Gram-Negative, Xylem-Limited Bacteria in Plant Disease

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ABSTRACT

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Studies that led to the isolation in axenic culture of the Gram-negative, xylem-limited bacteria associated with Pierce's disease (PD) of grapevine, almond leaf scorch, alfalfa dwarf, periwinkle wilt, phony disease of peach, and plum leaf scald are reviewed and discussed. The PD bacterium, shown by serological techniques to be present in citrus, could not be isolated in pure cultures directly from citrus, but was isolated in pure culture by using grapevines as selective hosts to separate the PD bacterium from bacterial contaminants. Epidemiological studies in Florida and California have found at least six wild plant species that serve as alternate hosts of the PD bacterium. Many characteristics of the PD bacterium, such as colony type, aggregation of bacterial cells in H_2O suspension, and virulence, are unstable during maintenance of the bacterium in axenic culture for 6–12 mo. Avirulent isolates that developed in culture were shown to infect grapevines without producing symptoms. Bacterial aggregates blocking leaf veins were correlated with leaf-necrosis symptoms of PD. Tolerance of grape cultivars to the PD bacterium is discussed.

Gram-negative, xylem-limited bacteria were first reported to be associated with a plant disease in 1973 (9,14). Electron microscopy coupled with chemotherapy or thermotherapy was used to demonstrate the association of the bacteria with Pierce's disease (PD) of grapevine. Because of some of their characteristics, these xylem-limited bacteria were referred to as "rickettsialike." They now appear to be only superficially similar to members of the Rickettsiaceae since the G + C content of the DNA of the PD bacterium is very different from the G + C content of rickettsial DNA (6).

Since 1973, there has been considerable progress on the characterization of these xylem-limited bacteria and the diseases they cause. Several reviews of these organisms have been written (6,11,21,23). Since the PD bacterium was the first of these bacteria to be cultured, more work has been done on PD than on others. This report covers some of the more recent work on the Gram-negative, xylem-limited bacteria. This communication presents the results of work on the isolation, and maintenance in culture, and on the pathological aspects of these bacteria with special reference to the PD bacterium.

Isolation of the xylem-limited, Gram-negative bacteria. An important step in studying the pathology and taxonomy of a plant pathogen is the isolation of the agent in axenic culture. The first of the xylem-limited bacteria to be isolated and shown to be pathogenic through the fulfillment of Koch's postulates was the bacterium that causes Pierce's disease of grapevine, almond leaf scorch, and alfalfa dwarf (4). Since this first success in 1978, efforts to culturing these bacteria has intensified tremendously and has resulted in the isolation of the bacteria associated with periwinkle wilt, phony disease of peach (PDP), plum leaf scald (PLS), and others (2,16,29).

The PD 2 medium developed by Davis et al (5) for the isolation and growth of the Pierce's disease bacterium (PDB) contained hemin chloride and bovine serum albumin as key growth-promoting factors. Early descriptions referring to the Gram-negative, xylem-limited bacteria as rickettsialike (9,14) led to the inclusion of hemin chloride and bovine serum albumin in the PD 2 medium, since they were components of a medium used for *Rochalimaea quintana*—a member of the Rickettisaceae that has been cultured (20). It has been suggested that the role of the hemin may be to protect the bacteria from peroxides in early stages of growth rather than to fulfill an essential nutrient requirement (5). The role of the bovine serum albumin appears to be as a detoxicant, since starch or activated charcoal may be substituted (6). PD 2 medium also contained the organic acids citrate and succinate rather than glucose.

PW medium was developed for the isolation of the xylem-limited bacterium associated with periwinkle wilt disease (2). The PW medium is similar to the PD 2 medium except for concentration changes of some ingredients and for the substitution of glutamine for citrate and succinate. The bacteria associated with PDP and PLS were also isolated and maintained in axenic culture on PW medium. Wells et al (29) also cultured the bacteria associated with PDP and PLS on a charcoal-yeast extract medium (BCYE) buffered with ACES (2-[(2-amino-2-oxoethyl) amino]-ethanesulfonic acid). The BCYE medium contains yeast extract, activated charcoal, L-cysteine $HCl \cdot H_2O$, ferric pyrophosphate, ACES buffer, and agar. A xylem-limited bacterium was found associated with elm, sycamore, and oak leaf scorch (10). The bacterium from elm was cultured in liquid S8 medium (3) and transferred to PD 2 medium for subculturing (16). While these bacteria appear to differ from the PD bacterium in cultural requirements, they are related serologically and cannot be distinguished by immunofluorescent techniques with currently available antibodies.

The leafhopper, *Oncometopia nigricans*, collected in a citrus grove in Florida was found to be carrying a xylem-limited bacterium that would infect ragweed but not grape, rough lemon citrus, or periwinkle (27). The ragweed bacterium reacts with PD antisera and PDP antisera in immunofluorescence tests and ELISA. The bacterium could not be maintained on PD 2 medium, but grew well on BCYE medium formulated for the culture of PDP bacteria. This ragweed bacterium may be the PDP bacterium or it may be a new member of the Gram-negative, xylem-limited bacteria.

Gram-negative, xylem-limited bacteria, serologically related to the PD bacterium, were shown by immunofluorescence techniques to be present in the rough lemon roots of blighted citrus (17). By using *O. nigricans*, the PD bacterium was transmitted from citrus with blight to cultivar Carignane grapevines, which subsequently developed typical PD symptoms. The bacterium was cultured from the grapevines on PD 2 medium, but could not be cultured directly

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from the blighted citrus (13). Vacuum infiltration of root and stem segments (8) with succinate-citrate-phosphate buffer was used to extract bacteria from the xylem of citrus trees (*unpublished*). Attempts to culture the PD bacterium from the extracts often yielded bacterial contaminants that grew on PD 2 and grew very slowly or not at all on nutrient agar, but were not serologically related to the PD bacterium. However, indirect immunofluorescence tests with PD antisera of some of these contaminant colonies in midsummer of 1980 revealed the presence of a few brightly fluorescent bacterial rods mixed in with the predominant nonfluorescent rods. Two of these mixed colonies were used to inoculate grapevines, which developed severe PD symptoms 10 wk later. Pure cultures of the PD bacterium were then obtained from these grapevines. Perhaps, the PD bacterium only survived the vacuum extraction procedure when aggregated with other bacteria and in this instance the grapevine was used to select out the pathogen. While this technique allowed the successful culture of the PD bacterium from citrus, it is very inefficient and time-consuming. It was also fortuitous that the contaminant colonies were relatively slow growing; rapid-growing contaminants would make it very difficult to detect the PD bacterium by immunofluorescence. An extraction technique is needed that would result in the growth of pure cultures from citrus as occurs with grapevines.

Occurrence of Gram-negative, xylem-limited bacteria in various plant hosts. Epidemiological studies on alternate hosts of the PD bacterium have been conducted in California and Florida. In California, enzyme-linked immunosorbent assay (ELISA) was used to test 28 wild plant species in areas of high PD incidence for the presence of bacteria, which gave a positive reaction to PD antisera (26). Dallis grass (*Paspalum dilatatum*), tall umbrella plant (*Cyperus eragrostis*), and poison-hemlock (*Conium maculatum*) gave positive tests. The PD bacterium was isolated from Dallis grass, but not from the tall umbrella plant or poison hemlock.

In Florida, 20 wild plant species were tested by ELISA with PD antisera (1). ELISAs were positive and the PD bacterium was cultured on PD 2 medium from pepper vine (*Ampelopsis arborea*), Virginia creeper (*Parthenocissus quinquefolia*), and American elder (*Sambucus canadensis*). The ELISA tests were positive from American beautyberry (*Callicarpa americana*) and a bacterium was cultured, but the bacterium did not produce PD symptoms in cultivar Carignane grapevines. The ELISA tests were positive, but nothing could be cultured from eastern baccharis (*Baccharis halimifolia*), goldenrod (*Solidago fistulosa*), and sumac (*Rhus* sp.). The beautyberry isolate may be a PD isolate that is avirulent, or weakly virulent, to grapevine or it may be some other serologically related bacterium. The positive ELISA tests with failure to culture the PD bacterium may be due either to a failure in isolation technique or to the presence of a different, but serologically related, bacterium.

The results of work in Georgia indicate that johnsongrass (*Sorghum halepense*) as well as several *Prunus* species may be alternate hosts of the PDP bacterium (28,30).

Cultural and pathogenic characteristics of the PD bacterium. Primary isolation of the PD bacterium often yields a range of colony types from domed colonies with entire margins to flat colonies with irregular margins. Weekly serial subculturing of the PD isolates results in all domed colonies after 2–3 mo. Our attempts to select for the flat, irregular colonies have always failed. The flat colony type seems to be unstable in serial subculturing. Transfers from isolated flat colonies yielded both colony types even after six to eight single-colony transfers.

Cells of recently cultured isolates also tend to aggregate or autoagglutinate in buffer or water suspensions. This makes it very difficult to adjust the cell concentration in inoculum by optical density and also interferes with microagglutination tests for the serological identification of the bacterium. Urea prevents this autoagglutination, but the effect on pathogenecity is not yet known. This aggregation characteristic of PD isolates usually is lost after 2–3 mo of serial subculturing.

The virulence of PD isolates obtained from grapevines in Florida is quite variable. In addition, many of the isolates become weakly virulent, or avirulent, after serial subculture for 6–12 mo (*unpublished*). Therefore, virulence of PD isolates must be monitored closely. Storage of lyophilized stock cultures may be used to maintain virulence.

The characteristics of the PD bacterium that are altered during maintenance of the bacterium in culture may be controlled by plasmids. Alternatively, these changes could reflect the adaptation of the bacterium to its new habitat on the laboratory media from the normal grape xylem vessel habitat. The development of a range of virulence levels indicate that the latter may be the best explanation for the loss of virulence.

Virulent, weakly virulent, and avirulent PD isolates are valuable in studies on the mechanism of pathogenesis, especially when virulent and avirulent strains of the same isolate are available. I first determined whether or not the avirulent strains could multiply and infest grapevine tissue (*unpublished*). Single points on petioles were inoculated, and at various times after inoculation populations of bacteria in the petioles were determined by dilution plating. Bacterial populations of virulent isolates reached 10^7–10^8 cells per centimeter of petiole and the maximum population of avirulent isolates was 10- to 100-fold less than this. With all isolates, bacterial populations in the petiole decline for the first 4–6 days and then increase for the next 10–14 days. With virulent and avirulent isolates, populations are maximal at this time. However, populations of weakly virulent isolates slowly increase for several weeks until PD symptoms develop 2–6 mo after inoculation. Populations of virulent isolates stabilized at the maximum level for the life of the inoculated leaf, whereas those of avirulent isolates started to decline at 4 wk after inoculation. All isolates had similar multiplication curves in liquid PD 2 medium.

While avirulent isolates did infect plants without causing symptoms, virulence appeared to depend on the maximum population of bacteria in the plant tissue. The avirulent strains never reached the concentration necessary for symptom production. The weakly virulent isolates required a much longer time span to reach this necessary concentration than did the avirulent isolates; therefore, symptoms took much longer to develop, were milder, and were very slow to spread from the inoculated leaf. There are several possible explanations for these results, including the bacteria may physically block xylem vessels in the petioles or leaf veins to produce the marginal leaf necrosis symptoms and a minimum population of bacteria is required for blockage; a minimum population may be required to produce sufficient toxin to produce the symptoms; or a combination of the above.

While bacterial aggregates block vessels in grapevines, rarely do more than 40% of the vessels in a leaf petiole or vein contain bacteria (19). Plants develop no water stress symptoms with higher percentages of nonfunctioning vessels (7). However, serial sectioning of 0.5-cm lengths of grape tissue indicated that vessel plugging values over the 0.5 cm length were 4–12 times greater than values per cross section (12). Almost 80% of the vessels of leaf veins from leaves with marginal necrosis were completely obstructed within the 0.5-cm length of tissue. Bacterial concentrations in the leaf veins correlated well with symptoms in leaves. This plugging in leaf veins could account for the leaf marginal necrosis symptom by causing water stress in the leaves. However, the Pierce's disease bacterium produces a phytotoxin in culture, which also could be involved in symptom production (18).

The failure to detect the PD bacterium in the current season plant tissue during the first 4 wk after budbreak (12) and the acropetal leaf symptom development indicated to us that the bacterium may multiply in older tissue better than younger tissue. To test this idea I inoculated 3- to 4-mo-old rooted cultivar Carignane cuttings in different age stem and leaf tissues (Table 1). Symptoms developed most rapidly after inoculation of the lower internodes of the stem, with leaf marginal necrosis (MN) occurring within 28 days. Symptoms developed within another week after petiole inoculations from the midpoint of the stem or the base. It

TABLE 1. Symptom development after single-point inoculations of cultivar Carignane grapevine with PD bacterium[a]

Inoculation point	Days to first symptoms[b]	Days to systemic symptoms[c]
Basal internode	28	28
Petiole (one of three basal leaves)	34	48
Petiole (mid-point of plant)	34	42
Leaf vein (mid-point of plant)	42	48
Stem (one of three top internodes)	48	48
Petiole (youngest expanded leaf)	55	76

[a] Inoculation was by pin-pricking the plant tissue at a single point through a drop of bacterial suspension containing 10^7 cells per milliliter. The inoculum of 8×10^5 bacterial cells per plant was pulled into the xylem vessels. Treatments were replicated three times.
[b] Days from inoculation to symptoms in inoculated leaves.
[c] Days from inoculation to symptoms in leaves other than the inoculated leaf.

took an additional 2–3 wk for symptoms to develop after inoculation of young stems or petioles. Since symptoms usually correlate with bacterial concentration (*unpublished*), the PD bacterium appeared to develop best in older tissue. This may be the reason that symptoms in many hosts do not develop until late fall when plants are senescing.

PD symptoms in grape seedlings take longer to develop than in rooted cuttings (Table 2). Perhaps this is due to the more juvenile seedling tissue being more resistant to the PD bacterium than the rooted cutting tissue. After the first symptoms appear, symptom development is as rapid and as severe in the seedling as in the rooted cutting. There simply seems to be a latent period for the bacterium while the seedling tissue ages for 3–4 wk, and then the disease develops.

In contrast, older foliage of many *Vitis vinifera* cultivars was more resistant to infection with PD after inoculation by leafhoppers than was young foliage (24). The reason for the difference in results using leafhoppers and mechanical inoculation is not obvious. However, it may result from a difference in vector efficiency on old and young grapevine leaves.

Resistance to PD in grapevines. The only feasible control for PD is resistance. In California, different levels of tolerance have been observed in *V. vinifera* cultivars (22). In the southeastern United States, muscadine cultivars have different levels of tolerance while European-type grapes are not grown because of PD (15). With muscadine cultivars I have found a correlation between time from inoculation to symptom production in the greenhouse and field tolerance to PD. Field tolerance also correlates with PD severity in greenhouse inoculations.

In California, differences in natural tolerance of cultivars of *V. vinifera* to spread of PD appeared not to be influenced much by vector preference or by susceptibility to acute infection. The differences appeared to be mostly due to overwinter recovery rates of cultivars from acute infections (24). However, ELISA tests also indicated that in California the PD bacterium multiplies to higher titers in cultivars that are more susceptible to PD in the field than in tolerant ones (25). It appears that field tolerance may be governed by several factors.

Summary and concluding remarks. The bacterium causing Pierce's disease of grapevine, almond leaf scorch, and alfalfa dwarf has been cultured and used to fulfill Koch's postulates. The Gram-negative, xylem-limited bacteria associated with periwinkle wilt, phony disease of peach, plum leaf scald, and elm leaf scorch also have been isolated in axenic culture. These successes will facilitate the determination of the pathogenic and taxonomic relationships among this group of bacteria.

The tremendous variability in virulence of the PD isolates needs to be studied further in relationship to host specificity of the bacterium. For example, only the most virulent isolate may cause disease in muscadine cultivars, or survive in citrus trees. Since even the avirulent PD isolates infest grapevine tissue, protection of grapevines against virulent isolates by inoculating with avirulent ones could be studied. The relationship of bacterial concentrations

TABLE 2. Inoculation of Concord grape seedlings and rooted cutting with PD bacterium[a]

	Symptom development[b]			
Host	4 wk	6 wk	8 wk	10 wk
Rooted cuttings	6/10	10/10	10/10	10/10
Seedlings	0/10	0/10	5/10	10/10

[a] Cuttings were inoculated 1 mo after rooting and seedlings 2 mo after emergence. Both were inoculated by pin-pricking two lower internodes through a drop of inoculum containing 10^7 cells per milliliter.
[b] Numerator is number of plants with symptoms and the denominator is the total number of inoculated plants.

in the vascular system to symptom development indicates a role for physical blockage of xylem vessels as a mechanism of pathogenesis. However, more work needs to be done on the roles of xylem blockage and phytotoxin activity in Pierce's disease.

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