, we want to discuss how different culturing and non-culturing systems may be useful to study plant pathogens, particularly CLas. We hope to help clarify and standardize these concepts for use in future publications by members of the HLB research community.

Historical perspective: the importance of axenic cultures for plant pathology

CLas is not the first plant pathogenic fastidious prokaryote that has been difficult to culture axenically. *Xylella fastidiosa* is a successful case study of how culturing notably accelerates studying a bacterial pathogen and aids in the development of management strategies to control the diseases it causes; even though in this case the time elapsed between disease reports and axenic culturing was nearly 100 years (Chatterjee et al. 2008; Hopkins and Purcell 2002). It is worth noting that HLB is also a century-old plant disease, however efforts into studying CLas have been greatly delayed in comparison to *X. fastidiosa* (Bové 2006; Kruse et al. 2019).

X. fastidiosa colonizes a wide range of plant hosts and causes substantial losses in economically important crops worldwide, including grapevine, citrus and olive (Chatterjee et al. 2008; Hopkins and Purcell 2002; Saponari et al. 2013). Similar to CLas, *X. fastidiosa* is limited to the vascular system (in this case xylem vessels) of plant hosts and foregut of insect vectors, which are mainly sharpshooter leafhoppers and spittlebugs (Chatterjee et al. 2008; Hopkins and Purcell 2002). The first report of Pierce's disease of grapevine in California occurred in the 1880s, although its causal agent was not known at the time (Hopkins and Purcell 2002). Culturing of *X. fastidiosa* only happened much later in the 1970s (Davis et al. 1978), while its genome sequence was published in 2000 (Simpson et al. 2000), being the first sequenced genome of a plant-associated bacterium. With these data at hand, research on *X. fastidiosa* quickly increased, and this bacterium was even considered one of the top 10 plant pathogenic bacteria in molecular plant pathology (Mansfield et al. 2012). Studies including functional genomics (Chen et al. 2017; Kandel et al. 2018), assessment of resistance and tolerance mechanisms to antimicrobials (Kuzina et al. 2006; Merfa et al. 2016), evaluation of colonization pattern of plant hosts and of pathogenicity and virulence mechanisms (Nascimento et al. 2016; Newman et al. 2003; Niza et al. 2015), extensive genomic analyses to examine recombination among subspecies of this bacterium (Potnis et al. 2019; Vanhove et al. 2019), and inspection of innovative strategies to control *X. fastidiosa* (Baccari et al. 2019; Muranaka et al. 2013), among many other studies, were made possible thanks to widely available axenic cultures. They all had remarkable contributions in

aiding the control and avoidance of the diseases caused by this bacterium, despite the fact that a cure for plants infected by *X. fastidiosa* is still not available (EFSA Panel on Plant Health 2016).

Another example of a plant pathogenic fastidious bacterium being cultured is *Spiroplasma citri*. This organism is the causal agent of the citrus stubborn disease, which significantly reduces fruit quality and production in infected trees, and was the first phloem-limited fastidious prokaryote to be axenically cultured (Saglio et al. 1971; Shi et al. 2014). *S. citri* is a pathogenic mollicute transmitted in a circulative, persistent manner by the leafhoppers *Circulifer tenellus* (in the U.S.) and *C. haematoceps* (in the Mediterranean area) (Bové et al. 2003; Fos et al. 1986; Liu et al. 1983). As described for *X. fastidiosa*, the in vitro culturing of *S. citri*, and more recently its genome sequencing (Davis et al. 2017), have enabled significant studies on the morphology and motility of this plant pathogen, and have also elucidated the relationships among *S. citri* and its plant hosts and insect vector. In addition, the cellular and molecular features of *S. citri* have been investigated through the development of functional genomics tools for this bacterium (Bové et al. 2003). Hence, these two examples of culturing fastidious prokaryotes (*X. fastidiosa* and *S. citri*) show the importance of axenic cultures in plant pathology allowing more rapid research progress geared towards controlling the diseases caused by these bacteria.

Definitions of culture and axenic culture

The technical definitions of "culture" and "axenic culture" are presented here to aid in reviewing the contributions of each study on CLas culturing performed to date. These definitions were taken from well-known textbooks and a biology dictionary. Although these terms may slightly vary among authors, they share great consistency. Culture is defined as "a particular strain or kind of organism growing in a laboratory medium" (Madigan et al. 2014), more specifically "in a container of culture medium" (Tortora et al. 2019). Broadly, a culture is any "batch of cells, which can be microorganisms or of animal or plant origin, that are grown under specific conditions of nutrient levels, temperature, pH, oxygen levels, osmotic factors, light, pressure, and water content" (Martin and Hine 2008). These cultures "are prepared in the laboratory for a wide spectrum of scientific research", and "a culture medium provides the appropriate conditions for growth" (Martin and Hine 2008).

On the other hand, axenic culture (synonym: pure culture) is defined as "a culture containing a single kind of microorganism" (Madigan et al. 2014), which contains "only a single

strain or species of microorganism" (Slonczewski and Foster 2016). Ultimately, an axenic culture will contain "a large number of microorganisms that all descend from a single individual cell" (Slonczewski and Foster 2016). These axenic cultures may be used to "determine the basic growth requirements or degree of inhibition by antibiotics or other chemicals of a particular species" (Martin and Hine 2008).

From these definitions it is evident that a bona fide bacterial culture only includes cells growing through the conditions defined by the culture medium and incubation settings, and thus it excludes any ex vivo systems that include the host cells or tissues. Moreover, an axenic culture only considers the clonal population of a single strain (Shrestha et al. 2013). Therefore, not even culturing of mixed strains from a same species constitute an axenic culture. Fundamentally, axenic means culturing free of any contaminants (Pinevich et al. 2018; Shrestha et al. 2013).

Applications of different culturing systems and non-culturing systems

Because of the failure to culture CLas axenically, researchers need to be creative and use innovative ideas and approaches to maintain this bacterium growing in vitro – at least partially. Thus, different culturing systems, and even non-culturing systems, may be used. However, the range of analyses that may be performed in each system is limited. Hence, the applications and limitations of some systems that may be useful for CLas are presented in Table 1. The broad definition of culture medium is defined here as "a nutrient material, either solid or liquid, used to support the growth and reproduction of microorganisms" (Martin and Hine 2008) in "a laboratory" (Tortora et al. 2019). Although different types of culture medium, such as defined, complex and enriched media (Madigan et al. 2014; Tortora et al. 2019) are available, these distinctions are not the focus of this review.

Table 1. Comparison among different culturing and non-culturing systems for bacterial growth.

System *		Description	Applications	Limitations	Koch's postulates	References
Culturing systems	**Solid medium**	Culture medium containing agar, or other inert solidifying agent, at a concentration of 1.0 to 2.0%	Useful for isolating bacteria in pure culture and determining the colony characteristics of the isolate. Also used in the following assays: - Screening of antimicrobials - Bacterial motility	Does not allow renewal of nutrients over time, thus access to nutrients may be limited	Yes	(Anjum 2015; Balouiri et al. 2016; Bonnet et al. 2020; Kandel et al. 2017; Madigan et al. 2014; Naranjo et al. 2020)

			- Counting of colony forming units (CFU)			
	Semisolid medium	Cultured medium prepared with agar, or other inert solidifying agent, at a concentration of 0.1 to 0.4%	Enables culturing microaerophilic bacteria. Also useful for determining bacterial motility, specifically swarming and swimming motilities, and chemotaxis		Yes	(Hashsham 2007)
	Liquid medium	Also called culture broth. Contains all required nutrients for growth of the desired bacteria dissolved in water, however without the presence of any solidifying agent	Used to propagate large number of cells and for specific assays including: - Biofilm formation - Growth curve [x] - Time lapse assays, including antimicrobials screening [x]	- Not suitable for isolating bacteria from a mixed sample - Does not allow identification of the morphological characteristics of bacterial species	Yes/No [y]	(Anjum 2015; Balouiri et al. 2016; Bonnet et al. 2020; Kandel et al. 2017; Naranjo et al. 2019; Naranjo et al. 2020)
	Co-culture	Two or more distinct bacterial species or strains are cultured together with some level of contact among them	- Allows studying metabolic interactions among co-cultured bacteria - May be used to culture fastidious and unculturable bacteria that rely on other organisms to grow - Different organisms may be axenically cultured by physical separation through a membrane that allows only metabolic interactions	- Physical separation among organisms can only be performed at a small scale and with a limited number of members - Although possible, axenic cultures are not easily achieved	Yes/No [z]	(Goers et al. 2014; Hashsham 2007; Merfa et al. 2019; Tanaka and Benno 2015)
	Liquid medium in flow conditions	Bacterial cells are cultured in flow systems or microfluidic chamber (MC) mimicking the plant vasculature, in which fresh culture medium broth is continuously supplied through a current flow	MC allows real-time observations in a microscope. MCs and flow systems are suited for the following assays: - Bacterial motility - Biofilm formation - Measurement of adhesion force to a surface - Screening of antimicrobials	Not suitable for isolating bacteria. Cells must be previously cultured axenically for downstream analysis using this system	Not applicable**	(De La Fuente et al. 2007; Meng et al. 2005; Naranjo et al. 2019; Naranjo et al. 2020)
Non-culturing systems	**Intracellular culture**	Intracellular bacteria are grown within host cells, which are cultured in vitro	- Ability to grow bacteria that would otherwise be unculturable - Allows assessing the interaction of the target bacterium with its host	- Do not allow a bona fide axenic culturing of bacteria - Number of assays that can be performed is limited	No	(Lagier et al. 2015a)
	Detached leaves	Leaves are detached from a desired plant host, surface-sterilized, inoculated with the respective bacterial pathogen to	Allows screening the pathogenicity/virulence of different strains/species of the pathogen; and screening resistance/susceptibility of		No	(Francis et al. 2010; Randhawa and Civerolo 1985)

		be studied and incubated in 0.5% water-agar plates	different genotypes of the plant host(s)			
	Leaf discs	Infected leaves of plant host are detached, surface-sterilized and leaf discs of 5 mm dia. are taken. Leaf discs are pooled and inoculated into test media to assess bacterial growth in planta over time by quantitative PCR (qPCR)	Allows evaluating unculturable bacteria growth in planta over different physicochemical and nutritional conditions		No	(Attaran et al. 2020)
	Hairy root	Hairy root formation is induced in infected roots of plant host by *Rhizobium rhizogenes*. Infected hairy roots are then inoculated into test media to assess bacterial growth in planta over time by quantitative PCR (qPCR)			No	(Mandadi et al. 2017)

* The differentiation between culturing and non-culturing systems was made based on the culture definition presented here. Thus, only systems in which cells are consistently grown in vitro were considered a culturing system; while systems that depend on other living organisms that do not rely directly on a culture medium were considered as non-culturing. Take note that non-culturing does not mean no growth, but that microorganisms rely on ex vivo tissues to survive and multiply.

** Studies in flow conditions using MCs are usually not aimed for subsequential analysis in planta.

[x] Assays performed by measuring turbidity of culture over time.

[y] Bacteria grown in liquid media may be used for fulfillment of Koch's postulates only if they have been previously isolated in pure culture, usually by plating in solid media.

[z] Co-culture system may be used for Koch's postulates when cells are physically separated by a membrane, allowing only for exchange of metabolites. Therefore, each member of the co-culture is considered axenic.

Among the systems described in Table 1, culturing in solid medium is the most desired for CLas due to ease of work and ability to isolate cells in axenic cultures (Bonnet et al. 2020). The conditions established in this system could then be applied in liquid medium to increase the range of assays to be performed. However, because of the recalcitrant nature of CLas to culturing, other systems may also be explored (Table 1). It has been suggested that CLas may have a microaerophilic respiration (Wang and Trivedi 2013), which would require a semi solid medium and/or incubation in controlled oxygen conditions to grow. Moving further, transient co-cultures of CLas with other bacteria show the potential of the co-culture system to (co-)culture CLas in vitro (Davis et al. 2008; Fujiwara et al. 2018; Ha et al. 2019; Parker et al. 2014; Sechler et al.

2009). Moreover, the use of microfluidic chambers mimicking the plant phloem system may allow optimizing the culturing conditions for this phloem-limited pathogen (Jagoueix et al. 1994; Naranjo et al. 2020). Finally, the use of the non-culturing systems is based on the intracellular nature of CLas, which is obligatory in planta but transitory in ACPs (Ghanim et al. 2017; Merfa et al. 2019). Although these systems do not constitute an actual culture, since they are ex vivo systems, they are valuable resources to grow CLas and study this bacterium.

We would like to note that there are other culturing systems being used to grow hitherto unculturable bacteria from different environmental sources. However, since these systems have not been tested so far with CLas, they are not the focus of this review. These systems include, but are not limited to: (i) growing marine bacteria in microtiter plates using extinction culturing with in situ concentrations of substrate, coupled to sensitive detection methods of microbes to assess growth and determine microbial diversity (Connon and Giovannoni 2002); (ii) establishing pure cultures of marine bacteria by encapsulating cells in gel microdroplets, which allows parallel microbial culturing in low nutrient flux conditions (Zengler et al. 2002); (iii) growing previously uncultured microorganisms by encapsulating them in polysulfone-coated agar spheres and incubating in simulated or natural environments (Ben-Dov et al. 2009); and (iv) culturing bacteria using a device, called I-tip, which allows cells and natural chemical compounds to diffuse into it and promote bacterial growth (Jung et al. 2014). For a more thorough review of different culturing systems, we suggest referring to other reviews published elsewhere (Lagier et al. 2015a; Lewis et al. 2020; Overmann et al. 2017).

CLas culturing studies

With all the technical definitions and culturing systems detailed above, following we will assess the contribution of each CLas culturing study to reach the ultimate long-sought goal of obtaining an axenic culture of CLas. These studies will be detailed here separated by culturing and non-culturing systems.

Culturing systems. To our knowledge, the first report on CLas culturing was published in 2008. In that study, CLas was co-cultured with an accidental skin commensal contaminant Actinobacteria commonly inhabiting citrus and ACPs, *Propionibacterium acnes*. The CLas/*P. acnes* co-culture was able to survive multiple passages. However, attempts to purify CLas in

axenic cultures were non successful. The authors concluded that the relationship among CLas and *P. acnes* was mutually beneficial, in which CLas would likely obtain nutrients and/or chemical signals, thus not allowing its axenic growth (Davis et al. 2008). Shortly after, Sechler and collaborators claimed in 2009 to successfully culture all three '*Ca.* Liberibacter spp.' suspected as causal agents of HLB in axenic conditions (CLas, CLam and CLaf). A culture medium mainly composed of citrus vein extract, and named Liber A, was able to maintain bacterial growth for four to five passages before viability started to decline. In addition, two isolates of CLas and one of CLam cultured in this system displayed pathogenicity on citrus plants and were isolated from noninoculated tissues of inoculated plants. The authors thus declared a partial fulfillment of Koch's postulates (Sechler et al. 2009). However, results of this study are controversial, since no other research lab could reproduce these cultures and no follow-up studies have even been published by the authors. A few years later, Parker and collaborators (2014) used a similar approach of mimicking the natural environment of CLas to culture this bacterium. They used culture media containing commercial grapefruit juice and were able to maintain viable CLas co-cultures in vitro with other microflora from grapefruit seeds (source of CLas inoculum of that study) for several months in biofilm. The growth pattern of CLas resembled cryptic growth over time, with oscillations in the population numbers. This indicates that the persisting population of CLas could partially grow using the content of dead cells (nutrients and/or signaling components) as growth stimulators, in an ongoing cycle of growth followed by death and release of nutrients. Thus, since there was no continuous nor axenic CLas growth, no bona fide culturing of this bacterium was claimed (Parker et al. 2014). For some years, these three reports were the main studies to contribute for achieving the goal of culturing CLas. However, new studies and strategies have been reported on recent years.

In 2018, co-culturing of the Japanese CLas strain Ishi-1, which bears no phages in its genome, was reported in association with phloem-associated microbiota (Fujiwara et al. 2018). The population of CLas was followed over time by quantifying DNA through qPCR, however with no direct quantification of viability. Moreover, distinctive colonies of CLas were not present in agar plates, but few cells were found under microscopic investigations. The presence of the phloem microflora was deemed as essential for CLas growth, since suppression of certain bacterial families by antibiotics decreased CLas survival. Furthermore, CLas was resistant to oxytetracycline and multiple other antibiotics (Fujiwara et al. 2018), contradicting previous reports (Zhang et al. 2014).

Therefore, reproducibility of these findings by other research groups is still needed, as we have already noted elsewhere (Merfa et al. 2019). Another report of CLas being grown in co-culture with citrus-associated microflora has been published recently in 2019 (Ha et al. 2019). By developing a novel culture medium based on BM7 medium, which is used to culture *Liberibacter crescens*, the only culturable species of the *Liberibacter* genus (Fagen et al. 2014a; Leonard et al. 2012), a long-term co-culture of CLas was established, with many successful sub-cultures, using a membrane biofilm reactor system. The authors argue that the long-term growth of CLas in their system is an advantage to the other studies on CLas culturing. However, even though that is an important contribution of their study, the number of CLas cells assessed by qPCR as genome equivalents only reached the order of 10^3 cells per mL of culture (Ha et al. 2019). This constitutes only a minor fraction of the entire biofilm population and is likely a bottleneck to study CLas using this system. By comparison, Parker and collaborators (2014) obtained CLas titers in their culturing system in the order of 10^5 to 10^6 cells per mL of culture. In addition, Ha and collaborators (2019) erroneously employ the term “axenic” to describe their cultures. As defined in this review, axenic means culturing a single clonal population of an isolate or strain. Therefore, the culture described by them is not axenic. Finally, a host-free co-culture of CLas with ACP-associated microbiota has been established recently using the same culture medium developed by Ha and collaborators (2019). In this study, mixed cultures of CLas were treated with different antibiotics, which were previously shown to not affect CLas in ex vivo assays (Zhang et al. 2014), to alter their composition (Molki et al. 2020). Authors were able to show that the presence of bacteria from the *Pseudomonadaceae* family has a positive correlation with CLas growth, while an abundance of *Bacillus aureus* decreased the CLas population to below the detection limit. The study thus suggests that enriching *Bacillaceae* within CLas-infected trees could possibly be a biocontrol strategy for HLB, which is currently being addressed by them (Molki et al. 2020).

Unfortunately, no follow-up studies have been published to date using any of these culturing systems described here. This shows their practical limitation to study the cellular and molecular features of CLas. However, they remain as seminal contributions to the effort of axenically culturing this organism. These studies have shown that CLas may grow in vitro outside either its plant or insect hosts, and more remarkably, that CLas usually required the host’s microbiota to grow, since most studies reported co-cultures of this bacterium. This is an usual particular feature of intracellular pathogens with reduced genomes, since they rely on the

ecological services provided by the host and associated microbiota to grow, while scavenging for nutrients and energy (Merfa et al. 2019).

Non-culturing systems. The first report of a non-culturing system to grow CLas cells is a patent application in the U.S. published in 2017 (Mandadi et al. 2017). In that application, the authors describe the induction of hairy roots by *Rhizobium rhizogenes* in plants already infected by CLas. These roots can then be propagated and inoculated into test media to evaluate CLas growth in planta over time in different conditions by DNA quantification through qPCR. In 2020, Zuñiga and collaborators used this system to validate their predictions about nutrient requirements of CLas, which were obtained through a genome-scale metabolic model of this bacterium. Among the metabolic model predictions and validation using the hairy root system, the authors conclude that CLas requires essential compounds form their hosts to survive, including aromatic amino acids, vitamins, saccharides and fatty acids (Zuñiga et al. 2020). However, both of these studies (the patent and the manuscript) call the hairy root system a culture of CLas, even though the manuscript clearly states that this is an ex vivo system (Mandadi et al. 2017; Zuñiga et al. 2020). Based on the definitions presented in this review, we propose that a better definition would be calling hairy roots an ex vivo system to grow CLas cells in planta, similar to what has been done previously (Yang et al. 2018), but enabling higher throughput assessments. In addition, neither of these publications show the titer that CLas has reached in hairy roots. Zuñiga and collaborators (2020) present their results as relative growth rate of CLas. However, the lack of data concerning uniformity of infection of root cells and actual number of CLas cells, as assessed by qPCR, precludes the analysis of how feasible this system is to grow this bacterium. Nevertheless, a recent published study has shown the applicability of the hairy root system to establish a relatively fast high throughput screening method of antimicrobials against '*Ca.* Liberibacter spp.'. By using this system, authors were able to determine a range of antimicrobial peptides and chemicals that inhibit CLas, and thus have the potential to be used as therapies to control HLB (Irigoyen et al. 2020).

Finally, another ex vivo assay to grow CLas was described in 2020 using leaf discs from infected citrus plants (Attaran et al. 2020). In this system, leaf discs are inoculated into test media to assess CLas growth in planta over time in different physicochemical and nutritional conditions by DNA quantification through qPCR. As the main findings, the authors observed CLas growth in the presence of glucose only when grown in microaerophilic conditions (10% O_2), while the

presence of the antibiotic amikacin further increased CLas growth (Attaran et al. 2020). The authors suggest that glucose may be either used directly by CLas or after glucose oxidation by the leaf tissue, through ATP uptake from the plant host by the bacterium. Additionally, the authors argue that, although CLas lacks the enzyme glucose 6-phosphate isomerase (PGI) of the glycolytic pathway, it could reroute its metabolism to generate glyceraldehyde-3-phosphate through the pentose phosphate pathway (PPP), which would then allow production of pyruvate from glucose (Attaran et al. 2020). However, in addition to the probable absence of the enzyme transaldolase of the PPP (Fagen et al. 2014b), another study has noted that, in combination to an incomplete glycolytic pathway, CLas also lacks a methylglyoxal detoxification system to eliminate this cytotoxic byproduct of glycolysis (Jain et al. 2017). Thus, it is more likely that CLas uses the metabolic products of glucose metabolism performed by the host, possibly by directly importing ATP from its hosts through its ATP/ADP translocase (Jain et al. 2017; Vahling et al. 2010). In addition, the authors provide the results of CLas growth in leaf discs only after three days of incubation (Attaran et al. 2020). It would be useful to know how the bacterium behaves during a longer time of growth and why this fastidious prokaryote presented optimal growth in such a short time. To conclude, similarly to the hairy root system (Mandadi et al. 2017; Zuñiga et al. 2020), the authors also call the leaf disc system a culture of CLas (Attaran et al. 2020), which we again propose that it would be more suitable to classify it as an ex vivo system to grow CLas cells in planta.

These non-culturing systems described here have the potential to be powerful tools to assess the CLas response to different conditions in planta. Although they may be similar to keeping CLas-infected plants in greenhouse conditions to perform a range of assays (Yang et al. 2018), they possibly represent a faster screening method to assess this bacterium. Additionally, they are focused on analyzing the bacterium itself, and not the plant host. However, although they are well-suited to evaluate CLas in planta, the analysis of compounds required for CLas growth is masked, since it is not possible to determine whether CLas may use the provided nutrients directly or after they are metabolized by the plant host and/or associated microbiota. To sum up, we reinforce that these systems do not fit the culture definition presented here, since growth of CLas occurs in planta using ex vivo tissues and may not directly rely on the culture medium to multiply and survive.

Final considerations

Although an axenic culture of CLas has not been established to date, researchers have been using different methods to study the features of this bacterium and its interactions with its insect and plant hosts. Some of these strategies include the employment of genomic tools, performance of in vivo assays with plant hosts and ACP, and use of surrogate bacteria, including *Sinorhizobium meliloti* and *Agrobacterium tumefaciens*, which are also bacteria of the Rhizobiaceae family (Andrade and Wang 2019; Vahling-Armstrong et al. 2012), and *L. crescens*, that has the highest genome synteny with CLas (Jain et al. 2019). A few of the studies made possible by using these methods include: (i) antimicrobial screening of CLas in planta (Yang et al. 2018; Zhang et al. 2014); (ii) assessment of the colonization patterns of CLas in plant and insect hosts (Achor et al. 2020; Ghanim et al. 2017); (iii) determination of the energetic requirements of CLas (Jain et al. 2017); (iv) performance of indirect functional genomics using surrogates (Andrade and Wang 2019; Vahling-Armstrong et al. 2012); and (v) analysis of pathogenicity factors (Clark et al. 2018; Jain et al. 2018).

However, lack of axenic culture still precludes many studies to be performed with CLas itself, and many hypotheses cannot be tested directly with this bacterium. Thus, the key question that we had in this review was: are researchers culturing CLas already? Considering the literal definition of culture presented here, the answer is yes, but only as co-cultures. Some studies have shown growth of CLas in vitro with only the aid of the nutrients present in the culture medium and of the incubation conditions, which means without the presence of any host, either plant or insect. However, another question arises: are these CLas culturing systems entirely suitable for answering standing questions on CLas biology? This time, the answer is no. An axenic culture of CLas has yet to be available and current culturing systems do not allow continuous growth of this bacterium (Davis et al. 2008; Parker et al. 2014). In addition, subsequent transfers of CLas cannot either be performed, or its titer only reaches a small proportion of the entire microbial population (Ha et al. 2019). Hence, with so many different strategies to culture CLas being reported and different concepts being presented, we hope that this review may help authors to standardize the terminology used in their publications on CLas culturing. We strive to avoid misunderstandings of the audience and/or disseminate erroneous concepts about CLas cultures and axenic culturing. Ideally, an axenic culture would be able to grow to high titers in both solid and liquid medium, be pathogenic to citrus plants, amenable to store as glycerol stocks at -80ºC and survive indefinite sub-cultures.

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