Association of a Bacterium and Not a Phytoplasma with Papaya Bunchy Top Disease

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ABSTRACT

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Papaya bunchy top (PBT), a major disease of papaya (*Carica papaya* L.) in the American tropics, was thought to be caused by a phytoplasma. However, 95 papaya plants with symptoms of PBT from 12 countries throughout the American tropics were assayed by polymerase chain reaction for the presence of 16S rRNA genes of phytoplasmas, but none were found. Examination of transverse sections of petiole tissue by epifluorescence microscopy revealed the presence of fluorescing materials associated with PBT on the periphery of the phloem, between the phloem and xylem, and sometimes extending along the phloem rays as far as the cortex. Bacteria were detected within the same region by transmission electron microscopy and were found consistently in three PBT-affected plants from Puerto Rico and Costa Rica, but not in a healthy plant from Florida. The bacteria were rod-shaped, measuring 0.25 to 0.35 μm in width and 0.8 to 1.6 μm in length. Their cell wall ultrastructure resembled that of Gram-negative bacteria, except that a peptidoglycan layer was not evident in the periplasmic space. The bacteria appeared to colonize laticifers. The bacteria were consistently observed by light microscopy in expressed sap from fresh papaya petioles of plants with PBT symptoms, but not in similar preparations from healthy plants. All attempts to isolate the bacteria in axenic culture were unsuccessful. If the bacterium causes PBT, it would be the first example of a leafhopper-transmitted, laticifer-inhabiting, plant pathogenic bacterium.

Papaya (*Carica papaya* L.) is native to the American tropics, but is now cultivated widely throughout tropical and subtropical areas of the world for both local consumption and export of fruit and, to a lesser extent, for production of papain. Papaya bunchy top (PBT) is one of the most economically important diseases of papaya in the American tropics (12). PBT was first reported in Puerto Rico in 1931 (9); papaya diseases of unknown etiology, but similar in symptomatology, were reported at about the same time in Jamaica (41) and the Dominican Republic (7). Diagnosis of PBT has relied exclusively on symptomatology and, therefore, has been presumptive. Presently, papaya diseases resembling PBT are known to occur on numerous other Caribbean islands from Grand Bahama southward to Trinidad and in Central and South America (4,22,34,44; R. T. McMillan, *personal communication*).

Two leafhopper species, *Empoasca papayae* Oman (1) and *E. stevensi* Young (22), have been identified as vectors of PBT. The disease was present wherever *E. papayae* was found to exist in the Antilles (34), but has not been found in Florida, where this principal insect vector does not exist (34,43). The identification of *E. stevensi*, which was first taxonomically described based on museum specimens from Florida (45), as a vector of bunchy top in Trinidad (22) is cause for concern about further spread of the disease to unaffected areas such as Florida.

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Although a viral etiology was first suspected (5,9), a phytoplasma (formerly called a mycoplasmalike organism) was later reported to be associated with PBT (42). Bodies resembling phytoplasmas were observed by transmission electron microscopy in the phloem of PBT-affected papaya plants, and infected plants treated with tetracycline-type antibiotics exhibited a remission of symptoms.

Initial PBT symptoms (2,5,12,28,29; B. R. Brunner, *unpublished data*) include diffuse chlorosis in young leaves accompanied by reduction in normal leaf blade expansion and petiole and internode elongation. Small (about 0.5 mm) discrete water-soaked spots appear on affected petioles and stems, later developing into irregular blotches about 1 to 2 mm in diameter. Petioles are rigid, extending more horizontally from the main stem than normal. Leaf blades are thickened, stiff, and may exhibit downward cupping, marginal and interveinal chlorosis, and/or necrosis. Flowering and fruit set rarely occur in PBT-affected plants. In advanced stages, the plants become denuded except for a tuft of small leaves at the apex. Symptoms and their rate of development, however, are variable and severity of symptom expression may be affected by host genotype. Dieback of papaya in Puerto Rico (2) may be a more severe form of PBT. The absence of latex exuding from fresh wounds of affected tissues is considered characteristic of PBT and Puerto Rican dieback (2), but is also symptomatic of two other papaya diseases of unknown etiology in Australia referred to as mosaic (38) and dieback (23). Webb and Davis (44) questioned the reliability of the latex-flow test for diagnosis of PBT because bodies resembling phytoplasmas were observed by

transmission electron microscopy in samples from symptomatic plants that exhibited a normal flow of latex upon wounding.

In an effort to develop more specific means to diagnose PBT, numerous attempts were made to isolate and clone DNA of the putative PBT phytoplasma (M. J. Davis and N. A. Harrison, *unpublished data*). However, all attempts failed, even though several different approaches which had been used successfully to clone DNA of other phytoplasmas (16,25,27,31,32) were tried. Furthermore, phytoplasma DNA was not detected in diseased papaya by hybridization assays using a general phytoplasma-DNA probe for the 16S rRNA gene of phytoplasmas (30) provided by B. C. Kirkpatrick. These results led us to question the role of a phytoplasma in the etiology of PBT.

The present study was initiated to investigate the etiology of PBT. Recently, Deng and Hiruki (18) described the application of polymerase chain reaction (PCR) technology to detect the 16S rRNA genes of phytoplasmas and others of the class *Mollicutes*. We applied this technology to survey papaya plants with PBT symptoms from different locations in the American tropics for the presence of phytoplasmas, but none were detected. Further investigation led to the discovery of a fastidious bacterium associated with PBT.

MATERIALS AND METHODS

Plant material. Papaya petiole samples, mostly from plants with typical PBT symptoms, were collected from commercial and experimental orchards in Puerto Rico near the cities of Isabela, Juana Diaz, Canóvanas, Mayaguez, Lajas, Cabo Rojo, and Guánica. Samples from papaya plants with PBT symptoms were also obtained from different countries throughout the American tropics with the aid of contributors (Table 1). Petioles from plants in Florida, where PBT is not known to exist, were used as PBT-free controls. Samples from Puerto Rico and Florida were either processed while fresh, stored at –80°C, or cut into 1- to 2-cm segments and preserved in an aqueous solution of 5% sodium borate (39) until use. Petiole samples from other locations were received as segments in 5% borate solution. Petiole samples from two papaya plants with yellow crinkle disease were kindly provided by R. Peterson, Department of Primary Industries, Mareeba, Australia. Petiole samples from two papaya plants with Australian dieback disease were kindly provided by D. Teakle, University of Queensland, Queensland, Australia. Samples in borate solution were kept at 4°C, except when in transit at ambient temperatures. In preliminary tests, borate solution proved to be an effective, easy, and efficient short-term preservative with no subsequent adverse effect on diagnostic PCR. Preliminary tests were performed with phytoplasma-infected periwinkle, healthy papaya, and mixtures of both; phytoplasma DNA was successfully amplified in all cases where expected. Furthermore, preservation in borate solution was also compatible with examination of stained tissues by epifluorescence microscopy as previously reported by Sinclair et al. (39).

Nucleic acids extraction. Total nucleic acids were extracted from healthy and infected plant materials using a method modified from that of Saghai-Maroof et al. (37). Samples in borate preservative were rinsed in deionized water. Preserved and fresh plant tissue samples were held at –80°C. Approximately 1 g of each frozen sample was thoroughly ground to a fine talcum powder consistency under liquid nitrogen using a mortar and pestle and transferred directly, without thawing, into a 30-ml centrifuge tube containing 15 ml of hexadecyltrimethylammonium bromide (CTAB) buffer (2% CTAB, 100 mM Tris-HCl [pH 8.0], 1.4 M NaCl, 20 mM EDTA, 0.2% 2-mercaptoethanol) preheated to 65°C. The preparation was incubated for 60 min at 65°C with gentle mixing by slow inversion every 10 min. Approximately 20 ml of chloroform/isoamyl alcohol (24:1) was added and mixed gently with the aqueous phase by slow inversion to complete emulsion. The phases were separated by centrifugation at 1,600 × *g* for 5 min, and 12 ml of the aqueous supernatant was gently mixed with 8 ml of cold (–20°C) isopropyl alcohol. After cooling to –20°C, precipitated nucleic acids were collected by centrifugation for 10 min at approximately 500 × *g*, washed (76% aqueous ethanol, 10 mM ammonium acetate), dried, and resuspended in approximately 10 volumes of deionized distilled water. DNA concentrations in solution were estimated by spectroscopy, and aliquots of the solutions were diluted to a working concentration of 25 ng/μl and stored at 4°C.

DNA from periwinkle infected with the phytoplasmas causing eastern aster yellows, western aster yellows, and western-X disease, and from coconut palm infected with the phytoplasma causing palm lethal yellowing were provided by N. A. Harrison.

Primer pairs and PCR conditions. PCR primers developed for the class *Mollicutes* generically and phytoplasmas specifically were used in attempts to amplify diagnostic DNA fragments from papaya extracts. The P1 and P6 primers of Deng and Hiruki (18) were used as the standard *Mollicutes* generic primers. These primers enabled amplification of an approximately 1,500-bp DNA fragment within the 16S rRNA gene of all *Mollicutes*. This has been confirmed in our studies, by published results, and by personal communications with other researchers. Universal phytoplasma rRNA intergenic region primers, O4 and O7, provided by B. C. Kirkpatrick, were tested similarly. In addition, the palm lethal yellowing specific primer pair, LYF-1 and LYR-1, (26) was tested. For all PCR amplification experiments, one or more DNA extracts from plants infected with the phytoplasmas were used as positive controls. Distilled deionized water was used as a negative control.

All PCR were performed in a Coy ThermoCycler II Model 110S with the in-sample probe (Coy Corporation, Grass Lake, MI). A standard 50-μl reaction mixture (20 mM Tris [pH 8.3], 1.5 mM $MgCl_2$, 25 mM KCl, 0.01% Difco Gelatin [Difco Laboratories, Detroit], 0.05% Tween 20, 100 μM of the four dNTPs, 1 ng/μl each of primer and template DNA) in 0.6-ml tubes was used for all amplifications. All mixture components were precooled to 4°C and kept on ice while mixed and prepared for amplification. Each reaction mixture was overlaid with one drop (about 50 μl) of certified DNase/RNase-free mineral oil (Sigma Chemical Co., St. Louis). The temperature cycling schedule was 90 s predenaturation at 94°C, followed by 35 cycles of 30 s at 94°C, 50 s at annealing temperature, and 80 s for extension at 72°C. The reactions were held at 4°C immediately subsequent to the cycling. The annealing temperature varied with the primers used and was

TABLE 1. Petiole samples from papaya plants with foliar symptoms of bunchy top disease tested by polymerase chain reaction (PCR) for phytoplasmas and by epifluorescence microscopy for internal symptoms associated with the disease

Country	No. samples[a]	No. with internal symptoms[b]	Contributor
Antigua	7	1	N. Roberts-Samuel
Barbados	4	2	M. Phillips
Belize	4	3	J. Link
Costa Rica	5	5	F. Elango
Dominican Republic	4	3	J. Borbon
Dominica	4	3	U. Martin
Ecuador	4	0	R. McMillan, Jr.
Grenada	4	2	C. Persad
Puerto Rico	44	42	(This study)
Saint Lucia	4	3	F. Henry
Saint Lucia	3	3	E. Ambrose
Saint Vincent	4	2	F. Gynsam
Venezuela	4	1	F. Leal
Total	95	70	

[a] All 95 samples were negative by PCR when using the P1/P6 primer pair.
[b] Transverse sections were stained with DAPI or acridine orange prior to microscopic examination.

set as that of the lower of the primer pair as calculated by the Breslauer et al. (6) thermodynamic nearest neighbor base stacking energy model. Thus, the annealing temperatures were 60, 55, and 58°C for the P1/P6, O4/O7, and LYF-1/LYR-1 primer pairs, respectively.

Amplification products (15 to 20 µl) were separated by electrophoresis in 2% agarose gels in Tris-acetate-EDTA (TAE) buffer (40 mM Tris-acetate, 1 mM EDTA [pH 7.8]). After separation at 1.5 V/cm for 18 h, gels were stained with ethidium bromide and then photographed with UV illumination. *Hin*dIII/*Eco*RI-digested lambda DNA fragments were used as molecular weight markers.

Fluorescence and electron microscopy. Aqueous DAPI (4′,6-diamidino-2-phenylindole·2 HCl) (Sigma Chemical Co.) was used at 0.4 µg/ml (40), and aqueous acridine orange (Fisher Scientific Co., Fair Lawn, NJ) was used at 20 µg/ml (10). Cross-sections were prepared by hand (about 100-µm thick) or by a freezing microtome (30- to 50-µm thick) from papaya petiole segments preserved in borate solution. Sections were either not stained, stained with DAPI for 30 to 45 min or with acridine orange for 3 min, rinsed with water, and mounted on microscope slides in 50% aqueous Karo white corn syrup. Sections were examined by epifluorescence microscopy. UV and blue light excitation were used for DAPI and acridine orange stained sections, respectively.

Epifluorescence microscopy was also used to examine sap from petioles for the presence of microorganisms. Sap was expressed with pliers from freshly cut transverse surfaces near the base of petioles, mixed with an equal volume of acridine orange or DAPI staining solution, incubated in the dark for 3 min for acridine orange or 30 min for DAPI, and examined as wet mounts by epifluorescence microscopy at 1,000× magnification.

Papaya petioles from two plants in Puerto Rico and one plant in Costa Rica with typical symptoms of PBT and from one healthy plant in Florida were examined by transmission electron microscopy. Petiole sections from the plants, which had been preserved in borate solution as described above, were resuspended in a solution containing 2% glutaraldehyde in 0.01 M PO_4 buffer (pH 7.2) and sent to the University of Florida, Interdisciplinary Center for Biotechnology Research, Electron Microscope Core Laboratory. Samples were further processed and examined essentially as described by Norris and McCoy (35). Latex flowing from freshly wounded, healthy papaya fruit was collected, mixed at 35°C in an equal volume with a molten solution of 2% low-melting-point agarose in deionized water, allowed to solidify at room temperature, and then prepared for electron microscopy in the same manner as petiole tissues.

Bacterial isolations. Approximately 50 g of petioles from symptomatic leaves were surface disinfested by dipping in 95% ethanol, submerging in 0.5% sodium hypochlorite for 5 min, and rinsing three times with sterile deionized water. The petioles were then homogenized in a sterile blender jar for 30 s with 4 volumes (wt/vol) of filter-sterilized (0.2-µm-pore size) 0.01 M potassium phosphate buffer (pH 6.9) containing 5% BBL Trypticase peptone (Becton Dickinson, Cockeysville, MD). The homogenate was filtered through four layers of cheesecloth and centrifuged for 10 min at 10,000 × *g*. The resultant pellet was resuspended in 10 ml of extraction buffer at 250 rpm for 20 min at room temperature. Three 100-fold serial dilutions in 0.01 M phosphate buffer (pH 6.8) were prepared from the suspension and then spotted onto different culture media in three to five replicate 10-µl aliquots per plate. Petioles from different plants were processed separately. Numerous media were tested including agar-solidified preparations and various modifications of the PD2 medium (15), SC medium (14), PW medium (13), L20 medium (17), and C3G medium (33). Inoculated media were incubated aerobically at 28°C and examined periodically for growth for 3 to 4 weeks.

RESULTS

PCR tests for phytoplasmas. Ninety-five papaya plants with symptoms of PBT from 12 countries throughout the American tropics (Table 1) were assayed by PCR for the presence of 16S rRNA genes of phytoplasmas using the P1 and P6 primer pair, but all were negative. No differences were detected between DNA samples from healthy and PBT-affected papaya plants (Fig. 1). Negative results were obtained when leaf midveins, petioles, stems, and shoot-tips from PBT-affected plants were examined. All positive control amplifications produced appropriately sized amplimers and all negative controls produced no correctly sized amplimers. Nonspecific amplifications of chloroplast and mitochondrial 16s rRNA genes were obtained with all samples. Negative results were also obtained with other generic and specific PCR primer pairs when petiole sections from PBT-affected plants were examined (data not shown). Amplification of positive controls was not diminished by thousand- and million-fold dilutions with relative quantities of papaya DNA extracts, indicating that low titer and/or interfering substances were not the reason for the failure to detect phytoplasma DNA in the diseased papaya samples. Furthermore, appropriately sized amplimers indicative of the presence of phytoplasmas were obtained for each sample from papaya plants with yellow crinkle and dieback diseases from Australia (Fig. 1).

Epifluorescence microscopy. When examined by epifluorescence microscopy, differences were observed between petiole sections from healthy papaya plants and those taken from PBT-affected plants (Fig. 2). In nonstained sections of diseased petioles, abnormal autofluorescence was observed on the periphery of the phloem on the innermost side near the xylem (Fig. 2A). This autofluorescence sometimes extended into the phloem rays as far as the cortex. The autofluorescence was a dull yellow under both blue light and UV light excitation. Similar autofluorescence was not observed in healthy petiole tissue or petiole tissue from plants infected with papaya ringspot virus (Fig. 2B; and data not shown). When petiole sections from PBT-affected papaya were stained with DAPI, regions of bright bluish white fluorescence were observed intermixed with the dull yellow autofluorescence associated with PBT-affected tissues (Fig. 2E). When sections were stained with acridine orange, these autofluorescing regions exhibited a brighter yellow fluorescence that sometimes had a

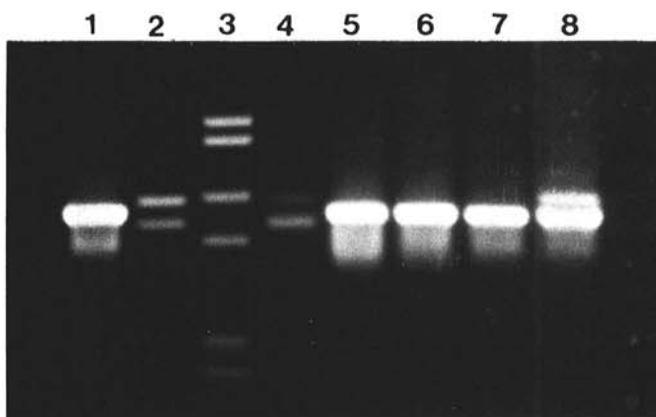

Fig. 1. Polymerase chain reaction (PCR) amplified 16S rRNA gene fragments obtained using the P1/P6 primer pair with total DNA extracts of healthy papaya plants from Puerto Rico, papaya plants with bunchy top (PBT) disease from Puerto Rico, yellow crinkle disease from Australia, and dieback disease from Australia. The healthy papaya and PBT-affected papaya DNAs were bulked samples from several plants each. An extract from a periwinkle infected with the eastern aster yellows phytoplasma was used as a positive control. The template DNAs were as follows: lane 1, eastern aster yellows; lane 2, healthy papaya; lane 4, PBT; lanes 5 and 6, yellow crinkle; lanes 7 and 8, dieback. Lane 3, *Eco*RI/*Hin*dIII-digested lambda DNA molecular weight standards.

fringe of orange fluorescence (Fig. 2C). Similar staining was not observed in sections from healthy plants and plants affected by papaya ringspot virus (Fig. 2D and F; and data not shown).

The fluorescence associated with PBT was observed in petiole sections stained with DAPI from all 37 plants with PBT symptoms sampled in commercial groves in the vicinity of Isabela, Juana Diaz, and Canóvanas, Puerto Rico, during March 1993. Concurrently, fluorescence associated with PBT was not observed in sections from 14 plants without PBT symptoms, five of which had symptoms due to papaya ringspot virus. In November 1993, PBT-associated fluorescence was observed in petiole sections stained with acridine orange from 38 of 39 plants with PBT symptoms taken from commercial groves near Cabo Rojo (six of six) and Guánica (15 of 15), Puerto Rico, and two experimental plantings (17 of 18) at the Lajas Substation of the University of Puerto Rico. PBT-associated fluorescence was not observed in sections from 62 of 68 plants without PBT symptoms from Lajas. The six symptomless plants with positive staining came from a variety collection in which the reaction of some varieties to PBT was unknown. The plantings at Lajas were sampled again in June 1995 and similar results were obtained (Table 2). Of the 95 samples from 12 countries which tested negative for phytoplasmas in the PCR assays, 70 exhibited the abnormal fluorescence associated with PBT when stained with DAPI or acridine orange (Table 1).

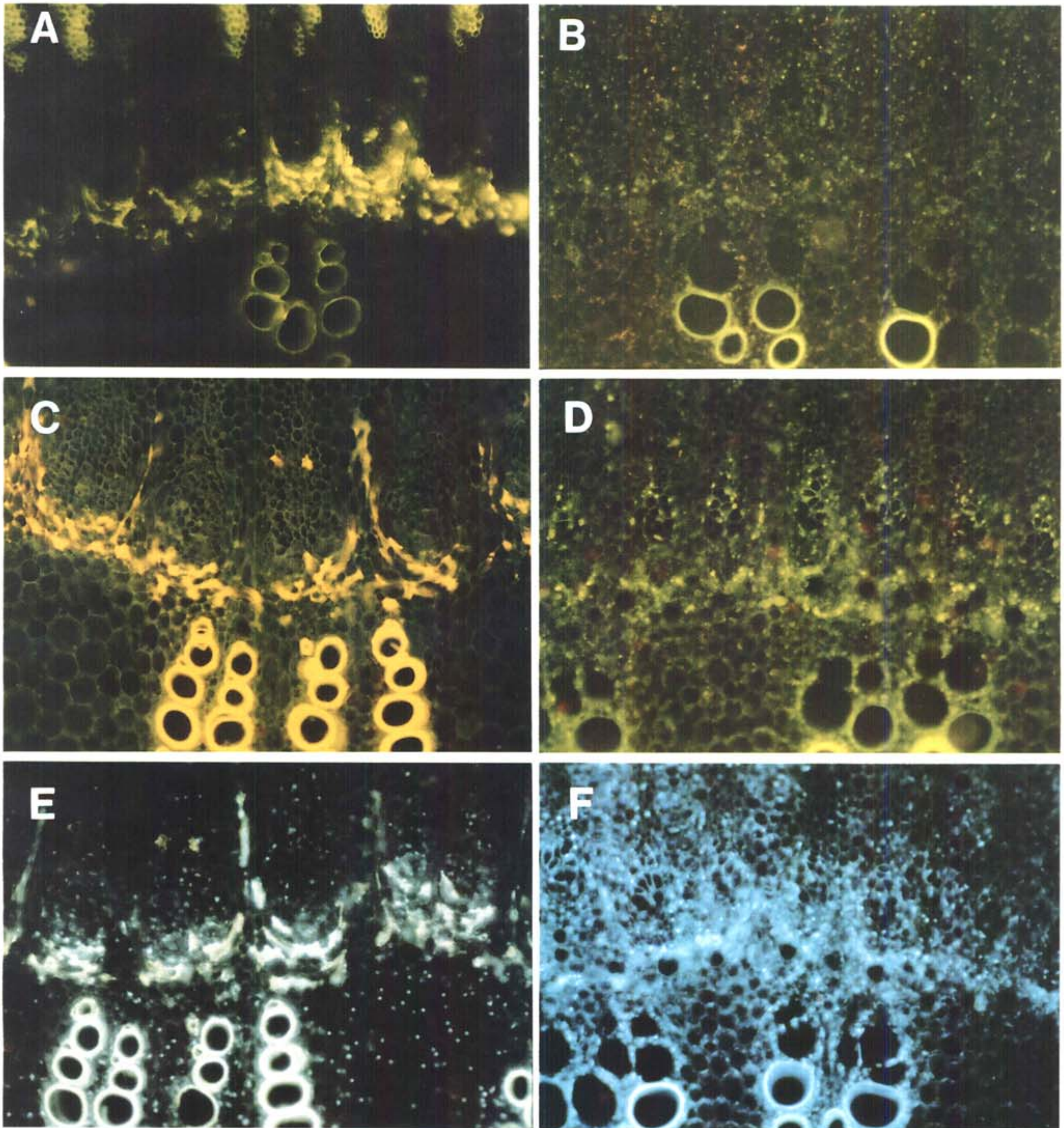

Fig. 2. Epifluorescence microscopy of transverse sections of papaya petioles from papaya bunchy top (PBT)-affected (**A**, **C**, and **E**) and healthy (**B**, **D**, and **F**) plants. Sections were not stained (**A** and **B**), stained with acridine orange (**C** and **D**), or stained with DAPI (**E** and **F**). Nonstained and acridine orange-stained sections were photographed using blue light excitation. DAPI-stained sections were photographed using UV light excitation.

Electron microscopy. Small bacteria were discovered by electron microscopy within papaya petiole cells on the periphery of the phloem (Figs. 3 to 5). The bacteria were found in all three PBT-affected plants from Puerto Rico and Costa Rica, but were not found in a healthy plant from Florida. The bacteria were rod-shaped, measuring approximately 0.25 to 0.35 µm in width and 0.8 to 1.6 µm in length. Their cell walls resembled those of Gram-negative bacteria, except that a peptidoglycan layer was not evident in the periplasmic space delimited by both an inner (cytoplasmic) and an outer trilaminar unit membrane (Fig. 4). The thickness of the bacterial cell wall ranged between 25 and 50 nm, primarily because of variation in the width of the periplasmic space; however, the topography of the cell wall was not prominently rippled. The bacterial cytoplasm contained electron dense particles and fibrils presumed to be ribosomes and DNA, respectively, and sometimes also contained electron lucent inclusion bodies.

TABLE 2. Comparison between the presence of papaya bunch top (PBT) symptoms, abnormal fluorescence of petiole tissues when stained with acridine orange, bacteria in expressed sap, and papaya ringspot virus (PRV) in one papaya planting in Lajas, Puerto Rico, sampled June 15, 1994

PBT symptoms[a]	No. plants	No. positive in fluorescence assay	No. with bacteria in expressed sap	No. positive for PRV[b]
Present	19	16 (84.2%)	15 (78.9%)	14 (73.7%)
Absent	15	0	2 (13.3%)	11 (73.3%)

[a] Symptomatic plants all had foliar symptoms of PBT and greatly reduced latex flow upon wounding of the affected portion of stems. Nonsymptomatic plants lacked foliar symptoms of PBT and had free-flowing latex upon wounding of the stem.

[b] Enzyme-linked immunosorbent assay (ELISA) for PRV conducted by R. T. McMillan, Jr., at the University of Florida, Homestead.

The plant cells in which the bacteria were found appeared to be laticifers (Fig. 5). Infected cells were in various stages of partial to complete collapse. In collapsed cells, bacteria and cell debris were embedded in an electron dense matrix, and the bacteria appeared crushed or otherwise misshapen. An electron lucent zone often surrounded the bacteria. The cytoplasm of the infected plant cells appeared extensively disrupted and mostly devoid of intact organelles. Remnants of what appeared to be latex vesicles and organelles were present in some of the infected cells (Fig. 3). Normal laticifers were not prevalent in the samples from PBT-affected plants, but were readily found in the same location within samples from the healthy plant (Fig. 5). Normal laticifers contained numerous intact vesicles (latex particles) which were almost totally devoid of any electron dense materials, and similar vesicles were found in sections of the agar-solidified latex exudate from a healthy plant (data not shown). Cells containing unidentified bodies similar to those presumed to be phytoplasmas in an earlier study (44) were observed in samples from the two PBT-affected plants from Puerto Rico, but not in the similar sample from Costa Rica and the healthy sample from Florida. The unidentified bodies were mostly spherical, measuring 0.8 to 1.0 µm in diameter, and contained varying amounts of fibrilar or granular materials. The density of the contents varied from cell to cell, but

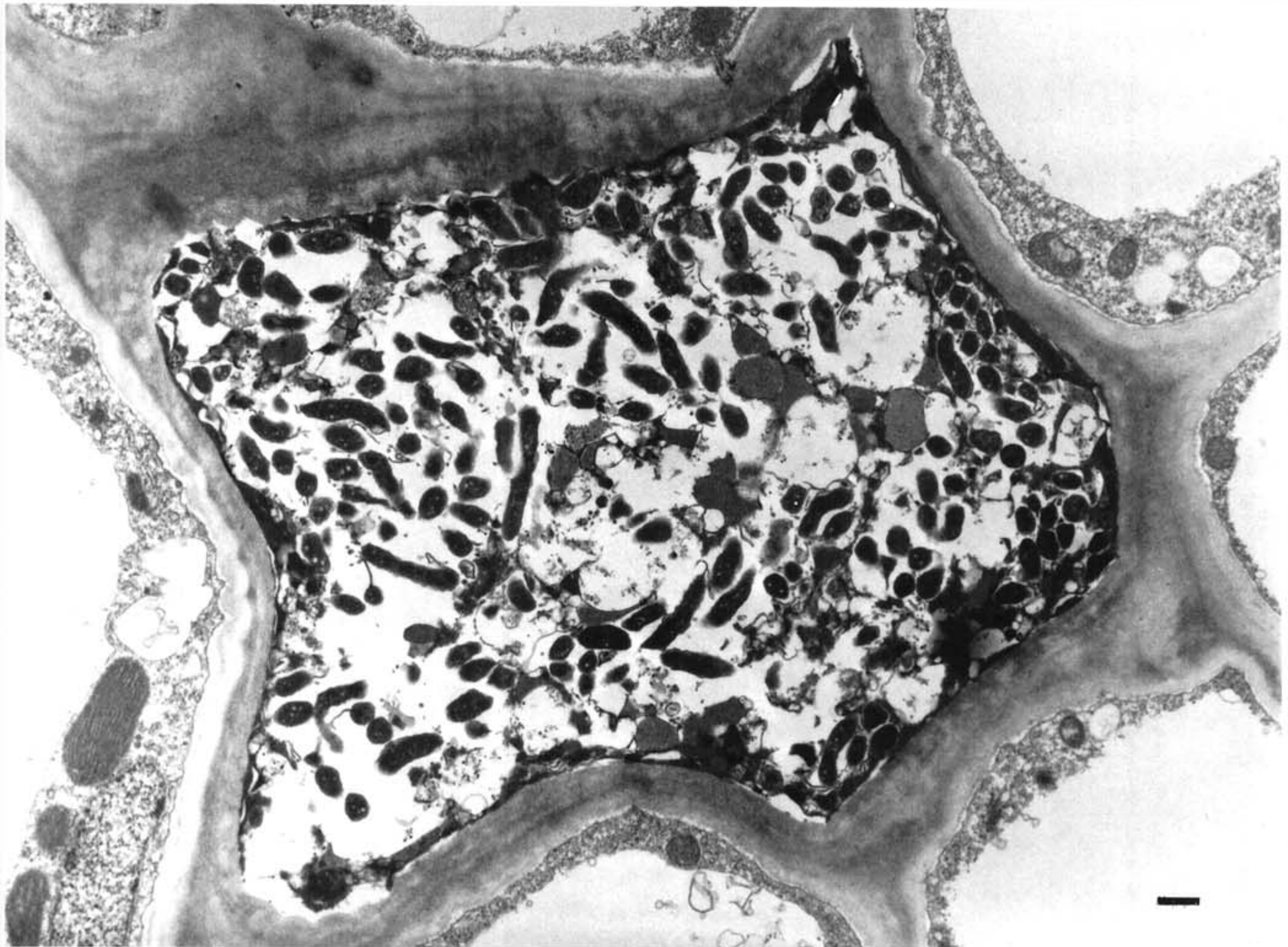

Fig. 3. Transmission electron micrograph of a transverse section of a papaya petiole showing a laticifer colonized by the rod-shaped bacteria associated with papaya bunchy top disease. Remnants of organelles and latex vesicles appear interspersed among the bacteria. Bar = 0.5 µm.

was more consistent among bodies within a particular cell. The bodies did not appear to be bound on the outside by a unit membrane similar to the cytoplasmic membrane of phytoplasmas. The bodies were observed in cells with thick cell walls that might have been laticifers, but the bodies appeared smaller on the average than normal latex vesicles and had more electron dense materials. Although the unidentified bodies and bacteria were never observed in the same cell, both were observed in thick-walled cells that had anastomosed and were devoid of any intervening cell wall or other visible structure separating the cells.

Association of bacteria with PBT. Numerous small, rod-shaped bacteria, often greater than 10 per microscope field (1,000×), were consistently observed in expressed sap from fresh papaya petioles from leaves with PBT symptoms, but were not observed in similar preparations from plants without PBT symptoms (Table 2). When examined by epifluorescence microscopy, the bacteria were bright greenish yellow or red when stained with acridine orange and bluish white when stained with DAPI. The bacteria could also be observed by phase-contrast microscopy (1,000×), but interference from cell debris made observation more difficult. Preliminary attempts on several occasions to similarly detect the bacteria in expressed liquids from preserved petioles were unsatisfactory; the bacteria were detected less frequently and usually were present in low numbers when detected.

Isolation attempts. All attempts to isolate the bacteria in axenic culture were unsuccessful. Undiluted inoculum preparations sometimes contained as many as 50 to 100 bacterial cells/microscope field, but only low numbers of fast-growing bacterial contaminants whose colony and cell morphology closely resembled those of bacteria similarly isolated from healthy papaya tissues were isolated from the undiluted preparations, and nothing was usually isolated from the 10^4-fold or greater dilutions.

DISCUSSION

Constant failure to detect phytoplasma DNA by PCR in papaya with PBT symptoms indicated that a phytoplasma was not associated with PBT, contrary to previous reports (42,44). PCR has proven to be a highly sensitive technique for the detection of phytoplasmas (3,18,26). In the present study, the 16S rRNA gene of different known phytoplasmas was consistently detected in PCR assays, but similar DNA was not detected in total DNA extracted from papaya with PBT. PCR inhibitors were apparently not responsible for the failure to detect the putative PBT phytoplasma. Further indication that phytoplasmas would have been detected had they been present in samples from PBT-affected

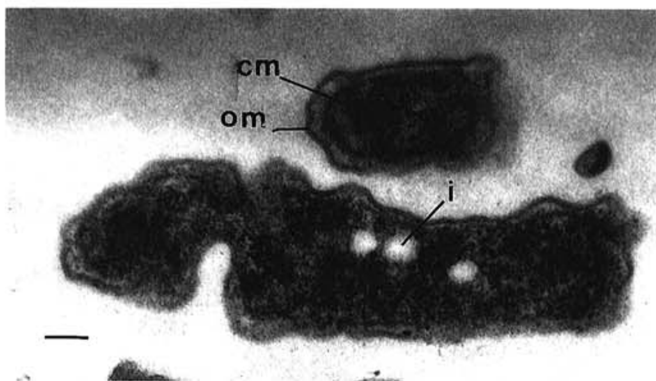

Fig. 4. Transmission electron micrograph of bacterial cells within papaya affected by bunchy top disease. The bacteria have a Gram-negative type cell wall structure with a outer unit membrane separated from the cytoplasmic membrane by a periplasmic space. Electron lucent inclusion bodies are evident in the bacterial cytoplasm. Abbreviations: om = outer membrane; cm = cytoplasmic membrane; i = inclusion body. Bar = 50 nm.

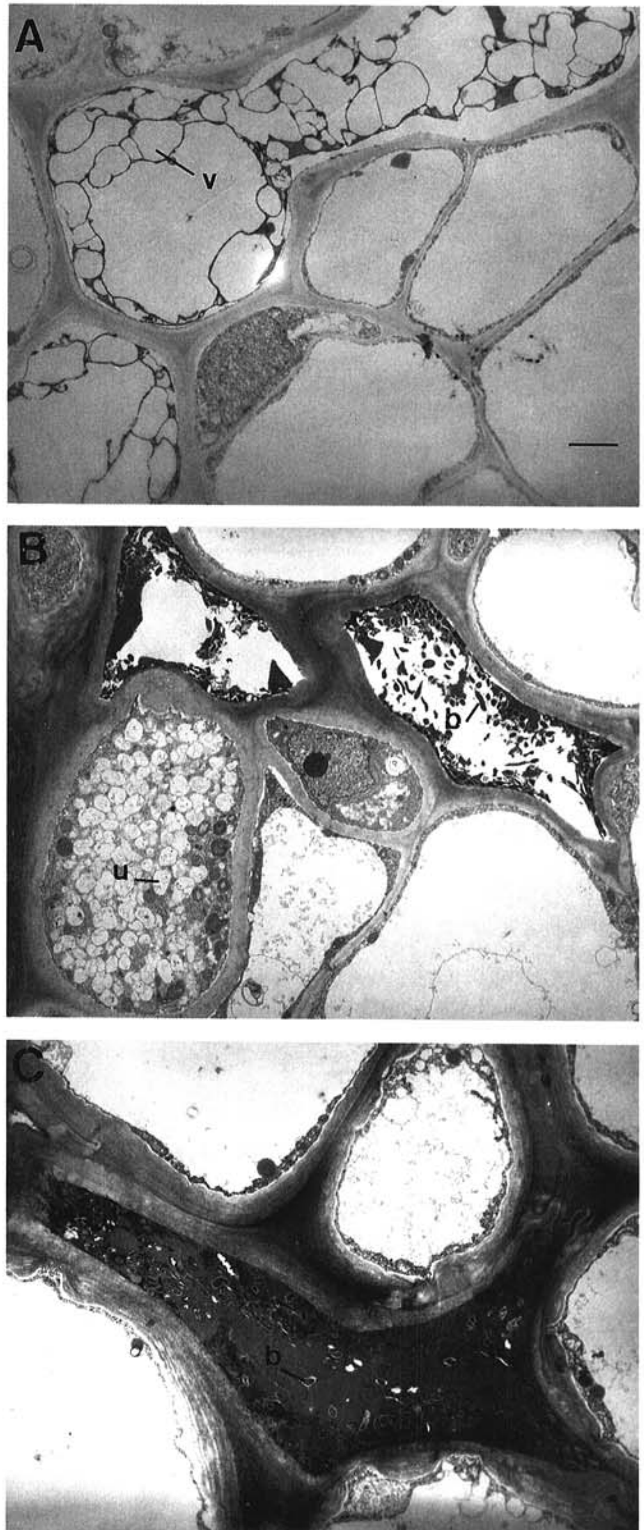

Fig. 5. Transmission electron micrographs of transverse sections of papaya petiole tissues from a healthy plant and a plant affected by papaya bunchy top (PBT) disease. **A,** Healthy plant tissues with laticifers containing latex vesicles. **B,** Similar section of PBT-affected plant tissue showing what appear to be partially collapsed laticifers colonized by bacteria and another laticifer containing numerous unidentified spherical bodies that might be altered latex vesicles. **C,** Collapsed laticifer containing an electron dense matrix with PBT-associated bacteria embedded in the matrix. Some bacteria are separated from the matrix by an electron lucent zone. Abbreviations: v = latex vesicle; b = bacteria; u = unidentified spherical body. All micrographs presented at the same magnification. Bar = 2 μm.

plants was obtained with the positive PCR results for phytoplasmas in papaya with yellow crinkle disease, which is possibly caused by the same phytoplasma that causes big bud of tomato in Australia (21,36). The etiologic agent of Australian dieback (20,24) and mosaic (36) diseases of papaya are unknown, and phytoplasmas have not been previously reported in association with these diseases. However, further investigation into the etiology of Australian dieback and mosaic diseases had established an association for both diseases with phytoplasmas, based on PCR detection of 16S rRNA genes of *Mollicutes* (M. J. Davis and D. S. Teakle, *unpublished data*). The possibility that a phytoplasma was responsible for PBT symptoms in some geographic locations in the American tropics, but not in others, seemed remote, because phytoplasma DNA was not detected by PCR in samples from plants with PBT symptoms from 12 different countries, including four samples from the Dominican Republic where phytoplasmas were first reported as being associated with PBT (42).

The previous reports by Story and Halliwell (42) and Webb and Davis (44) associating phytoplasmas with PBT appear to have been erroneous. The bodies resembling phytoplasmas in the electron micrograph presented by Story and Halliwell (42) could alternatively be interpreted as being degenerate mitochondria. No micrographs showing phytoplasmas were published in the brief note by Webb and Davis (44); however, 100 micrographs taken during the study were available and examined again. Bodies which had been previously identified as phytoplasmas were present in the majority of the micrographs and were identical to the unidentified bodies observed in tissue from PBT-affected papaya during the present study (Fig. 5B). No other bodies resembling phytoplasmas were observed. A relationship between PBT and these unidentified bodies has not been established, and similar bodies were not observed in sections from healthy plants in either study. One possible explanation is that the bodies were latex vesicles that had become more uniformly spherical and more dense in response to disease. The major function of latex within plants is commonly believed to be protection against biological stresses. The reduction or complete absence of latex flow upon wounding has been considered characteristic of PBT by most accounts (2,5,8,28), but Webb and Davis (44) reported that plants with foliar symptoms of PBT consistently exhibited normal latex flow upon wounding. In the present study, foliar symptoms of PBT were highly correlated with a reduction in latex flow. Therefore, the disease studied by Webb and Davis (44) might not have been PBT, although it had foliar symptoms resembling those of PBT, and the unidentified bodies, possibly altered latex vesicles, might have developed as a response to disease in general rather than PBT specifically.

Although a reduction or absence of latex flow is not a symptom that has been associated with other diseases of papaya in the American tropics, it is a symptom of dieback (23) and mosaic (38) diseases in Australia. Furthermore, autofluorescence of necrotic phloem cells and laticifers is also characteristic of both Australian diseases (23; M. J. Davis and D. S. Teakle, *unpublished data*). In plants affected by PBT, laticifers were often observed to be deformed, collapsed, and occluded (Fig. 5). Harding and Teakle (23) reported that the autofluorescence associated with Australia dieback appeared to arise from both phloem cells and laticifers that were often distorted, collapsed, and occluded, and noted that phloem cells were often necrotic. We found that phloem cells did not appear to be similarly affected in plants with PBT, and that the fluorescence associated with PBT arose from within the immediate region of laticifers on the periphery of the phloem and not from within phloem bundles. The autofluorescence associated with each of the diseases was presumably because of the accumulation of phenolic compounds in response to disease. Staining with DAPI and acridine orange, both DNA-binding fluorochromes, indicated that high concentrations of DNA were also present in the same region within PBT-affected plants that exhibited abnormal autofluorescence. In healthy papaya, similar concentrations of DNA were not evident, but high densities of plant nuclei were observed in the region of laticifers. The accumulation of plant DNA because of the collapsing of plant cells within PBT-affected tissues may have accounted for the intense staining with DNA-binding fluorochromes; however, the presence of microbial DNA may also have been responsible to a greater extent. In respect to the latter possibility, DNA-binding fluorochromes have been used for diagnosis of diseases caused by phytoplasmas (40), and, therefore, were initially used with this intention in the present study. Histochemical studies with DNA-binding fluorochromes have not been reported for the Australian papaya diseases. A comparison of anatomical and histochemical characteristics of PBT-affected tissues and tissues affected by the Australian diseases might help determine if the diseases have internal symptoms that are indicative of common host responses or if only superficial resemblances exist.

When electron microscope examinations were concentrated on the region in which the abnormal fluorescence associated with PBT had been observed, small, rod-shaped bacteria were discovered inside cells that appeared to be degenerating laticifers (Fig. 5). The bacteria were consistently detected in PBT-affected papaya from both Puerto Rico and Costa Rica, but not in healthy papaya from Florida. This association of bacteria with PBT was further supported when the bacteria were also detected by epifluorescence microscopy in expressed sap from PBT-affected plants and not healthy plants. Methods have not been developed to specifically ascertain whether a common identity existed between the bacteria seen in preparations from different diseased plants; however, no significant inconsistencies in size or shape of the bacteria were observed. The bacteria appeared to be Gram-negative based on cell wall ultrastructure, except that a peptidoglycan layer was not observed within the periplasmic space. However, failure to detect an existing peptidoglycan layer by electron microscopy has been encountered before in studies on bacterial ultrastructure, including studies on fastidious bacterial pathogens of plant vascular tissues (11,19). Therefore, the present evidence was insufficient to conclude that the PBT-associated bacteria have an unusual cell wall structure.

The results of the present study suggested that a previously unknown bacterium might be the causal agent of PBT. The causative agent of PBT is probably not a phytoplasma or closely related member of the *Mollicutes*. The remission of PBT symptoms after application of tetracycline antibiotics (42) would still tend to support a prokaryotic etiology of PBT, even after ruling out phytoplasmas. Our failure to isolate the bacterium on culture media which support the growth of fastidious prokaryotic plant pathogens, as well as other less fastidious bacteria, had precluded any attempts on our part to fulfill Koch's postulates for pathogenicity. However, if the bacterium does cause PBT, the situation would be highly unusual, since no other leafhopper-transmitted, laticifer-inhabiting, plant pathogenic bacterium exists to our knowledge.

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