Nitrogen Fixation by Bacterial Isolates from Decay in Living White Fir Trees [*Abies concolor* (Gord. and Glend.) Lindl.]

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INTRODUCTION

Hymenomycetous fungi are reported to be the major cause of decay in living trees (Boyce, 1961). Invasion and decay by many of these fungi are associated with or follow colonization of wood by bacteria and non-hymenomycetes (Shigo, 1967). During preliminary identification of bacterial isolates from decay in white fir trees, characteristics similar to those of some nitrogen-fixing klebsiellas were observed. Consideration of these observations and the low nitrogen content of wood (Cowling & Merrill, 1966) prompted an examination of these bacteria for N_2 -fixing capabilities. Twenty-eight per cent of 145 isolates tested exhibited positive nitrogenase activity.

METHODS

Most cultures used in this study were isolated in 1971 during a study of decay in white fir trees located in nine widely spaced locations in the Rogue River National Forest in southwestern Oregon. Thirty-two isolates were from a similar investigation on this forest in 1969.

In each of the nine areas, two decay samples were obtained from trees exhibiting an old basal injury or bearing conks of *Echinodontium tinctorium* Ell. and Ev. or *Phellinus pini* var. *abietis* (Karst.) Pilát. The trees were felled and dissected at or near the apparent infection site and near the lower or upper end of the decay column to obtain sample blocks for culturing. The blocks were split and chips from incipient decay, advanced decay and from a discoloured, wet-appearing zone adjacent to the bright wood were aseptically transferred to tubes of malt, malt and yeast extract, potato dextrose and nutrient agars (Difco). Inoculated tubes were incubated at laboratory temperatures until growth was observed and then stored at 2 °C.

Diagnostic tests on pure cultures were performed as recommended in the Clinical Manual of Microbiological Methods (Blair, Lennette & Truant, 1970) except that incubations were at 30 °C. DNA was extracted and purified from isolates w-1 and w-3 using conventional techniques (Marmur, 1961; Seidler, Starr & Mandel, 1969). The guanine-cytosine base

composition (%GC) of duplicate samples were determined by the thermal melting procedure using equation 5 of Mandel, Igambi, Bergendahl, Dodson & Scheltgen (1970) for calculations. DNA from *Escherichia coli* B with a known base composition of 51 % GC was used as a control.

Modified Pankhurst tubes ('H' tubes) were utilized in experiments in which acetylene reduction and N₂ fixation were investigated (Campbell & Evans, 1969). The major modifications concern the two 'H' tube arms (62 ml total volume) which are identical in size and attached to each other by a connecting tube fitted with a ground glass ball joint and a clamp to facilitate manipulation. One arm of each 'H' tube contained 10 ml of modified Hino and

Table 1. *Characteristics of bacteria isolated from decay in living white fir trees*

Source	Isolates ^a		
	W-1	W-3	W-15
Locality ^a ...	Rye Flat	Rye flat	Cinder pit
Tree no. ...	12	15	9
Fungal association ...	<i>Echinodontium tinctorium</i> ^b	<i>Phellinus pini</i> var. <i>abietis</i> ^b	<i>E. tinctorium</i> and <i>P. pini</i> var. <i>abietis</i> ^c
Characteristics			
TSI:			
Butt	A	A	A
Slant ^d	K	A	K
MR	+	+	+
VP	—	—	—
Indole	—	—	—
Citrate	+	—	+
Glucose ^e	Ag	A	Ag
Lactose	—	—	—
Ornithine decarboxylase (4 days)	+	—	±
Urease (2 days)	+	+	—
% GC	55.1	53.4	N.D.
Extinction increase during initial 48 h of incubation	0.06	0.03	0.10
C ₂ H ₂ reduced (nmol/culture/24 h) ^g	3151	645	5908

^a These isolates collected from Ashland Ranger District.

^b From discoloured wet-appearing zone between incipient decay and bright wood.

^c Incipient decay.

^d Acid (A) or alkaline (K) reaction.

^e Gas produced (g).

^f All isolates failed to produce acid from sucrose and maltose, all were Gram-negative, rods, H₂S negative, oxidase negative, gelatinase negative, and arginine and lysine decarboxylase negative; all fermented mannitol and fructose, were catalase positive and weakly motile at room temperature; maximum growth temperature about 35 °C.

^g Exposed to acetylene for 24 h after initial 48 h incubation.

Wilson medium (Hino & Wilson, 1958) (50 mg CaCl₂·2H₂O/l of medium instead of CaCO₃ and 2 % mannitol instead of sucrose). The procedure for sterilization, inoculation, flushing with N₂, maintenance of anaerobic conditions and sampling have been described (Campbell & Evans, 1969). Ethylene in each sample was assayed by using an Aerograph model 600D gas chromatograph fitted with a Porapak R column and flame ionization detector.

RESULTS

One hundred and forty-five cultures (113 from the 1971 study and 32 from 1969) representing six different fungal associations from 44 trees in 26 localities were tested for acetylene-reducing capability. Forty isolates from 21 trees in 14 different areas reduced acetylene at rapid rates when cultured anaerobically. When more than two successive transfers were made to a 'nitrogen-free medium', consistent growth occurred only with isolates that reduced acetylene. Of 80 isolates tested, none reduced acetylene when cultured aerobically. Fifteen bacterial cultures which reduced acetylene were isolated from decays associated with *Phellinus pini* var. *abietis*, 10 each with *E. tinctorium* and *Pholiota adiposa* (Fr.) Kumm, and four with *Hericiium abietis* (Weir ex Hubert) K. Harrison. Forty-three percent of the N_2 -fixing isolates were from the discoloured, wet-appearing zone between incipient decay and bright wood, 45% from the incipient zone and only 12% from advanced decay.

Properties of three representative acetylene reducing isolates (w-1, w-3, and w-15) are presented in Table 1. After incubation in H tubes for 24 h ten ml cultures reduced acetylene to ethylene at rates ranging from 645 to 5908 nmol in 24 h. These rates were comparable to those for the other 37 positive isolates. Tubes inoculated with all isolates lacking nitrogen-fixing capabilities and control tubes inoculated with sterile water produced less than 2 nmol of ethylene under comparable conditions. Nitrogen analyses of a series of cultures of w-1 showed that each 10 ml culture fixed about 4 μ mol of N_2 during a growth period of 90 h. Acetylene-reducing activity of w-1 also proceeded at a rapid rate during growth over 96 h although the presence of acetylene in the culture for 25 h inhibited growth as expected.

From reactions on Triple Sugar Iron agar (TSI), methyl red-Voges Proskauer (MRVP) tests, % GC content, and the N_2 -fixing capability, these three isolates seem similar to the *Klebsiella* group (Blair *et al.* 1970; Edwards & Ewing, 1962). However, these cultures are atypical of the *Klebsiella* group in that they do not grow at 37 °C, do not ferment lactose, sucrose, and maltose, and two out of three showed no active decarboxylase activities. Culture w-1 however is ornithine positive. Nitrogen-fixing enterics have been isolated from plants and soil (Line & Loutit, 1971; Evans, Campbell & Hill, 1972) which, because of their typical biochemical properties, could be assigned to the genera *Klebsiella* or *Enterobacter*. The maximum growth temperature, decarboxylase reactions and MRVP tests reported for an atypical N_2 -fixing bacterium described as *Escherichia intermedia* (Line & Loutit, 1971) are similar to those of our isolates. Differences exist, however, in the urease reaction and capacity to ferment lactose and sucrose. There are no previous descriptions of such biochemically deficient klebsiellas and hence there is no justification for classifying our isolates into any known species of *Klebsiella* or *Enterobacter*.

In view of the unique phenotypic properties of our isolates and the present status of the systematics of the nitrogen-fixing enterics, we feel it would be premature to make specific nomenclatorial recommendations.

To our knowledge this is the first report of nitrogen-fixing bacteria in decays in living trees. The possible role of these bacteria in the ecology of the decay process is obvious and is being investigated further.

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