Distribution, Enumeration, and Identification of Nitrogen-Fixing Bacteria Associated with Decay in Living White Fir Trees

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ABSTRACT

Of 130 fermentative, gram-negative bacterial isolates from the major decays in white fir trees, 68 were shown to be capable of fixing atmospheric nitrogen as determined by the acetylene reduction technique. Five isolates were also tested quantitatively by use of $^{15}N_2$. The N_2-fixing bacteria were isolated from 31 trees in 16 widely scattered locations and were associated with all decay stages caused by the most important fungal associations in white fir heartwood. High populations (10^5 to 10^6 cells/ml expressed sap) of these important bacteria were associated with the early and incipient decay stages caused by *Hericium abietis* and *Phellinus chrysoloma,* but not with *Echinodontium tinctorium*. The bacteria were characterized and identified as *Enterobacter agglomerans, E. aerogenes, Klebsiella pneumoniae,* and other atypical *Enterobacter* sp. This is the first report of N_2 fixation by the species *E. agglomerans*.

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Additional key words: Acetylene reduction, $^{15}N_2$ analysis, bacterial populations, deterioration, and heart rot.

Wood decay fungi are estimated to be directly or indirectly responsible for annual losses of $3.1 \times 10^6 m^3$ (110 million cubic ft) of lumber in Pacific Northwest forests (6). Most of the decayed wood in living trees is heartwood (2), which is known to contain low concns of nitrogen (27). Decay fungi are capable of utilizing sapwood and heartwood as their sole source of nutrients for growth; however, in vitro rates of decay may be significantly increased by addition of nitrogen (15, 30). Also, the addition of various forms of nitrogen to test blocks increases rates of deterioration caused by molds and soft rot fungi (9).

Wood-decay fungi have been speculated to either decompose large volumes of wood or obtain nitrogen

from outside sources to meet the needs of their comparatively nitrogen-rich vegetative mycelium, sporophores, and spores (27). Postulated mechanisms for obtaining required nitrogen include high efficiency in the use of wood constituents, use of nitrogen obtained from wood by a dynamic and continuous process of autolysis and reuse, and use of nitrogen from sources outside wood (27). Evidence supporting the view that fungal growth within decaying wood is derived from the nitrogenous constituents of the wood has been presented in several papers (20, 21, 26).

There is no conclusive evidence that wood-decay fungi can fix nitrogen from the air. A brief paper by Seidler et al. (31) described the isolation and characteristics of nitrogen-fixing fermentative bacteria from the major decays in living white fir, *Abies concolor* (Gord. and Glend.) Lindl., trees in Oregon. Nitrogenase activity in decaying American chestnut logs has been detected by use of the acetylene reduction technique (7); bacterial and fungal densities were estimated, but the microorganisms responsible for the nitrogen fixation were not identified. Nitrogenase activity also was detected in beech, oak, and Scots pine veneer pieces which had been buried in soil, but the causal microorganisms were not isolated or identified (33). The purpose of this paper is to provide more detailed information on the character and distribution of N_2-fixing bacteria in decaying white fir trees, and to present quantitative data on the capability of certain representative bacterial isolates from decaying wood to fix N_2.

MATERIALS AND METHODS.—*Isolation of bacteria.*—Bacterial cultures were isolated during two studies of microorganisms associated with decays in white fir trees in the Rogue River National Forest in southwestern Oregon. During the period June through September of 1969 (the first study), 501 white firs in 53 widely scattered localities with and without decay indicators were felled and dissected. When decay columns were encountered, 8-cm cubes of wood containing incipient and advanced decay were removed from the decay columns at approximately 2.4 m intervals. The blocks were labelled, placed in plastic bags, and kept in an ice chest. At the end of each day, the blocks were taken to the laboratory and refrigerated at 3 C overnight. Two isolations were made aseptically from both the incipient and advanced decay within each block on the following day. Blocks were carefully split with a mallet and sterile chisel. Small chips (approximately $5 \times 5 \times 3$ mm) of decayed wood were removed with a sterile gouge from freshly exposed surfaces, and, with a dissecting needle, placed in test tubes containing 2.5% malt agar (Difco). Inoculated tubes were incubated at laboratory temp (range 22-26 C) until growth was observed; the microorganisms were then transferred to screw-capped tubes and stored at 2 C until removed for purification.

In a second study in the Rogue River National Forest during July, August, and September of 1971, microorganisms were obtained from an intensive study of various stages of the most important decays of white fir. Twenty-seven trees were sampled in nine locations. Trees had also been studied in these locations in 1969. From each area, a tree with one of the following signs of decay was felled and dissected: (i) an old basal injury, (ii) sporophores of *Echinodontium tinctorium* Ell. and Ev., or (iii) sporophores of *Phellinus chrysoloma* (Fr.) Donk. (*Fomes pini* var. *abietis* Karst.). A 7.6-cm cross-section was cut from each tree at or near the apparent infection site and near the lower or upper end of the decay column. Each cross-section was split into eight 7.6 cm cubes, seven were labelled, placed in plastic bags, and stored in an ice chest. Cultures were taken immediately from the eighth block in the field by the technique used in the 1969 study, except that each block was split four times and a chip was aseptically taken from incipient and advanced decay and from a discolored wet-appearing zone surrounding the decay column. These were placed in tubes of malt, malt and yeast extract, potato-dextrose, and nutrient agars (Difco). The remaining seven blocks from each location within the study trees and the inoculated tubes were taken to a laboratory where the blocks were refrigerated at 3 C, and the tubes with chips were incubated at room temp. A block from both decay locations within study trees was removed from the refrigerator at 4, 16, 24, 48, 72, 96, and 168 h after the tree was felled. Culture techniques in the laboratory were the same as those used in the field. Inoculated tubes were incubated as previously described.

Identification of bacteria.—Pure cultures of bacteria were obtained from three sequential streaks of isolated colonies growing on Difco nutrient agar. During the purification procedure, cultures were incubated at room temp or at 30 C. Cultures of isolated bacteria were subjected to various diagnostic tests as recommended in the *Manual of Clinical Microbiology* (1) except that most cultures were incubated at 30 C. *Klebsiella pneumoniae* was incubated at 37 C. The pectate liquefaction test was according to Dye (11). Cellulose utilization was tested by growth on 1.0% Bacto cellulose containing 3.0 ml of Bacto phenol red broth base. Cellulose and pectin hydrolysis tests were examined periodically for a period up to 2 wk. Determination of guanine plus cytosine base composition (%GC) was made on representative cultures. DNA was extracted and purified by a modification of the Marmur technique (24), which involves the use of phenol in deproteinization (32). Percent GC was determined by the thermal denaturation technique (25), and calculations were based on equation 5 of Mandel et al. (23). DNA from *Escherichia coli* B with a known base composition was used as the standard.

Acetylene reduction capabilities.—Randomly selected purified gram-negative, fermentative bacterial cultures were tested for ability to reduce acetylene to ethylene, using a method slightly modified from those described by Campbell and Evans (5) and by Raju et al. (29). Modified Pankhurst tubes ("H" tubes) were used. The two H-tube arms (62 ml total volume) are identical in size and attached to each other by a connecting tube fitted with a ground glass joint and a clamp to facilitate manipulation. One arm of each H-tube contained 10 ml of a medium described by Hino and Wilson (17) except that 50 mg of $CaCl_2 \cdot 2H_2O$ per liter of medium was added instead of $CaCO_3$ and 2.0% mannitol was used instead of sucrose. The procedure for sterilization, inoculation, flushing with N_2, maintenance of anaerobic conditions, exposure to acetylene, and sampling have been described (5, 31). After sterilization of the tubes with medium in one arm and nonabsorbent cotton in each connecting tube, sterile

stoppers were inserted into each arm and the tubes flushed with sterile N_2. Alkaline pyrogallol (2.0 ml) was injected into the arm not containing medium. Each culture tube was inoculated with a sterile syringe and needle. Appropriate control tubes were inoculated with sterile water. Duplicate samples from each culture were assayed for ethylene with an Aerograph Model 600 D gas chromatograph fitted with a Porapak R column and flame ionization detector. Turbidity measurements of cultures were made in a Bausch and Lomb Spectronic 20 photoelectric colorimeter at 540 nm.

TABLE 1. Sources of fermentative isolates tested for nitrogenase activity by the acetylene reduction technique

Determination	Year of study		Total
	1969	1971	
Fraction of bacterial isolates fixing N_2	20/29	48/101	68/130
Fraction of localities where N_2-fixing isolates were found	12/18	6/6[a]	16/22[b]
Fraction of trees where N_2-fixing isolates were found	16/24	15/17	31/41
Proportion of isolates fixing N_2 (%)	69	47.5	52.3

[a]Bacteria isolated from trees in only six of the nine localities sampled in 1971 were tested for their nitrogen-fixing capabilities.
[b]Two localities were duplicated in the 1969 and 1971 studies.

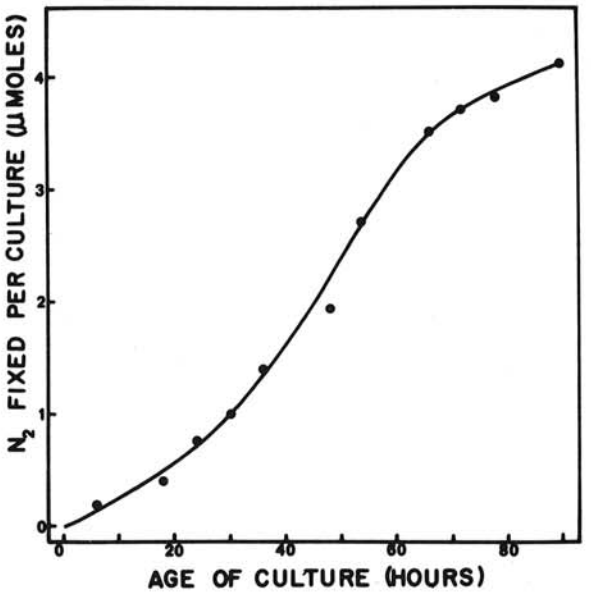

Fig. 1. Time-course of anaerobic nitrogen fixation by isolate W-1 identified as *Enterobacter* sp. A series of H-tubes each containing 10 ml of modified Hino-Wilson medium were inoculated with 0.5 ml of a 72-h culture of W-1 (optical density 0.17 at 540 nm) and incubated statically at 25 C. At the times indicated, duplicate aliquots were removed from each of two tubes and analyzed for nitrogen by the microkjeldahl method. Each value is a mean of four replicate determinations and has been corrected for the nitrogen content of the medium and inoculum.

A series of tubes was used for nitrogen determinations of a selected nitrogen-fixing isolate (*Enterobacter* sp.) at 6-h intervals starting from the time of inoculation. Each H-tube containing 10 ml of modified Hino-Wilson medium was inoculated with 0.5 ml of a 72-h-old culture of W-1 (O.D.-0.17 at 540 nm). At 6-h intervals duplicate aliquots were removed from each of two tubes and analyzed for nitrogen by the microkjeldahl method (36). Each nitrogen value is a mean of four replicate determinations and has been corrected for the nitrogen content of the medium and inoculum.

$^{15}N_2$ analyses.—Representative isolates were cultured for 48 h in H-tubes containing the modified Hino-Wilson medium. Each culture was flushed with sterile argon, 0.1 atm of argon was removed, and 0.1 atm of 99% $^{15}N_2$ was added with a sterile syringe. After exposure to $^{15}N_2$ for 70 h the bacteria in each culture were collected by centrifugation and digested by the microkjeldahl procedure. NH_3 in each digestate was distilled into 0.012 N H_2SO_4 by use of a microdiffusion method (4). An aliquot of the 0.012 N H_2SO_4 containing the NH_3 was used for the determination of the N content and the remainder was analyzed for $^{15}N_2$ excess by Professor R. H. Burris (Department of Biochemistry, University of Wisconsin). Since the total amount of bacterial cells from some cultures provided insufficient NH_3 for accurate analysis, it was necessary to add 200 μg of N as nonlabelled NH_4Cl. $^{15}N_2$ excesses were corrected for the dilution.

Bacterial populations.—Total bacterial populations and most probable numbers of nitrogen-fixing bacteria were determined for early and incipient decay in a tree bearing *E. tinctorium* sporophores, another with *P. chrysoloma* sporophores, and a third with a basal wound. Trees with these decay indicators were selected in the Green Creek area, Rogue River National Forest. Each tree was felled and an 8 cm cross-section was removed near the apparent infection site. A wood "plug" 7.5 cm long by 1.2 cm in diam containing half early and half incipient decay was removed with a sterile tubular chisel (18). The surface of the section from which the sample was taken was wiped with 70% ethyl alcohol before sampling. The plug was flamed at each end and placed in a section of sterile rubber tubing. Each tube with samples was placed in a hydraulic press and the sap expressed into a sterile bottle at 20.7 to 34.5 MPa (3,000-5,000 psi). Six sap dilutions (10^{-1} to 10^{-6}) were immediately made using modified Hino-Wilson medium. Because both total bacterial and N_2-fixing bacterial populations from decay associated with *E. tinctorium* conks were so low, the study was repeated two times for decay associated with this indicator. The third time 1 ml of sap was expressed directly into 9 ml of modified Hino-Wilson medium and quickly diluted as previously described.

One-tenth ml of sap and of each dilution was pipetted on each of three plates of Difco nutrient agar. The inoculum was spread over the surface with a sterile glass rod. The plates were incubated at 25 C and population counts were made after 2, 4, and 9 days.

Populations of N_2-fixing bacteria were determined by the most probable number (M.P.N.) technique (28). Dilutions of expressed sap in modified Hino-Wilson medium from 10^{-1} to 10^{-6} were pipetted into one arm of the previously described H-tubes. Each dilution was

replicated five times. Inoculated tubes were incubated at 25 C for 8 days and tested periodically by the acetylene-reduction technique for nitrogenase activity. The tubes not exhibiting nitrogenase activity were assumed not to have N_2-fixing bacteria present. The M.P.N. was the estimated most probable number of N_2-fixing bacteria in the nondiluted sap (28).

RESULTS AND DISCUSSION.—*Evidence of nitrogen fixation by bacterial isolates.*—Twelve hundred bacterial isolations were obtained from the 1969 and 1971 studies. From these, 572 isolates were purified. Forty percent were identified as aerobic pseudomonads, only 2.0% were *Bacillus* spp., and 58% were gram-negative, fermentative bacteria. In our earlier report (31), 145 fermentative and strictly aerobic bacteria were tested for nitrogenase activity. In the present study, 130 isolates of the fermentative bacteria were tested for acetylene-reduction capabilities. More than 52% reduced acetylene at rapid rates when cultured anaerobically (Table 1). In the qualitative assessment of nitrogenase activity, the 10-ml cultures were exposed to acetylene for 24 h. Positive activities range between 496 and 9,507 (mean 3,099) n moles of ethylene produced per culture per 24 h. These measurements of nitrogenase activities are similar to those for other nitrogen-fixing bacteria (5, 22, 29). None of the 80 fermentative isolates tested reduced acetylene aerobically. Only those isolates that reduced acetylene showed appreciable growth on the modified Hino-Wilson medium after two successive transfers. Nitrogen analyses of a series of cultures of isolate W-1, an *Enterobacter* sp. exhibiting positive nitrogenase activity, showed that each 10-ml culture fixed about 4 μmoles of nitrogen during a growth period of 90 h (Fig. 1).

Measurements of rate of acetylene reduction in large samples of decaying wood from living white fir trees was attempted. Intact blocks (30.5 cm long by 45.7 cm in diameter) which included sapwood evolved appreciable ethylene, and thus results of the acetylene reduction tests were inconclusive. Preliminary tests with blocks (20-cm diam by 20-cm length) of decayed heartwood produced up to 142 n moles of acetylene-dependent ethylene per day during a growth period of 6 days.

Further evidence of N_2 fixation was obtained by use of $^{15}N_2$. Five representative isolates that had shown positive results in acetylene reduction tests were grown for 48 h and provided with 0.1 atm of 99% $^{15}N_2$ as the sole source of nitrogen. All cultures incorporated $^{15}N_2$ into cells as indicated by the fact that cells contained from 5 to 33.5% excess ^{15}N (Table 2).

Occurrence of N_2-fixing bacteria.—The 130 isolates of fermentative bacteria tested by the acetylene reduction technique for nitrogenase activity were from 41 trees in 22 widely scattered localities (Table 1). The nitrogen-fixing bacteria were isolated from 31 trees occurring in 16 areas. They were found in 16 of 24 trees and in 12 of 18 localities in the 1969 study and in 15 of 17 trees and in all localities in 1971. Sixty-nine percent of the gram-negative, fermentative bacteria from the 1969 study, tested for acetylene reducing capabilities were positive for nitrogenase activity, while 47.5% were positive from the 1971 study (Table 1).

Although more culture attempts were made in the 1969 study, fewer bacteria were isolated because malt agar was the only culture medium used and no attempts were made to isolate microorganisms from the earliest stage of decay. Malt agar and malt agar with yeast extract added have been used as the culture medium in numerous studies attempting to isolate microorganisms from decay in living trees because most studies were undertaken to isolate decay fungi only. Other microorganisms isolated were often considered to be "contaminants" and discarded. In the 1971 study where four different media were used to isolate microorganisms, more bacteria as well as more nitrogen-fixers were obtained on nutrient agar than on other media (Table 3). During the 1971 study, isolations were also attempted from the early decay stage. Higher numbers of the fermentative and nitrogen-fixing bacteria were associated with the early and incipient stages of decay (Table 4). Of the N_2-fixing isolates, 25 were from the dark, wet-appearing zone surrounding decay columns, 28 were from incipient decay, and only 15 were from advanced decay. However, a higher percentage of fermentative isolates from advanced decay fixed nitrogen.

The N_2-fixing bacteria were associated with the major decay fungi in white fir (Table 4). *E. tinctorium, Pholiota adiposa,* (Fr.) Kumm., *Phellinus chrysoloma,* and *Hericium abietis* (Weir ex Hubert) K. Harrison are probably associated with more than 90% of all decay in living white firs in the study area. No N_2-fixing and only a few other types of bacteria were isolated from 14 decay columns associated with *Fomitopsis annosa* (Fr.) Karst. and from eight decay columns caused by *Armillariella mellea* (Vahl) Quél. These so-called "pioneer fungi" are known to be capable of invading wood in living trees before colonization by bacteria and non-hymenomycetous fungi (34).

Bacterial populations associated with decay.—Total

TABLE 2. Quantitative determination of $^{15}N_2$ incorporated into representative bacterial[a] isolates from decay in white fir trees in the Rogue River National Forest in Oregon

Isolate number	Bacterial species	Group	Fungal association	Atoms % excess $^{15}N_2$ in bacterial cells
09	*Enterobacter agglomerans*	I	*Echinodontium tinctorium*/ *Hericium abietis* mix	33.5
25	*Enterobacter* sp.	XA	*Echinodontium tinctorium*	22.7
34	*Enterobacter* sp.	XA	*Hericium abietis*	21.4
44	*Enterobacter* sp.	XB	*Phellinus chrysoloma*	28.4
57	*Enterobacter* sp.	XA	*Pholiota adiposa*	5.0
Control (NH_4Cl)				0.005

[a]Criteria for selection were based on fungal association and high rates of acetylene reduction capability. Selection was made before taxonomic identifications were completed.

TABLE 3. Effect of medium on isolation of gram-negative fermentative N_2-fixing bacteria from decaying wood in trees in the 1971 study

Isolation medium	Isolates tested (no.)	N_2-fixers (% of total)
Malt agar	5	0.0
Malt and yeast extract agar	13	53.8
Potato dextrose agar	19	63.2
Nutrient agar	64	45.3
Total	101	47.5

bacterial populations were determined from the early stages of decay in trees infected with three of the most important decay fungi in white fir (Table 5). There were 39.3×10^6 bacterial colonies per milliliter of expressed sap from wood infected with *P. chrysoloma* and 7.0×10^6 per milliliter of sap from *H. abietis* decay (Table 5). These populations are high compared with total bacterial counts from other habitats. Although colony counts from trembling aspen sapwood, heartwood, and wetwood increased with increasing degree of discoloration, none was as high as 1.0×10^6 per milliliter of expressed sap (19). Bacterial populations from decaying chestnut logs ranged from 9.4×10^3 to 3.9×10^6 per gram dry wt of wood (7).

Total concns of N_2-fixing bacteria were 3.5×10^5 colonies per milliliter of expressed sap from early decay associated with *H. abietis* and 1.6×10^6 colonies from *P. chrysoloma* decay (Table 6). The high concns of N_2-fixing bacteria indicate that they probably play an important role in decay of heartwood in living trees.

Cosenza et al. (8) have speculated that the relation between bacteria and wood-decay fungi may be mutualistic. They suggest that the bacteria benefit from decomposition of cellulose and hemicelluloses to simple sugars and from production of organic acids by decay fungi, while the fungi may obtain thiamine from bacteria that are able to produce this vitamin. To this list it may be possible to add that decay fungi obtain from bacteria the nitrogen needed to decompose nitrogen-meager wood.

Total and N_2-fixing bacterial populations in early decay associated with *E. tinctorium* were very low compared with those associated with *P. chrysoloma* and *H. abietis* decay (Tables 5 and 6). Although this experiment was repeated twice (samples were taken from three different trees bearing *E. tinctorium* sporophores) for decay in trees with this decay indicator, total bacterial

TABLE 5. Bacterial populations from early decay associated with three major decay fungi in white fir

Tree	Fungal association[a]	Number of colonies per ml of expressed sap[b]
A	*Hericium abietis*	7.0×10^6
B	*Phellinus chrysoloma*	39.3×10^6
C_1	*Echinodontium tinctorium*	1.4×10^3
C_2	*E. tinctorium*	49.3×10^4
C_3	*E. tinctorium*	6.6×10^5

[a]Decay fungi were identified by examination of cultures isolated from each decay column.

[b]Bacterial colonies were counted on serial dilution plates of nutrient agar, replicated three times, and incubated at 25 C for 9 days.

counts never reached 1.0×10^6 per ml of sap (Table 5), nor were N_2-fixing bacteria detected at any dilution (Table 6). In the third attempt to obtain bacterial counts from *E. tinctorium* decay, sap was expressed directly into the culture medium and dilutions were made immediately. With this precaution, bacterial counts did increase over previous attempts, but they were still low compared with those from other decays (Table 5). Shortly after serial dilutions were made for the third tree with *E. tinctorium* decay, a distinct "browning" was observed in every dilution, indicating the presence of phenolic compounds. High concns of phenols were detected, using the technique of Feldman and Hanks (14), even at the greatest dilutions. Possibly these compounds retarded growth of, or killed, bacteria in sap expressed from *E. tinctorium* decay.

Taxonomy of N_2-fixing bacteria.—Characteristics of the 130 gram-negative, fermentative bacteria tested for N_2 fixation are summarized in Table 7. The isolates were tested for 20 biochemical properties. All cultures did not produce H_2S on TSI agar, were catalase positive, oxidase negative, and fermented glucose in the Hugh-Leifson oxidative vs. fermentative test (1). Variable reactions were observed in the 16 remaining tests. Isolates were placed in 14 groups based primarily on the arginine dihydrolase, ornithine and lysine decarboxylase tests and production of acetylmethyl carbinol (VP reaction). Only 20 isolates were found to be biochemically typical of previously described species. Twelve cultures were identified as anaerogenic *Enterobacter agglomerans,* six were *E. aerogenes,* and two were *Klebsiella pneumoniae.*

TABLE 4. Occurrence of nitrogen-fixing bacteria in fungal associations and decay stages in white fir wood

Fungal association	Early decay[a]		Incipient decay		Advanced decay	
	Isolates tested (no.)	N_2-fixers (no.)	Isolates tested (no.)	N_2-fixers (no.)	Isolates tested (no.)	N_2-fixers (no.)
Echinodontium tinctorium	21	8	17	8	4	1
Pholiota adiposa	14	3	10	5	6	3
Phellinus chrysoloma	13	5	12	8	7	6
Hericium abietis	9	9	1	1	1	0
Mixed decays	...	...	8	6	7	5
Total	57	25	48	28	25	15

[a]A discolored, wet-appearing zone between incipient decay and bright wood. No isolations were attempted from this zone in the 1969 study.

TABLE 6. Most probable numbers of nitrogen-fixing bacteria in expressed sap from important decays in white fir wood

Fungal association	N_2-fixing bacteria[a] (no.)	Total bacteria isolated (%)
Hericium abietis	3.5×10^5	5
Phellinus chrysoloma	$>1.6 \times 10^{6c}$	>4.1
Echinodontium tinctorium[b]	0	0

[a]Concentration of N_2-fixing bacteria determined by most-probable-number method. Serial dilutions were prepared in 9-ml volumes of Hino-Wilson N_2-free medium, and incubated anaerobically in H-tubes at 25 C for 8 days.

[b]Three different attempts were made to determine the N_2-fixing bacterial population.

[c]Nitrogenase activity was measured in all five replicas at the greatest dilution (10^{-6}).

TABLE 7. Groups of fermentative, gram-negative bacteria from decayed trees

Group	Classification	No. of isolates	Property[a,b]														
			I	M	VP	C	U	A	L	O	Gel	Lac	Mann	Gly	Suc	Ara	Rha	N_2
I	*Enterobacter agglomerans*, Biogroup 2 or 3	9	0	100	0	66	0	0	0	0	55	44	100	66	44	100	100	56
II	*E. agglomerans*, Biogroup 1 or 6, mannitol-negative	3	0	67	100	67	0	0	0	0	33	33	100	67	100	100	100	0
III	*E. agglomerans*, Biogroup 2 or 3, mannitol-negative	17	0	94	0	44	0	0	0	0	25	19	0	31	6	100	100	56
IV	*E. agglomerans*, Biogroup 1 or 6	1	−	+	+	+[c]	−	−	−	−	−	−	−	−	+	+	+	−
V A	*Enterobacter aerogenes*, Voges-Proskauer negative	6	0	0	100	83	0	0	100	100	100	33	50	33	83	100	100	17
V B	*E. aerogenes*	7	0	100	0	86	0	0	100	100	86	57	29	29	43	100	100	71
VI	*Klebsiella pneumoniae*	2	100	0	100	100	100	0	100	0	0	100	100	100	100	100	100	100
VII	*Enterobacter cloacae*, ornithine-negative	13	0	23	100	77	0	100	0	0	87	92	100	92	54	100	100	23
VIII	*E. cloacae*, metabolically atypical	11	0	66	45	72	0	100	0	100	54	54	73	82	55	91	91	36
IX	*E. aerogenes*, lysine-negative, or *E. cloacae*, arginine-negative	2	0	100	100	50	0	0	0	100	100	0	100	50	100	50	100	0
X A	*Enterobacter* sp.	12	0	100	0	100	0	0	0	100	0	0	9	9	9	86	86	67
X B	*Enterobacter* sp.	13	0	100	0	100	0	0	0	100	100	16	56	0	8	100	100	100
XI A	*Enterobacter* sp.	11	0	100	0	0	0	100	0	0	91	27	72	18	72	100	100	36
XI B	*Enterobacter* sp.	4	0	100	0	100	0	100	0	0	100	0	75	50	25	100	100	100
XII	*Enterobacter* sp.	2	0	100	0	50	0	0	100	0	50	0	0	0	50	100	100	50
XIII	*Enterobacter* sp.	7	0	100	0	71	0	100	100	100	29	0	57	29	14	100	100	86
XIV	Unknown genera, glucose fermented	10	0	0	0	0	0	80	30	20	20	0	30	0	10	10	10	20

[a]I, M, V, P, C = (respectively) indole production, methyl red and Voges-Proskauer tests, and citrate utilization; U = urease production; A = arginine dihydrolase; L, O = lysine and ornithine decarboxylase; Gel. = gelatin liquefaction; Lac., Mann., Gly., Suc., Ara., and Rha = (respectively) fermentation of lactose, mannitol, glycerol, sucrose, arabinose, rhamnose; and N_2 = nitrogenase activity.

[b]Numbers indicate percent positive reactions.

[c]Since only one isolate is in this biogroup, a + and − system is used for the reactions.

TABLE 8. Percent guanine plus cytosine DNA base composition of selected N_2-fixing bacteria

Isolate no.	Classification	ΔTm[a]	% GC
21213234	*Enterobacter* sp.	2.0	55.0
21513353	*E. agglomerans*	1.2	53.4
2980-9	*Enterobacter* sp.	1.3	53.6
32713314A	*Enterobacter* sp.	3.3	57.6
4064-19	*Klebsiella pneumoniae*	2.1	55.2
4082-20A	*K. pneumoniae*	3.0	57.0
WP2	*Escherichia coli*	0	51.0

[a]ΔTm is the difference in the thermal midpoint of the *E. coli* DNA and the test sample. DNA samples were melted in 0.1 × SSC. The %GC base composition was calculated from equation 5 of Mandel et al. (23).

Systematics of the *Erwinia herbicola* group have recently been revised. According to Ewing and Fife (13), this group should be reclassified into the genus *Enterobacter,* and the correct specific epithet is *E. agglomerans*. Perhaps the most reliable phenotypic properties exhibited by over 500 isolates of this species is their ability to ferment mannitol and their inability to decarboxylate arginine, lysine, and ornithine. It is on these bases that the 12 isolates of groups I and II were classified into the otherwise hererogeneous *E. agglomerans* species. The 18 cultures of groups III and IV are atypical *E. agglomerans* since they failed to ferment mannitol. Isolates of groups X-XII are not included in *E. agglomerans,* because they exhibited one or more activities in decarboxylase and/or dihydrolase reactions.

Isolates in some groups differ from typical species by one to several significant taxonomic traits. For example, 13 cultures in group VII would be classified as *E. cloacae* if they exhibited ornithine decarboxylase activity. Ninety-six percent of *E. cloacae* isolates studied by Edwards and Ewing possessed this enzyme activity (12). Group VIII has 11 cultures which we have called metabolically atypical *E. cloacae*. About half these cultures exhibited aberrant reactions in the fermentation of lactose, sucrose, and the production of acetylmethyl carbinol.

Since none of the 130 isolates was capable of hydrolyzing pectin, all are excluded from three species of the genus *Pectobacterium* (11). However, *P. rhapontici* and *P. cypripedii* do not hydrolyze pectin (11). These two *Pectobacterium* species differ significantly from phenotypically similar isolates of groups X-XIV by producing acetylmethyl carbinol (VP test) and by the lack of dihydrolase or decarboxylase activities (11).

Knowledge of the %GC of representative isolates has been used to add molecular significance to phenotypic identifications (Table 8). The 53-58%GC range accurately reflects values reported by others for this group (16, 25). Published values for *E. agglomerans* (*E. herbicola*) are 53-55%GC (35). The agreement in %GC values is reassuring because of the phenotypic heterogeneity of the *Enterobacter* isolates encountered and the skepticism which might be associated with the isolation of *K. pneumoniae* from within living trees. With respect to the latter situation, recent reports have clearly demonstrated a common colonization of *K. pneumoniae* on various plants, vegetables, and bark of trees (3, 10). The present study, however, represents the first isolation of *K. pneumoniae* from within living trees.

Representatives of N_2-fixing *Klebsiellae* have been described in the two genera *Klebsiella* and *Enterobacter*. Some isolates of the species *K. pneumoniae, E. aerogenes,* and *E. cloacae* have been reported to possess nitrogenase activity (3, 22, 29, 31). This study represents the first report of N_2 fixation by the species *E. agglomerans*.

In this taxonomic study of bacteria from this unique habitat, we have decided to put relatively little significance into specific carbohydrate fermentation patterns. The classical taxonomic scheme for the Enterobacteriaceae was derived from studies of human or clinical isolates or both (1, 12). Since our cultures have evolved in a very different habitat, it should not be surprising that many isolates exhibited "atypical" biochemical responses. Many atypical reactions resulted from negative responses to carbohydrate fermentation tests. For example, many isolates of groups X-XII do not ferment lactose, mannitol, or glycerol. These compounds would not be expected to be prevalent in the heartwood of living trees. For these reasons we have acted conservatively in assigning a specific epithet to many isolates. Uniqueness of habitat and lack of typical phenotypic properties are not acceptable reasons in this case for assigning these isolates to a new species. The specific epithet must await further studies of a molecular nature, such as additional nucleic acid characterization.

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