Uptake and depuration of the opaque and translucent morphotypes of *Vibrio vulnificus*, and the effects of oyster passage on virulence

Absorption et purification des morphotypes opaque et translucide de *Vibrio vulnificus*, et effets du passage sur l'huître sur leur virulence

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Abstract

*Vibrio vulnificus* is an estuarine bacterium which is known to be a significant human pathogen. It occurs in high numbers in oysters and other molluscan shellfish, where it is part of the animals' normal flora. *V. vulnificus* occurs in two colony morphotypes; the opaque, encapsulated variety is virulent, whereas the translucent, non-encapsulated variety is avirulent. Studies were carried out to determine if the opaque and translucent morphotypes were taken up by oysters (*Crassostrea virginica*) at different rates, and whether UV-assisted depuration proceeded at different rates. Possible interconversion of the opaque and translucent morphotypes was also examined, as was the possibility that the virulence of *Vibrio vulnificus* would be modified following passage through the oyster. All studies were carried out using a strain of *V. vulnificus* which harbors the transposon, *TnphoA*, which allowed the added cells of *V. vulnificus* to be readily differentiated from other bacteria naturally present in the oysters.

Results indicated little difference in the rate of uptake or depuration of *V. vulnificus* by oysters (figure 1). Uptake in either case was rapid (saturation appeared within 30-60 minutes), and depuration by the laboratory-infected oysters appeared complete within 48 hours. Conversions for opaque and translucent morphotypes in oysters were not significantly different from *in vitro* rates (table I). No conversion from the translucent (avirulent) to the opaque (virulent) form was seen. LD50 values in mice after oyster passage were unchanged (table II).

Our results suggest that the presence of the capsule on *V. vulnificus* cells does not markedly contribute to its ability to be taken up or depurated by oysters, and that oyster passage neither increases nor decreases the virulence of this human pathogen.

Résumé

*Vibrio vulnificus* est une bactérie estuarienne reconnue comme pathogène pour l'homme. Il apparaît en très grand nombre dans les huîtres et autres mollusques où il fait partie de la flore normale des coquillages. *V. vulnificus* se présente sous forme de deux types morphologiques de colonie : une variété opaque, avec capsule, qui est virulente, et une variété translucide dépourvue de capsule, non virulente. Des études ont été réalisées pour déterminer si les morphotypes opaque et translucide étaient absorbés par les huîtres (*Crassostrea virginica*) à des vitesses différentes, et si la purification par ultraviolets induisait des différences.

L'éventualité d'une interconversion entre les morphotypes opaque et translucide a également été étudiée, de même que la possibilité de modification de la virulence de *Vibrio*
Vibrio vulnificus is one of the most invasive and rapidly lethal of human pathogens. This organism is part of the normal microflora of estuarine waters and occurs in high numbers in molluscan shellfish. Ingestion of raw seafood, primarily oysters, may result in primary septicemia. Septicaemia leads rapidly to cutaneous lesions of the extremities involving localized edema and cellulitis. The necrosis which ultimately occurs often requires surgical debridement of the tissue or amputation of affected limbs. Approximately 60% of all septicemia cases result in fatality. The majority of victims have some underlying chronic disease, typically involving the liver, that causes a serum iron overload (Oliver, 1989).
Table I: Conversion rates between morphotypes

<table>
<thead>
<tr>
<th></th>
<th>Before passage</th>
<th>After passage</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Opaque to Translucent</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total colonies counted</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><strong>Translucent to Opaque</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total colonies counted</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>

LD50 Data for *Vibrio vulnificus*

Table II: Lethality of *Vibrio vulnificus* CVD713 in 4-6 week old ICR white mice

<table>
<thead>
<tr>
<th></th>
<th>Prior to Oyster Passage</th>
<th>After Oyster Passage</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Opaque LD50</strong></td>
<td>3.2 x 10^6</td>
<td>5.0 x 10^7</td>
</tr>
<tr>
<td><strong>Translucent LD50</strong></td>
<td>2.6 x 10^7</td>
<td>3.5 x 10^7</td>
</tr>
<tr>
<td></td>
<td>3.2 x 10^7</td>
<td>1.8 x 10^7</td>
</tr>
<tr>
<td></td>
<td>7.2 x 10^8</td>
<td>9.5 x 10^8</td>
</tr>
<tr>
<td></td>
<td>5.5 x 10^7</td>
<td>9.5 x 10^7</td>
</tr>
<tr>
<td></td>
<td>1.8 x 10^8</td>
<td>6.9 x 10^7</td>
</tr>
<tr>
<td></td>
<td>5.5 x 10^7</td>
<td>3.2 x 10^6</td>
</tr>
<tr>
<td></td>
<td>5.4 x 10^7</td>
<td>2.7 x 10^6</td>
</tr>
<tr>
<td></td>
<td>5.4 x 10^7</td>
<td>8.4 x 10^6</td>
</tr>
<tr>
<td>Average</td>
<td>4.9 x 10^7</td>
<td>3.2 x 10^7</td>
</tr>
</tbody>
</table>

*Vibrio vulnificus* occurs in both an opaque and a translucent morphotype. The difference is due to an acidic polysaccharide surface capsule (Amako et al., 1984) which is lacking on the translucent morphotype. 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 (Kreger et al., 1981;
Shinoda et al., 1987; Simpson and Oliver, 1987; Simpson et al., 1987). The bacterium shifts from the opaque to the translucent morphology at a frequency of 0.1 – 1%; our studies have not found reversion from the translucent to the opaque morphotype, however (Simpson et al., 1987).

In recent years, the seafood industry has become increasingly concerned with the presence of *Vibrio vulnificus* in shellfish. Thus, the purpose of this study was to examine the relationship between *V. vulnificus* and the eastern oyster (*Crassostrea virginica*). In particular, the objectives were to:

1) compare uptake and UV-assisted depuration of opaque and translucent morphotypes of *V. vulnificus*,

2) to examine the effects of oyster passage on conversion rates between the opaque and translucent morphotypes,

3) to determine if there are changes in virulence of the opaque and translucent morphotypes upon oyster passage.

A problem with such studies in the past is that they have always employed such routine methods as enrichment culture, MPN determination of cell numbers, and the requirement for isolation and subsequent taxonomic identification of those bacteria believed to be *V. vulnificus*. Anyone who has carried out such studies realizes how time consuming and inherently inaccurate these methods are, especially the final taxonomic analysis. *V. vulnificus* is quite difficult to identify from environmental samples, at least in part due to the significant variation this bacterium exhibits in a number of key taxonomic traits (Oliver, 1987; Oliver et al., 1992). The studies we report here have been greatly facilitated by the use of a genetically marked strain of *V. vulnificus* which allows its specific detection and enumeration against a natural microflora background of $10^6$ bacteria per gram of oyster. We employed *V. vulnificus* strain CVD713, carrying a stable TnphoA transposon that confers kanamycin resistance and alkaline phophatase activity to the cells (Wright et al., 1990). The transposon causes the bacteria to produce blue colonies on a selective and differential medium containing kanamycin and the chromophore, BCI-phosphate. Strain CVD713 was assayed for production of hemolysin, lipase, mucinase, DNase, hyaluronidase, chondroitin sulfatase, collagenase, and albuminase activity to insure that insertion of this transposon had not affected any putative virulence factors. Both the opaque morphotype and a spontaneously derived translucent morphotype of CVD713 were found to respond the same as the parent strain.

The uptake of both the opaque and translucent morphotypes of *V. vulnificus* was rapid. The average of four uptake trials for the opaque morphotype of *V. vulnificus* was $4 \times 10^5$/g oyster tissue, and the average of 7 uptake trials of the translucent morphotype was $8 \times 10^5$/g tissue (figure 1). These values were not significantly different, indicating that these oysters do not differentiate between the opaque and translucent morphotypes of *V. vulnificus* upon uptake in the laboratory.

Ultraviolet-assisted depuration of both the opaque and translucent morphotypes of CVD713 from the oyster (figure 1) occurred at rates similar to those reported by others for the depuration of artificially introduced *V. vulnificus*. The rate of depuration of the two morphotypes was not significantly different. In
both case, *V. vulnificus* levels were reduced to non-detectable levels within 48 hours in those oysters exposed to ultraviolet-treated seawater.

When uptake and depuration of *E. coli* was compared to that of *V. vulnificus* (figure 2), essentially the same result was seen. These data suggest that artificially introduced *E. coli* and *V. vulnificus* appear not to be incorporated into the oysters' normal flora, and these bacteria are readily depurated.

In contrast, the depuration of total vibrios, as indicated by plate counts on TCBS, revealed decreases only to those levels present before uptake of *V. vulnificus* was initiated (figure 3). The slight decrease seen here is thought to reflect only the decrease caused by the depuration of the artificially introduced CVD713.

![Figure 2: Comparison of uptake and depuration rates for *V. vulnificus* CVD713 (■) and *E. coli* (●)](image)

![Figure 3: Depuration of total vibrios from the eastern oysters. Shown are UV-assisted depuration (■) and unassisted depuration (□) as determined by plate counts](image)
The greater depuration of the artificially infected oysters over naturally infected oysters suggests that naturally introduced vibrios have become part of the oysters' normal flora and are not depurated. These vibrios may be able to evade depuration due to a survival characteristic that allows them to remain associated with the oysters even though the oysters are actively pumping water. It has also been suggested that the failure of vibrios to depurate from shellfish may be caused by an ability to attach to oyster tissue or to grow at rates exceeding those of depuration (Greenberg et al., 1982; Jones et al., 1991). Other than the laboratory introduced V. vulnificus, all bacteria present in the oysters used in our studies were obtained naturally and were part of the oysters' normal flora.

Results similar to ours have also been reported by others (Son and Fleet, 1980; Timony and Abson, 1984; Vasconcelos and Lee, 1972), and suggest that artificial infection of oysters in the laboratory may not equate to natural infection of oysters by marine bacteria.

Conversion between the two morphotypes of V. vulnificus was compared before and after oyster passage (table I). Conversion of the translucent to the opaque morphotype was not observed in our study either before or after oyster passage. Although the conversion rate of the opaque morphotype to the translucent morphotype was found to be 0.13% prior to oyster passage and 0.075% after passage, these values were not significantly different. This suggests that a brief presence in the oyster does not affect the morphotype of V. vulnificus.

As with conversion rates, passage of V. vulnificus through the oyster was not found to affect the virulence of the two morphotypes (table 2). For the opaque morphotype, the average LD50 in ICR white mice, injected intraperitoneally, was 1.5x107 cells prior to oyster passage. This was not significantly different from that of 3.2x107 after oyster passage. The average LD50 of 4.9x108 cells prior to passage and 1.8x108 after passage for the translucent morphotype was also not significantly different.

In our studies, the use of a transposon-containing strain of V. vulnificus was essential because it allowed us to rapidly and specifically identify those bacteria that were fed to the oysters in the laboratory as opposed to the naturally introduced V. vulnificus cells which might already be present in the oysters. The use of the kanamycin medium to isolate the transposon-containing cells of V. vulnificus from oysters allowed us to detect the presence of these cells without the use of such imprecise methods and alkaline peptone enrichment and MPNs. The use of strain CVD713 allowed us to conclude that the findings of others who had artificially introduced V. vulnificus cells into oysters and found them to be depurated within a 48 hours period were, in fact, correct. We were also able to show that there is no difference between uptake and depuration of the two morphotypes of V. vulnificus. The use of 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 oyster passage could be determined. In both cases, no changes in colony type or virulence was detected. Without the use of CVD713, it would have been virtually impossible to determine if the bacteria obtained after oyster passage were the same as those introduced to the oyster.
REFERENCES


