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Effects of Physicochemical Factors and Bacterial Colony Morphotype on Association of *Vibrio vulnificus* with Hemocytes of *Crassostrea virginica*

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***Vibrio vulnificus* is a naturally occurring marine bacterium that causes invasive disease of immunocompromised humans following the consumption of raw oysters. It is a component of the natural microbiota of Gulf Coast estuaries and has been found to inhabit tissues of oysters, *Crassostrea virginica* (Gmelin 1791). The interaction of *V. vulnificus* with oyster host defenses has not been reported in detail. We examined the interaction of *V. vulnificus* with phagocytic oyster hemocytes as a function of time, temperature, bacterial concentration, pretreatment with hemolymph, and *V. vulnificus* translucent and opaque colony morphotypes. Within these experimental parameters, the results showed that the association of *V. vulnificus* with hemocytes increased with time, temperature, and initial *V. vulnificus*/hemocyte ratio. Pretreatment of *V. vulnificus* with serum or an increased serum concentration did not enhance *V. vulnificus*-hemocyte associations, a result suggesting the absence of opsonic activity. More than 50% of hemocytes bound the translucent, avirulent morphotype, whereas 10 to 20% were associated with the opaque, virulent form, a result indicating that the degree of encapsulation was related to resistance to phagocytosis, as previously described for mammalian phagocytes. Understanding these cellular interactions may, in part, explain the persistence of *V. vulnificus* in oyster tissues and the ecology of *V. vulnificus* in estuarine environments.**

It is well documented that raw-shellfish consumption increases the risk of human vibrio diseases (5, 16, 19). Among the *Vibrio* spp., *Vibrio vulnificus* is unusual in that it causes fulminant and severe systemic human illness after the ingestion of raw oysters (4, 29). Consequently, its occurrence in aquatic environments is a significant concern of shellfish industries and public health agencies. In an effort to reduce health risks, commercial methods of controlled purification (e.g., depuration) have been used in an attempt to eliminate *V. vulnificus* from oyster meats but have been unsuccessful (17, 18, 25).

The seasonal occurrence of *V. vulnificus* in marine environments is favored by high temperature and low to moderate salinities (20, 27). Further investigations have explored its ecology in oyster tissues and described seasonally changing levels in hemolymph, mantle, gills, adductor muscle, and digestive tissues (6). Tamplin and Capers (25) have reported the persistence of *V. vulnificus* in specific tissues of oysters exposed to UV-disinfected seawater and shown that *V. vulnificus* replicates in tissues and is released at numbers of over 100,000 cells per oyster per hour at 23°C.

Detailed explanations of the persistence of *V. vulnificus* in shellfish tissues are lacking. Its resistance to oyster defense mechanisms, such as phagocytic hemocytes, may partially explain its retention in oyster tissues. Hemocytes move throughout the oyster, functioning in host defense, nutrition, digestion, and excretion (11). In previous studies, research on hemocyte phagocytic activities focused on interactions with allochthonous particles, including mammalian erythrocytes, polystyrene beads, and human enteric bacteria (1, 14). Some investigations have examined interactions with protozoan parasites of adult oysters (*Perkinsus marinus* and *Haplosporidium nelsoni*) (3, 7–9).

The objective of this study was to define the effect of various environmental parameters on the association of *V. vulnificus* and oyster hemocytes. In addition, we tested *V. vulnificus* colony morphotypes previously shown to be related to virulence to determine whether they showed differential reactions with hemocytes. Understanding these relationships with oyster defenses may aid in explaining the persistence of *V. vulnificus* in oyster tissues under environmental and postharvest purification treatment conditions.

MATERIALS AND METHODS

Collecting and maintaining oysters. Oysters were harvested during the summer and fall of 1990 and 1991 from a reef near Mobile, Ala., and transported to the laboratory within 1 h of collection. Oyster shells were brush scrubbed under deionized water before placement in 30-liter aquaria filled with 20 liters of artificial seawater (ASW) (Instant Ocean; Aquarium Systems, Mentor, Ohio) at 25°C and

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15-ppt salinity. ASW was recirculated by a pump (Little Giant Pump Co., Oklahoma City, Okla.) through a 30-W UV light chamber (Aquanetics Systems, San Diego, Calif.) and filtered with a pump and filter containing diatomaceous earth (Magnum 300; Marineland Aquarium Products, Simi Valley, Calif.). After 48 h of acclimatization, specimens were used in experimental protocols.

Preparation of bacteria. The following isolates were used: environmental (4965) and clinical (E4125) *V. vulnificus* isolates; a transposon-derived translucent morphotype of E4125 (Etr5), kindly provided by Debra Cutter, Wake Forest University, Winston-Salem, N.C.; and a spontaneous translucent variant of 4965 (4965-T7) isolated following culturing of 4965 on tryptic soy agar (Difco) containing 1% NaCl at 25°C.

Except as noted otherwise, *V. vulnificus* was cultured for 24 h on tryptic soy agar–1% NaCl at 35°C. A colony was transferred to 5 ml of tryptic soy broth containing 1% NaCl and incubated until the optical density at 420 nm equaled 0.64 (approximately 2×10^8 CFU/ml). For experimental comparisons of opaque and translucent morphotypes, parent and translucent strains were grown at 25°C to optimally differentiate morphotypes (33). Tryptic soy broth cultures were centrifuged at 3,000 × *g* for 5 min and washed twice with 15-ppt-salinity sterile ASW (15).

Collecting hemolymph. The edge of the oyster shell adjacent to the adductor muscle was notched with a grinder saw blade, avoiding contact with mantle tissue (23). With a 21-gauge needle, oyster hemolymph was withdrawn from the adductor muscle sinus into a sterile 5-ml syringe.

Hemocyte-*V. vulnificus* binding assay. The binding assay methods used were modifications of those of Fisher (12) and Robohn (23). Before each assay, the number of hemocytes per ml of hemolymph was determined to obtain specific experimental bacterium/hemocyte ratios. For enumeration of hemocytes, a separate aliquot of hemolymph was stained with 1% Gram's crystal violet in ASW (vol/vol), and cells were counted with a hemocytometer. Hemolymph was then added to prewashed sterile tissue culture slide wells (Lab Tek; Thomas Scientific, Swedesboro, N.J.), and cells settled and adhered to glass slide wells for 10 min (15). Wells were gently washed five times with 200 μl of ASW. For most experiments, except as noted otherwise, following the final wash, 100 μl of bacteria diluted in ASW or ASW controls without bacteria was added to glass slide wells containing 100 μl of hemocyte-free hemolymph obtained from the same oyster (final hemolymph concentration, 50%). Following incubation of hemocytes with *V. vulnificus* for specified periods of time, 100 μl of well supernatant was removed, and reactions were terminated by the addition of 100 μl of 2% formalin for 5 min at 25°C. Next, wells were washed and samples were stained with 0.01% acridine orange. The tissue culture slide was separated from the tissue culture slide well, rinsed with ASW, and air dried before microscopic analysis.

The percentage of individual hemocytes associated with *V. vulnificus* was determined by epifluorescence microscopy (21). At least 200 hemocytes were counted, and three replicates were tested per experiment. Statistical analysis (Student's *t* test) was used to determine differences between means of datum points for the various treatments. Each datum point represented the mean for three or more replicates.

Effect of *V. vulnificus*/hemocyte ratios. Predetermined concentrations of *V. vulnificus* were added to hemocytes in slide wells at initial *V. vulnificus*/hemocyte ratios of 2:1, 5:1, or 10:1 for 15, 30, 45, or 60 min at 25°C. Control wells received ASW instead of *V. vulnificus*, and data were used to determine background levels of endogenous bacteria. In a separate experiment done to determine the possible growth of *V. vulnificus* in cell-free hemolymph (100%), no growth was observed for up to 60 min at 25°C.

Effect of temperature. Hemocytes were allowed to adhere to slides at 25°C as described above. *V. vulnificus* suspensions were kept at the test temperature for approximately 10 min prior to being mixed with adherent hemocytes. *V. vulnificus* and hemocytes were incubated for 30 min at 4, 15, 20, 37, or 44°C at a 2:1 *V. vulnificus*/hemocyte ratio.

Effect of hemolymph. One to three milliliters of hemolymph was centrifuged (200 × *g*) for 5 min to separate lymph and cells. The lymph concentration was adjusted with 15-ppt-salinity ASW to 0, 25, 50, 75, and 100%. *V. vulnificus* was added to wells containing adherent hemocytes from the same oyster, and the mixture was incubated for 15 and 45 min at 25°C.

In a separate experiment, *V. vulnificus* was preincubated with 50% hemolymph at 25°C for 0, 15, 30, and 60 min and then added to wells at a 2:1 *V. vulnificus*/hemocyte ratio for 30 min at 25°C. Control bacteria were preincubated with ASW.

Association of translucent and opaque *V. vulnificus* morphotypes. Translucent (Etr5 and 4965-T7) and opaque (E4125 and 4965) *V. vulnificus* morphotypes were grown in tryptic soy broth containing 1% NaCl at both 25 and 37°C and

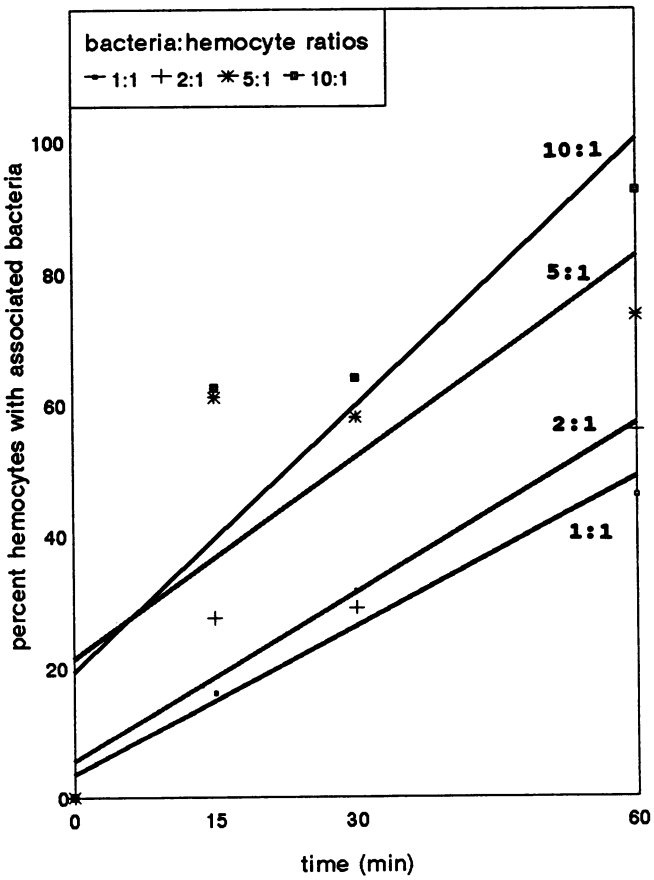

FIG. 1. Effect of initial bacterium/hemocyte ratio (1:1, 2:1, 5:1, and 10:1) and time (15, 30, and 60 min) on the percentage of hemocytes associated with *V. vulnificus* at 25°C.

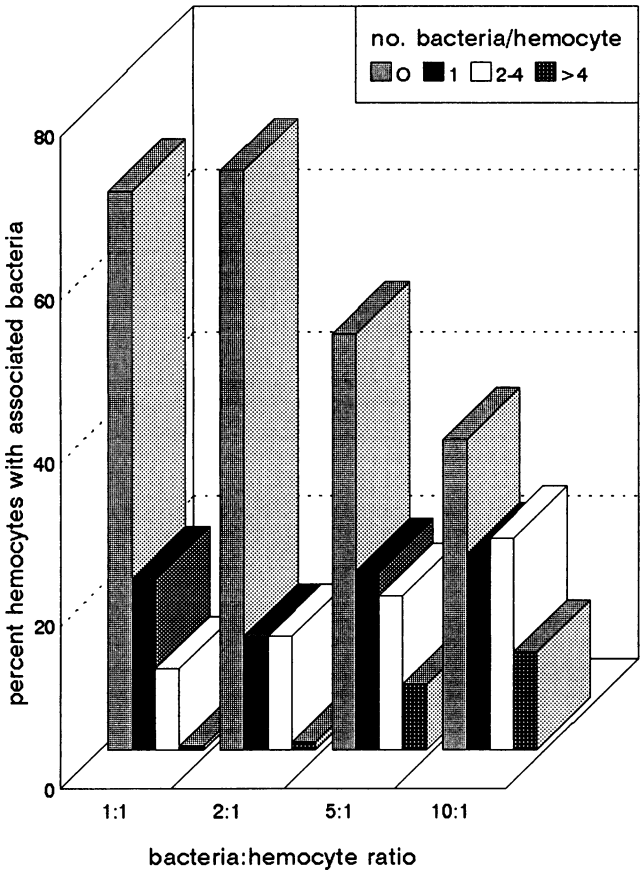

FIG. 2. Effect of initial bacterium/hemocyte ratio (1:1, 2:1, 5:1, and 10:1) on the number of *V. vulnificus* cells associated with hemocytes at 25°C for 30 min.

washed, concentrations were adjusted with 15-ppt-salinity ASW, and the organisms were then incubated with hemocytes (2:1 *V. vulnificus*/hemocyte ratio) for 30 min at 25°C.

RESULTS

The percentage of hemocytes associated with *V. vulnificus* increased with time and with the *V. vulnificus*/hemocyte ratio (Fig. 1); the association ranged from zero to more than four *V. vulnificus* cells per hemocyte (Fig. 2). Figure 1 represents variations in the ratio over time. An increase in the *V. vulnificus*/hemocyte ratio was related to a progressive increase in the percentage of hemocytes associated with two and more than four bacterial cells (Fig. 2).

An increased hemolymph concentration did not enhance the binding of *V. vulnificus* to hemocytes (Fig. 3) after 15 and 45 min ($P > 0.05$). After 15 min of incubation, the percentage of hemocytes with associated *V. vulnificus* decreased with increasing hemolymph concentration, especially at ≥25% hemolymph. Specifically, 28% of hemocytes bound *V. vulnificus* in 0% hemolymph, compared with 9% of hemocytes in 100% hemolymph. After a 45-min challenge, an increased hemolymph concentration had no observable effect on *V. vulnificus*-hemocyte associations (Fig. 3); i.e., 46% of hemocytes were associated with *V. vulnificus* in 0% hemolymph, compared with 39% in 100% hemolymph.

In a separate experiment to determine the effect of preincubation with hemolymph on binding and as a control for possible hemolymph viscosity affecting bacterial contact with hemocytes in the preceding experiment, *V. vulnificus* was preincubated with hemolymph or ASW for 0, 15, 30, or 60 min before exposure to hemocytes (Fig. 4). The results showed that preincubation with hemolymph did not markedly enhance the percentage of *V. vulnificus*-associated hemocytes at each time point ($P > 0.05$).

Increasing the temperature from 4 to 37°C correspondingly increased the number of *V. vulnificus* cells associated with hemocytes ($P < 0.05$) (Fig. 5). Specifically, following 30 min of incubation at 37°C, 49% of hemocytes bound *V. vulnificus* 4965 and 58% bound *V. vulnificus* C7184. In contrast, only 18 to 24% of hemocytes incubated at 4°C for 30 min bound either strain. In another study (data not shown), oyster hemocytes were incubated at 44°C. At this extreme temperature, associations could not be evaluated because hemocytes were disintegrated and damaged.

A larger percentage of hemocytes was associated with the translucent morphotype of *V. vulnificus* (Fig. 6) than with the opaque morphotype at both 25 and 36°C ($P < 0.05$). Approximately 40 to 50% of hemocytes bound translucent strains Etr5 and 4965-T7, whereas approximately 10 to 20% of hemocytes were associated with opaque strains E4125 and 4965. No differences were observed when morphotypes Etr5 and E4125 were cultured at 25 versus 36°C ($P > 0.05$).

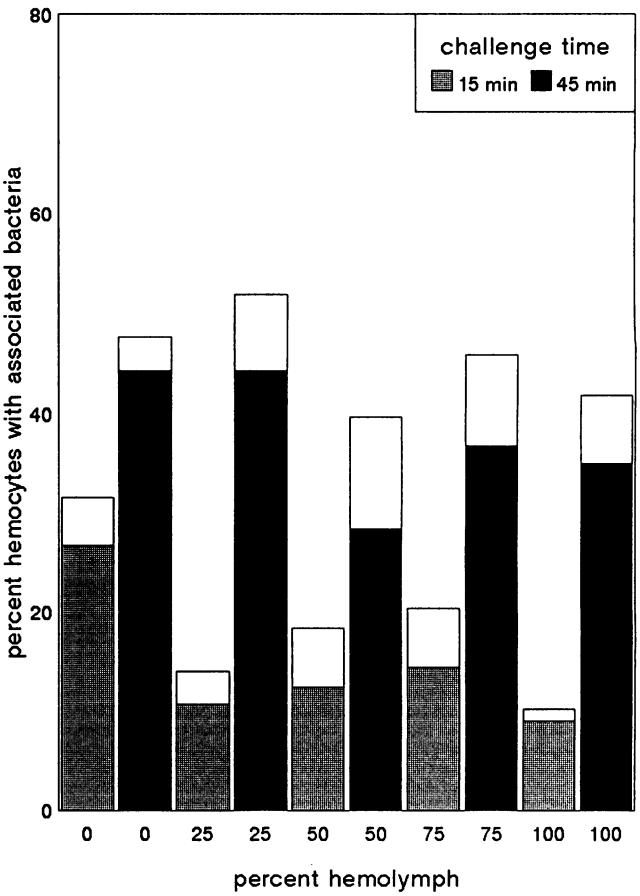

FIG. 3. Effect of hemolymph concentration on the percentage of hemocytes associated with *V. vulnificus* at 25°C after 15 and 45 min. Open bars indicate the standard error of the mean.

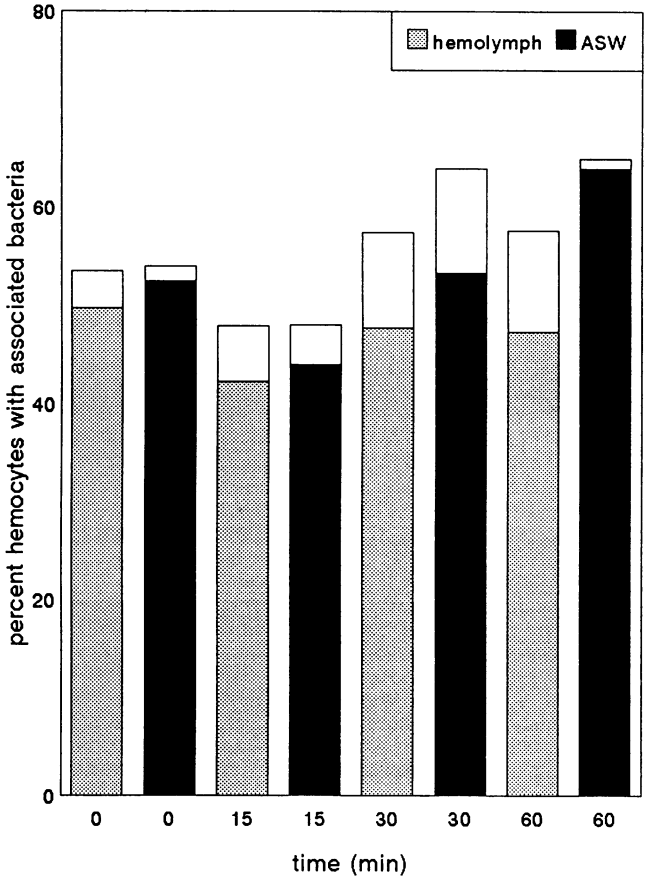

FIG. 4. Effect of preincubation of *V. vulnificus* with hemolymph or ASW for 0, 15, 30, and 60 min on the percentage of hemocytes associated with *V. vulnificus* at 25°C. Open bars indicate the standard error of the mean.

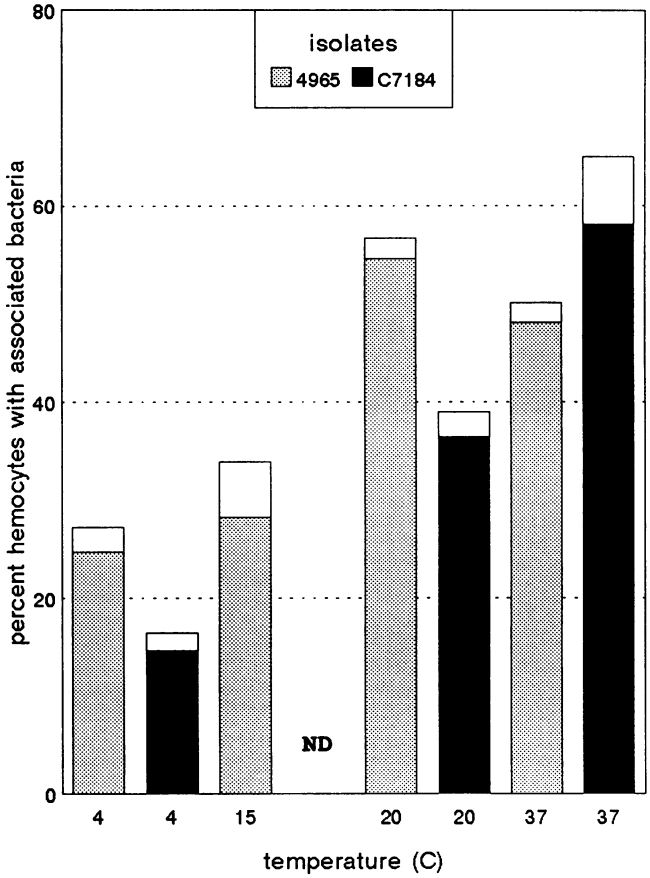

FIG. 5. Effect of temperature (4, 15, 20, and 37°C) on the percentage of hemocytes associated with *V. vulnificus* after 30 min. No data (ND) were collected for C7184 at 15°C. Open bars indicate the standard error of the mean.

DISCUSSION

Oyster hemocytes function in defense, nutrition, digestion, excretion, and repair of tissue and shell (11). The phagocytic process, first described by Bang in 1961, has been divided into four stages: attraction (chemotaxis), adherence (binding), endocytosis (ingestion), and intracellular digestion (destruction) of particles recognized as non-self (3, 10). Phagocytic hemocytes recognize a variety of particles, including bacteria, as non-self and have been shown in in vitro studies to phagocytize bacteria such as *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus* species (10, 32). The phagocytic response to *V. vulnificus*, however, has not been thoroughly investigated. Only the effects of temperature on the phagocytosis of *V. vulnificus* have been reported (24). This study provides evidence that oyster hemocytes bind *V. vulnificus* in response to time, temperature, bacterial concentration, and bacterial morphotype.

The number of *V. vulnificus* cells associated with hemocytes was decreased at 4 and 15°C compared with at 20 and 37°C. Rodrick and Ulrich (24) observed similar results at 4, 22, and 37°C. Fisher et al. (13) observed altered hemocyte activities in response to varied environmental temperatures and salinity.

Soluble hemolymph factors (e.g., lectins) of oysters are sometimes found to enhance the rate of phagocytosis because of agglutination properties (2, 22, 30, 31). Soluble factors from *Crassostrea virginica* have been shown to agglutinate several *Vibrio* species (14, 26). However, we observed that preincubation of *V. vulnificus* with hemolymph or an increased hemolymph concentration did not enhance *V. vulnificus*-hemocyte associations. High concentrations of hemolymph (e.g., 100%) reduced *V. vulnificus*-hemocyte associations, and preincubation with serum for up to 60 min showed no enhancing effect.

The outer polysaccharide capsule material has been shown to contribute to the antiphagocytic properties of *V. vulnificus* in mammalian models (28, 33). This characteristic has not been demonstrated in invertebrates, in which capsules could also contribute to bacterial persistence in host tissues. These data indicate that the encapsulation of *V. vulnificus* may also increase resistance to phagocytosis by oyster hemocytes. Lower environmental temperatures (e.g., ≤25°C) may result in fewer naturally encapsulated forms, thereby increasing the potential for phagocytosis and resulting in lower levels of *V. vulnificus* in oysters.

The interaction between *V. vulnificus* and hemocytes may be a critical event in the ecology of *V. vulnificus* in oysters and other estuarine animals. Understanding this interaction may explain the retention of *V. vulnificus* in oyster tissues and may aid in the design of postharvesting processes to reduce human risks from seafood-borne disease.

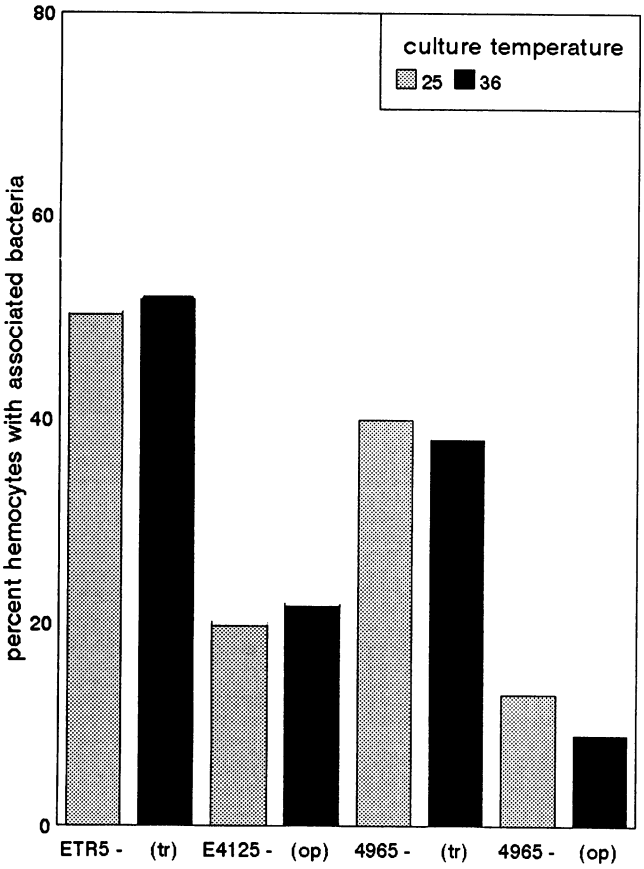

FIG. 6. Percentage of hemocytes associated with opaque (op) and translucent (tr) morphotypes of *V. vulnificus* at 25°C for 30 min.

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REFERENCES

1. **Alvarez, M., F. Friedl, J. Johnson, and G. Hinsch.** 1989. Factors affecting *in vitro* phagocytosis by oyster hemocytes. J. Invertebr. Pathol. **54:**233–241.
2. **Anderson, R. S., and R. A. Good.** 1976. Opsonic involvement in phagocytosis by molluscan hemocytes. J. Invertebr. Pathol. **27:**57–64.
3. **Bang, F. B.** 1973. A survey of phagocytosis as a protective mechanism against disease among invertebrates, p. 2–10. *In* W. Braun (ed.), Non-specific factors influencing host resistance. S. Karger, Publishers, Basel.
4. **Blake, P. A., M. H. Merson, R. E. Weaver, D. G. Hollis, and P. C. Heublein.** 1979. Disease caused by a marine vibrio. N. Engl. J. Med. **300:**1–7.
5. **Blake, P. A., R. E. Weaver, and D. G. Hollis.** 1980. Diseases of humans (other than cholera) caused by vibrios. Annu. Rev. Microbiol. **34:**341–367.
6. **Capers, G. M., M. L. Tamplin, and L. H. Hopkins.** 1990. Distribution and retention of *Vibrio vulnificus* in tissues of the eastern oyster, *Crassostrea virginica*, abstr. Q-99, p. 301. Abstr. 90th Annu. Meet. Am. Soc. Microbiol. 1990.
7. **Cheng, T. C., and E. Rifkin.** 1970. Cellular reactions in marine mollusks in response to helminth parasitism. Am. Fish. Soc. Spec. Publ. **5:**443–496.
8. **Chintala, M. M., and W. S. Fisher.** 1991. Disease incidence and potential mechanisms of defense for MSX-resistant and -susceptible eastern oysters held in Chesapeake Bay. J. Shellfish Res. **10:**439–443.
9. **Chu, F. E.** 1988. Development and evaluation of techniques to study acquired immunity to *Perkinsus marinus* in the oyster, *Crassostrea virginica* (Gmelin). J. Shellfish Res. **7:**51–55.
10. **Feng, S. Y.** 1988. Cellular defense mechanisms of oysters and mussels. Am. Fish. Soc. Spec. Publ. **18:**153–168.
11. **Fisher, W. S.** 1986. Structure and functions of oyster hemocytes, p. 25–35. *In* M. Brehelin (ed.), Immunity in invertebrates. Springer-Verlag KG, Berlin.
12. **Fisher, W. S.** 1988. Environmental influence on bivalve hemocyte function. Am. Fish. Soc. Spec. Publ. **18:**225–237.
13. **Fisher, W. S., M. M. Chintala, and M. A. Moline.** 1989. Annual variation of estuarine and oceanic oyster *Crassostrea virginica* Gmelin hemocyte capacity. J. Exp. Mar. Biol. Ecol. **127:**105–120.
14. **Fisher, W. S., and A. R. DiNuzzo.** 1991. Agglutination of bacteria and erythrocytes by serum from six species of marine molluscs. J. Invertebr. Pathol. **57:**380–394.
15. **Fisher, W. S., and R. I. E. Newell.** 1986. Salinity effects on the activity of granular hemocytes of American oysters, *Crassostrea virginica*. Biol. Bull. **170:**122–134.
16. **Janda, J. M., C. Powers, R. G. Bryant, and S. L. Abbott.** 1988. Current perspectives on the epidemiology and pathogenesis of clinically significant *Vibrio* spp. Clin. Microbiol. Rev. **1:**245–267.
17. **Jones, S. H., T. L. Howell, and K. O'Neill.** 1991. Differential elimination of indicator bacteria and pathogenic *Vibrio* sp. from eastern oysters (*Crassostrea virginica* Gmelin, 1791) in a commercial controlled purification facility in Maine. J. Shellfish Res. **10:**105–112.
18. **Kelly, M. T., and A. DiNuzzo.** 1985. Uptake and clearance of *Vibrio vulnificus* from Gulf Coast oysters (*Crassostrea virginica*). Appl. Environ. Microbiol. **50:**1548–1549.
19. **National Academy of Science.** 1991. Seafood safety report. Microbiological and parasitic exposures and health effects, p. 33–94. National Academy Press, Washington, D.C.
20. **Oliver, J. D., R. A. Warner, and D. R. Cleland.** 1983. Distribution of *Vibrio vulnificus* and other lactose-positive fermenting vibrios in the marine environment. Appl. Environ. Microbiol. **45:**985–998.
21. **Rainard, P.** 1986. Assessment of a fluorochrome microassay of phagocytic killing of group B streptococcus adherent to glass. J. Immunol. Methods **94:**113–118.
22. **Renwrantz, L., T. Yoshino, T. Cheng, and K. Auld.** 1979. Size determination of hemocytes from the American oyster, *Crassostrea virginica*, and the description of a phagocytosis mechanism. Zahrb. Zool. Abt. Physiol. Zoomorph. **83:**1–12.
23. **Robolm, R. A.** 1984. *In vitro* phagocytosis by molluscan hemocytes: a survey and critique of methods, p. 147–172. *In* T. C. Cheng (ed.), Comparative pathobiology, vol. 6. Plenum Press, New York.
24. **Rodrick, G. E., and S. A. Ulrich.** 1984. Microscopic studies on the hemocytes of bivalves and their phagocytic interaction with selected bacteria. Helgol. Meeresunters. **37:**167–176.
25. **Tamplin, M. L., and G. Capers.** 1992. Persistence of *Vibrio vulnificus* in tissues of Gulf Coast oysters, *Crassostrea virginica*, exposed to seawater disinfected with UV light. Appl. Environ. Microbiol. **58:**1506–1510.
26. **Tamplin, M. L., and W. S. Fisher.** 1989. Occurrence and characteristics of agglutination of *Vibrio cholerae* by serum from the eastern oyster, *Crassostrea virginica*. Appl. Environ. Microbiol. **55:**2882–2887.
27. **Tamplin, M. L., G. E. Rodrick, J. H. Blake, and T. Cuba.** 1982. Isolation and characterization of *Vibrio vulnificus* from two Florida estuaries. Appl. Environ. Microbiol. **44:**1466–1470.
28. **Tamplin, M. L., S. Specter, G. E. Rodrick, and H. Friedman.** 1985. *Vibrio vulnificus* resists phagocytosis in the absence of serum opsonins. Infect. Immun. **49:**715–718.
29. **Tison, D., and M. T. Kelly.** 1986. Virulence of *Vibrio vulnificus*

strains from marine environments. Appl. Environ. Microbiol. **51**:395–401.
30. **Tripp, M. R.** 1966. Hemagglutinin in the blood of the oyster *Crassostrea virginica*. J. Invertebr. Pathol. **8**:478–484.
31. **Tripp, M. R., and V. E. Kent.** 1967. Studies on oyster cellular immunity. In Vitro (Rockville) **3**:129–135.
32. **Weinheimer, P. F., R. T. Acton, and E. E. Evans.** 1969. Attempt to induce a bactericidal response in the oyster. J. Bacteriol. **97**:462–463.
33. **Yoshida, S. I., M. Ogawa, and Y. Mizuguchi.** 1985. Relation of capsular material and colony opacity to virulence of *Vibrio vulnificus*. Infect. Immun. **47**:446–451.