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Susceptibility and Resistance of the Rock Crab, *Cancer irroratus*, to Natural and Experimental Bacterial Infection[1]

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The incidence of naturally occurring bacterial infection in a Connecticut population of rock crab, *Cancer irroratus*, varied between 10 and 60% of the population. The fluctuation in incidence was correlated significantly with hemocyte concentrations. Twelve percent of the crabs sampled during January were found to be infected with a previously undescribed *Vibrio*. The *Vibrio* was demonstrated as pathogenic over a wide range of temperatures. High mortalities in experimentally infected crabs appear to result from hemocyte clumping and extensive intravascular clotting triggered by an endotoxin. The lobster pathogen, *Aerococcus viridans* var. *homari*, was found to be pathogenic at 25°C to *C. irroratus*, a prey species of the lobster. Results suggest that this species can act as a reservior host for *A. viridans* var. *homari*.

KEY WORDS: *Cancer irroratus; Aerococcus viridans* var. *homari; Vibrio* sp.; bacterial pathogens; susceptibility; resistance; endotoxin; hemocytes.

INTRODUCTION

Since the early work of Metchnikov (1905) on the water flea, *Daphnia* sp., many bacterial infections of crustaceans have been reported (Cantacuzène, 1923; Snieszko and Taylor, 1947; Bang, 1956; Krantz et al., 1969). However, few have been studied as thoroughly as gaffkemia. Described first by Snieszko and Taylor (1947), the causative agent of this disease is the Gram-positive micrococcus, *Aerococcus viridans* var. *homari* (formerly *Gaffkya homari*). Rabin and Hughes (1968) report that once *A. viridans* var. *homari* gains entry into the lobster, *Homarus americanus*, the host defense mechanisms are unable to eliminate the pathogen. Death results from the ability of the pathogen to deplete energy reserves of the host; utilization of hepatopancreatic glucose and glycogen by the bacteria causes dysfunction of the hepatopancreas. Death follows cessation of the biosynthetic and detoxification processes of this organ (Stewart and Cornwick, 1972; Stewart and Aries, 1973).

A. viridans var. *homari* has been found in the shrimp, *Penaeus aztecus* (Liuzzo et al., 1965), spider crab, *Libinia emarginata* (Rabin and Hughes, 1968), green crab, *Carcinus maenas* (Rabin and Hughes, 1968), and rock crab, *Cancer irroratus* (Cornwick and Stewart, 1968; Stewart and Rabin, 1970). The results of experimental injection in various crustaceans have suggested that this bacterium may be pathogenic for the dungeness crab, *Cancer magister* (Bell and Hoskin, 1966), blue crab, *Callinectes sapidus* (Tubiash and Krantz, 1970), and spider crab, *Libinia emarginata* (Rabin and Hughes, 1968). However, the effects of the infection on and the defense mechanisms of the host have been investigated only in the lobster.

Vibrio spp. as pathogens of marine arthropods have been reported from the horseshoe crab, *Limulus polyphemus* (Bang, 1956) and the blue crab, *C. sapidus* (Krantz et al., 1969; Tubiash and Krantz,

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1970). In contrast to gaffkemia, infections caused by *Vibrio* spp. are characterized by a reduction in hemocyte numbers and extensive intravascular clotting apparently triggered by an endotoxin (Bang, 1956; Levin, 1967).

During the winter of 1974, a *Vibrio* sp. was isolated by the authors from a naturally infected *C. irroratus*. The symptoms of infection, sluggish behavior, extremely low hemocyte numbers, high numbers of *Vibrio* sp. in the hemolymph and eventual death, were very similar to those produced by the *Vibrio* pathogenic to *Limulus* (Bang, 1956).

The purpose of this study is to establish whether *A. viridans* var. *homari* and the *Vibrio* sp. isolate are pathogenic to the rock crab, *C. irroratus*, and to investigate some of the aspects of the host–pathogen–environment interactions which allow these diseases to become established. Additionally, the *Vibrio* sp. isolate was characterized.

MATERIALS AND METHODS

Crabs. C. irroratus were collected using SCUBA off Ram Island, Mystic, Connecticut. Within 1 hr after capture, the crabs were placed in a 1200-liter flow-through seawater tank. They were not fed during this maintenance period or the experimental period. Crabs collected from Ram Island were used within 2 days after capture to determine the natural bacterial burden. Crabs designated for the remaining experiments were used by the end of the first week of captivity.

Crabs from Ram Island were sampled throughout the year to study the effect of molt status, temperature, and hemocyte numbers on the total bacterial burden of *C. irroratus* hemolymph. The presence of wounds, molt status, and sex of each crab was recorded. The crabs were classified by molt status using the criteria of Haefner (1976). For statistical analysis, the crabs were grouped as intermolt crabs (C_1-C_4) or molt crabs (D_1-B_2). Water temperature was measured to the nearest degree on each sampling date.

Hemolymph. The administration of inocula and collection of hemolymph were performed aseptically. Inoculum was introduced through the coxal membrane of the third walking leg with a sterile 1-ml Tuberculin syringe equipped with a 27-gauge needle. Hemolymph was collected through the coxal membrane (previously swabbed with 70% ethanol) with a 1-ml Tuberculin syringe. If crabs had previously been injected, the hemolymph was withdrawn through a coxal membrane on the opposite side of the crab.

The presence of bacteria in the hemolymph was determined five times during the year. One-tenth milliliter of hemolymph was withdrawn from each crab, transferred to tubes of sterile marine broth (Difco), and incubated at room temperature. Growth was recorded after 7 days. Bacteria isolated from the January sample were characterized to determine the percentage of *Vibrio* sp. infection in the winter crab population.

Vibrio *sp. isolate.* The *Vibrio* sp. was isolated from a naturally infected *C. irroratus* and maintained with weekly transfer in marine broth (Difco) at room temperature. Standard bacteriological methods were employed to characterize the *Vibrio* sp. All tests were done at least in duplicate.

In preparing *Vibrio* sp. inocula, 24-hr marine broth cultures (20°C) were used. The cultures were centrifuged at 580*g* for 20 min and the resulting pellet washed twice with 3% (w/v) sterile saline. The *Vibrio* pellet was resuspended with sterile saline to give a concentration of 4.8×10^6 bacteria/ml.

The *Vibrio* isolate was tested for its ability to fulfill Koch's postulates. One-tenth milliliter of hemolymph was taken from each of two crabs which were previously injected with 0.2 ml of an original *Vibrio* isolate and showing symptoms of the infection. The hemolymph sample was spread on a marine agar and incubated at room temperature for 24 hr. Two colonies which were morphologically similar to the original *Vibrio* sp. isolate (irridescent, buff colored, raised colonies) were isolated on marine

agar streak plates and later transferred to marine broth. The resulting cultures (designated as isolates 1 and 2) were shown to be identical to the original *Vibrio* isolate (Table 1).

In testing the pathogenicity of these isolates, four groups of five crabs each were injected with 0.2 ml of a 24-hr culture of isolate 1, isolate 2, the original isolate, and sterile marine broth, respectively. The crabs were then placed in running seawater (7 ± 1°C). Deaths were scored at 6-hr intervals. A crab was considered dead when it no longer responded to touch stimulus. Four samples of hemolymph from the experimentally infected crabs were aseptically withdrawn and analyzed as before for the presence of the *Vibrio* sp.

Aerococcus viridans *var.* homari. The *A. viridans* var. *homari* culture was obtained from Dr. James E. Stewart of the Fisheries Research Board of Canada (Halifax Laboratory, Halifax, Nova Scotia) and maintained in tryptic soy broth (Difco). At three times during this study, it was injected into lobster to detect any change in its virulence. No change was found in the median time to death (MTD) for the gaffkemic lobster.

The *A. viridans* var. *homari* inocula were prepared from 24-hr (20°C) tryptic soy broth cultures following the procedure used for the preparation of *Vibrio* sp. inocula. The inocula were adjusted to 7.2×10^6 bacteria/ml.

Median time to death for Vibrio-*injected crabs*. Median times to death (MTD) were determined for *C. irroratus* injected with the *Vibrio* sp. at three temperatures. 5 ± 2°, 15 ± 2°, and 20 ± 2°C. Twenty male crabs, 10 experimental and 10 control, were used in each experiment. The mean weights for each experimental group were 58, 55, and 56 g, respectively. The animals were gradually (5°C change per day) acclimated to the experimental temperature 2 days before the experiment. The experimental animals were injected with 4.8×10^6 *Vibrio*/kg of body wt, while the control animals were given 1.0 ml of 3% (w/v) sterile saline/kg of body wt. They were held in glass aquaria containing 25 liters of aerated sea water. The sea water was changed daily. Deaths were recorded at half-day intervals for 1 week.

Course of infection in crabs injected with Vibrio. The ability of rock crabs to clear the *Vibrio* sp. from their hemolymph was determined at 5 ± 2°, 15 ± 2°, and 25 ± 2°C. The mean weights of the crabs in these temperatures were 70, 70, and 85 g, respectively, for the experimental group and 72, 78, and 80 g for the control group. The crabs were placed in four 25-liter aerated aquaria immersed in a constant temperature bath. The sea water was changed daily. The acclimation and maintenance of the crabs were carried out as in the previous experiment.

The experimental crabs received 4.8×10^6 *Vibrio*/kg of body wt, while the control crabs received 1 ml of 3% (w/v) sterile saline/kg of body wt. Hemolymph samples were taken from one control and three experimental crabs at 0.04, 0.17, 0.25, 0.5, 1, 2, 3, and 7 days postinjection.

The number of bacteria in the hemolymph samples was determined following serial dilution, plating, and incubation of the inoculated marine agar plates at room temperature for 3 days. Dilutions were plated in triplicate.

In hemocyte enumeration, cysteine hydrochloride was used as an anticoagulant for crab hemolymph (McKay and Jenkin, 1970). Fifty milligrams of cysteine hydrochloride were dissolved in 1 ml of 3% (w/v) saline; the *p*H of the solution was adjusted with sodium hydroxide to 7.4, the *p*H of *C. irroratus* hemolymph (Stewart and Dingle, 1968). One-tenth of a milliliter of hemolymph was drawn into a syringe containing 0.3 ml of this anticoagulant solution, gently mixed, and a drop of the preparation was loaded onto a Levy and Levy-Hausser haemocytometer. All counts were made in duplicate.

Course of infection in A. viridans *var* homari-*injected crabs*. The clearance of *A. viridans* var. *homari* by rock crabs was also

investigated at 5 ± 2°, 15 ± 2°, and 25 ± 2°C. The crabs were maintained in a 1200-liter flow-through seawater tank. The mean weights of the crabs at each temperature were 69, 68, and 109 g for the experimental groups and 71, 67, and 115 g for the control groups. Experimental crabs received 7.2×10^6 *Aerococcus*/kg of body wt, while control crabs received 1 ml of 3% (w/v) sterile saline/kg of body wt.

One control and three experimental crabs were taken at 0.17, 1, 3, 7, and 14 days postinjection. Bacterial and hemocyte numbers of the hemolymph samples were monitored as in the *Vibrio* experiments with the exception that tryptic soy agar was used in pour plates.

Phagocytosis. The ability of the hemocytes to phagocytize the two bacteria was studied and compared in an in vitro experiment. Using 24-hr cultures (27°C) of both bacteria, suspensions of 3×10^8 bacteria/ml in lobster hemocyte medium (Paterson and Stewart, 1974) were made. The lobster hemocyte medium (LHM) was modified by deleting the phenol red, amino acids, and vitamins and adjusting the *p*H to 7.4. Prior to bleeding the crabs, 10-ml siliconized (Siliclad, Clay–Adams) syringes containing 1 ml of modified LHM were chilled under ice for 20 min. Two milliliters of hemolymph were then drawn into the syringe. Following gentle mixing, the needle (20 gauge) was removed and 2 drops of the hemocyte suspension were placed on each of 25 slides. To ten of the slides was added 1 drop of *Aerococcus* suspension and to another ten, 1 drop of *Vibrio* suspension. The hemocytes and bacteria were mixed immediately by rocking the slide which was then placed in a moisture chamber kept at 20°C. The five remaining slides received no bacteria and served as controls. One control slide and two slides from each experimental group were removed at 10, 20, 30, and 60 min incubation. They were flooded with cold LHM, fixed with cold absolute methanol for 5 min, and then stained with Giemsa stain. Only hemocytes which remained unclumped were used in determining the parasitic and phagocytic indices.

Toxin of the Vibrio *sp. isolate*. Since the previous work with *Vibrio* sp. (Bang, 1956) has indicated that the extreme virulence of the *Vibrio* sp. isolate may be partially caused by a toxin, the following experiments were initiated.

The exotoxin experiment employed 10 experimental crabs (mean weight = 55 g) and 10 control crabs (mean weight = 51 g). The filtrate obtained by passing a 24-hr *Vibrio* sp. culture through a 0.45-μm millipore filter was used as a crude preparation of exotoxin. The filtrate was administered at a dosage of 0.2 ml/100 g of body wt. Deaths were tallied after 4 days.

The endotoxin experiment also employed 10 control crabs (mean weight = 62 g) and 10 experimental crabs (mean weight = 53 g). The crude endotoxin was prepared by the technique of Rabin (1965). A 48-hr *Vibrio* sp. culture in 3% (w/v) NaCl tryptic soy broth was centrifuged and resuspended with 3% saline to give a 5% (v/v) suspension of the bacteria. This suspension was then boiled for 20 min. After centrifugation for 30 min (580*g*), the supernatant was ultrafiltered and used as a crude endotoxin. One-half milliliter of the crude endotoxin was administered per 100 g of body wt. In both experiments, crabs were held in a 1200-liter flow-through seawater tank at 15 ± 2°C. A second endotoxin experiment was conducted with 20 experimental crabs (mean weight = 50 g) and 20 control crabs (mean weight = 67 g) at 10 ± 2°C.

RESULTS

Variation in Natural Infection

In examining the sterility of crab hemolymph as an indicator of the frequency of natural infection, it was found that the percentage of the crabs with nonsterile hemolymph was highest from December through February (50–60%, $N = 98$) and lowest in May and June (ca. 10%, $N = 146$). Although hemocyte levels, molt status, and temperature could affect the frequency of

the natural infection in a population (Krantz et al., 1969; Tubiash et al., 1975), only hemocyte number is positively correlated significantly ($P < 0.05$) with the percentage of crabs with nonsterile hemolymph. Based on the large January and February samples, the presence of bacteria in the hemolymph was shown to be independent of molt status ($P > 0.05$, $N = 42$, $\chi^2 = 1.15$) and the presence of wounds ($P > 0.05$, $N = 47$, $\chi^2 = 2.27$).

Characterization and Pathogenicity of the Vibrio *sp. Isolate*

The optimum temperature and salt concentration for the growth of the *Vibrio* sp. isolate are 15–25°C and 0.5–4% (w/v) NaCl, respectively. Table 1 summarizes the characteristics of the *Vibrio* sp. isolate and several other *Vibrio* sp. which are pathogenic for marine shellfish or finfish. Of the *Vibrio* spp. listed, the one reported by Bang (1956) as pathogenic for *Limulus polyphemus* appears to be the closest to the *Vibrio* sp. isolated from infected *C. irroratus*. The *Vibrio* sp. reported by Bang, however, was only partially characterized.

When bacteria isolated from the hemolymph of the January crab samples were characterized, 14 of the 25 isolates grew at 25°C but not at 37°C on TCBS agar and they were all oxidase positive. Of these 14 isolates, five produced yellow colonies on TCBS agar and, therefore, were designated as presumptive *Vibrio alginolyticus* or *Proteus* sp. The remainder were all Gram-negative motile rods which produced raised, green colonies characteristic of the *Vibrio* sp. isolate. Two isolates of this group were omitted from further characterization when they produced hydrogen sulfide in TSI agar.

Of the remaining seven isolates, five appeared to be very similar to the original *Vibrio* sp. isolate (Table 2). This represents 20% of the isolated bacteria. The *Vibrio* sp. was present in 12% ($N = 42$) of the winter *C. irroratus* samples.

Injection of the original *Vibrio* sp. isolate and two isolates (isolates 1 and 2) recovered from experimentally infected crabs resulted in death of all injected crabs within 3 days. None of the five crabs injected with a marine broth placebo died within this time period or produced isolates showing characteristics similar to the *Vibrio*. Therefore, it was demonstrated that isolates 1 and 2 upon reinoculation into healthy crabs caused similar disease symptoms and death as those produced by the original *Vibrio* sp. Additionally, the bacteria recovered exhibited taxonomic characteristics identical to those of the original isolate. Thus, Koch's postulates were fulfilled.

Median Times to Death—Vibrio *sp.*

The MTD of *C. irroratus* injected with the *Vibrio* sp. varied with temperature (Fig. 1). While the MTD was 2.5 days at 5°C; it decreased to 1.25 days at 15°C and to 0.75 day at 20°C. A general increase in the total number of deaths by 7 days with increasing temperature was also evident. When the results of all three MTD experiments were pooled, the difference in mortality of experimental (23 of 30) and control crabs (1 of 30) was significant ($P < 0.01$).

Course of Infection

The course of infection of *A. viridans* var. *homari* in *C. irroratus* is temperature dependent. At 5°C, a temperature at which *A. viridans* var. *homari* displays no growth, there is a steady clearance, although a low level of bacteria (10 bacteria/ml) persisted in the hemolymph for 2 weeks (Table 3). Hemocyte numbers of experimental crabs (10^4–10^5 hemocytes/ml) did not differ significantly from those of the control crabs throughout the experiment (Table 4). At 15° and 25°C, an approximately one order of magnitude increase in the bacterial population was noted on day 3 followed by a steady decrease. Again, low numbers of *A. viridans* var. *homari* (100/ml at 15°C; 10/ml at 25°C) persisted to the termination of the experiment. Hemocyte numbers in these experimental crabs remained relatively

TABLE 1

CHARACTERISTICS OF THE *Vibrio* ISOLATE IN COMPARISON WITH SEVERAL MARINE *Vibrio*

Test	*Vibrio* isolate	*Vibrio* sp. (Bang, 1956)	*Vibrio parahaemolyticus*		*Vibrio fischeri*[a]	*Vibrio* 79-087[b]
			parahaemolyticus	*alginolyticus*		
Ferm. of glucose	+	+	+	+	+	+
Oxidase test	+	+	+	+	+	+
Flagella: one polar	+	+	+	+	d[c]	
lophotrichous	−	−	−	−	d	
Indole production	+	+	+	−	−	−
Methyl red test	+		+	−	+	−
V-P test	−		−	+	−	−
Citrate utilization	−		+	+	−	+
0/129 sensitivity	−		+	+	+	+
H_2S production	−	−	−	−	−	
Growth at:						
0% NaCl	−		−	−	−	
7% NaCl	+		+	+	+	+
10% NaCl	−		−	+	−	+
Growth at:						
5°C	+	−	−	−	+	
37°C	−	−	+	+	−	
42°C	−	−			−	
Arginine–alk. reaction	−		−	−	+	
Lysine decarboxylase	−		+	+	+	−
Ornithine decarboxylase	−		+	+	+	−
Luminescence	−	−	−	−	d	
Gelatin hydrolysis	+	+	+	+	+	
Typical colony:						
TCBS	+		+	+		−

Marine Agar	+	+	+	+	+	+
Pathogenic	+	+	+	+	−	+
Acid from:						
Mannose	+		+	+	+	
Sucrose	−	−	−	+	d	
Mannitol	+	+	+	+	d	
Dextrose	+	+	+	+		
Cellobiose	+		−[a]			
Lactose	−	−	−[d]	−	−	
Arabinose	−	−	d	−	−	
Inositol	−	−	−	−	−	
Xylose	−	−	−[d]	−	−	
Maltose	−	+	+	+		
Galactose	−	+	+[c]	+		
Fructose	−					
Starch hydrolysis	+		+	+	d	
Casein hydrolysis	−		+	+	+	
Nitrate reduction	+					
Litmus milk	−					
Hemolysis	+		d	d	−	
Urease activity	−		−	−		
Percentage of tests not in agreement with the *Vibrio* isolate	0	13.6	26.3	39.5	20.0	42.8

Note. Unless otherwise noted, all data were taken from Breed (1957) or Shewan and Vernon (1974).

[a] Fishein et al. (1970).

[b] Bowser et al. (1981).

[c] d denotes variation between strains.

[d] Carpenter et al. (1968).

[e] Sizemore et al. (1975).

TABLE 2
CHARACTERISTICS OF SELECTED *Vibrio* ISOLATES FROM WINTER CRABS (JANUARY)

	Original *Vibrio* isolate	Isolate No.						
		3	13	33	35	11	32	16
Ferm. of glucose	+	+	+	+	+	+	+	+
Oxidase test	+	+	+	+	+	+	+	+
Flagella: one polar	+	+	+	+	+	+	+	+
lophotrichous	−	−	−	−	−	−	−	−
Indole production	+	+	+	+	+	+	−	+
Methyl red test	+	+	+	+	+	+	+	−
V-P test	−	−	−	−	−	−	−	−
Citrate utilization	−	−	−	−	−	−	−	−
0/129 sensitivity	−	−	−	−	−	−	−	+
H_2S production	−	−	−	−	−	−	−	−
Growth at:								
0% NaCl	−	−	−	−	−	−	−	−
7% NaCl	+	+	+	+	+	+	+	−
10% NaCl	−	−	−	−	−	−	−	−
Growth at:								
5°C	+	+	+	+	+	+	+	+
37°C	−	−	−	−	−	−	−	−
42°C	−	−	−	−	−	−	−	−
Lysine decarboxylase	−	−	−	−	−	−	−	−
Ornithine decarboxylase	−	−	−	−	−	−	−	+
Luminescence	−	−	−	−	−	−	−	−
Gelatin liquidification	+	+	+	+	+	+	+	−
Typical colony:								
TCBS agar	+	+	+	+	+	+	+	+
Marine agar	+	+	+	+	+	+	+	−
Pathogenic for *C. irroratus*	+	+	+	+	+	+	+	+
Acid from:								
Mannose	+	+	+	+	+	+	+	
Sucrose	−	−	−	−	−	−	+	−
Mannitol	+	+	+	+	+	+	+	
Dextrose	+	+	+	+	+	+	+	+
Cellobiose	+	+	+	+	+	+	+	
Lactose	−	−	−	+	−	−	+	−
Arginine−alk. reaction	−	+	+	+	+	+	−	−
Percentage of tests not in agreement with the original *Vibrio* isolate		3.4	3.4	6.9	3.4	3.4	13.8	23.1

constant and showed little variation from those of the control crabs. While there was no mortality in either group at 5° and 15°C, several experimental crabs (yet no control crabs) at 25°C died from the fourth day on. When mortality data from the 25°C experiment were analyzed, *A. viridans* var. *homari* injected crabs showed significantly higher mortality than placebo-injected crabs ($P < 0.01$, $N = 49$, $\chi^2 = 6.63$).

The course of vibriosis in *C. irroratus* contrasts sharply with that of the *A. viridans* var. *homari* infection both in the clearance of bacteria and hemocyte counts (Tables 3, 4). At 5°C, although there was a brief period (7 hr) of clearance, the *Vibrio* increased rapidly to a steady level of approximately 10^2-10^3 *Vibrio*/ml of hemolymph before decreasing slowly to less than 10^2 *Vibrio*/ml of hemolymph at the termination of the experiment. Unlike *A. viridans* var. *homari*, the *Vibrio* can grow at 5°C. At

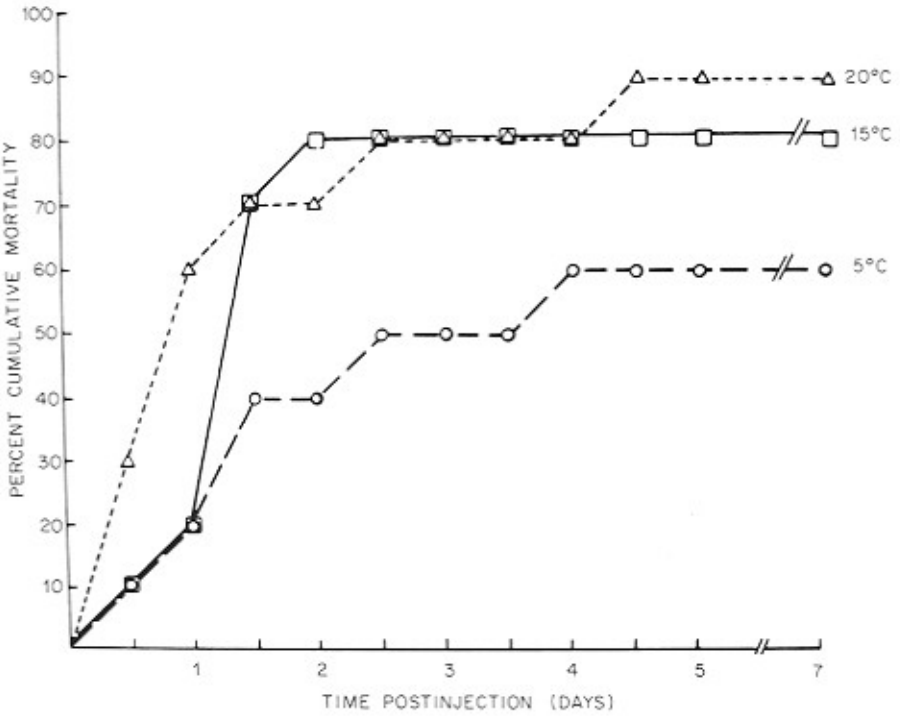

FIG. 1. Percentage cumulative mortality of *Vibrio*-injected crabs at three temperatures. Shorter median times to death and higher final percentage mortalities were noted at higher temperatures.

TABLE 3

COURSE OF INFECTION FOR *Vibrio* sp. AND *Aerococcus viridans* var. *homari*-INJECTED CRABS—BACTERIAL CONCENTRATIONS

Injected bacterium	Temperature (°C)	Bacterial concentration[a] on day								
		0.04	0.17	0.25	0.50	1	2	3	7	14
A. viridans var. *homari*										
	5	—[b]	3.24 (0.14)	—	—	2.04 (0.53)	—	0.88 (0.69)	0.76 (0.56)	0.27 (.038)
	15	—	3.93 (0.35)	—	—	3.28 (0.11)	—	4.72 (0.76)	2.25 (0.44)	1.77 (0.05)
	25	—	2.03 (0.45)	—	—	2.32 (0.03)	—	3.79 (1.24)	1.70 (1.22)	0.49 (0.41)
Vibrio sp.	5	1.04 (0.45)	—	0.45 (0.41)	2.77 (0.79)	1.94 (0.26)	2.72 (0.23)	2.76 (1.33)	1.54 (0.30)	—
	15	4.46 (0.49)	—	3.58 (0.93)	2.23 (0.49)	3.93 (1.41)	2.13 (0.14)	2.78 (0.88)	3.85 (0.00)	—
	25	3.71 (0.09)	—	2.34 (1.03)	3.50 (0.61)	3.77 (0.00)	—	—	—	—

[a] Log of mean number of colony-forming units per ml of hemolymph (1 SD).

[b] Indicates no data.

TABLE 4
HEMOCYTE COUNTS OF *Cancer irroratus* INFECTED WITH *Aerococcus viridans* var. *homari*

Injected bacterium	Temperature (°C)	Hemocyte concentrations[a] on day									
		0.04	0.17	0.25	0.50	1	2	3	7	14	Control
A. viridans var. *homari*	5	—[b]	4.98 (0.40)	—	—	5.01 (0.11)	—	4.88 (0.20)	4.81 (0.31)	4.95 (0.28)	4.93 (0.08)
	15	—	5.31 (0.01)	—	—	5.74 (0.08)	—	5.78 (0.18)	5.40 (0.01)	5.37 (0.19)	5.49 (0.16)
	25	—	5.56 (0.21)	—	—	5.55 (0.02)	—	5.47 (0.18)	5.65 (0.03)	5.47 (0.19)	5.68 (0.18)
Vibrio sp.	5	4.22 (0.55)	—	4.38 (0.40)	4.71 (0.50)	4.77 (0.23)	4.09 (0.90)	3.46 (0.28)	4.79 (0.19)	—	5.68 (0.17)
	15	5.05 (0.12)	—	5.28 (0.28)	4.93 (0.25)	4.52 (0.66)	5.12 (0.25)	5.10 (0.28)	4.00 (0.00)	—	5.81 (0.19)
	25	4.53 (0.47)	—	4.27 (0.23)	3.50 (0.35)	4.84 (0.00)	—	—	—	—	5.88 (0.11)

[a] Log of mean hemocytes per ml of hemolymph (1 SD).
[b] indicates no data.

15°C an initial clearance of the *Vibrio* lasted 14 hr. However, for the rest of the week, *Vibrio* remained relatively high at above 10^2 *Vibrio*/ml of hemolymph. At 25°C, the situation was similar to that at 15°C except that excessive deaths necessitated the termination of the experiment after 1 day when the *Vibrio* was present at approximately $10^{3.5}$/ml of hemolymph.

In all *Vibrio* experiments, the hemocyte numbers of experimental crabs (Table 4) decreased markedly as compared with those of the control crabs (log hemocytes/ml = 5.80) and remained low (log hemocytes/ml = 4.50) for the duration of the experiment.

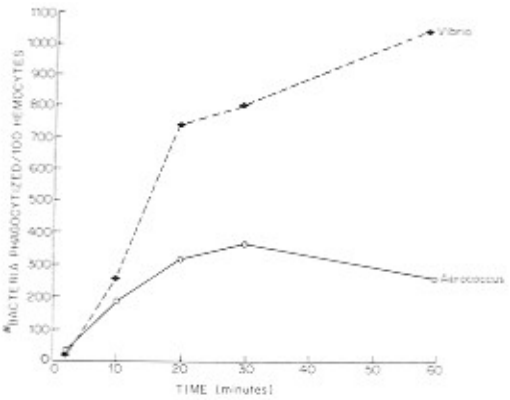

FIG. 2. Phagocytic indices of *Cancer irroratus* hemocytes exposed to *Aerococcus viridans* var. *homari* and *Vibrio* sp. at 20°C. The *Vibrio* sp. was phagocytized more rapidly than *A. viridans* var. *homari*.

Toxins of the Vibrio *sp.*

No mortality was observed in the crabs inoculated with a filtrate of the *Vibrio* isolate culture indicating that either there was no exotoxin or the exotoxin, if produced, was not lethal to the host. However, endotoxin was found to be present. A χ^2 statistic calculated using the mortality of control (4 of 30 crabs) and experimental (14 of 30 crabs) crabs showed that the difference between these two groups was significant at $P = 0.025$ ($N = 60$, $\chi^2 = 6.42$).

Phagocytosis

Based on the criteria of Wood and Visentin (1967), the hemocytes of *C. irroratus* consist of large and small granulocytes as well as hyaline cells with a ratio of 6:3:1. Forty-eight percent of the hemocytes had basophilic granules and 18% had eosinophilic granules. The hyaline cells appeared to be less active in phagocytosis than the granulocytes.

Both phagocytic and parasitic indices reveal that the *Vibrio* is more avidly phagocytized than *A. viridans* var. *homari* by *C. irroratus* hemocytes (Figs. 2, 3). Phagocytosis of *Vibrio* sp. proceeded rapidly in the first 20 min (70% hemocytes phagocytizing and 50 bacteria/100 hemocytes) and continued at a moderate rate up to 60 min when the indices reached 85% and 1050 bacteria/100 hemocytes. In contrast, both indices for *Aerococcus* were highest (33% and 365 bacteria/100 hemocytes) at 30 min after contact and were followed by a slow decline. It should be noted that the indices given here are likely overestimates due to the difficulty of estimating whether a bacterium was actually within or merely adhering to a hemocyte.

On slides containing hemocytes and the *Vibrio* sp., extensive clumping similar to that described by Bang (1956) for *Limulus polyphemus* hemocytes exposed to a *Vibrio* sp. was noted. *Vibrio* were seen adhering to

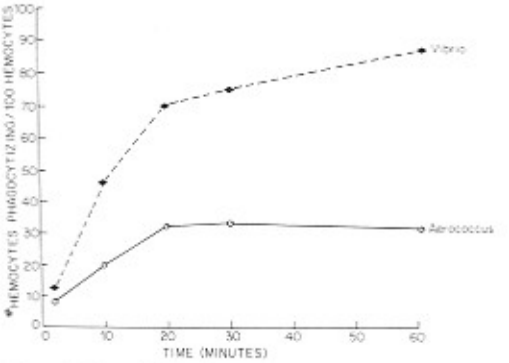

FIG. 3. Parasitic indices of *Cancer irroratus* hemocytes exposed to *Aerococcus viridans* var. *homari* and *Vibrio* sp. at 20°C. More hemocytes were involved in phagocytizing the *Vibrio* sp. than *A. viridans* var. *homari* throughout the course of the experiment.

cytoplasmic strands extending between clumps of hemocytes.

DISCUSSION

Natural Infection

The high level (10–60%) of natural infection in *C. irroratus* is not unexpected; Tubiash et al. (1975) have reported levels of natural bacterial infection in blue crabs to approximate 82%. The seasonal variation of such infection for the blue crab is opposite to that the rock crab (highest during December–February, lowest during May–June). The reason may be apparent from a review of their molting behavior. The molting of *C. irroratus* is associated with decreasing temperature (approximately September) (Haefner and Van Engel, 1975) while it is associated with increasing temperature (approximately April) in *Callinectes sapidus* (Van Engel, 1968). Since this study demonstrated that the high level of infection for *C. irroratus* was correlated with the low level of hemocyte concentrations which also occurred during molting (Bauchau and Plaquet, 1971), it is inferred that susceptibility of the two species is probably associated with the number of hemocytes. In addition to the reduction in hemocytes, the integrity of the integument is weakened and agglutinin titers often decrease (Bang, 1967) during molting. Indeed, mortalities for shedding blue crabs held in commercial tanks have been documented as 50% or higher (Krantz et al., 1969; Johnson, 1976). Many of the shedding crabs had large numbers of bacteria in their hemolymph (Krantz et al., 1969).

Twelve percent of the *C. irroratus* taken in January from Ram Island were naturally infected with a bacterium found to be a previously undescribed pathogenic *Vibrio* sp. This level of infection is similar to levels recorded for other crustaceans. Large numbers of the blue crab have the pathogen *Vibrio parahaemolyticus* in their hemolymph (Krantz et al., 1969; Tubiash and Krantz, 1970) and 32% of the lobster taken off the coast of Massachusetts are infected with *A. viridans* var. *homari* (Rabin, 1965). A Gram-negative rod pathogenic for *Carcinus maenas* is present in 11% of the green crabs examined by Sprindler-Barth (1976).

Experimental Infection

As with all poikilothermic organisms and their infectious agents (Tripp, 1969; Bang, 1973; Cooper, 1976), temperature plays an important role in the course of bacterial infections of the rock crabs studied. Growth of the bacteria within the host, rate of clearing the invaders by the host, susceptibility, hemocyte numbers, molting, and mortality of the host are affected.

In *A. viridans* var. *homari* infection of *C. irroratus*, a steady decline of the bacteria occurred at 5°C. However, higher temperatures apparently favored the growth of the bacteria which persisted at higher levels than at 5°C and even caused death of the host at 25°C.

Vibrio sp. in the rock crab recovered from an initial brief decline and continued to occur at relatively high level at 5° and 15°C; at 25°C large numbers of deaths of the host ensued as early as 1 day after the injection. It should be noted that *Vibrio* sp., in contrast to *A. viridans* var. *homari*, can grow at 5°C and this probably contributed to its higher persisting level relative to *A. viridans* var. *homari* at this temperature. Higher temperatures also shortened the median time to death of the host from 2.5 days at 5°C to 1.25 days at 15°C and 0.75 day at 20°C. The short median times to death reported here are similar to those found in other *Vibrio*-infected organisms. Bang (1956) observed that many adult *Limulus* which received small inocula died within 12 hr. Bowser et al. (1981) reported rapid death of lobster injected with a *Vibrio* isolate. Blue crabs injected with a *Vibrio* isolated from moribund crabs also had short median times to death (Tubiash and Krantz, 1970). In gaffkemic *C. irroratus*, temperature has been shown to determine the survival of the host.

Infection with *A. viridans* var. *homari*

does not affect hemocyte concentration of the rock crab. Although the same infection in lobsters does cause a decrease in hemocyte concentration (Stewart and Rabin, 1970), the bacterial concentration in *C. irroratus* hemolymph never reached the levels observed in the lobster hemolymph at the time of their decline (approximately 10^6-10^8 bacteria/ml of hemolymph). These findings, coupled with the low mortalities for gaffkemic rock crabs, indicate that *A. viridans* var. *homari* is less pathogenic for the lobster prey species, *C. irroratus*, than for the lobster itself. The results also suggest that *C. irroratus* can act as a reservior host for the bacterium as proposed originally by Cornwick and Stewart (1968).

The *Vibrio* sp. is more avidly phagocytized by hemocytes than *A. viridans* var. *homari*. The clumping of and the extension of cytoplasmic strands by the hemocytes could conceivably enhance phagocytosis of the *Vibrio* since immobilized *Vibrio* are more susceptible to the process than freely moving individuals (Levin, 1967). This avid phagocytosis of the *Vibrio* likely contributes to the more rapid initial clearance of the *Vibrio* compared to *A. viridans* var. *homari* but may also lead to the rapid death of the host through the release of endotoxin resulting from intracellular degradation of ingested *Vibrio*. The release of endotoxin likely induces more extensive hemocyte clumping and intravascular clotting. Blockage of blood flow could cause tissue necrosis and eventual death. This Schwartzmann-like reaction has been described for other endotoxin-producing *Vibrio* spp. pathogenic for invertebrates (Levin, 1967). The high mortalities of endotoxin-injected *C. irroratus* indicate the importance of this factor in determining the pathogenicity of the *Vibrio* sp. isolate. In *C. irroratus*, the presence of *Vibrio* and its endotoxin, as well as, the reduction of hemocytes would no doubt render the host more susceptible at all temperatures. This is consistent with the findings of Bang (1956), Levin and Bang (1964a, b), and Shirodkar et al. (1960).

Unpublished data of the authors indicate that the *Vibrio* sp. isolate may be pathogenic to a lesser degree for the American lobster, *Homarus americanus* (4 of 10 lobster dying after injection with 5×10^6 bacteria/kg of body wt at 15°C; 7 days).

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