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Ecological Relationships Between *Vibrio cholerae* and Planktonic Crustacean Copepods

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Strains of *Vibrio cholerae*, both O1 and non-O1 serovars, were found to attach to the surfaces of live copepods maintained in natural water samples collected from the Chesapeake Bay and Bangladesh environs. The specificity of attachment of *V. cholerae* to live copepods was confirmed by scanning electron microscopy, which revealed that the oral region and egg sac were the most heavily colonized areas of the copepods. In addition, survival of *V. cholerae* in water was extended in the presence of live copepods. Attachment of viable *V. cholerae* cells to copepods killed by exposure to −60°C was not observed. Furthermore, survival of *V. cholerae* was not as long in the presence of dead copepods as in the live copepod system. A strain of *Vibrio parahaemolyticus* was also seen to attach to copepod surfaces without effect on survival of the organism in water. The attachment of vibrios to copepods was concluded to be significant since strains of other bacteria, including *Pseudomonas* sp. and *Escherichia coli*, did not adhere to live or dead copepods. Attachment of *V. cholerae* to live copepods is suggested to be an important factor of the ecology of this species in the aquatic environment, as well as in the epidemiology of cholera, for which *V. cholerae* serovar O1 is the causative agent.

Evidence is rapidly accumulating which indicates that pathogenic *Vibrio* species, including *Vibrio cholerae*, are naturally occurring members of the aquatic environment (3, 4, 9, 15–18). The ecological studies reported to date have mainly described the spatial and temporal distribution of pathogenic vibrios in the water column of the aquatic environment.

All pathogenic *Vibrio* species elaborate an extracellular chitinase (2), and to date, only one study has fully investigated the significance of any association between these pathogenic vibrios and the chitin-containing zooplankton in the water column. In the study of Kaneko and Colwell (14), *Vibrio parahaemolyticus* was shown to adsorb onto copepods, with the efficiency of this effect being dependent on pH and salinity. Furthermore, both pH and salinity were concluded to be major factors influencing the distribution of *V. parahaemolyticus* in estuarine ecosystems such as Chesapeake Bay (15).

Colwell et al. (8) recorded the isolation of *V. cholerae* from plankton samples from Bangladesh waters and Chesapeake Bay and suggested that an association between *V. cholerae* and chitinous plankton may exist. The study reported here was designed to examine any association between *V. cholerae* and zooplankton and to determine whether the presence of copepods influences the survival of *V. cholerae* in the aquatic environment.

MATERIALS AND METHODS

Sample collection. Both adult and immature copepods were collected by hand trawl with a plankton net (no. 20, 77 μm mesh size) from waters of the Patuxent River, Sunderland, Md. (salinity, 2‰); the Chesapeake Bay near Solomons Island, Md. (salinity, 15‰); the Chesapeake Bay near Annapolis, Md. (salinity, 22‰); and the Buriganga River, Dacca, Bangladesh (salinity, 0.2‰). Samples were retained in sterile Nalgene bottles containing surface water collected at each site, and transported at ambient temperature to the laboratory where processing began within 3 h of collection. In Chesapeake Bay waters, the predominant copepod species were *Acartia tonsa, Eurytemora affinis*, and *Scottolana* spp. Samples from Bangladesh contained two predominant, but unidentified, copepod species.

Bacterial strains used and culture methods. The strains of bacteria used are listed in Table 1 along with their source. *Vibrio* species were grown in alkaline peptone water consisting of 1% (wt/vol) peptone (Difco Laboratories, Detroit, Mich.), and 1% (wt/vol) NaCl at pH 8.5. Other strains were grown in T_1N_1 broth containing 1% (wt/vol) Trypticase (BBL Microbiology Systems, Cockeysville, Md.), and 1% (wt/vol) NaCl at pH 7.2. All strains were incubated statically

TABLE 1. Source of strains used

Species	Strain no.	Source
V. cholerae O1 (classical Inaba)	CA401	Clinical isolate from Calcutta, India (1953)
V. cholerae O1 (El Tor Ogawa)	D-18050	Environmental isolate from river water, Dacca, Bangladesh (1980)
V. cholerae non-O1	OSU116	Environmental isolate from algae, Tillamook Bay, Oregon (1980)
V. parahaemolyticus	TK18136	Environmental isolate from brackish water, Teknaf, Bangladesh (1980)
E. coli	E/C744	Environmental isolate from river water, Dacca, Bangladesh (1980)
Pseudomonas sp.	PS11361	Clinical isolate from International Centre for Diarrhoeal Disease Research, Bangladesh hospital, Dacca, Bangladesh (1981)

for 12 h at 30°C and then washed three times in phosphate-buffered saline before use.

Procedures for attachment and survival studies. Copepods were washed by gently pouring 4 liters of filter-sterilized water from the same collection site over copepods retained on a metal filter to remove surface bacteria. Throughout the experiments, all water was filter-sterilized by passage through a 0.22-μm pore size filtration system (Millipore Corp., Bedford, Mass.). Dead copepods were prepared by placing a glass flask containing 100 ml of water and copepods in a freezer at −60°C for 30 min. In the experiments, approximately 500 washed copepods of all species collected were placed into each of several 2-liter flasks (see below) containing 500 ml of filter-sterilized river or bay water. For experiments with *V. parahaemolyticus*, the salinity of the water was adjusted to 30‰ by addition of NaCl. The salinity of all waters was recorded with a salinity refractometer (American Optical Corp., Buffalo, N.Y.). Where indicated, flasks were inoculated with each bacterial strain to a final concentration of approximately 10^4 colony-forming units (CFU)/ml. Dead bacteria were obtained by heating a suspension of cells in phosphate-buffered saline for 15 min in a water bath set at 54°C. Death was indicated by the loss of cell motility in hanging-drop preparations observed by light microscopy and the loss of growth on gelatin agar (24). In selected flasks, a nonaxenic algal culture, *Pseudoisocrysis* sp., was added to a final concentration of 10^3 cells/ml to serve as food supply for the live copepods. Addition of the algal culture did not result in an increase in the number of copepods in the flask. Washed copepods were cultured before use, using the method described below for *V. cholerae* serovar O1, and were consistently negative.

The following flask combinations were prepared for each experiment: (i) live copepods, live algae, and live bacteria; (ii) live copepods and live bacteria; (iii) live copepods and dead bacteria; (iv) dead copepods and live bacteria; (v) live algae and live bacteria; (vi) live bacteria; and (vii) live copepods and live algae.

After inoculation, flasks were incubated under static conditions at ambient temperature (23 to 26°C) for 336 h. For sampling, five copepods were removed along with 2 ml of water and homogenized manually in a Teflon-tipped tissue grinder (Wheaton Scientific, Millville, N.J.) until all copepods were completely and uniformly disintegrated. After appropriate decimal dilutions in phosphate-buffered saline had been prepared, 0.1-ml portions of each dilution were spread onto duplicate plates of selective medium. This method counted the bacteria associated with the copepods as well as the bacteria living free in the water. Thiosulfate citrate bile salts sucrose (TCBS) agar (BBL) was

TABLE 2. Influence of the presence of live copepods on the multiplication and survival of *V. cholerae* in waters from Chesapeake Bay and Dacca, Bangladesh

Site	Salinity (‰)	Test strain	Copepods present	Organisms per ml		Time (h) to reach >1 organism per ml
				Initial count	Maximum count	
Patuxent River	15.0	OSU116	Live	5.70×10^3	9.61×10^6	ND[a]
			Dead	5.60×10^3	1.90×10^5	ND
Patuxent River	22.0	CA401	Live	1.15×10^5	3.10×10^7	288
			Dead	9.0×10^4	3.15×10^6	168
Buriganga River	0.2	D-18050	Live	2.39×10^3	7.91×10^5	ND
			Dead	6.30×10^3	2.81×10^4	ND

[a] ND, Not determined as sampling stopped after 36 h.

used for *V. cholerae* and *V. parahaemolyticus*, McConkey agar (Difco) was used for *E. coli*, and Mueller-Hinton agar (Oxoid, Columbia, Md.) was used for *Pseudomonas* sp. Viable, culturable, heterotrophic bacteria were enumerated by using plate count agar (Difco) amended to contain 1% (wt/vol) NaCl. All plates were incubated at 35°C for 18 h. When strains of *V. cholerae* O1 were employed, colonies were picked from TCBS agar, streaked on gelatin agar, and incubated at 35°C for 18 h. Colonies on gelatin agar were serotyped by slide agglutination, using polyvalent *V. cholerae* O1 antiserum (Difco) to confirm identification of the culture.

SEM. For scanning electron microscopy (SEM), five copepods were removed from each flask, using wide-bore glass pipettes to avoid damage to the copepods as much as possible. Copepods were fixed in Bouin solution (1), which was added in the ratio of 1 part fixative to 20 parts water sample. A 2-ml water and copepod sample was mixed with the set volumes of fixative and stored at room temperature until samples could be processed further. After fixation, samples were washed exhaustively in filter-sterilized river or bay water to remove all traces of fixative. Samples were dehydrated in isopropyl alcohol gradients and fixed with a mixture of 2% osmium tetroxide (six parts) and saturated mercuric chloride (one part) (19). Samples were freeze-dried in a freeze-drying apparatus (Edwards Ltd., Crawley, England) and coated with gold-palladium alloy (6:4) in a Denton DV503 vacuum apparatus (Denton Ltd., Cherry Hill, N.J.). Stubs were examined in an AMR-1000A scanning electron microscope (Advanced Metals Research Corp., Bedford, Mass.) at an accelerating voltage of 20 KV. Photographs were prepared with PN 55 black-and-white positive-negative film (Polaroid Corp., Cambridge, Mass.).

RESULTS

Survival studies. Both non-O1 and O1 serovars of *V. cholerae* increased in number up to 100-fold in the presence of live copepods compared with cells in the presence of cold-killed copepods. Figures 1 and 2 illustrate the phenomenon observed when *V. cholerae* CA401 was added to a Patuxent River water sample (salinity, 2‰). In the presence of cold-killed copepods, *V. cholerae* counts rose from 1.45×10^4 to 3.02×10^6 CFU/ml within 36 h and then fell to below

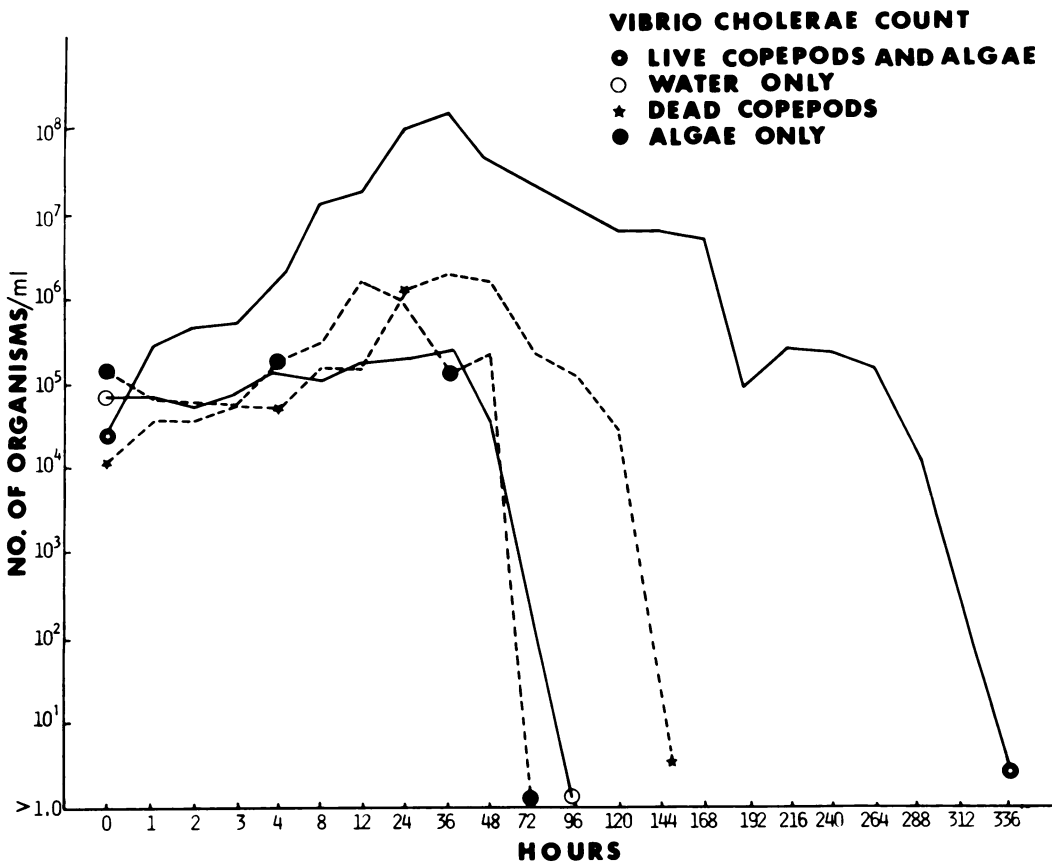

FIG. 1. Count of *V. cholerae* CA401 in flasks of Patuxent River water in the presence of live copepods and algae, dead copepods, and algae. Counts are per milliliter of water or homogenate.

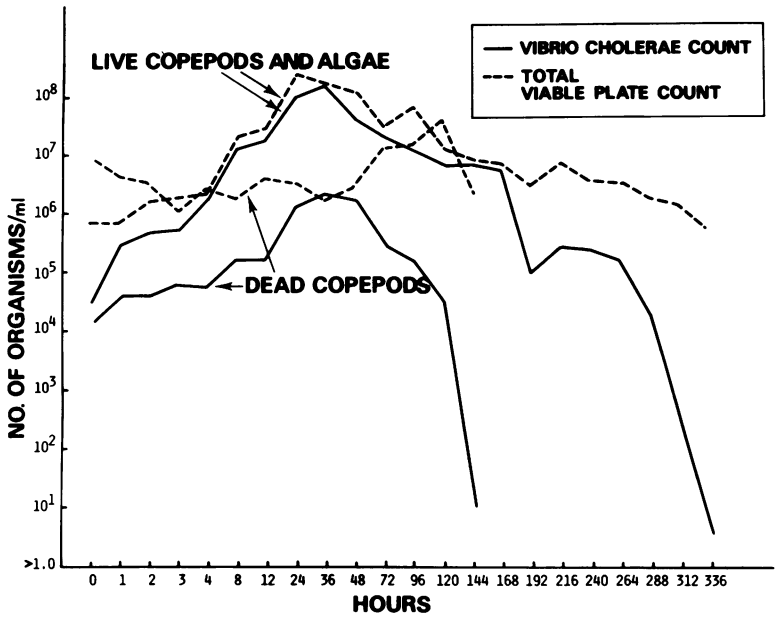

FIG. 2. Count of *V. cholerae* CA401 and total viable plate count in Patuxent River water in the presence of live copepods and algae, and dead copepods. Counts are per milliliter of water or homogenate.

detectable numbers by 144 h. The viable, heterotrophic plate count remained around 10^6 CFU/ml for the first 36 h, gradually rising to approximately 1 log increase before the sampling was terminated at 144 h. The source of these bacteria was presumbaly the gut of the cold-killed copepods.

In contrast, *V. cholerae* counts increased in the presence of live copepods, i.e., from 4.30×10^4 to 1.99×10^8 CFU/ml within 36 h, and did not decrease to below detectable numbers until after incubation for 336 h. The viable, heterotrophic plate count increased similarly as the *V. cholerae* count.

The contribution of a nonaxenic algal culture and copepod gut flora to the total viable plate count was not apparent until numbers of *V. cholerae* decreased near the end of the experiment. Appropriate controls showed that extended survival of *V. cholerae* CA401 was associated with the presence of live copepods. Similar results were obtained with all other strains of *V. cholerae* and water samples when tested over a shorter incubation period of 36 h. Thus, a striking trend toward increased cell number and survival of *V. cholerae* in the presence of live copepods was observed and confirmed by data from other test systems (Table 2).

Studies were also made of water collected from the Buriganga River, Dacca, Bangladesh, employing an incubation period of 36 h. In these experiments, *V. parahaemolyticus* demonstrated approximately a single logarithmic increase in numbers in the presence of live copepods, but not when incubated in the presence of dead copepods, for 12 h. Little change in count was detected when the experiments were repeated with incubation for 36 h with bay water.

The strain of *Pseudomonas* sp. increased in population size in the presence of both dead and live copepods. Results obtained in control flasks indicated that these increases were not associated with copepods. Interestingly, the counts of *E. coli* were higher at 12 h (1.91×10^5 CFU/ml) and 36 h (4.91×10^5 CFU/ml) in the presence of dead copepods compared with 12 h (8.40×10^4 CFU/ml) and 36 h (7.30×10^4 CFU/ml) in the presence of the live copepods.

SEM. SEM revealed that the washing procedure for cleaning freshly caught copepods effectively removed surface bacteria before contamination with test strains in our experiments (Fig. 3). Examination of copepods collected from the Patuxent River and incubated for 36 h in flasks amended with the bacterial cultures used in this study revealed that only *V. cholerae* attached to the live copepods. Attachment was observed on all species of copepod employed in this study. No attachment was seen when cold-killed copepods were employed. Attachment to live copepods appears to be selective, since the heaviest concentrations of bacterial cells were observed in the oral region (Fig. 4) and on the egg sac (Fig. 5) of the copepods. In some cases, dividing cells

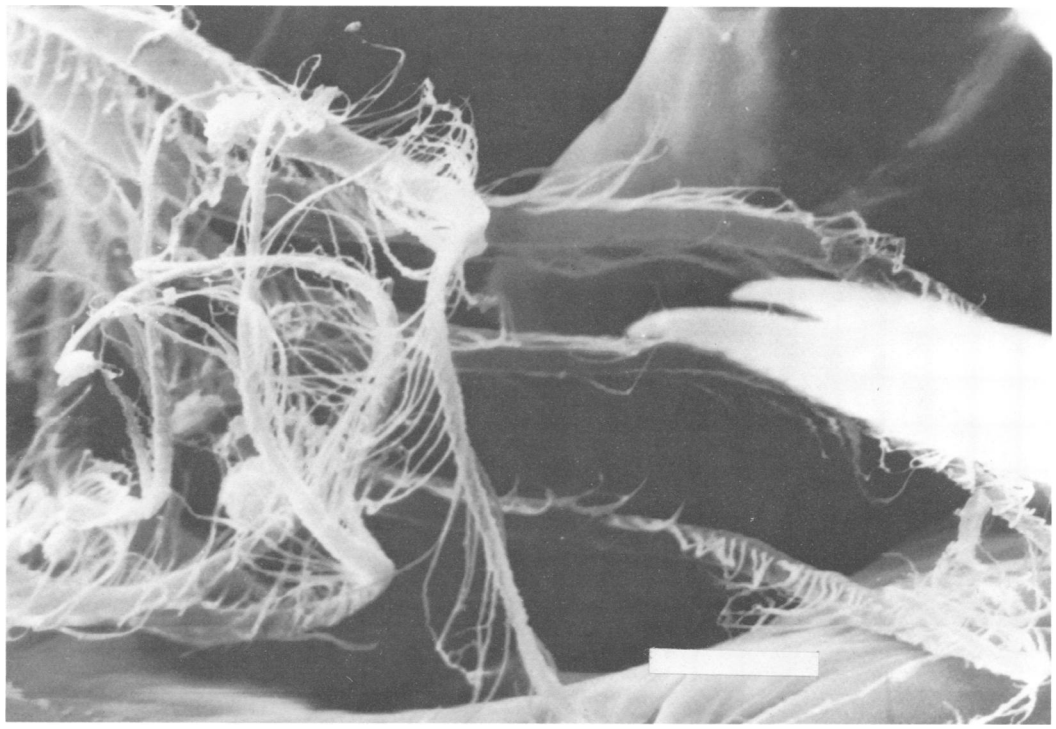

FIG. 3. View of a copepod oral region and feeding apparatus after washing in filter-sterilized Patuxent River water to show removal of surface bacteria. Bar, 10 μm.

were observed on the copepod egg sac surface (Fig. 6).

V. parahaemolyticus was observed to adhere to live copepods, but without selectivity; i.e., the cells covered the whole copepod. Strains of *Pseudomonas* sp. and *E. coli* did not attach to live or dead copepods.

Attachment was not observed for dead copepods seeded with heat-killed bacteria when examined by SEM.

DISCUSSION

The adsorption of bacteria to surfaces in aquatic environments has been investigated by microbial ecologists for several decades. The majority of these studies have observed colonization of geological or man-made surfaces (5, 28). In comparison, the significance of adsorption by bacteria onto the surfaces of living aquatic organisms has largely been neglected.

The study of Sochard et al. (25) demonstrated, by culture on marine agar 2216, that marine copepods carry a bacterial flora both on their surface and in the gut, the predominant bacterial group being members of the genus *Vibrio*. These observations were, in part, confirmed by SEM examination of copepods collected from Bangladesh aquatic environments by Colwell et al. (8) who demonstrated abundant numbers of diverse types of bacteria colonizing copepod surfaces. The predominance of a *Vibrio* population with zooplankton also has been described by Simidu et al. (22) who found differences between the generic composition of bacteria from zooplankton and seawater samples.

The prolonged survival of *V. cholerae* observed to occur in the presence of live copepods prompted the present investigation of specificity of attachment of vibrios and other bacteria to live and dead copepods. The study reported here reveals a significant association between *V. cholerae* and live copepods, with selective attachment taking place on the copepod surface. Highest counts of *V. cholerae* were obtained in the presence of live copepods. It is not possible to completely distinguish between copepod-associated multiplication of *V. cholerae* and the concentration, but no multiplication, of *V. cholerae* from the water onto copepod surfaces. Nevertheless, SEM data strongly suggest that multiplication takes place on the copepod surface. An interesting aspect of our observations is the lack of attachment of these strains to dead copepods. If chitin is the important factor in attachment by chitinolytic vibrios, as suggested by results of studies with *V. parahaemolyticus*

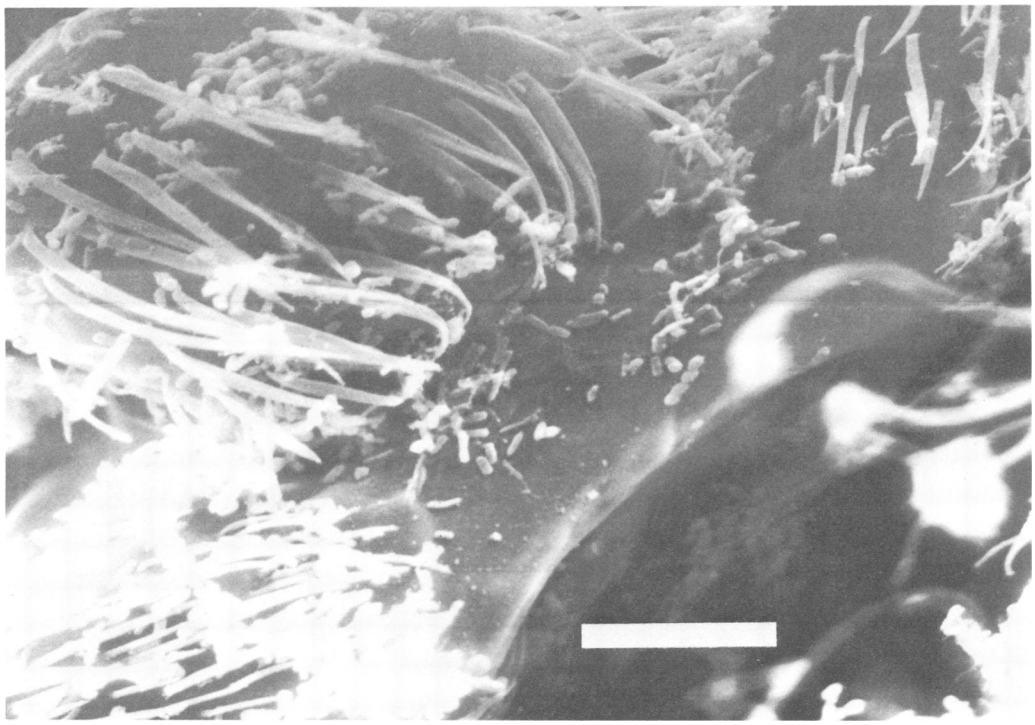

FIG. 4. View of a copepod oral region and feeding apparatus after incubation for 36 h in Patuxent River water and *V. cholerae* CA401. Bar, 10 μm.

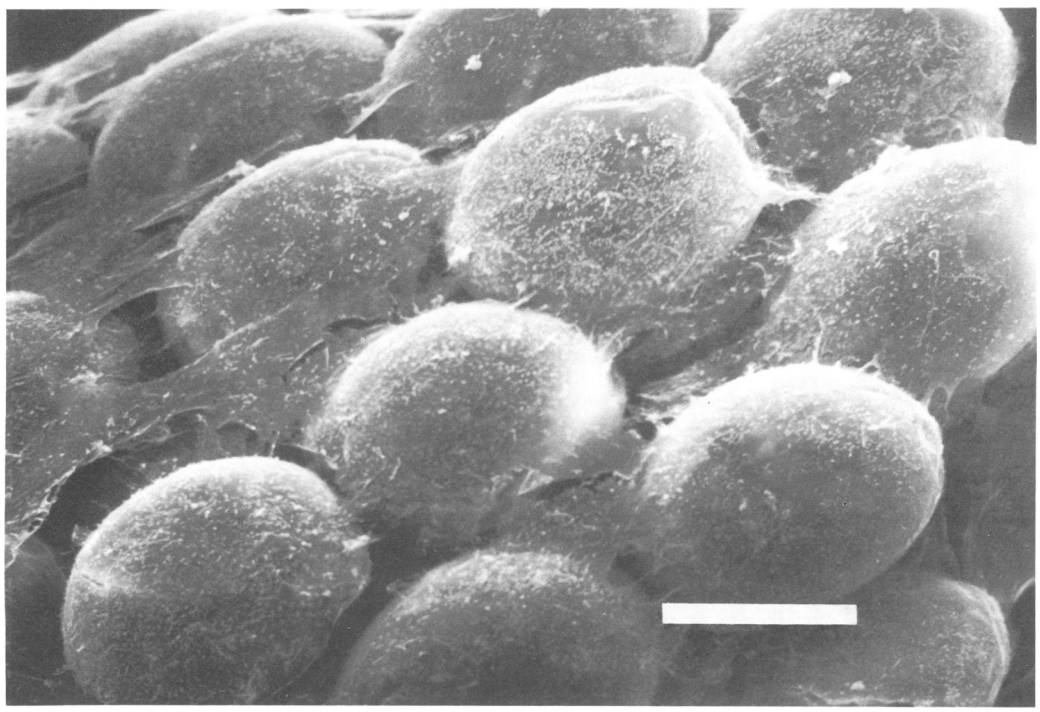

FIG. 5. Colonization of a copepod egg sac after incubation for 36 h in Patuxent River water and *V. cholerae* CA401. Bar, 50 μm.

FIG. 6. Attachment of *V. cholerae* CA401 to a copepod egg sac surface and the presence of dividing cells (arrows). Bar, 1 μm.

(14), it might be expected that *V. cholerae* would adhere to dead copepods equally as well as they would adhere to live copepods. This was not observed, and the possibility that live copepods excrete growth-promoting or chemical attractant compounds specific for *V. cholerae* warrants investigation. In addition, the outermost surface of copepods is covered with a epicutical of waxes (12). The net charge of this layer has been suggested as a limiting factor for attachment of chitinolytic vibrios to copepods.

The attachment phenomenon observed to occur between *V. cholerae* and live copepods has ecological as well as epidemiological significance. Since *Vibrio* species produce an active chitinase, it is suggested from the results of the present study and studies of the adsorption of *V. parahaemolyticus* to chitin particles and copepods (14) that a primary role of vibrios could be the colonization and initiation of degradation of chitinous material in aquatic ecosystems. Such a role was postulated by Hood and Meyers (13) who demonstrated the role of *Vibrio* species in chitin turnover and metabolism in marine crustaceae.

Traditionally, cholera epidemiologists considered that the only reservoir of *V. cholerae* was the human intestine and that survival of the organism outside the human body is brief (10, 11). This supposition may not be justified. A single case of cholera in Texas in 1973 (29) was followed in 1978 by an outbreak involving 11 persons in Louisiana (3). The phage type of the Texas and Louisiana isolates was identical, implying that the causative organism had been able to survive in the Gulf Coast environment between outbreaks (3). More recently, studies have demonstrated the ability of nontoxigenic *V. cholerae* strains to survive and multiply in aquatic ecosystems in England (30). Thus, data have accumulated supporting the original hypothesis of Colwell et al. (7) that *V. cholerae* is an autochthonous member of natural aquatic ecosystems.

The present study revealed an association between *V. cholerae* and another member of the aquatic environment, the copepod. Since this association appears to influence significantly the survival time of *V. cholerae* in water, epidemiological implications can be made. From SEM examination, the egg sac of copepods was found to be densely covered with bacteria, some undergoing binary division indicating multiplication. Since the majority of planktonic copepods spawn their mature eggs free in water (26), shedding of eggs would provide a suitable vehicle for dissemination and multiplication of *V. cholerae* in the aquatic environment. Moreover,

counts of free-living *V. cholerae* in 500-ml volumes of natural waters are typically at least 5 log less than the number required to induce cholera in normochlorohydric humans (6, 16, 17, 30). It is therefore interesting to hypothesize that the ingestion of similar volumes of unfiltered water, i.e., 500 ml, containing large numbers of copepods contaminated with toxigenic *V. cholerae* could result in clinical disease, since the organism can achieve a large enough population on copepods to induce cholera. The adsorption and growth of *V. cholerea* on chitinous material has previously been suggested as contributing significantly to the epidemiology of cholera (20).

The attachment of *V. cholerae* to the oral region of copepods may indicate that the organism serves as a food source. If multiplication of *V. cholerae* occurs in the gut, with subsequent excretion via fecal material, the organism would become widely dispersed in the aquatic environment (27). The study of Sochard et al. (25) has already clearly demonstrated that marine copepods have a predominant gut flora of *Vibrio* spp., reflecting the bacterial content of their aquatic environment. The contribution of proliferation of *V. cholerae* CA401 in the copepod gut to the increased count and survival of this strain in the presence of live copepods was not determined in the present study, but is the subject of studies in progress.

In addition to growth of *V. cholerae* in association with planktonic copepods, the seasonality of cholera outbreaks in endemic areas such as Bangladesh may well be related to this association. Almost every year in that country, an epidemic of cholera occurs in September or October. According to Oppenheimer et al. (21), the zooplankton population decreases during the monsoon season (May to July) in response to a reduction in nutrient concentrations in the water as a result of the heavy influx of rain water. Subsequently, the zooplankton population increases during August and September, being preceded by a phytoplankton bloom. An increase in the copepod population is invariably paralleled, or shortly followed, by the appearance of cholera cases initiating the annual epidemics which occur in Bangladesh. Colonization of copepods and other chitinous zooplankton may explain, in part, why *V. cholerae* is abundant in the water at the same time that the zooplankton are abundant.

A serious question currently under study in this laboratory is the infrequent isolation of *V. cholerae* serovar O1 from environmental samples during the inter-epidemic period in Bangladesh. A possible explanation lies in the demonstration of a nonculturable state for *V. cholerae*, in which cells remain viable and substrate-responsive but fail to grow on routine laboratory media (23). This finding throws doubt on the efficacy of the alkaline-peptone water and TCBS agar isolation regime, originally developed for clinical specimens but currently employed for environmental samples. It is possible that *V. cholerae* may be present ubiquitously and continuously in brackish and freshwater environments during the months of the monsoon when nutritional conditions of the environment change. Under such conditions, *V. cholerae* cells may become unculturable, hence not detected by isolation techniques currently employed in clinical laboratories. In this state, it is hypothesized that *V. cholerae* may survive attached to copepods and subsequently multiply and outgrow in the water column and on the copepod surface when conditions revert to optimum at the end of the monsoon period and the start of the cholera epidemic season.

Clearly, the implications of the association between *V. cholerae* and planktonic copepods on the epidemiology of cholera will remain speculative until field studies, designed to detect and assess the significance of nonculturable *V. cholerae* cells, are completed. Work is in progress, as part of a collaborative University of Maryland-World Health Organization study, to elucidate the ecology of *V. cholerae*.

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