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A Comparative Study of the Bacterial Flora of the Hemolymph of *Callinectes sapidus*

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*ABSTRACT—The bacterial flora of blue crabs (*Callinectes sapidus*) from Chesapeake Bay was examined. Hemolymph of normal, healthy blue crabs was found non-sterile, with total bacterial counts ranging from <100 to >3.0 × 10^5 on a seawater-based medium and 0 to >10^5 on Standard Methods Agar. Counts of 0-10^3 were observed on TCBS agar.* Vibrio *spp., including* Vibrio parahaemolyticus, *were the major taxonomic groups found in the crab hemolymph. A comparison of crab data with that of Chesapeake Bay oysters revealed a qualitative difference in that the crab hemolymph flora was almost entirely* Vibrio *spp., whereas the oyster flora included species of a variety of other genera.*

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INTRODUCTION

The normal bacterial flora of blue crabs (*Callinectes sapidus*) from Chesapeake Bay has not been extensively studied. Interest in the microbiology of shellfish, traditionally, has focused on human pathogens found in commercially important species, as in the case of the blue crab. Many of the papers published on the microflora of the blue crab have concerned pathogens rather than the normal microbial flora present in the healthy animal (Couch, 1967; Fishbein et al., 1970; Krantz et al., 1969; Rosen, 1967; Sawyer, 1969; Williams-Walls, 1968). In addition, information has been gathered concerning the bacteriology of processing, handling, packaging, or storage of crabmeat (Loaharanu and Lopez, 1970; Ulmer, 1961; Ward and Tatro, 1970).

This study was conducted to determine the total viable, aerobic, heterotrophic, bacterial flora of blue crab hemolymph in freshly caught, aquarium-held, and market crabs during the summer months and to identify and classify the bacterial strains isolated, using the methods of numerical taxonomy. Numerical taxonomy as a method for the analysis of taxonomic data for bacteria was first proposed by Sneath (1957) and has been used, with modifications of the original methods, by various investigators (Society for General Microbiology, 1962; Beers et al., 1962; Silvestri et al., 1962; Sokal and Sneath, 1963). Colwell and Liston (1961), in one of the earliest numerical taxonomy studies, examined marine bacteria. Subsequently, several other investigators examined the taxonomy of marine bacteria (Floodgate and Hayes, 1963; Hansen et al., 1965; Liston et al., 1963; Pfister and Burkholder, 1965; Qualding and Colwell, 1964). Results of these studies proved helpful in identifying and classifying the bacterial flora from blue crab hemolymph.

MATERIALS AND METHODS

Healthy adult blue crabs (*Callinectes sapidus*) weighing 150-230 g were obtained from seafood markets or collected from several areas in Chesapeake Bay and Chincoteague Bay during the summer of 1970. Male and female crabs possessing hard carapaces ("soft-shell" crabs undergoing ecdysis were not included) were sampled to determine total numbers and types of aerobic, heterotrophic bacteria comprising their normal microflora during the summer months when the water temperature ranged between 23°-32°C. Sources of crabs, bacterial isolates, and dates of sampling are given in Table 1.

The isolates from the holding tanks were obtained from animals maintained in the laboratory for more than 1 week. Crabs obtained from the fish market were held in a tank for 1 to 2 days before sampling. Crabs from the Rhode River in Chesapeake Bay were sampled immediately after capture. Crabs from Marumsco Bar were also sampled in the field without delay after capture. The largest sample of animals examined was a catch made off Franklin City, Va. The hemolymph was sampled 12-16 h after capture.

Total viable counts of aerobic, heterotrophic bacteria in blue crab hemolymph were carried out as follows. After removing the fluid with a sterile 25-gauge needle and syringe wetted with a sterile solution of 5 percent sodium polyanethol sulfonate (GROBAX)[1,2], an anticoagulant reportedly effective for isolating bacteria from blood (Morello and Ellner, 1969), 1 ml of the hemolymph was withdrawn from each crab, either through the posterior hinge of the carapace or from the base of the coxa of the flippers. A dilution series was made, using a sterile diluent composed of four salts and distilled water in the concentration: NaCl, 24.0 g; KCl, 0.78 g; $MgCl_2$, 5.3 g; $MgSO_4 \cdot 7H_2O$, 7.0 g; and distilled water, 1 liter. One-tenth ml of each dilution was inoculated onto agar plates, in triplicate, and spread evenly with a sterile, bent glass rod. The three media used were: MSYE (Proteose peptone, 1 g; yeast extract, 1 g; agar, 15 g; four salts solution, as above; pH adjusted to 7.4-7.6 with 0.1 N NaOH), SMA, the Standard Methods Agar (Casein digest, 5.0 g; yeast extract, 2.5 glucose, 1.0 g; agar, 15 g; distilled water, 1 liter; pH adjusted to 7.0 with NaOH) and TCBS, the Thiosulfate-citrate-bile salts-sucrose agar[3]

[1]Roche Diagnostics, Hoffman-LaRoche, Nutley, N.J.
[2]Reference to trade names does not imply endorsement by the National Marine Fisheries Service, NOAA.

Table 1.—Source and date of isolation of bacterial strains included in the numerical taxonomy analysis.

Strain number	Date of isolation	Source[1]
1-10	7/15/70	HT
11-27	7/19/70	FM
28-33	7/21/70	RR
34-39	8/22/70	MB
40	8/27/70	FC
41	8/27/70	FC
42	8/27/70	FC
43	8/27/70	FC
44	8/27/70	FC
45	8/27/70	FC
46	8/27/70	FC
47	8/27/70	FC
48	8/27/70	FC
49	8/27/70	FC

[1]HT – Holding tank, Georgetown University; FM = Fish market, District of Columbia; MB = Marumsco Bar, Md. (Chesapeake Bay); RR = Rhode River, Md. (Chesapeake Bay); FC = Franklin City, Va. (Chincoteague Bay).

(yeast extract, 5.0 g; polypeptone peptone, 10 g; sodium citrate, 10.0 g; sodium thiosulfate, 10.0 g; oxgall, 5.0 g; sodium cholate, 3.0 g; sucrose, 20.0 g; NaCl, 10.0 g; iron citrate, 1.0 g; thymol blue, 0.04 g; bromthymol blue, 0.04 g; agar, 14.0 g; distilled water, 1 liter; pH 8.6). The inoculated media were incubated at 25°C for 48 h, after which counts were made and the media again incubated for an additional 5 days for a repeat count.

Forty-nine cultures were isolated, purified, and subjected to the testing procedure used for numerical taxonomy. Colonies were selected randomly and were streaked three times to ensure purity of the cultures. After purification, all cultures were tested for ability to grow on a medium (MSYE) containing the major ionic constituents of seawater. All cultures isolated from the SMA and TCBS media also grew on MSYE and were, therefore, maintained on MSYE.

Taxonomic tests were carried out on each pure culture for morphology and motility, using wet-mount preparations of 24-h broth cultures under phase contrast; gram stain; growth characteristics on MSYE agar after 48 h incubation at 25°C; growth at 4, 15, 25, 37, and 45°C; growth at NaCl concentrations of 0.0, 3.0, 7.0, and 10.0 percent; growth at pH 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0; hemolysis of human blood; utilization of citrate; aerobic and anaerobic utilization of glucose and sucrose, employing MOF medium[4] (Leifson, 1963); utilization of galactose, mannitol, ribose, lactose, mannose; production of catalase, oxidase (Kovacs, 1956), indole, acetyl methyl carbinol, and urease; production of ammonia from peptone, NO_2, and NO_3; hydrolytic activity on starch, gelatin and casein; methyl red test; and utilization of alanine, proline, glutamic acid or methionine as sole carbon and nitrogen sources.

Drug sensitivity of each pure culture was determined using penicillin, 10 units; Chloromycetin, 30 μg; tetracycline, 30 μg; dihydrostreptomycin, 10 μg; and colimycin, 10 μg[5]. Sensitivity was recorded without quantification of the diameter of the inhibition zone. Sensitivity to the pteridine compound (0/129) was also recorded (Shewan et al., 1954).

All media, with the exception of the blood agar, were prepared with the salt solution, described above, as diluent. In most cases, dehydrated medium was mixed directly with the salt solution as diluent, but some media required special preparation. Media for the casein hydrolysis, methyl red, Voges-Proskauer, and carbohydrate utilization tests were prepared double strength in distilled water, and a double strength, sterile salt solution was added (1:1 vol/vol) after sterilization.

A total of 126 features used in the computer analysis were scored, with the code: 0 (negative), 1 (positive), or 3 (not tested or not applicable). The coded data were entered for analysis in an IBM 360/40 system equipped with discs and magnetic tape drives. Georgetown University Taxonomy Programs (GTP-1, 2, 4, and 5) were used in the analysis (Colwell, 1964; Colwell and Liston, 1961; Moffett and Colwell, 1968). The programs have been documented for the IBM Computer Users Library.

RESULTS

A wide range in bacterial counts for the blue crab hemolymph was observed. On the MSYE medium, counts ranged from <100/ml (five animals) to $>3.0 \times 10^5$/ml (five animals). Counts on SMA were lower, 0/ml (11 animals) to $>10^5$ ml (two animals), indicating that many of the bacteria found in the hemolymph require the major salts found in seawater for growth. On TCBS, the counts were from 0 (14 animals) to 10^3 (3 animals). The dominant colony type appearing on TCBS agar was yellow, 2 mm in diameter, and similar to *Vibrio alginolyticus*. Results of total viable counts are given in Table 2. No change in counts was observed between 48 h and 7 days.

Table 2.—Bacterial counts of the hemolymph of crabs collected at several locations on the media employed in the study. Counts given are per milliliter hemolymph of individual crabs. Figures given for each sample set are mean values, except where otherwise indicated.

Source	No. sampled	MYSE	SMA	TCBS
Laboratory holding tank	3	1.8×10^5	3×10^2	10
Fish market	2	6×10^4	4×10^3	1
Rhode River	6	2.1×10^5	60	0
Marumsco Bar	4	1.3×10^5	2×10^5	100
Franklin City, Va.	33	3.1×10^5	2×10^2	33
Total range	48	<100->3.0×10^5	0->10^5	0-10^3

The strain clusters obtained from the computer analysis of the taxonomic data are shown in Figure 1. All strains grouped at $S \geq 57$ percent and the major phenons detected are shown in Figure 1, A through I. Strains clustered in Phenon A were not found to be closely related, indicated by the low similarity values (62-64 percent). Phenon B consisted of strains more closely related to each other (S = 72-74 percent). Phenon C comprised the largest group, consisting of strains 41 through 11 (Figure 1) clustering at $S \geq 75$ percent. Three subgroups (I, II, III) were observed in Phenon C (Figure 1).

Phenon D, strain 9-24 (Figure 1) formed at S values $\geq$ 71-75 percent, indicating relatively high relationship among the strains. Phenon E formed at 72 percent S and was considered distinct from Phenon D. Phenons F and G formed at relatively low S values, 65-67 percent S and 68 percent S, respectively. Phenon H (intra S values of 62-63 percent) and Phenon I completed the groupings obtained in the analysis.

DISCUSSION

Counts on MSYE were comparable among sampling locations. Greater variations were noted for counts on SMA, with the lowest counts on SMA noted for Rhode River animals. Crabs from Marumsco Bar gave highest counts on SMA. The salinity of the water at Rhode River was lower than at Marumsco Bar and both the Rhode River and Marumsco Bar crabs were sampled immediately after capture. Hence, no correlation was noted between time of sampling after capture

[3]Baltimore Biological Laboratories, Baltimore, Md.
[4]Difco Laboratories, Detroit, Mich.
[5]Baltimore Biological Laboratory, Bioquest Division of Becton-Dickinson, Inc., Sensi-Disks.

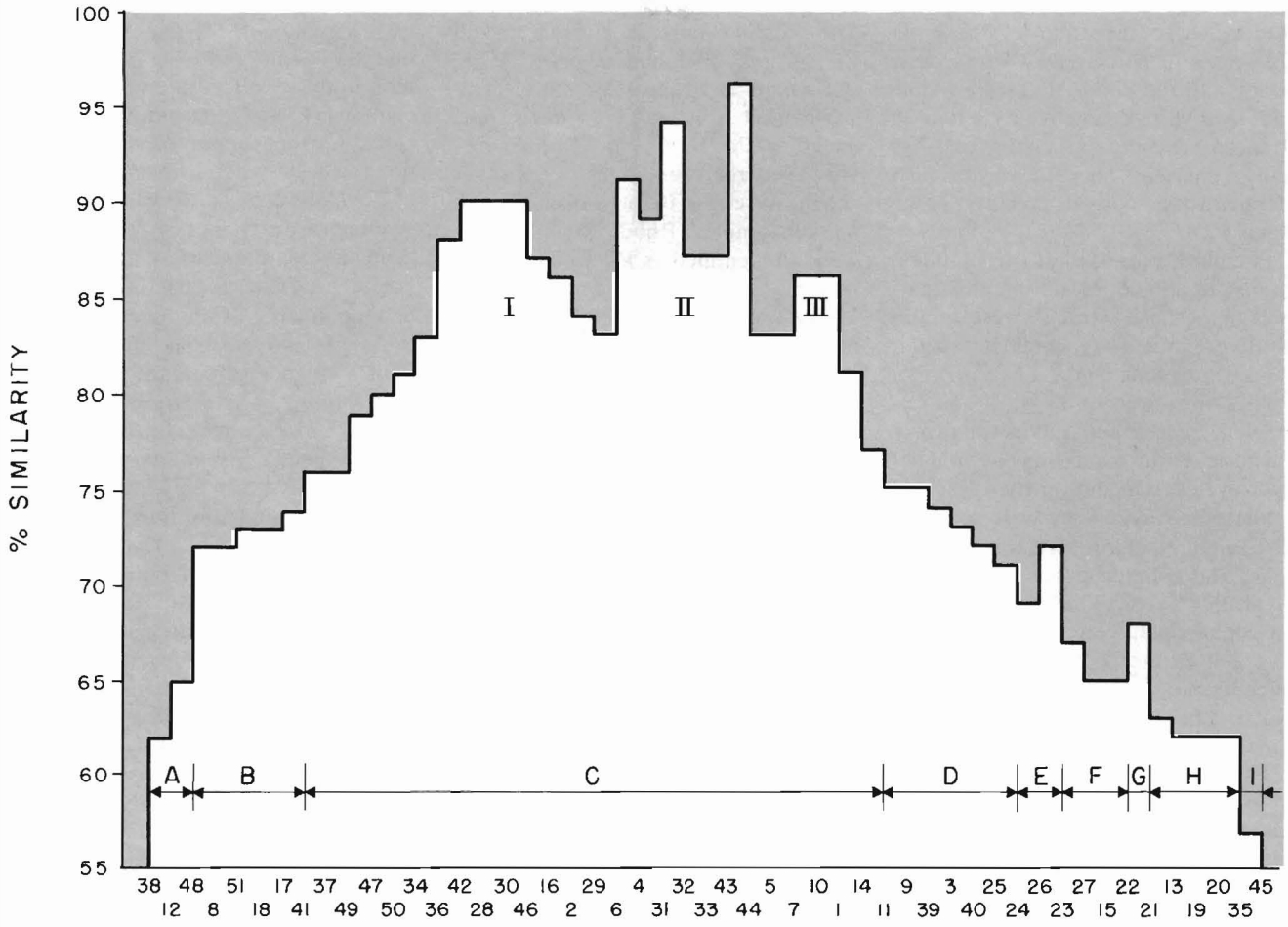

Figure 1.—Sorted output from the taxonomic analysis by computer. Groups were formed by highest linkage sorting.

and the salinity and the number of bacteria in crab hemolymph detected on SMA, a medium employed for detecting nonmarine bacteria. Also, no correlation between sex and total viable count on SMA was detected. However, it was clear that the proportion of nonmarine bacteria in crab hemolymph was higher for fish market and Marumsco Bar crabs. The holding tank and Franklin City samples exhibited approximately the same proportion of nonmarine bacteria (bacteria capable of growth on SMA).

The genera of bacteria found in crab hemolymph were obtained from the taxonomic data analyses, and each of the eight phenons were classified. Strains 38 and 12 (Phenon A) were gram-positive, motile rods, 1.3-3.0 μm long; capable of utilizing glucose and sucrose aerobically and anaerobically; growing at temperatures of 4°-37°C and pH 7.0-9.0; sensitive to penicillin, Chloromycetin, and colimycin; resistant to dihydrostreptomycin; being oxidase positive, indole negative, and citrate positive; utilizing alanine and proline as sole carbon and nitrogen sources; growing in 7-10 percent NaCl; and were classified as *Bacillus* sp.

Phenon B included five strains, one of which was the reference strain of *Vibrio parahaemolyticus* (strain Sak-3).

Phenon C was the largest group, with 27 strains comprising the cluster. All of the strains of Phenon C were round ended, motile, gram-negative rods, 1.3-3.0 μm long × 0.5-1.3 μm wide and occurring singly, not as pairs or chains. These strains grew at pH 4.0-9.0 and at temperatures of 15°-45°C, with half of the isolates capable of growth at 4°C and in 3-10 percent NaCl. However, 92 percent of the isolates were capable of growth without added NaCl. The strains formed convex, entire, translucent colonies yellow to white in color on MYSE agar. All fermented glucose anaerogenically and, in general, produced acid in sucrose, galactose, mannose, mannitol, and ribose and were lactose negative. All strains hydrolyzed starch, produced NH_3 from peptone, reduced nitrates, produced catalase, hydrolyzed casein, utilized citrate as a sole carbon source, were oxidase positive and methyl red positive. None of the strains of Phenon C produced urease or acetylmethylcarbinol, but half produced indole. All were sensitive to Chloromycetin; 64 percent were sensitive to dihydrostreptomycin; 36 percent to colimycin, and 16 percent to penicillin. Phenon C was classified as *Vibrio* spp.

Phenon D strains were straight, gram-negative rods, 1.3-3.0 μm long, occurring singly and in short chains (less than five cells). Colonies 2-5 mm in diameter and a translucent yellow or white were formed on agar. Glucose and sucrose were utilized aerobically and anaerobically, without gas. Acid was produced from mannose; nitrate

was reduced and NH_3 produced from peptone. All of the strains of Phenon D grew in 3-10 percent NaCl and most strains utilized citrate and were indole and methyl red negative. Phenon D strains were catalase positive and hydrolyzed casein. This phenon was also identified as comprising members of the genus *Vibrio*.

Phenon E consisted of two cultures, strains 23 and 26, clustering at 72 percent *S*. These strains were gram-positive, motile rods, occurring singly, 1.3-3.0 μm long $\times$ 0.7-1.5 μm wide. Both strains grew at 15°-45°C and fermented glucose and sucrose and produced acid from mannose. They did not hydrolyze casein and were oxidase and catalase negative. They were sensitive to penicillin, chloromycetin, tetracycline, and colimycin, but not to dihydrostreptomycin. The strains did not produce indole, were methyl red negative and citrate positive, did not produce urease, and grew in 7-10 percent NaCl. Phenon E was identified as a *Bacillus* sp.

The two strains, 15 and 27 (Phenon F) were identified as *Acinetobacter* sp. based on their inability to utilize the carbohydrates tested, their oxidase negativity, and catalase positivity.

The distinquishing features of Phenon G (strains 21 and 22) which clustered at 68 percent *S* were variable gram stain, rod shape and yellow colonies. The strains fermented glucose and sucrose, but did not reduce nitrates nor hydrolyze casein. They were oxidase positive, sensitive to dihydrostreptomycin, indole and methyl red negative, citrate positive, urease negative and grew in the presence of 0-10 percent NaCl. Phenon G was identified as a *Flavobacterium* sp.

Phenons H and I were not identified. They were motile, gram-negative rods which produced yellow to white colonies on MSYE agar at 15°-37°C and at pH 7.0-9.0. Strains in Phenons H and I fermented glucose and sucrose. The other characteristics tested varied among the strains of the two groups.

In summary, the isolates from the hemolymph of the blue crab belonged to the genera *Vibrio*, *Acinetobacter*, *Flavobacterium*, and *Bacillus*. The largest number of isolates, 38 of the 49 isolates examined, were identified as *Vibrio* spp.

A taxonomic distribution by source of the animals proved to be interesting. The holding tank isolates were all *Vibrio* spp., including phenons B, C (subgroups I, II and III), and D. The fish market isolates were *Vibrio* spp., including the B, C, and D phenons. However, representatives of the other genera were also isolated from the fish market samples. Rhode River isolates were all identified as *Vibrio* spp. (Phenon C, subgroups I and II). The Marumsco Bar isolates included phenons A, C, D, and H (*Vibrio*, *Bacillus*, and unidentified strains). The Franklin City isolates were predominantly of Phenon C (subgroups I and II) with some B and D phenons also observed to comprise the flora. Thus, the common feature of the crab hemolymph was the preponderance of *Vibrio* spp., particularly Phenon C vibrios which were present in crab hemolymph from all locations. A significant difference observed between the Marumsco Bar and fish market crabs and crabs from other locations in Chesapeake Bay was the presence of *Bacillus*, *Acinetobacter*, *Flavobacterium*, and other unidentified bacteria in the hemolymph.

The analysis was carried further in that the data for the 49 strains of this study were combined with data obtained from 181 other isolates from Chesapeake Bay blue crabs (unpublished data). The data for the 230 strains were subjected to computer analysis and the results showed 26 clusters at $S \geq 61$ percent with the largest cluster containing 68 strains. The 49 strains from the study reported here showed 22 strains clustered in the large, 68 strain phenon, identified as the genus *Vibrio*.

Thus, the results of the extended taxonomic analysis substantiates the observation that the predominant bacteria in the hemolymph of blue crabs are *Vibrio* spp.

An additional comparison was made of the data for the 49 crab isolates with data for 161 strains isolated from the Eastern oyster, *Crassostrea virginica*, which is found in Chesapeake Bay (unpublished data). The entire set of data for the 210 strains was analyzed by computer and it was found that the crab isolates, for the most part, did not cluster with and were distinct from the oyster strain clusters. Seven of the crab strains did cluster with oyster strains at $S \geq 61$ percent. These seven strains were largely of one phenon, Phenon D, members of which did not predominate crab hemolymph. The results suggest that the Phenon D *Vibrio* is more common in oysters than in crabs, with the phenon C *Vibrio* being more common in crabs. Further support for this observation can be drawn from the observations of Lovelace et al. (1968) who did a bacteriological analysis of oysters collected from the Marumsco Bar and Eastern Bay areas of Chesapeake Bay and found a predominance of *Vibrio*, *Pseudomonas*, *Achromobacter* spp., in that order, with a smaller percentage of *Corynebacterium*, *Cytophaga-Flavobacterium*, *Micrococcus-Bacillus*, and enteric species. *Pseudomonas*, *Achromobacter* and *Corynebacterium* spp. were not isolated from crab hemolymph in this study and only a few *Flavobacterium*, *Bacillus*, and other unidentified species were noted. However, the number of strains included in this study was relatively small. Nevertheless, the indication is that the composition of the microbial flora of the blue crab does not reflect geographical differences so strongly nor is so varied as that of the oyster (Lovelace et al., 1968).

In summary, the total number of viable, aerobic, heterotrophic bacteria found in hemolymph of healthy blue crabs is large and variable. Analysis of taxonomic data by computer revealed the predominant genus present in the hemolymph to be *Vibrio*, with members of *Bacillus*, *Acinetobacter* and *Flavobacterium* present in much smaller numbers. Clearly the hemolymph of most healthy blue crabs is not sterile.

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