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Bacteria in the Wood of Living Aspen, Pine, and Alder²

Introduction

The investigations on the microflora in the wood of living trees have up to now dealt almost entirely with the microorganisms associated with diseased trees. However, D. M. Knutson and E. Sucoff (in press) in 1968 reported large numbers of bacteria in the aseptically expressed sap of trembling aspen, *Populus tremuloides*, and Etheridge (1961) observed bacteria in the branches and stems of aspen. Basham and Taylor (1965) found fungi and bacteria in normal and discolored heartwood of second growth sugar maple in Ontario. Bacterial attack and deterioration of pine logs in pond storage had been described by Ellwood and Ecklund (1959) and Knuth and McCoy (1962). Lutman and Wheeler (1948) and Samisa and Etinger-Tulczyuska (1963) reported on the distribution of bacteria in tissues of healthy potatoes and tomatoes.

The present investigation consisted primarily of a study of the bacteria occurring in non-distressed wood of living trees, *Populus tremuloides* Michx., *Pinus ponderosa* Dougl. ex. Lawson, and *Alnus tenuifolia* Nutt. Aspen was chosen for the project because some research had already been done on the microflora contained in its wood. The other tree species were chosen because of their availability and occurrence in the same habitat with aspen.

The investigators were especially concerned with determining what species of bacteria occur in wood; the known habitats or sources of original isolation of these species as recorded in the literature might be a key to the discovery of the processes by which wood bacteria are introduced into trees.

The present project was regarded as a preliminary work with many possible informative and useful ramifications. Therefore, the investigators were also interested as to whether the species of bacteria that were isolated belong to genera known or suspected to include plant pathogens or decomposers of materials from dead or dying trees. Since Knutson and Sucoff reported that isolates of *Bacillus* and *Erwinia* from wood grew on Ashby's nitrogen-free medium the isolates in the present study were tested on the same kind of medium.

Methods and Materials

Location and description of the collection area. Wood samples were collected from an area located 2.9 miles from Cheney, Spokane County, Washington. The altitude

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here is approximately 2300 ft. (700 mi). The average yearly temperature is about 48°F (9°C) and the average monthly rainfall is 1.54 in. (3.9 cm). The collection area is mostly of basaltic scabrock formation. It is moderately forested with ponderosa pine, and has occasional stands of aspen and alder associated with a small eutrophic pond.

Field collection techniques. Cylinders of wood were secured from the trunks of living trees in the field by removing a portion of bark and cambium, approximately six mm square, and quickly inserting an increment borer. The bark and cambium were removed to decrease the possibility of contamination from the air and exposed surfaces. The hole in the tree from which the sample was taken was stoppered with a piece of fitted wood which had been presterilized in a 10 percent hypochlorite solution. The site of cylinder removal was approximately 1.2 m from the ground, a distance chosen empirically. Upon removal, the sample cylinder was transferred immediately to a sterile petri dish, which was placed in a polyethylene bag to lessen changes in moisture content, and stored at 5°C until assayed. The diameter of the sample boring was four mm and the average diameter of the trees sampled was approximately 15 cm. The length of the sample cylinder represented the radius of the tree sampled, from the cambium to the pith cylinder. Only samples containing apparently nondiseased wood were selected for testing.

Laboratory procedures. The work room where all grinding and transfers were carried out was first steamed and the working area was thoroughly washed with a 10 percent hypochlorite solution. The sample boring was surface-sterilized by washing in a solution of 10 percent hypochlorite for four minutes. This specific amount of time was selected through experiments with wooden cylinders coated with cultures of *Bacillus* sp. and manured soil. After sterilization, the boring was given three, two-minute rinses in presterilized water contained in petri dishes. A control to check the efficiency of the sterilization procedure was attained by pouring sterile melted double-strength trypticase soy agar (BBL) into the rinse water plates and incubating this agar-rinse water mixture.

With sterile forceps, the sample boring was then transferred to a sterile Waring Commercial Blender (Eberbach Corporation) with 150 ml of sterile water and ground for four minutes at low speed to attain the degree of fragmentation considered necessary for practical optimum yield of microbial colonies. Petri dishes with approximately 20 ml of agar were inoculated with 0.5 ml of fragmented wood suspension. The inoculum was positioned near the edge of the plate with a sterile pipet and then streaked with a sterile inoculating loop to obtain isolated bacterial colonies.

Three types of agar plates were used for the preliminary isolation of organisms. Eosin methylene blue agar, EMB agar (BBL), was used for the isolation and differentiation of gram-negative bacilli; trypticase soy agar, TSA (BBL), was used as a general-purpose medium for the growth of most aerobic bacteria; malt agar (Difco) was used for the isolation of yeasts and mycelial fungi. Kligler's iron agar (Difco) was used in an attempt to isolate hydrogen sulfide-forming anaerobic bacteria. Tubes of this medium were inoculated with fragmented wood suspension, and incubated in a GASPAC anaerobic jar (BBL) at 30°C. Duplicate culture plates from each sample were incubated, one at 30°C and one at 14°C. The 14°C incubation temperature was employed to promote growth of psychrophilic bacteria.

After 72 hours of incubation, plates were observed to determine the number and types of colonies present. The cultures were examined at regular intervals after 72 hours to check for new colony growth. Plates were not discarded until after at least seven days of incubation. Colonies were observed for difference in color, size, texture, and gram-staining properties of the cells. The colonies were examined with the aid of a Quebec colony counter although no definitive quantitative study was attempted here. An inoculum from each type of bacterial colony on a given plate was transferred to a tube of TSA; fungi were transferred to Sabouraud's dextrose agar (Difco). Isolates were maintained at room temperature.

All of the bacterial isolates were tested for the following properties using the media listed: Fermentation with production of acid or acid and gas of glucose, maltose, lactose, sucrose and mannitol: phenol red broth (Difco). Gelatin digestion: nutrient gelatin (Difco). Citrate utilization: Simmon's citrate medium (Difco). Urease production: urea broth (Difco). Ammonia production: peptone broth (Difco). Motility: motility medium (BBL). Indol production: tryptone broth (Difco). Hydrogen sulfide production: Kligler's iron agar (BBL). Acetyl methyl carbinol production: methyl-red-Voges-Proskauer medium (Difco). Determinations of the bacterial isolates were made by referring to *Bergey's Manual of Determinative Bacteriology*.

Tests were also conducted on the isolates for starch digestion using 1 percent soluble starch in nutrient agar (Difco); pectin digestion using a pectate medium described by Hungate and Fletcher (1961); cellulose utilization using a variant of a medium described by Hungate (1957); and for growth on Ashby's nitrogen-free medium.

Results

Microorganisms isolated. The bacterial species isolated were identified as follows: *Achromobacter parvulus* (Conn, 1922) Breed, 1953, five isolates; *Arthrobacter urea-faciens* (Krebs and Eggleston, 1939) Clark, 1955, three isolates; *Brevibacterium ammoniagenes* (Cooke and Keith, 1927) Breed, 1953, five isolates; *Brevibacterium insectiphilium* (Steinhaus, 1941) Breed, 1953, four isolates; *Brevibacterium linens* (Weigmann, 1910) Breed, 1953, seven isolates; *Cellulomonas acidula* (Kellerman *et al.*, 1913) Bergey *et al.*, 1923, one isolate; *Corynebacterium humiferum*, Seliskar, 1952, six isolates; *Erwinia amylovora* (Burrill, 1882) Winslow *et al.*, 1920; four isolates; *Erwinia salicis* (Day, 1924) Chester, 1939, one isolate.

Sources of the isolates. The numbers and sources of wood samples positive for each of these species are shown in Table 1. Although no definitive study for the quantitative distribution of bacteria was carried out here, the wood of aspen appeared to contain more bacteria than that of the pine or alder. All nine of the identified species of bacteria were found to occur in aspen.

Isolation temperatures. Of the two incubation temperatures used, 30°C and 14°C, that of 30°C was the better for obtaining good growth, and no species were isolated by culturing at 14°C which were not also obtained under mesophilic conditions. No bacterial isolations were obtained through the anaerobic culture procedures.

Tests with gelatin, pectate, cellulose, nitrogen-free and starch media. Table 2 shows the results of the tests for gelatin digestion, pectin digestion, cellulose utilization, and growth on a nitrogen-free medium. All of the isolates tested negative for starch hydrolysis.

TABLE 1. Sources of bacterial isolates. Numbers of samples from each tree species which were positive for bacteria.

Bacterial isolates	Aspen ^a	Pine ^b	Alder ^c
<i>Achromobacter parvulus</i>	2	3	1
<i>Arthrobacter ureafaciens</i>	3		
<i>Brevibacterium ammoniagenes</i>	3		2
<i>B. insectiphilium</i>	4		
<i>B. linens</i>	1	5	1
<i>Cellulomonas acidula</i>	1		
<i>Corynebacterium humiferum</i>	4		2
<i>Erwinia amylovora</i>	1	3	
<i>E. salicis</i>	1		

^a Number of samples—52.

^b Number of samples—41.

^c Number of samples—29.

TABLE 2. Results of testing bacterial isolates from wood with nutrient gelatin, pectate, cellulose, and nitrogen-free media.

Bacterial isolates	No. of strains tested	Gelatin digestion	Growth on Ashby's nitrogen-free medium	Cellulose utilization	Pectin digestion
<i>Achromobacter parvulus</i>	5	+	+	-	-
<i>Arthrobacter ureafaciens</i>	3	+	-	-	+
<i>Brevibacterium ammoniagenes</i>	5	-	+	-	-
<i>Brevibacterium insectiphilium</i>	4	+	-	-	-
<i>Brevibacterium linens</i>	7	+	-	-	+
<i>Cellulomonas acidula</i>	1	+	+	+	+
<i>Corynebacterium humiferum</i>	6	-	+	-	+
<i>Erwinia amylovora</i>	4	+	+	-	-
<i>Erwinia salicis</i>	1	-	+	-	+

Fungi isolated. Mycelial fungi were isolated from all three tree species. The wood samples tested for fungi were identical with those tested for bacteria. Fourteen isolates were made including four different types of fungi.

A phycmycete, probably in the order Mucorales, was isolated from all three tree species. It was found to be a pectin digester, but had no effect on cellulose. An ascomycete resembling the genus *Penicillium* was isolated from aspen and alder. In addition to these, two unidentified types of fungi with white mycelia were isolated from aspen.

Two yeast isolates were made from aspen. Colonies of both were white to cream in color, both had small oval cells which reproduced by budding. Neither of these isolates was identified further.

Discussion

As it has been established that bacteria may be present in the wood of living trees, one of the first questions to arise involves their mode of entry. The original sites of isolation and/or habitats of the bacterial species isolated in the present study had been reported as follows: *Achromobacter parvulus*—isolated from manure and found generally in the soil (Breed *et al.*, 1957). *Arthrobacter ureafaciens*—widely distributed in nature. Isolated from frozen foods (Komagata and Ogawa, 1966). *Brevibacterium ammoniagenes*—isolated from feces of infants and known to be associated with diaper rash of infants. Widely distributed in nature and found extensively in putrefying material (Breed *et al.*, 1957). *B. insectiphilium*—isolated from the body wall of bagworm, *Thyridopteryx ephemaeriformis* Haw. Habitat unknown (Breed *et al.*, 1957). *B. linens*—found in dairy products, in various grains, silage, green plants, water pools, manure, soil and air (Breed *et al.*, 1957). Has been isolated from frozen foods (Komagata and Ogawa, 1966). *Cellulomonas acidula*—isolated from soil in Utah (Breed *et al.*, 1957). *Corynebacterium humiferum*—previously isolated from *Populus tremuloides* and reported to be pathogenic to the Lombardy poplar, *Populus nigra* var. *italica* (Breed *et al.*, 1957). *Erwinia amylovora*, isolated from blossoms and leaves of pear and apple trees. Reported to attack a large number of species in several tribes of the family Rosaceae (Elliot, 1951). *E. salicis*, isolated from the cricket-bat willow, *Salix caerulea*, and from the white willow, *S. alba*. Reported to cause a water-mark disease of willow in England (Dowson, 1957).

Therefore, all of these bacterial species except *Brevibacterium insectiphilium*, *Erwinia amylovora*, and *E. salicis* may be regarded as primarily soil bacteria or of such diverse distribution that they may be assumed to occur in soil. This suggests the possibility that the presence of these in wood may be due to transport from the soil by way of the tree roots. This entrance might be facilitated by mechanical injury to the roots through burrowing insects, nematodes, and other small animals inhabiting the soil. Possibly the bacteria may also enter the wood through wounds in the leaves and stems made by aphids and other insects.

Possibly the bacteria in wood of nondiseased trees are reproducing at a very low rate or not at all, but are kept viable because the tree sap prevents their desiccation and may provide minimal nutrition. But, in itself, their presence in the wood suggests their possible significance in the ecology of diseased, nondiseased, and dead plant materials and the soil-plant association which should warrant extensive and diverse future investigations.

Five of the species of bacteria from the present study were shown to be pectin digesters and one, *Cellulomonas acidula*, utilized cellulose (Table 2); six species showed proteolysis. These properties may indicate potentials for invasion of plant cells.

It has been suggested that if digesters of pectin and cellulose found in soil and water gain entrance into wood they may cause a reduction in its strength. According to Haygreen and Wang (1966), bacteria could be the cause of reduced strength in aspen wetwood, assuming that wood is a suitable growth substrate. Bacteria in aspen lumber have been studied by Clausen, Rees, and Kaufert (1949) in relation to wood collapse. *Bacillus polymyxa* (Prazmowski) is a strong digester of pectin and cellulose, but, although Knuth and McCoy (1962) found this species occurring in pine logs in pond storage, their study produced no evidence that it reduced wood strength.

The presence in wood of microorganisms which digest pectin and cellulose also suggests that wood from freshly cut trees or branches may contain the agents of its own decomposition. It was the hypothesis of Springer and Hajny (1966) that release of heat in wood chip piles sometimes to the point of ignition might be due to oxidation of various wood constituents by microorganisms. But they found that the bacterial population in wood chips was very low, not high enough to cause sufficient oxidation for ignition. This assumption, however, does not preclude the possibility that microorganisms already present in wood can be important in its eventual decomposition. The small numbers of bacteria in sawdust reported by Springer and Hajny could be due to the drying out of the wood before sawing as bacterial cells in the vegetative stage seldom withstand slow desiccation.

Six of the bacterial species studied grew on a nitrogen-free medium (Table 2). However, growth on Ashby's medium is by itself not a sufficient proof that a bacterium fixes nitrogen, and further tests with the isolates are in order.

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