The Viable but Nonculturable State for Bacteria: Status Update

This dormant form of bacteria was first appreciated in 1982; now skeptics recognize this state as a bacterial response to stress and a strategy for survival

James D. Oliver

The notion of microorganisms living in a viable but nonculturable (VBNC) state originates from studies by Rita Colwell at the University of Maryland and her collaborators, whose focus was on *Vibrio cholerae*. Here, I summarize the early history of this field as well as some of the later studies on the genetics of the VBNC state, the role of quorum sensing in resuscitating dormant cells, and the effect global climate change is having on the spread and incidence of pathogenic vibrios. Much of this material was discussed during the symposium, “Healthy Waters, Healthy People: A Tribute to Rita Colwell,” convened during the 2015 ASM General Meeting in New Orleans, La.

The VBNC state, now identified in numerous bacterial species, including important pathogens, as well as in some eukaryotes, is induced by a variety of environmental stresses. It is now widely believed that VBNC likely accounts for the seasonality of some bacteria in natural environments, and that these dormant cells resuscitate when the inducing stress is removed, possibly aided by the presence of other actively growing bacteria and the quorum sensing molecules that they produce. Further, VBNC cells typically are resistant to antibiotics as well other environmental stresses, allowing the dormant cells to persist despite these otherwise lethal stresses.

Despite greatly reduced metabolic activity, which allows dormant cells to persist, these cells continue to express genes and show other signs of activity. We are also beginning to understand how the VBNC state and resuscitation from it is regulated genetically. Of special concern from a public health perspective is that global warming appears to be spreading *Vibrio* spp. and human vibriosis, adding yet another important concern related to global warming. The VBNC state, first recognized by Colwell and her collaborators in 1982 and then applicable only to two bacterial species subsequently became a major area of research worldwide, leading to my collecting more than 600 publications on this topic during the course of several decades.

Defining the Viable but Nonculturable State

The viable but nonculturable state refers to a microbial cell that fails to grow on nutrient media on which it would normally grow and develop into a colony but is alive and can return to a metabolically active and culturable state under appropriate circumstances. A variety of environmental stresses, ranging from suboptimum growth temperatures to the presence of heavy metals to elevated osmotic concentrations, can induce cells into the VBNC state, with the cells becoming fully nonculturable (Fig. 1a).

However, a large percentage of the original

---

**SUMMARY**

- The viable but nonculturable (VBNC) state was recognized in 1982 by Rita Colwell of the University of Maryland and her collaborators.
- VBNC, now identified in numerous bacterial species, as well as in some eukaryotes, is induced by a variety of environmental stresses.
- Many different types of bacteria, including human pathogens, are capable of entering this state, maintaining cellular structure and biology, and continuing significant gene expression while otherwise being nonculturable by standard laboratory methods.
- Cells that enter the VBNC state eventually exit this state of dormancy to become fully culturable again, a shift called “resuscitation” that may be aided by quorum sensing signal molecules.
- Global climate change appears to be resuscitating dormant forms of *Vibrio*, leading *Vibrio*-related infections to increase worldwide.
population retains viability, demonstrating that culturability cannot be equated to viability. Indeed, while these cells exhibit greatly reduced but detectable metabolism, they can be resuscitated to a more metabolically active and culturable state. This phenomenon is not restricted to the laboratory, but can be seen among microbial cells in estuarine waters. It is most striking when measured across varying water temperatures (Fig. 1b), suggesting that seasonality for some microorganisms in natural environments is a result of the VBNC phenomenon.

Huai-Shu Xu (1936–2001) conducted the first VBNC studies between 1980 and 1982 in Colwell’s laboratory (Fig. 2). As pointed out in a tribute to him during the 2015 International Marine Microbiology Conference in China (http://immc2015.csp.escience.cn/dct/page/70002), “Professor Xu was one of the first Chinese scientists to visit the USA after the Cultural Revolution. During this visit at the University of Maryland during July 1980 to November 1982, he participated in marine microbiological research, and was instrumental in recognizing the viable but nonculturable (VBNC) state of Vibrio cholerae in the aquatic environment. This work resulted in a landmark publication co-authored with Professor Rita Colwell.”

Colwell and Xu’s proposal for a viable form of a serious pathogen that could not be cultured in the laboratory was “a revolutionary concept for its time, and the work polarized scientific opinion, which was then immersed in the dogma that culturability was akin to viability,” according to that tribute. Although this first published report about V. cholerae and Escherichia coli in 1982 did not use the term VBNC, it soon appeared in a 1985 report from Colwell and her collaborators when they described dormant cells.

**Subsiding Skepticism about the Reality and Importance of the VBNC State**

Whereas some microbiologists remained skeptical about the VBNC state for many years, the phenomenon is now widely accepted as legitimate, reflecting in part extensive molecular studies that indicate such cells continue to express genes even after there are significant decreases in overall metabolic activity. Many different types of bacteria, including human pathogens, are capa-
ble of entering this state, maintaining cellular structure and biology, and continuing significant gene expression while otherwise being nonculturable by standard laboratory methods. According to UKEssays of Nottingham, United Kingdom: “That they can exit from this state, and become culturable again, is also undeniable... The VBNC state plays a critical role in the survival of important human (and other) pathogens, and possibly in their ability to produce disease” (see www.ukessays.com/essays/biology/viable-but-non-culturable-bacteria-biology-essay.php).

The number of bacterial types known to enter the VBNC state includes nearly 60 genera and more than 100 species. The list includes many human pathogens, including Mycobacterium tuberculosis, Helicobacter pylori, V. cholerae, Legionella pneumophila, Salmonella spp., and Campylobacter spp. As noted by Gengenbacher and Kaufmann of the Max Planck Institute for Infection Biology in Berlin, “Latent tuberculosis is the result of solid granulomas containing M. tuberculosis and because of decreased culturability, they are considered to be VBNC cells.” Moreover, “Chlamydia persistence is defined as a long-term association between Chlamydia and their host cell in which these organisms remain in a viable but culture-negative state,” add Yasser Abdel-Rahman and Robert Belland of the University of Tennessee Health Sciences Center. More recently, researchers reported the VBNC state in a number of eukaryotes, most notably the yeasts Saccharomyces cerevisiae and Brettanomyces spp.

Studies examining in situ gene expression, along with providing definitive evidence for the validity of the VBNC state, also provide evidence for why some cells appear less virulent while in the VBNC state. For example, we found that V. vulnificus no longer produces its antiphagocytic capsule when in a low temperature-induced VBNC state. This observation likely accounts, at least in part, for why this highly fatal pathogen causes so few infections during the winter. However, cells in the VBNC state are remarkably durable. For example, in this dormancy state V. vulnificus cells have an increased resistance to antibiotics, ethanol, high temperature, alkaline and acidic conditions, and heavy metals as well as to other potentially lethal stresses.

Earlier, researchers did not understand the genetic basis for the VBNC state. More recently, researchers studying “persister” cells, another state of dormancy that we believe is related to the VBNC state, described the important role that toxin/antitoxin (T/AT) modules play during dormancy. Cells produce two proteins in T/AT systems, with the antitoxin protein inactivating the toxin protein. Stress leads to selective proteolysis of the antitoxin, freeing the toxin molecule and
thus inhibiting cellular metabolism, including by blocking protein translation, which in turn leads to cell stasis. We found that both the hipA and relE systems, known to be involved in the persister response, occur in *V. vulnificus*. When cells are subject to low temperature stress, they induce the VBN€ C state following increases in activity of both these T/AT systems (Fig. 3).

**Resuscitating Cells from the VBN€ C State**

Cells that enter the VBN€ C state eventually exit this state of dormancy to become fully culturable again, a shift called “resuscitation.” This phenomenon occurs in the laboratory, in vivo, and in situ. Indeed, entry into the VBN€ C state followed by resuscitation likely accounts for the fluctuating levels of individual species typically observed in natural environments (Fig. 1b).

To resuscitate VBN€ C *V. vulnificus*, we use the membrane diffusion chambers that Gordon Mcfeters of Montana State University in Bozeman developed. These chambers allow full exchange of temperature, salinity, pH, nutrients, and bacteriophage, while retaining the bacterial cells within the chamber. Such studies show a cold temperature-induced loss of cell culturability despite maintenance of high numbers of viable cells. At warmer temperatures, the cells quickly resuscitate to become culturable again. Many vibrios become nonculturable at cold temperatures, whether growing freely in water or within a shellfish host. We routinely culture *V. vulnificus* from oysters, the primary vector for the potentially fatal food infections caused by this species during warm water months.

However, we are unable to isolate *V. vulnificus* from oysters during winter months. Assuming these bacteria remain present in oysters but in the VBN€ C state, we reasoned that warming winter oysters would lead to increases in vibrio culturability as the VBN€ C cells underwent resuscitation. To our surprise, however, we saw no increase after we incubated such oysters for two months at 20° C. However, when we added other, genetically marked *V. vulnificus* cells to these oysters, the levels of background *Vibrio* spp. then increased significantly. This new vibrio population, however, did not consist of the added *V. vulnificus* cells, but derived instead from resuscitated vibrios that were originally present in the oysters.

Those results led us to wonder about the mechanism and signal that enabled the added
bacteria to resuscitate the dormant *V. vulnificus* cells. A clue came from Slava Epstein of Northeastern University and his collaborators, who suggest that “scout cells” derived from the dormant cells trigger them to resuscitate.

To test this possibility, we added cell-free supernatants (CFS) from known quorum sensing-producing cells to *V. vulnificus* cells in the VBNC state. Whereas we typically resuscitate *V. vulnificus* cells by shifting them to room temperature for about 8 hours, adding CFS along with that same temperature upshift led to an almost immediate increase in culturability (Fig. 4). The same was observed when using supernatant material from other quorum sensing cells, including *V. parahaemolyticus* or *E. coli*, or when the quorum sensing molecule AI-2 was added to the dormant cells. However, CFS from a mutant that cannot produce the AI-2 quorum sensing molecule failed to resuscitate the dormant cells. Together these findings suggested to us that the dormant microflora of the oysters sensed the AI-2 molecules, which were interpreted as a signal for the presence of a large population of actively metabolizing cells. The dormant cells responded by resuming full metabolic activity.

**Climate Change Seems To Be Accelerating Vibrio Resuscitation Rates**

If temperature is so critical to the presence of free-living vibrio bacteria in water and in oysters, will increases in water temperature lead to increased geographic distribution and incidence of infections resulting from resuscitated cells? The answer to that question is not “if,” but “how soon?” *Vibrio*-related infections are indeed increasing worldwide, corresponding to rising global sea surface temperatures, according to Luigi Vezzulli and Carla Pruszzo of Università degli Studi di Genova in Genoa, Italy, and their collaborators. Indeed, as reported by Craig Bak-
er-Austin and colleagues at the Cefas Laboratory in the UK, fatal cases of *V. cholerae* are being reported in northern Finland, and episodes of vibriosis occurred in Denmark following unusually warm summers. Such occurrences are likely because VBNC vibrio cells are being resuscitated in warmer waters.

James D. Oliver is Bonnie E. Cone Distinguished Professor for Teaching in the Department of Biological Sciences, University of North Carolina at Charlotte.

**Suggested Readings**


