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Brett Froelich & James D. Oliver

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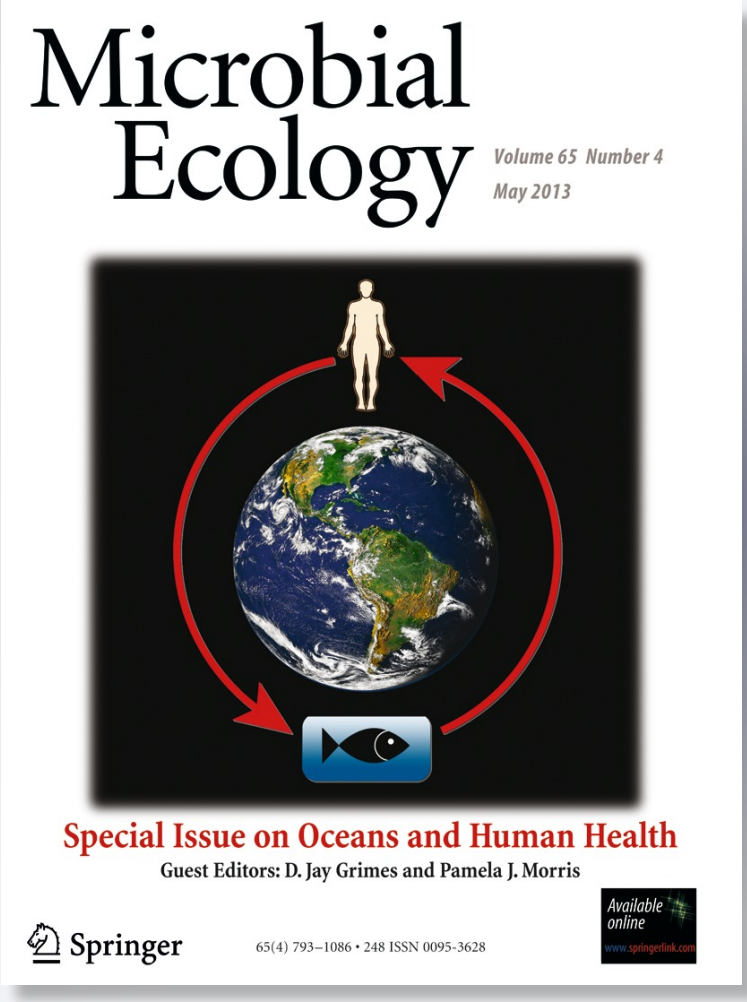

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MINIREVIEWS

The Interactions of *Vibrio vulnificus* and the Oyster *Crassostrea virginica*

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Abstract The human bacterial pathogen, *Vibrio vulnificus*, is found in brackish waters and is concentrated by filter-feeding molluscan shellfish, especially oysters, which inhabit those waters. Ingestion of raw or undercooked oysters containing virulent strains of *V. vulnificus* can result in rapid septicemia and death in 50 % of victims. This review summarizes the current knowledge of the environmental interactions between these two organisms, including the effects of salinity and temperature on colonization, uptake, and depuration rates of various phenotypes and genotypes of the bacterium, and host–microbe immunological interactions.

Introduction

Vibrio vulnificus is a Gram-negative, halophilic bacterium capable of causing gastroenteritis, wound infections, and fatal septicemia in humans [1–3]. This organism is routinely found in waters of estuarine environments as part of the normal microflora, as well as in oysters and other shellfish inhabiting those estuaries [3]. Wound infections caused by *V. vulnificus* are usually the result of exposure of an open wound to seawater containing the bacterium and can progress to necrotizing fasciitis [4, 5]. Mortality in wound infection cases has been previously reported at 24 % [5]. Even more striking, *V. vulnificus* infection is the leading cause of seafood-borne deaths in the USA, usually resulting from the consumption of raw or undercooked oysters [3]. Infections caused by ingesting oysters harboring *V. vulnificus* commonly result in primary septicemia, almost always require hospitalization, and have a fatality rate greater than 50 % [3, 6]. Thus *V. vulnificus*–shellfish interactions are important to consider when developing strategies to reduce infection risk. While causing rapid and fatal infections, most often *V. vulnificus* opportunistically targets those individuals with underlying diseases that make them more susceptible to this organism. These can include liver diseases (such as cirrhosis) or immune dysfunction (e.g., diabetes). A full review on the pathogenesis of this bacterium has been conducted by Jones and Oliver [4].

V. vulnificus exhibits a great deal of genotypic and phenotypic variation [3]. The species is divided into three biotypes, all of which are able to cause human infection, but biotype 1 is of greatest import to oyster producers and consumers [2, 7]. Biotype 2 strains routinely infect eels, especially those grown in aquaculture, while biotype 3 strains have only been isolated in Israel in association with handling of tilapia [4, 7–9].

Biotype 1 strains of *V. vulnificus* have been further divided into two genotypes, a difference discovered by RAPD-PCR analysis of strains from both clinical and environmental sources [10]. In this classification system, a gene identified as *vcg* (virulence correlated gene) was found to have two variations [11]. One variation correlates with strains obtained from clinical isolation, while the other variation is correlated with environmentally isolated strains [11, 12]. The gene has two alleles, *vcgC* and *vcgE*, representing clinical and environmental strains, respectively. Separation of clinical and environmental strains has also been performed by comparison of 16s rDNA sequences. This technique classifies environmental isolates as “A-type” and clinical isolates as “B-type” [13]. The *vcg* and rRNA

B. Froelich (✉)
The University of North Carolina at Chapel Hill,
Institute of Marine Sciences,
3431 Arendell Street,
Morehead City, NC 28557, USA
e-mail: bafroeli@unc.edu

J. D. Oliver
The University of North Carolina at Charlotte, Charlotte, NC, USA

methods agree on most strains, though strains exist that have conflicting classifications. Until recently, only two strains of *V. vulnificus* had been sequenced, both C-genotype strains of clinical origin [14, 15]. More recent studies and sequencing projects have added many more strains, including those of the E-genotype, revealing the incredible diversity of this species [16–18].

The Eastern oyster, *Crassostrea virginica*, used as a food source for thousands of years, survives in a wide array of habitat conditions but prefers salinities between 5 ‰ and 40 ‰ and temperatures from 20 °C to 30 °C [19–21]. This species of oyster is found naturally along the western Atlantic Ocean from the Canadian Maritime Provinces down to the Gulf of Mexico, Panama, and the Caribbean Islands [21]. *C. virginica* pumps water with a remarkable filtration rate that allows oysters to concentrate *Vibrio* spp., reportedly to levels as great as 6×10^4 CFU/g, from surrounding waters containing only 7 CFU/ml [22], making oysters an important reservoir for *V. vulnificus.* The interactions between these two organisms are complex and still largely unknown. Even oysters collected from the same location at the same time can have widely varying *V. vulnificus* concentrations [23]. While the study of *V. vulnificus* alone is, at times, difficult due to the large amount of phenotypic and genetic variation from strain to strain, the addition of a second organism with even greater diversity further complicates the matter.

Populations and Seasonality

V. vulnificus Population Dynamics in Oysters

Studies separating the natural *V. vulnificus* populations present in *C. virginica* by genotype, or other similar classification, agree that environmental strains of the species outnumber the clinical strains [13, 24–27]. Environmental (E-type) strains can range from 50 % to almost 85 % of the total *V. vulnificus* population in oysters, depending on the season [24, 26, 28]. A similar disparity between the two genotypes has been reported for the Pacific oyster, *Crassostrea gigas* [29]. This unequal distribution could reflect the population differences of the bacteria in the water, or could potentially reflect a selective advantage of environmental type strains as a result of differential uptake by, or survival within, oysters [26]. A study of the uptake of laboratory-grown C- and E-type bacteria showed little selective advantage of one type of *V. vulnificus* in oysters to the populations in the waters surrounding those oysters [30]. However, a report by Warner and Oliver found that the water column had a nearly even mix of C- and E-genotypes while oysters were predominated by E-types, and research by Høi et al. showed the same phenomenon, albeit in mussels [26, 31]. Such studies suggest that oyster bacterial populations are not directly dependent on the bacterial abundance or types present in the surrounding waters. This independence possibly explains why there can be oysters with high concentrations of *V. vulnificus* adjacent to oysters with almost none. It might also explain why there are individual oysters, within a single clutch, that can contain a greater proportion of clinical types to environmental types [24–26].

Seasonality of *V. vulnificus* in Oysters

It is generally accepted that *V. vulnificus* populations fluctuate seasonally, regardless of their environment (e.g., shellfish or estuarine waters). Multiple studies have shown that oysters harvested from the summer months have a greater likelihood of containing *V. vulnificus* cells, and at higher concentrations, than oysters from the winter months [21, 24, 26, 28, 32–36]. Studies that have separated *V. vulnificus* into the clinical and environmental subtypes concur that the clinical type strains show a greater seasonal shift than the environmental type strains [24, 26, 33]. This appears to be true regardless of the method used to determine strain type [24, 26, 33]. One study designed to observe *V. vulnificus* diversity in oysters found that, during summer months, there was a major shift in structure and intraspecific diversity in the *V. vulnificus* populations [28]. Such studies suggest there is a population of *V. vulnificus* that is more suited for oyster colonization, but that the summer season can reduce this advantage and allow the less capable populations to proliferate. Thus, the question arises, “are the higher numbers of oyster-associated *V. vulnificus* diseases in the summer months [37, 38] due to increased concentrations of *V. vulnificus* in oyster meats or an increased percentage of infectious strains?”

The Effects of Temperature on Oyster *V. vulnificus* Populations

The fact that warmer water temperatures are associated with increased *V. vulnificus* concentrations in oysters has been well established, and temperature may account for as high as 50 % of *V. vulnificus* density variability [26, 28, 31, 32, 35, 36, 39–44]. The lowest range of temperatures for finding culturable *V. vulnificus* in oysters generally ranges from 12 °C to 17 °C, and the density of the population increases as temperatures increase, with no maximum temperature being observed in estuarine environments [35, 36, 39, 41, 42, 45]. There have been studies where no correlation with temperature was found [34, 46], but these observations occurred in tropical climates where seasonal temperature changes are not as dramatic as they are in temperate climes [34]. In fact, the effect of temperature may not be visible in these tropical

waters if temperatures are consistently above 26 °C, as reported in India by Parvathi et al. [34]. Similarly, Motes et al. [41] found that *V. vulnificus* populations exhibited no additional increase above this temperature.

The lower temperature limit for *V. vulnificus* in oysters varies considerably from report to report. Tamplin et al. [35], as well as Tilton and Ryan [36], found no *V. vulnificus* below 17 °C, while Fukushima and Seki [39] and O'Neill et al. [45] suggest 15 °C as the minimum temperature. An extensive study by Randa et al. [42] found, in vivo, that the lowest temperature of *V. vulnificus* recovery was 12 °C, while the lowest in vitro temperature was 13 °C. In contrast, Wright et al. [47] reported relatively high numbers of *V. vulnificus* in oysters harvested from the Chesapeake Bay area when water temperatures were as low as 7.6 °C and suggested that the bacteria were adapting to the colder climate of this area. However, studies from regions farther north, such as New Jersey, New York, New Hampshire, and Maine, do not support this explanation, and unusually high numbers may be due to other factors [36, 42, 45].

Temperature is not only a major factor in the distribution of *V. vulnificus* in the environment, but appears to affect the rate at which *V. vulnificus* is depurated from oysters. At very cold temperatures, the bacteria may have sharply stunted replication rates, yet depuration has little effect on the density of *V. vulnificus* as oysters slow their pumping activity [48, 49]. Kelly and Dinuzzo [48] reported that slightly cool temperatures allow oysters to increase pump rates, causing a net decrease in the density of *V. vulnificus* contained within their tissues. Warm temperatures usually show no change in *V. vulnificus* numbers as replication and depuration rates become balanced, and sufficiently high temperatures can actually bring about an increase in the number of *V. vulnificus* cells within the oyster, with as many as 10^5 *V. vulnificus* cells being released per hour per oyster, which may act as a source for the pathogen during hotter weather [48, 50, 51].

The Effects of Salinity on Oyster *V. vulnificus* Populations

V. vulnificus is an obligate but moderate halophile which has only been recovered from water with a salinity of at least 5 ‰ but never from the open ocean [3]. Conclusions on the role of salinity on the density of *V. vulnificus* populations within oysters are mixed, having been reported as a positive correlation, negative correlation, or no correlation [28, 32, 34, 35, 42, 46, 47]. Zimmerman et al. [52] stated for *Vibrio parahaemolyticus* that a non-linear relationship between cell density and salinity would not be identified if the variation in salinity lies only on one side of the optimum salinity level, and the same may apply for *V. vulnificus*. If studies were conducted, or samples collected, in a salinity range that is too narrow, it would likely disguise such correlations [32, 52]. Parvathi et al. [34] and Johnson et al. [32] sampled in salinities ranging from <3 ‰ to >30 ‰, and both found a correlation between salinity and *V. vulnificus* density in oysters. On the other hand, Lin et al. [28] sampled oysters in salinities ranging from 5 ‰ to 25 ‰ and found no correlation, though the authors pointed out that this span was completely within the non-limiting range for *V. vulnificus*. Furthermore, two other teams found a correlation in *V. vulnificus* densities in water, but not in the oysters inhabiting those waters, when the salinity in these environments ranged from 0 ‰ to >30 ‰ [32, 35]. As such ranges should be wide enough to detect differences in *V. vulnificus* population changes if they existed, it is possible that other factors such as temperature, dissolved oxygen, or pH interact with salinity to make direct salinity correlations difficult. Evidence of this is discussed in the following section.

While moderate salinity appears to be the most permissive for *V. vulnificus* populations in *C. virginica*, some interesting phenomena occur at the extreme ends of the range. Reports of high numbers of recovered *V. vulnificus* at low salinities were detailed by Fukushima and Seki as well as Parvathi et al. [34, 39]. Both groups found *V. vulnificus* in oysters from brackish water (~6 ‰) as well as at very low salinity (<2.6 ‰), with the study by Parvathi et al. reporting the maximum density of cells, during a monsoon season in India, being at salinities close to 2 ‰ [34, 39].

Reports regarding high salinities (~25 ‰ or greater) agree that such conditions have an inhibitory or detrimental effect on *V. vulnificus* populations in oysters. Motes and his coworkers noticed in their *V. vulnificus* studies that they obtained much lower concentrations in years coinciding with unusually high salinity [41]. Parvathi found that at 25 ‰, *V. vulnificus* numbers decreased and disappeared completely above 30 ‰ [34]. When oysters harvested from one location, salinities ranging from 15 ‰ to 25 ‰, were relayed to another location with higher salinity, ranging from 32 ‰ to 35.3‰, *V. vulnificus* counts were reduced from as high as 14,000 CFU/g to less than 10 CFU/g [53]. Such a reduction is far better than is traditionally achieved by conventional depuration in moderate salinity waters (detailed below) and appears to significantly reduce the natural populations of *V. vulnificus* which are notoriously difficult to depurate. We found that oysters experiencing long durations of elevated salinity, even at levels less than the non-permissive limit of 25‰, show dramatically reduced *V. vulnificus* levels, even many months after the salinity of the surrounding waters returned to a permissive level for this pathogen [16]. It appears that extreme salinity events near the upper regions of the limit actually cause death or perhaps depuration of *V. vulnificus* from *C. virginica* and not simply a retardation of growth.

Interactions of Salinity and Temperature

While there is some agreement that temperature can affect how *V. vulnificus* responds to different salinities, there is no consensus on what that effect is. Randa et al. published that *V. vulnificus* densities were greater in salinities above 15 ‰ when the temperature was greater than 22 °C, and this was even more pronounced at or above 30 °C [42]. The opposite effect was claimed by Fukushima and Seki, who state that lower temperatures increase the tolerance to higher salinities [39]. Both of these publications cite as evidence an earlier work by Kaspar and Tamplin [40], which reported that when temperatures were above 22 °C, *V. vulnificus* levels remained unchanged or only dropped slightly, while at 14 °C, *V. vulnificus* populations encountering higher salinities exhibited sparser densities, supporting Randa et al. [40, 42]. However, Kaspar and Tamplin [40] also showed that *V. vulnificus* numbers were higher at lower temperatures over a broader range of salinities, giving support to Fukushima and Seki [39, 40]. The extent to which temperature affects the ability of *V. vulnificus* to survive at various salinities remains unclear, but is likely influenced by other environmental factors often not documented, or even spatial or temporal differences between the studies. A multi-year study of the effects of salinity and temperature on *V. vulnificus* loads in the Neuse River Estuary of eastern North Carolina is currently being conducted. While the findings may be specific to the region, the salinities in this study range from completely fresh (zero parts per thousand) to full strength seawater (>31 parts per thousand). After examining the effects of salinity and temperature on the distribution of over 1,500 *V. vulnificus* isolates, confirmed via PCR targeting species-specific genes, we found them to have a salinity optimum of 12–14 ‰, with infrequent isolation below 2 ‰ or above 24 ‰, and an optimum temperature range of 28–30 °C, with no isolation below 14 °C or above 33 °C regardless of salinity (Taylor et al., in preparation).

Uptake and Depuration of *V. vulnificus* in *C. virginica*

As a filter feeder, *C. virginica* pumps water through its gills, straining food particles from the flow [54]. Incredibly, *C. virginica* is able to pump water at a rate of 10 $Lh^{-1}g^{-1}$ dry tissue weight [54]. Depuration is the process where filter feeders are placed into clean water to purge bacteria over time [48, 55]. Experiments in which oysters were placed into water inoculated with cultured *V. vulnificus* bacteria typically result in reports that the oysters take up the cells quite rapidly, often within a few hours. However, such laboratory-grown bacteria are quickly flushed once the oysters are placed into clean water, often in less than 72 h [30, 55–57]. Two studies describe depuration of lab-grown bacteria taking longer, with one reporting 6 days and another 2 weeks [58, 59]. Those studies may be flawed in that, differing from other studies of this type, these researchers treated oysters with a strain of *V. vulnificus* that was indistinguishable from the background microflora, whereas most studies employ "marked" strains of *V. vulnificus* so that uptake rates of only the bacteria added by the investigators are measured. Such marked cells contain (e.g.) antibiotic resistance, alkaline phosphatase activity, or natural luminescence to distinguish them from the background natural flora [60–63]. In the two studies that did not use marked laboratory strains, the oysters sampled prior to bacterial exposure may have appeared to be free of *V. vulnificus*, but it is conceivable that the bacteria were dormant, or "viable but non-culturable" (VBNC) [64]. Cells in the VBNC state cannot be grown on their routine media but still retain viability [3]. *V. vulnificus* cells that enter into this state can return to a culturable, actively metabolizing, state in a process termed "resuscitation" [3]. Thus, cells existing in the VBNC state would not be counted at the beginning of these experiments, but if these endogenous cells resuscitated upon the addition of exogenous bacteria (see below), the researchers may have actually been enumerating re-emerging natural flora rather than the added strains.

It has been suggested that laboratory-acquired vibrios are purged quickly from experimental oysters because the bacteria become trapped in the feces of the oyster and are rapidly passed through the digestive tract [65]. Regardless, close associations of vibrios with the oyster's hepatopancreas cells could allow colonization within those cells, creating "persistently infected shellfish" [65–68]. The constant presence of *V. vulnificus* in surrounding waters could allow these closer associations with the intestinal tissues, but these processes would likely occur quite slowly and would not be observed in laboratory experiments where uptake of the *V. vulnificus* strains occurs only for a short time. This could help explain why oysters depurate laboratory-introduced *V. vulnificus* cells more easily than naturally acquired cells [65]. Conversely, neither incidence nor loads of *V. vulnificus* were shown by Sokolova et al. to increase with oyster age, suggesting there are yet undiscovered factors involved in the depuration process [69].

While the in vitro uptake of laboratory-grown cultures of *V. vulnificus* is very different from the in situ uptake of natural bacterial populations, several factors that affect uptake or depuration rates in the lab may also affect populations in the environment. The role of pili in oyster uptake has been examined primarily by two laboratories, with conflicting conclusions. Paranjpye et al. used *V. vulnificus* strains that were pili deficient (mutations in either the *pilA* or *pilD* genes) and found that these strains were taken up by oysters with the same efficiency as the wild type strain [70]. In contrast, when Srivastava et al. performed a similar

experiment using the *pilA* mutant, they reported a reduction in uptake in whole oyster and hemolymph samples compared to the wild type strain [56]. They did not see this difference in gill or digestive tissue, and concluded that *pilA* is important for oyster uptake but not for dissemination to the tissues [56]. This same study examined both non-motile and rugose strains, the latter being copious biofilm producers as well as being non-motile, and concluded that while motility was not involved in uptake, rugose *V. vulnificus* cells did show decreased uptake in whole oysters, gills, and hemolymph but not digestive tissue [56, 71]. This experiment also used a novel technique in which oysters were treated with tetracycline to reduce background levels of bacteria. While allowing for better observation of bacterial uptake in oysters, this method may have also eliminated competing bacteria, resulting in unnaturally high uptake rates [56]. Pili have been recently indicated as indicators for determining which *V. vulnificus* strains pose human health risks [72, 73]. As pili play important roles in both oyster colonization and human infectivity, this should be an area of focus for further research. Complicating matters further in oyster uptake experiments is that *V. vulnificus* is not likely to be found in natural waters as high numbers of planktonic cells, but rather associated with particles.

Oysters have the ability to select food particles based on size, with the gills acting as a sieve. Particles of the optimum size are caught and shunted toward the oyster mouth, while particles too small to be selected pass through the gills and are excreted from the oyster. The optimum particle size in *C. virginica* is between 5 and 7 μm in diameter, with particles of this size having a 90 % retention rate [74]. This rate drops to only 16 % for particles the size of a single *V. vulnificus* cell [74]. Thus, traditional bacterial uptake experiments in oysters are likely inefficient and are poor models of the natural environmental process. We found that *V. vulnificus* cells that were attached to marine aggregates exhibited significantly greater uptake than free-living cells (submitted), and this type of uptake study is likely a much better mimic of what occurs in situ.

Many studies have been conducted to determine what factors affect the rate of depuration of *V. vulnificus* by *C. virginica*. Most agree that laboratory-grown bacterial strains exhibit rapid depuration from oysters while there is a persistent, depuration-resistant, natural microflora [30, 55, 57]. A study reported by Lewis et al. used a unique flow-through depuration system and concluded that this type of depuration employing rapid (60 L/min.) flow was effective at reducing *V. vulnificus* numbers in oysters [59]. However, this experiment employed water that was at >30 ‰ salinity, and a similar experiment using salinity within the preferred range of *V. vulnificus* actually showed an increase of natural *V. vulnificus* cells [59]. Depurating oysters in water near or above 30 ‰ has been shown to be effective in reducing the persistent, naturally present *V. vulnificus* cells [16, 53], and the rapid flow may not have been a significant factor.

Two studies have concluded the clinical variant of *V. vulnificus* is depurated at the same rate as the environmental variant, although Lewis et al. concluded this even after finding more of the environmental (Type-A) strains before depuration and more of the clinically associated (Type-B) strains after depuration in their experiments [30, 59]. Our laboratory found that "pre-depuration," i.e., allowing oysters to depurate before the addition of laboratory-grown strains, had no effect on subsequent depuration rates of the introduced bacteria [55].

Despite the benefits of an antiphagocytic capsule, some strains of *V. vulnificus* exhibit a reduction in capsule expression [4]. Strains with full capsule production are termed "opaque," and those with reduced or without a capsule are referred to as "translucent" [4]. Opacity is phase variable, and strains can revert to either morphology [4]. Groubert et al. and Srivastava et al. both found that opaque and translucent strains have similar uptake and depuration rates [55, 56]. The latter study also examined a translucent strain that had lost the ability to revert to the opaque phenotype and reported significantly ($p<.05$) lower concentrations of this phenotype from oysters that were treated with exogenous bacteria. Whereas oysters infected with the opaque strain retained 3.2 log CFU *V. vulnificus* cells per gram of oyster tissue, the non-encapsulated form was reduced to 2.8 log CFU/g oyster tissue [56].

The role of pilus-based attachment was determined to be important in oyster colonization by Paranjpye et al. [70]. In contrast with the effects on uptake, pilus mutants (*pilA* and *pilD*) were depurated more rapidly than the wild type, a phenomenon that was confirmed by Srivastava et al. [56]. The latter authors additionally reported that the rugose phenotype of *V. vulnificus* exhibited a significant increase in depuration rate [56, 70]. It was proposed by Paranjpye et al. that *V. vulnificus* pili may bind specifically to carbohydrate-containing receptors on oyster cells [70].

The Response of Endogenous Bacterial Populations to Exogenous Bacteria in Oyster Uptake Experiments

We recently observed that when oysters with low numbers of culturable vibrios were inoculated with *V. vulnificus*, a greater number of culturable cells than were added would rapidly emerge from the oysters (submitted). One hypothesis is that the concentration of *Vibrio* bacteria in these oysters was so low that it could not be detected, but that the addition of exogenous bacteria caused these cells to rapidly divide and become detectable. The previously existing, oyster-adapted, *Vibrio* population may be important in preventing colonization by exogenous cells, explaining the

rapid depuration of these added bacteria. An alternate hypothesis is that the endogenous vibrios had been present in a nonculturable/dormant state, but resuscitated in response to the exogenous addition of culturable bacteria. This phenomenon occurred whether *V. vulnificus* or *Escherichia coli* was added to the oysters. A similar phenomenon has been documented by other researchers as well. Groubert et al. documented (but did not discuss) nearly the same results, using strains similar to those we employed [55]. Murphree and Tamplin [75] saw an increase of *V. vulnificus* cells in oysters that were inoculated with *Vibrio cholerae*, and Srivastava et al. [56] described an effect whereby the addition of *V. vulnificus* caused an increase in total bacteria in the oysters. This effect was not seen by Paranjpye et al., but their study examined total aerobic bacteria, and not *V. vulnificus* specifically [70]. Whether this phenomenon is a result of direct cell contact or a product of dividing cells is currently under investigation.

Fate of Bacteria Inside the Oyster

It is known that *V. vulnificus* does not exist only on oyster surfaces, but within tissues as well [50, 69, 76]. For example, Sun and Oliver found that over 95 % of oyster-associated *V. vulnificus* cells are within tissues rather than on meat surfaces [76]. Environmental *V. vulnificus* cells that have been taken up by *C. virginica* appear to concentrate primarily in the digestive gland, followed by the adductor muscle, then the mantle and gills [30, 50, 56, 65, 66]. A study by Aldrich et al. that utilized immunoelectron microscopy, however, asserted that free-living bacteria are not found in the adductor muscle, but are contained specifically in hemocytes within the adductor muscle [77]. Interestingly, as oyster size increases, the concentration of *V. vulnificus* inside the oyster decreases, possibly due to a decrease in tissue surface area as compared to volume [69].

As noted above, *V. vulnificus* cells with various degrees of encapsulation (opaque and translucent strains) have been employed in several uptake/depuration studies. Phase variation of these cells, while inside the oyster, from the translucent phenotype to the opaque phenotype was not observed by Groubert and Oliver [55] but was reported by Srivastava et al. [56]. They reported that the rate at which opaque cells phase shifted to the translucent phenotype was unaffected by oyster passage [55].

The ratio of clinical (C-genotype) strains to environmental (E-genotype) strains does not shift in natural oysters according to one study [25], nor is virulence affected by oyster passage [55]. However, it should be noted that, in these studies, virulence was only examined in laboratory-grown bacteria; natural populations have not been investigated.

Oyster Immune System and *V. vulnificus*

Oysters remove bacteria from the environment using phagocytic cells, known as hemocytes, present in the hemolymph [77]. This process begins with bacteria being phagocytized by hemocytes in the hepatopancreas/digestive gland and digested in the lysosome of the hemocyte, with residual material being discharged into the periphery of gills, muscle, and mantle tissues [77, 78]. Fisher termed this process "diapedesis" [78].

Oyster hemocytes are multifunctional, serving defensive, nutrition, excretion, repair, and digestive roles [78, 79]. Agglutinins traditionally increase phagocytosis by aggregating bacteria, but this process was not found to occur with *V. vulnificus* in oyster hemolymph [79, 80]. The oyster hemocytes ingest the *V. vulnificus* cells independently of bacterial contact with humoral factors [51, 81]. Multiple studies have shown that the encapsulated phenotype of *V. vulnificus* is more resistant to phagocytosis than the less or non-encapsulated forms, and it is suggested that encapsulation also allows for resistance to degradation and the ability for this pathogen to survive within the hemocyte [51, 79, 82]. No differences in the amount of hemocyte lysozymal or acid phosphatase activity were seen following ingestion of encapsulated vs. non-encapsulated strains [82].

Another explanation for *V. vulnificus* persistence inside these phagocytic cells is the inability of some hemocytes to kill *V. vulnificus*. While granular hemocytes are quite lethal to *V. vulnificus*, agranular hemocytes are not, possibly due to the lack of the lysosomal enzymes responsible for bacterial degradation [82–84]. In the summer, the percentage of agranular hemocytes in oysters is greater than granular hemocytes, possibly contributing to the increase of *V. vulnificus* seen within oysters in the summer months [82, 85]. This would appear, however, to be in contrast to the observation that increased temperatures lead to increased phagocytosis [51, 83]. Nevertheless, the increased growth rates of *V. vulnificus* in the water column during warmer summer weather must not be discounted when considering bacterial abundance in oyster matrices.

Oliver found that *V. vulnificus* incubated in the presence of homogenized oysters exhibited a rapid decline at low temperatures and suggested the presence of antimicrobials within the oyster tissues [86]. Pelon et al. showed that an oyster extract component was lethal to *V. vulnificus* cells [87], and Seo et al. extracted a protein from *C. virginica* that was able to inhibit *V. vulnificus* [88]. Because these studies used similar extraction techniques, it is likely that both teams were reporting on the same antimicrobial polypeptide, a protein that Seo and team have named "American oyster defensin." They suggested this might be the basis of the mechanism for the anti-*V. vulnificus* properties of oyster hemocytes [87–89]. More recently, additional proteins with

inhibitory effects on *V. vulnificus* have been discovered. Seo et al. described histone 2B proteins with strong activity on *V. vulnificus* and speculated that the significant concentrations of these proteins inside oyster tissues might be involved in regulating the prevalence of *V. vulnificus* in oysters [90]. The recent availability of the *C. gigas* genome combined with the existing knowledge of the Pacific oyster defensins could rapidly provide some insights into the *C. virginica* antimicrobial peptide, a potential target for oyster decontamination techniques [91, 92].

Pollution and *V. vulnificus* in Oysters

It has been reported that the numbers of *V. vulnificus* in oysters do not correlate to contaminants or pollution [59, 93]. A recent study, however, reported that oysters exposed to a cadmium-hypoxia double challenge had increased *V. vulnificus* concentrations as compared to controls [94]. *Vibrio* species are known to be tolerant to heavy metals, including cadmium, in comparison to other bacteria [95–99]. Oyster hemocytes, and subsequently their phagocytic and cytotoxic abilities, are disturbed by cadmium and other pollutants [100–105]. The disruption of these functions could conceivably allow for more rapid *V. vulnificus* growth within oyster matrices.

Conclusion

The interplay between oysters and *V. vulnificus* is sufficiently complex that while we have a general understanding of such interactions, there remain numerous unanswered questions. Researchers conducting culture-based bacterial uptake experiments in oysters commonly find that the cells are rapidly taken up, but are not incorporated into the microflora and are rapidly eliminated. It is likely that part of the reason for this is the common use of free-living (planktonic) bacterial cells in these experiments. The cells are simply too small for efficient uptake by oyster gills, and therefore, the exogenously added cells are only transiently present within the oysters. A further complication is that oyster-adapted, natural bacterial populations occupy all of the colonizable space available in the oyster gut, thus outcompeting the incoming cells. Because oysters harbor (often large) natural *V. vulnificus* populations, laboratory experiments involving exposure to water spiked with bacteria can be problematic, but the trend towards the use of molecular, rather than culture, based techniques is likely to prove useful in filling these knowledge gaps. Moreover, advances in genomic sequence data collection and analysis can allow us to examine the metagenome of oysters and thus delve deeper into the *V. vulnificus*–oyster interactions. As we continue to discover the biological workings that govern bacterial colonization, oyster immunology, and even location-specific interactions between oysters and their natural microflora, we can gain greater insight into *V. vulnificus* physiology and in the process keep seafood safer for human consumption.

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