The Biology of *Vibrio vulnificus*

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ABSTRACT  *Vibrio vulnificus*, carrying a 50% fatality rate, is the most deadly of the foodborne pathogens. It occurs in estuarine and coastal waters and it is found in especially high numbers in oysters and other molluscan shellfish. The biology of *V. vulnificus*, including its ecology, pathogenesis, and molecular genetics, has been described in numerous reviews. This article provides a brief summary of some of the key aspects of this important human pathogen, including information on biotypes and genotypes, virulence factors, risk factor requirements and the role of iron in disease, association with oysters, geographic distribution, importance of salinity and water temperature, increasing incidence associated with global warming. This article includes some of our findings as presented at the "Vibrios in the Environment 2010" conference held in Biloxi, MS.

DISEASE CHARACTERISTICS

*Vibrio vulnificus* is the single most fatal foodborne pathogen in the United States, and possibly in the world, accounting for 95% of all seafood-related deaths in the US with a fatality rate of ca. 50% (1). This greatly exceeds that of other foodborne pathogens (2), such as *Salmonella* (0.6%), *Escherichia coli* (3–5%), and *Clostridium botulinum* (<8%). The bacterium occurs naturally in estuarine waters worldwide and the food vehicle is primarily raw/undercooked oysters, which account for 93% of ingestion cases. While the concentration of *V. vulnificus* in estuarine waters is typically quite low (<10 CFU/ml), it becomes concentrated in such molluscan shellfish as oysters and clams due to their efficient use of filter-feeding to obtain food. The resultant levels in shellfish can reach 10^5 CFU/g of tissue or more (3, 4, 5). While the infectious dose is not known, it has been estimated to be as few as 100 cells or less (6). Most cases (86%) occur in males over the age of 40, with 95% of victims having one or more preexisting risk factors (see below). These statistics, based on US cases, appear not to differ from those in other countries, even where raw seafood is widely consumed. For example, Ito et al. (7) recently reported that the average age of patients of 166 *V. vulnificus* cases is 60 years, with approximately 90% being male; 42% had liver cirrhosis and 11% diabetes mellitus. The majority of cases (85%) reported in the US occur in the summer months of May to October, which correlates to the higher water temperatures common during that period. The incubation period for ingestion cases is quite rapid, averaging only 26 h. Symptoms of infection following ingestion of infected food include fever (94%), chills (86%), nausea (60%), abdominal pain (44%), hypotension (43%), and the development of secondary lesions (69%), which typically develop on the extremities (1). The reason for the latter may be the temperature optimum for growth of the pathogen (ca. 30°C) and the lower temperature of such extremities as the legs (8).

It has been estimated that 80,000 people contract *Vibrio* infections each year in the USA, contributing to 500 hospitalizations and 100 deaths (9). Despite at least 12 *Vibrio* spp. being human pathogens (10), the great majority of hospitalizations and deaths are due to *V. vulnificus*.

The reason for the highly significant gender difference (ca. 86% of infections are in males) is due, at least in part, to the role that estrogen plays in protecting against the bacterium’s endotoxin (11). However, why those over the age of 40 are predominantly affected is not known (Fig. 1). It might be thought that older people...
are the prime consumers of raw oysters, but in fact this honor goes to those under 29 years (12), and this aspect of the infection remains a mystery.

In addition to primary septicemia resulting from V. vulnificus infection, this bacterium is also capable of causing potentially fatal wound infections (13, 14, 15, 16). Most of these involve exposure of a preexisting wound to seawater or shellfish, or acquisition and infection of a wound during saltwater-associated recreational activity. Like ingestion cases, some 89% of wound cases are in males. This may reflect a greater occurrence of commercial and recreational fishermen of this gender, but given the large number of females involved in coastal swimming activities, such a gender difference is surprising. Major symptoms include fever (85% of patients), chills (68%), with edema (91%) and cellulitis (94%) at the wound site. The incubation period for this form of infection is even more rapid, averaging only 16 h. Chronic disease does not appear to be a prerequisite to these infections (over 80% have no underlying syndrome), but the 22% fatality rate that occurs in wound infections is likely a consequence of the fact that these victims generally have some form of liver or other serum iron-elevating condition. Nonfatal cases are often serious enough to warrant surgical intervention, including limb amputation. Interestingly, the incidence of wound infections is increasing in the USA; between 1988 and 1999 there was an average of 24 cases/year reported to the Cholera and Other Vibrio Illness Surveillance (COVIS) system, whereas between 2000 and 2010, the rate had increased to 52/year. Indeed, wound infections caused by V. vulnificus are now the predominant form of infection caused by this pathogen in the USA, exceeding nonwound isolations (17, 18).

BIOTYPES AND GENOTYPES

Despite the need for underlying chronic disease, a national health survey estimates that as many as 36 million Americans have one or more of these predisposing syndromes (19), so the question arises, why are there not more cases? There are undoubtedly several reasons for this, including the need to consume raw shellfish harboring this pathogen, human physiological conditions both natural (male, age over 40 years) and pathological (liver or immunocompromising disease), and the production of essential virulence factors by the infecting V. vulnificus cells. There are likely more human host factors we have yet to discern, but it is certainly the case that we currently understand little of the essential virulence traits needed. Despite extensive study by many laboratories around the world, we know very little of this critical aspect. We do now know, however, that there are at least three biotypes, and of the major human biotype, the existence of two genotypes. Biotype 1 was the first described and is the type found in virtually all human infections (15). Biotype 2 causes a rapidly fatal septicemia in eels, especially those raised in aquaculture farms (20, 21, 22, 23, 24), and it has, on rare occasions, been isolated from human cases. Biotype 3 was the most recently described and it has been proposed to be a genetic mosaic of biotype 1 and 2 strains (25). To date, this biotype has only been isolated from human wound infections associated with tilapia aquaculture, and only in Israel (26). The latter observation is rather incredible, given the ease of disease spread around the world.

In 1999, we reported results of a RAPD-PCR analysis of numerous clinical and environmental (oyster, seawater) strains of V. vulnificus. This revealed the presence of a PCR amplicon, which appeared to be unique to the human clinical isolates (27). On sequencing this DNA fragment, we discovered that the base sequence of the human isolate form was dramatically different (up to 30% of the bases) from that found in a homologous gene in the environmental isolates (28). A similar separation of V. vulnificus isolates using 16S rRNA sequencing was reported by Aznar et al. (29), and subsequently, by Nilsson et al. (30) and Gutacher et al. (31), and a 100% correlation between the B/C and C/E genotyping schemes has been reported (28). Realizing that two different genotypes of this pathogen existed, we examined over 50 strains of V. vulnificus and determined that 90% of isolates with the “C” (clinical) genomic pattern had come from human clinical cases, while 93% of those possessing the “E” (environmental) pattern had come from oysters or water (28). We subsequently designed a multiplex PCR protocol that simultaneously identifies an
isolate as *V. vulnificus* (through analysis of the *vvhA* gene, which is unique to this bacterium) and reveals whether it possesses the C or E genomic pattern (33). Compared to conventional phenotypic analyses, this PCR method is highly accurate and requires only 3 h from colony selection to species confirmation and genotype determination, which is much faster than other typing methods (34). Subsequent studies using extensive phenotypic analysis (35), multilocus sequence typing (34, 36), pulsed field genomic analysis (34, 37), and ultimately, whole genome sequencing (38), confirmed the existence of the two biotype 1 genotypes, and the highly significant correlation of the C-genotype with human disease-causing ability. That two distinct genotypes are present in this species was confirmed by the genomic studies of Gulig et al. (39) and when we recently sequenced three E-genotype strains and compared them to three previously published C-genotype strains (38). We found numerous genes to be unique to the E-genotype, likely allowing their enhanced survival in the environment, as well as in the C-genotype strains, undoubtedly in some cases responsible for the increased human virulence of this genotype. While strains of the C-genotype are better able to resist serum killing (40, 41), why this genotype is better able to cause disease is only beginning to be understood.

This striking and consistently observed difference in genome structure in multiple *V. vulnificus* strains led us to propose that *V. vulnificus* exists as two distinct ecotypes (42). How such diversity in a single species evolved is a fascinating question but may well have involved horizontal gene transfer that is ubiquitous among vibrios, including *V. vulnificus* (43, 44).

**VIRULENCE FACTORS**

The one absolute requirement for virulence that is known is possession of an antiphagocytic capsule (45, 46, 47). Several capsule types exist (48, 49), and these are capable of undergoing phase variation/conversion (50, 51, 52). Interestingly, phase variation occurs at a very high rate (50), and on occasion, cells spontaneously mutate. In both instances capsule loss, either temporary or permanent, results. Whether the cells produce a capsule or not is easily determined, as encapsulated cells produce an opaque colony, while those lacking capsular polysaccharide appear translucent. Such a colony morphology change is easily seen on routine media (Fig. 2). Only the opaque (encapsulated) strains are virulent (45, 46) but capsule serotype does not appear to correlate with virulence (53).

Our studies have indicated that the factor that is the likely cause of human fatality is the lipopolysaccharide (LPS) endotoxin (54). This is not unusual for a Gram-negative pathogen (50), and when the nitric oxide-inducing role of LPS was inhibited, we no longer observed death in laboratory animals (56).

In contrast to the significant roles played by capsule and LPS to pathogenesis, most of the numerous factors that have been proposed to be important to virulence have typically been found not to be essential for disease production, at least in animal models (1). An exception may be the toxin, which several studies have recently shown to be essential to virulence in mouse models (57, 58, 59, 60, 61).

Several excellent reviews exist on the known or suspected virulence factors possessed by *V. vulnificus* and the reader is referred to these (1, 62, 63, 64).

**RISK FACTOR REQUIREMENTS AND ROLE OF IRON IN DISEASE**

While possessing an overwhelming case fatality rate, infections caused by *V. vulnificus* are quite rare, averaging ca. 50 ingestion and 50 wound infections per year in the US (1, 19). This is likely due to the fact that *V. vulnificus* is an opportunistic pathogen, requiring one or more of several predisposing factors in order to initiate disease. Bross et al. (65) stated that 97% of patients have some chronic disease, including liver disease (80%).
alcoholism (65%), diabetes (35%), malignancy (17%), or renal disease (7%). A major reason liver disease is such a prominent factor is the elevated serum iron that results from the chronic damage to this major reservoir of iron in the human body. Iron has been shown in numerous studies to be critical to the ability of *V. vulnificus* to survive and grow in the body, and the serum iron overload that results from chronic liver disease is central to its ability to cause fatal infections (40, 41, 66, 67, 68, 69). It must be noted, however, that the likely importance of such underlying syndromes has been described almost exclusively in primary septicemia (ingestion) cases. The situation might be quite different in wound infections (63).

### ASSOCIATION OF *V. VULNIFICUS* WITH OYSTERS

*V. vulnificus* occurrence is associated with all estuarine organisms (15, 70, 71, 72), as well as particulates and plankton (73). It occurs in the highest levels in molluscan bivalves (including mussels and clams) (74) due to their filter-feeding of particles from seawater. However, oysters are of greatest importance in the transmission of this most fatal seafood-borne pathogen in the US and considerable effort has been made to understand the interactions of *V. vulnificus* with the Eastern oyster, *Crassostrea virginica*; for a recent review, see Froelich and Oliver (75). We examined the uptake and depuration of both genotypes of *V. vulnificus* by adult oysters, but found no significance differences in these interactions (76). In a novel variation, we obtained aseptically removed eggs and sperm from female and male oysters and, following *in vitro* fertilization, examined the uptake of both *V. vulnificus* genotypes at the various larval stages as development progressed. Again, no significant differences in uptake were observed, at least through the veliger larval stage (K. Doyle, A.H. Ringwood, J.D. Oliver, unpublished data).

In such studies, researchers have invariably added *V. vulnificus* cells to tanks containing oysters, and examined uptake/depuration of these cells. In fact, oysters undertake a sophisticated size discrimination of particles they ingest, and particles of bacterial size are filtered out with extremely low efficiency (ca. 10%). We recently reported that if *V. vulnificus* cells are allowed to incorporate into marine aggregates (“snow”) and then added to oyster tank water, the efficiency of uptake is greatly increased, and C/E-genotype differences are seen (77).

It is well known that great oyster-to-oyster variations exist in the levels of *V. vulnificus* (3), but the reasons for this are not as obvious. It may be that, as in all organisms, significant variation exists in the genetic makeup, and thus the physiological and innate immune response, of individual oysters. Indeed, we (78) have identified several genes that appear to correlate to *V. vulnificus* levels. Similarly, we have found *V. vulnificus* loads to correlate with oyster size, but interestingly, not with level of *Perkinsus* infection (79).

When we employed our multiplex PCR method to determine which genotype was present in oysters, we were surprised to find that ~85% of the ~900 *V. vulnificus* cells we isolated from 85 different oysters were of the (relatively avirulent) E-genotype (80). This finding is likely one more factor accounting for the relative rarity of these infections; in order to develop a *V. vulnificus* infection, a person presumably must consume a sufficient number of C-genotype cells, and these are a minority of those found in oysters.

The question of how many oysters must be consumed to put a person at risk of *V. vulnificus* infection can only be indirectly addressed. Using Food and Drug Administration data on the number of oysters eaten by people who developed *V. vulnificus* infection led us to conclude that a single oyster may contain enough *V. vulnificus* cells to cause disease, and to cause death. While we cannot know the number of *V. vulnificus* cells present in an oyster that is consumed, it is likely that there must be a sufficient number of C-genotype cells. It may be significant that, in our study examining the occurrence of C- and E-genotypes in oysters (80), we found that only two of the 85 oysters examined had more C than E-type cells, suggesting it may be sufficient to consume a “more dangerous” oyster to allow initiation of infection (1). Interestingly, Jackson et al. (81) provided evidence that, despite there being 10⁷ *V. vulnificus* cells/g of oyster associated with infection, presumably of significant genetic variety, only a single *V. vulnificus* strain was subsequently isolated from human tissue. A recently developed real-time PCR method (77) capable of detecting and enumerating C-genotype cells in oysters may allow a more rapid and definitive method to help define the numbers of such cells needed for human infection.

### GEOGRAPHIC DISTRIBUTION

*V. vulnificus* and *V. vulnificus* infections have been reported throughout Europe, Scandinavia, South America, the Far East, South Pacific, as well as on all coasts of the United States (15). Along with oysters and other molluscan bivalves and estuarine/coastal waters, *V. vulnificus* has been reported in fish (82, 83, 84), sediments (85, 86),
and plankton (73). The presence of this pathogen appears to be spreading to areas where it was not previously reported, and this is likely due to global warming (see below).

**IMPORTANCE OF SALINITY**

A number of environmental parameters have been examined for their role in determining the ecology of *V. vulnificus* in estuarine waters, including dissolved oxygen, coliform levels, pH, turbidity, and dissolved organic carbon (see references 15, 70, 71, 87, 88). The two factors that are routinely reported to have the greatest significance, however, are salinity and temperature (88, 89, 90, 91, 92). These two parameters are discussed here.

Although its salt requirements are not high, *V. vulnificus* is an obligate halophilic bacterium, restricted to estuarine/brackish waters of moderate salinity. An ongoing study conducted in the Neuse River estuary of North Carolina indicates that the salinity limits for isolation of this pathogen are ca. 2‰ to 25‰, with an optimum of ca. 10‰ to 18‰ (C. Taylor and J.D. Oliver, unpublished data). Such findings are consistent with the numerous and multiyear studies we have conducted in estuarine waters (80, 87, 88). The organism is not isolated from open ocean waters, suggesting that the high salinities found in such waters (typically ca. 35‰) are not permissive to the growth of this organism. Indeed, during the period 2007 to 2009, a severe drought occurred in NC, resulting in an increase in the salinity of the estuarine waters we had sampled for many years to increase from a normal of ca. 15‰ to ≥22‰. The result was a nearly total loss of our ability to isolate *V. vulnificus* from these waters and from oysters taken from those waters (93) as might be predicted from Fig. 3. When the drought ended in 2010, the salinity returned to ca. 15‰, and we were again able to routinely isolate *V. vulnificus* from these waters and oysters (93). Such findings are consistent with studies suggesting “relaying” oysters from low to high salinity waters significantly reduces the *V. vulnificus* load (94, 95).

We have conducted several studies (96, 97) wherein cells of *V. vulnificus* were incubated in environmental chambers placed in natural estuarine waters of various salinities (11‰ to 31‰) in order to examine *in situ* gene expression of a variety of genes involved in stress responses and virulence, as they might be regulated by this critical parameter. Expression of *rpoS*, the “stress” sigma factor, was observed for up to 108 h at 11‰, suggesting that even lower salinities may present a stress to the cells. Another gene of interest was *viuB*, which is required for siderophore-mediated iron acquisition. As noted earlier, the ability of *V. vulnificus* to acquire iron is essential for growth of this pathogen in the human host, and the fact that expression of *viuB* was no longer observed by 12 h in both salinity environments may again help explain the relative rarity of *V. vulnificus* infections. If cells in the environment are unable to rapidly scavenge iron on entrance into the human host, they might be unable to cause human infection. However, whether cells within oysters respond in a similar manner to salinities ≥21‰ is not known. Such *in situ* studies have also identified other genes that are differentially expressed, and which respond to the natural estuarine environment in a quite different manner (Fig. 4).

**IMPORTANCE OF WATER TEMPERATURE**

Water temperature is a parameter critical to the ecology of *V. vulnificus* and in the incidence of human infection (15, 90, 91, 92). As shown in Fig. 5, cases of *V. vulnificus* over a 12-year period demonstrate a distinct seasonality, with almost all cases (97%) occurring in the months of April through November when water temperatures in the Gulf of Mexico are at or above 20°C. Indeed, our field studies have regularly suggested that water temperatures of 20°C indicate the point where a human health concern resulting from elevated *Vibrio* levels may exist (88). In contrast, temperatures of 13°C or lower induce the viable but nonculturable state in this pathogen (see below).

The two *V. vulnificus* genotypes appear to respond in a similar manner to seasonal temperature variations (Fig. 6), although again the level of the E-genotype is
**In situ Gene Expression**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Expression (hours)</th>
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<tbody>
<tr>
<td>vvhA</td>
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<tr>
<td>trkA</td>
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<tr>
<td>viuB</td>
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consistently 5- to 6-fold greater than the C-genotype, both in estuarine water and oysters. It is evident that *V. vulnificus* prefers warmer temperatures (>20°C) but appears to be adversely affected by temperatures above 30°C.

**TEMPERATURE AND THE VIABLE BUT NONCULTURABLE STATE**

The lower line in Figure 5 temp indicates 13°C, which is the temperature that our *in situ* studies indicate is the lower limit of growth for *V. vulnificus*. In fact, below this point, cells of *V. vulnificus* become dormant, undertaking a physiological response known as the viable but nonculturable (VBNC) state. VBNC bacteria are organisms that fail to grow and develop colonies on the media they are normally cultured on, but their metabolic activity capabilities indicate that they are still alive (98). Several reviews of this dormancy state have been published (99, 100, 101, 102, 103, 104, 105, 106, 107, 108), and the list of bacteria known to enter this state is constantly growing. We have also employed diffusion chambers to demonstrate that this phenomenon occurs not only in the lab, but also *in situ* in estuarine waters (105). We have also found cells of *V. vulnificus*, when present in the VBNC state, to be more resistant to a variety of potentially lethal environmental factors than are the culturable cells of this pathogen (109).

**INCREASING INCIDENCE DUE TO GLOBAL WARMING**

Recent dramatic rises in seawater temperature may well lead to significant increases in the geographic distribution of estuarine *Vibrio* spp., and thus, a likely increase in the incidence of infection (110, 111, 112, 113). Increases in *Vibrio* infections have already been noted in northwest Spain and the Baltic Sea (114), as well as in Israel (115) and New Caledonia (116), with correlations made to global warming. In the United States, while food-borne *Vibrio* infections are increasing, this does not appear to be due to global climate change. However, wound infections caused by *V. vulnificus* are increasing worldwide, including in the US (19), and in these cases, global warming and the spread of vibrios may be a factor. It also cannot be excluded that strains of *V. vulnificus*, as well as other pathogens in this genus (e.g., *V. parahaemolyticus*), may be exhibiting increased virulence (117, 118). Significantly more study is needed to elucidate the factors causing this rise.

**CONCLUSIONS**

*V. vulnificus* is an exceptional bacterial pathogen, carrying the highest case-fatality rate of any food-borne...
pathogen, and causing extremely rapid infections in those consuming raw oysters. Of interest is its gender specificity, its predisposition for those over the age of 40 years, and the need for underlying disease in its victims; liver cirrhosis is an especially dangerous predisposing factor. Added to this is its ability to cause potentially fatal wound infections. While the virulence factors essential to the disease are little understood, we now realize there are more than one biotype and genotype, and this should prove of value in deciphering its ecology. Temperature plays a critical role in its occurrence and distribution, including in its entrance into the viable but nonculturable state. Its incidence, both in the US and worldwide, is increasing and global warming is undoubtedly a factor in its worldwide spread. Along with temperature, salinity is a determining factor in its ecology, and extreme weather events, such as droughts, have proven to at least temporarily eliminate this pathogen from regions stricken with high salinities. Recent genome sequencing is likely to help us understand both its pathogenesis and ecology and the near future promises to be a most exciting time in this study of this fascinating bacterium.

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REFERENCES


