

Bacterial Flora of the Hemolymph of the Blue Crab, *Callinectes sapidus*: Numerical Taxonomy

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Bacteria isolated from the hemolymph of normal blue crabs were found to be predominantly *Vibrio* sp., *Pseudomonas* sp., *Acinetobacter* sp., *Bacillus* sp., *Flavobacterium* sp., and coliforms. *Vibrio parahaemolyticus*, a cosmopolitan facultative pathogen widely implicated in outbreaks of gastroenteritis related to the consumption of improperly processed seafood, was present in crabs collected between the months of May to November and was identified in up to 21% of the hemolymphs sampled.

A causative agent of gastroenteritis in humans, *Vibrio parahaemolyticus*, has been reported as a public health problem in the commercial preparation of crab meat (6, 14). *V. parahaemolyticus* can be isolated from Chesapeake Bay water and sediment and demonstrates a characteristic association with plankton, particularly during the warmer months (7). Moribund blue crabs have also been shown to contain *V. parahaemolyticus* (9). Several outbreaks of gastroenteritis due to the consumption of crabs contaminated with *V. parahaemolyticus* have been reported (12, 13, 18). Clearly, the information available suggests that the bacteria associated with blue crabs, *Callinectes sapidus*, includes potential human and crab pathogens. In light of these facts and the recent report of bacterial flora in the hemolymph of freshly caught, apparently healthy blue crabs (19), a study of the taxonomy of this flora seems of obvious public health significance. The present study concerns the numerical taxonomy of these bacteria with particular emphasis on pathogens such as *V. parahaemolyticus*. The aerobic, heterotrophic bacteria isolated from the hemolymph of a large number of crabs captured over an 18-month period comprised the test set of strains subjected to numerical taxonomy analysis.

MATERIALS AND METHODS

Bacterial cultures. A total of 99 bacterial strains were isolated on 28 different sampling dates from 99 apparently normal blue crabs. The crabs were bled aseptically and the hemolymph was subjected to bacteriological analysis using the most probable num-

ber method as described previously (19). Agar plates were streaked from tubes of the highest dilution showing growth. Isolates were selected from the streak plates by picking the colony representing the predominant colony type on the plate. Usually the highest dilution appeared to be a pure culture of a single organism. Control microorganisms *Escherichia coli* ATCC 11303, *V. parahaemolyticus* Sak 3 (3), *V. parahaemolyticus* Sak 4 (3), *Pseudomonas bathycetes* ATCC 23599, *Pseudomonas* sp. Ox-Sawyer (3), *Achromobacter fischeri* (3), and *Vibrio marinus* ATCC 15382 were included in the analysis.

The *E. coli* control strain was maintained on a modified yeast extract medium containing 1 g of proteose peptone (Difco), 1 g of yeast extract (Difco), and 1 liter of distilled water (pH 7.2). The crab isolates and the marine reference strains were maintained on a modified yeast extract medium prepared with a four salts solution (NaCl, 24 g; KCl, 0.78 g; MgCl \cdot 6H $_2$ O, 5.3 g; MgSO \cdot 7H $_2$ O, 7.0 g; and 1 liter of distilled water) as the diluent.

Tests and testing methods. Biochemical and morphological tests routinely employed (2, 3, 11) were used in this study. Morphology, staining characteristics, motility, and growth characteristics in broth culture were determined using broth cultures incubated for 24 h at 25 C. Colony morphology and pigment production were evaluated after 4 days of incubation at 25 C on yeast extract medium solidified by the addition of 2% agar (Difco). Growth at various temperatures (4, 15, 25, and 37 C) and pH (4.0, 5.0, 6.0, 7.5, 8.0, and 9.0) were evaluated after incubation for 4 days and 3 weeks, respectively. The ability to utilize glucose or sucrose aerobically or anaerobically was tested using MOF medium (10). The production of acid and/or gas from glucose, galactose, mannitol, ribose, sucrose, and lactose was examined in broth cultures containing a pH indicator and Durham vial.

The ability of the organism to utilize alanine, proline, glutamic acid, methionine, glucose, ribose, sodium acetate, or sodium citrate as sole carbon and/or nitrogen source was determined. Cultures were

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also tested for ability to utilize starch, gelatin, casein, and agar, as well as production of lipase and lecithinase. Additionally, the following tests were performed using previously cited methods (3, 4, 11): methyl red, Voges-Proskauer, oxidase (8), catalase, lysine decarboxylase, ornithine decarboxylase, arginine dihydrolyase, phosphatase, indole production, growth on Simmons citrate medium, nitrate reduction, denitrification, production of ammonia from peptone, and production of hydrogen sulfide from sodium thiosulfate or cysteine.

Sensitivity to penicillin (10 U), chloramycetin (30 μ g), tetracycline (30 μ g), dihydrostreptomycin (10 μ g), and colimycin (10 μ g) was determined using sensitivity disks (BBL, BioQuest, Cockeysville, Md.). Susceptibility to pteridine 0/129 compound was tested by dropping crystals onto a plate inoculated with the test organism.

All media, with the exception of those used for *E. coli*, were made up with the four salts solution substituted for distilled water. A few media, such as skim milk broth, methyl red-Voges-Proskauer broth, and the carbohydrate broths, were prepared double strength in distilled water and added (1:1 vol/vol) to double strength four salts solution (after autoclaving, to avoid problems of precipitation).

The methods of numerical taxonomy originally proposed by Sneath (15) and subsequently modified (1, 16) were applied in this study. A total of 188 tests were recorded as follows: 0 (negative), 1 (positive), or 3 (not tested or inappropriate). The Georgetown Taxonomy Programs (GTP-1, 2, and 3) were used in the analysis. Only positive correlations (S_{ij}) were used and clusters were formed by the single linkage methods. The methods and programs employed have been published (2, 11).

RESULTS

All of the strains tested and subjected to numerical taxonomy analysis were found to group at a similarity value of 61% ($S > 60\%$). A similarity matrix was obtained which is shown in Fig. 1. All of the strains examined were catalase positive and capable of growth at room temperature (20 to 25 C) over a wide pH range (pH 5.0 to 7.5). All strains demonstrated a requirement for Ca^{2+} and K^{+} added to the growth medium. No branching or filamentous forms were encountered and none of the motile forms exhibited gliding motility, when tested on Cook's cytophaga medium.

Characters used to differentiate groups were those which were positive, i.e., present in 90% of the strains in a given group, and present in at least one of the groups obtained in the analysis. All positive characters were included in the original calculations, with subsequent elimination of nondiscriminatory characters. The clusters were selected by scanning the similarity matrix (Fig. 1) and a dendrogram was prepared from results of the single linkage analysis.

Those groups which lacked homogeneity, i.e., did not form a well-defined cluster as viewed from the matrix, were either dropped from further analysis, or were treated by removing strains until good internal homogeneity was obtained. By this analysis the organisms were placed into 13 groups.

Cluster A (Fig. 1), which was classified as an *Aeromonas* sp., formed at 67% S and was comprised of three motile gram-negative strains, two of which were curved rods and one was spiral-shaped. The three strains produced acid from glucose, under both aerobic and anaerobic conditions in MOF medium. Gas was also produced from glucose. Members of this group were positive with respect to nitrate reduction, indole production, and oxidase test, and were sensitive to penicillin, tetracycline, and chloramycetin (Table 1).

Group B consisted of seven strains forming a cluster at 75% S. The phenon comprised three rather loosely linked subgroups (Fig. 1) which represented five species in the genus *Pseudomonas*. All strains in this group were oxidase positive, gram-negative rods not fermenting glucose or producing gas from glucose (Table 1). The strains did not reduce nitrate or produce indole and were negative for the methyl red and Voges-Proskauer tests. None of the strains were sensitive to either the vibriostatic agent 0/129 or penicillin. Two strains, FC 249 and FC 298, clustered with phenon B (Fig. 1) but were not included in the cluster because they demonstrated an anaerobic utilization of glucose, whereas strains comprising phenon B utilized glucose only aerobically.

Twenty-four organisms comprised group C, including *V. parahaemolyticus* strains Sak 3 and Sak 4, *V. parahaemolyticus* strain FC 1011 (all three of these strains have been shown to share high deoxyribonucleic acid/deoxyribonucleic acid homology, i.e., 89% and similar guanine + cytosine content [46%]) (5, 17). All strains in this group were motile gram-negative rods which formed off-white colonies and were considered to be in the species *V. parahaemolyticus*. Individuals in this cluster utilized glucose aerobically and anaerobically, without production of gas, and were positive for oxidase reaction, indole production, nitrate reduction, starch and casein hydrolysis, phosphatase, and H_2S production. Acid was produced from galactose, ribose, and mannitol. Sixteen of the 24 strains in group C clustered at 79% S. Strain FC 296, joining group C at 72% S, was included because of its high similarity with strains comprising the group. Also, it shared characters describing the group. *Achromobacter fischeri*

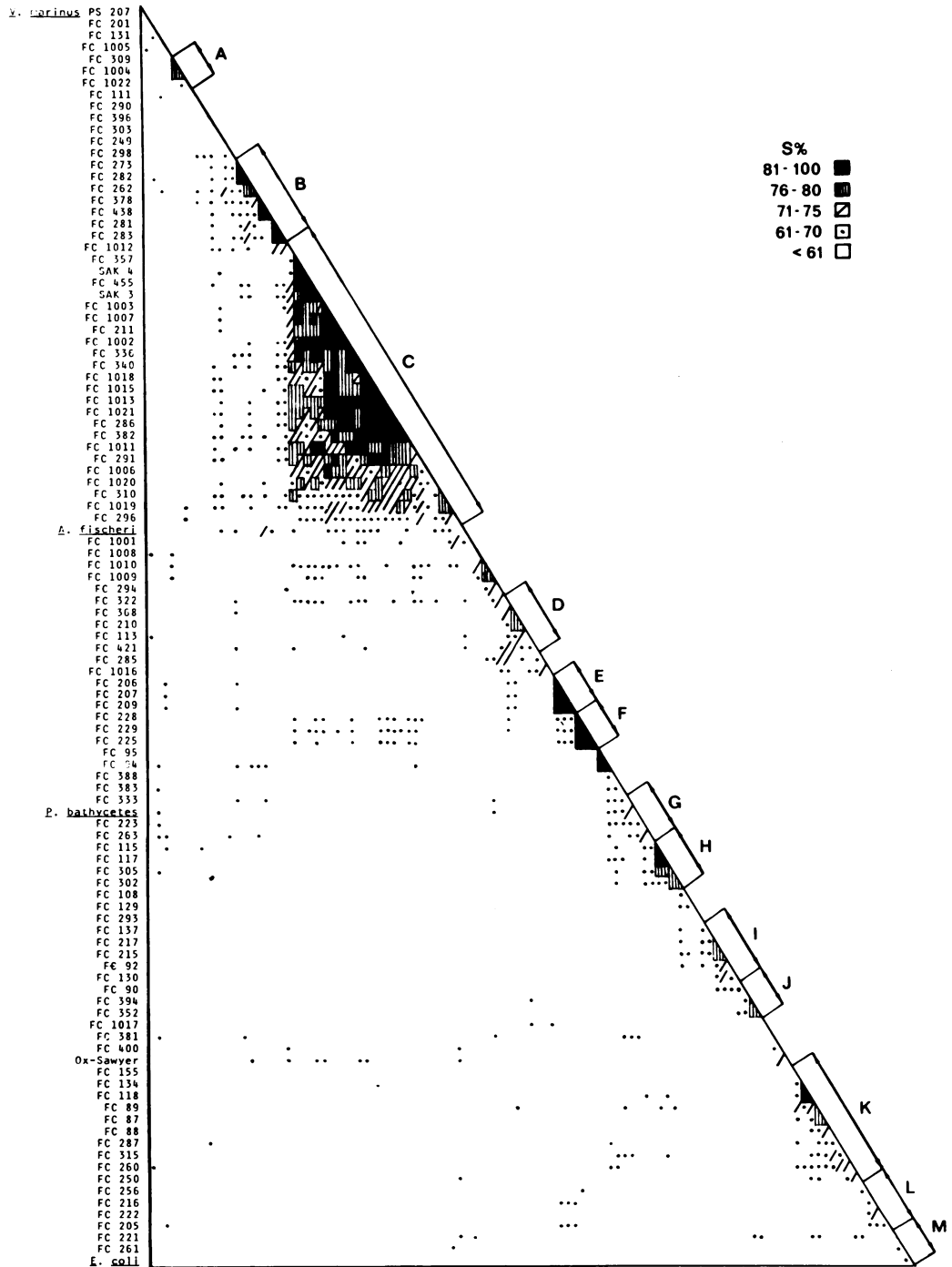


FIG. 1. Similarity matrix prepared by single linkage analysis of bacteria isolated from crab hemolymph.

appeared to be closely related to strains of group C, joining the cluster at 71% S, in keeping with the recent observation that this organism be renamed *Vibrio fischeri* (3).

Five loosely related ($S \geq 74\%$) fermentative, oxidase positive, gram-negative rods comprised group D. These microorganisms, which were identified as *Vibrio* sp., were able to reduce

TABLE 1. Characters used in identification of the clusters obtained in the analysis^a

| Character | Group | | | | | | | | | | | | |
|-----------------------------------|----------------|----|----|---|---|-----|----|----|---|---|----|----|-----|
| | A | B | C | D | E | F | G | H | I | J | K | L | M |
| Taxon | Ae | Ps | Vp | V | V | Bac | Ps | Ac | V | V | UK | F1 | Col |
| No. of organisms | 3 | 7 | 24 | 5 | 3 | 3 | 4 | 4 | 5 | 3 | 10 | 4 | 3 |
| Gram stain | - ^b | - | - | - | - | V | - | - | - | - | - | V | - |
| Motility | + | V | + | V | - | + | V | - | - | - | V | - | V |
| MOF acid aerobic | + | V | + | + | + | + | - | - | V | + | + | + | + |
| MOF acid anaerobic | + | - | + | + | + | + | - | - | V | + | + | + | + |
| Glucose gas | + | - | - | - | - | - | - | - | - | - | - | - | V |
| Oxidase | + | + | + | + | + | + | + | - | - | V | - | + | - |
| Indole | + | - | + | - | - | - | - | - | - | V | - | - | + |
| Methyl red | V | - | V | + | + | V | - | - | V | V | - | V | + |
| Voges-Proskauer | - | - | V | V | V | V | - | - | V | - | - | - | - |
| Simmon's citrate | NT | V | V | - | - | - | - | - | - | - | - | - | - |
| NO ₃ → NO ₂ | + | - | + | + | + | + | + | - | V | V | V | - | + |
| Peptone → NH ₃ | V | + | + | V | V | - | V | - | V | V | V | - | V |
| Cysteine → H ₂ S | NT | NT | + | + | + | - | V | + | V | + | V | V | + |
| Starch hydrolysis | V | - | + | + | + | + | - | - | - | - | + | + | - |
| Casein hydrolysis | + | V | + | - | + | + | - | - | V | - | V | + | V |
| Alanine ^c | V | + | + | - | - | - | V | V | - | - | - | + | V |
| Proline ^c | V | V | + | V | - | - | V | - | - | V | V | + | V |
| Glutamic ^c acid | + | + | + | V | - | V | + | V | - | + | V | + | + |
| Spores | - | - | - | - | - | + | - | - | - | - | - | - | - |
| Lactose acid | NT | V | V | - | - | - | - | - | - | V | - | - | V |
| Lactose gas | NT | - | - | - | - | - | - | - | - | - | - | - | V |
| Sucrose acid | NT | V | V | V | - | - | - | - | - | V | - | - | V |
| Galactose acid | NT | V | + | + | - | - | - | - | - | V | - | - | V |
| Mannitol acid | NT | V | + | - | - | - | - | - | - | V | - | - | V |
| Ribose acid | NT | V | + | - | - | - | - | - | - | V | - | - | V |
| 0/129 sensitive | V | - | V | V | - | - | - | + | - | - | V | V | - |
| Tetracycline sensitive | + | + | + | + | + | + | + | + | V | V | V | + | + |
| Penicillin sensitive | + | - | - | V | + | V | + | + | + | + | V | + | V |
| Chloromycetin sensitive | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Dihydrostreptomycin sensitive | - | V | V | V | + | + | V | V | V | + | V | + | - |

^a Ae, *Aeromonas* sp.; Ps, *Pseudomonas* sp.; Vp, *Vibrio parahaemolyticus*; V, *Vibrio* sp.; Bac, *Bacillus* sp.; Ac, *Acinetobacter* sp.; UK, unknown; F1, *Flavobacterium* sp.; Col, coliform-like.

^b +, 100 to 90% positive; V, 89 to 1% positive; -, 0% positive; NT, not tested.

^c Utilization as sole carbon and nitrogen source.

nitrate and produce H₂S from cysteine, and were methyl red positive. Neither casein, starch, nor other carbohydrates (with the exception of galactose and sucrose) were utilized by strains of this cluster. Strains FC 1009, FC 1010, and FC 1008 were clearly associated with group D in the similarity matrix (Fig. 1). Although the three strains joined phenon D by single linkage at S values of 73%, they were not included in the phenon because of the low internal similarity between these organisms and other strains of the cluster. The three strains were motile, oxidase positive, fermentative, gram-negative rods resembling strains of cluster D in several of the differential tests (see Table 1). However, the three strains grew poorly on the media em-

ployed in the study; hence, results were often inconclusive.

Group E proved to be a small tightly linked (S ≥ 94%) group of three strains of atypical *Vibrio* sp. The strains were nonmotile, fermentative, oxidase positive, gram-negative rods. Members of this group reduced nitrate, utilized glucose, and hydrolyzed starch, but were unable to metabolize the other carbohydrates tested. They were sensitive to tetracycline, penicillin, chloromycetin, and dihydrostreptomycin, but not to the vibriostatic agent, pteridine 0/129.

Group F was also a small, tightly linked group of three organisms, grouping at 85% S. The strains were sporeforming, motile, gram-positive rods of the genus *Bacillus*. They fermented

glucose but utilized no other carbohydrates. Group F strains were oxidase positive, reduced nitrate, formed H_2S , and hydrolyzed starch and casein.

Pseudomonas bathycetes and three crab isolates classified as *Pseudomonas* sp. linked at 70% S to form group G. All of the group G strains were oxidase positive, gram-negative rods unable to utilize any of the carbohydrates tested. Three of the four strains were motile and all reduced nitrate. Group H strains shared many of the characters of group G, evidenced by high intergroup similarity values (Fig. 1). Group H, strains of *Acinetobacter* sp., showed some differences from cluster G in that group H strains were nonmotile and oxidase negative. Strains FC 95, FC 94, FC 388, and FC 383 joined strains of groups G and H at 68% S. These strains were not included in either group G or H because of differences in their characteristics. These four strains formed a relatively diverse grouping and were not considered to be a species cluster.

Strains FC 108, FC 129, and FC 293 were not included in group H, even though these strains demonstrated 69% S with strains of group H, especially with strain FC 302 (Fig. 1). Strains FC 108, FC 129, FC 293, and FC 302 appeared to be gram-negative cocci, whereas strains of groups G and H were rod shaped. Strains FC 108 and FC 293 fermented glucose and were oxidase negative. Strain FC 129 did not utilize any of the carbohydrates tested and was oxidase positive.

Groups I and J were heterogenous arrays of strains, most probably of the genus *Vibrio*, grouping at S = 70%. Some strains in groups I and J resembled strains FC 108, FC 129, and FC 302 since they appeared to be gram-negative cocci. Strains FC 137 and FC 217 in group I were similar to strains in groups G and H in their inability to attack carbohydrates, including glucose. Both strains were oxidase negative, a character held in common with strains of group H. Strains FC 215, FC 92, and FC 130, comprising the remainder of group I, were similar to FC 137 and FC 217 in lack of ability to utilize many of the carbohydrates tested and in the oxidase reaction. Strains FC 215, FC 92, and FC 130 fermented glucose when tested in MOF medium. Strains in group J, i.e., FC 90, FC 394, and FC 352, fermented glucose and utilized several of the other carbohydrates tested. Two of the three strains were methyl red and oxidase positive, in contrast to the majority of strains comprising group I.

Group K which clustered at 71% S was an

unidentified taxon comprising 10 heterogenous strains. Strains in this group were straight or curved, oxidase negative, gram-negative rods, frequently arranged in pairs (Table 2). They fermented glucose but not the other carbohydrates tested. Indole, methyl red, Voges-Proskauer, and citrate tests were negative, as were the lysine decarboxylase, ornithine decarboxylase, and arginine dihydrolase tests. All the strains in group K were capable of hydrolyzing starch and produced yellow or off-white colonies. Only four of the 10 strains reduced nitrate, one produced ammonia from peptone, whereas six of the 10 strains were sensitive to the 0/129 vibriostatic agent.

The four strains comprising group L, which was very heterogenous (67% S), are best classified as *Flavobacterium* spp. One strain, FC 205, was gram variable, whereas the others were gram-negative. All were nonmotile and oxidase positive, fermented glucose, produced yellow or orange colonies on agar plates, and hydrolyzed starch. Strains of this group were negative for the indole, methyl red, Voges-Proskauer, and citrate tests, with the exception of strains FC 216 and FC 222 which were methyl red positive and did not reduce nitrate. They fermented glucose, but no other carbohydrates; however, this group utilized proline, alanine, and glutamic acid as sole carbon and nitrogen sources.

The remaining three strains of the analysis, FC 221, FC 261, and *E. coli*, group M, clustered at 62% S, representing a heterogenous group of coliforms. These strains produced acid from glucose aerobically and anaerobically, were oxidase negative, and reduced nitrate. Indole and methyl red tests were positive and the Voges-Proskauer and Simmon citrate tests were negative.

DISCUSSION

Identification and classification of the groups of strains isolated in the course of this study was somewhat difficult because of the diversity of the strains. Only a few groups, namely C, E, and F, showed very high intragroup similarity. The other groups observed in the analysis most likely represent generic groupings or closely related genera. A rather low intragroup homogeneity was the rule, with several species clusters being noted.

V. parahaemolyticus was the most common species of bacteria (21% of the total strains isolated) found in crab hemolymph. An interesting correlation was also noted between the occurrence of *V. parahaemolyticus* and the sampling season. Eighteen of the 22 strains

TABLE 2. Characters useful in describing cluster K of the analysis

| Character | Organism | | | | | | | | | |
|-----------------------------------|----------|--------|--------|-------|-------|-------|--------|--------|-----------------|-----------------|
| | FC 155 | FC 134 | FC 118 | FC 89 | FC 87 | FC 88 | FC 287 | FC 315 | FC 260 | FC 250 |
| Gram stain | - | - | - | - | - | - | - | - | - | - |
| Cells in pairs | - | + | + | + | - | + | + | + | + | + |
| Motility | + | - | - | - | - | - | - | - | + | + |
| MOF acid aerobic | + | + | + | + | + | + | + | + | + | + |
| MOF acid anaerobic | + | + | + | + | + | + | + | + | + | + |
| Glucose gas | - | - | - | - | - | - | - | - | - | - |
| Oxidase | - | - | - | - | - | - | - | - | - | - |
| Indole | - | - | - | - | - | - | - | - | - | - |
| Methyl red | - | - | - | - | - | - | - | - | - | - |
| Voges-Proskauer | - | - | - | - | - | - | - | - | - | - |
| Simmon's citrate | - | - | - | - | - | - | - | - | - | - |
| NO ₃ → NO ₂ | - | + | + | + | - | - | - | + | - | - |
| Peptone → NH ₃ | - | - | - | - | - | + | - | - | - | - |
| Cysteine → H ₂ S | - | + | + | - | + | + | - | - | NT ^a | NT ^a |
| Starch hydrolysis | + | + | + | + | + | + | + | + | + | + |
| Casein hydrolysis | - | - | - | - | - | + | + | - | - | - |
| Alanine ^b | - | - | - | - | - | - | - | - | - | - |
| Proline ^b | - | - | - | - | - | - | - | - | - | + |
| Glutamic acid ^b | + | - | - | + | - | + | - | - | - | + |
| Spores | - | - | - | - | - | - | - | - | - | - |
| Lactose acid | - | - | - | - | - | - | - | - | - | - |
| Lactose gas | - | - | - | - | - | - | - | - | - | - |
| Sucrose acid | - | - | - | - | - | - | - | - | - | - |
| Galactose acid | - | - | - | - | - | - | - | - | - | - |
| Mannitol acid | - | - | - | - | - | - | - | - | - | - |
| Ribose acid | - | - | - | - | - | - | - | - | - | - |
| 0/129 sensitive | - | - | - | + | + | + | + | - | + | + |
| Tetracycline sensitive | + | - | - | + | - | - | + | - | + | - |
| Penicillin sensitive | + | + | + | + | + | + | - | + | - | - |
| Chloromycetin sensitive | + | + | + | + | + | + | + | + | + | + |
| Dihydrostreptomycin sensitive | + | - | - | - | - | + | + | - | - | - |

^a NT, Not tested.^b Utilization as sole carbon and nitrogen source.

falling in the *V. parahaemolyticus* cluster (group C) were isolated from crab hemolymph collected during May, June, and July, the season shown by Kaneko and Colwell (7) to be associated with high incidence of *V. parahaemolyticus* in Chesapeake Bay. The remaining four strains were found between the months of August and November.

The observation that *V. parahaemolyticus* and related *Vibrio* sp. occur in the normal crab hemolymph shed new light on the occurrence of *V. parahaemolyticus* food poisoning traced to crabs or crabmeat. Furthermore, seafood handling practices and seafood regulations must be reviewed with this new information concerning the incidence of *V. parahaemolyticus* in shellfish.

In summary, the bacteria isolated from nor-

mal blue crab hemolymph were found to be predominantly *Vibrio*, *Pseudomonas*, and *Acinetobacter* sp. *Aeromonas*, *Bacillus*, and *Flavobacterium* sp., and coliforms were also isolated. Several yellow pigmented, fermentative, oxidase-negative rods not classified to genus or species were found in crab hemolymph. The major taxonomic group, with respect to frequency of occurrence, was *V. parahaemolyticus* and related *Vibrio* sp.

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