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

Trudi Groubert, James D. Oliver

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

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*

vulnificus par passage dans l'huître. Toutes les études ont été réalisées avec une souche de *V. vulnificus* comportant le transposon TnphoA, ce qui a permis de différencier aisément les cellules ajoutées des autres bactéries présentes naturellement dans les huîtres.

Les résultats révèlent peu de différence dans les cinétiques d'absorption ou de décontamination de *V. vulnificus* par les huîtres (figure 1). Dans les deux cas, l'absorption se fait rapidement (saturation atteinte au bout de 30-60 minutes), et la décontamination des huîtres infectées en laboratoire semble totale après 48 heures. Les conversions entre morphotypes opaque et translucide ne sont pas significativement différentes des résultats *in vitro* (tableau I). Aucune conversion du type translucide (non virulent) en type opaque (virulent) n'a été observée. Les valeurs DL50 chez les souris après passage sur l'huître restent inchangées (tableau II).

Nos résultats suggèrent que la présence de la capsule sur les cellules de *V. vulnificus* ne contribue pas de façon notable à la capacité de celui-ci d'être absorbé ou purifié par l'huître, et que son passage sur l'huître ne diminue ni n'accroît la virulence de cet organisme pathogène.

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 septicaemia. 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 septicaemia 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).
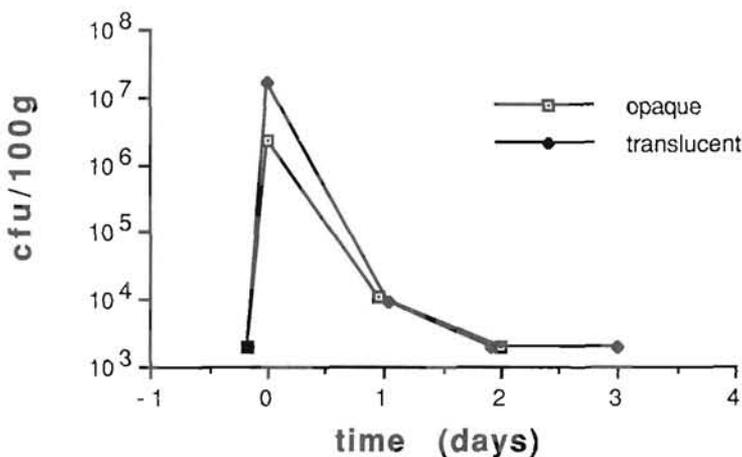

Figure 1: Comparison of uptake and depuration rates for opaque (■) and translucent (♦) *V. vulnificus*. Shown are plate counts on the kanamycin-containing medium specific for strain CVD713

Table I: Conversion rates between morphotypes

	Before passage	After passage
Opaque to Translucent		
Total colonies counted	10,633	12,000
Conversion rate	0,13%	0.075%
Translucent to Opaque		
Total colonies counted	10,015	12,584
Conversion rate	<0.01%	<0.01%

LD50 Data for *Vibrio vulnificus*

Before and After Oyster Passage

	Opaque	Translucent
Before Oyster Passage	1.5×10^7 (n=2)	4.9×10^8 (n=2)
After Oyster Passage	3.2×10^7 (n=10)	1.8×10^8 (n=10)

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

Prior to Oyster Passage		After Oyster Passage	
Opaque LD50	Translucent LD50	Opaque LD50	Translucent LD50
3.2×10^6	3.5×10^7	5.0×10^7	7.2×10^8
2.6×10^7	9.5×10^8	1.7×10^7	6.9×10^8
		1.3×10^8	2.2×10^7
		5.0×10^7	6.4×10^6
		6.8×10^5	5.8×10^7
		1.1×10^7	5.4×10^7
		6.5×10^5	7.6×10^7
		5.2×10^7	9.3×10^3
		3.2×10^6	2.7×10^7
		9.0×10^6	8.4×10^7
average 1.5×10^7	4.9×10^8	3.2×10^7	1.8×10^8

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 Tn*pho*A 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 4×10^5/g oyster tissue, and the average of 7 uptake trials of the translucent morphotype was 8×10^5/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.
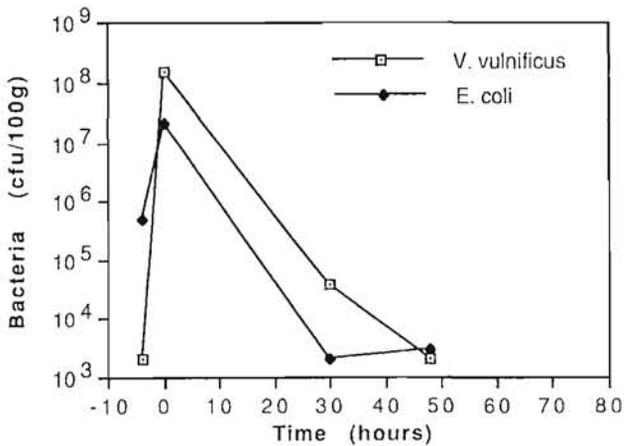

Figure 2: Comparison of uptake and depuration rates for *V. vulnificus* CVD713 (☐) and *E. coli* (♦)
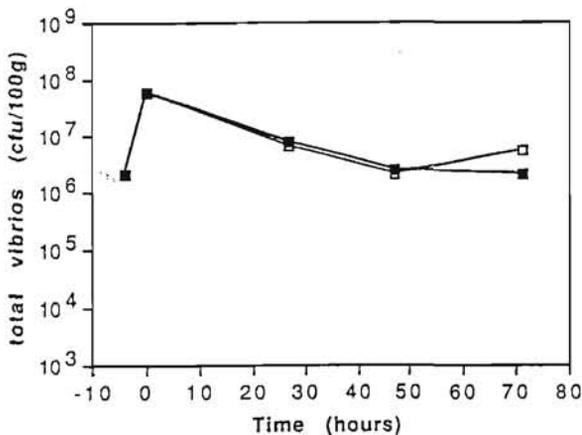

Figure 3: Depuration of total vibrios from the eastern oysters. Shown are UV-assisted depuration (■) and unassisted depuration (☐) as determined by plate counts

The greater depuration of the artificially infected oysters over naturally infec-
ted 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 cau-
sed 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 labora-
tory introduced *V. vulnificus*, all bacteria present in the oysters used in our stu-
dies 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 artifi-
cial 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 translu-
cent 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 1.5×10^7 cells prior to oyster passage. This was not significantly different
from that of 3.2×10^7 after oyster passage. The average LD50 of 4.9×10^8 cells
prior to passage and 1.8×10^8 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 intro-
duced *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. vulni-
ficus* 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 pas-
sage 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

Amako K., K. Okdada, S. Miake, 1984. Evidence for the presence of a capsule in *Vibrio vulnificus. J. Gen. Microbiol.*, **130**, 2741-2743.

Greenberg E.P., M. Dubois, B. Palhof, 1982. The survival of marine vibrios in *Mercenaria mercenaria*, the hardshell clam. *J. Shellfish Res.*, **10**, 105-112.

Jones S.H., T.L. Howell, K.R. O'Neill, 1991. Differential elimination of indicator bacteria and pathogenic *Vibrio sp.* from eastern oysters (*Crassostrea viginica* Gmelin, 1791) in a commercial controlled purification facility in Maine. *J. Shellfish Res.*, **10**, 105-112.

Kreger A., L. De Chatelet, P. Shirley, 1981. Interaction of *V. vulnificus* with polymorphonuclear leukocytes: association of virulence with resistance to phagocytosis. *J. Infect. Dis.*, **144**, 244-248.

Oliver J.D., 1989. *Vibrio vulnificus*, in: Foodborne Bacterial Pathogens, M. Doyle ed., Marcel Dekker, Inc., N.Y., 569-600.

Oliver J.D., K. Guthrie, J. Preyer, A. Wright, L.M. Simpson, R. Siebeling, J.G. Morris Jr., 1992. Use of colistin-polymyxin ß-cellobiose agar for isolation of *V. vulnificus* from the environment. *Appl. Environ. Microbiol.*, **58**, 737-739.

Shinoda S., M. Kobyashi, H. Yamada, S. Yoshida, M. Ogawa, Y. Mizuguchi, 1987. Inhibitory effect of capsular antigen and *Vibrio vulnificus* on bactericidal activity of human serum. *Microb. Immunol.*, **31**, 393-401.

Simpson L.M., J.D. Oliver, 1987. Ability of *V. vulnificus* to obtain iron from transferrin and other iron-binding proteins. *Curr. Microbiol.*, **15**, 155-157.

Simpson L.M., V.K. White, S.F. Zane, J.D. Oliver, 1987. Correlation between virulence and colony morphology in *V. vulnificus. Infect. Immun.*, **55**, 269-272.

Son N.T., G.H. Fleet, 1980. Behavior of pathogenic bacteria in the oysters, *Crassostrea commercialis*, during depuration, relaying and storage. *Appl. Environ. Microbiol.*, **40**, 994-1002.

Timoney J.F., A. Abston, 1984. Accumulation and elimination of *E. coli* and *Salmonella typhimurium* by hard clams in an *in vitro* system. *Appl. Environ. Microbiol.*, **47**, 986-988.

Vasconcelos G.J., J.S. Lee, 1972. Microbial flora of Pacific oysters (*Crassostrea gigas*) subjected to ultraviolet-irradiated seawater. *Appl. Microbiol.*, **23**, 11-16.

Wright A.C., L.M. Simpson, J.D. Oliver, J.G. Morris Jr., 1990. Phenotypic evaluation of acapsular transposon mutants of *Vibrio vulnificus. Infect. Immun.*, **50**, 1769-1773.