Reinvestigation of the Role of a Lactobacillus Associated with Leafhopper Vectors of Pierce's Disease of Grapevines

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ABSTRACT

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Transmission of Pierce's disease to healthy grapevines occurred only after its leafhopper vectors were first allowed to feed on diseased vines. Noninfective adult males and nymphs of *Hordnia circellata* or *Draeculacephala minerva* that were injected with or given access feeding to several cultures of a *Lactobacillus* sp. isolated from *H. circellata* that had transmitted the Pierce's disease agent, did not transmit the disease to test plants. Infective and noninfective *H. circellata*, when surface-sterilized, triturated, and plated on medium 523 agar, produced isolates of this bacterium about equally. The bacterium was not recovered from *D. minerva* that had fed on grapevines with Pierce's disease and was recovered only infrequently from *D. minerva* that had fed on broth cultures of the bacteria. These results contradict previous reports that this bacterium is the causal agent of Pierce's disease.

Additional key words: alfalfa dwarf, lucerne dwarf, rickettsialike organisms.

Recent work with chemotherapy (8), electron microscopy (5, 7), and heat therapy (5) suggested a nonviral etiology for Pierce's disease (PD) of grapevines. Earlier work (6) had shown that the causal agent is transmitted by xylem-feeding leafhoppers after access to xylem tissues of affected plants for both acquisition and transmission (9).

Auger et al. (1, 2) recently reported isolating a rod-shaped, Gram-positive, acid-producing bacterium from *Draeculacephala minerva* Ball that had fed on diseased vines. When noninfective *D. minerva* were injected with cultures of this bacterium and placed on healthy test plants, these plants developed PD symptoms, and no symptoms were observed when infective leafhoppers were injected with sterile broth and placed on healthy plants (1). Auger (1) also reported that characteristic bacterial colonies developed on agar media streaked with the triturated bodies and excreta of leafhoppers that were allowed to feed on PD-affected vines but never in similar isolation attempts from leafhoppers not given access to diseased grapevines. Latorre et al. (11) recently characterized the bacterium and identified it as a *Lactobacillus* sp. nov.

The investigations that are presented in this paper were conducted separately at Berkeley and Davis and the results are corraborative. We report here our failure after repeated attempts to isolate the pathogenic bacterium described by Auger et al. (1, 2) from infective *D. minerva*. Attempts to transmit Pierce's disease using *Hordnia circellata* (Baker) that were injected with or fed on cultures of *Lactobacillus* isolated from inoculative leafhoppers also were unsuccessful.

MATERIALS AND METHODS

Draeculacephala minerva leafhoppers were collected near Sacramento, California in 1973 and colonized on barley (*Hordeum vulgare* 'Atlas') following the method of Freitag (4). *Hordnia circellata* was collected from native vegetation in Berkeley and the Napa Valley, California. Population samples of *D. minerva* used in these studies were tested periodically on grape plants for freedom from the PD causal agent and were always noninfectious. In addition, *H. circellata* exposed for 4 days or longer on test plants were presumed to be noninfectious if the test plants remained healthy for at least 6 mo. Test plants were rooted cuttings of *Vitis vinifera* 'Pinot Noir', 'Mission', or 'Carignane' from vineyards free of PD at the University of California, Davis campus. Leafhoppers were caged in the laboratory or greenhouse for acquisition feeding periods of 2 or more days on PD-infected grapevines for use in tests requiring infectious leafhoppers.

Isolations of bacteria were attempted from the titurated bodies of surface-sterilized leafhoppers (1, 2) on medium 523 (10). Gram-positive, catalase-negative rods that formed distinctive small, white convex colonies with entire margins on medium 523 were identical to the bacterium reported by Auger et al. (2). These were isolated and transferred to broth to test for acid production. Bacteria with these physical and cultural characteristics were designated as "HC-bacterium"; they appear to be members of the genus *Lactobacillus* (11, 12). These isolates were maintained on medium 523 or

lyophilized in skim milk or a mixture of medium 523 and lactose broth. At Berkeley, only HC-bacterium isolates from infective *H. circellata* were used in transmission studies.

Injections of insects with 36- to 48-hr-old medium 523 broth cultures of HC-isolates were made with glass needles connected to a low-pressure aspirator or micrometer syringe as described by Auger (1). Noninfective leafhoppers were allowed to feed on Parafilm M® (American Can Co., Greenwich, Connecticut) sachets (13) of medium 523 broth cultures for acquisition feedings of several hours to overnight. After injection, and in most cases after membrane feeding, treated leafhoppers were placed on healthy test plants for at least 2 wk except in a few cases as noted. Test plants were held in closed, forced-ventilation greenhouses for 6 mo for symptom expression.

At Berkeley, seven different HC-bacterium isolates were injected into a total of 12 adult male and 29 nymphal *D. minerva* and 73 adult male and 116 nymphal *H. circellata*. A total of five adult male and 31 nymphal *H. circellata* survived acquisition feeding on one of three different isolates and were placed on healthy grape test plants. At Davis, similar attempts also were made with 39 *D. minerva* and five *H. circellata* that survived injection or membrane feeding in eight different experiments using separate isolates of the HC-bacterium. Lyophilized cells of a bacterial culture identified by J. Auger as the causal agent of PD and deposited by him in the Department of Plant Pathology at Davis in 1974, were used in one experiment.

Direct inoculation of susceptible host plants with bacteria in broth media was attempted to induce alfalfa dwarf or Pierce's disease using five plants each of Hubam clover (*Melilotus alba*), and 'Carignane' and 'Thompson Seedless' grape plants by slitting the petioles half-way with a scalpel dipped into 12- to 24-hr broth cultures of HC-bacteria. Alfalfa (*Medicago falcata* also 'Caliverde 65') and Hubam clover seedlings with shoot terminals removed also were immersed in a water suspension of 3.5×10^8 cells/ml for 24 hr at 30 C and then planted in sterile potting mix. In a third series of tests, Mission grapevines, almond (*Prunus amygdalus* 'Texas') seedlings, Caliverde 65 alfalfa, and Hubam clover were inoculated by vacuum infiltrating an inoculum of broth cultures sedimented by centrifugation and resuspended in 0.05 M potassium phosphate buffer, pH 7.2, containing 0.85% NaCl and 0.1% Triton X-100 (Sigma), which improved inoculum infiltration into plant tissues without reducing cell viability. The plants were individually immersed in the inoculum suspension and vacuum applied to the entire plant for 10 min, released, and then reapplied. Three concentrations of HC-bacterium cells: 22, 44, and 220×10^6 cells/ml in 0.1 M K_2HPO_4 buffer, pH 7.2, and 1×10^8 cells/ml in buffer with 0.1% sucrose were infiltrated under vacuum into Mission grape (three to five nodes) and 'Texas' almond cuttings. The grape cuttings were placed into sterile sand flats for rooting and buds were taken from the almond cuttings and grafted onto peach rootstocks. Samples of the liquid exuded from the cut end of the woody plant cuttings were plated on medium 523 agar to confirm the presence of the HC-bacterium.

RESULTS

Only plants exposed to leafhoppers that had fed on PD-infected plants developed PD symptoms. All other attempted transmissions by needle injection, by membrane feeding of noninfective leafhoppers, or by mechanical inoculations with cultures of the same Gram-positive, rod-shaped, acid-producing, catalase-negative bacteria originally isolated from infective leafhoppers failed to produce PD symptoms in any test plant.

The bacteria (HC-bacteria) were reisolated from 14 of 26 nymphs and seven of 17 adults of *H. circellata* 4 days after acquisition feeding through parafilm. Recoveries were made from three of eight nymphs and one of five adult *D. minerva* after membrane feeding on one occasion and from each of 14 *D. minerva* after a second feeding trial. These were the only instances in which we isolated the HC-bacterium from *D. minerva*. Contrary to the report of Auger et al (2), none of 127 *D. minerva* fed on PD source plants for 24 hr nor 235 others collected

TABLE 1. Comparison of Pierce's disease (PD) transmission to grapevines to the isolation of HC-bacterium[a] from the leafhopper vector, *Hordnia circellata*

Transmission of PD by *H. circellata*:	Isolation of HC-bacterium from *H. circellata*: +[b]	−[b]	Total
Fed on PD-infected vines 2 days or more:			
Positive transmission[c]	40 (44.7)[d]	185 (180.3)	225
Negative transmission	13 (8.3)	29 (33.6)	42
Total	53	214	267
adj chi-square (with Yate's correction for continuity) = 3.07 (not significant at $P > .05$)			
Not fed on PD-infected vines, but some naturally infective:			
Positive transmission	6 (3.5)	3 (5.5)	9
Negative transmission	12 (14.6)	26 (23.4)	38
Total	18	29	47
adj chi-square = 3.10 (not significant at $P > .05$)			

[a]Gram-positive, catalase-negative, acid-producing bacteria forming characteristic colonies on Kado's medium 523.

[b]Symbols: + = isolation of HC-bacterium; − = failure to isolate HC-bacterium.

[c]Positive transmission = definite symptoms of Pierce's disease on test plants within 6 mo after inoculation access by tested leafhoppers.

[d]Figures in parentheses are expected numbers if the associations of + and − with "Positive" and "Negative" were random; e.g., 225/267 (fraction of leafhoppers which transmitted) × 53 (total number from which HC-bacterium isolated) = 44.7.

from Butte, Napa, and San Joaquin counties in California yielded HC-bacteria. No HC-bacteria were isolated from 21 *D. minerva* that had fed on PD-infected vines for 3 days and then on test plants for 4 days, including 12 such insects that had transmitted PD to the test plants.

As shown in the contingency table (Table 1) there was no significant association between the isolation of HC-bacteria from individual *H. circellata* and the likelihood that those individuals would transmit the PD agent. This applied both to groups of leafhoppers in which a high percentage (84%) of leafhoppers were infectious (Table 1) and to natural leafhopper populations in which the percentage of transmitting individuals was much lower (19%, Table 1). Of 71 *H. circellata* from Berkeley not exposed to PD source plants, 139 (50%) yielded the HC-bacterium.

A variety of different types of bacteria (mostly Gram-negative rods and cocci) and yeasts were cultivated on medium 523 from the titurated bodies of surface-sterilized *H. circellata;* many fewer types were cultivated from *D. minerva.* None of the isolated and unidentified bacteria cultures showed a consistent association with leafhoppers that transmitted PD. Certain bacterial types, however, were more consistently isolated from leafhoppers that had been caged together on particular plants.

DISCUSSION

Our results contradict the bacterial etiology of PD proposed by Auger (1) and Auger et al. (2) in three important respects: (i) we could not demonstrate the pathogenicity of any Gram-positive, catalase-negative, acid-producing, rod-shaped bacteria isolated on medium 523 from infective leafhoppers, even with the culture of Auger's original isolate; (ii) we were able to isolate *Lactobacillus* from noninfectious as well as from infectious *H. circellata;* and (iii) we never isolated the same *Lactobacillus* from *D. minerva* that had fed on PD-diseased source plants.

The histological and therapeutic evidence (5, 7, 8, 14) that a prokaryotic organism is the incitant of PD is suggestive but not final proof that it is the causal organism. Auger (1) and Auger et al. (2) could not isolate a bacterium directly from naturally diseased plants on artificial media and could not cause PD in test plants by direct inoculation (1, 2, 5, 7, 12). The failure to complete Koch's postulates would be understandable of a virus or other fastidious parasite because of the current lack of media on which such pathogens can be cultivated, but the HC-bacterium grows well on several media (1, 2, 12). With corn stunt (3) and citrus stubborn (15), injection of spiroplasma cultures into noninfectious leafhoppers is so far the only method of infecting plants with those agents from cultures, but the presumed causal agents of these two diseases can be cultured directly from diseased plants.

Differences in pathogenicity among various isolates or the attenuation of virulence in culture might be possible explanations to account for the failure to verify pathogenicity in any of the HC-isolates which we tested. However, such genetic differences would not seem to explain our failures to isolate any HC-bacteria from *D. minerva* following access feeding on PD-infected plants, the low percentage (20%) of infectious *H. circellata* from which HC-bacteria were isolated, or the frequent isolation of HC-bacteria from noninfective leafhoppers.

Isolates selected only on the basis of colony appearance on agar media and the morphology of Gram-stained bacteria by Latorre, including one original isolate of Auger's and two of Purcell's, were not serologically different in agar double-diffusion tests (13) and also showed a high degree of homogeneity in their utilization of carbohydrates, temperatures for optimum growth, and failure to induce hypersensitive reaction in tobacco. Latorre (12), however, showed significant basic differences in the cell wall structure between HC-bacteria grown in culture and the presumed causal organism of PD observed in thin sections of diseased tissues (5, 7, 14).

Our results are in direct contrast to the proposal of Auger et al. (2) that a Gram-positive, rod-shaped bacterium isolated from the excreta and crushed bodies of vector leafhoppers and cultivatable on Kado's medium 523, blood-dextrose agar, as well as other nutrient media is the causal agent of Pierce's disease. We cannot explain this wide discrepancy. The HC-bacterium is apparently intimately associated with *H. circellata,* but seems to have no direct role in inciting Pierce's disease. Our results suggest that the causal organism of PD is a fastidious parasite of xylem tissues morphologically resembling bacteria (5, 7, 14) other than the one described by Auger (1, 2).

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