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Bacteria Associated with the Surface and Gut of Marine Copepods

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Little is known about the nature of bacteria associated with the surface and gut of marine copepods, either in laboratory-reared animals or in the natural environment. Nor is it known whether such animals possess a gut flora. The present report deals with studies of microorganisms isolated from healthy, laboratory-reared copepods of the species *Acartia tonsa* Dana, from several species of wild copepods collected from a marine or estuarine environment, and from laboratory dishes containing moribund copepods. Evidence for a unique gut flora in laboratory-reared animals is presented; the predominant bacteria were represented by the genus *Vibrio*. Other organisms such as *Pseudomonas* and *Cytophaga* were found less abundantly associated with the copepods and not specifically associated with the gut.

Acartia tonsa Dana, an estuarine and neritic calanoid copepod, has proved to be useful as an experimental animal in standardized laboratory culture (25). However, little is known about the effects of associated microorganisms on such marine invertebrates, or, in fact, about the actual associations themselves, either in the natural environment or in laboratory cultures. Ecological relationships and changes in such relationships that might occur when wild copepods are brought into culture should be understood, especially if there is a unique association between copepods and bacteria, such as symbiosis or commensalism. Mass mortalities frequently observed in laboratory culture of copepods may be caused by specific pathogens or opportunistic bacteria, but little is known about bacterial pathogens infecting copepods. In addition, the gut flora of the copepod may confer protection on the animals, but until the existence of a commensal gut flora in the copepod is established, such an hypothesis cannot be proven.

Research for the project reported here was initiated on a cruise in the Gulf of Mexico aboard the U.S.N.S. MIZAR. Several species of copepods were collected at four different stations, including sites in the open ocean, off the continental shelf, and in the Mississippi Delta. Samples were also collected in the Anclote River, Tampa, Fla.; in Bayboro Harbor, Bayboro, Fla.; and from copepod cultures at the Naval Research Laboratory, Office of Naval Research, Washington, D.C. Cultures of bacterial isolates were preliminarily grouped by selected biochemical tests, and strains comprising the predominant flora were subjected to numerical taxonomy. The relationships noted between bacteria and copepods and the evidence indicating the existence of a unique gut flora in *A. tonsa* Dana reared under defined laboratory conditions are reported here.

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MATERIALS AND METHODS

Sample collection. A total of ca. 50 water and 50 copepod samples were collected. Samples were obtained at 1 m below the surface at four stations in the Gulf of Mexico, located as follows: station 1—Lat. 27°00'N, Long. 86°00'W; station 2—Lat. 27°30'N, Long. 88°30'W; station 3—Lat. 29°19'N, Long. 88°30'W; and station 4—Lat. 28°35'N, Long. 89°00'W, in the Atlantic Ocean. Surface water samples were also collected from the mouth of the Anclote River, Tampa, Fla. and off the seawall at Bayboro Harbor, Fla. Approximately 20 water samples were taken from the tanks in which copepods are maintained in continuous culture at the Naval Research Laboratory by methods previously documented (10, 25, 26). Approximately 12 water and copepod samples from copepod culture dishes showing animal mortality and presence of slimes and/or ropy filaments attached to dead or dying copepods were also included in the analyses. Microscopic examinations were made of live and dead copepods for epibionts or parasites (i.e., dinoflagellates, fungi, or peritrichous ciliates), but none were observed in the copepods examined.

Processing of samples. Water was collected with a sterile Niskin sampler (General Oceanics, Miami, Fla.). Fifty-milliliter water subsamples were filtered through 0.22-μm membrane filters (Millipore Corp., Bedford, Mass.). Duplicate filters were each placed on

TCBS agar (BBL Microbiology Systems, Cockeysville, Md.), on mycology agar (Difco Laboratories, Inc., Detroit, Mich.) made up in distilled water or 80% seawater, and on 2216 agar (Difco). In the field, copepods were collected by plankton tow, using a 0.5-m, 250-μm-mesh plankton net during normal trawling operations at approximately 2 knots. Three copepods of each species were washed twice in 1.0 ml of sterile sea water, and the second wash was plated on the media listed above. Bacterial isolates from these specimens were termed "copepod surface isolates." The copepods were washed a third time, the wash was discarded, and the animals were ground in 1.0 ml of sterile seawater in a tissue grinder. Homogenized copepod tissue was plated on the media listed above. Bacterial isolates from these samples were termed copepod "gut-surface" isolates, since there was no assurance that such isolates truly represented gut flora alone. It was felt, however, that differences among isolates from gut-surface specimens and those from surface specimens might indicate which strains most probably were of gut origin. The question was answered, in part, by experiments involving aseptic removal of the entire gut from laboratory-reared copepods (see below).

Identification of copepods. Wild copepods were identified on board ship by members of the Marine Science Institute of the University of South Florida. Copepod species so identified included *A. tonsa*, *Pontellopsis regalis*, a species of *Pleuromamma*, *Labidocera aestiva*, and *Centropages furcatus*.

Copepod gut dissections. The preliminary work with washes of copepods showed that aseptic dissection of copepod gut needed to be examined. Thus, gut samples were prepared by aseptic dissection of laboratory-reared *A. tonsa*. Dissection was performed on healthy adult copepods, employing sterile technique. Animals were each embedded head first in marine agar 2216. The entire gut was removed by hooking a fine (3.0-mil; ca. 0.076-mm) sterile tungsten wire embedded in a glass rod into the caudal rami. The rami were dissected away with another sterile wire, leaving the gut entirely free. The gut was macerated with a sterile bacteriological loop and plated on TCBS and marine agar 2216 (see below).

Media employed for total viable counts. Total counts of *Vibrio*-like organisms were made on TCBS. Total viable counts of aerobic, marine, heterotrophic bacteria were made on marine agar 2216. Counts of fungi were made on mycology agar (Difco) by employing duplicate plating procedures. One set of plates were made up with distilled water, and the other contained 80% filtered seawater and 20% distilled water. The seawater medium was employed for the purpose of isolating fungi requiring seawater for growth.

Cultures were picked from all plates, with only representative strains picked from TCBS agar, since the purpose of picking colonies from TCBS was to verify the selection for *Vibrio* by this medium and the selection of colonies from the other media was to establish the generic distribution of the bacterial flora associated with the copepod. A total of 329 isolates were collected, of which 93 were subjected to numerical taxonomy analysis.

Biochemical tests on bacterial isolates. Bacterial isolates were tested for reaction in Hugh and Leifson medium (MOF, Difco) with glucose. Moeller medium (Difco) was used to determine arginine dihydrolase, ornithine, and lysine decarboxylase reactions. Requirement for sodium chloride for growth was determined by inoculating T broth medium consisting of 1.0% (wt/vol) Trypticase (Difco), 0.2% (wt/vol) yeast extract (Difco), and none, 3% (wt/vol), 7% (wt/vol), or 10% (wt/vol) NaCl added. Kovac's oxidase test (N-discs, BBL) was performed on 18-h cultures grown on marine agar 2216 (Difco).

API 20 strips (Analytab Products, Inc., Plainview, N.Y.) were used, with a three-salt solution (7) as the diluent. Lecithinase tests were done on marine agar 2216, and lipase activity was also determined (7). Chitinoclastic activity was assessed by the method of Skerman (21) on marine agar 2216.

Antibiotic susceptibility tests. Antibiotic sensitivity tests were performed using antibiotic disks (BBL) placed on seeded marine agar 2216 plates. Antibiotics tested included tetracycline (Te_{30}), bacitracin (B_{10}), erythromycin (E_{15}), terramycin (T_{30}), polymyxin B (PB_{50}), chloramphenicol (C_5), novobiocin (NB_5), dihydrostreptomycin (DS_{10}), penicillin (P_{10}), and aureomycin (A_5). The antibiotic vibriostat 0/129 (2,4-diamino-6,7-diisopropyl pteridine) was prepared by a modified procedure of Hendrie et al. (11).

Cultural characteristics of bacterial isolates. Bioluminescence was determined by growing the bacterial strains on marine agar 2216 or on photobacterium agar (Difco), followed by inspection in a darkened room. Fluorescent and other pigments were detected on Pseudomonas F or P medium (Difco), prepared with 80% filter-sterilized seawater and 20% distilled water, or on marine agar 2216. Characteristics recorded included turbidity, motility, flagellar stains, temperature requirement for growth, and morphology as viewed by electron microscopy of negatively stained preparations grown in T broth of the following composition: Trypticase (Difco), 0.3% (wt/vol); yeast extract (Difco), 0.3% (wt/vol); filtered seawater, 75%; distilled water, 25% (pH 7.9). One drop of trace elements solution was added to each 100 ml of the T medium. The solution consisted of the following: $CuSO_4 \cdot 5H_2O$, 1.96% (wt/vol); $ZnSO_4 \cdot 7H_2O$, 4.4% (wt/vol); $CoCl_2 \cdot 6H_2O$, 2.2% (wt/vol); $MnCl_2 \cdot 4H_2O$, 36% (wt/vol); $Na_2MoO_4 \cdot 2H_2O$, 1.26% (wt/vol); iron sequestrene, 1.0% (wt/vol).

Morphology of isolates. The primary isolates transferred to marine agar 2216 slants were Gram stained. Also, flagellar stains were processed using standard methods (14). Electron microscopy, employing negative staining, was also done (4), with the following modification in methodology: potassium phosphotungstate was prepared in three-salts solution brought to pH 7.25 with 1 N KOH to prevent lysis of the marine bacteria. Bovine serum albumin was added to T broth cultures to a concentration of 0.05% (wt/vol). An RCA EMU 2B electron microscope (RCA, Princeton, N.J.) was used for the electron micoscope studies.

Numerical taxonomy. Preliminary grouping of the strains was possible using the scheme shown in Fig. 1 and a composite of schema employed by other

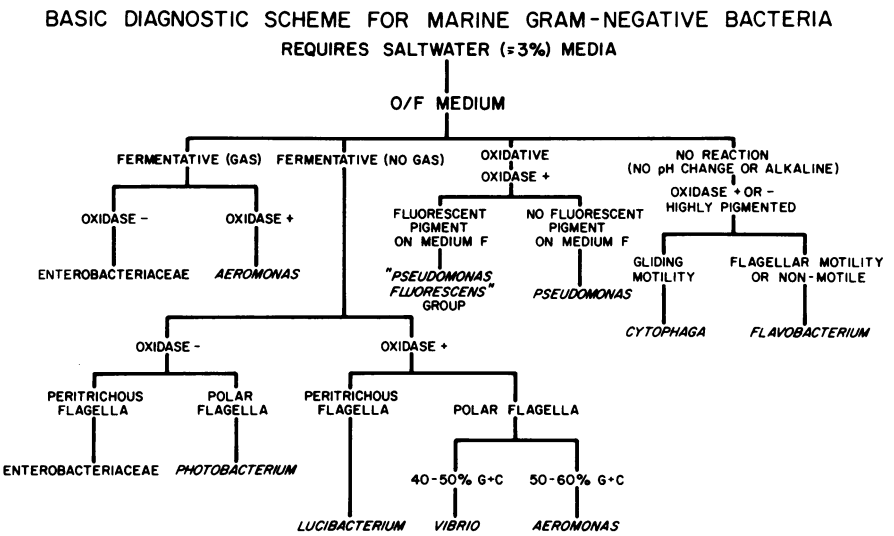

FIG. 1. *Basic diagnostic scheme for marine gram-negative bacteria (adapted from reference 2).*

investigators and in our laboratory (2, 3, 11). Strains included in the major groups were subjected to numerical taxonomy analysis, with a total of 85 characteristics recorded for each strain. *Pseudomonas*, *Cytophaga*, *Flavobacterium*, and *Chromobacterium* spp. were also isolated (11, 12, 23) but at a very low frequency of occurrence. The very few fungal isolates obtained among the isolates were not identified to species level.

Reference cultures. Bacterial strains included in the analyses as reference were: *Vibrio parahaemolyticus* strains FC1011 and 163, isolated by R. Colwell and R. Sakazaki, National Institute of Health, Tokyo, Japan, respectively; strain PCP, a freshwater isolate provided by D. McKay and C. R. Jenkin, University of Adelaide, Adelaide, Australia; *Vibrio fischeri*, from Carolina Biological Supply Co., Gladstone, Ore.; *Aeromonas liquefaciens* (*A. hydrophila*), provided by Peter K. Chen, Biology Department, Georgetown University, Washington, D.C.; and *Vibrio cholerae*, a noncholeragenic strain obtained from James Hawley, Analytab Products Inc.

Computer analyses. The methods for numerical taxonomy were those routinely employed in our laboratory. The final matrix included 93 strains and 85 characters. The data were analyzed using the simple matching coefficient (S_{SM}) (22), which includes both positive and negative matches, and the Jaccard coefficient (S_J) (23), which excludes negative matches. Programs used, including the UMTAXON 3 and 1SP53 program packages, are available on the University of Maryland UNIVAC 1108 computer.

Serology. Selected isolates, resembling *V. parahaemolyticus* as defined (16), were serotyped with anti-K serum. Selected isolates resembling *Vibrio anguillarum* (2, 5) were serotyped with *V. anguillarum* group antiserum.

RESULTS

The station locations and the total viable counts of bacterial heterotrophs and vibrio-like organisms isolated from water samples from which copepods were collected are given in Table 1. Plate counts were 3 or less colony-forming units per ml on both the 2216 and TCBS agar. The source of the copepods recorded by species, along with comparative data on total viable counts of heterotrophic bacteria versus vibrio-like organisms isolated from the external surface of copepods and from gut-surface samples, are given in Table 2. The latter could not be determined accurately as being of gut origin alone, because of the nonselective procedure for isolation of the bacteria (see Materials and Methods), and so the term gut-surface isolate was used. Comparison of data presented in Tables 1 and 2 shows that many more bacteria were found associated with copepods than were found to be free-living in the water column, indicating a preferential adsorption to or association of the bacteria with the animals. Fewer bacteria were isolated from gut surface samples than from the copepod surface samples (Table 1).

The taxonomic distribution of microorganisms in water samples from laboratory dishes in which normal, healthy copepods were reared, in preparations of moribund animals reared in the laboratory and aboard ship, and in estuarine water and ocean water samples from the stations at which the copepods were collected is given in Table 2. Of the 329 strains examined, *Vibrio* spp. were the most abundant of the bacterial taxa present in the pelagic samples, with *Pseudomonas* spp. the most abundant in laboratory-reared, healthy copepods. The bacterial genera represented in water samples from moribund copepods reared in the laboratory or in shipboard culture dishes were similar.

Data for estuarine, pelagic, and laboratory-reared animals are given in Table 3, showing bacterial strains isolated from water, from the surface of copepods, and from the copepod gut surface samples. The estuarine copepods were found to have the highest representation of *Vibrio* spp., with laboratory-reared and pelagic animals sharing a lower count for the vibrios. On the other hand, the pelagic copepods possessed more *Pseudomonas* spp. in the gut flora than copepods collected from the estuarine environment or copepods reared in the laboratory.

The distribution of bacterial genera comprising the associated or commensal flora of the

TABLE 1. *Total viable counts of marine heterotrophs and Vibrio-like organisms recovered from surface versus gut-surface samples from copepods collected during cruise*

Copepod	Source	Surface[a]		Gut-surface[a]	
		Heterotrophs	*Vibrio*	Heterotrophs	*Vibrio*
Acartia sp.	Anclote R., Fla.	60	3	ND	
Acartia sp.	Anclote R., Fla.	ND		1×10^3	26
Pleuromamma sp.	Station 1	ND		ND	
Pleuromamma sp.	Station 1	ND		3×10^2	0
P. regalis	Station 1	1×10^3	13	4.7×10^2	3
L. aestiva	Station 2	1×10^3	1.2×10^2	1.37×10^3	70
L. aestiva	Station 3	3×10^2	30	1.4×10^2	16
L. aestiva	Station 3	3×10^4	2.9×10^3	1×10^5	28
C. furcatus	Station 3	>100	25	1×10^5	1×10^5
Acartia sp.	Station 3	1×10^3	1×10^3	1×10^3	1×10^3
L. aestiva	Station 4	3×10^2	<1	20	<1
Acartia sp.	Station 4	10^2	<1	1.4×10^2	<1
L. aestiva	Station 4	20	<1	23	3

[a] Total counts of heterotrophs were made on marine agar 2216; total counts of *Vibrio*-like organisms were made on TCBS agar. Counts are average of three animals. ND, Not done.

TABLE 2. *Taxonomic distribution of microbial isolates*

Microorganisms	No. (% of total) of microbial isolates from source:				
	Laboratory-reared, normal copepods ($N = 12$)[a]	Moribund laboratory-reared copepods ($N = 19$)	Moribund copepods in culture aboard ship ($N = 11$)	Estuarine water ($N = 8$)	Pelagic water ($N = 39$)
Vibrio spp.	2 (16.7)	10 (52.6)	5 (45.5)	8 (100)	29 (74.3)
Pseudomonas spp.	9 (75.0)	8 (42.1)	5 (45.5)	0 (0)	3 (7.7)
Cytophaga/Flavobacterium spp.	1 (8.3)	1 (5.3)	0 (0)	0 (0)	4 (10.3)
Chromobacterium spp.	0 (0)	0 (0)	1 (9.0)	0 (0)	0 (0)
Fungal species	0 (0)	0 (0)	0 (0)	0 (0)	3 (7.7)

[a] *N*, Number of strains included in the analysis.

TABLE 3. *Taxonomic distribution of the microbial flora of estuarine, pelagic, and laboratory-reared animals*[a]

Microorganisms	Estuarine samples ($N = 54$)				Pelagic samples ($N = 135$)				Laboratory reared ($N = 35$)	
	Water	Surface	Gut-surface	%	Water	Surface	Gut-surface	%	Gut-surface	%
Vibrio spp.	8	22	21	94.4	29	41	15	63.0	29	82.9
Pseudomonas spp.	0	1	0	1.9	3	12	25	29.6	4	11.4
Cytophaga/Flavobacterium spp.	0	1	0	1.9	4	0	2	4.4	2	5.7
Chromobacterium spp.	0	1	0	1.9	0	0	0	0.0	0	0
Fungal species	0	0	0		4	0	0	3.0	0	0

[a] Water samples, copepod surface washes, and combined gut-surface samples are shown. A total of 224 strains were included in the analysis. *N*, Number of strains in each analysis.

copepod species sampled during this study is given in Table 4. One striking finding was that when samples of 23 laboratory-reared animals were collected aseptically and cultured, all of the isolates were *Vibrio* spp. (see Table 3). Bacterial strains obtained from all the animals studied were *Vibrio* and *Pseudomonas* spp., with *Vibrio* spp. being three times more abundant.

The overall distribution of bacterial genera represented among strains comprising the bacterial flora of copepods is given in Table 5. *Vibrio* spp. clearly were the most abundant of the bacterial species found associated with the copepod, with *Pseudomonas* spp. the next most abundant. Fungal species were rarely encountered and only on pelagic copepods.

Of the *Pseudomonas* spp., a large proportion were *Pseudomonas fluorescens*. Of greatest interest was the large number of vibrios found associated with the copepod. The vibrios were further tested for numerical taxonomy analysis.

The *S*-value matrix computed for the *Vibrio* isolates, including the reference strains of the genus *Vibrio*, is shown in Fig. 2. The sorted similarity matrix is based on the S_J coefficient. Eight groups emerged from the data analyses. Figure 3 provides a simplified dendrogram, prepared using the S_J coefficient, with the eight groups of vibrios represented as phena. Similarity (*S*) for the eight phena was at the 75% level.

Table 6 presents the characteristics, or positive responses, as percentage of the unit char-

TABLE 4. *Distribution of bacterial genera associated with species of copepods*[a]

Copepod species	Source	Surface	Gut-surface	Aseptic gut dissection	Fecal pellets
A. tonsa	Anclote R. (estuarine)	15 *Vibrio* spp. 1 *Chromobacterium* sp.	7 *Vibrio* spp. 1 *Pseudomonas* sp. 1 unknown[c]	—[b]	—
	MIZAR[d] Station 3 (pelagic)	5 *Vibrio* spp.	3 *Vibrio* spp.	—	—
	MIZAR Station 4 (pelagic)	2 *Pseudomonas* spp.	3 *Pseudomonas* spp. 1 unknown[c]	—	—
	Bayboro Harbor (estuarine)	7 *Vibrio* spp. 1 *Cytophaga* or *Flavobacterium* sp.	15 *Vibrio* spp. 1 unknown[c]	—	3 *Pseudomonas* spp.
	Laboratory reared	—	9 *Vibrio* spp. 10 unknowns[c] 9 *Pseudomonas* spp. 1 *Cytophaga* or *Flavobacterium* sp.	23 *Vibrio* spp.[e]	—
L. aestiva	MIZAR Station 2 (pelagic)	5 *Pseudomonas* spp. 2 *Vibrio* spp.	8 *Vibrio* spp. 2 *Pseudomonas* spp.	—	—
	MIZAR Station 3 (pelagic)	11 *Vibrio* spp. 2 *Pseudomonas* spp. 5 unknown[c]	13 *Vibrio* spp. 7 *Pseudomonas* spp. 2 *Cytophaga* or *Flavobacterium* spp. 2 unknown[c]	—	—
	MIZAR Station 4 (pelagic)	3 *Pseudomonas* spp. 1 unknown[c]	3 *Pseudomonas* spp.	—	—
P. regalis	MIZAR Station 1 (pelagic)	No growth	5 *Pseudomonas* spp. 4 *Vibrio* spp.	—	—
Pleuromamma sp.	MIZAR Station 1 (pelagic)	No growth	6 *Pseudomonas* spp.	—	—
C. furcatus	MIZAR Station 3 (pelagic)	8 *Vibrio* spp. 1 *Pseudomonas* sp.	2 *Vibrio* spp. 2 *Pseudomonas* spp. 2 unknown[c]	—	—

[a] Samples are from a three-animal composite sample except for aseptic gut dissections (see the text).
[b] —, Not done.
[c] Isolate not viable on subculture.
[d] MIZAR, Cruise of U.S.N.S. MIZAR.
[e] A total of 23 animals were sampled.

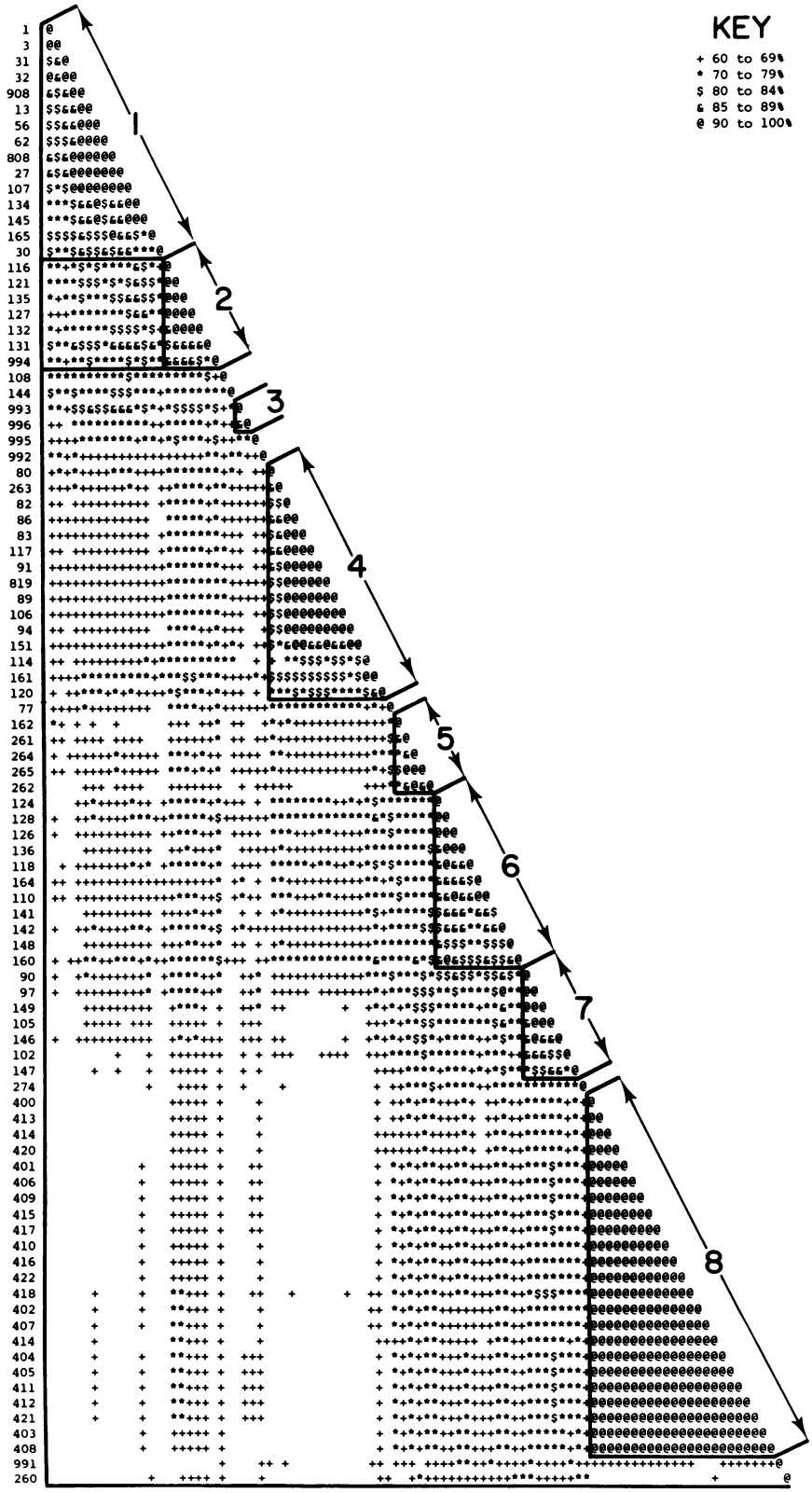

FIG. 2. *Shaded diagram of a sorted similarity matrix based on the S_J coefficient and unweighted average linkage clustering.*

TABLE 5. *Distribution of bacterial genera among 329 strains isolated from copepods*

Organism	No.	Percent
Vibrio spp.	182	55
Pseudomonas spp.	73	22
Cytophaga or *Flavobacterium* spp.	11	3
Chromobacterium spp.	2	<1
Unidentified[a]	55	17
Fungi	6	2

[a] Strains not viable on subculture or not identified to genus and/or species.

acters for phena 1 to 8.

All of the bioluminescent vibrios grouped in phenon 1, which, incidentally, excluded the single luminescent reference strain, *V. fischeri*. The bioluminescent isolates were examined further, employing additional biochemical tests as well as electron microscopy. Phenon 1, comprising the bioluminescent strains, grew well on TCBS agar and, by electron microscopy, possessed a single polar flagellum. These strains grew at both 37 and 5°C. Unfortunately, this group could not be identified to any of the species described by Hendrie et al. (11).

Very few of the isolates could be serotyped. Of these, two isolates, 33 and 108, were typable with anti-*V. parahaemolyticus* K sera K-18 and K-19. Strain 108 clustered with phenon 3, which contained the reference strains of *V. parahaemolyticus*. Strains 124 and 125 reacted with anti-*V. anguillarum* serum. Strain 124 fell into phenon 6, whereas strain 125 was lost before sufficient data could be gathered for computer assignment.

Figure 4 shows typical adult male and adult female *A. tonsa* Dana reared in captivity. Females are generally larger and more plump than males, being adapted to carrying eggs. The caudal rami may easily be observed.

DISCUSSION

It has been concluded by many investigators that, like many, if not all, terrestrial animals, the digestive tracts of most marine animals contain a commensal microbial flora (1, 6, 8, 9, 20, 24). Exceptions have been recorded, with results of light and scanning electron microscopy providing evidence for the absence of an associated microbial flora in the gut of the crustacean species *Limnoria*, *Chelura*, and *Oniscus* spp. (3, 18, 19). However, in the study of the copepod, a zooplankton member, the presence of a commensal flora, comprising several genera of heterotrophic bacteria in the digestive tract of several species of marine copepods, i.e., crustaceans, is clearly established. Interestingly, after elimination of fecal pellets by the copepods, it was found, on subsequent culture of the dissected gut, that much lower numbers of bacteria were found in the gut. Thus, the release of copepod gut flora bacteria via fecal pellets provides a mechanism for the wide distribution of these bacteria in the water column and sediment of estuaries and the oceans.

The significant number of marine vibrios found to be associated with marine copepods is indeed noteworthy, as is the monotypic flora in the gut of the copepod *A. tonsa*, maintained under defined laboratory conditions in the laboratory. Although culture conditions for the copepod have been discussed elsewhere (25, 26), it is well to reiterate some of the details. Copepods in culture through multiple generations are maintained in Millipore-filtered water, i.e., seawater is passed through a bacteria-retaining Millipore filter before entering the aquaria, and food, in the form of three species of bacteria-free algal preparations, is also provided aseptically, since the algae are cultured aseptically under artificial lighting with temperature and other physical parameters carefully controlled (19, 20). Thus, a unique association between bacteria and copepods appears to establish itself. The unique gut isolates have not separated, or grouped in the computer analyses. Thus, they are referred to as "marine vibrios."

It is conceivable that maintaining *A. tonsa* in laboratory culture imposes special restrictions on the expression of the normal flora, with the result that selective pressures are imposed by the artificial conditions of the laboratory environment. For example, *A. tonsa* are in nature herbivores. The choice of three algal forms for

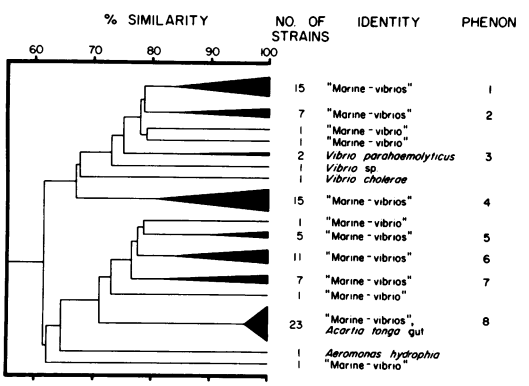

FIG. 3. *Simplified dendrogram prepared using the S_J coefficient and average linked method of clustering.*

TABLE 6. *Summary of the characteristics of the phena obtained in the analyses, expressed as percent positive responses*

Characteristic	Phenon							
	1	2	3	4	5	6	7	8
Source								
Seawater	27	0	0	7	20	18	0	0
Copepod	73	86	0	93	80	82	100	100
Colonial morphology								
Small colony, 1-mm diameter	13	86	0	0	0	0	0	100
Spreader	0	0	0	87	0	0	0	0
Opaque	100	14	100	0	0	0	0	0
Translucent	7	86	0	100	100	100	100	100
Off-white	0	100	100	100	100	100	100	100
Bioluminescent	67	0	0	0	0	0	0	0
Micromorphology								
Curved rods	7	0	50	0	0	0	29	100
Coccobacilli	53	71	50	93	0	91	57	100
Motile	100	100	100	100	100	100	100	100
Biochemistry								
Arginine dihydrolase	7	0	0	0	0	36	0	64
Lysine decarboxylase	100	100	100	100	0	0	0	0
Ornithine decarboxylase	100	100	100	0	0	0	0	0
Fermentative metabolism	100	100	100	100	100	100	100	100
Indole production	100	100	100	100	100	100	100	100
NO_3-NO_2	100	100	100	100	100	100	100	100
O-Nitrophenyl-β-D-galactopyranoside	60	29	0	7	100	0	100	100
Oxidase	87	100	100	100	100	82	71	100
TCBS, alkaline	73	86	100	20	60	100	86	100
Tryptophan deaminase	0	0	0	87	100	91	0	0
Voges-Proskauer reaction	0	0	0	100	20	73	29	0
Degradation of:								
Chitin	100	100	0	93	100	50	14	100
Gelatin	100	100	100	100	80	100	100	100
Lecithin	100	100	100	100	100	100	100	100
Tween 20	100	100	100	100	100	100	100	100
Tween 40	100	100	100	100	100	100	86	100
Tween 60	100	100	100	100	100	100	100	100
Tween 80	100	100	100	100	100	100	100	100
Urea	20	14	50	0	0	0	0	0
Sensitivity to:								
0/129	7	14	50	0	60	91	29	100
Aureomycin	40	0	100	0	100	64	100	80
Bacitracin	0	0	100	57	0	0	0	0
Chloramphenicol	100	100	100	100	100	100	100	100
Dihydrostreptomycin	100	100	0	100	100	100	71	20
Erythromycin	80	100	100	71	100	82	86	100
Novobiocin	100	100	0	100	100	91	100	100
Penicillin	0	0	100	0	75	9	86	100
Polymyxin B	20	50	100	14	100	91	71	100
Terramycin	60	100	100	86	100	100	100	100
Tetracycline	60	100	100	93	100	100	86	100
Growth at 43°C	7	0	100	67	0	0	0	0
Growth in:								
3% (wt/vol) NaCl	100	100	100	100	100	100	100	100
7% (wt/vol) NaCl	100	100	50	100	20	91	43	
10% (wt/vol) NaCl	0	0	0	67	0	0	0	0
Utilization of:								
Amygdalin	100	100	100	100	80	64	0	0
Arabinose	0	14	0	7	0	0	0	0
Mannose	93	86	100	87	100	82	0	0
Saccharose	13	29	0	73	60	0	0	100
Sodium citrate	100	100	100	100	0	82	14	0

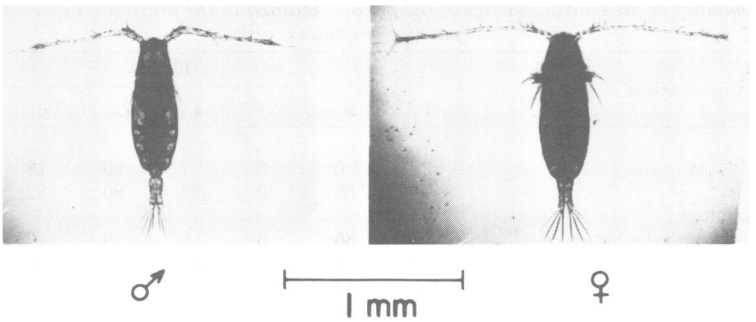

FIG. 4. *Typical male and female specimens of A. tonsa Dana. The females, adapted for egg bearing, are typically larger than the males. The latter are further differentiated by their longer, slightly more curved antennae.*

laboratory "fodder" was a conscious choice for optimum propagation. In the wild, the animals come into contact with many bacterially contaminated algal forms also serving as food (8).

Interestingly, the flora of *A. tonsa* organisms isolated during cruises, from estuarine samples, and from normal as well as moribund copepods revealed that the bacterial flora does not vary significantly.

The gut flora organisms formed a cohesive group at $S = 60$ to 100%. The *A. tonsa* gut isolates can be seen in Fig. 2 and 3 as phenon 8. Reference strains included in the computer analysis grouped separately with no observable relationship with the fresh isolates. *V. fischeri*, for example (Fig. 2), grouped separately from bioluminescent isolates. However, phenon 2 may be nonluminescent strains of *V. fischeri*, if one chose to group phena 1 and 2 as *V. fischeri*.

Two strains of *V. parahaemolyticus*, strains FC 1011 and 163, grouped as phenon 3, again separate from the marine isolates under study. *V. cholerae* (non-choleragenic strain) and *Vibrio* strain PCP (a freshwater isolate) were separated from the marine vibrios at the species level. *A. liquefaciens* (*A. hydrophila*) was not related to any of the marine *Vibrio* spp. of the study.

Several strains of *Photobacterium phosphoreum* were examined during the research work reported here. These bacteria were relatively easy to distinguish from the marine vibrios, since *P. phosphoreum* could be isolated from the light organs of deep-sea fish and required lower incubation temperatures. The characteristic light emitted by them was of a different wavelength from that of the marine bioluminescent vibrios found in this study, i.e., phenon 1 (see Fig. 2 and 3).

Several aspects require further study. It is not known what ecological parameters affect the gut flora of the copepods when they are brought into laboratory culture. The commensal bacterial flora does not vary much from the wild. However, differences, possibly associated with changes in salinity, were observed in the flora of pelagic versus estuarine versus laboratory-reared copepods (Tables 2 and 3).

Such differences in the bacterial flora in otherwise similar copepods collected from different locations can be speculated to arise from feeding habits, since only *A. tonsa* is truly herbivorous, the others being omnivorous.

Significant association of bacterial species with copepod species was not observed.

A very difficult problem is the taxonomy of marine vibrios. Only a few *Vibrio* spp. are available in culture collections, yet vibrios often are the dominant organisms in the marine and estuarine environment. *V. cholerae, V. parahaemolyticus, V. alginolyticus, V. anguillarum, V. fischeri*, and *V. metchnikovii* are the only relatively completely characterized species (14). From the taxonomic results obtained in this study, it is clear that there are several new species of marine *Vibrios* that will need to be described and named.

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