Interaction of Vibrio vulnificus and the Eastern Oyster, Crassostrea virginica

TRUDI N. GROUBERT and JAMES D. OLIVER*

Department of Biology, University of North Carolina at Charlotte, Charlotte, North Carolina 28223

(Received April 2, 1993/Accepted September 18, 1993)

ABSTRACT

The estuarine bacterium, Vibrio vulnificus, is a human pathogen associated with the consumption of raw oysters. To date, no effective means exists for the elimination of this health hazard in oysters meant for raw consumption. The purpose of this study was to investigate the interaction between V. vulnificus and the eastern oyster. These studies were facilitated through the use of a strain of V. vulnificus containing a TnphoA transposon that allowed specific identification of the bacterium on a selective and differential medium. In studies employing ultra-violet assisted (UV-assisted) depuration, no differences were found in the oysters of the encapsulated (virulent) and nonencapsulated (avirulent) morphotypes of V. vulnificus. Both types were readily depurated from the oysters, while a naturally obtained microflora was shown not to depurate. Virulence of V. vulnificus and conversion rates between the virulent and avirulent morphotypes of this bacterium were found to be unchanged by oyster passage.

Vibrio vulnificus is one of the most invasive and rapidly lethal of human pathogens (for a recent review, see ref. 13). This organism is part of the normal microflora of estuarine waters and occurs in high numbers in molluscan shellfish (9,10,14,15,17). Infection is known to occur by two portals of entry (3,28). Ingestion of raw seafood, primarily oysters, may result in primary septicemia. Septicemia generally leads to secondary cutaneous lesions and necrotic ulcers of the extremities; approximately 60% of known cases result in fatality (13). The majority of victims have some underlying chronic disease, typically involving the liver, that causes a serum iron overload (13,32). A second portal involves wound infections resulting from exposure of skin lesions to V. vulnificus in seawater and/or shellfish. Localized edema and cellulitis usually occur, with surgical debridement of affected tissue or amputation of limbs often required. Mortality is 20% for these cases (13).

Vibrio vulnificus occurs in both an opaque and a translucent morphotype, with opaque forms being characterized by an acidic polysaccharide capsule which is lacking in the translucent morphotype (1,25). Only the opaque colony type has been found to be virulent, resistant to the bactericidal activity of serum, and able to utilize transferrin-bound iron for growth (11,23-25,34). The bacterium has been reported to shift from the opaque to the translucent morphology at a frequency of 10^-4; studies show that

reversion from translucent to opaque generally does not occur (25,34).

In recent years, the seafood industry has become increasingly concerned with the presence of V. vulnificus in shellfish, as no effective means of treating oysters meant for raw consumption exists. Thus, the focus of this study was to examine the interaction of V. vulnificus and the eastern oyster (Crassostrea virginica). In particular, the objectives were i) to compare uptake and UV-assisted depuration of the opaque and translucent morphotypes of V. vulnificus; ii) to compare depuration rates of V. vulnificus from oysters both with and without prior UV-assisted depuration to determine the effects of the natural microflora on depuration; iii) to examine the effects of oyster passage on conversion rates between the opaque and translucent morphotypes; and iv) to determine if there are changes in virulence of the opaque and translucent morphotypes upon oyster passage. Examination of these points was facilitated by the use of a novel method which employed a genetically marked strain of V. vulnificus. This method allowed the specific detection and enumeration of this bacterium against the natural microflora present in high numbers in oysters.

MATERIALS AND METHODS

Maintenance of shellfish

Oysters (Crassostrea virginica) from Chesapeake Bay were obtained from a local seafood distributor. Shells were scrubbed and the animals placed in 55-gal (ca. 208.27 L) holding tanks for 3 to 4 days to allow for acclimation to laboratory conditions. All tanks were equipped with a canister filter pump (Magnum 200; Aquaria, Inc., Simi Valley, CA) with diatomite micron cartridge, an 8-watt ultraviolet sterilizer (Hawaiian Marine Imports, Inc., Houston, TX), and aeration. The tanks contained artificial seawater (ASW; Instant Ocean, Aquarium Systems, Mentor, OH) at 20 ppt salinity and pH 8.0 and were maintained at 23°C. Oysters were routinely fed 1 L of a phytoplankton culture initially and during the feeding periods.

Bacteria cultures and oyster feeding

A V. vulnificus strain, CVD713, carrying a stable TnphoA transposon that confers kanamycin resistance and alkaline phos-
phatase activity to the cells (12) was employed for this study. The transposon causes the bacteria to produce blue colonies on Tn agar, composed of L-agar amended with 0.2% glucose, 200 μg/ml kanamycin, and 40 μg 5-bromo-4-chloro-3-indolyl phosphate (BCIP) per ml. This medium is selective and differential due to the presence of kanamycin and the alkaline phosphatase substrate, BCIP, respectively (33). CVD713 was assayed for production of hemolysin, lipase, mucinase, DNase, hyaluronidase, chondroitin sulfatase, collagenase, and albuminase activity to insure that the insertion of the transposon had not affected any of these putative virulence factors. Both the opaque (encapsulated) morphotype and a spontaneously derived translucent (acapsular) morphotype of CVD713 used in our studies were like the parent strain in possessing these activities.

Opaque and translucent cells of *V. vulnificus* were grown and maintained on heart infusion (HI, Difco Laboratories, Detroit, MI) agar containing 50 μg/ml kanamycin (Sigma Chemical Co., St. Louis, MO). Isolated colonies were inoculated into HI broth containing 50 μg/ml kanamycin and grown at 22°C standing overnight for oyster uptake studies.

**Uptake and fate of *V. vulnificus***

Groups of approximately 80 oysters were allowed to acclimate in a 55-gal (ca. 208.27 L) holding tank equipped as described above. For uptake studies, oysters were transferred to a separate tank to which a mixture of phytoplankton culture (1 L) and 50 ml of either opaque or translucent *V. vulnificus* cells (at a concentration of 10^9 cells per ml) was added. Oysters were allowed to filter the algal-bacterial mixture for 4 h, then returned to the 55 gal (ca. 208.27 L) tanks with UV-treatment system for depuration studies.

**Sampling procedures**

For each sample, 8 to 10 oysters were removed from the tank, the shells rinsed with 70% ethanol, and opened with an alcohol-flamed oyster knife. The oyster contents were aseptically re-moved, weighed, and placed into sterile blender jars with an equal weight:volume of sterile ASW. The resulting mixture was homogenized for 2 min using 15-s bursts with 5-s pauses between bursts and serially diluted in ASW. Homogenate dilutions were plated onto HI agar to determine total bacterial counts, thiosulfate-citrate-bile salts-sucrose (TCBS; Difco) agar to determine total *Vibrio* counts, and Tn agar to enumerate *V. vulnificus* CVD713. Oyster samples were taken before inoculation of oysters to determine background bacterial levels, immediately after feeding, and at daily intervals thereafter.

**Effect of oyster passage on virulence and opaque/translucent conversions**

Oysters were fed either opaque or translucent cells of *V. vulnificus* then recovered from oysters at various times. Oyster homogenates were plated onto Tn agar and incubated at 37°C overnight to determine the number of *V. vulnificus* cells present. The numbers of opaque and translucent colonies resulting on the plates were used to determine the conversion rates between the two colony morphotypes as well as the depuration rates for each colony type. Opaque/translucent conversion rates were compared to those of cells grown as described above then plated onto Tn agar but without oyster passage.

Several of the resulting colonies, both opaque and translucent, were also randomly selected from the plates and grown in HI broth with 50 μg/ml kanamycin. These cultures were then examined for virulence by injecting 0.5 ml of phosphate-buffered saline dilutions intraperitoneally (i.p.) into 4- to 6-week-old ICR white mice. LD_{90} values were calculated according to the method of Reed and Muench (19). These values were compared to LD_{20} values calculated for the same colony morphotype injected into mice prior to oyster passage.

**RESULTS AND DISCUSSION**

Oysters are filter-feeders, trapping food particles in mucus on their gills which are then drawn into the mouth. The particles eventually accumulate in the gut. During this process, oysters concentrate bacteria and viruses from the surrounding waters. The accumulation of *V. vulnificus* in oysters was enhanced in our laboratory studies by the use of the "piggy-back" method in which the bacteria were presented to the oysters along with phytoplankton cells. This method causes a concentration of bacteria within the oyster rather than an equilibrium between the oyster and the surrounding seawater (27).

The average of four individual uptake studies for the opaque morphotype of *V. vulnificus* was 4.0 × 10^7/100 g oyster tissue and the average of seven uptake studies of the translucent morphotype was 8.0 × 10^7/100 g oyster tissue. These values were found not to be significantly different (p > 0.05 from a one-way analysis of variance), suggesting that *C. virginica* does not differentiate in its uptake of the opaque and translucent morphotypes of *V. vulnificus* in the laboratory. These values are also comparable to those obtained by Steslow (27) using the same uptake method. The total number of vibrios present in these oysters following uptake of *V. vulnificus*, as measured by plate counts on TCBS, averaged from seven separate experiments, was 4.3 × 10^7/100 g oyster tissue. The number of total bacteria present in the oysters, as measured by plate counts on HI agar, averaged 4.9 × 10^7/100 g oyster tissue in nine separate experiments.

A variety of methods, including relaying and depuration, have been employed in an attempt to purify oysters of bacterial contaminants. Relaying involves the transfer of shellfish from "contaminated" to "clean" waters to allow self purification before harvesting and sale. Depuration is a process of purification whereby shellfish are placed in disinfected, recirculating seawater and allowed to actively filter feed. Chlorine, ozone and UV irradiation have been used in some studies to disinfect water employed in depuration of shellfish. Ultraviolet irradiation is the most commonly used method (5) for this purpose, however, and the method we chose to investigate. Depuration is controlled by the oyster pumping rate and filtration efficiency, both of which are affected by such environmental parameters as temperature, salinity and water turbidity (5,18). Rates of depuration can also be affected by initial bacterial levels (5).

In our experiments, ultraviolet-assisted depuration of both the opaque and the translucent morphotypes of CVD713 from the oyster (Fig. 1) occurred at rates similar to those reported by others for the depuration of laboratory introduced *V. vulnificus* (3,4,22,27). The rates of depuration for the opaque and the translucent morphotypes were not significantly different (Fig. 1). In both cases, *V. vulnificus* was reduced to nondetectable levels within 48 h in those oysters exposed to ultraviolet-treated seawater.

In contrast, both the depuration of total vibrios and the depuration of total bacteria (Fig. 2) showed only slight decreases, to levels approximately the same as before uptake of *V. vulnificus* was initiated. These decreases are thought...
Conversion between the two morphotypes of *V. vulnificus* was compared before and after oyster passage (Table 1). Conversion of the translucent to the opaque morphotype was not observed in our study either prior to or after oyster passage, consistent with previous reports (25,34). The conversion rates of the opaque morphotype to the translucent morphotype prior to oysters passage (0.13%) and after means for significantly reducing bacterial numbers (6-8,20,21,27). The greater depuration of the artificially infected oysters over naturally infected oysters suggests that naturally introduced bacteria, including vibrios, have become a part of the oysters’ normal flora. The failure to depurate normal flora bacteria may be a result of the ability of such bacteria to attach to oyster tissue, to colonize oyster tissue previously inhabited by other bacteria, or to grow at rates exceeding that of depuration (7,8). Thus, our findings are in concurrence with previous studies which have suggested that artificial infection of oysters in the laboratory may not equate to infection of oysters in the natural environment (3,6,18,26,27,31).

To further explore the possible role of the natural oyster microflora in the depuration process, we examined the depuration of *V. vulnificus* from oysters that were exposed to ultraviolet-irradiated seawater for 3 days prior to feeding and from those which were not previously exposed. We found prior depuration to have little effect on the depuration of *V. vulnificus* (Fig. 3), suggesting again that a persistent, depuration-resistant microflora is present in oysters which prevents the establishment of the newly introduced *V. vulnificus* cells. We suggest that the inability of the laboratory-infected cells of *V. vulnificus* to become part of this resident microflora apparently leads to their rapid depuration.

Conversion between the two morphotypes of *V. vulnificus* was compared before and after oyster passage (Table 1). Conversion of the translucent to the opaque morphotype was not observed in our study either prior to or after oyster passage, consistent with previous reports (25,34). The conversion rates of the opaque morphotype to the translucent morphotype prior to oysters passage (0.13%) and after

**Figure 1.** Uptake and UV-assisted depuration of opaque (●) and translucent (○) morphotypes of *V. vulnificus* from the eastern oyster as determined by plate counts on Tn agar. Time zero values are bacterial levels following 4 h of uptake of *V. vulnificus*. Results are shown as cells of *V. vulnificus* per 100 g of oyster tissue, and are representative of several such experiments performed.

**Figure 2.** Ultra-violet-assisted depuration of total vibrios (●), as determined by plate counts on TCBS agar, and of total bacteria (○), as determined by plate counts on HI agar, from the eastern oyster following uptake of *V. vulnificus*. Time zero values are bacterial levels following 4 h of uptake of *V. vulnificus*.

to reflect the depuration of the newly introduced CVD713 strain of *V. vulnificus*. As we observed for *V. vulnificus* (Fig. 1), previous studies have shown artificially introduced bacteria to be rapidly reduced when the oysters are exposed to UV-irradiated seawater (6,22,30). However, as we observed with those vibrios and total bacteria (Fig. 2) naturally present in the oysters we studied, UV-assisted depuration has generally been found to be an ineffective
TABLE 1. Conversion rates between morphotypes.

<table>
<thead>
<tr>
<th></th>
<th>Before passage</th>
<th>After passage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Opaque to Translucent</td>
<td>10,633</td>
<td>12,000</td>
</tr>
<tr>
<td>Conversion rate</td>
<td>0.13%</td>
<td>0.075%</td>
</tr>
<tr>
<td>Translucent to Opaque</td>
<td>10,015</td>
<td>12,584</td>
</tr>
<tr>
<td>Conversion rate</td>
<td>&lt;0.01%</td>
<td>&lt;0.01%</td>
</tr>
</tbody>
</table>

Table 2. Lethality of Vibrio vulnificus CVD713 in 4 to 6 week-old IC white mice.

<table>
<thead>
<tr>
<th>Prior to Oyster Passage</th>
<th>After Oyster Passage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Opake LD&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Translucent LD&lt;sub&gt;50&lt;/sub&gt;</td>
</tr>
<tr>
<td>3.2 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>3.5 x 10&lt;sup&gt;7&lt;/sup&gt;</td>
</tr>
<tr>
<td>2.6 x 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>9.5 x 10&lt;sup&gt;8&lt;/sup&gt;</td>
</tr>
<tr>
<td>1.3 x 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>6.4 x 10&lt;sup&gt;8&lt;/sup&gt;</td>
</tr>
<tr>
<td>5.0 x 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>5.4 x 10&lt;sup&gt;9&lt;/sup&gt;</td>
</tr>
<tr>
<td>6.5 x 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>7.6 x 10&lt;sup&gt;9&lt;/sup&gt;</td>
</tr>
<tr>
<td>1.3 x 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>2.7 x 10&lt;sup&gt;9&lt;/sup&gt;</td>
</tr>
<tr>
<td>Average</td>
<td>1.5 x 10&lt;sup&gt;9&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

In our studies, the use of a transposon-containing strain of V. vulnificus was essential because it allowed us to follow specifically those bacteria that were fed to the oysters in the laboratory as opposed to those V. vulnificus cells which might be present naturally in the oysters. The use of a special medium (Tn) to isolate the transposon-containing V. vulnificus allowed us to quickly and easily detect and quantitate these cells without the use of such imprecise methods as alkaline peptone enrichments, most probable numbers, and complex and time consuming taxonomic testing. Indeed we, as well as others, have reported on the difficulty in accurately identifying V. vulnificus taken from natural sources such as oysters (14-16, 29). The use of strain CVD713 allowed us to confirm the findings of others who had artificially introduced V. vulnificus into oysters and found it to be depurated within a 48-h period. We were also able to show that there is no difference between the uptake and depuration of the two morphotypes of V. vulnificus. The use of strain CVD713 allowed us to track the passage of the cells through the oyster so that conversion rates and lethality of the opaque and translucent morphotypes upon passage could be determined. Without the use of this strain, it would have been virtually impossible to determine if the bacteria obtained after oyster passage were the same as those introduced into the oyster. The use of this strain also allowed us to examine whether prior UV-assisted depuration had an effect on V. vulnificus depuration. The results of our studies again point out, however, that at present a suitable model for examining the depuration of laboratory-infected oysters does not exist. We are currently attempting to adapt our transposon-marked bacterium model to this end.

ACKNOWLEDGMENTS

These studies were supported, in part, by grants from the Florida Sea Grant Program (R/LR-Q-15) and the North Carolina Sea Grant Program (R/ME-R-20).

REFERENCES


