Techniques

Isolation Media for the Pierce's Disease Bacterium

M. J. Davis, A. H. Purcell, and S. V. Thomson

Former graduate research assistant and assistant professors, respectively, Departments of Plant Pathology and Entomological Sciences (second author), University of California, Berkeley 94720. Present addresses of first and third authors: Department of Plant Pathology, Cook College, Rutgers University, New Brunswick, NJ 08903; and Department of Biology, Utah State University, Logan 84322, respectively.

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ABSTRACT

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Media supporting the first isolations of the xylem-limited bacterium causing Pierce's disease of grapevines and almond leaf scorch contained PPLO broth base, hemin chloride, bovine serum albumin or starch, and agar. Modifications of these media led to the formulation of the PD2 medium containing (in grams per liter): Tryptone (4.0), Soytone or Phytone (2.0), trisodium citrate (1.0), disodium succinate (1.0), hemin chloride (0.01), $MgSO_4 \cdot 7H_2O$ (1.0), KH_2PO_4 (1.0), K_2HPO_4 (1.5), Bacto-agar (15.0), and bovine serum albumin fraction five (2.0). Bacterial strains from grapevine and almond had a high viability on the PD2 medium as indicated by comparison of total cell counts to counts of colony forming units, and had a generation time averaging 9.2 hr at 29 C in PD2 broth medium. Ninety percent of the isolation attempts from naturally infected grapevines in vineyards in California and Florida were successful, and the strains were all agglutinated by antisera produced against a California Pierce's disease strain.

Additional key words: rickettsialike bacteria, alfalfa dwarf disease.

In 1973, a xylem-limited bacterium (XLB) was independently discovered in Florida (13) and California (10) in association with Pierce's disease (PD) of grapevines. Similar XLB subsequently were found associated with other plant diseases including almond leaf scorch disease (ALS) (20), alfalfa dwarf disease (10), phony peach disease (14,22), plum leaf scald (8,16), elm leaf scorch (25), and periwinkle wilt (19). Vector and graft transmission studies indicated that PD, ALS, and alfalfa dwarf are caused by the same agent (11,20), and a XLB, which was shown to cause PD and ALS, subsequently was isolated in pure culture from all three hosts (5–7,26). Koch's postulates have only been partially completed for alfalfa dwarf disease, since symptoms in inoculated alfalfa were not consistent with those reported for the disease (26). There are some similarities between the XLB and rickettsiae (10,12,13) but these similarities may be only superficial, since no actual taxonomic relationship has been established.

Pierce observed "bacteria-like bodies" in infected grapevine leaf tissue and attempted unsuccessfully to isolate the causal bacteria (23). Subsequent attempts by others to isolate the causal agent of PD using common bacteriological media also failed (9,12,13,18,20,24). Auger et al (1) reported the isolation of a Gram-positive bacterium, later characterized and named *Lactobacillus hordiniae* (18), from *Draeculacephala minerva* Ball leafhopper vectors of PD but not directly from diseased grapevines. They further reported that *D. minerva* injected with *L. hordiniae* transmitted PD to grapevines, but direct inoculation of grapevines with *L. hordiniae* was not reported. Subsequently, the etiological role of *L. hordiniae* in PD has been refuted (12,24).

This report describes our progress in developing a medium for the isolation and growth in pure culture of the causal organism of

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PD and ALS. Media developed in this study were designated PD1 and PD2 to avoid confusion with the JD2 and JD3 media (5). The formulations of these media are slightly different modifications of the JD2 and JD3 media which were unfortunately published with errors. We also describe here the use of the culture media developed in this study to confirm field diagnosis of PD.

MATERIALS AND METHODS

Source of inoculum. Rooted cultivar Mission, Ruby Cabernet, and Pinot Noir grapevine cuttings (*Vitis vinifera* L.) that had been inoculated by infective leafhopper vectors, *Grapholacephala atropunctata* (Signoret) (= *Hordnia circellata*) or *D. minerva,* were used as a source of inoculum for testing media. Petioles from leaves showing PD symptoms were surface sterilized in 1% sodium hypochlorite for 2.5 min and rinsed with four changes of sterile distilled water. Sap from 1.0–1.5 cm petiole sections was extracted by placing each section in a sterile tube and centrifuging it in an Eppendorf Microfuge Model 3200 at 8,000 *g* for 2.5 min. The extract from each petiole section was blotted from the end of the section onto a medium leaving four to six slight impressions in the agar to mark the spot of inoculation. At least one petiole section from 3–4 different leaves was used to inoculate each plate of medium. Inocula for testing modifications of the PD1 medium were obtained by removing growth from the agar with a spatula and suspending it in sterile tap water. Each plate of medium was spot-inoculated with 0.1 ml of a bacterial suspension ($A_{560} = 0.1$).

For isolation attempts from field-collected grapevine specimens in California, 1–2 canes from each plant were transported to the laboratory in plastic bags on ice, and isolations were attempted within 24 hr of collection. In Florida, isolations were made from naturally infected grapevine immediately after collection in the laboratory of D. L. Hopkins at the University of Florida, Institute of Food and Agricultural Sciences, Research Center at Leesburg, FL. In all isolations from field-collected specimens, the sap was expressed with forceps and blotted from the petiole section onto the medium. Approximately 3–4 petiole sections from each of three symptomatic leaves from a diseased plant or 3–4 older, mature leaves from each nonsymptomatic plant were sampled.

Cultural conditions and media evaluation. Solid media formulations were evaluated in 60 × 15 mm petri plates using approximately 15 ml of medium. Inoculated media were incubated aerobically at 28 C and examined periodically for colonies with a dissecting microscope (× 15). Media were modified to increase the number and rate of colony development and the most promising formulations were selected for reevaluation and comparison with subsequent modifications of the media. Several bacterial strains from California, usually one grape strain each from Napa and Tulare Counties and one almond strain from Contra Costa County were used to evaluate each medium modification. The almond strains were obtained from trees with ALS as described elsewhere (5,7). Media were ranked visually for ability to support growth from uniform inoculum.

TABLE 1. Effect of different supplements to the basal medium on isolation of the Pierce's disease bacterium from grapevines

	Media[b]							
Supplements[a]	JD1	A	B	C	D	E	F	G
Glucose		2	2					
Sucrose					5	5	5	
Starch			1	1	1			
Bovine serum albumin	0.5	0.5						
Hemin chloride	0.04	0.04	0.04	0.04	0.04	0.04		0.04
Colony formation[c]	+	+	−	+	+	−	−	−

[a] Glucose, sucrose, and starch were added to the basal medium before autoclaving. The basal medium was autoclaved after adjusting to pH 6.5. Bovine serum albumin was filter sterilized as a 0.5% aqueous solution and added to the autoclaved portion of the medium at 45–50 C. Hemin chloride was autoclaved separately at 0.04% in 0.05 N NaOH.

[b] The basal medium contained 10 g/L of PPLO broth base and either Bacto-agar at 15 g/L (JD1 medium and media A-D) or Noble agar at 20 g/L (Media E-G).

[c] Colonies were first observed on some media 2 wk after inoculation with xylem extracts from diseased plants. Presence (+) or absence (−) of colonies after 3 wk of aerobic incubation at 28 C.

Media. The C-3 spiroplasma medium (3) modified by the addition of 5 g/L Soytone, 5 g/L gum arabic, and 20 g/L Noble agar, by the omission of sucrose, and with the pH adjusted to either 5.0, 6.0, 6.5, or 7.0 was used in the initial attempts to isolate the Pierce's disease bacterium (PDB). Media formulations were developed using a basal medium containing a peptone or combination of peptones and supplementing these basal media with a variety of ingredients. The peptones included: Biosate (Baltimore Biological Laboratory [BBL], Division of Becton, Dickinson and Co., Cockeysville, MD 48232), Lactalysate (BBL), PPLO Broth Base (Difco Laboratories, Detroit, M176101), Proflo (Traders Oil Mill Co., Ft. Worth, TX 76101), Proteose Peptone No. 3 (Difco), Soytone (Difco) or Phytone (BBL), Thiotone (BBL), and Tryptone (Difco). Media were prepared by dissolving the peptone and autoclavable supplements in double distilled water at a volume so that after the addition of filter sterilized ingredients the desired final volume was obtained. The pH of the media was adjusted with 1N NaOH or HCl prior to sterilization. Heat labile ingredients were filter-sterilized by vacuum through a 0.22-μm Millipore membrane filter and added to the autoclaved protion of media at 45–50 C.

Bovine blood derivatives were evaluated as media supplements. Hemin chloride (Sigma Chemical Co., St. Louis, MO 63178 or Calbiochem, San Diego, CA 92112) was dissolved at 0.1% in 0.05 N NaOH and generally added to the media prior to autoclaving except in some experiments where it was autoclaved separately. Hemoglobin (bovine, A-grade; Calbiochem) at 2% in distilled water was autoclaved separately. Bovine serum albumin (A grade; Calbiochem) and bovine serum albumin fraction five (Sigma) were filter sterilized in 0.5–20.0% aqueous stock solutions.

Other supplements included Difco Supplement B which was added aseptically to the autoclaved medium at 1%, and grapevine xylem sap. The xylem sap was collected from freshly cut canes attached to vacuum flasks. Root pressure was adequate in the spring but later in the year partial vacuum was necessary to obtain significant quantities of sap. The sap was filter sterilized and stored frozen. Tergitol 7 (Sigma) and cycloheximide (Sigma) were incorporated into some media in an effort to prevent contamination. Bromthymol blue was used at 0.05 g/L in some media as a pH indicator.

Viability and generation time. The mean of eight replicate plate counts from suspensions in sterile tap water of a grapevine and an almond strain were compared with the mean of 40 hemacytometer counts for the same suspensions. Generation times were determined for grapevine and almond strains growing in 100 ml of PD2 broth in 250-ml sidearm flasks on a gyrotory shaker at 150 rpm at 29 C. The absorbance at 560 nm of the cultures was monitored every 6 or 12 hr, and the generation times were calculated using the doubling time of the abosrbance during log-phase growth.

Serological and pathogenicity tests. Agglutination tests using rabbit antiserum (5) produced against the VN1 Napa Valley PD strain was used to check the identity of different strains of the PDB and to compare the PDB with strains of other bacterial species. Strains of known bacterial species, grown on nutrient agar for 48 hr, and of the PDB grown on PD2 agar for 6 days were removed from the medium and suspended in sterile saline (0.85%), and 0.1 ml of the suspension ($A_{560} = 0.1$) was mixed with 0.1 ml antiserum which had been diluted 1:50 with saline. The agglutination tests were performed for 30 min at 25 C in microtiter plates. Standards within the tests included the homologous reaction using the VN1 strain and also substitution of the antiserum with normal serum or saline alone. The pathogenicity of some strains was tested in grapevines using the xylem infiltration technique (5).

RESULTS

Initial isolation of the PDB. Small, rod-shaped bacteria, presumably the PDB, were consistently observed with a phase-

contrast microscope (× 1,000) in sap extracted from the petioles of diseased grapevines. They characteristically appeared to be slightly curved with darkened ends and were seen either singly or in pairs free in the sap but more often larger numbers of these bacteria were observed as aggregates.

Initial attempts to isolate the PDB were made on the modified C-3 spiroplasma media. Visible colony formation was not evident on any of the media 3–4 wk after inoculation. However, thin films of bacteria became evident on modified C-3 media containing PPLO broth base, Soytone, gum arabic, and 2.5 or 5.0 g/L yeast extract. The number of PDB observed with a phase-contrast microscope in smears from the films when compared with the number of bacteria observed in samples of different extracts used for inoculum suggested that limited multiplication had occurred. All attempts to subculture these bacteria failed. Further examination of the agar medium revealed that PPLO broth base at 10 g/L alone supported the development of thin films of bacteria but not of discernible colonies.

Readily discernible colonies developed after 2–3 wk on a basal medium of 10 g/L PPLO broth base and combinations of 15 g/L Bacto-agar or 20 g/L Noble agar, supplemented with various combinations of 40 mg/L hemin chloride, 0.5 g/L bovine serum albumin, 1.0 g/L starch, and 5.0 g/L sucrose (Table 1). These colonies were approximately 0.5 mm in diameter and were closely spaced on the original inoculation site. The colonies coalesced, forming films that were larger, and thicker than the thin films from which bacteria were recovered in our initial experiments using only PPLO broth base. We failed to establish subcultures from 3-wk-old primary isolations by streaking the bacteria with an inoculation loop or blotting them with a sterile toothpick onto fresh media of the same kind.

Development of the PD1 and PD2 media. The JD1 medium (Table 1) was modified to support consistent primary isolations and continuous subculturing of the PDB. Growth of the PDB on the JD1 medium was improved by the addition of grapevine xylem sap (25%) or a combination of $(NH_4)_2SO_4$ (1 g/L), $MgSO_4 \cdot 7H_2O$ (1 g/L), $MnSO_4 \cdot 4H_2O$ (2 mg/L), $FeCl_3 \cdot 6H_2O$ (2 mg/L), and disodium succinate (1 g/L) as employed in the PSS medium (2) for *Spirillum volutans*. Effective substitutions in the JD1 medium included Difco Supplement B (1%) or hemoglobin (2 g/L) for hemin chloride, bovine serum albumin fraction five (2 g/L) for bovine serum albumin, and a combination of Tryptone (7 g/L) and Soytone (3 g/L) for PPLO broth base. The PD1 medium (Table 2) was formulated by a synthesis of results obtained while modifying the JD1 medium, and at pH 6.8 it was superior to previous media formulations both for primary isolations and subculturing of the PDB. Subsequent media modifications were evaluated using inocula obtained by subculture rather than primary isolations. Strains both from grapevines and almonds were used in subsequent media experiments with very similar results. Successful subcultures were first obtained by using heavy inoculum, thus slightly turbid suspensions of bacteria were used as inoculum for evaluation of modifications of the PD1 medium.

Trisodium citrate (1.0 g/L), but not sucrose (0.5-4.0 g/L) or glucose (0.5–4.0 g/L), effectively replaced succinate in the PD1 medium, and adding trisodium citrate to the PD1 medium enhanced growth of the PDB. Modifications of the PD1 medium containing individual peptones at 5, 10, and 15 g/L all supported growth, with Soytone at 5 g/L appearing best and supporting growth equal to that on the PD1 medium without modification. A medium base containing 1 g/L trisodium citrate, 1 g/L disodium succinate, 10 mg/L hemin chloride, 1 g/L $MgSO_4 \cdot 7H_2O$, 1 g/L KH_2PO_4, 1.5 g/L K_2HPO_4, 15 g/L Bacto-agar, and 2 g/L bovine serum albumin fraction five was used to examine the effects of combinations of Tryptone and Soytone at 0,1,2,4, and 8 g/L each on the growth of the PDB. The medium without Tryptone and Soytone supported very limited growth. All other combinations supported moderate to excellent growth of the PDB with a combination of Tryptone at 4 g/L and Soytone at 2 g/L (PD2 medium, Table 2) appearing to be best.

The addition of 50 mg/L cycloheximide to the PD2 medium was used to reduce fungal contamination without imparing growth of the PDB. Tergitol 7 at 0.1 ml/L in the PD2 medium was not toxic to the PDB, however, without bovine serum albumin fraction five in the PD2 medium the slow growth of the PDB which normally occurred was prevented by Tergitol 7. Tergitol 7 did not appear to substantially reduce bacterial contaminants in the PD2 medium.

Viability and generation times. Mean colony counts (1.5×10^9 colonies/ml) for the grapevine strain of the PDB were not significantly different from the hemacytometer counts (1.7×10^9 cells per milliliter) from the inoculum, while those counts (1.9×10^9 colonies per milliliter and 7.7×10^9 cells per milliliter) for an almond strain were significantly different ($P = 0.01$; Student's t-test). The generation time for the strains in liquid PD2 medium ranged from 7.07 hr to 11.21 hr and averaged 9.2 hr (Table 3).

Isolations from naturally infected grapevines. The PDB was isolated from 27 of 30 (90%) of the symptomatic grapevines from vineyards in California and Florida. Bacteria were successfully isolated from 14 known and three unknown cultivars of European bunch grapes with PD symptoms. Single attempts to isolate bacteria from 12 of 13 grapevines in the Napa Valley of California and from 10 of 12 grapevines in the San Joaquin Valley of California were successful. In California, PDB were isolated on 16 of 19 attempts using the PD1 medium and six of six attempts using

TABLE 2. Composition of the PD1 and PD2 agar culture media for isolation and growth of the Pierce's disease bacterium

Constituent[a]	Concentration (g/L)	
	PD1 medium	PD2 medium
Tryptone (Difco)	7.0	4.0
Soytone (Difco) or Phytone (BBL)	3.0	2.0
Trisodium citrate	···	1.0
Disodium succinate	1.0	1.0
Hemin chloride[b]	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
Bovine serum albumin fraction five[c]	2.0	2.0

[a] All constituents except bovine serum albumin fraction five were added to 980 ml of double distilled water prior to autoclaving at 121 C and 1.27 kg/cm^2 for 20 min. The pH's of the PD1 and PD2 media were adjusted to 6.8 and 7.0, respectively, after dissolving the agar.

[b] Hemin chloride at 0.1% in 0.05 N NaOH was added at 10 ml/L.

[c] Bovine serum albumin fraction five at 20% in double distilled water was filter-sterilized and added at 10 ml/L to the autoclaved portion of the medium at 45–50 C.

TABLE 3. Generation times of Pierce's disease and almond leaf scorch bacterial strains from California

Source of strain	Strain no.	Experiment no.	Generation[a] time (hr)
Grapevine, Napa County	1	1	11.21
	2	2	9.93
	3	1	10.61
Grapevine, Tulare County	1	1	8.05
		2	7.16
Almond, Contra Costa County	1	1	9.04
		2	7.07
		2	10.17
	2	1	8.31
	3	1	9.82
			Mean 9.20

[a] The generation time for each culture during log-phase growth was calculated from the regression equation: $y = mx + b$, where y = log absorbance at 560 nm and x = time in hours. The bacterial strains were cultured in broth PD2 at 29C.

the PD2 medium. All six isolation attempts on the PD2 medium in Florida were successful, and no differences were observed between any of the California and Florida strains in culture. The PDB was not isolated in more than 13 attempts in California from nonsymptomatic, field-collected grapevine specimens.

All of the strains from California and Florida agglutinated in the agglutination test using antisera for the VN1 Napa PD strain. Highly turbid suspensions of the PDB autoagglutinated and upon standing the clumps of bacteria settled out and a slightly turbid suspension remained. Serological agglutionation tests were most accurately performed when suspensions of low initial turbidity or the slightly turbid supernatant from an autoagglutinated suspension were used. Antisera for the PDB did not agglutinate *Erwinia amylovora, E. herbicola, E. carotovora* var. *carotovora, E. carotovora* var. *atroseptica, Pseudomonas phaseolicola, P. syringae, Xanthomonas campestris, Agrobacterium tumefaciens,* or a number of unknown bacterial contaminants of the PD1 and PD2 media, nor did any of these bacteria exhibit autoagglutination.

Three strains each from the Napa and San Joaquin Valleys of California and two Florida strains were tested for pathogenicity in grapevines. All of the strains were pathogenic, inciting typical PD symptoms 2–3 mo after inoculation.

DISCUSSION

The PDB causing PD and ALS was isolated for the first time on media developed in this study. The addition of hemin to the media resulted in the first successful growth of discrete colonies. Bovine serum albumin was also found to be very effective in improving growth. However, even through both ingredients enhance growth of the PDB, they were not essential; the PDB grew but poorly on the PD2 media without either hemin or bovine serum albumin. The effectiveness of hemin was most apparent when inoculating with a small number of bacteria. Similar observations for *Pasteurella pestis* suggested that rather than fulfilling an essential requirement for tetrapyroles as hemin (X factor) does for some *Haemophilus* spp. and other bacteria (2,17) hemin helped to protect the bacteria from peroxides in early stages of growth (17).

The role of bovine serum albumin may be that of a detoxifying agent (21). Without bovine serum albumin in the PD1 medium, which contained both an organic and inorganic nitrogen source, the PDB grew poorly. Also, the addition of 0.01% Tergitol 7 to the PD2 medium without bovine serum albumin completely inhibited growth, but with bovine serum albumin the growth of the PDB was unaffected. Hemin and bovine serum albumin were incorporated into the JD1 medium at the same concentrations as used to support optimal growth of *Rochalimaea quintana,* the etiological agent of trench fever belonging to the family Rickettsiaceae (2,21). The PD bacterium also resembles *R. quintana* in that both have a preference for succinate and citrate over glucose (15); are small, nonmotile, Gram-negative, rod-shaped bacteria; have arthropod vectors; and grow slowly in culture, forming colonies usually less than 1 mm in diameter (2,5,21,27).

The stimulatory effect of xylem sap may have been due to organic or inorganic compounds present in the sap (4,28). Xylem sap alone in agar media did not support growth of the PDB. Thus, it seems that the media formulated for the PDB contained substances for its in vitro growth which were not present, at least in sufficient quantities, in xylem sap. It was not determined whether sap would enhance growth on the PD1 and PD2 media.

Colony and hemacytometer counts of the grapevine and almond strains indicated that the PD2 medium was capable of supporting high viability of individual bacterial cells. The difference in colony and hemacytometer counts may partially reflect the propensity of these bacteria to autoagglutinate. The isolates often demonstrated a loss of the tendency to autoagglutinate in culture.

The media and techniques developed in this study were useful for confirming field diagnosis of PD based on symptomology. The PDB was readily isolated from diseased grapevines both in California and in Florida. Although the slow growth and colony morphology in addition to the general lack of contaminating bacteria were generally sufficient for recognizing the PDB in culture, the additional application of the serological agglutination test was useful for rapid verification of isolates. Pathogenicity tests may be used as an ultimate means of identifying an isolate, but should not be necessary for routine diagnosis.

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