Citrus Variegated Chlorosis Bacterium: Axenic Culture, Pathogenicity, and Serological Relationships with Other Strains of *Xylella fastidiosa*

John S. Hartung, Julia Beretta, Ronald H. Brlansky, Joan Spisso, and Richard F. Lee

First and fourth authors: United States Department of Agriculture, Agricultural Research Service, Room 111 Building 004, Beltsville Agricultural Research Center-West, Beltsville, MD 20705; second author: Instituto Biológico, São Paulo, Brazil; and third and fifth authors: University of Florida, IFAS, Citrus Research and Education Center, 700 Experiment Station Road, Lake Alfred 33850.

ABSTRACT

Hartung, J. S., Beretta, J., Brlansky, R. H., Spisso, J., and Lee, R. F. 1994. Citrus variegated chlorosis bacterium: Axenic culture, pathogenicity, and serological relationships with other strains of *Xylella fastidiosa*. Phytopathology 84:591-597.

A xylem-limited bacterium serologically related to strains of *Xylella fastidiosa* has been associated previously with citrus variegated chlorosis, a new and potentially serious disease of citrus in Brazil. When isolated and grown on PW (periwinkle wilt) medium, this gram-negative bacterium measured 0.4 × 4 μm and was indistinguishable based on colony appearance from reference strains of *X. fastidiosa* obtained from the American Type Culture Collection, Rockville, MD. The bacterium also had a rippled cell wall typical of *X. fastidiosa* and induced symptoms typical of citrus variegated chlorosis in sweet orange after artificial inoculation. The bacterium was reisolated from petioles of symptomatic artificially inoculated plants, and its identity was confirmed by membrane entrapment immuno-fluorescence and Western blotting with antiserum UF-26 prepared against the original strain and extracts of petioles and midribs from inoculated plants. The organism was observed in large numbers in xylem vessels of diseased, but not healthy, plant petioles and in extracts of diseased, but not healthy, petioles, using a gold label with antiserum UF-26. The bacterium reisolated from symptomatic plant tissue was culturally, morphologically, and serologically indistinguishable from the strain used to inoculate the plants, completing Koch's postulates. Antiserum UF-26 reacted most strongly with strains of *X. fastidiosa* that cause diseases of grapevines, almond, ragweed, and oak. The citrus strain of *X. fastidiosa* also reacted as strongly as the homologous strain from plum to antiserum 1609-PP. This strain of *X. fastidiosa* may represent a new serological group intermediate between previously described serogroups of *X. fastidiosa*.

Additional keywords: *Citrus sinensis*, pecosita.

Fastidious, gram-negative bacteria with rippled cell walls cause diseases of numerous woody plants, including grapevine (14), peach and plum (11,45), almond (17), elm, sycamore, and oak trees (10,20), and mulberry (27). Similar bacteria cause periwinkle wilt (16) and ragweed stunt diseases (43). The bacteria are typically shown by electron microscopy to be tightly packed in the lumen of xylem vessels (32,33) and are transmitted in a persistent manner by various leafhopper vectors (7,35). Strains isolated from various host plants were considered genotypically and phenotypically similar and were classified as *Xylella fastidiosa* (44). The literature on this group of pathogens has been reviewed (24,38,42).

A new disease of sweet orange (*Citrus sinensis* (L.) Osbeck) has been associated with strains of *X. fastidiosa* in Brazil (8,40). The authors of initial reports suggested that the disease, citrus variegated chlorosis, posed an immediate threat to the Brazilian and world citrus industry (30,39). Pierce's disease, caused by another strain of *X. fastidiosa*, already limits grapevine production in the southeastern United States and Central America (18). The preliminary epidemiological data presently available from Brazil are inadequate to forecast the future course of the citrus variegated chlorosis epidemic (19). However, it has been estimated that in the absence of remedial measures a disease incidence of 90% in a grove could occur 12 yr after introduction of a single infected tree (19). There are no data available concerning yield reductions that result from infection. Affected fruit, however, are hard and undersized and damage juicing machinery, causing entire lots of fruit to be rejected at processing plants (30). Symptoms also include a brilliant chlorosis, stunting, and canopy dieback. The disease has already spread widely within the citrus-producing region of Brazil (19,30). A similar disease associated with *X. fastidiosa* called 'pecosita' occurs in Argentina (2,5).

We now report the isolation and characterization of *X. fastidiosa* from infected citrus, as well as the completion of Koch's postulates for *X. fastidiosa* as the causal agent of citrus variegated chlorosis. We also present serological data to characterize the relationships of the citrus variegated chlorosis strain to other strains of *X. fastidiosa*. Preliminary accounts of some of this work have been presented (3,29). Our results confirm those of Chang et al (9) who also completed Koch's postulates for citrus variegated chlorosis and published while this manuscript was in preparation.

MATERIALS AND METHODS

Bacterial strains. Strains of *X. fastidiosa* were obtained from the American Type Culture Collection, Rockville, MD (Table 1), except for strain B, which was isolated in Beltsville, MD, from twigs collected from citrus variegated chlorosis-infected Pera sweet orange trees near Bebedouro, São Paulo, Brazil. *Xanthomonas campestris* pv. *vesicatoria* strain 084-1275 was provided by J. Miller, Division of Plant Industry, Gainesville, FL. *Pseudomonas solanacearum* strain K60, *P. cichorii* strain Z1, *Agrobacterium tumefaciens* strain Chry 9, and *Clavibacter michiganense* subsp. *nebraskense* strain PF4 were provided by R. E.. Stall, University of Florida, Gainesville.

Isolation and characterization of the pathogen. Symptomatic leaves were surface-disinfested with 10% bleach for 5 min, followed by two rinses in sterile distilled water. Strain B was obtained after midribs were excised and sap was expressed from 2-mm sections onto periwinkle wilt (PW)-medium plates (16). Plates were incubated at 28 C. Colonies were selected, and cells were

streaked on fresh plates to obtain single colonies. Identification of *X. fastidiosa* was based on in vitro fastidious growth habit, gram-stain reaction, size measurements made by light microscopy, electron microscopy, and serological assays. Cultures were stored at -70 C in PW broth + 40% glycerol. Strain B was subsequently used for plant inoculations. Reisolations from inoculated plants were done on PW or SPW plates that contained cycloheximide and naladixic acid at 100 and 20 $\mu g/ml$, respectively. SPW is PW medium (16) supplemented with 5 g of malt extract, 10 g of sucrose, 0.1 g of myo-inositol, 0.01 g of thiamine HCl, 0.01 g of pyridoxine HCl, 0.005 g of nicotinic acid, and 0.002 g of glycine per liter. These ingredients are components of a citrus cell suspension culture medium that stimulated growth of the *X. fastidiosa* isolated from sweet orange (3).

Cultural characteristics of strains of *X. fastidiosa*. Inoculum from 21-day-old cultures on buffered charcoal yeast extract (BCYE) (44) agar was dispersed in sterile distilled water and then streaked in triplicate onto different growth media reported to support growth of *X. fastidiosa*. Agar media tested included BCYE, Pierce's disease medium #2 (PD-2) (15), and SPW. The gram reaction of the inoculum also was determined, and the average length and width of each strain was estimated by bright-field microscopy and gram-stained cells. After 3 wk of growth at 27 C, diameters of 30 colonies, 10 on each of three SPW and PD-2 plates, were determined with a binocular microscope equipped with an ocular micrometer. Analysis of variance was performed on the data by the general linear model with SAS computer software (SAS Institute, Cary, NC). The entire experiment was repeated one time.

Plant material and inoculations. We initially maintained citrus plants for experimental inoculation in 15-cm pots containing Metromix with Peter's fertilizer in the irrigation water and Osmocote (Grace Sierra Horticultural Products Co., Milpitas, CA) with trace elements in the pots. Insecticides were used as necessary. Subsequently, plants were put in 25-cm pots of Metromix containing composted sewage sludge (2:1) to better control an unrelated root rot. Sixteen plants that included five Valencia sweet orange on sour orange (*C. aurantium* L.) rootstocks, seven self-rooted Madame Vinous sweet orange, and four Schaub rough lemon (*C. jambhiri* Lush.) seedlings were inoculated as follows. Bacteria were scraped from PW plates or pelleted from PW broth and washed in phosphate buffered saline (20 mM sodium phosphate, pH 6.8; 0.85% NaCl; PBS) with 0.1% Tween 20 (PBST). Plants were inoculated by two methods. First, we applied 10 μl of a concentrated suspension ($A_{600nm} = 1.0$) of bacteria to the surface of citrus branches and pierced the surface of the branches through the inoculum droplets with a 21-gauge syringe needle. Twenty-five inoculation sites were used per plant. We performed inoculations two times, 10 wk apart. To insure that sufficient inoculum reached the vascular system, in subsequent inoculations we used a razor blade to slash vertically part of the way through the branch to be inoculated, so the upper half of the branch could imbibe the inoculum solution from a microcentrifuge tube. In the bacterial cell suspensions used for these inoculations, $A_{600nm} = 0.1$–0.3. The slashed branch was held in the microcentrifuge tube with laboratory tape, and after 2 h, the inoculum tubes were removed, and the wounds were wrapped with grafting tape. The volume of liquid taken up by the plants varied but averaged 200–300 μl. These inoculations were performed three times, 4, 8, and 22 wk after the second needle-prick inoculations. We also inoculated 15 plants of the same varieties in a similar manner with PBST to serve as controls. These inoculations were repeated on a second set of Madame Vinous sweet orange, with an isolate of *X. fastidiosa* (BARC-5) from a symptomatic Valencia tree from the first inoculation series and distilled water instead of buffered Tween 20 as the suspension medium for the inoculum. During the course of this inoculation series, a second, fresh isolate of *X. fastidiosa* from citrus variegated chlorosis-diseased Brazilian Pera sweet orange was obtained (BARC-6) and was included in the inoculum for some of these plants. Five sweet orange plants received bud grafts from plant five, a symptomatic inoculated plant that was the source of strain BARC-5. Five plants received bud grafts from healthy citrus as a control.

Preparation of antisera and serological assays. Antiserum 948-LH, against the Pierce's disease bacterium (*X. fastidiosa*, ATCC 35881), and antiserum 1609-PP, against the phony peach bacterium (*X. fastidiosa* strain PPDB-5 [16]), were prepared previously in rabbits, using the method of Raju et al (37). Antiserum UF-26 was made against strain B similarly isolated from diseased citrus, except the bacterium was cultured on SPW agar plates. The immunoglobulin G (IgG) fraction of all antisera was isolated by protein A column chromatography (31) and used in double antibody sandwich–enzyme-linked immunosorbent assays (DAS-ELISA) (13). In these assays, both the sample and conjugate buffer were PBS plus 0.1% Tween 20, 0.5% bovine serum albumin (fraction V; Sigma Chemical Co., St. Louis), and 2.0% polyvinylpyrrolidone 40 (Sigma). IgG was conjugated with alkaline phosphatase, using glutaraldehyde. Immulon II plates (Dynatech, Chantilly, VA) were coated with IgG in buffer (5 $\mu g/ml$). Conjugate was diluted in conjugate buffer so the homologous antigen/antiserum would give an $A_{405nm} = 1.0$ in 1 h.

Strains of *X. fastidiosa* (Table 2) were harvested while actively growing 14 days after plating on SPW agar for use as antigens. Strain B bacteria were harvested 21 days after inoculation because of the slower growth rate of this strain. Bacterial cultures used for negative controls (Table 2) were harvested during log phase from agar plates of appropriate media. Bacteria were suspended in 2 ml of sterile distilled water and transferred to a centrifuge tube for three washes with PBST. The A_{550nm} was adjusted to 0.13 ($\sim 1 \times 10^9$ cfu/ml) and diluted 10^{-1} and 10^{-2} in sample buffer. ELISA data were summarized as the ratio of the A_{405nm} of the tested strain to that of the strain used to prepare the antiserum (23).

Published procedures for dot immunobinding assays (DIBA) and Western blotting were used (28) with antiserum UF-26 as the primary antibody and with goat anti-rabbit conjugate. Extracts of diseased petioles from Brazil were included on each Western blot as positive controls. Serological identification and detection of *X. fastidiosa* was performed by membrane entrapment immunofluorescence (MEIF), as described previously, to detect *Xanthomonas campestris* pv. *citrumelo* and *Xanthomonas campestris* pv. *citri* (6) with the modification that single leaf petioles were used rather than leaf disks. Antiserum UF-26 to *X. fastidiosa* strain B was conjugated to tetramethylrhodamine isothiocyanate

TABLE 1. Source, host, and growth of strains of *Xylella fastidiosa* on supplemented periwinkle wilt (SPW) medium and Pierce's disease medium #2 (PD-2)

Strain[v]	Host[w]	Colony on medium[x,y]	
		SPW	PD-2
35869	Mulberry	0.88 a	0.37 b
35870	Almond	0.53 b	0.18 d
35876	Ragweed	0.48 bc	0.36 bc
35868	Mulberry	0.45 c	0.33 c
CVC-B	Sweet orange	0.36 d	0.15 de
BARC-5	Sweet orange	0.35 d	0.09 g
35879	Grapevine	0.34 d	0.42 a
35871	Plum	0.22 e	0.13 ef
35881	Grapevine	0.11 f	0.11 fg
35874	Red oak	0.08 fg	0.10 fg
35877	Grapevine	0.07 g	...[z]

[v] Strains are from the American Type Culture Collection, Rockville, MD, except strain B, which was isolated from citrus variegated chloris-diseased Pera sweet orange from Brazil. Strain B was used to inoculate sweet orange at Beltsville, MD, and BARC-5 was reisolated from an inoculated tree with symptoms of citrus variegated chloris.

[w] Host from which the strain was originally isolated.

[x] Average diameter in millimeters. Mean of 30 determinations (10 colonies each on three plates). Measurements were made with a binocular microscope equipped with a micrometer after 21 days incubation at 27 C.

[y] Means followed by the same letter are not significantly different according to Duncan's multiple range test ($\alpha = 0.05$, $Pr > F = 0.0001$). The experiment was repeated one time with similar results.

[z] Scant to no growth.

(TRITC) for this purpose. The negative-control antiserum used was prepared previously to *Xanthomonas campestris* pv. *citri* strain XC62 (12).

Electron microscopy. Citrus leaf petiole and midrib tissues were excised from symptomatic leaves and fixed with 3% glutaraldehyde in 0.066 M phosphate buffer (PB), pH 6.8. Pieces 4 mm in length were fixed for transmission electron microscopy (TEM) and larger pieces (8–10 mm) were fixed for scanning electron microscopy (SEM). Fixation was overnight at 4 C, and the samples then were washed three times (20 min each) in PB. Samples for TEM were postfixed in 1% osmium tetroxide in PB for 2–4 h and then washed three times (20 min each) in PB. All samples were dehydrated in an ethanol series (10–100%). Samples for TEM were infiltrated and embedded in L. R. White resin, (Ted Pella Inc., Redding, CA), using the hard formulation and polymerization at 65 C. SEM samples were mounted on stubs, critical point-dried, sputter coated with gold palladium, and viewed on a Hitachi S-530 scanning electron microscope (Nissei Sangyo American, Ltd. Mountainview, CA) operating at 20 kV. Ultrathin sections (60–85 nm) were obtained from TEM-embedded samples, and immunogold labeling was done as previously described (4) with antiserum against strain B.

RESULTS

Isolation and cultural characteristics. A fastidious, gram-negative bacterium that reacted positively in DAS-ELISA and DIBA with antisera raised against *X. fastidiosa* isolated originally from diseased plum, grapevines, and ragweed was isolated from xylem-containing tissue of citrus showing citrus variegated chlorosis symptoms but not from asymptomatic citrus. The growth rate of this bacterium, as judged by colony size, was in the middle of the range of 10 reference strains tested for comparison on SPW and PD-2 (Table 1). Similar results were obtained on BCYE medium, although the charcoal in the medium made it impossible

to precisely determine colony diameters. The average dimensions of the cells were 0.4×2–4 μm after growth on BCYE, similar to that of other *X. fastidiosa* reference strains tested under the same conditions. On SPW medium, isolated colonies were clearly visible within 7 days after streaking, as compared to 10–14 days on BCYE and PW medium. Colony diameters comparable to that on SPW generally were not observed on PD-2 (Table 1) or PW (data not shown). BCYE also supported growth of strains B and BARC-5, but colony diameters also were consistently much smaller than on SPW (data not shown).

Inoculation results. Twelve of the 16 plants inoculated with *X. fastidiosa* and 10 of the 15 plants inoculated with PBST as controls in the first inoculation series survived 10 mo after the first inoculations described here. The remainder of the plants died of an unrelated root rot disease. Eight months after the initial needle-prick inoculations, one asymptomatic, inoculated plant (plant 5, Valencia on sour orange [*C. aurantium*] rootstock) tested positive in dot-immunobinding (data not shown). Western blot assays of this plant also gave a weakly positive reaction, consistent with a low concentration of strain B in this asymptomatic tree (data not shown). Ten months after the first needle-prick inoculations, and consistently thereafter, a bacterium with the same cultural, serological, and morphological characteristics was reisolated from plant 5. Plant 5 had mild marginal leaf chlorosis at the time of the first isolation, which became progressively more pronounced (Fig. 1A). Symptoms were the same as observed on naturally infected trees in Brazil (Fig. 1B), including necrotic centers in chlorotic zones. *X. fastidiosa* was not isolated from other inoculated plants from the first inoculation series nor from any of the plants mock-inoculated with PBST as controls. No other plants gave a positive reaction in the DIBA or Western blot assays. In the MEIF tests, polycarbonate membranes containing petiole extracts from plant 5 contained gold-orange fluorescing bacteria when incubated with TRITC-labeled UF-26 antiserum (Fig. 2). Fluorescing bacteria were not observed on membranes incubated with the control antiserum raised against *Xanthomonas campestris* pv. *citri* nor in petiole extracts from healthy control plants or from other inoculated plants when incubated with either labeled antisera (data not shown).

In the second inoculation series, three of five plants that received bud grafts from plant 5 became symptomatic, and *X. fastidiosa* was detected in four of these plants by both Western blotting and MEIF of petiole extracts (Figs. 2 and 3). No symptoms of citrus variegated chlorosis or *X. fastidiosa* were detected in five
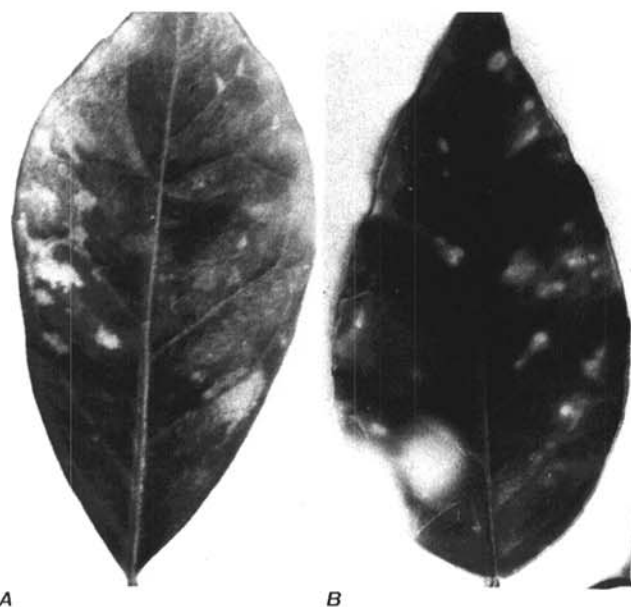
TABLE 2. Serological reactivities of cultured strains of *Xylella fastidiosa* isolated from various hosts

Antisera[w]	DAS-ELISA[x]			
	948-LH	UF-26	1609-PP	Group[y]
Strain/host[z]				
35868/Mulberry	96	28	79	II?
35869/Mulberry	33	44	36	II?
35870/Almond	50	140	39	I
35873/Elm	134	71	123	II?
35874/Red oak	29	101	40	II?
35876/Ragweed	56	120	45	II
35877/Grapevine	65	182	33	I
35879/Grapevine	90	166	46	I
35881/Grapevine	100	166	70	I
CVC-B/Sweet orange	59	100	102	?
35871/Plum	89	83	100	II
Negative controls				
Xylella vesicatoria	2	1	4	
Pseudomonas solanacearum	0	4	4	
Agrobacterium tumefaciens	0	1	4	
Pseudomonas cichorii	1	1	4	
Clavibacter michiganese	2	4	2	
Buffer only	0	1	2	
Healthy plant extract	0	0	0	

[w] Polyclonal antisera raised against *X. fastidiosa* strains originally isolated from diseased grapevine (948-LH), sweet orange (UF-26), and plum (1609-PP).

[x] Double antibody sandwich–enzyme-linked immunosorbent assay values are the ratio of the A_{405nm} of the tested strain to that of a homologous strain expressed as a percent. Homologous antigen/antiserum combinations were 35881/948-LH, UF-26/UF-26, and 35871/1609-PP. Means from two experiments with two determinations each.

[y] Groups within *X. fastidiosa* as designated by Purcell (34).

[z] Test strains are all from the American Type Culture Collection, Rockville, MD, except for strain B, which was isolated in the course of this work. All cultured bacteria were suspended in buffer and adjusted turbidimetrically to a final concentration of 10^9 cfu/ml.

A   B

Fig. 1. Symptoms of citrus variegated chlorosis on leaves **A,** from plant 5 artificially inoculated at Beltsville, MD, and **B,** from a naturally infected tree from Brazil.

plants that received bud grafts from a healthy Valencia sweet orange (Table 3). Eight of 17 Madame Vinous plants inoculated with strain BARC-5 or strains BARC-5 and BARC-6 became symptomatic 6 mo after the first inoculation by needle-pricking. Strains of *X. fastidiosa* indistinguishable morphologically and serologically from the strain used for inoculation were isolated from 14 of these plants, whereas 11 and 14 of these plants gave positive results in Western blotting and MEIF assays, respectively. No *X. fastidiosa* were isolated from the buffer-inoculated control plants, and none of these plants gave positive results in either the Western or MEIF assays (Table 3; Fig. 3).

Comparison to strains of *X. fastidiosa* by ELISA. Strains of *X. fastidiosa* were used as antigens for DAS-ELISA assays (Table 2). When assayed at 10^9 cfu/ml, the average of four determinations of the A_{405nm} for the homologous antigen/antiserum assay was 0.92 for strain 35881 and antiserum 948-LH, 0.77 for strain B and antiserum UF-26, and 1.04 for strain 35871 and antiserum 1609-PP (Table 2). The data from two separate ELISA tests run at different times with different preparations of antigen were combined, with each antigen run in duplicate for each test. The strains were serologically compared by expressing the raw absorbance data as a ratio of that of the tested strain to the homologous strain for the test antiserum. The three antisera showed different reactivities against the different strains, but all the tested *X. fastidiosa* strains were serologically related, and no significant cross-reactions to other plant-pathogenic bacteria were observed for any of the three antisera (Table 2). A high serological relationship of the strain from sweet orange to strains of *X. fastidiosa* isolated from diseased grapevine and almond (group I; [34]) was evident when antiserum UF-26, raised against strain B, isolated from diseased citrus was used as the test antiserum. The sweet orange strain did not react very strongly to antiserum against 948-LH (grapevine) in the reciprocal test but did react as strongly

as strains 35870 (almond) and 35877 (grapevine) with this antiserum. Two of three strains of *X. fastidiosa* isolated from grapevine, one strain from elm, and one of two strains from mulberry were highly related when antiserum 948-LH was used, and strains from elm, plum, and sweet orange were highly related when antiserum 1609-PP was used. Similar results were obtained with test antigen adjusted to 10^8 cfu/ml. Reactions generally were weak when the test antigen was at 10^7 cfu/ml.

Electron microscopy. Bacteria were readily observed in the xylem vessels of petioles and midveins from symptomatic tissues of plant 5 when viewed with SEM (Fig. 4). The bacteria measured 0.4×2.0 μM. Using TEM, the bacteria were morphologically similar to those reported previously for citrus variegated chlorosis (8,40) (Fig. 5A). Immunogold labeling with UF-26 antiserum was positive (Fig. 5B) for these TEM sections. No bacteria were found in the xylem of control tissues or in tissues from other inoculated plants.

DISCUSSION

At 10-mo postinoculation and thereafter, a fastidious bacterium with the morphological and serological characteristics of the in-

TABLE 3. Inoculation of Madame Vinous sweet orange with *Xylella fastidiosa* strains BARC-5 and -6

Plant number	Inoculation type[u]	Symptom[v]	Reisolation[w]	Western blot[x]	MEIF[y]
64	Graft plant-5	+	ND[z]	+	+
65	Graft plant-5	+	ND	+	+
66	Graft plant-5	+	ND	+	+
67	Graft plant-5	−	ND	+	+
68	Graft plant-5	−	ND	−	−
69	Graft control	−	ND	−	−
70	Graft control	−	ND	−	−
71	Graft control	−	ND	−	−
72	Graft control	−	ND	−	−
73	Graft control	−	ND	−	−
101	BARC-5	+	+	+	+
102	BARC-5	+	+	+	+
103	BARC-5	+	+	+	+
104	BARC-5	−	+	+	+
105	BARC-5	+	+	+	+
106	BARC-5	−	+	+	+
107	BARC-5	−	+	+	+
108	BARC-5	−	+	+	+
109	BARC-5 & -6	+	+	+	+
110	BARC-5 & -6	+	+	+	+
111	BARC-5 & -6	+	+	−	+
112	BARC-5 & -6	+	+	+	+
113	Buffer only	−	−	−	−
114	Buffer only	−	−	−	−
115	Buffer only	−	−	−	−
116	Buffer only	−	−	−	−
117	Buffer only	−	−	−	−
118	Buffer only	−	−	−	−
119	Buffer only	−	−	−	−
120	Buffer only	−	−	−	−
121	BARC-5 & -6	−	−	−	+
122	BARC-5 & -6	−	ND	−	−
123	BARC-5 & -6	−	−	−	−
124	BARC-5 & -6	−	+	−	−
125	BARC-5 & -6	−	+	−	+

[u] Plants were graft inoculated with budwood from plant number 5 or serially inoculated with cultured BARC-5 or a mixture of BARC-5 and -6 by both needle-prick and slash inoculations.
[v] + = bright irregular mottle on mature leaves with necrotic central zones; − = no symptoms 1 yr after the first inoculation.
[w] *X. fastidiosa* reisolated from surface-disinfested petioles and midribs more than 1 mo after last inoculation. + based on gram staining and dot immunobinding assays, and − based on failure to isolate presumptive *X. fastidiosa* from surface-disinfested petioles and midribs.
[x] Extracts of leaf midribs and petioles were separated electrophoretically and probed with antiserum UF-26 raised against strain B.
[y] Extracts of leaf midribs and petioles were evaluated by membrane entrapment immunofluorescence with antiserum UF-26.
[z] ND = Not done.

Fig. 2. *Xylella fastidiosa* strain B in petiole extracts of symptomatic plant 5 prepared by membrane entrapment immunofluorescence, using tetra-methylrhodamine isothiocyanate-labeled antiserum UF-26.

oculated strain B was consistently isolated on PW medium from a single Valencia plant, and MEIF assays gave positive results (Fig. 2). At this time, the plant presented a distinctive mild mottle similar to that observed on naturally infected sweet orange trees in Brazil (Fig. 1A and B). This mottle gradually became more distinct and spread throughout the plant. Similar symptoms were not observed on other inoculated or control plants, and none of the other plants gave a positive result in DIBA or electron microscopy assays. Bacteria of this type were not isolated from any other inoculated or control plants despite repeated attempts. Petiole sections from only this plant had xylem vessels that contained bacteria morphologically similar to *X. fastidiosa* when viewed by SEM (Fig. 4). The growth of this bacterium in xylem vessels (Fig. 5A) is typical of diseases caused by *X. fastidiosa* (24,42). In TEM sections, these xylem-limited bacteria were specifically labeled with gold-conjugated antiserum raised against the bacterium used for the inoculation (Fig. 5B), as observed in plants naturally showing symptoms of citrus variegated chlorosis (4). There is no possibility that this symptomatic and serologically positive plant was naturally infected with *X. fastidiosa*, because it was raised from seed in screened greenhouses in Beltsville, MD, and was isolated from other hosts of *X. fastidiosa*.

These results were confirmed in the second inoculation series, in which fresh isolates of *X. fastidiosa* were used as inoculum. *X. fastidiosa* was isolated from 82% (14/17) of these plants and infection was confirmed by symptomology, Western blotting, and MEIF assays (Table 3). These data demonstrate that the *X. fastidiosa* strain previously associated with citrus variegated chlorosis (4,8,40) is in fact the etiologic agent of this disease.
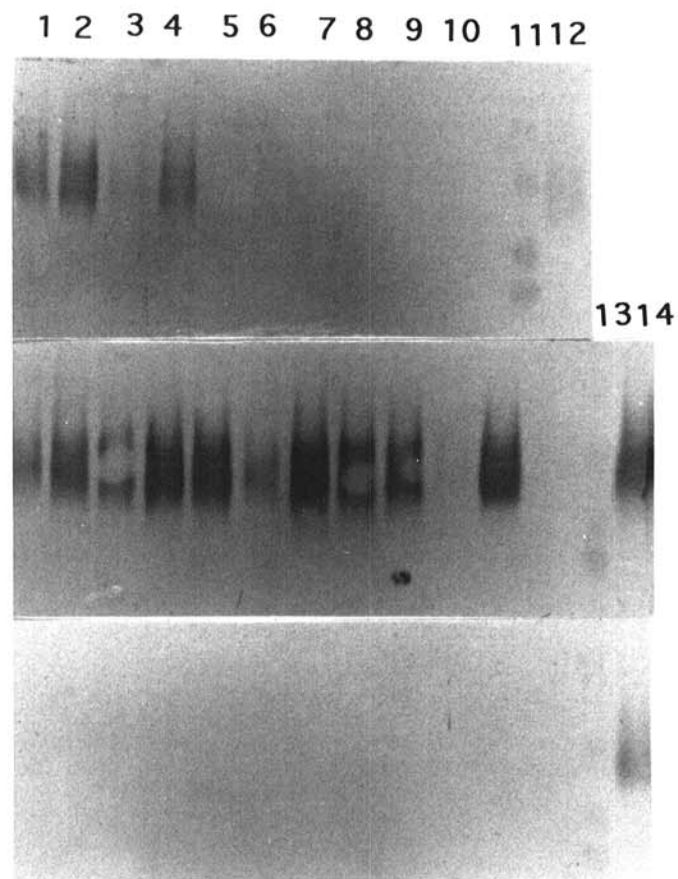

Fig. 3. Western blot of sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels developed with UF-26 polyclonal antiserum to strain B of *Xylella fastidiosa*. (Table 3 provides treatment details). Top: lanes 1–10, plants 64–74; lane 11, size standards; and lane 12, positive control. Middle: lanes 1–12, plants 102–113; lane 13 size standards; and lane 14, positive control. Bottom: lanes 1–12, plants 114–125; lane 13, size standards; and lane 14, positive control.

The inoculation efficiency in the initial experiment was, however, only 8% (1 diseased/12 inoculated survivors). Transmission efficiencies of less than 100% have been reported previously for artificial inoculations with *X. fastidiosa*. Inoculations with *X. fastidiosa* grown in vitro with susceptible ragweed (*Ambrosia artemisiifolia* (L.)) or red oak (*Quercus rubra* (L.)) had efficiencies that varied from strain to strain and were usually less than 100% (10,43). High inoculation efficiency has been reported for the Pierce's disease organism and grapevines (*Vitus vinifera* (L.)) (14) and, more recently, using strains of *X. fastidiosa* and elm (*Ulmus americana* L.) and sycamore (*Platanus occidentalis* L.) seedlings (41). Sherald attributed his success to several factors, including the short period that his isolates were maintained in culture prior to inoculation (24 days). *X. fastidiosa* strains that cause Pierce's disease of grapevines can lose pathogenicity in culture (21). Our isolate had been in culture for 1 yr prior to the initiation of the first inoculation series reported here. We also subsequently found that 0.1% Tween 20 was toxic to strain strain B of *X. fastidiosa*. We had included it in the initial inoculation buffer to obtain a uniform suspension, because strain B rapidly forms large aggregates in solution. It was omitted from the second inoculation series in which bacteria were suspended in sterile deionized water, in which the bacteria survive for more than 3 h without loss of viability. Our inoculation efficiency was apparently dramatically improved by the use of strains that have not been in culture for a long period of time and by the use of distilled water to prepare the inoculum. Chang et al (9) also easily obtained infections of inoculated sweet orange with this pathogen when using a fresh isolate, consistent with the results of our second inoculation series. These workers, however, used sweet orange seedlings rather than grafted plants and also inoculated with the bacterium in the PW growth medium. We are now performing a host-range study including several *Citrus* and non-*Citrus* species and varieties.

After serial transfers, other strains of *X. fastidiosa* adapt to culture and grow faster, and strains belonging to group II grow slowly on PD-3 medium. In contrast, strain B from citrus remains slow growing even after 18 mo of culture by serial transfer on PW medium. The additional nutrients in SPW stimulated growth in most of the strains tested compared to growth in PD-2 medium,

Fig. 4. Scanning electron micrograph of *Xylella fastidiosa* in xylem vessels. Magnification 13,500×.

which is similar in composition to PW medium. Perhaps significantly, this stimulation was particularly apparent with the freshly isolated strain BARC-5 compared with strain B (Table 1). This may relate to the adaptation to culture and loss of virulence reported previously for *X. fastidiosa* (21,41).

Based on the DAS-ELISA assays reported here, strain B is not easily assigned to either group I or II of *X. fastidiosa* as classified by Purcell (34). Group I strains react strongly with group I antisera but relatively weakly with group II antisera. The reverse is true for group II strains (16,43). The sweet orange strain tested appears to have greater serological affinity to the group I (34) strains than to the group II strains, although there was also a strong serological relationship to the strains from ragweed (group II) and oak (group II?) when antiserum UF-26 was used and to the plum strain (group II) when antiserum 1609-PP was used (Table 2). Our results confirm the close serological relationship observed (9) between the *X. fastidiosa* strain causing citrus variegated chlorosis and the strains that cause Pierce's disease of grapes and almond leaf scorch. Our ELISA data, however, also showed a strong serological relationship to the type strain of the oak leaf scorch strain, whereas the others workers did not observe this relationship (9). The reason for this discrepancy is unknown. These serological reactions with antisera to groups I and II and citrus variegated chlorosis B strains can be used as a rapid method to place an unknown *X. fastidiosa* isolate in groups I and II or a possible intermediate group for citrus strains.

The origin of the citrus variegated chlorosis disease in Brazil remains undetermined. Because of the wide host range of *X. fastidiosa*, it is probable that the *X. fastidiosa* strains that cause citrus variegated chlorosis were originally in weeds or crops and were moved into citrus by a Homopteran vector (34,36,39). *X. fastidiosa* from citrus has been reported previously and has been tentatively proposed as the etiologic agent of citrus blight (22,

25,26). Extracts from trees affected by citrus blight have a characteristic set of blight related proteins that include a 12-kDa protein that may be diagnostic of the disease. However, extracts from trees affected by citrus variegated chlorosis lack all of these proteins (1). Alternatively, the citrus variegated chlorosis disease could have existed without identification for a period of time until it began to limit production in an area in which the environment promoted rapid spread and extreme symptom expression. A citrus disease, pecosita, associated with *X. fastidiosa* with serological relationships similar to citrus variegated chlorosis has been reported in Argentina (2,5), but the disease is not a limiting factor of production in Misiones or other areas of Argentina where it occurs.

LITERATURE CITED

1. Beretta, M. J. G., Bach, E. E., Lee, R. F., and Derrick, K. S. 1991. Serological comparison of citrus blight from Florida with declinio and citrus variegated chlorosis in Brazil. (Abstr.) Summa Phytopathol. 17:51.
2. Beretta, M. J. G., Contreras, J., Lee, R. F., Chagas, C. M., Marlelics, L., DeNegri, J. D., and Derrick, K. S. 1992. Similarity between citrus variegated chlorosis (CVC) in Brazil and pecosita in Argentina. (Abstr.) Summa Phytopathol. 18:6.
3. Beretta, M. J. G., Lee, R. F., Derrick, K. S., Davis, C. L., and Barthe, G. A. 1992. Culture and serology of a *Xylella fastidiosa* associated with citrus variegated chlorosis in Brazil. Page 58 in: Book of Abstracts, 7th International Citrus Congress. International Citriculture Society, Acireale, Italy.
4. Brlansky, R. H., Davis, C. L., Lee, R. F., and Timmer, L. W. 1993. Immunogold localization of xylem-inhabiting bacteria affecting citrus in Argentina and Brazil. Pages 311-319 in: Proc. 12th Conf. Int. Org. Citrus Virol. P. Moreno, J. V. DaGraca, and L. W. Timmer, eds. IOCV, Riverside, CA.
5. Brlansky, R. H., Davis, C. L., Timmer, L. W., Howd, D. S., and Contreras, J. 1991. Xylem-limited bacteria in citrus from Argentina
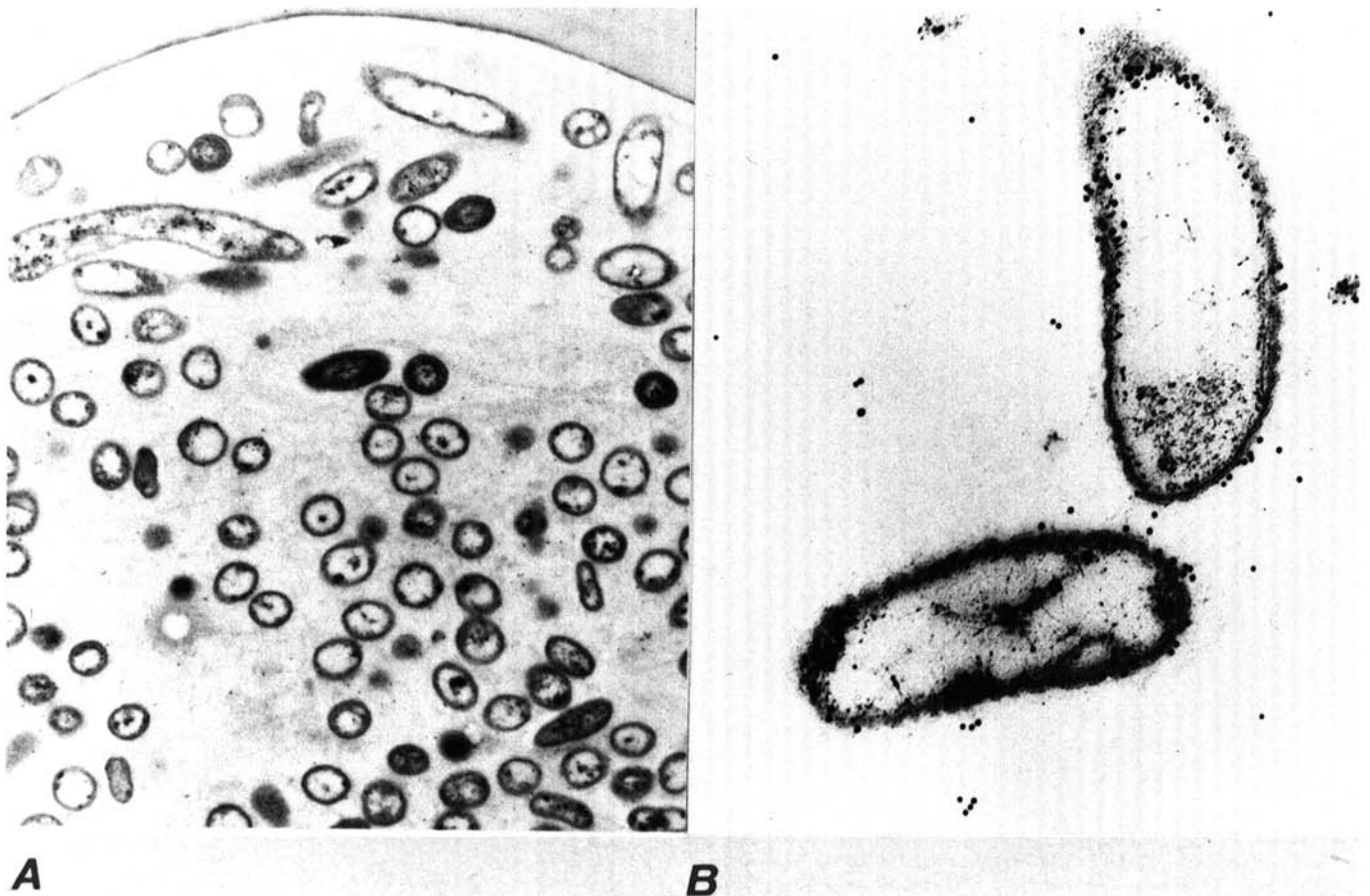
Fig. 5. Immunogold labeling of *Xylella fastidiosa* strain B **A,** in xylem vessels and **B,** in petiole extracts from sweet orange plant 5. **B,** shows the rippled cell wall. Magnification 37,000×.

with symptoms of citrus variegated chlorosis. (Abstr.) Phytopathology 81:1210.

6. Brlansky, R. H., Lee, R. F., and Civerolo, E. L. 1990. Detection of *Xanthomonas campestris* pv. *citrumelo* and *X. citri* from citrus using membrane entrapment immunofluorescence. Plant Dis. 74:863-868.

7. Brlansky, R. H., Timmer, L. W., French, W. J., and McCoy, R. E. 1983. Colonization of the sharpshooter vectors, *Oncometopia nigricans* and *Homalodiscus coagulata*, by xylem-limited bacteria. Phytopathology 73:530-535.

8. Chagas, C. M., Rossetti, V., and Beretta, M. J. G. 1992. Electron microscopy studies of a xylem-limited bacterium in sweet orange affected with citrus variegated chlorosis disease in Brazil. J. Phytopathol. 134:306-312.

9. Chang, C. J., Garnier, M., Zreik, L., Rossetti, V., and Bové, J. M. 1993. Culture and serological detection of the xylem-limited bacterium causing citrus variegated chlorosis and its identification as a strain of *Xylella fastidiosa*. Curr. Microbiol. 27:137-142.

10. Chang, C. J., and Walker, J. T. 1988. Bacterial leaf scorch of northern red oak: Isolation, cultivation, and pathogenicity of a xylem-limited bacterium. Plant Dis. 72:730-733.

11. Chang C. J., and Yonce, C. E. 1987. Overwintering of plum leaf scald bacteria in infected trees. Ann. Phytopathol. Soc. Jpn. 53:345-353.

12. Civerolo, E. L., and Fan, F. 1982. *Xanthomonas campestris* pv. *citri*, detection and identification by enzyme-linked immunosorbent assay. Plant Dis. 66:231-236.

13. Clark, M. F., and Adams, A. N. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for detection of plant viruses. J. Gen. Virol. 34:475-483.

14. Davis, M. J., Purcell, A. H., and Thomson, S. V. 1978. Pierce's disease of grapevines: Isolation of the causal bacterium. Science 199:75-77.

15. Davis, M. J., Purcell, A. H., and Thomson, S. V. 1978. Isolation media for the Pierce's disease bacterium. Phytopathology 70:425-429.

16. Davis, M. J., Raju, B. C., Brlansky, R. H., Lee, R. F., Timmer, L. W., Norris, R. C., and McCoy, R. E. 1983. Periwinkle wilt bacterium: Axenic culture, pathogenicity, and relationships to other gram-negative, xylem-inhabiting bacteria. Phytopathology 73:1510-1515.

17. Davis, M. J., Thomson, S. V., and Purcell, A. H. 1980. Etiological role of the xylem-limited bacterium causing Pierce's disease in almond leaf scorch. Phytopathology 70:472-475.

18. Goheen, A. C., Raju, B. C., Lowe, S. K., and Nyland, G. 1979. Pierce's disease of grapevines in Central America. Plant Dis. Rep. 63:788-792.

19. Gottwald, T. R., Gidtti, F. B., Santos, J. M., and Carvalho, A. C. 1993. Preliminary spatial and temporal analysis of citrus variegated chlorosis in Brazil. Pages 327-335 in: Proc. 12th Conf. Int. Org. Citrus Virol. P. Moreno, J. V. DaGraca, and L. W. Timmer, eds. IOCV, Riverside, CA.

20. Hearon, S. S., Sherald, J. L., and Kostka, S. J. 1980. Association of xylem limited bacteria with elm, sycamore and oak leaf scorch. Can. J. Bot. 58:1986-1993.

21. Hopkins, D. L. 1984. Variability of virulence in grapevine among isolates of the Pierce's disease bacterium. Phytopathology 74:1395-1398.

22. Hopkins, D. L. 1988. Production of diagnostic symptoms of blight in citrus inoculated with *Xylella fastidiosa*. Plant Dis. 72:432-435.

23. Hopkins, D. L. 1988. Xylem limited bacteria. Pages 95-104 in: APS Laboratory Guide for the Identification of Plant Pathogenic Bacteria. N. E. Schaad, ed. American Phytopathological Society, St. Paul, MN.

24. Hopkins, D. L. 1989. *Xylella fastidiosa*: Xylem limited bacterial pathogen of plants. Annu. Rev. Phytopathol. 27:271-290.

25. Hopkins, D. L., Bistline, F. W., Russo, L. W., and Thompson, C. M. 1991. Seasonal fluctuation in the occurrence of *Xylella fastidiosa* in root and stem extracts from citrus with blight. Plant Dis. 75:145-147.

26. Hopkins, D. L., Thompson, C. M., Bistline, F. W., and Russo, L. W. 1990. Relationship between xylem-limited bacteria and citrus blight. Proc. Fla. State Hortic. Soc. 102:21-23.

27. Kostka, S. J., Tattar, T. A., Sherald, J. L., and Hurtt, S. S. 1986. Mulberry leaf scorch, new disease caused by a fastidious, xylem-inhabiting bacterium. Plant Dis. 70:690-693.

28. Lee, R. F., Beretta, M. J. G., Derrick, K. S., and Hooker, M. E. 1992. Development of a serological assay for citrus variegated chlorosis—A new disease of citrus in Brazil. Proc. Fla. State Hortic. Soc. 105:32-34.

29. Lee, R. F., Beretta, M. J. G., Hartung, J. S., Hooker, M. E., and Derrick, K. S. 1993. Clorose variegada dos citros: Confirmacao da *Xylella fastidiosa*, como agente causal. Summa Phytopathol. 19:123-125.

30. Lee, R. F., Derrick, K. S., Beretta, M. J. G., Chagas, C. M., and Rossetti, V. 1991. Citrus variegated chlorosis: A new destructive disease of citrus in Brazil. Citrus Ind. 72:12, 13, 15.

31. Miller, T. J., and Stone, H. O. 1978. The rapid isolation of ribonuclease free immunoglobulin G by protein A-Sepharose affinity chromatography. J. Immunol. Methods 24:111-125.

32. Mircetich, S. M., Lowe, S. K., Moller, W. J., and Nyland, G. 1976. Etiology of almond leaf scorch disease and transmission of the causal agent. Phytopathology 66:17-24.

33. Mollenhauer, H. H., and Hopkins, D. L. 1974. Ultrastructural study of Pierce's disease bacterium in grape xylem tissue. J. Bacteriol. 119:612-618.

34. Purcell, A. H. 1990. Homopteran transmission of xylem-inhabiting bacteria. Adv. Dis. Vector Res. 6:243-266.

35. Purcell, A. H., and Finlay, A. 1979. Evidence for noncirculative transmission of Pierce's disease bacterium by sharpshooter leafhoppers. Phytopathology 69:393-395.

36. Raju, B. C., Goheen, A. C., and Frazier, N. W. 1983. Occurrence of Pierce's disease bacteria in plants and vectors in California. Phytopathology 73:1309-1313.

37. Raju, B. C., Goheen, A. C., Teliz, D., and Nyland G. 1980. Pierce's disease of grapevines in Mexico. Plant Dis. 64:280-282.

38. Raju, B. C., and Wells, J. M. 1986. Diseases caused by fastidious xylem-limited bacteria and strategies for management. Plant Dis. 70:182-186.

39. Roistacher, C. N. 1992. CVC: A warning and an appeal. Citrograph 77:17-18.

40. Rossetti, V., Garnier, M., Bové, J. M., Beretta, M. J., Teixeira, A. R. R., Quaggio, J. A., and DeNegri, J. D. 1990. Presence de bacteries dan le xylem d'orangers atteint de chlorose variegee une nouvelle maladie des agrumes au Brazil. C.R. Acad. Sci. (Paris) 310:345-349.

41. Sherald, J. L. 1993. Pathogenicity of *Xylella fastidiosa* in American elm and failure of reciprocal transmission between strains from elm and sycamore. Plant Dis. 77:190-193.

42. Sherald, J. L., and Kostka, S. J. 1992. Bacterial leaf scorch of landscape trees caused by *Xylella fastidiosa*. J. Arboric. 18:57-63.

43. Timmer, L. W., Brlansky, R. H., Lee, R. F., and Raju, B. C. 1983. A fastidious, xylem-limited bacterium infecting ragweed. Phytopathology 73:975-979.

44. Wells, J. M., Raju, B. C., Hung, H., Weisburg, W. G., Mandelco-Paul, L., and Brenner, D. J. 1987. *Xylella fastidiosa* gen. nov., sp. nov.: Gram-negative, xylem limited, fastidious plant bacteria related to *Xanthomonas* spp. Int. J. Sys. Bacteriol. 37:136-143.

45. Wells, J. M., Raju, B. C., Thompson, J. M., and Lowe, S. K. 1981. Etiology of phony peach and plum leaf scald diseases. Phytopathology 71:1156-1161.