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Microbiology of Wetwood: Importance of Pectin Degradation and *Clostridium* Species in Living Trees

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Wetwood samples from standing trees of eastern cottonwood (*Populus deltoides*), black poplar (*Populus nigra*), and American elm (*Ulmus americana*) contained high numbers of aerobic and anaerobic pectin-degrading bacteria (10^4 to 10^6 cells per g of wood). High activity of polygalacturonate lyase ($\leq$0.5 U/ml) was also detected in the fetid liquid that spurted from wetwood zones in the lower trunk when the trees were bored. A prevalent pectin-degrading obligately anaerobic bacterium isolated from these wetwoods was identified as *Clostridium butyricum*. Pectin decomposition by *C. butyricum* strain 4P1 was associated with an inducible polygalacturonate lyase and pectin methylesterase, the same types of pectinolytic activity expressed in the wetwood of these trees. The pH optimum of the extracellular polygalacturonate lyase was alkaline (near pH 8.5). In vitro tests with sapwood samples from a conifer (Douglas fir, *Pseudotsuga menziesii*) showed that tori in membranes of bordered pits are degraded by pure cultures of strain 4P1, polygalacturonate lyase enzyme preparations of strain 4P1, and mixed methanogenic cultures from the tree samples of wetwood. These results provide evidence that pectin in xylem tissue is actively degraded by *C. butyricum* strain 4P1 via polygalacturonate lyase activity. The importance of pectin degradation by bacteria, including *Clostridium* species, appears paramount in the formation and maintenance of the wetwood syndrome in certain living trees.

Pectin is a natural polymer common to the middle lamella and primary cell wall of plants. In general, pectin is composed of poly(1-4)-α-D-galactopyruanosiduronate and possesses a varying degree of methoxylation. The exact chemical nature of plant pectin is not known, and neutral sugars can be convalently bound to the polymer (9). Pectin is degraded by the action of several different types of enzymes that include demethoxylating (i.e., methylesterase) and depolymerizing (i.e., hydrolase and lyase) enzymes. The diversity and biochemical properties of pectinolytic enzymes were reviewed by Rexová-Benková and Markovic (21). The importance of pectinolytic activity in nature has been associated with the biological role pectin plays as a cementing substance in plants. Thus, microbial degradation of pectin leads to the weakening and maceration of plant tissues, which is of interest to such different processes as soft rot of harvested potatoes (16), industrial retting of flax and hemp (2), and invasion of symbiotic or pathogenic microorganisms into plant tissues (3).

Wetwood is an atypical condition of the heartwood of many living trees, including both hardwoods and conifers (32). The high moisture content of wetwood is often associated with a fetid liquid that emanates from increment core holes bored into certain tree species and with active microbial methanogenesis (37). The prevalent microbial population associated with wetwood in mature cottonwoods and elms was recently enumerated and generally characterized (23). The wetwood syndrome in these trees was part of an anaerobic microbial ecosystem composed mainly of fermentative *Clostridium*, *Bacteroides*, *Erwinia*, *Edwardsiella*, *Klebsiella*, and *Lactobacillus* species. Notably, these studies (23) demonstrated that the destruction of vessel to ray pit membranes in wetwood was associated with bacteria and that a prevalent bacterium isolated from the wetwoods of all of the examined trees was a pectinolytic *Clostridium* species.

The purpose of the present paper is to examine the importance of anaerobic pectin decomposition in wetwood. We report here on the pectinolytic activity associated with wetwood, the general and enzymatic characterizations of *C. butyricum* strain 4P1, and the influence of *Clostridium* species and pectinolytic enzymes on wood tissue.

MATERIALS AND METHODS

Location of study sites and sample collection, preparation, and analysis. Five standing trees were examined: one American elm, 79 (*Ulmus americana* L.); three eastern cottonwoods, 2, 4, and 19 (*Populus deltoides* Bartr. ex. Marsh.); and one black poplar, 72 (*Populus nigra* L.). The cottonwood and elm trees were located in Dane County, Wisconsin, and the black poplar was located in Wickrath, West Germany. The techniques used for sample collection and preparation and for the enumeration, isolation, and microscopic analysis of bacteria were as described previously (23). All experimental results presented were either duplicated or triplicated on separate occasions. All measurements were done at least twice in parallels of the same sample.

Enumeration and growth of pectinolytic bacteria. Pectin was autoclaved as a 10% solution in distilled water (pH 4.5) in sealed serum bottles. Before cooling, the bottles were gassed with oxygen-free nitrogen, and the pH was adjusted to 7.0 with sterile anaerobic NaOH. Only a relatively small amount (<15%) of the methyl esters was hydrolyzed by this procedure, whereas autoclaving at pH 7.0 hydrolyzed >60% of the ester bonds. The total methanol content of the pectin was 10.3% by mass and corresponded with a 60 to 65% degree of methoxylation. Polygalacturonic acid solutions were treated like pectin. Media employed for the growth and enumeration of pectinolytic microorganisms contained a low-phosphate-buffered (pH 7.2) minimal medium (36), a 0.5% final substrate concentration (added separately from the 10% stock solutions), 0.1% yeast extract, and 0.05% sodium sulfide for anaerobic cultures. Pectinolytic microorganisms were enumerated by the three-tube most-probable-number procedures (1). Pectinolytic activity was quantified by the detection of >2% conversion of ^{14}C-labeled pectin to $^{14}CO_2$. The use of a ^{14}C-most-probable-number test was a control to ensure that observed growth was not due to yeast extract consumption alone (14). Enumerations were performed in anaerobic culture tubes that contained 10 ml of the above medium with pectin as the substrate and 10 μl of a *U*-^{14}C-labeled pectin solution (1.15 mg/ml, 126 μCi/ml, 110 μCi/mg; ICN Pharmaceuticals Inc., Irvine, Calif., catalog no. 11 114, lot 892 172). $^{14}CO_2$ in the culture tube headspace was measured by the gas chromatograph-gas proportional counter technique described by Nelson and Zeikus (20). $^{14}CO_2$ in the culture tube headspace agreed well with increased amounts of $^{12}CO_2$ and an optical density at 660 nm of >0.3. Growth was quantified by direct insertion of the culture tubes into a Spectronic 20 colorimeter (Bausch & Lomb, Inc., Rochester, N.Y.).

Pectinolytic enzyme assays. Culture plate assays were used for the initial detection of pectin methylesterase (38), polygalacturonate hydrolase, and polygalacturonate lyase (15). For quantitative analysis, pectin methylesterase was assayed by measuring the pH change caused by the formation of free carboxylic groups (27), polygalacturonate hydrolase was assayed by the detection of reducing sugars formed in 50 mM potassium acetate buffer (pH 5.0) containing 1 mM $NaHSO_3$ (modified according to reference 18), and polygalacturonate lyase was assayed by a spectrophotometric assay (13) based on monitoring unsaturated products at 235 nm in a Gilford model 240 spectrophotometer. Enzyme assays were performed in the laboratory with fetid liquid samples obtained upon removal of the wood increment core from test trees. Polygalacturonic acid lyase was assayed by spectrometry (13), and pectin esterase and polygalacturonic acid hydrolase were determined by plate assays (15, 38) with 100 to 300 μl of fetid liquid. All assays were performed at 30°C; the plate assays were incubated for 48 h.

Pectin degradation in wood samples. Longitudinal cores (12 by 6 mm) of Rocky Mountain Douglas fir (*Pseudotsuga menziesii* var. *glauca* (Beissn.) Franco) green sapwood were used as samples. Wood samples were treated with enzyme preparations by infusion with a solution of polygalacturonate lyase (5.4 U/ml) in 100 mM tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 8.0)–2 mM $CaCl_2$. The polygalacturonate lyase was prepared by ammonium sulfate precipitation (90%) of the culture supernatant obtained from *C. butyricum* strain 4P1 grown on pectin medium. For long-term incubation with bacterial suspensions, wood samples were surface sterilized by immersion for 1 min in 1% sodium hypochlorite (Chlorox, Chlorox Co., Oakland, Calif.) and washed three times with sterile distilled water. The bacterial suspension consisted of either a late-exponential-phase *C. butyricum* strain 4P1 monoculture or a mixed methanogenic wetwood culture grown on pectin medium. Incubation was stopped by the addition of 10 volumes of ethanol. The treated samples were processed through three repeated ethanol dehydrations and were prepared for scanning electron microscopic analysis as previously described (22, 23, 33).

Chemicals. All chemicals were of reagent-grade quality and were obtained from Sigma Chemical Co., St. Louis, Mo., or from Mallinckrodt Inc., Paris, Ky. Agar, yeast extract, tryptone, and peptone were purchased from Difco Laboratories, Detroit, Mich. All gases were obtained from Matheson Gas, Joliet, Ill. Pectin was a gift of Sunkist Growers, Corona, Calif.

RESULTS

Pectinolytic activity of wetwoods. The fetid liquid of the poplars and elms contained a high activity of polygalacturonate lyase (i.e., pectate lyase or transeliminase) but no detectable polygalacturonate hydrolase (i.e., pectate hydrolase). The highest values observed for pectate lyase in total liquid samples from black poplar 72 and elm 79 were 0.5 and 0.4 U/ml, respectively. Pectate lyase activity in fetid liquids sampled from the three cottonwoods examined was ≤0.1 U/ml. Pectin methylesterase activity was also present in all fetid liquid samples analyzed by the plate assay method, but pectate hydrolase activity was not detected.

Pectinolytic microorganisms were enumerated in the wetwood samples and are compared with the total numbers of heterotrophic bacteria

in Table 1. In all trees examined, high populations of pectinolytic microbes were found in wetwood, and often in the same order of magnitude as the total number of heterotrophs. The same orders of magnitude of pectinolytic and total heterotrophic bacteria were obtained with three independent wetwood samplings from four different trees in the summer of 1975. The number of heterotrophs in these trees was also the same as that reported for wetwood samples obtained from 14 trees in 1975 and 1976 (23). The last positive dilution tube from each pectinolytic enumeration was examined by phase-contrast microscopy. End tubes of aerobic enumerations contained only moderately sized gram-negative straight rods. The anaerobic dilution end tubes always contained gram-positive and gram-negative short and fat rods and free oval spores. Identical *Clostridium* isolates were obtained from the last positive end tube of the anaerobic pectinolytic enumerations obtained from all five trees studied. In addition, pectinolytic clostridia were observed in enrichment cultures of wetwoods obtained from 10 other cottonwoods in Wisconsin and Wyoming. *C. butyricum* strain 4P1 was representative of all pectinolytic clostridia isolated from wetwoods. Eucaryotic microorganisms were never observed in the culture fluids of the pectinolytic enumeration tubes.

Taxonomic characterization of *C. butyricum* strain 4P1. The cellular properties of *C. butyricum* strain 4P1 varied slightly depending on the growth substrate. On glucose, cells were granular and 1.5 by 6 to 10 μm, and spores were not prominent, whereas on pectin, cells were slightly smaller, contained fewer refractile granules, and were more elliptical in shape, and oval spores (1.5 by 2 μm) were easily recognized in the early stationary phase.

TABLE 1. *Comparison of pectinolytic and heterotrophic microbial populations in representative wetwoods*

Tree	Population	Total no.[a]	
		Pectinolytic	Heterotrophic
P. deltoides 19	Anaerobes	3.0×10^6	3×10^6
P. deltoides 2	Aerobes	3×10^4	1.5×10^6
	Anaerobes	6×10^4	2×10^6
P. deltoides 4	Aerobes	5.6×10^5	5.6×10^6
	Anaerobes	2×10^5	2×10^6
U. americana 79	Aerobes	1.1×10^5	7×10^5
	Anaerobes	2.3×10^5	8×10^7

[a] Represents cells of wetwood tissue as determined by the three-tube most-probable-number analysis in TYEG medium (23).

C. butyricum strain 4P1 fermented glucose, lactose, sucrose, maltose, xylose, arabinose, cellobiose, mannose, raffinose, trehalose, galactose, salicin, glycerol, pectin, mannan, and starch. Mannitol, sorbitol, rhamnose, cellulose, xylan, and arabinogalactan were not energy sources for growth. Notably, galacturonic acid was not an energy source. Gelatin was not liquefied, but esculin was hydrolyzed. Catalase, oxidase, urease, and acetylene reduction, indole formation, and hydrogen sulfide production were not detected.

Fermentation products of *C. butyricum* strain 4P1 grown on glucose, pectin, or polygalacturonic acid are listed in Table 2. Butyrate, hydrogen, and acetate were prevalent products of glucose fermentation. However, acetate and hydrogen were prevalent products of pectin and polygalacturonic acid fermentation, and smaller amounts of butyrate and ethanol were formed. Isopropanol, butanol, propionate, and lactate were trace products, whereas methanol was a major reduced end product of pectin metabolism. Methanol was quantitatively produced during pectin fermentation (i.e., 1 mol of methanol per mol of methoxyl moiety).

Pectinolytic activity of *C. butyricum* strain 4P1. Figure 1 compares the growth and pectate lyase activity of *C. butyricum* strain 4P1 during fermentation of either glucose, polygalacturonic acid, or pectin. Notably, exponential growth on glucose (i.e., 2.1-h doubling time) was not significantly faster than growth on pectin or polygalacturonate (i.e., 1.7-h doubling time). The specific activity of pectate lyase correlated with growth on pectin or polygalacturonate, but the enzyme was not detected during growth on glucose. Pectate lyase was by and large an extracellular enzyme because equivalent amounts of activity were detected during growth in either cell suspensions or cell-free culture supernatants. In addition, during growth the culture contained extracellular pectin methylesterase activity at levels twice as high as pectate lyase activity.

The polygalacturonate lyase activity had an alkaline optimum near pH 8.5, and this is typical of pectate lyases which are not usually dependent on methylesterases to degrade pectin. Polygalacturonate hydrolase was not detected in the culture fluid or sonicated cell homogenates. This is in accord with the inability of *C. butyricum* strain 4P1 to ferment galacturonic acid, the terminal product of pectate hydrolase action.

Effect of pectinolytic activity on wood tissue. The occurrence of free pectate lyase and

TABLE 2. *Relationship between energy source and fermentation products of C. butyricum strain 4P1*[a]

Substrate	Fermentation product (total μmol/tube)								
	H_2	Methanol	Ethanol	Isopropanol	Acetate	Butanol	Propionate	Butyrate	Lactate
Glucose[b]	82	ND	23	7	75	2	ND	114	8
Pectin	150	160	2	16	462	ND	0.5	26	ND
Polygalacturonic acid	123	ND	2	7	463	0.5	1	16	ND

[a] Experiments were performed in anaerobic culture tubes that contained 10 ml of minimal medium, 0.1% yeast extract, and 0.5% substrate (i.e., 26 to 28 mM). Values reported represent the average of three separate experiments. Products were determined at the end of growth. CO_2 was an end product but was not quantified. ND, Not detectable.

[b] Values from reference 23.

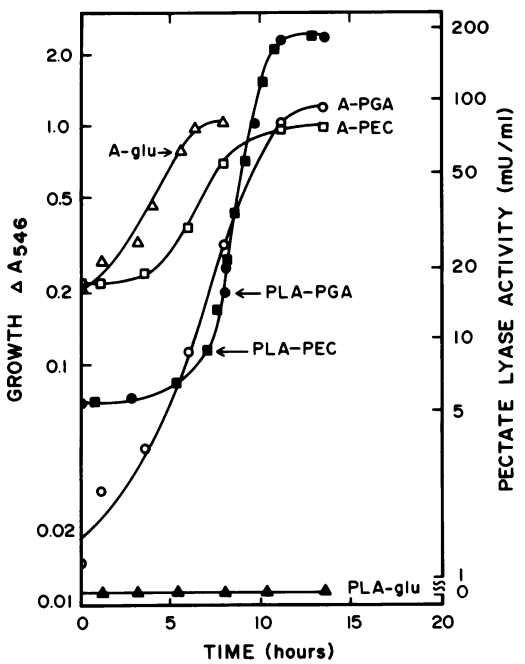

FIG. 1. *Relationship of pectinolytic activity to growth substrates of C. butyricum strain 4P1. Anaerobic culture tubes contained 10 ml of LPBB-YE medium (36) with 0.5% pectin or glucose and were incubated at 30°C after inoculation. Samples (100 μl) were removed by syringe for enzymatic analysis. Abbreviations: A, absorbance; glu, glucose; PGA, polygalacturonic acid; PEC, pectin; PLA, pectate lyase.*

pectin methylesterase activity in fetid liquid samples from different trees raised questions as to the effect of these enzymes on wood structure and function. Pectin in xylem tissue is contained in the middle lamellae, primary walls, and vessel to ray pit membranes. Green sapwood from Douglas fir, a conifer, was selected as the test wood because large amounts of pectin are concentrated in the central tori of bordered pit-pair membranes of conifers. Wood samples were incubated in short-term experiments (12 h) with *C. butyricum* strain 4P1 crude pectate lyase and in long-term studies (1 to 2 weeks) with *C. butyricum* strain 4P1 in either pure culture or in mixed methanogenic cultures obtained from enrichment of wetwood bacteria on pectin medium. The effect of wetwood pectinolytic activity on the ultrastructural features of tree tissue is shown in Fig. 2. A remarkable destruction of the amorphous material in the central torus was observed in samples incubated with mixed methanogenic wetwood cultures of pectinolytic clostridia (Fig. 2B) and with pure cultures of *C. butyricum* strain 4P1 (Fig. 2C). A similar destruction of the torus was observed with a 12-h treatment with crude pectate lyase concentrate (5.4 U/ml) from pure cultures of *C. butyricum* strain 4P1 (Fig. 2D).

DISCUSSION

Pectin decomposition appears as the most dynamic metabolic activity expressed by the microbial population associated with the wetwood syndrome in mature hardwood trees. This statement is supported by the following lines of evidence: high activity of pectate lyase expressed in fetid liquid, high numbers of aerobic and anaerobic pectinolytic bacteria detected in wetwood, production of pectate lyase and methylesterase by a prevalent obligately anaerobic bacterium isolated from wetwood, and transformation of intact sapwood vessel to ray pit membranes by wetwood bacteria or enzymes into tissues that are ultrastructurally characteristic of wetwood (22, 23). Thus, these data provide the first evidence to support the suggestions that microbial pectin decomposition is of importance to wetwood formation in living trees (4, 23, 29, 32). Microbial degradation of the structural polymers (i.e., cellulose and lignin) is not manifested in the wetwood syndrome (4, 23, 29), probably due to the encrustment of cellulose by lignin and the recalcitrance of lignin to anaerobic decom-

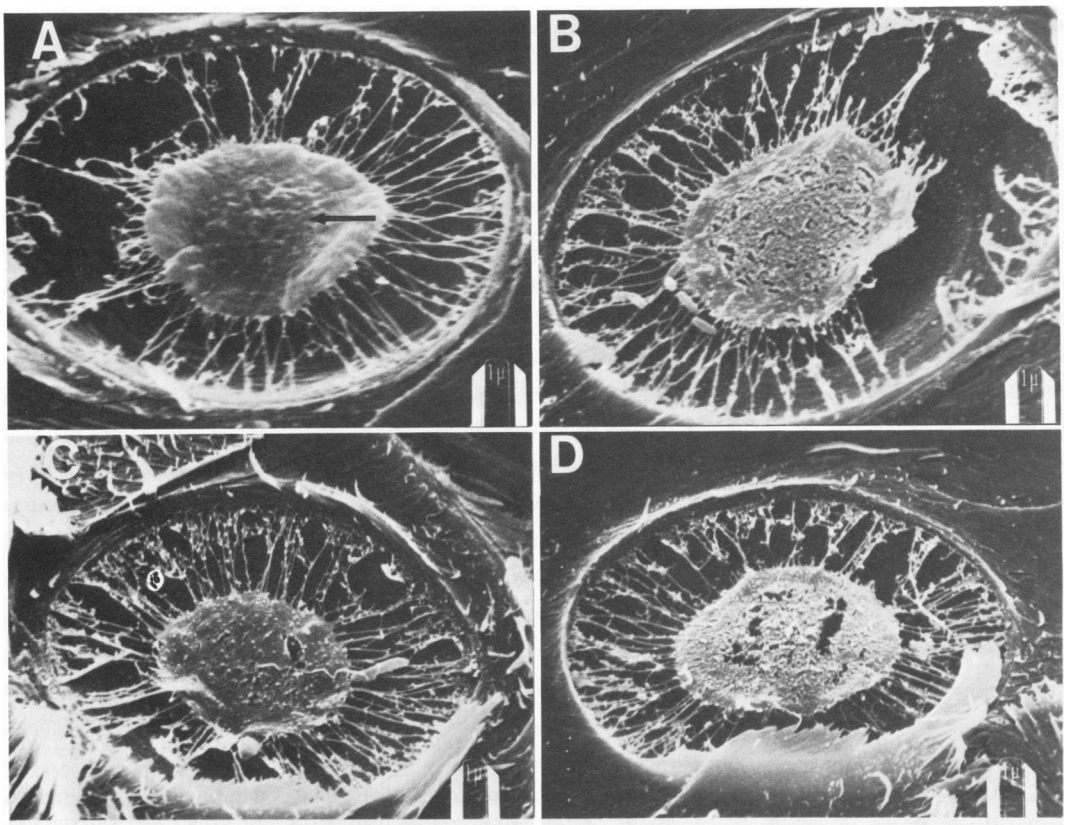

FIG. 2. *Degradation of bordered pit membranes from sapwood of P. menziesii var. glauca by pure and mixed cultures of C. butyricum strain 4P1 and by free polygalacturonic acid lyase from strain 4P1. (A) Untreated sample. The surface of the central torus appears amorphous. The arrow points to the torus. (B) After 2 weeks of incubation with a mixed pectinolytic and methanogenic culture containing C. butyricum strain 4P1 in LPBB-YE medium (36) which was obtained from inoculation of wetwood into pectin medium. (C) After 1 week of incubation with a pure culture of C. butyricum strain 4P1 in LPBB-YE medium. (D) After 12 h of incubation with 5 ml of an enriched polygalacturonic acid lyase preparation from strain 4P1 in 100 mM tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 8.0, containing 2 mM $CaCl_2$. All treated samples, B, C, and D, show tori that no longer appear amorphous.*

position (34). It should be noted that xylan- and cellulose-degrading bacteria were not detected in wetwood enrichment cultures (23).

The observed destruction (i.e., voids in the tori) of wood tissue after treatment with clostridial pectinolytic activity is in accord with previous studies which demonstrated torus erosion in coniferous sapwood after treatment with commercial pectinase (6, 30). It is not possible to obtain intact heartwood from poplar and elm species. Also, heartwood does not respond well to treatment with pectinolytic enzymes because additional lignification and deposition of heartwood extractives provide protective coatings to the pectin-rich torus (6, 12, 22). Tschernitz and Sachs (30) suggest that the observed pattern of voids in degraded tissue strongly implies an initial perforation of the torus by pectinases in and about the plasmodesmata. The intact cellulose microfibrils observed in the margo of sapwood treated with clostridial pectinolytic activity, which included a mixed culture, support the absence of detectable cellulolytic activity (23). In contrast, both the torus and the margo of Douglas fir sapwood were degraded after treatment with *Bacillus polymyxa*, which is frequently isolated from pond-stored logs (12).

The pectinolytic activity described here correlates with the known physical and chemical attributes of wetwood and suggests, but does not prove, a role for bacteria in wetwood formation. Wetwood in the hardwood tree species examined is distinguished in part from normal heartwood by decomposed vessel to ray pit membranes (22, 23), abnormally high moisture content (32), and a tendency to crack during drying (32). The

tendency to crack during drying has also been observed in wetwood of oaks from which *Clostridium* species were isolated (31, 33). Apparently, microbial pectate lyase activity transforms intact vessel to ray pit membranes of sapwood into degraded tissue that appears devoid of normal water translocatory function; hence, this process could lead to water soaking of heartwood and possibly the unusually rapid outward extensions of wetwood into the sapwood as described by Bauch et al. (5) for trees under environmental stress. Microbial destruction of wood pit membranes via log ponding is a well-known industrial process for the enhancement of sapwood permeability to preservatives (11, 17, 25). However, clear evidence for the involvement of microbial pectin decomposition in wetwood formation has not been reported; this is also a novel suggestion for the role of pectinolytic bacteria in a disease syndrome. The more common attribute of pectinolytic microorganisms in plant diseases is destruction of the middle lamella (3). This feature of pectinolytic activity also appears important to wetwood and may explain the tendency of wetwood to develop shake and frost cracks in trees (31, 32) and checking defects in drying lumber (31, 32, 33).

The prevalent obligately anaerobic pectin-decomposing bacterium isolated from wetwood is assigned to the species *C. butyricum* on the basis of similar morphological and physiological characters described for this species in the 8th edition of *Bergey's Manual of Determinative Bacteriology* (8). On the basis of cellular and metabolic features described here and previously (23, 24), *C. butyricum* strain 4P1 clearly differs from other recognized species (7, 8). In agreement with *C. butyricum* strains and other group I clostridia (10), strain 4P1 can grow in defined medium. Also, *C. butyricum* strain 4P1 appears distinct from both taxonomically recognized pectinolytic clostridia (8) and unassigned pectinolytic *Clostridium* species that have recently been shown to be important agents of soft rot in potatoes (16; A. Kelman, personal communication). The pectinolytic *Clostridium* strains examined previously can produce pectin methylesterase and either pectate lyase or pectate hydrolase (15, 19, 26). It is of interest to note here that more microbiological studies are needed to document the role of anaerobic bacteria in plant disorders, and at present this remains as a virgin area for research.

Definition of the exact roles for the prevalent pectinolytic *Clostridium* strains in the wetwood syndrome awaits more conclusive evidence. Nonetheless, it is worth discussing how the metabolic features of this prevalent wetwood bacterium may interact in this anaerobic ecosystem. The pH of wetwood in the tree species examined is usually neutral to alkaline, whereas the pH of normal heartwood is slightly acidic (32). The wetwood syndrome appears as a progressive disorder and starts in the base and spreads upward through the trunk and into the stem during tree maturation (5, 29, 31, 32). Undoubtedly, there are other species of prevalent pectinolytic bacteria in trees (e.g., the facultatively anaerobic species present in the end tubes for enumeration of aerobic pectin decomposers). The nearly similar number of *C. butyricum* and total wetwood heterotrophs, and the ability of the pectate lyase of this species to function best at alkaline but also at slightly acidic values, however, suggests a possible role for *C. butyricum* in the maintenance and transmission of the wetwood syndrome inside maturing hardwood trees. It should also be noted that the pectinolytic enzymes of *C. butyricum* appear largely extracellular, whereas major portions of the pectinolytic activity of gram-negative bacteria such as *Erwinia* species (28) are cell bound and thus not free to diffuse through the wood tissue. In addition, greater competitive value would be afforded to fermentative species such as *C. butyricum* than to prevalent facultatively anaerobic bacteria isolated from wetwood (23) that do not contain hydrogenase (i.e., *Edwardsiella*, *Klebsiella*, *Erwinia*, and *Lactobacillus* species). Namely, many fermentative bacteria are able to alter their catabolism when grown in association with H_2-consuming methanogenic bacteria, and this leads to increased H_2 formation in lieu of other reduced products (i.e., lactate, ethanol, and butyrate) and increased adenosine 5'-triphosphate gain (35). In this regard, when *C. butyricum* is grown on pectin in coculture with *Methanosarcina barkeri*, pectin is completely decomposed, and H_2, CO_2, methanol, and acetate are simultaneously metabolized to CH_4 (B. Schink and J. G. Zeikus, submitted for publication). Although methanol and acetate are transformed to methane by wetwood mixed cultures, we have not been successful at isolation of the causative methanogenic organism(s).

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