Co-cultivation of '*Candidatus Liberibacter asiaticus*' with Actinobacteria from Citrus with Huanglongbing

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ABSTRACT

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Huanglongbing (HLB), also known as citrus greening disease, is a devastating disease of citrus caused by phloem-limited bacteria that have not been grown in culture. Three species, '*Candidatus Liberibacter asiaticus*', '*Ca. L. africanus*', and '*Ca. L. americanus*', are known. '*Ca. L. asiaticus*' and its insect vector, the psyllid *Diaphorina citri*, have been recently introduced into Florida. We attempted to isolate '*Ca. L. asiaticus*' using media formulations developed in response to the growth of another bacterium that appears to be related to the liberibacters based on 16S rRNA gene identities. Cultures were obtained that were polymerase chain reaction (PCR) positive for '*Ca. L. asiaticus*'. However, transmission electron microscope examination of the culture, PCR using generic primers, and sequencing of the PCR products revealed the presence of other bacteria in the cultures. These were actinobacteria related to *Propionibacterium acnes* based on 16S rRNA identities. The co-cultures remained after attempts to purify the cultures by single-colony isolation, suggesting that the bacteria might be mutually beneficial to each other in culture. The co-cultures have survived more than 10 weekly passages to fresh medium. PCR using *P. acnes*-specific primers indicated that actinobacteria are common inhabitants of citrus and psyllids, whether or not '*Ca. L. asiaticus*' is present.

Huanglongbing (HLB), also known as citrus greening disease, is an extremely devastating disease of citrus (1,3,15). The disease has long been present in Asia, Africa, the Indian subcontinent, the Mascarene Islands, and the Arabian Peninsula (2), but has only recently been found in South and North America (14,25). The disease is apparently caused by phloem-limited gram-negative bacteria that have not been grown heretofore in culture. At least three closely related pathogens in the α -subdivision of the Proteobacteria individually cause HLB and are provisionally classified as '*Candidatus Liberibacter asiaticus*', '*Ca. L. africanus*', and '*Ca. L. americanus*' until they can be cultured and then formally classified (17,26).

Fastidious prokaryotes have been associated with plant disease since the pioneering work of Doi et al. (9), who demonstrated that phytoplasmas (mycoplasma-like organisms) were in the phloem of plants infected with mulberry dwarf, potato witches'-broom, aster yellows, and paulownia witches'-broom, and of Ishie et al. (16), who demonstrated symptom re-

mission in plants with mulberry dwarf disease when treated with tetracycline group antibiotics. In 1970, Laflèche and Bové (19) reported observing structures resembling phytoplasmas in the phloem of citrus with HLB, but these organisms were later identified as gram-negative bacteria (12,20). Attempts to isolate fastidious prokaryotic plant pathogens in culture have met with limited success. The phloem-limited spiroplasmas were first isolated in the early 1970s (10,24) but none of the phloem-limited phytoplasmas have been isolated in culture. All of the fastidious xylem-limited bacteria, including both the gram-negative *Xylella fastidiosa* and the gram-positive *Liefsonia xyli* (*Clavibacter xyli*), have been isolated (4,6). The laticifer-inhabiting rickettsia associated with papaya bunchy top disease has not been cultured (5,8). None of the fastidious, phloem-limited bacteria, including the '*Candidatus Liberibacter*' spp., have been cultured, although there have been unsubstantiated reports that both mycoplasmas (13) and bacteria (11) associated with HLB have been cultured.

During a previous study on papaya bunchy top disease in 1995 (M. J. Davis, unpublished), a bacterium was isolated from the petioles of a single potted babaco plant (a hybrid of *Carica stipulata* and *C. pubescens*), also known as mountain papaya, that was nearly defoliated and had a few chlorotic leaves remaining when sampled. The bacterium grew on a medium modi-

fied from the PD2 medium (7) developed for isolation of *X. fastidiosa* causing Pierce's disease of grapevines. The medium had been modified to support better growth of another fastidious bacterium that had been isolated originally on the PD2 medium from California buckeye, *Aesculus californica*, with leaf scorch symptoms (M. J. Davis, unpublished). After sequencing the 16S rRNA gene of the babaco bacterium, it was determined that the bacterium was not the same as the rickettsia associated with papaya bunchy top disease, and its 16S rRNA gene did not share significant DNA identity with the DNA of any other plant-pathogenic bacterium in databases at the time. However, when a subsequent database search was conducted in 2006, a 93% identity between the 16S rRNA genes of '*Ca. L. americanus*' and the babaco bacterium was discovered. The identities with '*Ca. L. africanus*' and '*Ca. L. asiaticus*' were 92 and 90%, respectively. This indicated that the babaco bacterium might be the closest relative to the liberibacters that can be grown in axenic culture.

The purpose of this study was to develop means to grow '*Ca. L. asiaticus*' in culture. Growth of the babaco bacterium was evaluated on different media formulations in attempts to develop a formulation that also would support growth of '*Ca. L. asiaticus*'. Each new formulation was inoculated separately with both the babaco bacterium or with '*Ca. L. asiaticus*' extracted from HLB-affected citrus and examined periodically for growth. Slow-growing bacteria were isolated sporadically from the citrus extracts. Examination of the cultures indicated that '*Ca. L. asiaticus*' was present in co-culture with other bacteria.

MATERIALS AND METHODS

Plant materials. Citrus shoots with blotchy mottle symptoms of HLB were collected either from naturally infected plants in a grove of Valencia sweet orange (*Citrus sinensis* (L.) Osbeck) near Lake Placid, Florida, or from greenhouse-grown plants of Valencia sweet orange, citron (*C. medica* L.), mandarin (*C. reticulata* Blanco), rough lemon (*C. jambhiri* Lush.), or pummelo (*C. grandis* L.) that had been graft inoculated with HLB-infected bud wood. Leaves were removed from the shoots before surface disinfections. Young angular green shoots (3 to 5 cm)

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were dipped into 70% EtOH for 30 s, rinsed two to three times with sterile deionized water, submerged in 1% hypochlorite for 10 min, and rinsed three to four times with sterile deionized water. The bark was removed aseptically from each shoot piece, and the phloem tissue removed by scraping the inner bark with a sterile razor blade. The phloem tissues from two to three shoot pieces were suspended in a small amount of broth culture medium and either diced into approximately 1-mm² pieces with a razor blade or homogenized with a mortar and pestle. The volume was brought to 2.5 ml and the homogenate was clarified by centrifugation at 1,380 × g for 2 min. The supernatant was transferred equally to two 1.5-ml tubes and centrifuged at 7,000 rpm for 5 min, and the supernatant was discarded. Each pellet was resuspended in 250 µl of broth medium to use as inoculum. Inoculum (100 µl) was used to inoculate 2.5 ml of broth medium.

Media and culture conditions. The babaco basal medium (BBM) consisted of 20.34 g of brain heart infusion broth, 3.39 g of α-ketoglutaric acid, 16.95 g of *N*-(2-acetamido)-2-aminoethane sulfonic acid (ACES), 6.37 g of KOH, and 33.9 g of D-mannitol per liter, and the pH was adjusted to 6.9 before autoclaving. The AD medium consisted of 47.5 ml of BBM supplemented with 2.5 ml of Insectagro DS2 insect cell culture medium (Mediatech, Inc., Herndon, VA). The 2G medium consisted of 28.5 ml of BBM supplemented with the following filter-sterilized ingredients: 12.5 ml of DS2 medium, 7.5 ml of fetal bovine serum, 0.5 ml of ferric pyrophosphate stock (1.25% wt/vol), 0.5 ml folic acid stock (2% wt/vol), and 0.5 ml L-arabinose stock (10% wt/vol). The BBM3 basal medium consisted of 16.22 g of brain heart infusion broth, 2.70 g of α-ketoglutaric acid, 13.51 g of ACES, 5.08 g of KOH, and 27.02 g of D-mannitol per liter, and the pH was adjusted to 6.9 before autoclaving. The complete BBM3 medium consisted of 37.5 ml of BBM3 basal medium supplemented with the following filter-sterilized ingredients: 1 ml of bovine serum albumin fraction five (20% wt/vol), 5 ml of fetal bovine serum, 5 ml of TMN-FH insect medium (Sigma-Aldrich, St. Louis), 0.5 ml of ferric pyrophosphate stock (1.25% wt/vol), 0.5 ml folic acid stock (2% wt/vol), and 0.5 ml L-arabinose stock (10% wt/vol). Broth media were aseptically distributed to 13-by-100-mm borosilicate glass tubes at 2.5 ml/tube. After inoculation, broth cultures were incubated at 29°C on a gyratory shaker at 180 rpm unless incubated in a modified atmosphere, where they were incubated without shaking. Modified atmospheres were created in GasPak jars using either GasPak Plus anaerobic systems or microaerophilic systems envelopes (Becton Dickinson and Co., Sparks, MD). Semi-

solid media were prepared by adding 20 g of agar to the basal medium before autoclaving.

Transmission electron microscopy. Bacterial cells grown in 2.5 ml of BBM3 broth medium were collected by centrifugation at 10,000 × g for 10 min and resuspended in 100 µl of molten 1% SeaPlaque GTG agarose (FMC Bioproducts, Rockland, ME) in phosphate-buffered saline. The preparation was fixed after solidification for 2 h in 3% glutaraldehyde in 0.1 M potassium phosphate buffer, pH 7.2, then washed two times in the same buffer. Post-fixation treatment was for 2 h at room temperature in 2% osmium tetroxide in the same buffer. The sample was then washed three times in the above buffer, dehydrated in an acetone series, and embedded in Spurr's resin (23). After curing, silver to gold sections were made with a diamond knife on an LKB Huxley ultramicrotome (LKB Instruments Inc., Rockville, MD). The section on grids were stained with 2% aqueous uranyl acetate for 15 min and post stained with lead citrate (22) for 5 min. The samples were observed on a Morgagni transmission electron microscope (FEI, The Netherlands) and photographed with an AMT digital camera (Advanced Microscopy Techniques Corp., Danvers, MA).

DNA extraction, polymerase chain reaction, cloning, and sequencing. Genomic DNA was prepared from 1 ml of culture or plant extract. The solution was centrifuged at 10,000 rpm for 5 min in a 1.5-ml tube, and the supernatant was discarded. The pellet was resuspended in 467 µl Tris-EDTA (TE) buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA) by vortexing, mixed with 30 µl of 10% sodium dodecyl sulfate and 3 µl of 20 mg/ml of proteinase K, and incubated at 37°C for 1 h. An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added to the tube, mixed, and centrifuged (12,000 rpm, 10 min). The upper aqueous phase was transferred to a clean tube, 6 µl of RNase A at 10 mg/ml was added, and the mixture was incubated at 37°C for 30 min. The phenol/chloroform/isoamyl alcohol extraction was repeated, and the supernatant was transferred to a new tube and mixed with 1/10 volume of sodium acetate (3 M, pH 5.2) and 0.6 volume of isopropanol. After centrifugation (12,000 rpm, 10 min), the supernatant was discarded and the precipitate washed once with 70% ethanol (12,000 rpm, 5 min). The air-dried DNA was dissolved in 20 µl of sterile milli-Q H₂O.

Psyllids (*Diaphorina citri* (Kuwayama)) were collected using an aspirator from psyllid colonies maintained on plants in the greenhouse or growth chamber. Psyllids were also collected from commercial citrus groves in the vicinities of Lake Placid, Avon Park, and Lake Wales, FL. Psyllids were stored in ethanol at -40°C

until extraction. Psyllids were transferred from ethanol to several layers of Kimwipes with a fine paint brush. They were next transferred to 1.5-ml tubes, either individually or in batches of up to six insects, to which 300 µl of nucleic acid buffer (1 mM Tris-HCl, pH 8.0; 10 mM NaCl; 1 mM EDTA; 2% sodium laurel sulfate) and 30 µl of Proteinase K (20 mg/ml) was added. The psyllids were homogenized using a plastic pestle. The homogenate was incubated at 50°C for 2 h with mixing by inversion every 15 min. The homogenate was extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1, pH 8.0), 200 µl of the extract was mixed with 20 µl of 3 M sodium acetate (pH 5.2) followed by 1 ml of ice cold ethanol (95 to 100%), and the mixture was held at -40°C for 18 h. The precipitated DNA was collected by centrifugation at 4°C for 15 min at 13,000 rpm and washed with 1 ml of 70% ethanol. The alcohol was removed and tube was vacuum centrifuged at 1,000 rpm for 10 minutes at 25°C. The dried pellet was resuspended in either 20 µl (one or two psyllids) or 50 µl (more than two batched psyllids) of nuclease-free water. The suspension was centrifuged for 2 min at 10,000 rpm (25°C) and stored at -40°C until used as a template for polymerase chain reaction (PCR).

Three pairs of primers, OI1/OI2C (18), fD1/rP2 (27), and Pas9/Pas11 (21), were used for PCR amplification in 25-µl volumes, containing 1× fast buffer, 0.2 mM dNTPs, 0.2 µM forward and reverse primer, and 0.625 U SpeedSTAR HS DNA polymerase (TaKaRa, Madison, WI). The PCR program was initialized at 94°C for 2 min; followed by 40 cycles of 94°C for 10 s, 66°C (OI1/OI2C and RP2/FD1) or 57°C (Pas9/Pas11) for 10 s, and 72°C for 1 min; and ending with a final step at 72°C for 10 min and at 4°C until use. PCR products were separated by electrophoresis and visualized by UV transillumination following staining with ethidium bromide.

PCR products were recovered from agarose by using an agarose gel DNA extraction kit (F. Hoffmann-La Roche Ltd., Basel, Switzerland), and ligated to pGEM-T Easy Vector (Promega Corp., Madison, WI). The ligation mixture was then transformed into *Escherichia coli* DH-5α competent cells via heat shock. Clones with the correct size of insert were chosen. Sequencing was done by the University of Florida, Interdisciplinary Center for Biotechnology Research.

RESULTS AND DISCUSSION

Two to three weeks after inoculating broth AD medium with an extract obtained from a symptomatic citron tree that had been graft inoculated with buds from an HLB-infected tree, a film of growth appeared on the bottom of the tube. Light microscope examination (×1,000) of the culture revealed small pleomorphic coco-

bacilliary cells. PCR examination of DNA from the culture using the OI2c/OI1 primer pair for detection of '*Ca. L. asiaticus*' produced positive results. The amplicon was cloned and sequenced, and a search of the National Center for Biotechnology Information (NCBI) nucleotide collection (BLAST search) indicated that it was from '*Ca. L. asiaticus*'. The culture was streaked onto semisolid AD and BBM3 media and incubated under aerobic and microaerophilic conditions. Single colonies were obtained on BBM3 medium under microaerophilic conditions that were approximately 1 mm in diameter and appeared opaque and convex with circular margins after 1 week of incubation. Under aerobic conditions, growth was barely visible with a stereomicroscope after 2 to 3 weeks on the BBM3 medium. Growth was poor on semisolid AD medium under microaerophilic conditions, and no growth occurred when incubated under aerobic conditions. Cultures were obtained by single-colony isolation under microaerophilic conditions, and all 35 cultures from single-colonies were positive in the PCR for '*Ca. L. asiaticus*'. However, when the amplicon was cloned and sequenced, the sequence did not match well with any sequence in the NCBI database. Further examination of the PCR amplification product indicated that two nearly comigrating amplicons were present. Cloning and sequencing of the faster-migrating gel band yielded a 16S rRNA sequence that matched that of '*Ca. L. asiaticus*'. Restriction digestion of the 1,160-bp '*Ca. L. asiaticus*' amplicon with *Xba*I yielded 640- and 520-bp fragments from a single cut as expected for '*Ca. L. asiaticus*', whereas the unknown DNA was not cut.

Cells from a culture obtained after single-colony isolation were embedded in agarose and examined by transmission electron microscopy (Fig. 1). The cells were cocobacilliary, had cell walls resembling those of gram-positive bacteria, and appeared to divide by septate division. This did not match previous descriptions

Fig. 1. Transmission electron micrograph of actinobacteria from co-culture with '*Candidatus Liberibacter asiaticus*'. Bar = 500 nm.

of '*Ca. L. asiaticus*' that showed the bacterium to be predominantly rod shaped and to have a gram-negative type of cell wall (12). To further examine the identity of the observed bacteria, the 16S rRNA gene was amplified using generic primers fD1/rP2 (27), cloned, and sequenced. A BLAST search revealed 100% identity with a 1,006-bp partial sequence of the 16S rRNA gene of *Propionibacterium acnes* GenBank accession AF154832 and the same degree of identity with the gene of three uncultured bacteria. Thus, '*Ca. L. asiaticus*' appeared to be growing in co-culture with an actinobacterium closely related to *P. acnes*.

A second co-culture of an actinobacterium and '*Ca. L. asiaticus*' was obtained after 31 more isolation attempts from HLB-infected trees. This culture in 2G medium was from a field-grown sweet orange tree with HLB symptoms. A third co-culture was obtained in AD medium from an HLB-infected pummelo tree in the greenhouse after 12 more isolation attempts. The actinobacteria were not isolated alone from citrus. Amplification using the OI2c/OI1 primer pair for detection of '*Ca. L. asiaticus*' produced positive results for both of these isolates, and the cloned 16S rRNA sequences matched the 16S rRNA of '*Ca. L. asiaticus*'. Amplification with the generic primer pair fD1/rP2 produced products with 90 and 98% identity (840/931 and 1,014/1,030 bp for the second and third co-cultures, respectively) with the 16S rRNA sequence of *P. acnes* GenBank accession AM157438, which was the closest named species. Following single-colony isolations, all six single-colony isolates tested from each co-culture were PCR positive for '*Ca. L. asiaticus*'. All three co-cultures have been maintained for more than 10 weekly passages in 2G medium, although '*Ca. L. asiaticus*' is often only weakly detected by PCR. In addition to the single-colony isolation performed with each co-culture, each subsequent transfer in 2G medium involved a 100-fold dilution; thus, it appears that '*Ca. L. asiaticus*' was multiplying in culture.

DNA extracted from two healthy rough lemon trees and one healthy Valencia sweet orange tree from the greenhouse and a healthy Valencia sweet orange tree from the field all had positive PCR results using the Pas9/Pas11 primer pair for detection of *P. acnes*. Furthermore, positive results were obtained for two periwinkle plants (*Catharanthus roseus*) that had been infected with HLB by dodder (*Cuscuta* spp.) transmission from HLB-infected citrus and for one healthy periwinkle plant.

PCR with DNA from 15 psyllids using the Pas9/Pas11 primer pair for detection of *P. acnes* produced positive results for all insects originally from the same colony. These insects included five psyllids that had fed on HLB-infected plants that tested PCR positive for '*Ca. L. asiaticus*', five

psyllids that had fed on HLB-infected plants that tested PCR negative for '*Ca. L. asiaticus*', and five psyllids that had not fed on HLB-infected plants. When field-collected psyllids were tested by PCR for *P. acnes*, 10 of 15 extracts were positive. These included three positive samples from two pooled psyllids each from Lake Placid, FL; five positive samples of eight samples from two to six pooled psyllids each from Avon Park, FL; and two positive samples of four samples from six pooled psyllids each from Lake Wales, FL. None of the extracts from the field-collected psyllids tested positive in PCR for '*Ca. L. asiaticus*'. When the PCR amplicon from a sample from each of the three locations was cloned and sequenced, the sequences all had greater than 99% identity with a 916- to 946-bp portion of the 16S rRNA gene of actinobacteria, among which *P. acnes* was the only named species.

In conclusion, although '*Ca. L. asiaticus*' was not isolated in axenic culture, it was isolated in co-cultures with actinobacteria closely related to *P. acnes*. The co-cultures appear to be long-standing, having been maintained through single-colony isolation and repeated passage to fresh medium. '*Ca. L. asiaticus*' and the actinobacteria remained together after single-colony isolation, suggesting that both bacteria benefit one another for survival in culture. PCR tests with primers designed for detection of *P. acnes* produced positive results for both citrus trees and psyllids, whether or not they were infected with '*Ca. L. asiaticus*', suggesting that actinobacteria are common inhabitants of these hosts. Whether or not there is any interaction between '*Ca. L. asiaticus*' and the actinobacteria in the plant or insect remains to be determined.

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