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Isolation of *Klebsielleae* from Within Living Wood†

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Previous studies from this laboratory have documented the presence of coliform bacteria emanating from wooden reservoirs containing finished drinking water. Coliforms were identified as *Klebsiella pneumoniae* and *Enterobacter* spp. In the present report, evidence is presented which suggests that the origin of these coliforms is from the wood used to construct the reservoirs. In liquid expressed from freshly cut redwood, total bacterial counts in the range of 10^5 to 10^6/ml were commonly observed. When present, coliform counts were over 10^3/ml of expressed liquid. *E. agglomerans* was the most prevalent coliform present, but *Klebsiella* was isolated from freshly cut logs. *Citrobacter freundii* was also occasionally isolated. No fecal coliform-positive *Klebsiella* were obtained from any of the samples. Highest total bacteria and coliform counts were observed in sapwood specimens. Coliforms were present throughout sapwood as evidenced by contact plating serial sections of freshly cut wood. Scanning electron micrographs illustrate the presence of bacterial colonies within sapwood tracheids. Other wood species also contained coliform bacteria but in numbers lower than found in redwood.

Coliform bacteria (2) of the genus *Klebsiella* have been detected in excessive numbers in redwood reservoirs used to store finished drinking water (27). In that survey, no obvious external source of *Klebsiella* was found. It was determined that *Klebsiella* were associated with slime accumulated on the redwood staves, but the primary source was unexplained.

The association of *Klebsiella* with other botanical material has been reported on several occasions. These organisms, which are also opportunistic pathogens for humans as well as animals (4, 6, 20, 21, 28), have been found in high numbers in effluents from pulp and paper mills (16, 18), sugarcane wastes (23), textile mill effluents (10), and on fresh vegetables (5, 11). Duncan and Razzell (11) reported the isolation of *Klebsiella* from tree needles and bark in a forest environment, along with another member of the *Klebsielleae* tribe, *Enterobacter*. Organisms from the *Klebsielleae* tribe have been isolated from within living white fir trees (1), from the sapwood of southern pines stored under a water spray (9), and from cotton (G. B. Michaels, D. J. Wofford, and I. L. Roth, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, Q8, p. 262).

Knittel et al. (18) examined the growth and survival of *Klebsiella* growing in and on environmental material. They found that these organisms are capable of multiplying to high numbers (10^6/ml) in aqueous extracts of sawdust and on the surfaces of vegetables (10^3/g of surface peel). No difference in growth characteristics was found between known pathogenic and environmental isolates.

Klebsiella isolated from environmental sources appear to be indistinguishable from pathogenic clinical isolates, based on biochemical reactions and mouse virulence tests (S. T. Bagley and R. J. Seidler, Health Lab. Sci., in press). In view of the health significance of environmental *Klebsiella* and their previous isolation from redwood water reservoirs and other wood-associated environments, the present study was undertaken to determine whether these organisms and other coliform bacteria could be isolated from within wood. Several types of wood, including redwood, were examined for total coliforms (TC) and total numbers of bacteria (TB) present by expressing liquid from the wood under pressure. Contact impressions of freshly cut wood surfaces were also made on selective media. Scanning electron micrographs (SEM) were prepared from sections of redwood sapwood (SW) in an attempt to determine the location of any bacteria within the wood structure.

MATERIALS AND METHODS

Wood samples. Samples of fresh and aged redwood were obtained from National Tank and Pipe Co., Portland, Ore., and Simpson Lumber Co., Arcata,

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Calif. Samples of other wood were obtained from mills in Oregon.

Liquid extraction from wood. All types of wood had outer surfaces removed by using a power circular saw. The sawblade and cutting table were surface sterilized with 95% ethanol. Swabs of these surfaces were routinely streaked onto m-Endo agar LES (Difco Laboratories, Detroit, Mich.) to ensure absence of external contamination. The wood samples were then cut into blocks approximately 8 cubic inches (ca. 131 cm^3) in size. Moisture content of the wood was determined by measuring sample weight both before and after drying at 60°C for 7 days.

Liquid was extracted from the wood blocks by using a Carver Laboratory hydraulic press (Fred S. Carver, Inc., Summit, N.J.) operated at up to 12,000 lb/in^2. Blocks were placed on sterile aluminum foil with upturned edges for liquid collection. During pressing, the blocks were flooded with 2 ml of sterile 1:10 (wt/vol) polyvinyl pyrrolidone to neutralize any released phenolic compounds. Expressed liquid was transferred to sterile tubes for bacterial testing.

Bacterial enumeration. Dilutions of collected liquid were made in sterile 0.01 M tris(hydroxymethyl)aminomethane (pH 7.5). Portions (0.1 ml each) of appropriate 10-fold dilutions were spread onto media for viable cell counts. TB counts were made on nutrient agar (Difco), with incubation at 30°C for 48 h. TC counts were made by using m-Endo agar LES, with incubation at 35°C for 24 h. In one experiment with redwood collected directly from a California mill, both TB and TC counts were made at 37°C to enhance selection of *Klebsiella* (5). With nonredwood samples, most-probable-number techniques were also used to obtain confirmed TC counts (2). Fecal coliform counts were made by using m-FC agar (Difco), with incubation at 44.5 ± 0.5°C for 24 h in a water-jacketed dry air incubator.

Cut surface contact impressions. Outside surfaces of wood samples were removed as described above, and impressions were made by bringing a freshly exposed surface into contact with m-Endo agar LES. With two samples of fresh redwood, six to nine successive 0.25-inch (ca. 0.64-cm) sections were cut through each block after outer surface removal. An impression of each successively exposed surface was made on m-Endo agar LES. All plates were examined for growth and for green-sheened colonies after 24 to 48 h of incubation at 35°C.

Coliform identification. Confirmed coliform isolates from all wood experiments were tested biochemically for identification. A green-sheened colony from m-Endo agar LES was considered to be a confirmed coliform if it produced gas in lactose broth within 24 h at 35°C. Presumptive speciation was made by using triple sugar iron, urea, and Simmons citrate agars, lysine and ornithine decarboxylase broths, and motility tests. Further identification and characterization were made by using standard biochemical tests (15) and the API 20E system (Analytab Products, Inc., Plainview, N.Y.). *Klebsiella* isolates were identified to species level according to Naemura et al. (L. G. Naemura, S. T. Bagley, and R. J. Seidler, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, I149, p. 179).

Electron photomicrographs of wood. Samples 0.2 to 0.5 cm^3 in size were cut from redwood blocks, processed through solutions of ethanol and trichlorotrifluoroethane in increasing concentrations, and dried by the critical point method of Cohen et al. (7). After drying, the cubes were split parallel to the longitudinal axis of the wood fibers to expose fresh internal surfaces. These pieces were fastened to aluminum planchets and coated with approximately 20 nm of 60:40 gold-palladium. Samples were observed at 15 kV in an ISI Mini-SEM MSM-2 scanning electron microscope (International Scientific Instruments, Inc., Santa Clara, Calif.), and images were recorded on type 55 Polaroid film.

RESULTS

Results of three separate experiments examining bacterial content of liquid aseptically extracted from redwood heartwood (HW) and SW samples are presented in Table 1. The data indicate that not only are significant levels of bacteria present, but high levels of TC are present as well. The levels of TC detected ranged from 3.2×10^2 to 1.4×10^6/ml. High TC counts also corresponded with high levels of TB.

The highest TB counts and all TC detected were associated with SW or SW and HW combined samples (experiment 2). There was no apparent correlation between percent moisture content of the HW or SW samples for the same log (experiment 3) and respective bacterial counts. Percent moisture content of SW samples, only, did seem to correspond with bacterial loading; e.g., bottom log SW redwood had the highest percent moisture content and highest TC and TB levels as well. In contrast to the SW results, most HW redwood tested was nearly sterile.

All coliforms identified in these experiments were of the *Klebsielleae* tribe (*Klebsiella* and *Enterobacter*). *E. agglomerans* was the only coliform identified from the redwood SW tested in experiment 1. Because *Klebsiella* spp. were the predominant coliforms present in contaminated drinking water held in redwood water storage reservoirs (27) and most common on tree needles and bark (11), samples in experiment 2 were incubated at higher temperatures to reduce the numbers of *Enterobacter* and enhance the isolation of *Klebsiella* (5). *Klebsiella* spp. were in fact recovered from all samples having detectable TC. In experiment 3, however, *Klebsiella* were also isolated from redwood samples, although incubation was at 35°C. No fecal coliforms were detected from any wood sampled by plating onto mFC agar.

Liquid expressed from other types of wood (Table 2) was examined similarly to the redwood samples to determine whether the same levels of TC and TB were present in these woods as well. With the exception of the cedar sample, all woods tested were SW and had levels of TB

TABLE 1. *Bacteria present in liquid expressed from redwood*[a]

Type of wood	No. of samples	Bacterial count/ml		% Moisture content[b]	Coliform(s) identified
		TB[c]	TC[d]		
Expt 1[e]					
Sinker HW	2	3.3×10^5	NG	NT	
Heavy stain HW	1	1.3×10^3	NT	NT	
Young growth HW	2	NG	NG	NT	
Wide ring count SW	2	4.5×10^6	TNTC	NT	*E. agglomerans*
Heavy SW	2	1.7×10^6	2.0×10^3	NT	*E. agglomerans*
Expt 2[f]					
Trim green HW	1	3.5×10^4	NG	NT	
HW	2	1.7×10^4	NG	NT	
HW and SW	1	5.0×10^5	4.0×10^3	NT	*K. pneumoniae*
HW and SW chips	2	1.3×10^5	2.0×10^3	NT	*K. pneumoniae*
HW and SW	1	1.0×10^5	5.0×10^2	NT	*K. pneumoniae, K. oxytoca*
HW and SW	1	1.3×10^5	3.0×10^4	NT	*K. pneumoniae, E. cloacae, E. agglomerans*
SW	1	NG	NG	NT	
Expt 3[g]					
Bottom log, HW	3	5.0×10^2	NG	230	
Bottom log, SW	3	1.4×10^7	1.4×10^6	300	*E. agglomerans*
Third cut, HW	3	NG	NG	150	
Third cut, SW	3	1.3×10^6	4.5×10^4	160	*K. pneumoniae, E. agglomerans, Enterobacter* sp.
Top log, HW	3	1.0×10^2	NG	90	*Enterobacter* sp.
Top log, SW	3	5.7×10^3	3.2×10^2	130	*K. pneumoniae, E. agglomerans*

[a] NG, No growth; NT, not tested; TNTC, too numerous to count.
[b] Determined by dividing wet weight by dry weight of wood.
[c] Average results after 48 h of incubation on nutrient agar at 30°C, except for experiment 2, in which incubation was at 37°C.
[d] Average results after 24 h of incubation on m-Endo agar LES at 35°C, except for experiment 2, in which incubation was at 37°C.
[e] Cut wood samples 1 to 2 months old.
[f] Freshly cut wood from a redwood sawmill.
[g] Freshly cut wood from a log pile.

TABLE 2. *Bacteria present in liquid expressed from nonredwood wood*

Type of wood	No. of samples	Bacterial count/ml			% Moisture content[a]	Coliform(s) identified
		TB[b]	TC[c]			
			MPN[d]	m-Endo[e]		
Cedar	2	$<1.0 \times 10^1$	<2.0	0	59.6	*E. agglomerans*[f]
Cottonwood SW	2	3.8×10^5	6.5×10^1	0	74.3	*E. agglomerans, C. freundii*
Douglas fir, freshly cut SW	3	4.5×10^2	0.9	0	113.5	*E. agglomerans*
Douglas fir, racked SW	2	7.9×10^5	1.3	0	120.0	*K. pneumoniae, E. agglomerans*
Hemlock SW	2	2.0×10^6	2.0	5	61.2	*K. pneumoniae, E. agglomerans, C. freundii*

[a] Determined by dividing wet weight by dry weight of wood.
[b] Results after 48 h of incubation at 30°C on nutrient agar.
[c] No fecal coliforms were detected.
[d] MPN, Most probable number. Average of confirmed counts after 24 h of incubation at 35°C on eosin-methylene blue agar.
[e] Average results using m-Endo agar LES after 24 h of incubation at 35°C.
[f] Isolated as a green-sheened colony on m-Endo agar LES after 48 h of incubation.

comparable to those found from redwood (Table 1). Although all woods but cedar also had recoverable TC, the counts were 10 to 100 times less than the TC counts obtained from redwood. Except for hemlock, TC were only recovered by using most-probable-number techniques. Percent moisture content, again, did not apparently correlate with levels of bacteria detected.

Coliforms identified were essentially the same as those from redwood, with the additional isolation of *Citrobacter freundii*. *K. pneumoniae* was isolated from two wood samples, racked Douglas fir and hemlock. No fecal coliforms were detected from any type of wood in these experiments.

Contact impressions of freshly exposed wood surfaces (Table 3) gave results paralleling TC counts for the same wood samples (Tables 1 and 2). Except for cedar, all wood types from which TC were obtained via pressing also had TC present on the inner surfaces. The two redwood SW samples tested from experiment 1 (Table 1) had confluent coliform growth on the test plate surface within 24 h at 35°C. No green-sheened colonies appeared from the other wood contact impressions until after another 24 h of incubation. All of these colonies, however, were identified as *C. freundii* or *E. agglomerans*. No *Klebsiella* spp. were detected in these experiments.

Table 4 presents the results of contact impressions made of successive serial cuts through the two redwood SW samples having confluent coliform growth (Table 3). The results generally indicate that the coliforms were present throughout the wood. One sample, wide ring count SW, had confluent growth at 24 h on all sections. This corresponds with the confluent TC growth on all dilution plates reported from the wood-pressing experiments (Table 1). Although the heavy SW sample had confluent coliform growth on several sections, the number of TC present at 24 h decreased in successive cuts until no TC were present in the innermost sections (sections 4–6). After 48 h of incubation, TC counts were nearly uniform for all sections. The only coliform identified from any sample section after 24 or 48 h of incubation was *E. agglomerans*. This was also the only coliform previously isolated from these same redwood samples (Tables 1 and 3).

SEM of wide ring count SW sections (Fig. 1–3) taken at increasing magnifications clearly indicate the presence of several types of bacteria (rods and cocci) within the wood structure itself. Figure 1 (×200) shows the bordered pits in sapwood tracheids. The upper tracheal layers have

TABLE 4. *Contact impressions of serial cuts through redwood*[a]

Type of redwood	No. of samples	Surface examined[b]	No. of coliforms[c]	
			24 h	48 h
Heavy SW	2	Upper surface	TNTC	TNTC
		2	12	150
		3	10	150
		4	NG	100
		Center	NG	100
		6	NG	100
		7	15	75
		8	30	75
		Lower surface	TNTC	TNTC
Wide ring count SW	2	Upper surface	TNTC	TNTC
		2	TNTC	TNTC
		Center	TNTC	TNTC
		4	TNTC	TNTC
		5	TNTC	TNTC
		Lower surface	TNTC	TNTC

[a] Blocks roughly 8 cubic inches in size (ca. 131 cm^3) were cut in 0.25-inch (0.64-cm) slices. In all cases, *E. agglomerans* was the only coliform identified and was present in all of the wood sections.

[b] Numbers refer to position of cut section from upper surface (section 1).

[c] Reported as average number of green-sheened colonies appearing on m-Endo agar LES at 35°C. TNTC, Too numerous to count; NG, no growth.

TABLE 3. *Contact impressions of freshly exposed wood surfaces*[a]

Type of wood	No. of samples	No. of coliforms[b]		Coliform(s) identified
		24 h	48 h	
Redwood, HSW	2	TNTC	TNTC	*E. agglomerans*
Redwood, WSW	2	TNTC	TNTC	*E. agglomerans*
Cedar	2	NG	NG	
Cottonwood, SW	2	NG	9	*C. freundii*
Douglas fir, freshly cut SW	6	NG	2[c]	*E. agglomerans*
Douglas fir, racked SW	4	NG	10	*E. agglomerans, C. freundii*
Hemlock, SW	2	NG	1[c]	*C. freundii*

[a] HSW, Heavy sapwood; WSW, wide ring count sapwood; TNTC, too numerous to count; NG, no growth.

[b] Reported as average number of green-sheened colonies appearing on m-Endo agar LES at 35°C.

[c] Number of colonies on only impression plate showing growth.

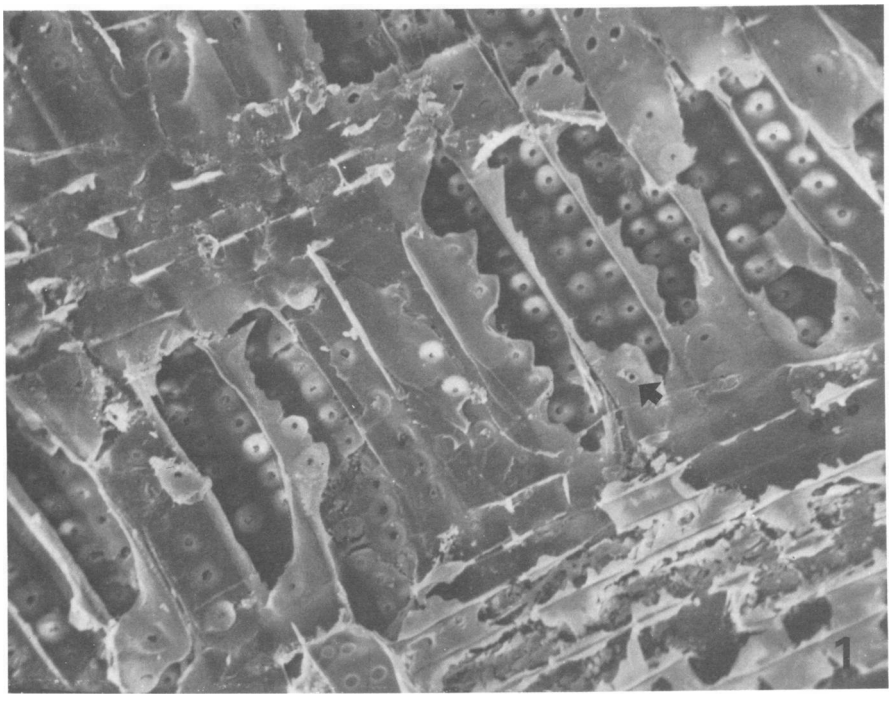

FIG. 1. *SEM (×200) of interior of redwood wide ring count SW. Tracheal tubes with bordered pits are shown. Arrow indicates portion of field enlarged in Fig. 2.*

been removed during sample preparation. Figure 2 (×1,000) is an enlargement showing numerous individual bacteria and microcolonies present on the walls and bordered pits. Bacteria of diverse morphologies, including short rods typical of coliforms, can be clearly seen in Fig. 3 (×5,000).

Further evidence that the TC and TB detected in these studies were actually within the wood itself is the fact that no bacteria (TB or TC) were detected on the cutting saw blade, table top, or other equipment after surface sterilization. Extraneous contamination, therefore, did not occur during sampling experiments.

The three genera of coliforms identified in these studies from all types of wood, *Klebsiella* spp., *Enterobacter* spp., and *C. freundii,* have few biochemical test differences from previously reported studies (14, 15). The only test reaction with possible significance was the increase in numbers of *Enterobacter* spp. able to ferment inositol. Forty-eight percent of *E. agglomerans* and 33.0% of *E. cloacae* fermented inositol, as compared with reference values of 18.3% (15) and 21.9% (12), respectively, for the two species. All of the *Klebsiella* spp. also fermented inositol.

DISCUSSION

Although there have been several reports of isolations of bacteria within wood, either living or cut (8, 9, 13, 19, 25, 26), there are few cases specifically identifying coliforms as being present (1, 8, 9). All of the coliforms identified, however, have been members of the *Klebsielleae* tribe, *Klebsiella* spp. and *Enterobacter* spp. Including *Citrobacter* spp., these three genera were most frequently isolated from tree needles and bark and from other botanical environments (11, 16, 23). *Klebsiella* spp. and *Enterobacter* spp. have also been frequently isolated from redwood water storage reservoirs (27). Because all possible outside sources of contamination were ruled out, the assumption might be made that the source of these coliforms was the redwood itself.

The high numbers of TC, *Klebsiella* spp., and *Enterobacter* spp. found in liquid extracted from the redwood SW samples (Table 1) clearly indicate that these organisms are indigenous to the wood. Coliforms also appear to be part of the normal flora of other trees such as Douglas fir, cottonwood, and hemlock. Investigators have noted heaviest bacterial contamination occurring in the SW of trees such as southern pine (9) and members of the poplar family (26). In balsam fir and black spruce, the greatest microbial activity was in the pith column, with little in the HW and none in the SW (13). Almost total absence of bacteria in redwood HW samples was

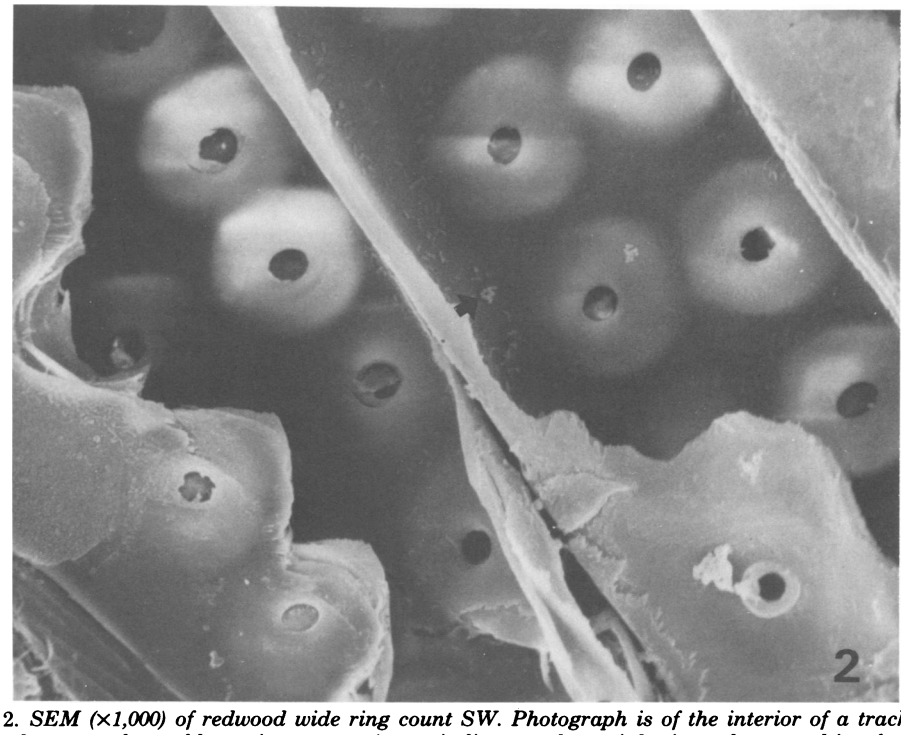

FIG. 2. *SEM (×1,000) of redwood wide ring count SW. Photograph is of the interior of a tracheal tube showing large numbers of bacteria present. Arrow indicates a bacterial microcolony resulting from in situ multiplication.*

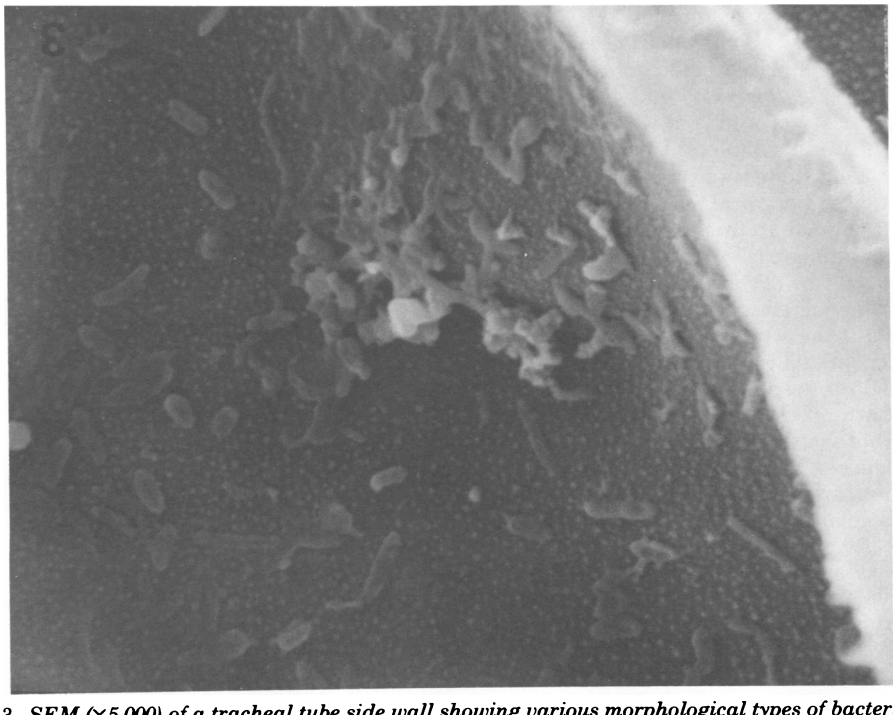

FIG. 3. *SEM (×5,000) of a tracheal tube side wall showing various morphological types of bacteria present within the redwood. Morphology of the plump rods is consistent with that of coliform bacteria.*

unexpected, since redwood water storage reservoirs are constructed exclusively of HW. It has been hypothesized, however, that bacteria in SW can progress into the HW tissue via ray cells (9, 25). Extending this theory to the source of *Klebsielleae* in redwood used for the tanks, low levels of coliforms in HW, causing water contamination, could have arisen from the high numbers of coliforms present in the SW of the same tree.

Although all redwood SW samples had recoverable coliforms, there was a broad range in cell densities (Table 1). Use of most-probable-number techniques for TC detection may have given even higher numbers of TC. These techniques, as evidenced in tests on wood other than redwood (Table 2), appear to be more sensitive in the liquid extraction experiments. The coliforms existing within some of the woods may have been in a stress situation, as evidenced by lack of growth at 24 h but green-sheened colonies after 48 h on the m-Endo agar LES (Tables 3 and 4). Prior enrichment in the lactose broth used in the most-probable-number techniques was probably the cause for better TC recovery.

There was also a variation in types of coliforms present within the redwood SW samples. Based on the results of these experiments, *Enterobacter* spp., particularly *E. agglomerans,* could be expected from any redwood SW examined. Although two species of *Klebsiella* could be isolated (*K. pneumoniae* and *K. oxytoca*), some SW apparently did not contain these organisms. *E. agglomerans* may be indigenous to a wide variety of woods; it was isolated from all types of wood sampled in this survey. This particular coliform has a history of being closely associated with botanical material and was only recently renamed from the yellow-pigmented, non-phytopathogenic *Erwinia* (15).

There could be several hypotheses as to the origin of the bacteria, and of TC in particular, found within the various wood samples. These TC are considered to be part of the normal soil microbial flora, even in "unpolluted" forest areas (8, 11). Entrance into living wood could occur through root systems and up into the trunks (25, 26). Evidence of this mode of entry is the SEM (Fig. 2), clearly showing numerous bacteria within tracheal tubes. A second method of entry into logs already felled and in contact with the soil or in water storage would be by penetration of ray cells and subsequent movement into SW and, later, HW tissues (9, 25). Some of the wood used in this survey had been cut several months before sampling (e.g., redwood in experiment 1 [Table 1] and cedar, racked Douglas fir, and hemlock [Table 2]). The greatly increased TB and TC counts of racked Douglas fir (from a woodpile) over freshly cut Douglas fir may be due to such an influx of bacteria. All other wood samples, however, were sampled within hours or days after cutting. Bacteria and TC isolated from these redwood samples were undoubtedly present within the wood itself when cut. The high numbers of TC and TB detected may have been due to additional bacterial growth after the wood was harvested.

The increase in bacterial numbers in SW and HW has been correlated with wood decay or wet wood (8, 25, 26). The wood used in the present experiments showed no evidence of decay, even though high levels of TB were detected. Previous studies with SEM have also shown bacterial destruction of pit membranes (9, 26). Although large numbers of bacteria are present in Fig. 2 and 3, alteration or destruction of pit membranes was possibly due to mechanical injury during sample preparation and not any direct microbial activity.

Klebsielleae which are able to fix atmospheric nitrogen have also been reported from within living wood (1) and in pulp mill effluents (22). Although the isolates from these studies were not tested for nitrogen-fixing capabilities, the presence of such a trait could help explain their increased numbers and survival in a high-carbon, low-nitrogen wood environment.

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