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External Microflora of a Marine Wood-Boring Isopod

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Bacteria associated with the marine wood-boring isopod *Limnoria lignorum* were enumerated by acridine orange epifluorescence microscopy and by plate counts on several media; the plate-viable bacteria were isolated and identified. Similar procedures were followed to enumerate and identify bacteria associated with the wood substrate from which the isopods were collected and with the surrounding water from the isopod habitat. Approximately 1.4×10^7 bacterial cells were associated with each individual *L. lignorum*. *Aeromonas hydrophila*, *Pseudomonas*, and *Vibrio* were the most common genera in the isopod microflora. Wood from *L. lignorum* burrows had an associated bacterial flora of 1.6×10^7 cells per mg (damp weight). *A. hydrophila* also predominated in the wood microflora. The water from which the isopod population was collected contained 2.3×10^6 bacteria per ml. *Pseudomonas* and *Vibrio* species were very common in the water microflora, but *A. hydrophila* was not detected. Interactions between the isopod, its associated microorganisms, and the microorganisms within the wood substrate are discussed in the light of the known absence of a resident digestive tract microflora in these animals.

Wood-boring isopods are ubiquitous in temperate and tropical marine habitats. They are responsible for substantial damage to equipment, structures, and boats in estuarine and coastal zones. Wood biodeterioration costs the U.S. Navy more than $200 million annually (20), and private losses are also large.

Species of *Limnoria* maintain a digestive tract which normally is free of any resident, attached microflora (6, 7, 45). An exception to the normal bacteria-free gut condition occurs in *L. tripunctata* removed from creosoted wood (58); bacteria, representative of the microflora found on aged creosote, were found within the digestive tract of these isopods.

The complete lack of a microflora in the normal *Limnoria* digestive system is unique in light of present assumptions concerning the microbiological status of animal digestive tracts (10). Conversely, the outer exoskeleton surfaces of these wood-borers are colonized densely by a diverse array of bacteria and other microorganisms (6, 7).

There have been few investigations of the microflora associated with marine invertebrates (52). This report contains the results of a study of the respective bacterial floras found in association with the exoskeleton of *Limnoria lignorum* (Rathke), with the wood from which the isopods were collected, and with the seawater in which they were found. The objective of this investigation was to determine whether a characteristic bacterial flora is associated with these isopods or with their wood substrate.

MATERIALS AND METHODS

Sample collection. Two samples of untreated pine (20 by 5 by 2 cm) were removed from the wood frame of a specimen tank which had been submerged for 7 months beneath the Marine Biological Laboratory dock in Eel Pond, Woods Hole, Mass. (salinity, 29‰). The wood contained a very active population of *L. lignorum* (Rathke), as well as the wood-inhabiting amphipod *Chelura terebrans* Philippi. No macroscopic fouling was present on the wood surface. Alcohol-rinsed (95% ethanol) latex gloves were worn during the collection of all samples. The samples were placed immediately in a polyethylene pail (prerinsed with 95% ethanol) filled with Eel Pond seawater. No attempt was made to exclude microorganisms from the air-water surface microlayer, since the wood containing the *Limnoria* population had been exposed continually to this microlayer while in situ.

Water samples were collected from the same site. Sterile Whirl-Pack bags (approximately 14 by 20 cm) were immersed in the water and held vertically 15 cm below the surface. While submerged, the perforated top was ripped from the bag and the wire mouth was opened, thus allowing the entrance of a water sample of approximately 400 ml. The bag was raised out of the water with its mouth still opened to include microorganisms from the surface microlayer. The bags were sealed with their integral wire mouths immediately upon removal from the water and were placed in a

large bucket of seawater to provide temperature control during the short trip to the laboratory. Analysis of the respective microfloras began within 30 min of *Limnoria*, wood, and water collection.

Bacterial isolation and culture media. Four solid media were utilized for the initial isolation and enumeration of plate-viable, heterotrophic aerobic bacteria. Except for the first (listed below), the media were made in artificial seawater, which consisted of NaCl, 24.0 g; $MgCl_2 \cdot 6H_2O$, 5.3 g; $MgSO_4 \cdot 7H_2O$, 7.0 g; KCl, 0.7 g; and glass distilled water, 1 liter.

The media were: (i) marine 2216 agar (Difco Laboratories, Detroit, Mich.) (2216 agar); (ii) marine standard methods agar (BBL Microbiology Systems, Cockeysville, Md.) made with artificial seawater (MSM agar); (iii) one-tenth strength MSM agar made with full-strength artificial seawater (MSM^{-1} agar); and (iv) natural seawater agar consisting of aged (2 months), unfiltered seawater from Nahant, Mass., plus agar (NSW agar). All media were sterilized by autoclaving and contained a final concentration of 1.5% agar.

After the initial isolation, bacteria were purified and maintained on plates and slants on 2216 agar incubated at room temperature (18 to 22°C).

Specimen preparation and bacterial plating. All dissection equipment, tissue grinders, and petri dishes were sterile; all fluids were sterilized by filtration (0.22-μm sterile Millex filters [Millipore Corp., New Bedford, Mass.]). Specimens of *L. lignorum* were removed from the wood by dissection under a Wild M-8 zoom stereomicroscope. The animals were placed in 20 ml of sterile seawater in a petri dish. The animals were allowed to swim in the filtered seawater for 10 min to remove bacteria that were not attached permanently to the exoskeleton.

After the sterile rinse, single *L. lignorum* individuals (1.5 to 3 mm, approximate length) were placed in each of 10 conical glass tissue grinders (3 ml nominal volume; Bellco Glass, Vineland, N.J.) containing 2.0 ml of filtered sterilized NSW collected at Woods Hole. The animals were homogenized for 2 min (clearance in the cylindrical portion of the grinder was 102 to 152 μm). Samples were ground slowly with frequent 5-s rests to prevent excess frictional heating of the sample. After homogenization, three serial 10-fold dilutions of each *L. lignorum* homogenate were made, using sterile artificial seawater with 0.1% (wt/vol) peptone as dilution water. A 0.5-ml subsample of the homogenate remaining in each tissue grinder was transferred to a separate glass vial containing 4.25 ml of filtered (0.22 μm) seawater and 0.25 ml of filtered (0.22 μm) 40% Formalin. These preserved samples were retained at 5°C for epifluorescence direct counting of bacteria.

Samples (0.1 ml) of each homogenate and its respective 10^{-1}, 10^{-2}, and 10^{-3} dilutions were inoculated individually onto duplicate plates of the four agar media (Fig. 1). Inocula were spread over the agar surface with an alcohol-flamed glass rod, using a standard petri dish turntable. After inoculation, plates were covered with their sterile tops, inverted, and incubated at 20°C.

During the aseptic collection of *L. lignorum*, three small samples of the heavily bored pine habitat were removed with a sterile scalpel and forceps. Each sample was taken from within a separate isopod burrow, but no *L. lignorum* individuals were included. The three wood samples were weighed in separate, sterile, preweighed aluminum foil envelopes. After weighing, the wood samples were homogenized in 2.0 ml of filter-sterilized NSW, as described for *L. lignorum* individuals. A 0.5-ml sample of each homogenate was preserved for acridine orange direct counting (AODC) as described. Samples (0.1 ml) of each 10^{-2}, 10^{-3}, and 10^{-4} dilutions were spread plated in duplicate on each of the four media, as described for isopod homogenates (Fig. 1).

The water samples were shaken to distribute the contents evenly within the Whirl-Pack bags. A 1.0-ml sample was removed aseptically from each of four sampling bags with separate sterile 1.0-ml pipettes. Three serial 10-fold dilutions were prepared from each water sample. Samples (0.1 ml) of the 10^{-1} to the 10^{-3} dilutions were spread plated in duplicate on each of the four media (Fig. 1). A 0.5-ml sample of each 10^{-1} dilution was preserved for AODC, as described.

Plates were stored in sealed plastic bags during incubation to prevent drying. Colonies were counted on the appropriate plates after a 3-day incubation and again after 10 days. The NSW plates were incubated for 1 month before colony counts were made to insure that all plate-viable cells had formed colonies. Colonies on the NSW plates were extremely small, and even after 30 days of incubation counting required the aid of a Wild-M8 zoom dissecting microscope at approximately ×40 magnification. The NSW plates were utilized for enumeration purposes only; no isolates were collected from these plates.

Epifluorescence enumeration. Preserved samples for AODC were stored at 5°C and were analyzed within 1 month. Methods for AODC were the same as those developed by Hobbie et al. (26).

Counts of AO-stained cells were made with an Olympus Vanox research microscope equipped with a 200-W mercury burner, a fluorescein isothiocyanate exciter filter, and a BG-12 exciter filter, a DM-500 dichroic mirror, an 0-515 filter, and a Y495 barrier filter. Fields for counting were obtained by random movement of the microscope stage from its initial, near-central location; stage movements were carried out while the operator's eyes were closed, to eliminate bias in the choosing of fields for counting. Two filters were prepared for each sample, and twenty fields were counted on each filter.

Selection and identification of bacterial isolates. Bacterial isolates were collected from all individual *L. lignorum*, gathered from widely separated burrows in two different wood samples, to provide information representative of the *L. lignorum* population as a whole.

The large number of colonies that grew on the spread plates precluded the isolation of every colony for identification. Since many bacteria have nearly identical colony appearance on agar plates, isolates for identification were selected as follows. For the first 2 of the 10 *L. lignorum* individuals that were sampled, virtually all of the bacterial colonies on the countable plates were isolated and purified for identification, including many that looked identical, to insure that none were overlooked simply because they had the morphology and color of another colony. Several col-

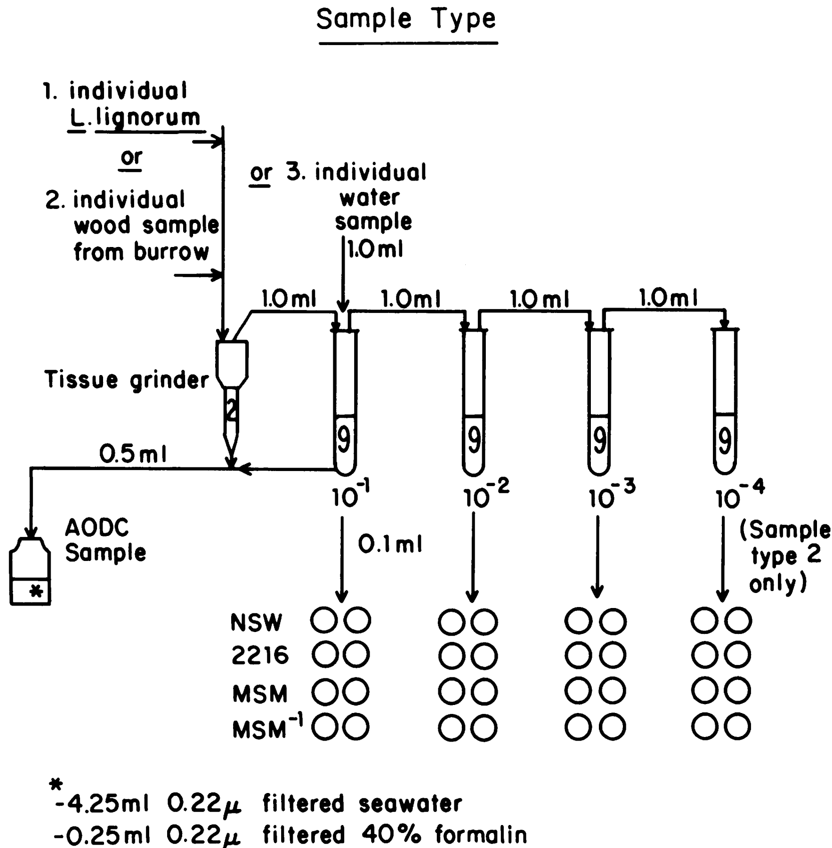

FIG. 1. *Sampling of bacterial microflora associated with the wood-boring isopod L. lignorum, wood from L. lignorum burrows, and water from the L. lignorum habitat. Numbers within tubes indicate volume of homogenization or dilution fluid.*

ony types had a very distinctive morphology and color pattern, and for these, obvious duplicates were not isolated repeatedly. Additional bacterial isolates were collected with the intent of gathering the full range of plate-viable bacterial types present. Consequently, the plates from the remaining eight *L. lignorum* were searched for obviously different colony types, and these were isolated and purified for identification.

Bacterial isolates from the wood and water sample plates were collected by the same methods outlined for isolate collection from *L. lignorum* plates. That is, almost all colonies were isolated from the appropriate plates of the first sample in a given series regardless of apparent duplication (e.g., almost all colonies from the first wood sample and the first water sample); plates from the remaining samples in a given series served to provide isolates representative of the range of plate-viable bacteria associated with the type of sample under study.

Isolates were collected on 2216 agar from distinct, well-separated colonies. Once isolated, bacteria were purified by streaking on 2216 agar plates. Cultures were Gram stained and observed microscopically for determination of cell morphology and cell arrangement. It was possible to place gram-positive isolates in a genus or major group with only a few additional tests. These included cell morphology parameters and tests for motility, pigment production, production of acid or gas when grown on glucose, the presence of cytochrome oxidase and catalase activity, and spore production. Gram-negative isolates were identified with the use of: (i) the API 20E system (Analytab Products, Plainview, N.Y.); (ii) Oxiferm tubes (Roche Diagnostic Laboratories, Nutley, N.J.); and (iii) additional, standard bacteriological tests not included in, but in some cases duplicating, the previously mentioned systems (8, 16, 33). Additional, peripheral tests included oxidase and catalase activity, motility, salt tolerance and requirement, growth on MacConkey agar, nitrate reduction, denitrification, agar liquefaction, utilization of lactose, pigment production, and duplication of tests contained in the prepackaged systems using laboratory-prepared media. Isolates that exhibited a salt requirement were suspended in 3.0% NaCl, rather than 0.85% NaCl, for inoculation into the API 20E system.

Comparison of the biochemical and morphological characteristics of the isolates with data and identification schemes contained in *Bergey's Manual of Determinative Bacteriology* (8), the *Color Atlas and*

Textbook of Diagnostic Microbiology (34), and *Sea Microbes* (49) allowed identification to the generic level for most isolates and to the species level for many. Several additional references aided in the identification of the bacterial isolates (2-4, 11, 14, 21, 32, 35, 47).

RESULTS

Bacteria associated with the isopod exoskeleton, the wood burrows, and the surrounding water were enumerated, isolated, and identified to allow a comparison of the plate-viable fraction of the respective microfloras.

Bacterial enumerations. The 2216 agar and the NSW agar gave nearly identical counts for all samples (Table 1). In all cases, both MSM and MSM^{-1} agars gave counts that were 10 to 100 times lower than those obtained with 2216 or NSW agar. The MSM agar and the MSM^{-1} agar had the additional drawback of drastically reduced colony diversity when compared with the NSW and the 2216 media. MSM and MSM^{-1} plates routinely showed only one or two colony types. For the reasons of lower numbers and reduced diversity, no isolates were collected from the MSM and MSM^{-1} plates. (The very few colony types seen on these plates also occurred on 2216 agar, and they were identified from that medium.)

The mean number of plate-viable bacteria associated with an individual *L. lignorum* was 1.7×10^5 ($\pm 0.9 \times 10^5$; 95% confidence level [conf]) on 2216 agar and 1.5×10^5 ($\pm 0.6 \times 10^5$; 95% conf) on NSW agar. These means share the same population variance ($F_{0.05(9,9)} = 4.03$; $F_{sample} = 2.10$), and in all cases (Table 1) there was no significant difference (95% conf) between the means for 2216 agar and for NSW agar (Student *t* tests and the Wilcoxon nonparametric signed rank test). Conversely, counts obtained with MSM and MSM^{-1} media did not share the same population variance with 2216 agar (*F* test), and Behrens-Fisher tests (for samples with different population variance) indicated that the mean for 2216 agar was significantly different (i.e., larger) from the means for MSM and MSM^{-1} agar (51); the significance of this difference was confirmed at the 1% level by the Wilcoxon nonparametric signed rank test (51).

Previous research (9) had indicated that higher counts might result with the NSW medium. However, the 2216 agar yielded counts that were statistically equivalent to those obtained with NSW agar. Colony development on NSW agar took over 1 month, and many colonies were extremely small, necessitating the use of a dissecting microscope for counting. All bacterial isolates for identification were collected from the 2216 agar plates. Isolates were not collected from NSW agar since the colonies on these plates were over 1 month old when they were ready for counting.

TABLE 1. *Enumeration of bacteria in L. lignorum individuals, wood samples, and water*

Medium[b]	No. of bacteria (CFU)[a] in: Individual *L. lignorum*	Wood[c] from *L. lignorum* burrow (per mg of wood)	Water from *L. lignorum* habitat (per ml of water)
2216	1.7×10^5 (1.23×10^5)	2.4×10^5 (0.42×10^5)	5.4×10^4 (1.45×10^4)
NSW	1.5×10^5 (0.85×10^5)	2.1×10^5 (0.32×10^5)	2.0×10^4 (1.16×10^4)
MSM	1.2×10^3 (0.51×10^3)	9.4×10^3 (0.72×10^3)	5.6×10^3 (2.75×10^3)
MSM^{-1}	1.2×10^3 (0.67×10^3)	3.3×10^3 (2.26×10^3)	4.4×10^3 (1.03×10^3)

[a] CFU, Colony-forming units. Counts shown are means (from *L. lignorum*, $n = 10$; *L. lignorum* wood, $n = 3$; *L. lignorum* habitat water, $n = 4$) of duplicate plate count ($n = 2$) means. Values in parentheses are standard deviation.

[b] See text, Bacterial isolation and culture media.

[c] Wood sample actual weights were 8.7, 7.3, and 8.6 mg.

Samples of seawater collected from the *L. lignorum* habitat contained 5.4×10^4 ($\pm 2.3 \times 10^4$; 95% conf) plate-viable bacteria per ml on 2216 agar (Table 1).

Samples of wood from *L. lignorum* burrows contained 2.4×10^5 ($\pm 1.0 \times 10^5$; 95% conf) plate-viable bacteria per mg on 2216 agar (Table 1).

AODC was carried out on a limited basis due to problems of bright background fluorescence of wood and animal tissue. AODC enumerations were conducted on two of the *L. lignorum* samples, two of the water samples, and two of the wood samples (Table 2). It is interesting, but not surprising, that in each case the AODC enumeration indicated a bacterial density approximately two orders of magnitude higher than that obtained by plate counts.

The enumeration data are summarized in Table 2, together with a normalization of bacterial density per unit weight for each sample type. Although this normalization is a somewhat artificial manipulation of the enumeration data, it provides a comparison of the relative bacterial densities of the various samples.

Bacterial identification. We compared the predominant heterotrophic, aerobic bacteria living on the exoskeleton of *L. lignorum* with the species distribution in the wood habitat and the surrounding water. The great majority of bacteria isolated from all samples and specimens in this study were gram-negative rods. Four generic groups comprised the predominant taxa, includ-

TABLE 2. *Bacterial enumeration summary and normalized bacterial densities of the bacterial floras associated with L. lignorum, wood samples, and water samples*

Sample type	Bacteria (CFU) per individual sample[a] determined by:		Bacteria (CFU) per g of sample[b] determined by:	
	Plate count[c]	AODC[d]	Plate count	AODC
L. lignorum ($\bar{x}$ = 0.15 mg/isopod)[e]	1.7×10^5	1.4×10^7	1.1×10^9	9.3×10^{10}
Wood from *L. lignorum* burrows (sample = 1 mg)	2.4×10^5	1.6×10^7	2.4×10^8	1.6×10^{10}
Eel Pond water (1-ml sample)	5.4×10^4	2.3×10^6	5.4×10^4	2.3×10^6

[a] Data summarized from Table 1 and text. CFU, Colony-forming units.
[b] Normalization per gram of sample calculated from respective counts obtained by plate count and AODC.
[c] Difco 2216 agar.
[d] Counts shown are means (n = 2) of individual sample means (n = 20).
[e] n = 26.

ing *Aeromonas*, *Pseudomonas*, *Vibrio*, and *Acinetobacter*. However, these genera did not occur with equal frequency in all of the sampled microfloras.

Aeromonas hydrophila predominated in the microflora associated with *L. lignorum* and with wood from *L. lignorum* burrows (Table 3). However, *A. hydrophila* was not found in any of the water samples collected from the *L. lignorum* habitat in Eel Pond (Table 3).

Pseudomonas species were numerous in all samples, and they predominated in the seawater samples collected from the *L. lignorum* habitat. *P. fluorescens* appears to be the most common species in this group, followed by *P. paucimobilis*, *P. stutzeri*, *P. cepacia*, *P. putrefaciens*, and 15 as yet identified (and not necessarily different) *Pseudomonas* species.

Vibrio species also occurred commonly in all of the sampled microfloras. *V. alginolyticus* was found in all samples.

Acinetobacter species occurred in all samples. *A. calcoaceticus* var. *anitratus* occurred only in the wood microflora and was a very common

TABLE 3. *Bacteria associated with L. lignorum exoskeleton, wood from L. lignorum burrows, and water from the L. lignorum habitat*[a]

Relative frequency of occurrence[b]	Bacteria associated with *L. lignorum*[a] exoskeleton	Bacteria associated with wood[a] from *L. lignorum* burrow	Bacteria associated with water[a] from *L. lignorum* habitat
M.P.O.	*A. hydrophila* (8)[c]	*A. hydrophila* (1)	*Pseudomonas* species (4), including *P. fluorescens* (1), *P. paucimobilis* (2), unidentified spp. (1)
V.C.O.	*Pseudomonas* species (12), including *P. fluorescens* (3), *P. paucimobilis* (2), *P. stutzeri* (1), *P. diminuta* (1), unidentified spp. (5)	*A. calcoaceticus* var. *anitratus* (1)	*Vibrio* sp. (1)
C.O.	*V. alginolyticus* (3), *Aerococcus* spp. (6)	*V. alginolyticus* (1), *Pseudomonas* spp. (3), *Bacillus* sp. (2)	*F. halmephilum* (1)
Also	*A. calcoaceticus* var. *lwoffi* (2), *Erwinia* sp. (1), *Micrococcus* sp. (2), *Planococcus* sp. (1), *Arthrobacter* sp. (2), *Alcaligenes* sp. (2), CDC group IV-C *Alcaligenes*-like (1),[d] *Hyphomicrobium*-like species (2), *Actinomyces* sp. (2)	*Arthrobacter* sp. (4), *Micrococcus* sp. (1), *Planococcus* sp. (1), *Actinomyces* sp. (1)	*A. calcoaceticus* var. *lwoffi* (1), *C. diffluens* (1), *Planococcus* sp. (1), *Bacillus* sp. (1), *Actinomyces* sp. (1)

[a] Collected at Eel Pond, Woods Hole, Mass., habitat of *L. lignorum*.
[b] Relative frequency of occurrence on 2216 agar plates: M.P.O. = most predominant organism (40 to 50% of total number of isolates); V.C.O. = very common organism (30 to 40% of total number of isolates); C.O. = common organism (10 to 20% of total number of isolates); also = one or two isolates only.
[c] Numbers in parentheses indicate the number of identified isolates that belonged to a particular generic grouping or unidentified class.
[d] CDC = Centers for Disease Control, Atlanta, Ga.

organism in this habitat. *A. calcoaceticus* is the only species designation within this genus. Two subspecies are recognized presently, *A. calcoaceticus* var. *lwoffi*, which is non-saccharolytic and is recognized as being the more common strain, and *A. calcoaceticus* var. *anitratus*, which is oxidatively saccharolytic on glucose (34). *A. calcoaceticus* var. *lwoffi* occurred infrequently in association with *L. lignorum* and in water samples from the *L. lignorum* habitat, but was not found in specimens of wood.

Gram-positive bacteria were isolated from all specimens sampled in this study. The gram-positive organisms were much less common in water samples from the *L. lignorum* habitat than in the microfloras associated with the isopods and the burrow wood. *Aerococcus* species were common in the *L. lignorum* flora. Other gram-positive cocci, including *Micrococcus* spp. and *Planococcus* sp., occurred also in association with *L. lignorum* and with the wood from *L. lignorum* burrows. Gram-positive coryneform bacteria were isolated from the isopod and wood microfloras where they occurred with low to moderate frequency. These isolates were assigned to the genus *Arthrobacter* on the basis of their being gram-positive, pleomorphic, nonfermentative rods. *Arthrobacter* species are among the dominant bacteria in soils (8), and their occurrence in a shallow tidal pond, like Eel Pond in Woods Hole, is not surprising. Gram-positive rods in the genus *Bacillus* were common in the wood microflora and less common in the water samples. *Bacillus* species were not found in the isopod microflora.

Additional bacteria occurring in low numbers included the gram-negative species *Erwinia* and *Alcaligenes*, in association with *L. lignorum*; the strongly agarolytic *Cytophaga diffluens* in water samples from the *L. lignorum* habitat; and *Flavobacterium halmephilum*, which occurred commonly in water samples. Two isolates of a distinctive, prosthecate bacterium were collected from the *L. lignorum* microflora. These isolates were assigned to the genus *Hyphomicrobium* on the basis of their very characteristic morphology (8).

Actinomycetes and yeasts occurred frequently in association with all of the samples collected for this study.

The major bacterial groups associated with each sample type are summarized in Table 3.

DISCUSSION

The quantitative and qualitative study of the surface bacterial population of *L. lignorum* and its wood substrate was designed to provide an insight into the comparative microbial ecology of these closely associated microbial habitats.

The bacterial enumerations in this study were conducted to provide information regarding the total microflora associated with *L. lignorum* and its wood substrate. The bacterial identifications, carried to the generic and, where possible, the species level, were carried out to provide a taxonomic and nutritional description of the platable portion of the total aerobic, heterotrophic bacterial population. These identifications were intended as a basis for comparison between the animal, wood, and water microfloras, to determine whether these wood-boring isopods possess a specific flora which is different from that of the surrounding environment, with the realization that the identified bacteria are those adapted for growth on plates.

The single most noticeable finding in this study was the clear predominance in the isopod and wood microfloras of *A. hydrophila*, an organism which was not found in any of the water samples from the isopod habitat.

A report on the occurrence of *A. hydrophila* in limnetic environments (48) suggested that this species may not be indigenous to the marine environment. Another recent study (31) showed that, although *A. hydrophila* occurs frequently in low-salinity estuarine waters, numbers of *A. hydrophila* were consistently low in waters with a salinity above 15‰. Data collected in the present study suggest that, although *A. hydrophila* may not occur commonly in marine water samples, it does enter into close association with marine wood-boring isopods.

The predominance of *A. hydrophila* in the microflora associated with wood-boring isopods and their burrows is a clear departure from the data of previous studies, in which *Aeromonas* species have been found to occur in relatively low numbers in association with invertebrates compared to *Pseudomonas* and *Vibrio* species (30, 52).

Interest in *A. hydrophila* has increased during the past few years since this potential pathogen (17) has been isolated from uncontaminated freshwater supplies, well water (36), and seawater (34), as well as sewage-contaminated water (8). In humans, *A. hydrophila* can cause transient diarrhea, wound infections, and rarely, septicemia (34), as well as meningitis, gastroenteritis, and peritonitis (17). This species is also a pathogen of snakes and causes red leg disease in frogs and infections in freshwater fish (8, 34), including salmon (49). However, *A. hydrophila* is not always a pathogen. The gut flora of salmonids is predominated by *Aeromonas* spp. when the fish are in freshwater (57), and in clam larval cultures (species unnamed) sickness can be associated with a drop in numbers of *Aero-*

monas and other bacterial species (36). Kaneko et al. (30) showed that *A. hydrophila* was nonpathogenic to the soft-shell clam *Mya arenaria*. *Aeromonas* sp. occurs in the gut of the sea urchin *Echinus esculentus* under normal circumstances (52), and *A. hydrophila* is associated with the freshwater green hydra (*Hydra viridis*; 5). *Bergey's Manual* (8) states that *Aeromonas proteolytica* (now designated as a subspecies of *A. hydrophila* [8]) was isolated originally from the alimentary tract of *L. tripunctata* (39, 40). This original isolate of *A. proteolytica* [sic] was most probably a contaminant from the exoskeleton microflora, since *Limnoria* species now are known to maintain a digestive tract that is free of bacterial colonization (6, 7, 45, 46).

In contrast to the absence of a resident microflora within the digestive tract, the marine wood-boring isopods possess a dense microflora on their external exoskeleton surface (6, 7). Attached microorganisms are particularly numerous on the posterior appendages, the pleopods (6, 7), which function in swimming, respiration, and excretion (43) (Fig. 2).

The bacteria found in association with *L. lignorum* quite possibly benefit in several ways from attachment to the pleopod surfaces, where exoskeleton bacterial density is highest (Fig. 2). These bacteria may utilize waste products released by the animal and water-soluble mono- and disaccharides released through the action of the cellulolytic flora found within the burrow system. Furthermore, *A. hydrophila* may be well adapted to maintaining a predominant position in the *L. lignorum* microflora, owing to its ability to produce enzymes that lyse a variety of other bacteria (1).

Ammonium is the major nitrogenous waste product of marine, freshwater, and terrestrial isopods (18, 25, 33, 54–56). These isopods release a large portion of their nitrogenous wastes across the respiratory surfaces (54). Bacteria attached to the respiratory appendages may utilize isopod wastes transported across the pleopod surfaces. The large number of bacteria associated with *L. lignorum* (Table 1), the predominance of *A. hydrophila* in the *L. lignorum* microflora (Table 3), and the especially dense colonization of rod-shaped bacteria on the pleopods of *L. lignorum* (6, 7, 50, and Fig. 2) together suggest that *A. hydrophila* occurs in large numbers on the surface of the pleopods. This suggestion is supported by the knowledge that *A. hydrophila* is able to utilize ammonium as a sole nitrogen source (8, 40). The association of *A. hydrophila* with *L. lignorum* pleopods is currently being studied in our laboratory by fluorescent-antibody techniques.

Limnoria individuals groom their appendages by passing them over the mouth parts (53); grooming of the pleopods may result in the ingestion of bacteria. The dense external microflora (Fig. 2) may represent a type of mixed, continuous culture that is provided with a surface for attachment and is supported by the animal wastes, while it is maintained in an actively growing state and prevented from overpopulation by the isopod grooming activities. Such a relationship could provide a conservative, closed-loop recycling of at least a portion of the dietary nitrogen, allowing subsistence on relatively nitrogen-poor food materials, such as the wood on which *Limnoria* feed. Experiments are in progress to determine whether such a recycling via the external microflora occurs.

Other bacteria that were common in the isopod and wood microfloras include *Vibrio*, *Pseudomonas*, and *Acinetobacter* species (Table 3), and these have been shown to hydrolyze cellulose (1, 8, 19, 27, 28, 44, 49). In addition to their cellulolytic capabilities, some *Vibrio* strains are known to hydrolyze chitin, the main component of isopod and other crustacean exoskeletons (11, 49).

Vibrio species have been isolated from a wide range of marine samples. Members of this genus were common associates of *L. lignorum*, and they occurred frequently in the isopod burrows, as well as in the water samples. *Pseudomonas* species and *Vibrio* species were the most common bacteria associated with the Pacific oyster, *Crassostra gigas* (12, 13), and similar findings were reported for the American oyster, *C. virginica* (38, 41, 42). Similar data were reported in a study of the bacterial associates of flatfish (37). *Vibrio* species are known to predominate in salmonid digestive tracts when the fish are in seawater (57), and *Vibrio* and *Pseudomonas* species are common gut inhabitants in mullet (22–24). In addition, *Vibrio* spp. have been found in the hemolymph of the blue crab, *Callinectes sapidus* (15), and are known to occur commonly in seawater (2), in association with copepods (29), and in coral mucus (19).

The presence of *A. calcoaceticus* var. *anitratus* (capable of oxidative utilization of glucose) among the common wood bacteria suggests the presence of soluble cellulose breakdown products within the wood matrix, possibly released by bacterial and fungal extracellular cellulolytic enzyme action and available to the microbial community in general and, perhaps, the boring isopods. It is interesting that *A. hydrophila*, *Pseudomonas* spp., *Vibrio* spp., and *Acinetobacter* spp., the four most common genera found in association with the marine wood-boring isopods and their burrows, all grew on MacConkey agar, which contains surface-active agents that

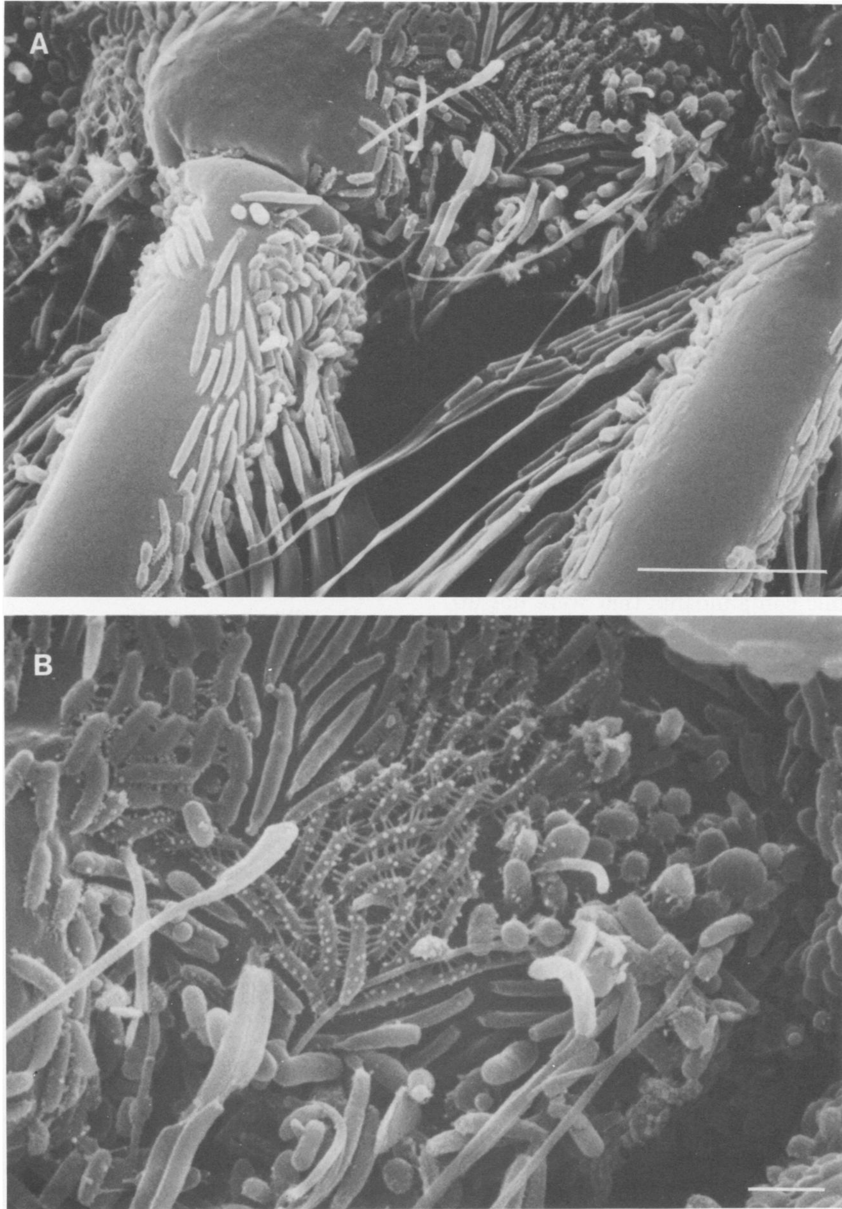

FIG. 2. *Scanning electron microscope view of the dense bacterial colonization typically found on the external exoskeleton surfaces of the posterior respiratory appendages (pleopods) of L. lignorum. (A) Low-magnification view showing the hair-covered spines that project from the edge of a pleopod blade and many attached bacteria (scale bar = 5 μm). (B) High-magnification view of bacteria on the surface of a pleopod blade, showing a diversity of cell morphologies and clear evidence of the extracellular polymer that aids in bacterial attachment (scale bar = 1 μm).*

inhibit the growth of many bacteria. The isopod digestive tract contains surface-active agents (unpublished data), and the release of these substances in fecal material may apply selection pressure for a microflora able to cope with surfactants.

The relatively low number of gram-positive bacteria found in all samples of this study is not surprising. The great majority of bacteria isolated from marine habitats are gram negative (4, 49). The exceptions to this rule are found in sediments and the marine surface microlayer,

where relatively high concentrations of organic matter occur.

It appears from data collected in this study that 2216 agar is a good medium for enumerating and isolating plate-viable marine, aerobic, heterotrophic bacteria. Bacterial numbers detected with 2216 agar were the same as with NSW agar. However, colonies grew much more rapidly on 2216 agar, facilitating the isolation of viable bacteria. Bacteriological agar contains, in addition to the cold-water-insoluble neutral galactans, numerous soluble polysaccharides, including pyruvated galactans and sulfated galactans, which marine bacteria reportedly utilize easily (49). It is surprising that colony formation on the NSW medium continued for over 1 month, during which time new colonies appeared regularly and grew very slowly. Although it contained undefined constituents, the NSW agar obviously presented a very different nutrient level than the 2216 agar. Apparently, the NSW agar contained either a very low concentration of nutrients or a set of organic contaminants that were not utilized readily by the bacteria under study. Further research is needed to determine whether or not 2216 agar yields the same diversity of bacterial types as NSW agar and other media.

The number of viable cells in all samples was approximately two orders of magnitude lower than the number of total cells detected by the AODC method. This 100-fold difference between total cell count and viable cell count is common in microbiological enumeration studies. Research on media modification may allow the laboratory growth of a greater proportion of the total flora present in sampled materials.

Limnoria boring activity greatly increases the surface area within the wood matrix. The extensive surface area within the burrows is made available to colonization by cellulolytic and noncellulolytic bacterial and fungal species. The complex network of the burrow system excavated by *Limnoria* species increases the availability of native cellulose to the burrow microflora. This flora is, in turn, available for grazing by *Limnoria* species as they move within the burrow system. The microflora of the burrow may represent an important source of available nitrogen for *Limnoria* spp. as they feed on a carbon-rich substrate that contains very little native nitrogen.

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