Bacteria Associated with Discolored and Decayed Tissues in Beech, Birch, and Maple

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ABSTRACT

Bacteria isolated from discolored and decayed tissues in a total of 27 beech, birch, and maple trees from New Hampshire were identified and characterized. Species of *Bacillus* and *Pseudomonas* were the most common among the 326 isolates examined. Members of the family Enterobacteriaceae, yellow-pigmented gram-negative rods, coryneforms, and yeasts accounted for the remainder. Phytopathology 60:1547-1551.

Additional key words: Hardwoods, defects, microorganisms, taxonomy.

Discoloration and decay are the major internal defects that lower the value of timber. Although the role of Hymenomycetes in the terminal stages of decay has been emphasized (1, 8, 21, 23), there is much evidence (9, 15, 20, 22, 24) which indicates that a succession of organisms is actually involved; e.g., bacteria and nonhymenomycetous fungi are considered to be the initial invaders in discoloration and decay of beech, birch, and maple. By various physical and biochemical actions, microorganisms apparently predispose the wood to eventual decay. While the role of nonhymenomycetous fungi has received some attention, the specific action of bacteria has been studied hardly at all. Evans & Halvorson (6) proposed that leucoanthocyanins of the wood condense in the presence of microbial enzymes to form soluble polymers. This functions as a brown stain precursor which migrates to the wood surface during drying, and oxidizes to produce a dark brown polymer. Pigmentation by several species of *Bacillus, Pseudomonas,* and other genera either singly or in combination may contribute also to discoloration, since soluble pigments are produced under certain cultural and environmental conditions.

This paper gives some information on the taxonomy of bacteria associated with discolored and decayed tissues in several species of trees, characterizes some of the principal bacteria, and considers some of their ecological aspects.

MATERIALS AND METHODS.—*Isolation of bacteria.*—Wood billets ca. 30 × 12 × 12 cm containing discolored, decayed, and healthy wood were cut from 27 trees; red maple *Acer rubrum* L., sugar maple *A. saccharum* Marsh., paper birch *Betula papyrifera* Marsh., yellow birch *B. alleghaniensis* Britt., and American beech *Fagus grandifolia* Ehrh. The trees, ranging from 15 to 40 cm diam at 1.4 m aboveground, were in the White Mountain National Forest, New Hampshire. Billets were split longitudinally with a sterile ax in the laboratory, and the freshly exposed surfaces were flamed lightly. Small chips of wood, ca. 0.3 × 1 cm, were removed with a sterile gouge. The chips were placed in an agar medium consisting of 10 g malt extract and 2 g yeast extract/liter of distilled water (23). Cultures were incubated at 25 C with subsequent subcultures in Difco-Bacto nutrient broth restreaked to establish purity. All 326 isolates were subjected to morphological, cultural, and physiological examination as specified by the Committee on Bacteriological Technic (3) unless otherwise stated.

Identification of gram-positive cultures.—All gram-positive rods growing aerobically and having endospores were placed in the genus *Bacillus*. Speciation was according to Smith et al. (26). Formation of vacuolated cytoplasm on 1% glucose agar was a criterion for separating *B. megaterium* and *B. cereus* from other species; cells were stained 20 sec with 10% aqueous basic fuchsin. Because some proteolytic bacilli produce an alkaline reaction in fermentation media containing an organic nitrogen source, Dowson's basal medium (4) plus 0.5% glucose, lactose, or mannitol was used for fermentation studies. Salt tolerance was examined using Bacto nutrient broth containing 7% NaCl. *Bacillus* isolates were streaked on egg-yolk agar and observed for opaque zones surrounding colonies.

Identification of gram-negative cultures.—All isolates were grouped according to Shewan et al. (19). The presence of cytochrome oxidase was determined by the Kovacs (14) test. Glucose metabolism was observed in the medium of Hugh & Leifson (11). Hydrogen sulfide production was detected in Bacto-SIM medium. Phenylalanine agar (7) was used for separating *Proteus* and *Providence* groups from other members of the Enterobacteriaceae.

Pseudomonas.—Because most of the pseudomonads had fluorescein pigments, identification followed Rhodes (17). All media were inoculated with one

loopful of broth (YE) containing 0.3% Bacto-Yeast Extract, 1% Bacto-Peptone, and 0.5% NaCl. Cultures were incubated at 25 C for 5 days and examined after 1, 2, 3, and 5 days. Bacto-Pseudomonas F and -Pseudomonas P media were used to observe pigment formation. The medium of Hugh & Leifson (11) with glucose separated oxidative and fermentative types. Antibiotic sensitivity was determined using discs containing 2.5 IU of potassium Penicillin G (Squibb), 10 μg Terramycin (Pfizer), and Erythromycin (Lilly).

Growth of all isolates was studied at 5, 12, 22, 30, 37, and 42 C in 5 ml of YE broth, using water baths. The effect of pH on growth was assessed by comparing visual turbidity in YE broth adjusted to pH 4, 4.5, 5.5, 6, 7, 7.5, and 8.0. Salt tolerance was determined by inoculating YE broth containing 3.5 and 6.5% NaCl.

The basal medium of Koser (13) was used to study the utilization of organic acids. In addition, the following carbon sources were added to Dowson's basal medium (4): D-glucose, D-galactose, D-fructose, D-arabinose, L-arabinose, D-xylose, L-rhamnose, sucrose, maltose, lactose, trehalose, cellobiose, dextrin, raffinose, cellulose, inulin, salicin, sorbitol, mannitol, dulcitol, inositol, ethanol, glycerol, phenol, cresol, and naphthalene.

Replacing citric acid in Koser's medium (13) with 0.5% gluconic acid and neutralizing allowed us to detect the formation of 2-keto-D-gluconic acid after addition of Benedict's reagent and heating (17). Urease activity was noted in Bacto-urea broth. Ammonia production was determined by adding Nessler reagent to 7-day 1% Bacto-peptone broth cultures after recording pH values. Pseudomonad cultures were spotted on an egg-yolk medium (16) and observed for opaque zones surrounding colonies. Stab inoculations were made in a pectate medium (28) and examined for growth and liquefaction, using *Erwinia carotovora* as a control. Lipolysis was examined in the medium of Sierra (25) using Tween 40, 60, and 80 (polyoxyethylene sorbitan monopalmitate, -stearate, and -oleate, respectively; Atlas Powder Co., Wilmington, Del.) as substrates.

RESULTS.—Discolored and decayed tissues associated with all ages and types of wounds yielded bacteria. Bacteria were associated with nonhymenomycetous fungi from discolored wood surrounding the decay columns. Bacteria were cultured infrequently from nondiscolored tissues contiguous to discolored tissues as well as from nondiscolored wood in trees without discoloration.

The majority of bacteria recovered were members of the genus *Bacillus,* constituting 42% (138 isolates). Pseudomonads (33%; 108 isolates) and yellow-pigmented, gram-negative rods (7%; 23 isolates) were also isolated. Seven cultures were either unidentifiable gram-positive rods or pink-pigmented yeasts, presumably *Rhodotorula* sp. Table 1 shows the percentage and number of major bacterial isolates from one group of trees examined. In general, little host specificity was seen, since nearly identical generic distribution of bacteria was noted in all species of trees examined.

Gram-positive cultures.—Bacillus isolates were motile, with peritrichous flagella and produced endospores. The genus was represented by two species, *Bacillus subtilis* and *B. cereus*. The former was most abundant and was characterized by vegetative cells $<0.9\ \mu$ in diam, lack of vacuolated cytoplasm when grown on glucose agar, and no lecithinase activity. Biochemical characterizations are given in Table 2. Neither organism produced indole or hydrolyzed urea, grew anaerobically in glucose broth, or produced acid from lactose. *Bacillus cereus* isolates had cells $>0.9\ \mu$ in diam that were highly vacuolated on glucose agar. In addition, all cultures grew in salt broth and produced acid from mannitol. Asporogenous rods, identified tentatively as coryneforms, and several pink yeasts constituted other gram-positive isolates.

Gram-negative cultures.—The family Enterobacteriaceae was represented by gram-negative rods which were fermentative with or without gas production in O-F Medium. All reactions are shown in Table 3. None of the isolates was oxidase- or methyl red-positive, produced indole or H_2S, or hydrolyzed urea.

Six isolates were identified as *Aerobacter aerogenes* which exhibited typical IMViC reactions. Twelve isolates were placed tentatively in the paracolon group because lactose was not fermented. Twenty-three cultures resembled *Xanthomonas*. They showed a yellow water-insoluble pigment, a single polar flagellum, oxidase-positive reaction, hydrolyzed casein and gelatin, and produced acid from glucose, lactose, and su-

TABLE 1. Number and percentage of 222 wood chips that yielded bacteria from discolored tissues in one collection of samples from 27 beech, birch, and maple trees

Bacteria	No. trees sampled							
	18 Red and Sugar Maple		3 Beech		3 Yellow Birch		3 Paper Birch	
	Chips that yielded bacteria							
	no.	%	*no.*	%	*no.*	%	*no.*	%
Bacillus subtilis	36	25	7	32	4	11	0	0
B. cereus	30	21	4	18	15	32	9	50
Pseudomonas spp.	44	30	7	32	17	36	3	17
Enterobacteriaceae	26	18	3	14	7	15	5	30
Yellow-pigmented forms	9	6	1	5	3	6	1	6

TABLE 2. Biochemical characteristics of *Bacillus* species isolated from beech, birch, and maple

Biochemical character	No. of positive cultures	
	B. subtilis (75 isolates)	*B. cereus* (63 isolates)
Vacuolation	0	63
Growth in 7% NaCl broth	75	0
Gelatin liquefaction	75	63
Starch hydrolysis	75	63
Nitrate reduction	75	63
Acetyl methyl carbinol production	75	0
Citrate utilization	75	63
Casein hydrolysis	75	63
Lecithinase production	0	63
Acid from:		
Glucose	75	63
Mannitol	75	0

crose. None of these reduced nitrate, produced indole or H_2S, or hydrolyzed starch or pectin. Eighteen isolates were unidentified.

Pseudomonas.—Individual isolates showed minor variation in pigmentation, colonial surface, and opacity. All pseudomonads isolated in this study were gram-negative, asporogenous rods ranging in size from 1 to 2.0 μ with polar flagellation. Fluorescein pigments were produced by all but three isolates which elaborated a brown water-soluble pigment. All cultures were resistant to penicillin, Terramycin, and Erythromycin as noted by Shewan et al. (19). A summary of biochemical reactions is given in Table 4.

Growth occurred in broth containing 3 and 6.5% NaCl. Only the fluorescent pseudomonads produced 2-keto-D-gluconic acid. Haynes (10) considered the ability to form reducing substances from gluconate, a useful characteristic in the identification of pseudomonads. After 7 days in peptone broth, pH values had increased to 8.2-8.4 from the initial value of 7.1. Ammonia was produced in all cases. While growth was evident in pectate medium, no hydrolysis occurred.

According to Rhodes (17), the green fluorescent isolates are members of *Pseudomonas*. The speciation of these and of the three brown-pigmented pseudomonads is uncertain.

DISCUSSION.—Although fungi and bacteria have been isolated frequently from discolored and decayed wood in a number of tree species (1, 5, 15, 20, 23), few attempts were made to identify the bacteria. Sheneman & Costilow (18) identified several hundred bacteria from tapholes made for the collection of sap for syrup in maple trees, and reported that over two-thirds of the isolates were members of the genera *Pseudomonas, Achromobacter,* or *Flavobacterium*. They (27) also found species of *Micrococcus, Bacillus, Sarcina,* and

TABLE 3. Biochemical characteristics of Enterobacteriaceae isolated from beech, birch, and maple

Biochemical character	No. of positive cultures		
	Aerobacter aerogenes (6 isolates)	Paracolon group (12 isolates)	Unidentified (32 isolates)
Citrate utilization	6	12	32
Acetyl methyl carbinol production	6	12	0
Acid and gas in:			
Glucose (OF)	6	12	32
Glucose (Durham tube)	6	12	32
Lactose (Durham tube)	6	0	0

TABLE 4. Biochemical characteristics of pseudomonads isolated from beech, birch, and maple

Biochemical character	No. of positive cultures	
	Green fluorescent forms (105 isolates)	Brown pigmented forms (3 isolates)
Indole production	0	0
Starch hydrolysis	105	3
Gelatin liquefaction	105	3
Methyl red test	0	0
Citrate utilization	105	3
Urease activity	0	0
Ammonia production	105	3
H_2S production	0	0
Gluconate production	105	0
Pectinase activity	0	0
Lipase activity	105	3
Growth at 5-42 C	105	3
Growth in 3-6.5% NaCl broth	105	3
Growth at pH 4.5-8.0	105	3
Carbon source:		
Na succinate	105	3
Malic acid	105	3
Lactic acid	105	3
Na acetate	105	3
Tartaric acid	105	3
Na formate	105	3
Oxalic acid	0	0
Glycerol	105	3
Phenol	0	0
Naphthalene	105	3
Cresol	0	0
Citric acid	105	3
Ethyl alcohol	105	3
Galactose	105	3
Glucose	105	3
Rhamnose	105	3
Arabinose	105	3
Fructose	105	3
Xylose	105	3
Sucrose	105	3
Maltose	0	0
Lactose	0	0
Trehalose	105	3
Cellobiose	105	3
Dextrin	0	0
Raffinose	0	0
Cellulose	0	0
Inulin	0	0
Salicin	0	0
Sorbitol	105	3
Mannitol	105	3
Dulcitol	0	0
Inositol	105	3
Acid from:		
Galactose	105	3
Glucose	105	3
Rhamnose	105	3
Arabinose	105	3
Inositol	105	3
Fructose	105	3
Xylose	105	3

Chromobacterium as well as asporogenous, gram-positive rods. In addition, yeasts were isolated commonly with *Rhodotorula* species among the dominant cultures. Several of these groups of microorganisms were recovered in the present study. Others did not appear, and some of our isolates were not noted by Sheneman & Costilow (18).

There is reason to believe that all the bacteria we isolated are usual members of a plant-soil ecosystem. A variety of species of *Bacillus, Pseudomonas,* coryneforms, and members of the Enterobacteriaceae are found commonly in forest soils as well as on or within a variety of plants. It is not difficult to speculate on mode of entry by these bacteria, as well as fungi, in view of the inevitable wounding that occurs in trees from time to time; e.g., fires, storms, animals, insects, birds, or man.

Species of *Bacillus* are isolated commonly from soil and are characterized as saprophytes that grow over a wide temp range. They are both fermentative and proteolytic, utilizing carbohydrates, alcohols, organic acids, and protein. Knight & Proom (12) showed that *B. subtilis* was capable of growth with ammonium salts as a sole source of nitrogen, while *B. cereus* required several amino acids. Pseudomonads are well recognized as common soil and water inhibitants. They are able to hydrolyze a wide variety of complex organic compounds and can grow in very simple media containing inorganic salts and a carbon source. Rhodes (17) found that conspicuous colony differences rarely correlated with other diagnostic criteria. However, Williamson (29) reported that colonial variants of *P. aeruginosa* showed differences in mouse virulence and carbohydrate utilization. Members of the Enterobacteriaceae represent microorganisms associated frequently with warm-blooded animals, insects, and plants. They are prevalent in forest soils. The yellow-pigmented gram-negative rods and nonpigmented asporogenous rods, tentatively considered as xanthomonads and coryneforms, respectively, are recovered routinely from plant and/or soil materials.

The pH of healthy sapwood in the trees studied is approximately 4.5 (20) and thus suited ideally for fungi. Discolored wood has pH values as high as 8 (20, 21), which would allow development of bacteria and nonhymenomycetous fungi. This high value may be a result of proteolytic activity of *Bacillus* or *Pseudomonas* species. In addition, the characteristic odor of freshly felled diseased trees may be attributed often to microbial action.

The association of bacteria and nonhymenomycetes may be mutualistic; i.e., bacterial metabolism of glucose which has accumulated from fungal hydrolysis of cellulose. Fungi produce organic acids (citric, lactic, and others) (2) which are readily utilizable by bacteria. Conversely, some pseudomonads and members of the genus *Bacillus* produce thiamine, which is essential for many species of decay fungi (2).

LITERATURE CITED

1. Basham, J. T., & L. D. Taylor. 1965. The occurrence of fungi and bacteria in normal and discolored heartwood on second-growth sugar maple in Ontario. Plant Dis. Reptr. 49:771-774.
2. Cochrane, V. W. 1958. Physiology of fungi. John Wiley & Sons, N.Y. 524 p.
3. Committee on Bacteriological Technic. 1957. Manual of microbiological methods. McGraw-Hill Co., N.Y. p. 140-167.
4. Dowson, W. J. 1957. Plant diseases due to bacteria, p. 43 [2nd ed.]. The University Press, Cambridge.
5. Etheridge, D. E., & L. A. Morin. 1967. The microbiological condition of wood of living balsam fir and black spruce in Quebec. Can. J. Bot. 45:1003-1010.
6. Evans, R. S., & H. N. Halvorson. 1962. Cause and control of brown stain in western hemlock. Forest Products J. 12:367-373.
7. Ewing, W. H., B. R. David, & R. W. Reavis. 1957. Phenylalanine and malonate medium and their use in enteric bacteriology. Public Health Lab. 15:153.
8. Good, H. M., J. T. Basham, & S. D. Kadzielawa. 1968. Respiratory activity of fungal associations in zones of heart rot and stain in sugar maple. Can. J. Bot. 46:27-36.
9. Good, H. M., & J. I. Nelson. 1962. Fungi associated with *Fomes igniarius* var. *populinus* in living poplar trees and their probable significance in decay. Can. J. Bot. 40:615-624.
10. Haynes, W. C. 1951. *Pseudomonas aeruginosa*—its characterization and identification. J. Gen. Microbiol. 5:939-950.
11. Hugh, R., & E. Leifson. 1953. The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various gram negative bacteria. J. Bacteriol. 66:24-26.
12. Knight, B. C. J. G., & H. Proom. 1950. A comparative survey of the nutrition and physiology of mesophilic species in the genus *Bacillus*. J. Gen. Microbiol. 4:508-538.
13. Koser, S. A. 1923. Utilization of the salts of organic acids by the colon-aerogenes group. J. Bacteriol. 8:493-520.
14. Kovacs, N. 1956. Identification of *Pseudomonas pyocyanea* by the oxidase reaction. Nature 178:703.
15. Maloy, O. C., & V. S. Robinson. 1968. Microorganisms associated with heart rot in young grand fir. Can. J. Bot. 46:306-309.
16. McGaughey, C. A., & H. P. Chu. 1948. The egg-yolk reaction of aerobic sporing bacilli. J. Gen. Microbiol. 2:334-340.
17. Rhodes, M. E. 1959. The characterization of *Pseudomonas fluorescens*. J. Gen. Microbiol. 21:221-263.
18. Sheneman, J. M., & R. N. Costilow. 1958. Identification of microorganisms from maple tree tapholes. Food Res. 24:146-151.
19. Shewan, J. M., W. Hodgkiss, & J. Liston. 1954. A method for the rapid differentiation of certain non-pathogenic asporogenous bacilli. Nature 173:208-209.
20. Shigo, A. L. 1963. Fungi associated with the discolorations around rot columns caused by *Fomes igniarius*. Plant Dis. Reptr. 47:820-823.
21. Shigo, A. L. 1965. The pattern of decays and discolorations in northern hardwoods. Phytopathology 55:648-652.
22. Shigo, A. L. 1967. Successions of organisms in discoloration and decay of wood, p. 237-299. Int. Rev. Forest Res. II. Academic Press, N.Y.
23. Shigo, A. L., & E. M. Sharon. 1968. Discoloration and decay in hardwoods following inoculations with Hymenomycetes. Phytopathology 58:1493-1498.
24. Siegle, H. 1967. Microbiological and biochemical aspects of heartwood stain in *Betula papyrifera* Marsh. Can. J. Bot. 45:147-154.
25. Sierra, G. 1957. A simple method for the detection

of lipolytic activity of microorganisms and some observations on the influence of the contact between cells and fatty substrates. Ant. van Leeuwen. J. Microbiol. Serol. 23:15-22.
26. SMITH, N. R., R. E. GORDON, & F. F. CLARK. 1952. Aerobic sporeforming bacteria. USDA Monogr. No. 16. 148 p.
27. SNEATH, P. H. A. 1956. Cultural and biochemical characteristics of the genus *Chromobacterium*. J. Gen. Microbiol. 15:70-98.
28. STARR, M. P. 1947. The causal agent of bacterial root and stem disease of guayule. Phytopathology 37: 291-300.
29. WILLIAMSON, C. K. 1956. Morphological and physiological considerations of colonial variants of *Pseudomonas aeruginosa*. J. Bacteriol. 71:617-622.