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Viability of *Vibrio vulnificus* in Association with Hemocytes of the American Oyster (*Crassostrea virginica*)

LINDA HARRIS-YOUNG,[1,2]* MARK L. TAMPLIN,[3,4] J. WALTER MASON,[1]
HENRY C. ALDRICH,[4] AND J. KEITH JACKSON[3]

School of Public Health, University of Alabama at Birmingham, Birmingham, Alabama 35241[1]; Biology Department, Motlow State Community College, Tullahoma, Tennessee 37388-8100[2]; and Department of Home Economics,[3] and Department of Microbiology and Cell Science,[4] University of Florida, Gainesville, Florida 32611

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Certain indigenous estuarine bacteria, such as *Vibrio vulnificus*, may cause opportunistic human infections after consumption of raw oysters or exposure of tissues to seawater. *V. vulnificus* is known to be closely associated with oyster (*Crassostrea virginica*) tissues and is not removed by controlled purification methods, such as UV light-assisted depuration. In fact, when live shellfish are subjected to controlled purification, the number of *V. vulnificus* cells can markedly increase. A review of previous studies showed that few workers have examined mechanisms in oysters which may influence the persistence of *V. vulnificus* in shellfish, such as the fate of *V. vulnificus* following phagocytosis by molluscan hemocytes. The objectives of this study were to define the intracellular viability and extracellular viability of *V. vulnificus* during the phagocytic process and to study the release of specific lysosomal enzymes. The viability of a virulent estuarine *V. vulnificus* isolate with opaque morphology was compared with the viability of a translucent, nonvirulent form, the viability of *Vibrio cholerae*, and the viability of *Escherichia coli* in phagocytosis experiments. Our results showed that the levels of phagocytosis and bactericidal degradation of the opaque *V. vulnificus* isolate were less than the levels of phagocytosis and bactericial degradation of the translucent morphotype. These findings indicate that encapsulation may contribute to resistance to ingestion and degradation by hemocytes. The rates of intracellular death of *V. cholerae* and *E. coli* exceeded the rate of intracellular death of the opaque *V. vulnificus* isolate, even though the ingestion or uptake rates did not differ significantly. The levels of lysozyme activity and acid phosphatase activity were not significantly different in hemocyte monolayers inoculated with *V. vulnificus*.

Raw shellfish have the potential to cause human disease because they are vectors of viral, bacterial, and parasitic pathogens (23). These infectious agents originate from sources in the natural environment or from fecal pollution of shellfish-harvesting waters. Since 1980, considerable attention has been focused on deaths caused by exposure to the endogenous vibrio flora. One indigenous aquatic bacterium, *Vibrio vulnificus*, almost exclusively infects humans suffering from preexisting liver disease who eat raw oysters (2, 6, 18).

Controlled purification of shellfish (e.g., depuration) in commercial operations has been used extensively worldwide to remove unwanted microorganisms from shellfish and to improve product quality. Fecal pollutants, such as *Escherichia coli* and *Salmonella* spp., are depurated from oysters more easily than *V. vulnificus* is (19, 22, 24, 25). Tamplin and Capers (30) have reported that *V. vulnificus* is not removed by controlled purification methods. *V. vulnificus* has been reported to persist at high levels and to even reproduce in oyster hemolymph and other oyster tissues (30). Selective retention in oysters was also demonstrated by the isolation of *V. vulnificus* from oysters when no *V. vulnificus* was detected in the surrounding seawater (30).

Oyster defense mechanisms, particularly hemocytes which possess strong phagocytic properties (13), may play a significant role in the survival of *V. vulnificus* in tissues. The phagocytic process of ingestion and degradation is enhanced by humoral factors, such as lectins and lysosomal enzymes. Phagocytized bacteria are initially enclosed within phagosomes, where enzymatic degradation begins (4); this is followed by release of digestive enzymes into serum within 30 min (14). Residual material or remnants of bacterial degradation, including glycogen granules, have been observed intracellularly in transmission electron micrographs (4).

It is possible that bacteria which survive in the presence of oyster humoral components establish more stable relationships with oyster tissues. In this regard, Tamplin and Fisher (31) observed that *V. vulnificus* was not agglutinated or killed by soluble hemolymph components.

Hemocytes are capable of ingesting *V. vulnificus* and other types of bacteria independent of bacterial contact with humoral factors (16, 35). The relationship of time, temperature, bacterial concentration, and bacterial morphology to binding of *V. vulnificus* by phagocytic hemocytes of *Crassostrea virginica* has been examined by Harris-Young et al. (16). In general, more hemocytes are associated with avirulent, translucent morphotypes than with the more virulent opaque forms.

The objective of this study was to measure the extracellular viability and intracellular viability of *V. vulnificus* associated with phagocytic hemocytes. Rates of ingestion and die-off were determined for opaque and translucent colonial *V. vulnificus* morphotypes, *Escherichia coli* (a fecal coliform bacterium), and *Vibrio cholerae* (another pathogenic vibrio). In addition, lysosomal enzyme contents (lysozyme and acid phosphatase contents) were determined in extracellular and intracellular samples following exposure to oyster hemocytes. Transmission electron microscopy was performed to visualize intracellular bacteria and to determine the location and integrity of bacteria. By further elucidating mechanisms which affect survival of *V. vulnificus* in oyster tissues, we gained additional information

* Corresponding author. Mailing address: Biology Department, Motlow State Community College, P.O. Box 88100, Tullahoma, TN 37388-8100. Phone: (615) 393-1709.

about transmission of pathogenic *V. vulnificus* to humans and the ecology of this organism in estuarine environments.

MATERIALS AND METHODS

Collecting and maintaining oysters. Oysters were harvested from Cedar Key, Fla., during the summer and fall of 1992 and winter and spring of 1993 and were transported overnight (within 24 h) to Jacksonville State University, Jacksonville, Ala., in a refrigerated (4°C) cooler. Upon receipt, the oysters were brush scrubbed with deionized water at 20°C and then placed in 30-liter aquaria filled with 20 liters of artificial seawater (ASW) (Instant Ocean; Aquarium Systems, Mentor, Ohio) at 20°C; the level of salinity was 15 ppt. The ASW was recirculated with a pump (Little Giant Pump Co., Oklahoma City, Okla.) and was disinfected with a 30-W UV light chamber (Aquanetics Systems, San Diego, Calif.). After 48 h, specimens were used in experiments.

Collecting hemolymph. The edge of an oyster shell adjacent to the adductor muscle was notched with a grinder saw; contact with the mantle tissue was avoided (26). Then oyster hemolymph was withdrawn from the adductor muscle sinus with a 21-gauge needle into a 5-ml syringe.

Preparation of bacteria. The following isolates were used in experiments: isolate 4965-O, an opaque, environmental *V. vulnificus* isolate; *V. vulnificus* 4965-T7, a spontaneous translucent variant of 4965-O; *V. cholerae* 01 (102) (biotype El Tor), which was isolated from Peruvian water in 1992; and *E. coli* ATCC 25922. The opaqueness of 4965-O and the translucency of 4965-T7 were maintained throughout the experimental period and after the experiments were completed. Bacteria were cultured for 24 h on tryptic soy agar containing 1% NaCl at 25°C. Colonies were transferred to 5 ml of a chemically defined ASW (12) until the optical density at 420 nm was 0.64 (approximately 2×10^8 CFU/ml). Bacterial suspensions were diluted with ASW to obtain the desired bacterium/hemocyte ratio in each well. Approximately 400,000 bacteria were added to wells which contained 2×10^6 hemocytes.

Phagocytosis assays. Before each assay, the number of hemocytes per milliliter of hemolymph was determined so that we could obtain the desired experimental bacterium/hemocyte ratio (1:5) (16). To enumerate hemocytes, a separate aliquot of hemolymph was stained with 1% (vol/vol) Gram's crystal violet in ASW, and the cells were counted with a hemocytometer. Hemolymph was then added to sterile tissue culture wells (Costar, Cambridge, Mass.), and the hemocytes were allowed to settle and form monolayers on the well surfaces (12). After 20 min, the hemocyte monolayers were gently washed 10 times with 500 μl of ASW to remove the endogenous bacteria present in the oyster tissue. Following the final wash, 100-μl portions of bacteria diluted in ASW or ASW controls without bacteria were added to monolayers containing 500 μl of filter-sterilized, cell-free hemolymph obtained from the same oyster.

Enumeration of viable extracellular and intracellular bacteria. To determine rates of ingestion (phagocytosis) and intracellular death of bacteria, the numbers of viable bacteria were determined at 15-, 30-, 60-, and 120-min intervals. Supernatants were collected from hemocyte monolayers and then washed twice with a 0.02% trypsin-EDTA solution (GIBCO) to remove bacteria that adhered to the surface. The supernatants and wash solutions were pooled to determine the number of extracellular *V. vulnificus* cells. Preliminary experiments showed that trypsin-EDTA had no detrimental effect on the viability of the bacteria (expressed as CFU) in these studies, on the viability of oyster hemocytes (as determined by the trypan blue exclusion test), or on adherence of oyster hemocytes to plastic surfaces (1). To enumerate viable intracellular bacteria, adhered hemocytes were treated as described above and then disrupted by flooding the preparation with cold (4°C) 0.05% Triton X-100 (Sigma Chemical Co., St. Louis, Mo.) for 10 min; this was followed by agitation and two washes with phosphate-buffered saline (PBS). In a separate experiment, we determined that the Triton X-100 solution did not affect bacterial viability. Tenfold serial dilutions of extracellular and intracellular samples were prepared in PBS and then plated onto tryptic soy agar containing 1% NaCl.

The level of bacterial uptake (or percentage of viability) was measured by determining the numbers of the initial viable bacteria (~400,000 bacteria per well) that remained in the extracellular milieu over time, with corrections for growth in controls as described below. The intracellular death rate was measured by determining the decrease in the number of viable ingested bacteria. Statistical analysis (Student's *t* test) was used to evaluate the differences between the means of datum points for the various treatments at $P < 0.05$. Each datum point represented the mean of six or more separate trials.

Control experiments. Control experiments were performed to ensure that any change in the number of bacteria was not due to bactericidal or growth-enhancing substances or reagents in supernatants or to detachment of hemocytes from monolayers.

To detect and correct for bacterial reproduction in hemolymph, separate wells were seeded with bacteria and 500 μl of sterile lymph. Levels of bacterial viability were measured over the same time intervals used in phagocytosis experiments. The corrected number (CN_t) of extracellular bacteria at time t was calculated as follows: $CN_t = N_t(B_0/B_t)$, where N_t is the number of extracellular bacteria at time t, B_0 is the number of bacteria in the control suspension (containing only bacteria) at zero time, and B_t is the number of bacteria in the control at time t (20).

Lysosomal enzyme assays. The levels of lysozyme and acid phosphatase activities in extracellular and intracellular samples were determined following exposure of hemocytes to bacteria for 15 and 120 min. Lysozyme activity was determined spectrophotometrically as the ability to lyse a standard suspension of *Micrococcus lysodeikticus* cells (27, 28). A decrease in absorbance (expressed as units per minute per milliliter) indicated that lysozyme had a lytic effect. Acid phosphatase activity was measured by using a commercial test kit (catalog no. 104-AL; Sigma). Acid phosphatase activity was proportional to color intensity in the *p*-nitrophenolphosphate hydrolysis test (expressed as units per milliliter).

Transmission electron microscopy. To establish the presence of intracellular bacteria, monolayers were prepared for examination with a transmission electron microscope (3). Briefly, hemocyte monolayers were fixed in a solution containing 2% formaldehyde and 2% glutaraldehyde and then fixed in 1% osmium tetroxide and dehydrated in ethanol. Cells were infiltrated with Epon epoxy resin, which was hardened at 60°C. Ultrathin sections were cut with an model RMC MT-6000 ultramicrotome, collected on Formvar-coated 75-mesh grids, poststained with 0.5% aqueous uranyl acetate, and then exposed to lead citrate. The sections were observed and photographed with a Zeiss model EM-10CR transmission electron microscope. Our interpretation and identification of intrahemocyte structures were based on the extensive transmission electron microscope studies performed by Cheng (4) and Feng et al. (10).

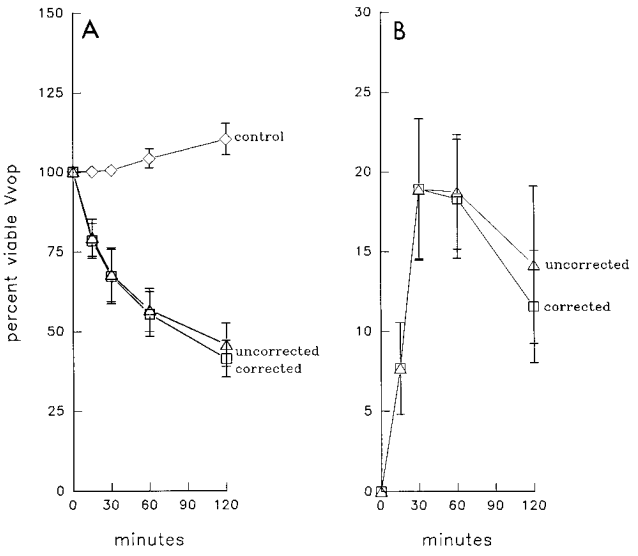


FIG. 1. Extracellular viability (A) and intracellular viability (B) of *V. vulnificus* 4965-O after 15, 30, 60, and 120 min of exposure to phagocytic hemocytes. The points on the curves represent percentages of viable bacteria (measured as number of CFU per well). The controls contained only bacteria and cell-free hemolymph. Corrections were made for growth of extracellular bacteria during the course of the experiment. The vertical lines represent standard errors of the means ($n = 11$). Vvop, *V. vulnificus* opaque strain.

RESULTS

Extracellular viability and intracellular viability. After 60 and 120 min, 55 and 41%, respectively, of the initial concentration of the *V. vulnificus* opaque strain added to wells remained viable in the extracellular milieu (Fig. 1). After 120 min, 59% of the *V. vulnificus* opaque strain cells were associated with phagocytes, while 12% remained viable inside hemocytes.

After 60 and 120 min, 60 and 40%, respectively, of the initial concentration of the translucent *V. vulnificus* strain remained in the extracellular milieu (Fig. 2). These results were not statistically different from the results obtained with the *V. vulnificus* opaque strain (Fig. 1). However, 32% of the initial *V. vulnificus* translucent strain concentration had been removed from the extracellular milieu after 15 min, compared with only 21% of the initial concentration of the *V. vulnificus* opaque strain ($P > 0.05$). After 120 min, 5% of the *V. vulnificus* translucent strain cells (Fig. 2) remained viable intracellularly,

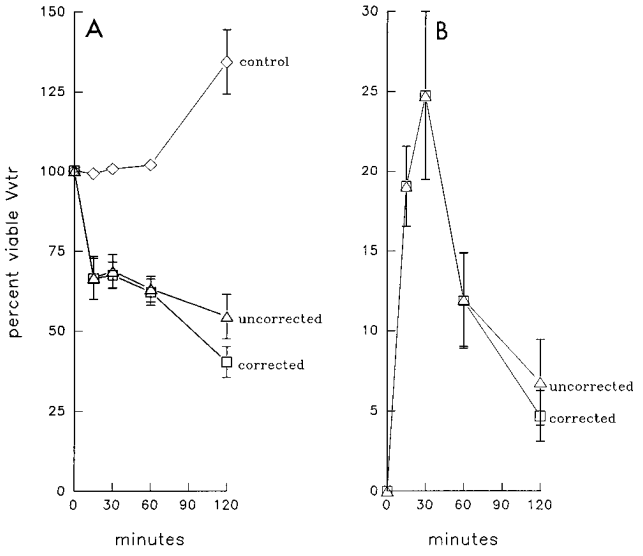


FIG. 2. Extracellular viability (A) and intracellular viability (B) of *V. vulnificus* 4965-T7 after 15, 30, 60, and 120 min of exposure to phagocytic hemocytes. The points on the curves represent the percentages of viable bacteria (measured as number of CFU per well). The controls contained only bacteria and cell-free hemolymph. Corrections were made for growth of extracellular bacteria during the course of the experiment. The vertical lines represent the standard errors of the means ($n = 6$). Vvtr, *V. vulnificus* translucent strain.

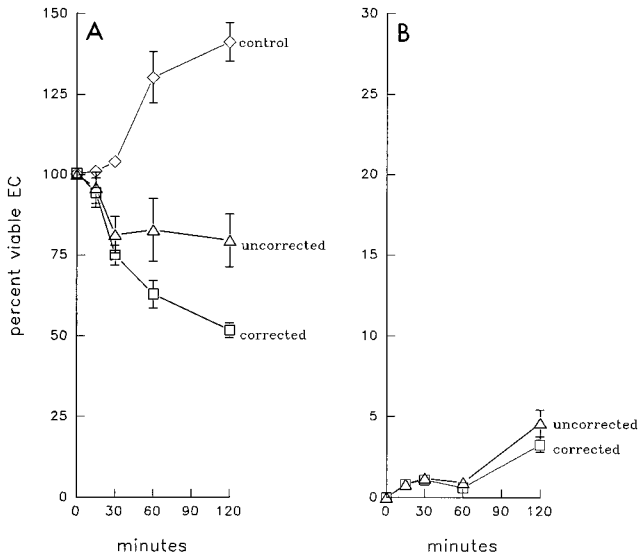


FIG. 4. Extracellular viability (A) and intracellular viability (B) of *E. coli* ATCC 25922 after 15, 30, 60, and 120 min of exposure to phagocytic hemocytes. The points on the curve represent percentages of viable bacteria (measured as number of CFU per well). The controls contained only bacteria and cell-free hemolymph. Corrections were made for growth of extracellular bacteria during the course of the experiment. The vertical lines represent the standard errors of the means ($n = 6$). EC, *E. coli*.

compared with 12% of the *V. vulnificus* opaque strain cells ($P < 0.05$) (Fig. 1).

The concentration of *V. cholerae* in the extracellular milieu decreased by 62% after 120 min (Fig. 3). The uptake of *V. cholorae* was not significantly different from the uptake of the *V. vulnificus* opaque strain or the *V. vulnificus* translucent strain. The greatest percentage of viable intracellular *V. cholerae* cells was observed after 15 min (3.3%); however, the percentage of viable *V. cholerae* cells decreased to 1.2% after 120 min (Fig. 3).

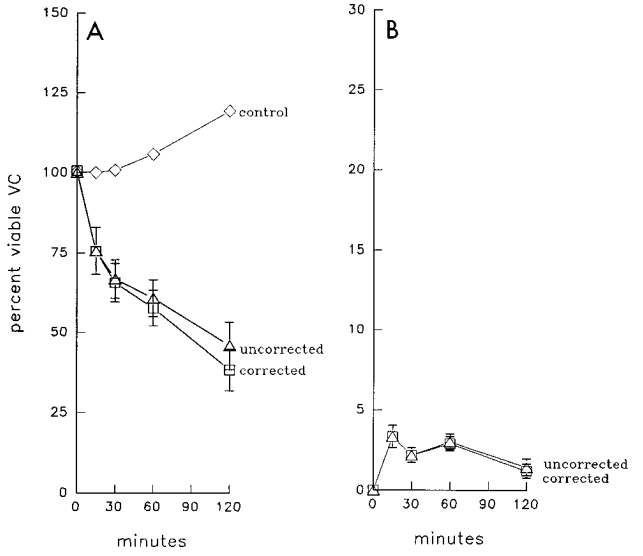


FIG. 3. Extracellular viability (A) and intracellular viability (B) of *V. cholerae* 102 after 15, 30, 60, and 120 min of exposure to phagocytic hemocytes. The points on the curves represent percentages of viable bacteria (measured as number of CFU per well). The controls contained only bacteria and cell-free hemolymph. Corrections were made for growth of extracellular bacteria during the course of the experiment. The vertical lines represent the standard errors of the means ($n = 5$). Vc, *V. cholerae.*

A total of 48% of the viable extracellular *E. coli* cells were removed at 120 min (Fig. 4); this value was less than the values obtained for the *V. vunificus* opaque strain and the *V. vulnificus* translucent strain. After 60 min, when 38% of the *E. coli* cells had been taken up by phagocytes, less than 1% of the cells remained viable intracellularly. An increase in the level of viable intracellular *E. coli* cells (3%) was observed after 120 min.

Figure 5 shows the intracellular viability data for the *V. vulnificus* opaque and translucent strains, *V. cholerae*, *E. coli* obtained after 15 and 120 min. After 15 min of exposure to hemocytes, 23% of the *V. vulnificus* translucent strain cells remained viable, compared with 8% of the *V. vulnificus* opaque strain cells, 3% of the *V. cholerae* cells, and 0.8% of the *E. coli* cells. After 120 min the number of viable *V. vulnificus* opaque strain cells was significantly higher than the numbers of cells of the other three bacterial strains tested ($P > 0.05$).

Lysosomal enzymes. Most levels of lysozyme and acid phosphatase activities were consistently higher in extracellular samples than in intracellular samples; the only exception was *V. cholerae* acid phosphatase activity, which was higher in intracellular environments than in extracellular environments after 120 min (data not shown). Overall, the levels of enzyme activity in control wells containing no bacteria were not significantly different from the levels of enzyme activity in wells containing bacteria. However, significantly higher levels of lysozyme activity were observed in extracellular samples of hemocytes exposed to *V. cholerae.*

Transmission electron microscopy. Figures 6 and 7 are transmission electron micrographs showing hemocytes from monolayers exposed to the *V. vulnificus* opaque strain (Fig. 6) and the *V. vulnificus* translucent strain (Fig. 7) for 120 min. The bacteria are contained in phagosomes. The phagosomes also

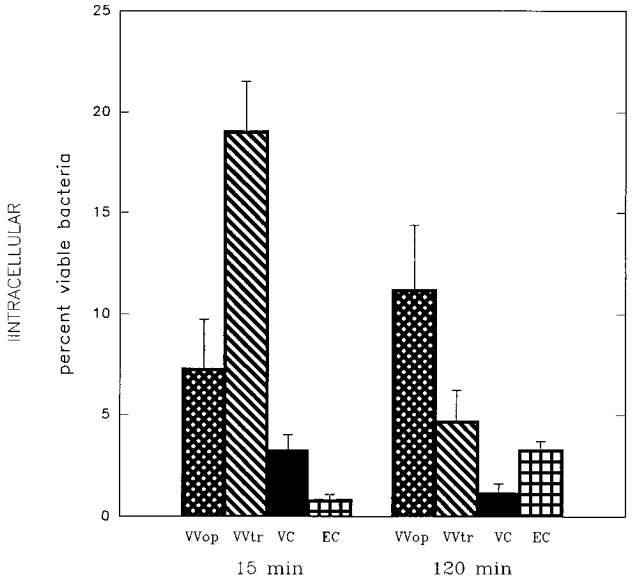


FIG. 5. Comparison of the intracellular viabilities of opaque ***V. vulnificus*** (VVop), translucent *V. vulnificus* (VVtr), *V. cholerae* (VC), and *E. coli* (EC) after 15 and 120 min of exposure to phagocytic hemocytes. The vertical lines represent the standard errors of the means.

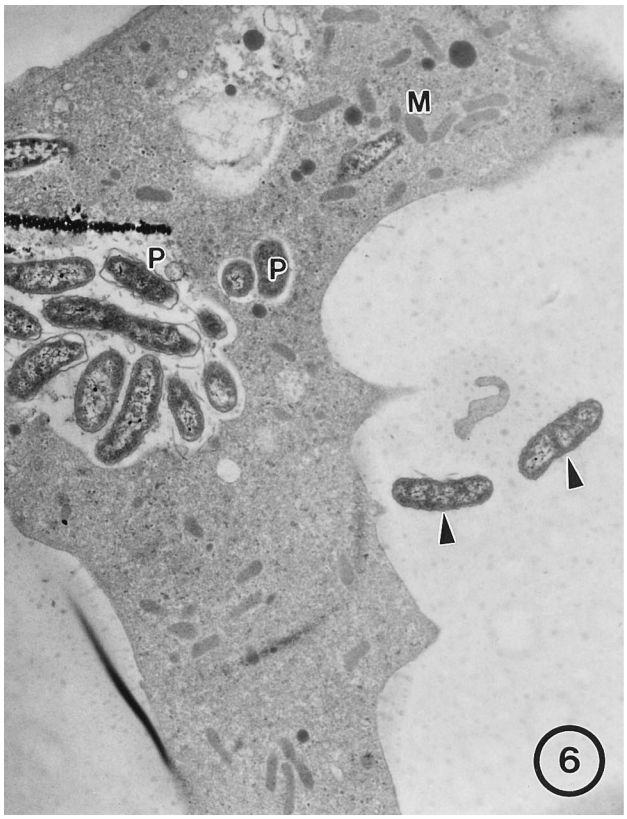
appear to contain glycogen granules, which indicates that bacterial degradation occurred. Other intracellular structures indicated on the micrographs are a nucleus, a mitochondrion, and an ingested diatoms.

DISCUSSION

In this study we found that *V. vulnificus* opaque strain 4965-O is more resistant to the bactericidal effects of the intracellular environment of oyster hemocytes than the translucent *V. vulnificus* morphotype (strain 4965-T7), *V. cholerae*, and *E. coli* are.

Previous light microscopy studies of Tripp (34) revealed that bacteria which are experimentally introduced into invertebrates, such as oysters, are phagocytized and intracellularly degraded by hemocytes. However, it was later found that the rates of phagocytosis and degradation varied depending on the type of hemocytes and the bacterial species. The results of in vivo and in vitro studies have shown that endogenous *Pseudomonas* spp. and *Vibrio anquillarium* isolates survive longer in oysters than allochthonous *Bacillus* spp. and *E. coli* isolates (7, 15). Howland and Cheng found that oyster hemocytes migrated toward *E. coli* cells, but not toward *Vibrio parahaemolyticus* cells (17).

In mammalian phagocytes, some bacterial species (including *Shigella*, *Listeria*, *Mycobacterium*, and *Brucella* species) survive intracellularly (11). The ability to resist intracellular bactericidins contributes to bacterial survival since surviving bacteria are liberated when a cell dies. Studies in which intracellular



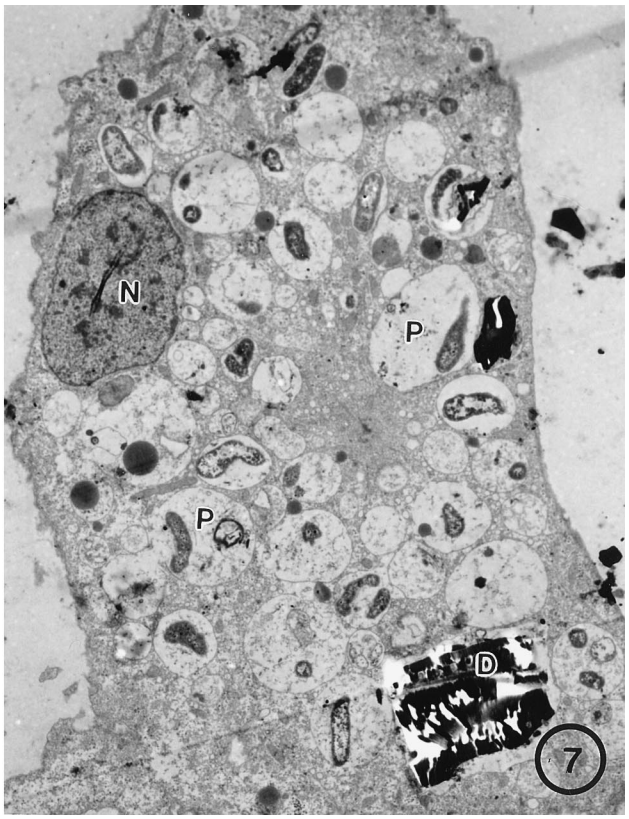


FIG. 6. Transmission electron micrograph of *V. vulnificus* opaque strain 4965-O, showing bacteria outside the cell (arrowheads) and within phagosomes (P) of a *C. virginica* hemocyte after 120 min of exposure. M, mitochrondron. The nucleus is out of the plane of the section. Magnification, ×5,000.

FIG. 7. Transmission electron micrograph of *V. vulnificus* translucent strain 4965-T7, showing bacteria within phagosomes (P) of a *C. virginica* hemocyte after 120 min of exposure. N, nucleus; D, ingested diatom. The scratches on the section were unavoidable because of silicaceous diatom skeletons in many cells. Magnification, ×6,250.

survival in mammalian cells has been examined have shown that microbial secretions and surface components seem to be involved (29). However, workers who have studied survival of bacteria within oyster cells and tissues have not described similar mechanisms of survival. In some cases, the bacterial numbers fluctuated, but they eventually decreased to low or nondetectable levels within 12 to 24 h (8, 34).

Cheng (4) proposed that bacteria and hemocytes have specific host-parasite interactions that depend on the genetic characteristics of both the bacteria and the hemocytes. In this regard, the outer polysaccharide capsule material of *V. vulnificus* may contribute to the interaction of this organism with hemocytes. Since the rate of phagocytosis of the opaque *V. vulnificus* strain was slower than the rate of phagocytosis of the translucent *V. vulnificus* strain, encapsulation may be related to resistance to phagocytosis and degradation. This characteristic has also been shown to contribute to the antiphagocytic properties of *V. vulnificus* in mammalian models (33). An *E. coli* mutant that lacked certain sugars in its cell walls was more susceptible to phagocytic uptake by oyster hemocytes than the wild-type strain containing these sugars (26).

Persistence of viable *V. vulnificus* cells within phagocytes might also be attributed to the inability of agranular hemocytes to kill, thereby allowing the bacterium a place to reside. Notably, the percentage of agranular hemocytes is higher than the percentage of granular hemocytes during the summer months (21). Hemolymph lysozyme and total protein levels are also higher in the winter months than in the summer months (5). Both granular and agranular hemocytes are capable of ingesting bacteria; however, agranular cells do not contain lysosomal enzymes that are capable of bacterial degradation (13). The life span of hemocytes is thought to be at least 25 days (9), which could lengthen the time that *V. vulnificus* resides intracellularly. These factors could be correlated with the prevalence of *V. vulnificus* in the estuarine environment during the warmer summer months (32) and with the persistence of this organism under controlled purification conditions (30). Our results did not reveal significant variation among oysters harvested at different times of the year; this may be attributed to the long period of oyster acclimation in the laboratory before our experiments were performed.

The fact that some bacteria within hemocytes survive while others are destroyed is presumably related to susceptibility to hemocyte lysosomes that act on different classes of bacterial macromolecules. Acid phosphatase and lysozyme activities have been detected in oyster hemocytes and supernatants both with and without bacterial challenge (14). In this study we did not observe increased levels of lysozyme or acid phosphatase activity with either the opaque or translucent *V. vulnificus* morphotype. The extracellular levels of both of these enzymes were higher than the levels found in intracellular samples. Rodrick and Cheng (27) also found that lysozyme levels were increased more by gram-positive bacteria than by gram-negative bacilli, such as *E. coli* and *Salmonella* spp.

We found that only supernatant samples containing *V. cholerae* exhibited increased lysozyme activity. Chu and La Peyre (5) have suggested that a bacterial cell wall component (e.g., a mucopolysaccharide) provides a specific stimulus that is responsible for increased hemolymph lysozyme levels, which would explain this observation. Cheng (4) proposed that certain bacterial surfaces are altered by serum enzymes before the bacteria are ingested. Other authors have proposed that bacterial surfaces altered by serum enzymes become more hydrophobic or are opsonized by lectins which make the bacteria more easily phagocytized (26, 35). This could explain the rapid intracellular die-off of *V. cholerae* and *E. coli* in our experiments.

Overall, our results indicate that the persistence of opaque *V. vulnificus* strains within phagocytes is probably related to the ability of these organisms to resist digestion by some unknown mechanism or the inability of some agranular hemocytes to digest them. The rate of destruction or elimination of *V. vulnificus* has public health significance, especially in this case of a commercially important shellfish species. Only when parameters that influence phagocytosis and degradation of *V. vulnificus* are understood can workers devise methods to enhance this self-cleansing process and bring about more efficient reduction of this potential pathogen to safe levels in edible shellfish.

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