Periwinkle Wilt Bacterium: Axenic Culture, Pathogenicity, and Relationships to Other Gram-Negative, Xylem-Inhabiting Bacteria

M. J. Davis, B. C. Raju, R. H. Brlansky, R. F. Lee, L. W. Timmer, R. C. Norris, and R. E. McCoy

First, sixth, and seventh authors: University of Florida, Institute of Food and Agricultural Sciences, Agricultural Research and Education Centers, 3205 S.W. College Avenue, Ft. Lauderdale 33314. Third, fourth, and fifth authors: 700 Experiment Station Road, Lake Alfred, FL 33850. Second author: Yoder Brothers, P. O. Box 68, Alva, FL 33920.

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ABSTRACT

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.

The Gram-negative, xylem-inhabiting bacterium associated with periwinkle wilt (PW) disease was consistently isolated on the PW, SC, and BC-YE media from PW-diseased periwinkle, but not from healthy periwinkle. Colonies of the bacterium were circular with entire margins, convex, opalescent-white, and reached 0.7–1.0 mm in diameter after 2–3 wk at 28 C. The periwinkle bacterium incited typical PW symptoms during July and August in periwinkle inoculated during the previous fall and winter. The periwinkle bacterium and the bacterium that causes Pierce's disease (PD) of grapevine were pathologically distinct. The periwinkle bacterium infected grapevine, but no symptoms developed which could be attributed solely to the infection. The PD bacterium incited slight chlorosis in periwinkle, but did not incite typical PW symptoms. The ultrastructure and morphology of the periwinkle bacterium in culture, inoculated periwinkle, and a sharpshooter vector, *Homalodisca coagulata*, were similar. The periwinkle bacterium was distinguishable from similar Gram-negative, xylem-inhabiting bacteria associated with PD, phony disease of peach (PDP), and plum leaf scald (PLS), in that it grew readily on the PW, SC, and BC-YE media, but not on PD2 medium. In an enzyme-linked immunosorbent assay (ELISA), the periwinkle bacterium was closely related to the PDP and PLS bacterium.

Periwinkle wilt (PW), a disease of Madagascar periwinkle (*Catharanthus roseus* (L.) G. Don) and its associated fastidious, xylem-inhabiting bacterium were described in 1978 (21). The etiological association of the periwinkle bacterium to PW was firmly established by graft and leafhopper transmissions, but attempts to culture the bacterium failed. Although PW has been found only once in several greenhouse-grown plants, the possible etiological relationship to other diseases could be significant.

The habitat, transmission, morphology, ultrastructure, and antigenicity of the periwinkle bacterium identify it with the so-called "rickettsialike" bacteria (21). These bacteria either have been associated with, or now are known to cause, Pierce's disease (PD) of grapevines (7,13,17), almond leaf scorch (10,22), alfalfa dwarf (13,29), phony disease of peach (PDP) (5,18,24,31), plum leaf scald (PLS) (5,19,28,31), elm leaf scorch (ELS) (14), and possibly other diseases (11). These Gram-negative, xylem-inhabiting bacteria form a distinct group and will probably be taxonomically classified as cogeneric species or subspecies (11).

The PD bacterium was the first of the fastidious, xylem-inhabiting bacteria to be grown in axenic culture and shown to be pathogenic, causing PD (6), almond leaf scorch (10), and probably alfalfa dwarf (29). Attempts to isolate the other xylem-inhabiting bacteria, including the periwinkle bacterium, on the medium developed for the PD bacterium were not successful. Subsequently, media were developed that supported growth of other fastidious, xylem-inhabiting bacteria (5,31). Pure cultures of these bacteria can now be used to determine pathogenicity and other characteristics of these bacteria.

We selected PW for further study because the causal agent might also cause diseases of economic importance, and information about the causal agent might be useful in the study of other xylem-inhabiting bacteria. During this study, a medium developed for the periwinkle bacterium was used to obtain axenic cultures of the PDP and PLS bacteria (5). Other media developed independently (28,31) for the PDP and PLS bacteria were subsequently used to isolate the periwinkle bacterium. The combined results of these studies of PW are reported herein.

The objective of this research was to determine the relationship of the periwinkle bacterium to PW disease, and to examine the pathological relationships between the periwinkle and PD bacteria. Serological relationships between the periwinkle bacterium and other xylem-inhabiting bacteria were examined.

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MATERIALS AND METHODS

Plant material. PW disease was maintained by graft or leafhopper transmission in Madagascar periwinkles (21). Dwarf

periwinkle seedlings of cultivars Bright Eyes, Little Blanche, Little Delicate, and Little Pinkie, open pollinated cultivar Pinot Noir grapevine seedlings, and rooted cuttings of cultivar Mission grapevine were used to test the pathogenicity of the PD and periwinkle bacteria.

Bacterial strains and media. The strain designation and source of xylem-inhabiting bacteria used in this study are listed in Table 1. Primary isolations of the periwinkle bacterium were made on PW medium (5) on SC medium (6), and on BC-YE medium (31). All strains were maintained aerobically on PW or BC-ZE (28) medium at 25–28 C and were transferred to fresh medium every 10–14 days.

Isolation procedure. Petioles, stems, and roots were used in isolation attempts. Plant material was surface sterilized by dipping it briefly in 95% ETOH and then submerging it in 1% sodium hypochlorite (20% commercial bleach) for 2.5 min. The plant material was then rinsed four times with sterile distilled water. Sap was expressed with sterile forceps from freshly cut surfaces and placed directly onto the agar medium. All inoculated plates were aerobically incubated at 25–28 C and observed 10–14 days after inoculation.

Inoculation procedure. Inoculum was scraped with sterile polyester swabs from heavily streaked plates after 7–10 days of incubation at 28 C. The bacteria were suspended in sterile 0.01 M phosphate buffer, pH 7.0, at an $A_{560\ nm} = 0.01$. Plants were inoculated with the suspensions by the needle puncture method (10) with a syringe and 0.89-mm-diameter (20-gauge) needle, or by the pin-prick method (15). The main shoot of 6- to 10-wk-old periwinkle and grapevine was punctured four or five times and the inoculum was forced into the wound with the needle. After inoculation, plants were kept in a greenhouse or screenhouse without additional shading and under moderate water stress.

Electron microscopy. Petioles from periwinkle infected with the PW and PD bacteria were prepared for transmission electron microscopy (TEM) and examined by previously described methods (21). Periwinkle bacteria from culture were negatively stained with ammonium molybdate and also embedded and thin sectioned for TEM (28,31). Ultrathin sections were made on a Huxley LKB ultramicrotome. Preparations were viewed with a Philips 201 transmission electron microscope.

Healthy and PW-diseased periwinkle and the periwinkle bacterium from culture were also examined by scanning electron microscopy (SEM) using previously described methods (2,28). Leafhoppers were fixed, dissected, and examined by SEM to determine the presence of bacteria in their cibaria and precibaria (3,4).

Serology. Direct (2) and indirect (9) fluorescent antibody staining, and enzyme-linked immunosorbent assay (ELISA) (23,28) were performed using antisera prepared against the periwinkle, PDP, PD, and PLS bacteria. All antisera had homologous titers of 1:1,024 to 1:2,048 in microagglutination tests against approximately 5×10^8 bacteria per milliliter. Immunofluorescence was used routinely to help identify the periwinkle bacterium upon primary isolation and the periwinkle and PD bacteria upon reisolation from inoculated hosts. Extracts from diseased and healthy periwinkle stems and petioles, and preparations of different strains of xylem-limited bacteria from culture were prepared (27) and tested by ELISA.

Virus transmission by leafhoppers. Twelve healthy, reared sharpshooters, *Homalodisca coagulata* (Say), that had been caged on PW-infected periwinkle for at least 14 days were transferred to a cage containing two healthy periwinkles. The sharpshooters were allowed to feed on the healthy periwinkle for 18 days. The six surviving sharpshooters were fixed and examined by SEM for bacteria in their cibaria and precibaria, and the two periwinkle plants observed for symptoms. The plants were also checked periodically for bacteria in smears of expressed xylem sap using methylene blue (4) and immunofluorescent staining. In addition, in situ immunofluorescent staining was also done (2).

TABLE 1. Strain and isolate designation, disease association, and source of bacterial strains

Disease association	Strain or isolate[a]	Source: Host	Source: Area
Periwinkle wilt	PWB-1, PW-R15, PW-S2, PW-S36, PW-P10, PW-P28, PW-100, PE-17, PE-22	Periwinkle	Florida
Pierce's disease	PD-N5, VP-4	Grapevine	California
	PD-LH	Grapevine	Florida
Almond leaf scorch	PD-A2	Almond	California
Alfalfa dwarf	MT-5, PD-AL3	Alfalfa	California
Phony peach disease	PPDB-5	Peach	Florida
Plum leaf scald	PL-57	Plum	Georgia

[a] All periwinkle bacterium designations listed are for isolates with a common origin. The isolates were obtained from different periwinkle plants or plant parts with periwinkle wilt disease transmitted from one original source plant. All other bacterial designations listed are for strains with no known common origin.

RESULTS

Isolations. The periwinkle bacterium was consistently isolated on BC-YE medium, SC medium, and PW medium from PW-diseased, but not from healthy periwinkle petioles and stems. In one test with five PW-diseased periwinkles and the BC-YE medium, the periwinkle bacterium was isolated in all attempts from each of 30 stems and petioles, and on six of 20 attempts from roots. Similar isolation attempts from each of 20 roots, stems, and petioles of five healthy plants were negative.

Colonies of the periwinkle bacterium were visible 10–12 days after plate inoculation on PW, SC, or BC-YE medium. Individual colonies upon subculture measured 0.7–1.0 mm in diameter after 2–3 wk of incubation and were circular with entire margins, convex, and opalescent-white under reflected light. Rod-shaped bacteria were observed in water-mounted specimens of colonies by phase-contrast microscopy. The bacteria appeared similar in size and shape to those expressed from wilt-affected periwinkle in this and a previous study (21).

Inoculations of periwinkle and grapevine. All isolates (PWB-1, PW-P10, PW-S2, PW-R15, PE-17, PE-22, and PW-100) of the PW bacterium tested incited PW symptoms in periwinkle. Forty-five of 46 inoculated plants developed symptoms and the periwinkle bacterium was reisolated from 43 of these plants after symptoms developed. Nine uninoculated control plants remained healthy and

TABLE 2. Disease severity in periwinkle and cultivar Pinot Noir grapevine seedlings which were inoculated with the periwinkle bacterium (PWB-1) and the Pierce's disease bacterium from grapevine (VP-4) and alfalfa (MT-5)

Plant inoculated[a]	No. of plants	Bacterial strain	Average severity rating[b]	No. positive reisolations
Periwinkle	14	VP-4	0.14 (1.0)[c]	4
	13	MT-5	0.0	0
	16	PWB-1	2.38	16
	6	None	0.0	0
Grapevine	12	VP-4	3.17	12
	12	MT-5	2.83	12
	12	PWB-1	0.83 (1.0)[c]	4
	12	None	0.58	0

[a] Periwinkle and grapevine were inoculated by needle puncture with portions of the same suspensions of the bacteria from axenic culture, and the data were recorded after 47 wk and 16 wk, respectively.

[b] Severity rating for periwinkle: nonsymptomatic = 0; slight marginal chlorosis of mature leaves = 1; general chlorosis throughout most of the plant = 2; severe chlorosis, wilting and defoliation = 3; dead = 4. Severity rating for grapevine; nonsymptomatic = 0; slight marginal necrosis of leaves = 1; marginal necrosis and interveinal chlorosis = 2; marginal necrosis, interveinal chlorosis and defoliation = 3; completely defoliated or dead = 4.

[c] Average severity rating for plants from which the VP-4 or PWB-1 strains were reisolated is given in parenthesis.

the periwinkle bacterium was not isolated from them. When 16 plants were inoculated in October, no symptoms were observed until July and August (9–10 mo) (Table 2) although the bacterium was reisolated from all plants 5 mo after inoculation. When inoculated three times at 3–4 wk intervals starting in December, periwinkle began exhibiting PW symptoms in July and August (6–7 mo) of the following year. A bacterium that was morphologically similar to the inoculated bacterium was observed by phase-contrast microscopy (×1,000) in expressed sap from petioles and stems of all symptomatic plants, but not in the sap from asymptomatic plants. No remission of symptoms was observed throughout the subsequent fall and winter. PW disease did not readily kill periwinkle and most plants, although severely diseased, remained alive more than 1 yr after inoculation.

In periwinkle inoculated with the PD bacterium and observed for 1 yr or more, only slight chlorosis of older leaves with no wilting was seen (Table 2). Bacteria morphologically similar to the PD bacterium were observed by phase-contrast microscopy in expressed sap of seven of 14 periwinkles 11 mo after they had been inoculated once in October with a grapevine strain (VP-4) of the PD bacterium. The bacterium was reisolated, however, from only four of the 14 plants. An alfalfa strain (MT-5) of the PD bacterium was neither observed in nor reisolated from inoculated periwinkle. In another experiment, all 10 periwinkle plants (three or four plants per strain) inoculated on three occasions at 3–4 wk intervals starting in December or January with either a grapevine (PD-N5), almond (PD-A2), or alfalfa (PD-AL3) strain of the PD bacterium became infected and exhibited slight chlorosis but no wilting. Characteristic bacteria were reisolated from all of the inoculated plants.

The periwinkle bacterium multiplied in grapevine, but was not pathogenic. No symptoms attributable to disease were incited by the PWB-1 isolate in inoculated cultivar Pinot Noir grape seedlings, although characteristic bacteria were reisolated from four of 12 plants 16 wk after inoculation (Table 2). Cultivar Mission grapevines inoculated with the PW-S2, PW-P10, PW-P28, and PW-S36 isolates of the periwinkle bacterium did not develop symptoms. No reisolations were attempted.

The pathogenicity of all PD strains to grapevine was confirmed. The same inoculum suspensions used to inoculate periwinkle were used to inoculate grapevine. Cultivar Pinot Noir grapevine inoculated with the VP-4 and MT-5 PD strains began exhibiting PD symptoms 8–10 wk after inoculation, and all plants were dead 8 mo after inoculation. The VP-4 grapevine strain appeared slightly more virulent than the MT-5 alfalfa strain as indicated by disease severity ratings made 16 wk after inoculation (Table 2). Typical PD symptoms developed in cultivar Mission grapevine 3–4 mo after inoculation with the other PD strains (PD-N5, PD-A2, PD-LH, and PD-AL3) used in this study.

Electron microscopy. The periwinkle bacterium was observed in the xylem vessels of PW-diseased periwinkle with SEM; no bacteria were found in vessels of healthy plants (Fig. 1B). The periwinkle bacterium from culture strongly resembled the bacteria previously described (2,21) in PW-diseased periwinkle when examined by TEM and SEM (Fig. 2). Cells negatively stained with ammonium molybdate measured 0.4–0.5 μm in width and 2.5–3.5 μm in length and exhibited the characteristic "rippled" topography of the cell wall (Fig. 2B and C). Periwinkle petioles from plants inoculated approximately 1 yr earlier with the VP-1 strain of the PD bacterium or with the PWB-1 isolate of the periwinkle bacterium had xylem vessels containing the bacteria (Fig. 3). Periwinkle inoculated with the PWB-1 isolate exhibited strong PW symptoms at the time of sampling for TEM. At the same time only mild chlorosis of older leaves was observed in periwinkle plants inoculated with the VP-4 strain; although the lumen of some vessel elements were completely occluded with bacteria.

Serology. All isolates of the periwinkle bacterium tested positive for immunofluorescent staining with antisera to the PD bacterium upon primary isolation from graft- or insect-inoculated periwinkle and upon reisolation from periwinkle and grapevine inoculated with bacteria from culture. Strains of the PD bacterium reisolated from periwinkle and grapevine also produced a positive

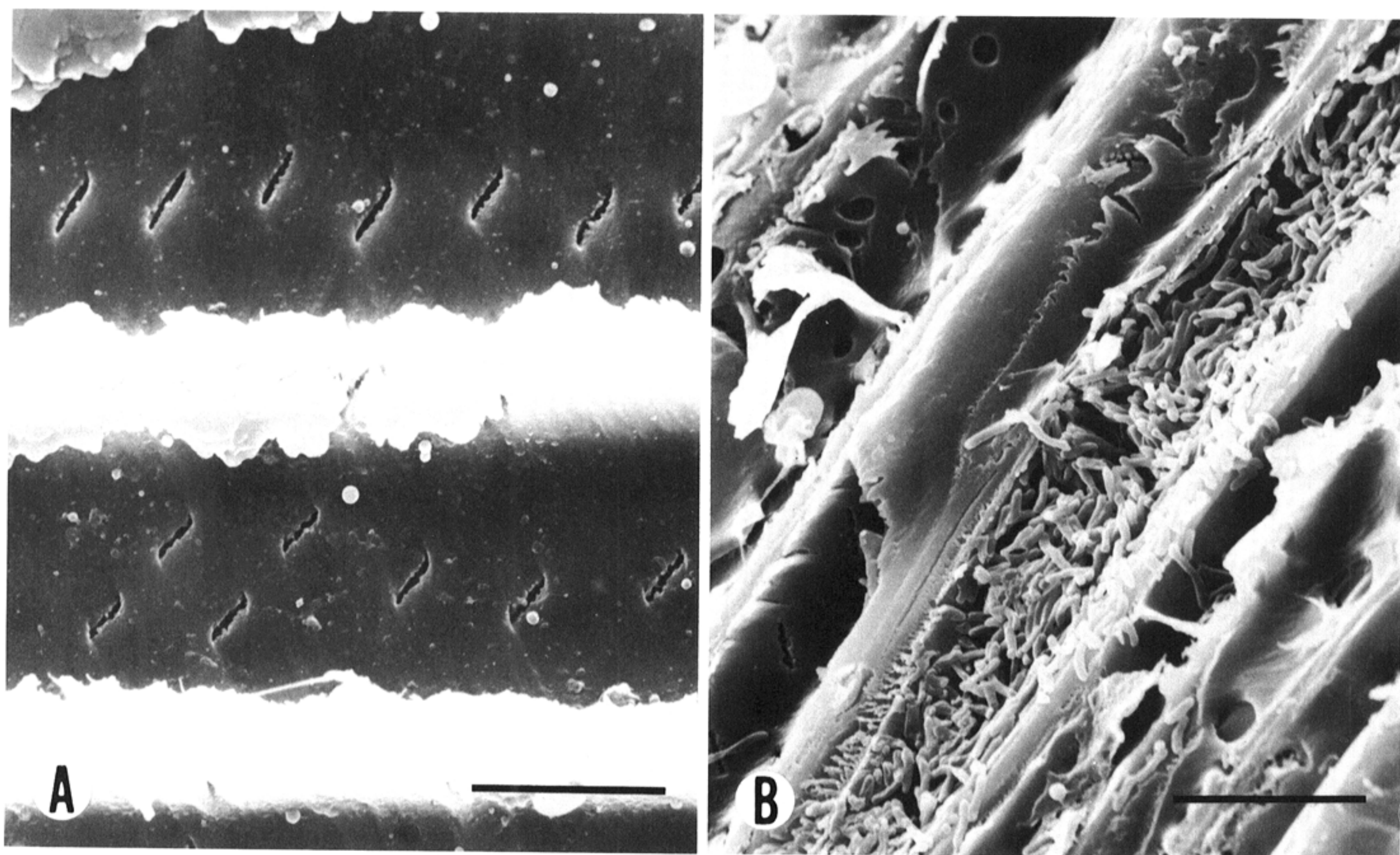

Fig. 1. Scanning electron micrographs of xylem vessels of **A,** healthy and **B,** diseased periwinkle. Bar indicates 10 μm.

A B C

Fig. 2. Periwinkle wilt bacteria from culture. **A,** Scanning electron micrograph of bacterial colony on agar. **B,** Bacteria stained with ammonium molybdate, showing characteristic "rippled" topography of the cell wall. **C,** Ultrathin section of bacteria. Bars indicate 1 μm.

A B

Fig. 3. Transmission electron micrographs of periwinkle inoculated with **A,** Pierce's disease and **B,** periwinkle wilt bacteria from culture. Longitudinal sections of petioles show uneven distribution or absence of bacteria in adjacent xylem vessels. Bar indicates 10 μm.

TABLE 3. Enzyme linked immunosorbent assay (ELISA) of the periwinkle wilt (PW), Pierce's disease (PD), phony disease of peach (PDP), and plum leaf scald (PLS) strains of the Gram-negative xylem-inhabiting bacteria in suspensions from pure culture and extracts from diseased plants

Sample material	Host source	No. Tested	Average $A_{405\ nm}$ measured by using antiserum to strains[a]				
			PW-100	PD-LH	PD-N5	PL-57	PPDB-5
Bacterium[b]							
PW	Periwinkle	5	0.61	1.06	0.31	0.71	>2.00
PD	Grapevine	2	0.05	0.60	1.05	0.35	0.38
PDP	Peach	1	0.22	0.09	...	...	1.31
PLS	Plum	1	...	...	0.41	1.10	...
Plant material[c]							
PW-diseased	Periwinkle	25	1.73	...	0.11	0.45	...
Healthy	Periwinkle	23	0.01	...	0.02	0.02	...
PD-diseased	Grapevine	3	0.58	...	...	...	...
Healthy	Grapevine	3	0.01	...	...	...	...
PLS-diseased	Plum	1	1.74	...	...	...	...
Healthy	Plum	3	0.12	...	...	...	...
Phosphate-buffered saline	N/A[d]	N/A	0.01	0.01	0.01	0.02	0.02

[a] PW-100, PD-LH, and PPDB-5 strains at 10^6 cells per milliliter assayed in reciprocal tests using antisera to each strain. PW-S2, PW-P10, PW-P28, PW-S36, PD-N5, and PL-57 strains at 10^9 cells per milliliter were assayed in tests using antisera to the PD-N5 and PL-57 strains, and the results for the PW strains were averaged since they did not differ significantly ($P = 0.01$).

[b] Twenty-two PW-diseased and 20 healthy periwinkle plants were assayed using antisera to the PD-N5 and PL-57 strains and the remaining three PW-diseased and three healthy periwinkle were assayed using antiserum to the PW-100 strain. Results obtained between replicates were averaged since they did not vary significantly ($P = 0.01$).

[c] Averages of three composite replicates per bacterial suspension or plant extract tested. Values among replicates were not significantly different ($P = 0.01$). ELISA reactions were run for 1 hr for bacterial suspensions from culture and for 30 min for plant extracts using the same coating and conjugate antiserum.

[d] N/A = not applicable.

immunofluorescent reaction.

Isolates of the periwinkle bacterium had positive reactions with antisera to strains of the periwinkle, PD, PLS, and PDP bacteria in ELISA (Table 3). The $A_{405\ nm}$ values using antisera to the PDP and PLS bacteria were significantly higher for the PW bacterium than for the PD bacterium, and the $A_{405\ nm}$ values obtained by using antiserum to the periwinkle bacterium were significantly higher for the PDP than the PD bacterium. Similar differences in the $A_{405\ nm}$ values in ELISA reactions were observed among antisera to the periwinkle, PD, and PLS bacteria when assaying extracts from PW-diseased periwinkle (Table 3). However, all three antisera differentiated PW-diseased from healthy periwinkle, and the antiserum to the periwinkle bacterium also gave positive reactions in ELISA when tested against PD-diseased grapevine extracts and PLS-diseased plum extracts as compared to extracts from healthy grapevine and plum (Table 3).

Leafhopper transmission. The sharpshooter, *H. coagulata*, transmitted the periwinkle bacterium in our preliminary tests to one of two periwinkles as confirmed by symptom development and examination of expressed sap from petioles by phase-contrast microscopy and by immunofluorescence. Three of six sharpshooters that survived the 14-day acquisition access period and 18-day inoculation access period contained bacteria upon examination of their cibaria and precibaria by SEM.

DISCUSSION

The periwinkle bacterium was shown to cause PW disease. Both the periwinkle and PD bacteria were pathogenic to periwinkle; however, the periwinkle bacterium was more virulent than the PD bacterium. The periwinkle bacterium incited typical PW symptoms including marginal and veinal chlorosis in mature leaves and wilting, while the PD bacterium incited only mild chlorosis of older, mature leaves. These same PD strains were highly virulent in grapevine inciting typical PD symptoms, while the periwinkle bacterium was avirulent in grapevine. Thus, the PD and periwinkle bacteria were pathologically distinct. Early investigations of PD and PDP involving graft and insect transmission of their disease agents, which are now known to be xylem-inhabiting bacteria, established that the bacteria have wide host ranges, but that most hosts are symptomless (11,12). The ability of the PD bacterium also to incite dieback symptoms in citrus seedlings under experimental conditions was recently reported (16). Xylem-inhabiting bacteria might be responsible, under natural conditions, for a lack of vigor or slow decline in some plants without the condition being recognized as a distinct disease.

Large numbers of the PD bacterium were observed by TEM in xylem vessels of inoculated periwinkle. The PD bacterium might have been unevenly distributed or less numerous throughout the plant than indicated by TEM. For TEM, only petioles from symptomatic leaves were examined, and the presence of the bacteria in expressed sap of each petiole was confirmed by phase-contrast microscopy before further sampling.

The PW bacterium was experimentally transmitted in this preliminary study by *H. coagulata* and by *Oncometopia nigricans* in previous studies (3,21). These sharpshooter leafhoppers are xylem-feeders and known vectors of PD and PDP (1,30). The mechanism of transmission of the PW bacterium by *H. coagulata* appears to be the same as reported with other xylem-inhabiting bacteria (3,4,25,26).

The relatedness among the Gram-negative, xylem-inhabiting bacteria examined in this study is evidenced by similarities in morphology, ultrastructure, host habitat, and mechanism of transmission. The bacteria also exhibit similar nutritional fastidiousness; they will grow on the PW and BC-YE media but not on common bacteriological media (5,21,31). However, the PW bacterium was distinguishable from the other strains since it grew on the SC medium, but not the PD2 medium (8,21). The PD bacterium is readily grown on both the SC and PD2 media, and the PDP and PLS bacteria cannot be grown on either medium (5). Serological relationships between these bacteria have also been demonstrated (5,9,28,31), mostly by fluorescent antibody procedures. ELISA, unlike immunofluorescence, might possibly be used to quantitatively measure the degree of serological relatedness between these bacteria. Our results suggested that the PW bacterium was more closely related to the PDP and PLS bacteria than the PD bacterium. Similarly, ELISA has been used to measure relationships between spiroplasma strains (20). It follows that serological interrelationships among these xylem-inhabiting bacteria and, consequently, the specificity of antisera to these bacteria will have to be considered when using ELISA to detect the bacteria in suspected hosts.

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