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Pierce's Disease of Grapevines: Isolation of the Causal Bacterium

Abstract. *A Gram-negative, rod-shaped bacterium has been consistently isolated from grapevines with Pierce's disease. Grapevines inoculated with the bacterium developed Pierce's disease, and the bacterium was reisolated from the plants. The bacterium was serologically and ultrastructurally indistinguishable from the one in naturally infected plants, and also indistinguishable from a bacterium isolated from almonds with almond leaf scorch disease.*

The etiological agent of Pierce's disease (PD), an important and often devastating disease of grapevines (*Vitis vinifera* L.) (*1*), is also considered to cause alfalfa dwarf (*2*) and almond leaf scorch diseases (*3*). Prior to 1971, PD was considered to be a viral disease (*4*), but chemotherapy, thermotherapy, and electron microscopy subsequently implicated the "rickettsia-like" organism seen in the xylem vessels of diseased grapevines as the etiological agent (*2, 5, 6*). Many investigators have reported failure to isolate the PD pathogen from diseased plants using artificial media (*2, 6, 7*). Similar insect-vectored bacteria have recently been associated with a number of other plant diseases, and although determination of their pathogenicity and taxonomic position has been delayed by the inability of investigators to culture these bacteria, they apparently constitute a new group of plant pathogenic bacteria (*8*).

Recently, a Gram-positive, catalase-negative bacterium that could be isolated from infectious leafhopper vectors but not from diseased plants was reported to be the etiological agent of PD (*7*). However, this bacterium did not infect healthy plants following direct inoculation, and contradictory evidence as to its causal role has been reported (*9*).

We now report the consistent culture of a Gram-negative, catalase-positive bacterium from grapevines with PD, and evidence that this bacterium causes PD.

A rod-shaped, Gram-negative bacterium was first isolated on our JD-1 medium (Table 1) from grapevines experimentally inoculated by the leafhopper vector, *Hordnia circellata* (Baker). Initially, the inoculum was collected by centrifugation of sap from surface-sterilized petioles; in later isolations, inoculum was obtained by expressing sap from petioles with forceps. The inoculum was blotted directly from the petioles onto the media. Colonies on the JD-1 medium appeared after 2 to 3 weeks of aerobic incubation at 28°C. Progressive refinements in the culture medium shortened the necessary incubation period. On the JD-3 medium (Table 1), small but distinct colonies are visible without magnification within 6 days. Colonies are circular with entire margins, white, smooth, and convex, and reach a diameter up to 1.0 mm within 2 weeks.

We have consistently isolated the PD bacterium from diseased grapevines. In one isolation experiment, single petioles from 195 rooted cuttings of eight European grapevine varieties (Pinot Noir, Mission, Ruby Cabernet, Flora, Cabernet Sauvignon, White Riesling, Barbera, and Thompson Seedless) were used. Pierce's disease had been transmitted with leafhopper vectors to 116 plants. Of the 79 remaining plants categorized as healthy, 52 had been subjected to feeding by leafhoppers but did not develop PD, and 27 were noninoculated controls. Positive isolations on the JD-2 medium (Table 1) of the PD bacterium determined on the basis of colony characteristics were obtained from 97.4 percent (111/116) of the diseased plants. Only one plant in each group of healthy plants, or a total of 2.5 percent (2/79), yielded bacteria with colonies resembling those of the PD bacterium. Other bacteria were rarely isolated from diseased or healthy plants.

The pathogenicity of the PD bacterium was tested by inoculating green stem cuttings of the grapevine varieties Pinot Noir, Mission, and Ruby Cabernet. The upper end of each two- or three-node cutting with leaves intact was attached to a vacuum pump, and 0.1 to 0.2 ml of a turbid suspension of the PD bacterium (approximately 5×10^8 bacteria per milliliter) in sterile tap water was drawn into each cutting. Controls consisted of noninoculated cuttings and cuttings inoculated with sterile tap water alone, or with suspensions of *Erwinia amylovora* (8×10^8 bacteria per milliliter). After inoculation, the cuttings were rooted on a heated bench under intermittent mist for 14 days and transplanted. Typical PD symptoms (*1*) developed in 86 percent (43/50) of the cuttings inoculated with the PD bacterium within 2 to 4 months, and many of these plants died within 5 months. All 47 of the control plants remained healthy throughout the study. Colonies characteristic of the PD bacterium were reisolated from 35 of 36 in-

oculated plants with symptoms, one of seven inoculated plants without symptoms, and none of 39 control plants. In subsequent inoculations, PD isolates that had been transferred weekly for at least 6 months remained pathogenic.

Mircetich *et al.* (*3*) have proposed that PD and almond leaf scorch disease (ALS) are caused by the same organism. We have transmitted the PD bacterium from diseased grapevines to five Mission almonds, using *H. circellata*. The PD bacterium was isolated from these almonds after they had developed symptoms of ALS. Subsequently, one of these isolates was inoculated by suction into 19 healthy grapevine cuttings. Typical PD symptoms developed in 16 of these rooted cuttings and the PD bacterium was reisolated from these plants. Bacterial isolates were obtained from almond trees naturally infected with ALS and from grapevines inoculated with ALS by leafhoppers. The ALS and PD isolates appear to have the same cultural characteristics, and in pathogenicity tests 19 of 19 grapevines inoculated with an ALS isolate developed PD.

The most distinctive features of the bacterium associated with PD, ALS, and alfalfa dwarf noted by other investigators (*2, 3, 6, 10*) are (i) confinement to the lumen of xylem vessels, (ii) rod-shape with a diameter of 0.25 to 0.50 μm and length of 1.0 to 4.0 μm, (iii) cell wall resembling that of Gram-negative bacteria, and (iv) outer portions of the cell wall corrugated similar to those of some rickettsial pathogens of vertebrates and invertebrates. We confirmed these ultrastructural features of the bacteria in suction-inoculated grapevines (Fig. 1A). Bacteria isolated from plants with PD and ALS and grown in revolving liquid cultures in the JD-3 medium without agar were also ultrastructurally similar to those in naturally infected plants (Fig. 1B). Pierce's disease bacteria from broth cultures were also negatively stained with 1 percent phosphotungstic acid and examined under the electron microscope. The diameter of the PD bacteria ranged from 0.25 to 0.50 μm and the length from 1.1 to 2.3 μm. The mean dimensions of 56 cells were 0.39 μm in diameter and 1.44 μm long. At no time were flagella or other indications of motility observed.

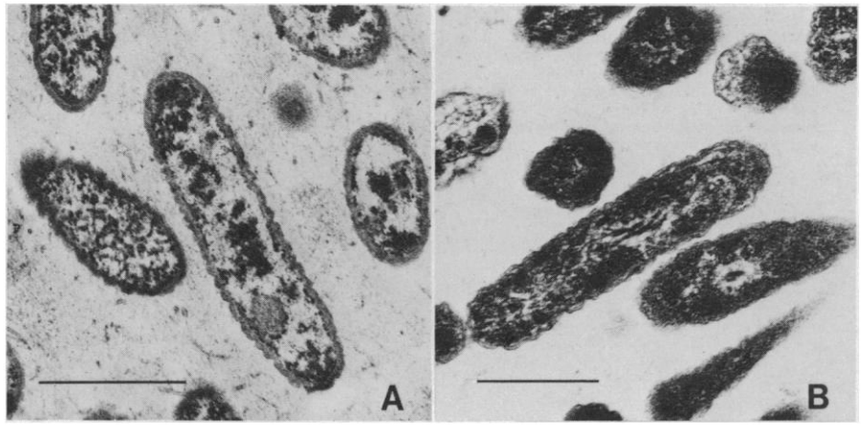

Fig. 1. Electron micrographs of thin sections of the Pierce's disease bacterium. (A) In the lumen of a xylem vessel of suction-inoculated grapevine. (B) In a pellet from 4-day liquid culture. Scale bars, 0.5 μm.

Table 1. Constituents (in grams per liter) of culture media for isolation and growth of the Pierce's disease bacterium.

Constituent*	Medium		
	JD-1	JD-2	JD-3
PPLO broth base (Difco)	10		
Pancreatic digest of casein		7.0	4.0
Papaic digest of soy meal		3.0	2.0
Trisodium citrate		2.0	2.0
Disodium succinate		2.0	0.01
Hemin chloride†	0.04	0.01	0.01
$MgSO_4 \cdot 7H_2O$		1.0	1.0
K_2HPO_4			1.5
KH_2PO_4			1.0
$FeCl_3 \cdot 6H_2O$		0.002	
$MnSO_4 \cdot 4H_2O$		0.002	
$(NH_4)_2SO_4$		1.0	
Bacto-agar (Difco)	15.0	15.0	15.0
Bovine serum albumin fraction V‡		3.0	2.0
Bovine albumin§	0.5		

*All constituents except those that were filter sterilized were dissolved in 890 ml of distilled water and then autoclaved at 121 and 1.27 kg/cm² for 20 minutes. The *p*H was adjusted after dissolving agar and before autoclaving with 1*N* NaOH or HCl. The *p*H adjustments were: JD-1 to 6.5, JD-2 to 6.8, and JD-3 to 7.0. †0.04 or 0.01 percent hemin chloride in 0.5*N* NaOH added at 10 ml per liter of medium. ‡BSA fraction V (Sigma) added to 100 ml of distilled water at *p*H 6.8 to 7.0, filter sterilized, and added to autoclaved medium at 45° to 50°C. §Bovine albumin added to 100 ml of distilled water, filter sterilized, and added to autoclaved medium.

Antiserums to the PD bacteria were produced in mice and rabbits. Petioles from Pinot Noir grapevines naturally infected with PD were collected and the PD bacterium was extracted from the xylem by centrifugation. The pelleted materials from each petiole were combined in 0.01*M* phosphate buffered saline, *p*H 7.2, to produce 1.0 ml of the suspension with a turbidity comparable to 10^9 bacteria per milliliter. Bacteria and debris in the extract were then pelleted by centrifugation and resuspended in 2 percent glutaraldehyde in phosphate buffered saline for 4 hours at 4°C followed by five washes in phosphate buffered saline. An extract was also collected from approximately the same number of petioles from healthy plants of the same variety and prepared in the same manner; however, a comparable turbidity was not obtained. Separate white mice were immunized by intraperitoneal injections with each of the extracts (*11*). New Zealand White rabbits were immunized against two PD bacterial isolates by intravenous injections (*12*). In addition, rabbits were immunized against an ALS isolate.

Three PD isolates from diseased grapevines in Napa County, California, and two isolates from diseased grapevines in Tulare County, California, were compared serologically with agglutination tests to determine dilution extinction titers (*13*). Mouse antiserums for the diseased grapevine extract had a titer of at least 1 : 512 with all of the PD isolates, while pooled normal mouse serums and the antiserum for the healthy plant extract failed to agglutinate any of the isolates. Both the rabbit antiserums for Napa and Tulare PD isolate agglutinated all five PD isolates having titers of at least 1 : 640 in every test. Again, normal rabbit serums did not agglutinate any of the PD isolates. Slide agglutination (*13*) with antiserums for both

PD and ALS isolates gave positive tests for 11 PD and five ALS isolates from different plants.

Previously noninfective (*9*) *H. circellata* placed for 2 days on symptomatic, suction-inoculated grapevines subsequently transmitted PD to healthy grapevine seedlings in five of five attempts. Transmission of the PD bacterium from cultures to grapevines via leafhopper vectors have been inconclusive. The mouthparts of 45 noninfective CO_2-anesthetized (*9*) *H. circellata* adults collected from natural populations were held in colonies of the PD bacterium until the insects regained mobility and were transferred to test plants. Only two leafhoppers treated in this manner transmitted the PD agent. In numerous other transmission attempts we injected suspensions of the PD bacterium into the body cavities of *H. circellata* as well as another leafhopper vector of PD, *Draeculacephala minerva* Ball, with fine glass needles or fed suspensions of the PD bacterium in sterile membrane sachets to both vector species (*9*). Neither of these methods resulted in PD transmission to grapevines.

The PD bacterium was also isolated from vector leafhoppers. Individual *H. circellata* were exposed to PD source plants for at least 2 days and subsequently transmitted PD to healthy grapevines. These leafhoppers were surface-sterilized, macerated, and then plated (*9*) on JD-2 medium. Of 21 isolation attempts, 11 yielded colonies resembling those of the PD bacterium. Seven of these isolates tested positively in slide agglutination tests (*13*) with antiserums to a cultured PD bacterium. No PD bacteria were isolated from *H. circellata* that failed to transmit PD to test plants.

We have cultured the "rickettsia-like" organism associated with PD by previous investigators (*2, 6, 10*), and we believe that this bacterium is the etiological agent of PD. The PD and ALS bacteria appear to be closely related if not identical.

MICHAEL J. DAVIS
ALEX H. PURCELL
SHERMAN V. THOMSON
Departments of Plant Pathology and *Entomological Sciences, University of California, Berkeley 94720*

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11. Young male white mice were injected intraperitoneally four times at weekly intervals with 0.25 ml of extract emulsified in an equal volume of Freund's incomplete adjuvant (Difco). The mice were bled 4 days after the final injection.
12. Six male rabbits weighing approximately 2 kg each were injected in pairs with one of three isolates. Two PD isolates, one each from Napa and Tulare counties, and one ALS isolate from Contra Costa County were grown in JD-2 medium without agar, pelleted by centrifugation, fixed with 2 percent glutaraldehyde, and washed in phosphate buffered saline. For intravenous injection, 10^9 bacteria per milliliter were suspended in physiological saline. Each rabbit was injected with increasing dosages of 0.5, 1.0, 2.0, 4.0, and 5.0 ml at 3-day intervals and bled 1 week after the final injection.
13. J. B. Kwapinski, *Methods of Serological Research* (Wiley, New York, 1965), pp. 133–137. Dilution extinction titers were determined in tube agglutination tests.
14. We thank A. H. Finlay for technical assistance in the insect transmission studies.

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Thyroid Gangliosides with High Affinity for Thyrotropin: Potential Role in Thyroid Regulation

Abstract. *Thyroid cell membranes contain a multiplicity of gangliosides, some of which inhibit thyrotropin binding to thyroid membranes. The most potent inhibitor is a ganglioside which is present in only trace amounts and appears to have a novel structure. Thyroid gangliosides may play a role in relaying the hormonal message to the thyroid cell.*

Gangliosides purified from brain inhibit the binding of thyrotropin (TSH) to thyroid membranes (*1, 2*). Since the thyroid gland itself contains a diversity of gangliosides, we postulated that gangliosides are involved in the interaction of TSH with thyroid membranes analogous to their role as receptors for cholera toxin (*3, 4*). In order to test this postulate, we examined the ability of gangliosides isolated from thyroid gland to inhibit the binding of TSH to thyroid membranes.

Fresh bovine thyroid glands (1.5 kg from about 75 adult animals) were homogenized in a stainless steel Waring blender with 10 liters of a mixture of chloroform and methanol (1 : 2, by volume). The homogenate was filtered, and the residue was extracted twice with 5 liters of the same solvent. A fourth extraction with 4 liters of buffered tetrahydrofuran (*5*) recovered little additional lipid-bound sialic acid (LBSA). The combined chloroform-methanol extracts were taken to dryness under reduced pressure and partitioned (*6*). The resulting lower phase was washed twice with theoretical upper phase; the combined upper phases were taken to dryness, dissolved in water, dialyzed, and lyophilized. The ganglioside mixture thus obtained contained 195 μmole of LBSA (*7*); analysis by thin-layer chromatography (*8*) revealed a complex pattern of gangliosides (Fig. 1A). Fractionation of the ganglioside mixture by column chromatography on diethylaminoethyl (DEAE)-cellulose (*9*) resulted in eight pooled fractions. When each of these pooled fractions was analyzed by thin-layer chromatography (Fig. 1B), at least 30 distinct resorcinol-positive bands could be visualized, that is, several minor ganglioside components present in the mixture become apparent only after enrichment by DEAE-cellulose chromatography. Each of these eight pooled fractions was further purified by silica gel column chromatography (*10*). This procedure resulted in 28 separate ganglioside fractions; some fractions contained a single pure ganglioside component when analyzed by thin-layer chromatography, others contained several ganglioside bands not further purified in this study. Several fractions discussed below are shown in the chromatogram in Fig. 2A.

Each of the 28 thyroid ganglioside fractions was tested for its ability to inhibit ^{125}I-labeled TSH binding to thyroid membranes. Binding was assayed by a filtration method with the use of cellulose acetate filters (Millipore, EHWP-02500) (*11*). ^{125}I-Labeled TSH and thyroid plasma membranes were bovine preparations (*11, 12*). In addition to the particular ganglioside fraction being tested, the incubation mixture contained (in 130 μl): 25 m*M* tris-acetate, *p*H 6.0, 0.5 percent bovine serum albumin, ^{125}I-labeled TSH, approximately 100,000 count/min (specific activity = 10.5 μc/μg, iodinated within 14 days of use, approximately 0.2 pmole), and 10 μg of membrane protein (*13*). Ganglioside fractions were first incubated with ^{125}I-labeled TSH for 30 minutes at 0°C prior to the addition of membranes. The percent inhibition of ^{125}I-labeled TSH binding was determined

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