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MINIREVIEW

Factors Affecting the Uptake and Retention of *Vibrio vulnificus* in Oysters

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***Vibrio vulnificus*, a bacterium ubiquitous in oysters and coastal water, is capable of causing ailments ranging from gastroenteritis to grievous wound infections or septicemia. The uptake of these bacteria into oysters is often examined *in vitro* by placing oysters in seawater amended with *V. vulnificus*. Multiple teams have obtained similar results in studies where laboratory-grown bacteria were observed to be rapidly taken up by oysters but quickly eliminated. This technique, along with suggested modifications, is reviewed here. In contrast, the natural microflora within oysters is notoriously difficult to eliminate via depuration. The reason for the transiency of exogenous bacteria is that those bacteria are competitively excluded by the oyster's preexisting microflora. Evidence of this phenomenon is shown using *in vitro* oyster studies and a multiyear *in situ* case study. Depuration of the endogenous oyster bacteria occurs naturally and can also be artificially induced, but both of these events require extreme conditions, natural or otherwise, as explained here. Finally, the "viable but nonculturable" (VBNC) state of *Vibrio* is discussed. This bacterial torpor can easily be confused with a reduction in bacterial abundance, as bacteria in this state fail to grow on culture media. Thus, oysters collected from colder months may appear to be relatively free of *Vibrio* but in reality harbor VBNC cells that respond to exogenous bacteria and prevent colonization of oyster matrices. Bacterial-uptake experiments combined with studies involving cell-free spent media are detailed that demonstrate this occurrence, which could explain why the microbial community in oysters does not always mirror that of the surrounding water.**

Oysters are an important food source and can be prepared many ways but are often consumed live, raw, or undercooked. Unfortunately, undercooked and raw oysters are implicated as the predominant source of seafood-borne death in the United States, with an overwhelming (>95%) majority of these deaths caused by the bacterium *Vibrio vulnificus* (1–3). These bacteria are ubiquitous in estuarine and coastal environments, and one study of Louisiana restaurants found that the majority (67%) of raw and even some (25%) cooked oysters contained this pathogen (4). Infections caused by ingesting *V. vulnificus* can result in gastroenteritis with associated abdominal pain, diarrhea, and vomiting but have the potential to quickly progress to primary septicemia (2, 5). When this occurs, the infected patient can exhibit blistering skin lesions or organ failure, sometimes occurring as rapidly as within 24 h after exposure (2, 5). Even with aggressive medical treatment, death occurs more than 50% of the time, distinguishing *V. vulnificus* as having the highest case fatality rate of any food-borne pathogen (2, 6). For more-specific information on the pathogenesis of *V. vulnificus* and its interactions with oysters, please see the reviews by Jones and Oliver (3) and Froelich and Oliver (7), respectively.

Because of the grievous nature of these infections and the speed with which they manifest, significant research effort is devoted to better understanding the role of oysters in the tripartite lifestyle of *V. vulnificus* as it moves from the water column to oyster tissues and, finally, into the human host. Some of this research is directed at simply understanding the mechanics of the underlying biological interactions that drive the incorporation of viable bacterial cells into oyster matrices. Other avenues of research deal with more directly applied science, such as the interest in postharvest processing of oysters in such a way that the bacteria are purged or inactivated, thus rendering the oyster less harmful for consumption.

There have been several methods that researchers have employed that allowed them to quantify bacterial uptake and elimination in oysters. The "core" method is described first, followed by examples of deviation from this formula. The core method, elegant in its simplicity, is to grow the bacterial strain of interest to the desired concentration and seed an aquarium with a specific concentration of those bacteria, while allowing oysters within that aquarium to bioaccumulate the cells (7–21). The use of this method often yields similar results in which the bacteria are rapidly and significantly taken up by the oysters but quickly depurated to minimal or nondetectable levels within a few days (11, 12, 19, 22–24). Similar results have been seen with other pathogenic and nonpathogenic *Vibrio* species and non-*Vibrio* species and in oysters and other types of shellfish. Examples include *V. parahaemolyticus* in clam species (*Ruditapes decussatus* and *R. philippinarum*) and oysters (*Crassostrea plicatula*, *C. gigas*, and *Tiostrea chilensis*), *V. cholerae* in mussels (*Mytilus galloprovincialis*) and oysters (*Crassostrea virginica*), and *Escherichia* in *C. virginica* and in *M. galloprovincialis* (8, 9, 13–15, 18, 21). The rapid elimination of these added bacteria is likely due to factors both biological and methodological. This transience of the added bacteria can be attributed partly to the presence of a competing, natural population of bacteria that had previously colonized the oysters (7, 11). These preexisting bacteria inhabit the available surface area within oyster tissues, preventing exogenous bacteria from establishing residency. Another explanation for the failure of exogenous bacteria to establish residency in oysters is that it might be due to how

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researchers attempt to add these bacteria to oyster aquaria. Both of these factors are discussed further here.

The natural *V. vulnificus* population in oysters can be as high as 6×10^4 CFU per gram of oyster tissue (25). At that concentration, it would take unnatural numbers of bacteria added to the oysters to visualize an increase in the population. Therefore, researchers often use oysters with reduced preexisting loads of *V. vulnificus*, which can be obtained in a variety of ways. The simplest technique is to begin with oysters that naturally contain few or no cells that would interfere with uptake, detection, and quantification. Oysters collected during the coldest months are often sufficient for bacterial-uptake experiments as there is a lower concentration of background *V. vulnificus* cells (9, 10, 19, 26–28). Other locations, such as in Europe, have oysters with naturally low bacterial concentrations year round (29). Experimental results obtained using this technique generally agree that exogenously added bacteria are rapidly taken up by the oysters but are still quickly depurated.

One alternative to using oysters with low concentrations of bacteria is the utilization of oysters that have been "predepurated," i.e., allowed to bathe in bacterium-free water for a short time (11, 13). Even though exogenous bacteria usually rapidly depurate around 72 to 96 h after introduction, the endogenous bacteria are notoriously resistant to depuration and using this technique has not been shown to increase the uptake of laboratory-grown bacteria (9, 11, 23, 28, 30). Allowing the oysters to bathe in high (>30‰)-salinity water before experimentation has proven successful in reducing the resident bacterial levels before beginning bioaccumulation experiments (8, 17). Further information on the effects of water with elevated salinity on oyster *V. vulnificus* populations is presented below.

A more radical method of clearing oysters for later use in uptake experiments involved the novel approach of bathing the oysters in the antibiotic tetracycline, followed by a soak in tanks with charcoal filters to clear the antibiotic, reducing the native *V. vulnificus* population of summer oysters from ~1,000 cells per gram to fewer than 10 (19). No differences between winter oysters and antibiotic-treated oysters in the levels of uptake were seen. However, the rapid decline normally observed in the bioaccumulated strains of bacteria was also not seen. This indicates that the added bacteria were establishing themselves in the oysters, a result not typically observed in oysters not treated with antibiotics. These experiments support the theory that an existing population of oyster-acclimated bacteria prevents colonization of exogenous cells, as the antibiotics successfully eliminated the preexisting bacteria, freeing surface area for the added strains to colonize.

While some researchers have used unmarked bacterial strains in oyster uptake experiments, most teams have in place some method of differentiating the laboratory-grown exogenously added cells from the naturally occurring resident population. These methods have included the use of *V. vulnificus* strains with specific antibiotic resistance, natural bioluminescence, radiolabeling, alkaline phosphatase activity, fluorescence, or a specific molecular marker (8, 13, 20–22, 31). These marked strains are critical for ensuring that the data collected on the uptake or depuration rates of the added cells are accurate and that factors such as outgrowth or a lingering population of similar bacteria are not confounding the results.

Oysters are filter feeders and have the remarkable ability to pump water through their gills at a rate of 10 liters per h per g of dry tissue weight, which might lead one to believe that the bacteria inside an oyster would mimic the bacterial population found in the surrounding water (7, 32). However, this has been repeatedly shown to not be the case. A study by Warner and Oliver (33) found that oysters harvested from Alligator Bay, NC, contained *V. vulnificus* cells that were predominately (>84%) of the E genotype, an allelic variant of *V. vulnificus* most often associated with environmental isolation. The water from which those oysters were collected had a near-even ratio of C-type cells, the allelic variant correlating with clinical sources, to E-type cells, providing evidence that the population of bacteria in the water does not necessarily reflect the population of bacteria found in shellfish inhabiting those waters. For further information on the C and E genotypes of *V. vulnificus*, we direct the reader to articles by Rosche et al. (34), Warner and Oliver (35), Chatzidaki-Livanis et al. (36), and Rosche et al. (37). In another example, after an extreme drought altered estuarine salinity levels to the point that *V. vulnificus* had all but disappeared from North Carolina water and oysters, the drought eased and salinity returned to normal, quickly followed by the reemergence of *V. vulnificus* in the water column (28, 38). Yet, in that study, which examined oysters of commercial size, ca. 2 years of age or older, *V. vulnificus* was still nondetectable, even when the oysters had, for many months, been filtering water that had abundant *V. vulnificus* cells (28). These reports demonstrate that it is not just the exogenous bacteria added in a laboratory setting that are blocked by resident cells but natural populations of environmental bacteria as well. If the bacteria within an oyster prevent further colonization by those cells taken up from the surrounding water, where then did the preexisting bacteria originate? It has been hypothesized that the microflora of oysters is established at the larval stage (K. Doyle and J. D. Oliver, unpublished data), as supported by experiments using bacterium-free oyster larvae. Further evidence comes from the oysters collected during the North Carolina drought. The oysters used in those studies were specifically chosen as they represented oysters of commercial age and size that were at least 2 years of age. Thus, we predicted that when *V. vulnificus* had returned to North Carolina estuaries but was not able to recolonize adult oysters, it would not be found in commercial sized oysters for at least 2 years. This appeared to be case when oyster sampling continued with still little recovery of *V. vulnificus* cells from North Carolina oysters in 2010 and 2011 and recovery increasing in 2012 (unpublished data).

If oysters have an established microflora that is resistant to depuration, then how was a new population of bacteria able to competitively displace the once-dominant *V. vulnificus* population during the drought? The answer is that elevated salinity appears to play an important role in oyster depuration and colonization with respect to the pathogenic *Vibrio* species. In areas where the salinity is typically permissible for *V. vulnificus*, brief spikes in salinity did not have any long-lasting or, in some cases, detectable effects on the abundance of *V. vulnificus* (28, 38–41). This is in agreement with experiments that highlight the tenacity of bacteria that are established as oyster flora. However, in locales where salinity is chronically above ~23‰ to ~25‰, such as the Mediterranean Sea, *V. vulnificus* is rarely found in oysters (42, 43). It has been seen, though, that when effects (e.g., drought or inundation) raise or lower the salinity for extended periods, oysters, in fact, have reduced or increased loads of *V. vulnificus*, respectively (27, 28, 44). Location appears to be irrelevant, as areas with little history of *V. vulnificus*, such as the Mediterranean Sea, and areas

with significant concentrations of these pathogens, such as the mid-Atlantic region of the eastern United States, both show the same effect (27, 42, 45, 46).

These examples are further validated by the case of the extreme North Carolina drought mentioned previously. After DNA sequencing was used to identify the bacteria that were newly inhabiting the oysters, it was found that bacteria that were relatively more salt tolerant than *V. vulnificus* had colonized the oysters and that these bacteria were preventing the *V. vulnificus* in the surrounding water from reestablishing residency, similarly to what occurs *in vitro* when researchers perform uptake experiments in oysters (28, 44). The same type of competition was observed in shellfish samples taken from the Mediterranean coast (43). In that harvest location, the salinity is consistently above that which would limit the growth or survival of *V. vulnificus*, and Macián et al. (43) also recovered relatively more salt-adapted vibrio cells than *V. vulnificus* cells.

Even artificial elevation of the salinity that the oysters are living in is capable of reducing the *V. vulnificus* concentration found in oysters many times over. This is evidenced by experiments relaying oysters from their original harvest sites to areas with much greater relative salinity. Motes and DePaola (24) took U.S. Gulf Coast oysters that originally contained $\sim 10^4$ (most probable number [MPN]) *V. vulnificus* per gram and relayed them to a site with full-strength (~32‰ salinity) seawater for at least a week. They found that *V. vulnificus* levels in most of the oysters had been reduced to <10 MPN/g and that longer periods of high-salinity exposure resulted in further reductions of the bacteria (24). A similar study performed in the Chesapeake Bay by Audemard et al. (47) reported equal success in their attempt to reduce *V. vulnificus* and *V. parahaemolyticus* loads by moving oysters to waters with salinity above 30‰. There have been modifications of these initial experiments used for continued research, including the use of flowthrough depuration systems at elevated salinities, and the future use of relay as an approved method for reducing *V. vulnificus* loads in oysters looks promising (48, 49).

While it seems evident that preexisting oyster-adapted bacteria are capable of preventing the colonization of oysters by exogenous bacteria, this leads to an interesting question: why do oysters collected during cold winter months, with apparently fewer cells, still exhibit the same resistance to being colonized by exogenous bacteria? One explanation requires discussion of a bacterial phenomenon termed the viable but nonculturable (VBNC) state. A comprehensive review of the VBNC state in bacteria was recently published by Li et al. (50), and we refer the reader to this document. Many bacteria, including *V. vulnificus*, enter the VBNC state in response to stress, such as low temperature (51–57). Cells in this state are defined as being alive, with viability confirmed by techniques that included the detection of uncompromised membranes or active RNA transcription, but are unable to be grown on the routine media normally employed for their culture (50, 58, 59). When cells become VBNC, they enter a state of reduced metabolic activity that has been shown to be protective against a variety of stressors, including heat, oxidation, osmotic challenges, and pH extremes (60, 61). Additionally, several toxins such as ethanol, antibiotics, or heavy metals have also exhibited reduced lethality (60, 62). The bacteria can exit this state, usually when the stressor that initiated the state has been removed, in a process termed "resuscitation," although there remain some bacteria in which the resuscitating factor is as yet unknown (50, 55, 56, 58, 63, 64).

Oysters harvested from winter months are often found to have reduced loads of *V. vulnificus* bacteria that in most cases are below the limit of detection, which makes them candidates for bacterial-uptake experimentation (22, 25, 27, 33, 65). As mentioned previously, though, experiments using these oysters still show rapid depuration of the laboratory-grown bacteria, even when the oysters appear to have reduced starting loads of microbes (66). A recent report proposes that these winter oysters are actually harboring VBNC bacteria that are not resuscitated by simply placing the oysters into warmer temperatures (65). These bacteria, even when nonculturable, could still be occupying the colonizable space within oyster matrices and preventing the exogenous cells from becoming resident. Evidence of this VBNC population is seen when addition of exogenous bacteria of one species causes the rapid and sustained appearance of a different species within oysters (11, 65). The cells appear to respond to the exogenous bacteria, including those added experimentally, by emerging from the VBNC state. A potential mechanism for this response has been found by Ayrapetyan et al. (67) where the utilization of cell-free spent media provided a similar response in the preexisting VBNC bacteria in oysters. This interaction appears to be mediated by the quorum-sensing molecule AI-2, which permits interspecies cell-to-cell communication (67–69). Thus, it seems that even when oysters appear to have relatively fewer bacteria, they are actually still heavily colonized with a population of microbes that is likely contributing to the transiency of bacteria taken in through biological filtration.

Another reason for the rapid depuration seen in oyster bioaccumulation experiments could be the methods employed for these tests. As stated previously, often the protocol used in this type of research is to add bacteria to a tank of water with the oysters to be investigated, allowing the oysters to filter the cells. This may not be the most efficient way to perform these types of experiments, however. Oysters select the particles they eat based on size, with the gills acting much like a sieve (32, 70). Particles that are too large are stopped and excreted as pseudofeces, while particles that are too small pass through the gills without capture. Ward and Shumway (70) found the optimum particle size for uptake by *C. virginica* to be between 5 and 7 μm in diameter, which is many times larger than the average planktonic *Vibrio* bacterium. Oysters would be expected to capture only ca. 16% of particles the size of *V. vulnificus*, which translates to loss of efficiency in oyster uptake experiments (70). To combat this loss, the bacteria can be incorporated with larger particles to increase uptake (70). This can be accomplished with the use of laboratory-generated marine aggregates, also known as marine snow. Marine snow in marine waters is formed naturally of phytoplankton, microbes, feces, larvacean houses, and inorganic materials that are aggregated via shear forces and Brownian motion (71, 72). These particles can be found to harbor *Vibrio* bacteria, even when there are no detectable *Vibrio* cells in the surrounding water, and are predicted to usually contain *Vibrio* pathogens (73, 74). Marine aggregates are notoriously fragile and difficult to capture from the environment (71, 75), although they can be formed *in vitro* with relative ease (76, 77). Kach and Ward (77) found that by allowing bacterial cells to integrate into these marine aggregates before the cells are fed to oysters, the ingestion rate of the cells increased significantly, as the bacteria had become part of a larger particle that can be captured with greater efficiency by oysters (77, 78). When this type of experiment was performed with *V. vulnificus*, known to associate

with aggregates, similar results were seen. The aggregate-integrated, exogenously added cells exhibited significantly increased uptake and slightly more retention in oysters than nonaggregated cells (75, 79). When different strains of *V. vulnificus* were grown in competitive coculture, aggregated, and fed to oysters, it was found that the less virulent E-genotype strains were more abundant in the oysters than the more virulent, C-genotype counterparts, even when starting with similar concentrations (79). This selectivity appeared to be a result of the difference in the rates of integration into these aggregates by these two clades of *V. vulnificus*, providing another possible mechanism to explain why the bacteria found in oysters often do not correlate with those in the surrounding water column.

The relationship between oysters and *V. vulnificus* is a complex one. It has often frustrated researchers that oysters harvested from the same clutch can often contain wildly differing concentrations and types of bacteria that simultaneously differ from the water that the oysters are actively filtering. There appear to be several layers of potential explanations, all of them likely working in concert to produce this effect. The oyster flora may not mimic that of their milieu because of various bacterial affinities for association into marine aggregates, which facilitate the uptake of bacteria by oysters. Furthermore, the bacteria that are captured by oysters encounter preexisting, oyster-adapted bacteria that prevent the exogenous cells from being anything but transient. Evidence suggests even that bacteria in a dormancy-like VBNC state react to incoming bacteria by resuscitation. These endogenous bacteria, which are normally quite resistant to depuration, can be displaced via extreme conditions. The best-studied condition is elevated salinity, which looks to be especially efficient at removing *V. vulnificus* cells, regardless of whether this change in salt concentration is caused naturally, such as under conditions of drought, or directly by relay or depuration with water of higher salinity. Even then, other vibrios fill the vacant space, preventing recolonization by *V. vulnificus*. While this could lead to reduced infections caused by that particular organism, the bacteria that now inhabit the oyster matrices could potentially present problems of their own.

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