Cultivation of '*Candidatus* Liberibacter asiaticus', '*Ca.* L. africanus', and '*Ca.* L. americanus' Associated with Huanglongbing

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ABSTRACT

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A new medium designated Liber A has been designed and used to successfully cultivate all three '*Candidatus* Liberibacter spp.,' the suspect causative agents of huanglongbing (HLB) in citrus. The medium containing citrus vein extract and a growth factor sustained growth of '*Ca.* Liberibacter spp.' for four or five single-colony transfers before viability declined. Colonies, positive for '*Ca.* L. asiaticus' by a 16s-based rDNA real-time polymerase chain reaction (RT-PCR) assay and sequencing, were irregular-shaped, convex, and 0.1 to 0.3 mm after 3 to 4 days. Suspect '*Ca.* L. asiaticus' and '*Ca.* L. americanus' cells were observed in infected tissue and on agar culture by scanning electron microscopy. The cells were ovoid to rod shaped, 0.3 to 0.4 by 0.5 to 2.0 µm, often with fimbriae-like appendages. Two strains of '*Ca.* L. asiaticus' and one of '*Ca.* L. americanus' grown on Liber A medium were pathogenic on citrus and could be isolated from noninoculated tissues of inoculated trees and seedlings 9 and 2 months later, respectively. The identity was confirmed by RT-PCR and 16s rDNA sequencing. This is the first report of the cultivation and pathogenicity of '*Ca.* L. asiaticus' and '*Ca.* L. americanus' associated with symptoms of HLB.

Additional keyword: greening.

Huanglongbing (HLB) disease of citrus (23,24,29), also known as "citrus greening," is one of over 20 known plant diseases where the suspected phloem-limited causal bacterium has yet to be cultured (6). The disease causes a rapid tree decline, characterized by yellowed shoots with blotchy mottled leaves, small, poor-quality, lopsided fruit with color inversion, and aborted seed (2). Management of the disease is difficult and, with the onset of severe symptoms, infected groves become economically unfeasible in 7 to 10 years (11). HLB is considered the most destructive disease of citrus species in the world and currently threatens the existence of the citrus industry (2). Diagnostic tests using 16s rDNA and the *rpoJL* loci as target sequences for both classical polymerase chain reaction (PCR) (17,18,25) and real-time PCR (RT-PCR) (23,31,37) have consistently identified a '*Candidatus* Liberibacter sp.' associated with the symptoms of HLB.

Three species of '*Ca.* Liberibacter' have been described as the cause of HLB in different countries and climates of the world, primarily by 16s rDNA and other gene sequences: '*Ca.* L. asiaticus' (17), '*Ca.* L. africanus' (17), and '*Ca.* L. americanus' (5,35). There is a suggestion that '*Ca.* L. africanus' has a closely related subspecies, '*Ca.* L. africanus subsp. capensis' (10), responsible for disease on cape chestnut, *Calodendrum capense.* It is unknown whether any of the proposed '*Ca.* Liberibacter spp.' associated with the disease are the actual causes of HLB or are parts of syndromes. '*Ca.* L. africanus' was reportedly cultivated from HLB-infected citrus in South Africa (9,33) and Koch's postulates completed (33) but reports have not been verified because cultivation could not be independently repeated. A recent report from Brazil found a phytoplasma to be associated with HLB-like symptoms (36).

Most traditional media for culturing bacteria favor organisms that grow in isolation, in high density, and in high concentrations of nutrients (21). Not all organisms can be cultured in these environments or conditions. Some recent techniques to culture fastidious organisms that do not grow in these conditions have attempted to simulate nutrient-poor bacterial environments with minimal nutrient media (19), to isolate single cells in an environmental nutrient extract (40), and to grow cultures in diffusion chambers that allow microorganisms to grow in communities (20). Minimal media enhanced with soil extracts have been used successfully to culture members of soilborne *Rhizobiaceae*, the closest relative to '*Ca.* Liberibacter spp.' based upon 16s rDNA sequence comparisons (18). Therefore, a media supplemented with an environmental extract may be useful for the culturing '*Ca.* Liberibacter spp.'

In this study, a new medium, Liber A, was designed and used to successfully cultivate all three '*Ca.* Liberibacter spp.' isolated from locations around the world. Two strains of '*Ca.* L. asiaticus' and one of '*Ca.* L. americanus' were found to be pathogenic on citrus and were reisolated from distant tissues of inoculated trees and seedlings 9 and 2 months after inoculation, respectively.

MATERIALS AND METHODS

Source of '*Ca.* Liberibacter' samples. Source material for cultivation of the '*Ca.* Liberibacter spp.' came from infected symptomatic citrus trees or leaf samples acquired from the People's Republic of China, Brazil, Thailand, India, Taiwan, the United

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States (Florida), and South Africa. The infected plants were maintained inside a containment suite at the Foreign Disease Weed Science Research unit (FDWSRU), Fort Detrick, MD in a Percival lighted dew chamber (model E 54U-DL; Perryville IA) at 31 and 27°C day and night, respectively, with a 16-h photoperiod. Healthy plants were grown and maintained in another building in a walk-in growth chamber (Environmental Growth Chamber, Chagrin Falls, OH) set as above and inspected and maintained free of insects.

Isolations, media, and culturing. For isolations, 3- to 4-cm-long sections of leaf midveins and petioles were cut from HLB-infected citrus and surface sterilized by soaking in a 0.6% sodium hypochlorite solution for 3 min, followed by two separate rinses in sterile water for 2 min each. The surface-sterilized tissue was cut into 1-cm sections with a sterile scalpel and, using cooled, flame-sterilized forceps, were squeezed at the fresh cut end of the tissue to express a drop of sap from each section. These drops were directly touched onto the surface of duplicate agar plates. A sterile loop was then used to streak each drop over the agar surface ("squeeze-drop method"). The squeezed tissue sections for each petiole and midvein were recombined, minced, and allowed to soak in sterile water (100 to 200 µl) for 10 to 20 min before pipetting 10 to 20 µl of the suspension onto two separate test agar plates and streaking the drop with a sterile loop ("mince-soak method"). The remaining suspension was collected and archived at –20°C for RT-PCR. The minced tissue pieces were collected and used for DNA extraction using a Plant DNeasy Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Each plate was wrapped with Parafilm M, inverted, and incubated at 28°C. Half of the plates were placed in a growth chamber under ambient conditions and half in a CO_2 chamber in a 5.0% CO_2 environment. At 2 and 4 weeks after plating, all resulting colonies were observed using a binocular dissecting microscope (Nikon SMZ1500) with oblique lighting from below, and portions of several single colonies were streaked onto similar test media. All isolations and transfers were done in a sterile laminar flow hood. Each colony was tested by RT-PCR to identify putative '*Ca*. L. asiaticus' colonies.

Initially, media C3G (4) and LD8A3 (22) for spiroplasms and PD2 (8), PW (7), and PW supplemental (32) for *Xylella fastidiosa* were tested for cultivation of '*Ca*. Liberibacter spp.' Based on preliminary observations that limited growth only occurred at the site of the expressed sap droplet, citrus vein extract (CVE) was added to both C3G and PD2, creating the modified media C3G+E and PD2+E, respectively. CVE was prepared from the petiole and midvein of young, fully expanded citrus leaves. The combined plant tissues were added to a volume of water equal to twice the tissue mass in a Waring Pro blender (model 51BL16; Torrington, CT) and blended at high speed for 2 min. The resulting slurry was filtered through three layers of cheese cloth, then further refined by centrifugation (1,000 × *g* for 20 min). The supernatant was decanted and sterilized by serial filtrations though sterile 1.0-, 0.2-, and 0.1-µm filters (Millipore).

The final Liber A agar medium contained 1.5 g of K_2HPO_4, 1.0 g of KH_2PO_4, and 15.0 g of purified agar per liter after autoclaving and cooling to 55°C in a water bath, followed by the addition of 10 mg of NADP, 20 ml of CVE, and 25 mg of cycloheximide. For liquid Liber A medium, purified agar was omitted.

Cryogenic storage. Cells of '*Ca*. L. asiaticus' washed from the surface of Liber A agar medium were stored at –80°C in either sterile 50% glycerol in water or sterile 50% glycerol in saline (0.85% NaCl). After 6 months, the cultures were removed from the freezer and streaked onto Liber A agar medium to assess viability.

Electron microscopy. Culture and petiole material of China1, China2, and Brazil1 were prepared at the FDWSRU for electron microscopy, killed, fixed with 2.0% glutaraldehyde, and then transported to the Agricultural Research Service Core Technology Unit, Eastern Regional Research Center, Wyndmoor, PA, where they were further processed and observed by scanning electron microscopy (SEM) using the freeze-fracture method of Haggis and Phipps-Todd (12) and transmission electron microscopy (TEM) using routine methods of negative staining (3) and embedding or thin sectioning of samples in epoxy resin (16). Culture material and petioles prepared in Bangkok, Thailand were processed at Kasetsart University for SEM and TEM.

RT-PCR. Two RT-PCR assays were used to detect the presence in tissues used for isolation and to confirm the identity of '*Ca*. Liberibacter spp.' The *rpoJL* locus assay (37) was done according to published protocols to detect '*Ca*. L. asiaticus'. The 16s rDNA assay was done using previously described primers and probes and conditions as described for the SmartCycler V2.0 (Sunnyvale, CA) (31). This assay was adapted and run on the Applied Biosystems Sequence Detection System 7700 (ABI 7700) and the Applied Biosystems Sequence Detection System 7900 (ABI 7900) in fast mode. Each platform used the recommended master mix for the PCR mix at a 1× final concentration of TaqMan Universal PCR Master Mix for the ABI 7700, TaqMan Faster Universal Master Mix (2×) for the ABI 7900, and 1 µM each primer and 0.2 µM probe; double-distilled water was used to bring the mix up to final volume, 23 µl for the ABI 7700 and 18 µl for the ABI 7900. A 2-µl sample was used in all RT-PCR reactions. The thermocycling profile for the ABI 7700 was as follows: 50°C for 2 min, 95°C for 10 min, and 60 cycles of 95°C for 15 s and 60°C for 1 min. The thermocycling profile for the ABI 7900 was as follows: 50°C for 20 s and 60 cycles of 95°C for 1 s and 60°C for 20 s. The ABI 7700 was used for direct PCR samples whose DNA was not extracted prior to RT-PCR, such as the archived minced tissue suspension and colonies directly from liquid or agar media. The ABI 7900 and SmartCycler V2.0 were used to test samples of extracted DNA.

'*Ca*. L. asiaticus' and '*Ca*. L. americanus' sequence similarity. The identity of the bacteria was further confirmed by sequencing the 16s rDNA with general prokaryote primers (38), specific '*Ca*. L. asiaticus' primers (18), and '*Ca*. L. americanus' primers (5) on a Perkin Elmer 310 Genetic Sequence Analyzer (San Jose, CA). Sequences were deposited into GenBank for strains China1 (EU999026), China2 (EU999027), and Brazil1 (EU999028) and aligned visually using BioEdit v. 7.0.9 (13) against previously sequenced 16s rDNA sequences for '*Ca*. L. asiaticus' from Florida (EU130553), Brazil (DQ471901), India (L22532), China (DQ432005, DQ432004, DQ432000, DQ432003, and DQ431998), Taiwan (DQ302750), and Malaysia (EU371107); '*Ca*. L. africanus' from South Africa (L22533); and '*Ca*. L. africanus subsp. capensis' (AF137368) and '*Ca*. L. americanus' from Brazil (AY742824).

Pathogenicity. Several inoculation methods were used to test pathogenicity using cells from the third single-colony transfer of a '*Ca*. L. asiaticus' strain China1. Cells were washed off Liber A agar in 100 µl of sterile distilled water. A 10-fold dilution in sterile distilled water was prepared and the resulting inoculum used to inoculate one 5-year-old alemow tree (*Citrus macrophylla*). Inoculation was done on separate main branches by several methods: (i) infiltration into three fully expanded leaves near the midvein using the blunt end of a syringe, (ii) multiple needle injections into petioles and secondary phloem using a 26-gauge needle, (iii) placing a drop of inoculum onto the surface of a fresh cut young branch, and (iv) using a carpet needle to penetrate the bark of a main branch and adding inoculum into the phloem. Inoculation of water by infiltration and injection were done as negative controls on another 5-year-old alemow tree. Plants were incubated in a lighted Percival growth chamber (model E-54B, Boone, IA) at 31 and 27°C day and night, respectively, with a 16-h photoperiod in a containment laboratory. All plants were tested for the presence of '*Ca*. Liberibacter spp.' by RT-PCR prior to inoculation.

For a second experiment, cells from the third single-colony transfer of '*Ca*. L. asiaticus' strains China1 and China2 and '*Ca*. L. americanus' strain Brazil1 were used. After growing in liquid Liber A medium for 10 days without shaking, cells were pelleted by centrifugation, carefully decanted, and suspended in 100 µl of sterile distilled water. After 10-fold dilution in sterile water, the inoculum was infiltrated into a cotyledon and first true leaf of three two- to three-leaf-stage sweet orange (*C. sinensis* cv. Madam Vinous) seedlings, as above. Plants were incubated as above in a Percival growth chamber. For controls, two seedlings were infiltrated with water and liquid Liber A medium.

RESULTS

Isolations, media, and culturing. Very limited growth occurred on C3G and PD2 media when the squeeze-drop method was used, with growth only at the site where the drop of sap was placed. No growth occurred along the streak and no growth occurred on any of the initial test media when the mince-soak method was used. The occurrence of growth only in the presence of relatively concentrated plant sap generated by the squeeze-drop method led to the addition of CVE to subsequent test media. With the addition of CVE, scattered '*Ca*. L. asiaticus' colonies were visible on both C3G+E and PD2+E media after 10 to 14 days. The strains survived for only one or two colony transfers (10 to 14 days each) on these media. The size of the colonies was similar on both media. Growth of '*Ca*. L. asiaticus' on C3G+E was

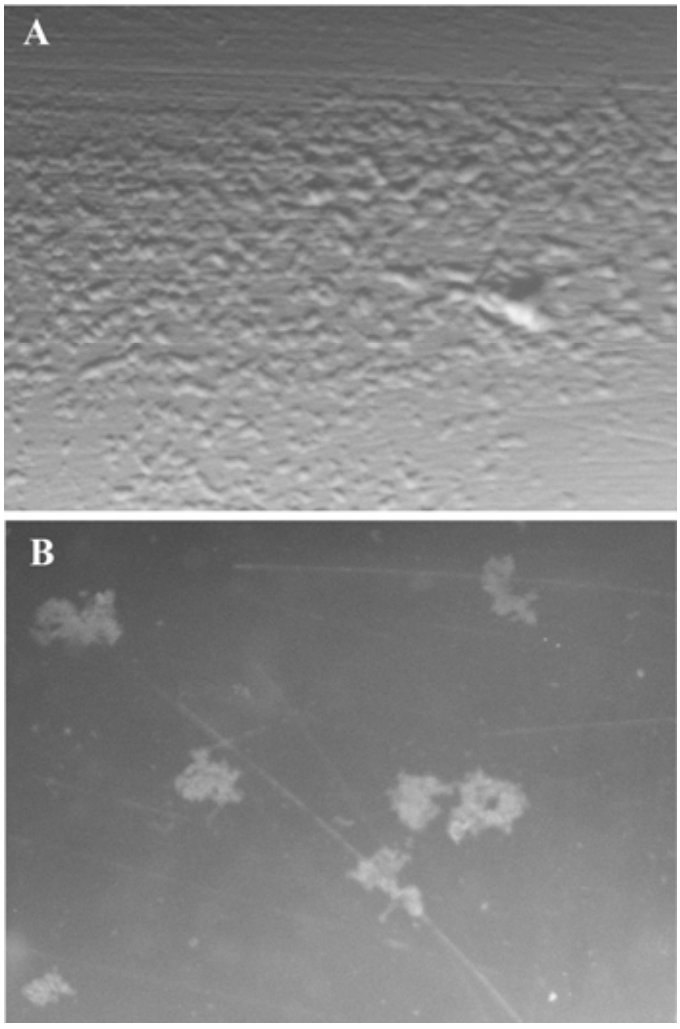

Fig. 1. A, Typical colonies of '*Candidatus* Liberibacter asiaticus' strain China1 in the first streak after 14 days at ×150 and **B,** '*Ca*. L. americanus' strain Brazil1 in liquid Liber A after 21 days at ×150.

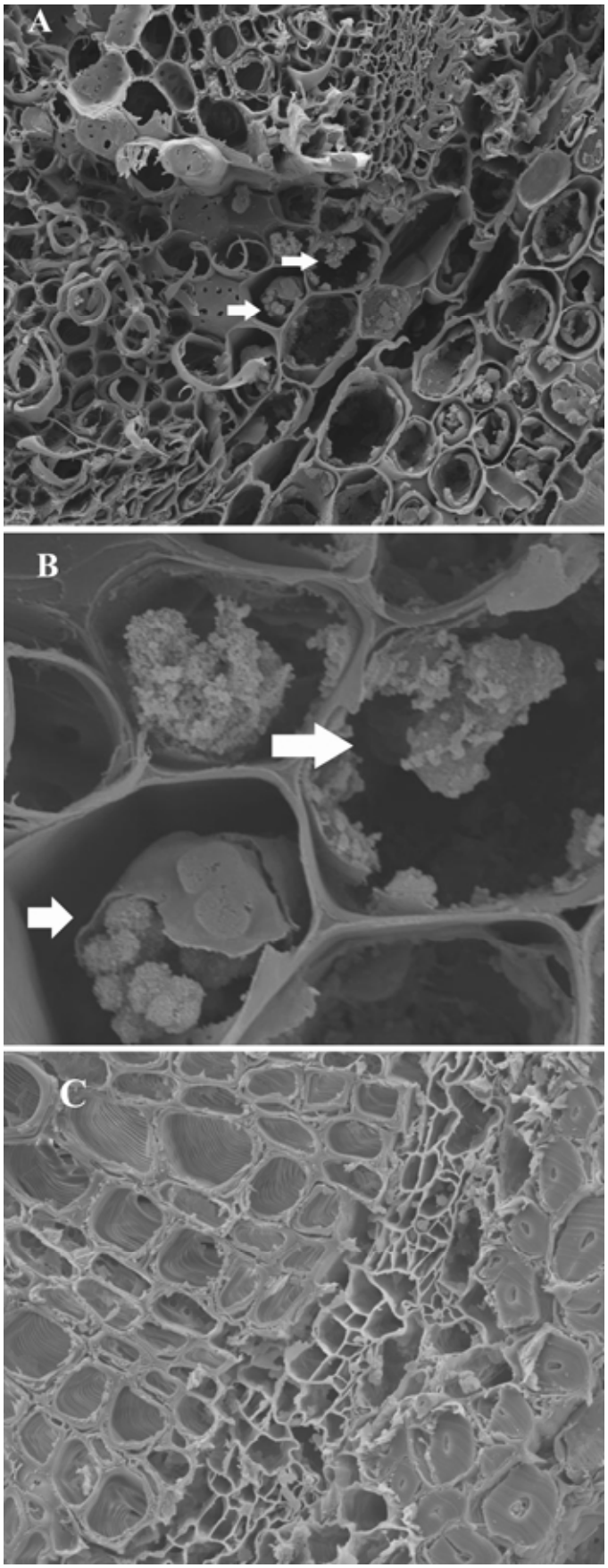

Fig. 2. Petiole cross section of infected tissue at **A,** ×2,500 and **B,** ×5,000 and healthy petiole tissue at **C,** ×2,500 viewed by scanning electron microscopy. Phloem fiber cells are clearly visible in **A** and **C**. Arrows indicate clumps of bacterial cells.

confirmed independently in Thailand by the isolation of similar shaped colonies, positive by RT-PCR, and 16s rDNA sequencing (data not shown). Medium C3G was not tested further due to the cost of serum.

Use of CVE at greater than 2% final concentration did not provide additional enhanced growth benefits based on observations in either PD2+E or Liber A agar medium. Observation showed that CVE prepared from only midvein and petiole tissue supported growth better then citrus extract prepared from whole leaves or leaves with the midvein removed. It also appeared that CVE was most beneficial when it was prepared from the midveins of young, fully expanded leaves. Preliminary results showed that the species of citrus from which the CVE was prepared did not affect growth of '*Ca*. Liberibacter spp.' Similar results were observed when CVE was prepared from lemon, lime, or orange. Autoclaved CVE did not support growth in either PD2+E or Liber A agar medium.

Compared with 28°C, little growth occurred at either 35 or 22°C. Colonies were visible 12 to 15 h sooner in a 5.0% CO_2 environment then in the ambient environment but, after 5 days, overall growth was no better. No growth was seen under anaerobic conditions or when plates were not sealed. The addition of NADP to basal Liber A agar medium resulted in an initial increase in growth; colonies became visible after only 3 or 4 days instead of 4 to 5 days. In contrast, NAD had no effect on growth.

Colonies of '*Ca*. Liberibacter spp.' on Liber A agar medium were irregularly shaped and ranged in size from 0.1 to 0.3 mm when observed after 3 to 4 days at 28°C (Fig. 1A). The colonies did not increase in size and viability was lost after four to five serial single-colony transfers (10 to 14 days each). In liquid Liber A, clumps of cells were visible after 7 to 10 days (Fig. 1B).

RT-PCR-positive strains were cultured and colonies transferred successfully from samples from the People's Republic of China, ('*Ca*. L. asiaticus'), Brazil ('*Ca*. L. asiaticus' and '*Ca*. L. americanus'), Thailand ('*Ca*. L. asiaticus'), India ('*Ca*. L. asiaticus'), Taiwan ('*Ca*. L. asiaticus'), Florida ('*Ca*. L. asiaticus'), and South Africa ('*Ca*. L. africanus') on Liber A medium. No differences in colony morphology were noted for any of the '*Ca*. Liberibacter' strains. Cells of '*Ca*. L. asiaticus' media were successfully cultured on Liber A agar medium after 6 months of archival storage at –80°C from solutions of both 50% glycerol and water and 50% glycerol and saline.

Electron microscopy. Cross-sections of infected (Fig. 2A and B) and healthy (Fig. 2C) petioles mounted for SEM revealed the presence of bacterial-like cells only in the infected tissue. This material was localized to cells in the vascular bundle and the presence of phloem fiber cells showed they were typical of secondary phloem cells of citrus (30). The bacterial-like cells appeared to be an aggregate of almost spherical bodies in the plant (Fig. 2A and B). Cells grown on agar were quite pleomorphic; the more typical cell was rod shaped, 0.3 to 0.5 µm wide by 0.7 to 2.0 µm long. The cells often had fimbriae-like appendages and a globular material attached (Fig. 3). Occasionally, cells as long as 4.0 µm or nearly spherical were observed. The cells appeared to have an outer membrane 20 to 30 nm wide (Fig. 4).

'*Ca*. L. asiaticus' and '*Ca*. L. americanus' identification. '*Ca*. L. asiaticus' sequences from China1 and China2 were identical to each other and had 98.5 to 98.7% similarity when compared with the 16s rDNA sequences from other China strains and 98.5 to 98.8% similarity when compared with all '*Ca*. L. asiaticus' sequences (Table 1). When China1 and China2 strains were compared with '*Ca*. L. americanus,' '*Ca*. L. africanus,' and '*Ca*. L. africanus subsp. capensis,' similarities were only 93.4, 96.4, and 93.5%, respectively. The '*Ca*. L. americanus' sequence Brazil1 was 100% identical to previously submitted '*Ca*. L. americanus' sequence AY742824 (Table 1).

Pathogenicity. One of three mature leaves of the 5-year-old tree infiltrated with cells of the China1 strain resulted in symptoms throughout the leaf. The inoculation site became brown and the surrounding tissue became mottled after 4 to 5 months. By 6 months, the entire leaf showed the typical mottling symptoms of HLB (Fig. 5A). Leaf tissue cut from the first 2 to 3 cm of the tip of the leaf, ≈2 cm from the inoculation site, was RT-PCR positive. Isolations from the same tissue resulted in growth of typical '*Ca*. L. asiaticus' colonies on Liber A agar medium. RT-PCR and 16s rDNA sequencing results of the isolated colonies confirmed the identity as the same organism used for inoculation. RT-PCR and isolation from leaves near the inoculation site and on other branches were negative at 6 months. After an additional 3 months, '*Ca*. L. asiaticus' strain China1 was detected in several leaves on the same branch 12 to 15 cm from the original inoculated leaf by RT-PCR and by isolation (Fig. 5B and C). Symptoms continued to develop distally along the same branch away from the inoculation

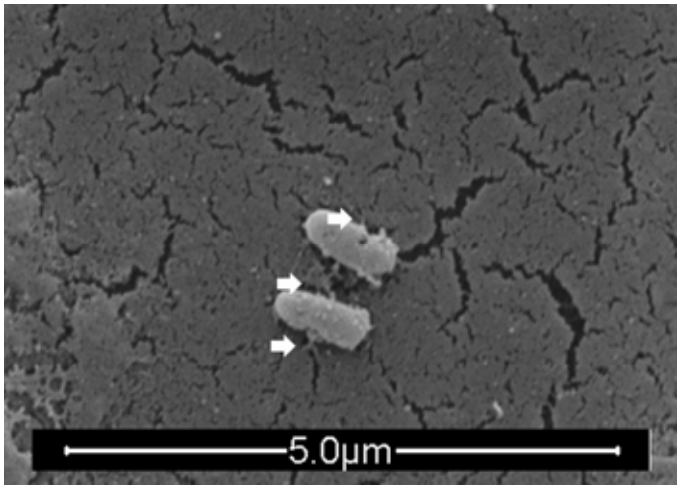

Fig. 3. Scanning electron micrograph of China1 cells (×10,000) taken from Liber A agar. Arrows indicate the presence of fimbriae-like structures and the globular material on the surface of the bacteria.

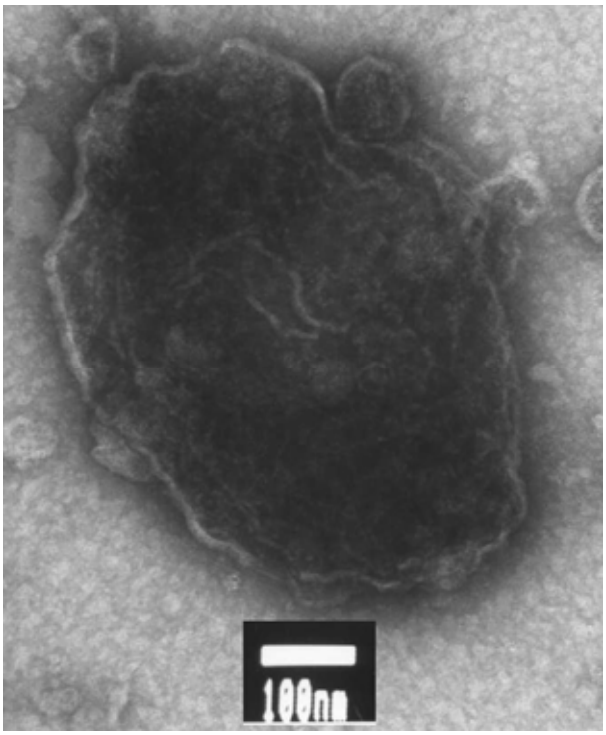

Fig. 4. Transmission electron micrograph of a single cell (×50,000) of the Thai sample isolated from *Citrus aurantiflora* cv. Pan grown in liquid PD2+E.

TABLE 1. Similarity matrix of the 16s rDNA gene region for the three strains used in the pathogenicity trials and strains of '*Candidatus* Liberibacter africanus' (*Ca.* afr), '*Ca.* L. africanus subsp. capensis' (*Ca.* cap), '*Ca.* L. americanus' (*Ca.* amer), and '*Ca.* L. asisaticus' (*Ca.* asia) from multiple regions of known infections

Strains	China1	China2	Brazil1	*Ca.* afr	*Ca.* cap	*Ca.* amer	Florida	Brazil	India	Taiwan	Malaysia	Guangdong	Chongqing	Guangxi	Jiangxi
China1[a]	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…
China2[a]	1.000	…	…	…	…	…	…	…	…	…	…	…	…	…	…
Brazil1[a]	0.934	0.934	…	…	…	…	…	…	…	…	…	…	…	…	…
Ca. afr	0.964	0.964	0.938	…	…	…	…	…	…	…	…	…	…	…	…
Ca. cap	0.935	0.935	0.907	0.953	…	…	…	…	…	…	…	…	…	…	…
Ca. amer	0.934	0.934	1.000	0.938	0.907	…	…	…	…	…	…	…	…	…	…
Florida[b]	0.988	0.988	0.944	0.972	0.941	0.944	…	…	…	…	…	…	…	…	…
Brazil[b]	0.988	0.988	0.944	0.972	0.941	0.944	1.000	…	…	…	…	…	…	…	…
India[b]	0.987	0.987	0.938	0.976	0.941	0.938	0.992	0.992	…	…	…	…	…	…	…
Taiwan[b]	0.987	0.987	0.943	0.971	0.940	0.943	0.999	0.999	0.991	…	…	…	…	…	…
Malaysia[b]	0.988	0.988	0.944	0.972	0.941	0.944	1.000	1.000	0.992	0.999	…	…	…	…	…
Guangdong[b,c]	0.986	0.986	0.941	0.969	0.938	0.941	0.997	0.997	0.989	0.996	0.997	…	…	…	…
Chongqing[b,c]	0.987	0.987	0.943	0.971	0.940	0.943	0.998	0.998	0.990	0.997	0.998	0.995	…	…	…
Guangxi[b,c]	0.986	0.986	0.940	0.968	0.937	0.940	0.996	0.996	0.988	0.995	0.996	0.993	0.994	…	…
Jiangxi[b,c]	0.987	0.987	0.944	0.970	0.939	0.944	0.998	0.998	0.990	0.997	0.998	0.995	0.996	0.994	…
Fujian[b,c]	0.985	0.985	0.940	0.968	0.937	0.940	0.996	0.996	0.988	0.995	0.996	0.993	0.994	0.992	0.994

[a] Strains used in the pathogenicity trials.
[b] '*Ca.* L. asiaticus' strains identified by geographic region.
[c] '*Ca.* L. asiaticus' strains from China.

site and main trunk; typical mottling was present in leaves 32 cm from the original inoculation site after 10 months. The other two infiltrated leaves abscised from the branch prior to symptom appearance and were not recovered for testing. The control leaves inoculated with water remained symptom-free, RT-PCR negative, and '*Ca*. L. asiaticus' could not be isolated (Fig. 5D).

From the second experiment, all three seedlings inoculated with strains China1, China2, and Brazil1 displayed a generalized mottling and an apparent reduction in growth after 2 months, with symptoms progressing to a distinct mottling by 3 months (Fig. 6). After 5 months, the seedlings began showing symptoms of dieback, especially the '*Ca*. L. americanus'-inoculated seedlings. By the end of 7 months, all '*Ca*. L. americanus'- and half of the '*Ca*. L. asiaticus'-inoculated seedlings were dead. Isolations from inoculated leaves and noninoculated leaves with symptoms resulted in typical colonies on Liber A agar medium. Positive RT-PCR results and 16s rDNA sequencing confirmed their identity as the same organisms used for inoculation. The seedlings inoculated with water and liquid Liber A medium failed to develop symptoms and remained negative by RT-PCR using inoculated and noninoculated tissues (Fig. 6).

DISCUSSION

With only several thousand bacteria isolated in pure culture and the total number of bacterial species estimated between 10^5 and 10^6 (20), methods for understanding the microbial world have moved beyond traditional culturing techniques to 16s rDNA sequencing (26), fluorescence in situ hybridization (21,27,28), genomic sequencing (15,34), and single-cell studies (1,39). Although using 16s rDNA and genomic sequencing to understand microbial diversity has led to the identification and classification of many previously undescribed bacteria, identifying pathogenic bacteria and correlating them to a disease still relies on obtaining pure cultures.

The best medium formulation was the simplest, incorporating both monobasic and dibasic potassium phosphate, CVE, and NADP. The use of Liber A agar medium improved the speed and overall growth relative to the slow and limited growth observed on C3G+E and PD2+E and increased the number of single-colony transfers to at least four before some strains began losing viability.

Although CVE could be made from any citrus species, autoclaving destroyed all activity. There appeared to be some heat-labile nutrient or protein that was deactivated or denatured by autoclaving. Nor was CVE alone able to sustain growth of '*Ca.* Liberibacter spp.' because water agar supplemented with up to 10% CVE did not support growth of the bacterium. The inclusion of CVE in Liber A agar medium allowed for growth of '*Ca.* Liberibacter spp.' from the mince-soak method whereas it did not for C3G+E and PD2+E. The requirement of CVE for the cultivation of this fastidious plant pathogen mimics the need of using soil-extract in media to successfully cultivate an *Actinomycetes* sp. from soil (14) and the observation of Garnett that '*Ca*. L. africanus' could grow on media conditioned by citrus callus (9).

Results showed that leaf infiltration was the only effective method for inoculation of citrus with '*Ca*. L. asiaticus' and '*Ca*. L. americanus'. All other methods of inoculation failed; however, the results could have been due to inoculum concentration rather than inoculation method. The time needed for development of systemic symptoms was much shorter for the seedlings than the development of local symptoms in the older tree. Symptom development and expression in a growth chamber may be more variable than symptom expression in a natural environment. The inoculated seedlings showed stunted growth and a generalized mottling at 2 months, with symptoms progressing to distinct mottling symptoms by 3 months after inoculation. The stunted growth symptom was consistent with symptoms noted in seedlings inoculated with '*Ca*. Liberibacter spp.' by psyllids in the greenhouse (V. D. Damsteegt, *unpublished data*). Younger leaf tissue or tissue on young trees seemed much more conducive to rapid spread of '*Ca*. L. asiaticus' and '*Ca*. L. americanus' than older leaf tissue or leaf tissue on older trees.

There remains the possibility that HLB is a syndrome with multiple causes, and '*Ca*. L. asiaticus' and '*Ca*. L. americanus' are only parts of syndromes and not entirely responsible for all symptoms seen in diseased trees (36). Because the trees used in this work were too young to set fruit, we were unable to see whether symptoms developed in fruit from the '*Ca*. L. asiaticus' and '*Ca*. L. americanus' inoculations. Thus, we cannot conclude that '*Ca*. L. asiaticus' and '*Ca*. L. americanus' are the only causal agents of HLB because Koch's postulates have not been completed by inoculating mature trees and reproducing the entire disease etiology. However, this partial fulfillment of Koch's postulates shows that '*Ca*. L. asiaticus' and '*Ca*. L. americanus' are pathogens of citrus, cause HLB-like symptoms in citrus, and are likely major components of the disease even under artificial growing conditions. Ideally, this work should be replicated in a natural field environment before '*Ca*. L. asiaticus' and '*Ca*. L. americanus' are confirmed as sole disease causing entities for HLB.

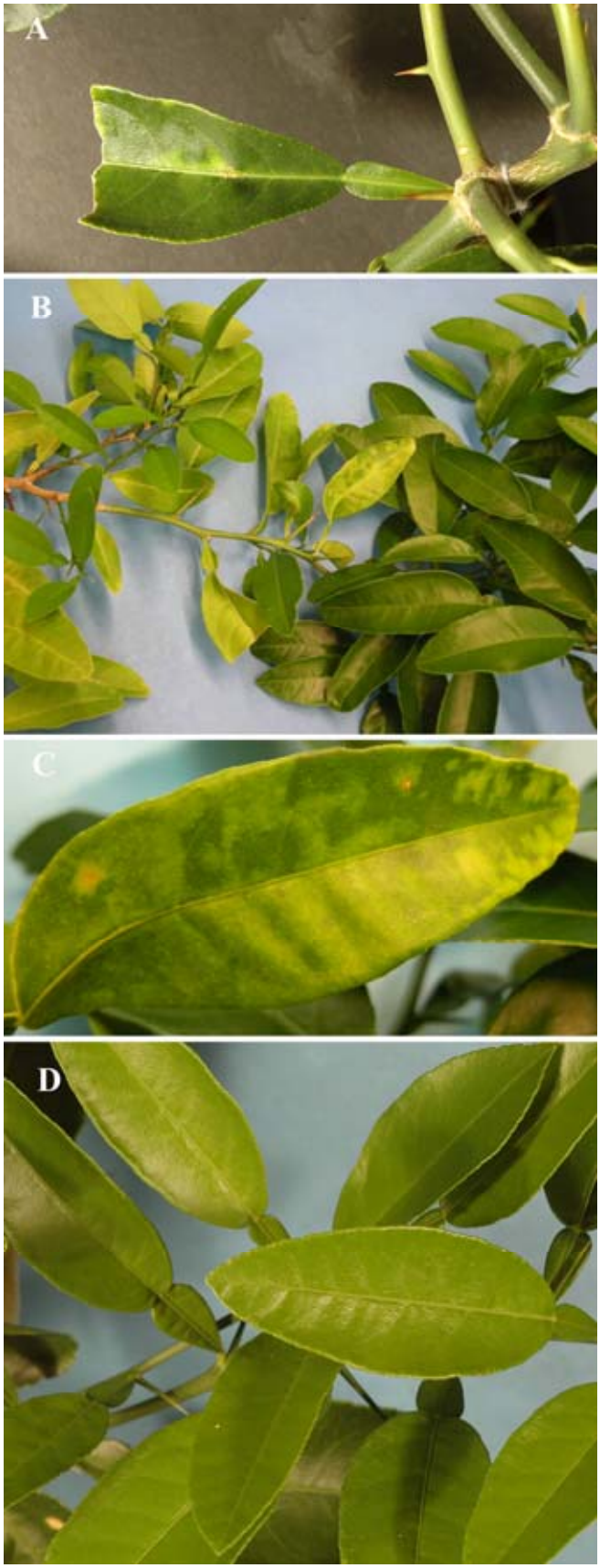

Fig. 5. A, Initial mottling symptoms on inoculated C*itrus macrophylla* leaf after six months. Distal portion of leaf was removed for a real-time polymerase chain reaction assay and isolation. **B,** Mottling and yellowing symptoms on noninoculated leaves 32 cm beyond the inoculated leaf shown in **A,** 9 months after inoculation. Note green healthy leaves to the right further out the branch. **C,** Close up of typical mottling symptoms on leaf shown in **B**. **D,** Control leaves of *C. macrophylla* inoculated with water.

Fig. 6. Mottling and stunted growth of *Citrus sinensis* cv. Madame Vinous 3 months after inoculation with '*Candidatus* Liberibacter americanus' strain Brazil1(left) and a water inoculated control (right).

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