The viable but nonculturable state and cellular resuscitation

J.D. Oliver

University of North Carolina, Charlotte, NC 28223 USA

ABSTRACT

Aquatic microbial ecologists have long recognized that portions of bacterial populations seem to disappear from natural water bodies during certain times of the year, only to reappear at other times. In the case of *V. vulnificus*, numerous studies have documented the difficulty researchers experience in culturing this bacterium during cold months, presumably due to "die-off" of the population. This decrease in culturability is thought to be due to the entrance of the cells into a viable but nonculturable (VBNC) state, reported to occur in at east 30 other bacterial species. It is thought that the VBNC state may represent a survival response of bacteria exposed to stressful environmental conditions and, in the case of *V. vulnificus*, is induced by temperatures below 10 ^{o}C. The ability of any bacterium to resuscitate from this dormant state would appear to be essential if the VBNC state were truly a survival strategy. Whether the culturable cells, which appear following stress removal, are a result of true resuscitation, or from re-growth of a few residual culturable cells, has long been debated. Our research, including dilution studies, time required for resuscitation to occur, and the effects of nutrient on recovery, indicates that, at least for *V. vulnificus*, true resuscitation does occur. Our studies have further suggested that nutrient is in some way inhibitory to the resuscitation of cells in the VBNC state, and that studies which add nutrient in an attempt to detect resuscitation are only able to detect culturable cells which might be present.

Introduction

Microbial ecologists have long recognized that large proportions of the microbial populations inhabiting natural habitats appear to be nonculturable. Indeed, plate counts of bacteria in soil and aquatic environments typically indicate that far less than 1% of the total bacteria observed by direct microscopic examination can be grown on culture media. It has also long been known that certain portions of bacterial populations in natural environments seem to "disappear" during certain seasons, only to "reappear" at other times. We now understand that at least part of the explanation for these observations is not due to seasonal die-off of the cells, but to their entry into what is most commonly called the "viable but nonculturable" (VBNC) state [11]. At least 30 species contained in16 genera have been demonstrated to enter the VBNC state [12]. This paper reviews primarily our own studies on the estuarine bacterium, *Vibrio vulnificus*, for which more is known regarding this state than any other bacterium.

A bacterial cell in the VBNC state may be defined as one which fails to grow on the routine bacteriological media on which it would normally grow and develop into a colony, but which is in fact alive. It should be emphasized that there is a good deal of disagreement regarding the terminology employed for this phenomenon. I would like to note, however, that we do not say that cells are "nonculturable", but that they have entered

Microbial Biosystems: New Frontiers
Proceedings of the 8th International Symposium on Microbial Ecology
Bell CR, Brylinsky M, Johnson-Green P (eds)
Atlantic Canada Society for Microbial Ecology, Halifax, Canada, 1999.

into a viable but nonculturable *state*. Thus, *while in this state*, they are nonculturable, but can exit this state when conditions are appropriate. Bacteria enter into this "dormant" state in response to one or more environmental stresses which might otherwise ultimately be lethal to the cell. Thus, the VBNC state should be considered a means of cell survival. Eventually, when the inducing stress is removed, these cells are able to emerge from the VBNC state, and again become culturable on routine media. Regarding my use of the term "dormancy", I would note that cells in the VBNC state appear to be metabolically inactive (i.e. dormant), or nearly so, but retain the potential to become metabolically active. Indeed, the assays typically employed to determine "viability" all involve incubation under permissive conditions. These assays thus determine activity not of cells still under the VBNC-inducing stress (e.g., low temperature), but while exposed to those conditions which favor metabolic activity. I would also note, however, that a number of researchers doubt the validity (or significance?) of the VBNC state, regardless of the terminology employed, and the reader is referred to two very recent papers which summarize much of these objections [1, 5]. The paper by Weichart in these proceedings also presents some philosophical thoughts about this state.

As is evident from Fig. 1, cells lose their ability to be cultured in a rather linear manner, eventually reaching a point where platings suggest a total lack of any living cells. However, whereas death of a bacterial population generally leads to lysis of the cells and loss of cell structure, direct examination of cells entering the VBNC state indicates that the cells remain intact. Such cells could, of course, have died, but simply not undergone lysis. The primary evidence that such cells are alive, even if nonculturable, is from data obtained when one of the "direct viability" assays is applied to such cultures. These assays allow the direct determination of the viability of individual cells in a population, without the need for culture. As seen in Fig. 1, such assays often indicate that a large proportion of the apparently dead population is indeed alive.

Characteristics of the Cells in the VBNC State

Cells entering the VBNC state generally undergo a reduction in size. In the case of *V. vulnificus*, for example, whereas log phase (actively growing) cells are 3 μm long, those in the VBNC state are coccoid, typically 0.6 μm in diameter. During this size reduction, significant changes in membrane structure, protein composition, ribosomal content, and possibly even DNA arrangement are experienced. Again using *V. vulnificus* as an example, we have found rapid and dramatic decreases in the levels of synthesis of DNA, RNA, and protein when these cells are exposed to a temperature downshift to 5 ^{o}C [9]. However, such decreases do not mean that all synthesis has ceased. Indeed, protein synthesis appears to be essential for entry into this state, and under these conditions *V. vulnificus* produces some 40 new proteins not seen during growth at "normal" temperatures [8]. At the same time, dramatic decreases in membrane fatty acid composition [7] and nutrient transport and respiration rates have generally been reported to occur as cells enter this dormant state. Cell wall synthesis, or at least metabolism of the constituents of these structures, also apparently continues, as addition of penicillin to VBNC cells has generally been observed to result in rapid cell death [11]. Interestingly, it is becoming increasingly apparent that changes (possibly even major) in the cells' chromosomal DNA may be occurring as cells enter the VBNC state [11].

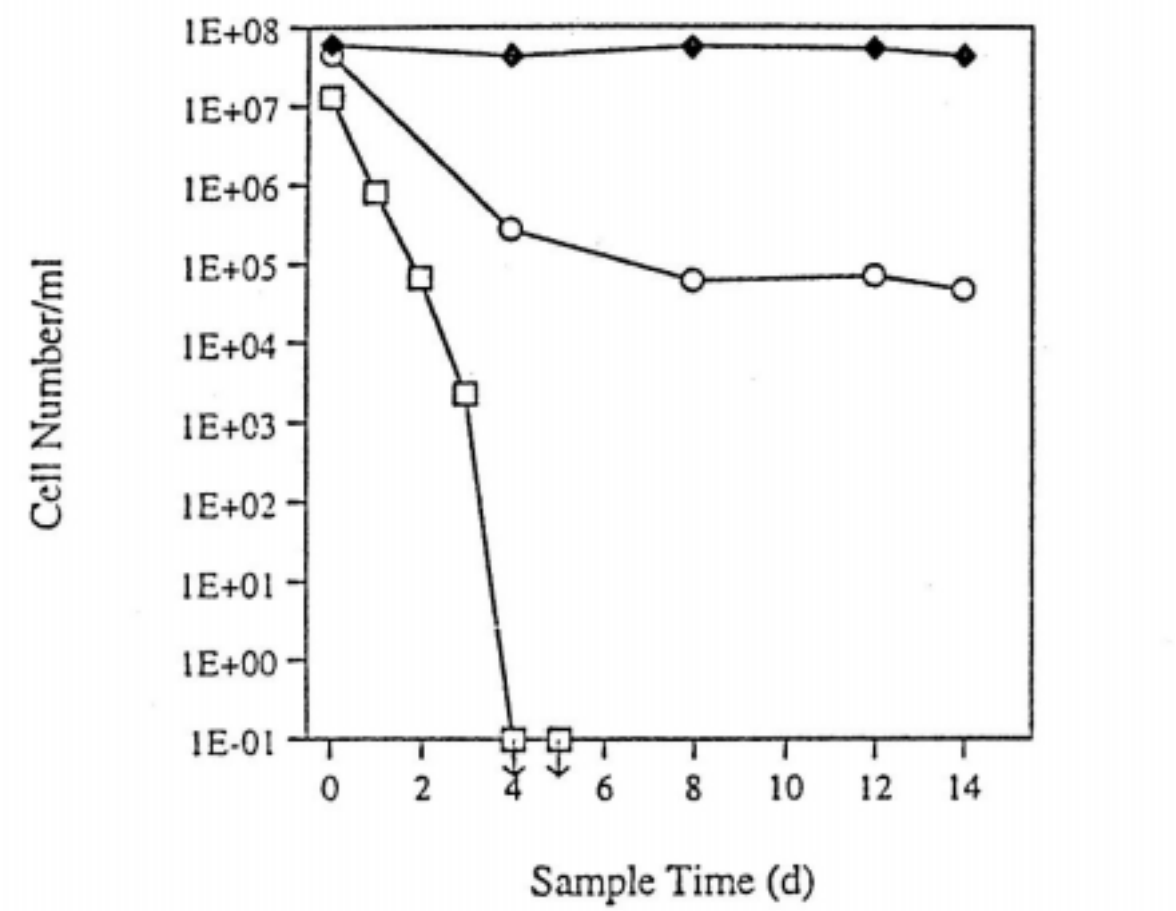

Fig. 1. Entry of *V. vulnificus* into the VBNC state in an artificial sea water microcosm at 5 ^{o}C. Shown are plate counts ( ) on HI agar in cfu/ml, total cell counts (♦) by the acridine orange staining method, and direct viable counts (o) by the substrate responsive method using yeast extract and nalidixic acid (from Whitesides and Oliver [17]).

Inducing Factors

Different bacteria are known to enter the VBNC state in response to different factors, all of which would normally be encountered in natural environments. These include such stresses as elevated or reduced temperature, elevated or reduced osmotic (e.g., salt) concentrations, nutrient starvation, levels of oxygen, and even certain intensities of white light [11]. In all cases, the inducers of the VBNC state appear to be environmental factors which are potentially injurious to a given bacterial species. For example, while entry into the VBNC state by *V. vulnificus* (T_{opt}=37 ^{o}C) is induced by low (<10 ^{o}C) temperature incubation [18], the reverse is true for *Pseudomonas fluorescens*. This bacterium, which prefers low temperatures, enters the VBNC state at 37 ^{o}C.

The time required for cells to enter the VBNC state varies markedly with the bacterium and the inducing conditions. Reports of months being required are not uncommon, while others have reported days for the same bacteria. One factor, which has been shown to have a dramatic effect on the time required for lab-grown cells to become nonculturable, is the "age" of the cells. We have shown that, whereas *V. vulnificus* cells from the logarithmic

phase of growth generally require less than ten days to become completely nonculturable at 5 °C, those taken from the stationary phase require over a month. Indeed, a direct correlation between time required to become nonculturable and the age of the population has been demonstrated [10].

Resuscitation from the VBNC State

For the VBNC response to represent a true survival response the cells must be able to exit this dormant state and return to a metabolically active and once again culturable state. Such a reversal in physiology is termed resuscitation, and is often triggered simply by the removal of the stress which initially induced the VBNC response. In *V. vulnificus*, for example, exit from the low temperature-induced VBNC state is triggered by a temperature upshift (e.g., from 5 °C to room temperature). After such a shift, culturable cells rapidly (typically within 8 hr) begin to appear, and population levels approximately equaling the original levels are generally observed within 12-24 hr (Fig. 2). During this time, the small coccoid cells which result as the cells enter the VBNC state are replaced by rods typical in size for *V. vulnificus* [9].

While entry into a VBNC state has been described by many researchers and for many different bacterial species, demonstrating resuscitation has not always been a simple matter. Indeed, while some bacteria like *V. vulnificus* can be resuscitated by simply reversing the inducing stress, in others it has been quite difficult to show. We now realize

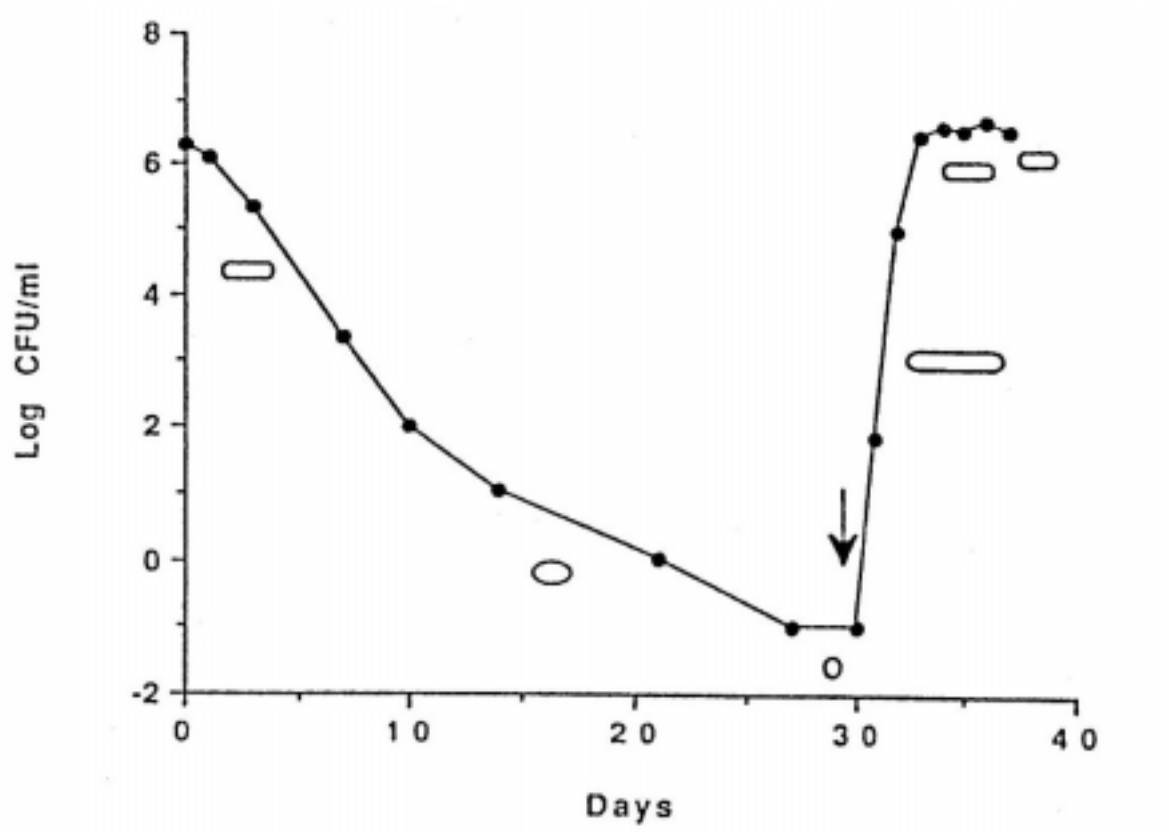

Fig. 2. Changes in culturable cell (plate) counts and cell morphology during temperature downshift to 5 °C and subsequent resuscitation of the nonculturable cells by incubation at ca. 22 °C (from Nilsson et al. [9].

that resuscitation may be an extremely complex event. For example, in the case of *L. pneumophila* (which enters the VBNC state in response to nutrient deprivation), simple addition of nutrient to the cells does not reverse the dormancy. However, the addition of certain amoebae, which are natural hosts to this bacterium in the aquatic environment, allows resuscitation of this causative agent of Legionnaire's disease [5].

That complex conditions must sometimes be employed to allow resuscitation of cells from the VBNC state is not the only problem in demonstrating this aspect of the VBNC state. It has also been difficult to overcome the argument that what is being observed in the name of "resuscitation" is, in fact, regrowth of a few culturable cells which had escaped detection during plating of the population under study. However, we have recently presented strong evidence that, at least in the case of *V. vulnificus*, true resuscitation does occur [17]. Our studies employed extensively diluted populations of VBNC cells in which it was extremely unlikely for any culturable cells to be present. Resuscitation of these populations occurred at such a rapid rate that, if it were due to regrowth of culturable cells, they would have to have had a doubling time of approximately six minutes. This is clearly an impossible generation time for cells incubated at a suboptimal temperature without nutrient or aeration (Fig. 3). We also

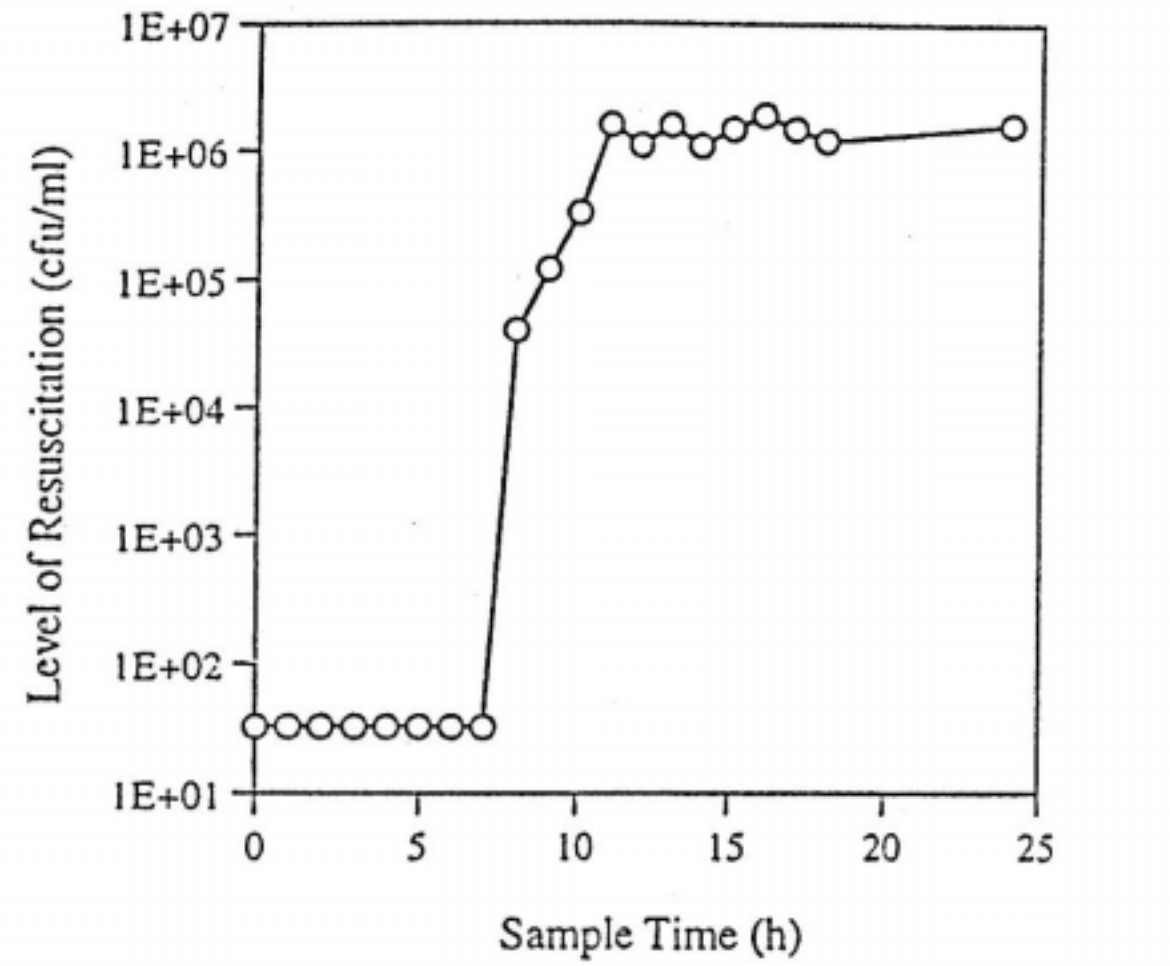

Fig. 3. Time required for resuscitation of VBNC *V. vulnificus* cells. Cells from a VBNC microcosm (<3.3 x 10^1 cfu/ml) were shifted to room temperature and aliquots removed at hourly intervals and plated onto HI agar (from Whitesides and Oliver [17]).

observed that nutrient appears to inhibit (but not kill) VBNC cells, and this may be the reason such cells are "nonculturable" when plated onto the high nutrient media routinely employed for bacterial culture. This point is discussed at the end of this paper.

In situ Evidence for the VBNC Response

One can validly question whether this response actually occurs in natural environments. We have conducted studies employing membrane diffusion chambers which clearly demonstrated the entry of *V. vulnificus* into the VBNC state when the estuarine waters in which they were submerged were cold [14]. Similarly, when cells in the VBNC state were placed into chambers which were suspended in warm (summer) estuarine waters, resuscitation from the VBNC state occurred rapidly and to levels approximating those of the original population. Thus, it is likely that our inability to isolate *V. vulnificus* during the winter months may be accounted for by entrance into the VBNC state, and that recovery from this state in natural environments may result from a temperature upshift. Taking these observations to their logical conclusions, it seems quite likely that the VBNC response may be a significant source of the variations seen in natural populations as they undergo various environmentally-induced stresses, and for our inability to culture many of the microorganisms observed in natural environments.

Why do Cells Enter the VBNC State?

Recently, a number of theories have been advanced to explain why cells enter this state of dormancy, and why they can no longer be cultivated on routine media. Koch [6] notes that when bacteria enter into a "shut down" mode, the nuclei of the cells compact due to a higher degree of coiling. Further, he notes that when mRNA synthesis is halted, the various DNA-binding proteins can more effectively bind to the genome. Our observations [3,16] that PCR amplification of *V. vulnificus* cells ceases as the cells enter the VBNC state is consistent with such modifications of the genome. Indeed, we concluded that either DNA-binding proteins (e.g., the cold-induced Csp series of DNA-binding proteins described in *E. coli*) and/or supercoiling might be responsible for decreased amplification [16]. Such binding and/or supercoiling might result in a shut down of transcription of any of a large number of genes essential for cell division. On removal of the inducing stress, proteolytic enzymes (e.g., Lon) might degrade the DNA-binding proteins, allowing transcription to occur and growth to resume.

The above hypothesis might explain why cells respond to a stress with entry into this dormant state. However, it does not explain why cells like *V. vulnificus* are no longer able to grow on routine nutrient media when incubated at a permissive temperature. In a recent study [17] investigating resuscitation of *V. vulnificus*, we observed that nutrient may be in some manner inhibitory to the resuscitation of these cells from the VBNC state. However, *V. vulnificus* cells which experience a temperature upshift in the *absence* of nutrient are able to exit the VBNC state, and when subsequently placed onto nutrient media, to develop into colonies. We therefore speculated that studies which add nutrient to a VBNC population in an attempt to detect resuscitation would only be able to detect any residual *cultural* cells which might be present. Such cells would not be inhibited by the added nutrient. A corollary of this would be that the presence of nutrient would, in fact, be bacteriostatic or bacteriocidal to VBNC cells, and would explain their inability to develop into colonies on routine media. In fact, this is essentially what Koch [6] proposed when he stated that dormant cells, on exposure to high nutrient, might so rapidly transport the

nutrient that they would experience a cell damaging internal nutrient concentration. He noted that such an event, which might occur within seconds, would likely be due to inadequate feedback inhibition or overflow metabolism occurring as the dormant cells transported the nutrient. Death, he speculated, could arise simply from the osmotic effects of the large internal nutrient concentration, or to the specific effects of one or more components of the nutrient.

More recently, Dodd et al. [4] and Bloomfield et al. [2] speculated that, on exposure to high nutrient levels, growth-arrested cells would likely undergo an imbalance in metabolism which would result in a near instantaneous production of super-oxide and other free radicals. In the absence of some pre-adaptation (e.g. starvation-induced stress proteins), such cells would not be able to detoxify these toxic radicals and would die. This theory could also account for the inability of VBNC cells to grow in nutrient media. Bloomfield et al. [2] ended by stating, "In conclusion there is every reason to accept the practical existence of a V(B)NC state, but, if we are finally to remove the non-culturable label from what are truly viable cells, we must better understand the biochemistry and physiology of the interactions between growth, respiration and the devastatingly destructive power of oxidation damage". The VBNC state has now been described in many bacteria by many investigators around the world. There seems little doubt that this state of dormancy represents another survival strategy. We now must identify the genetic basis of this phenomenon, and begin to better characterize the induction of cells into and out of this state.

References

1. Barer MR, Kaprelyants AS, Weichart DH, Harwood CR, Kell DB (1998). Microbial stress and culturability: conceptual and operational domains. Microbiology 144:2009-2010
2. Bloomfield SF, Stewart GSAB, Dodd CER, Booth IR, Power EGM (1998). The viable but nonculturable phenomenon explained? Microbiology 144:1-3
3. Brauns LA, Hudson MC, Oliver JD (1991) Use of the polymerase chain reaction in detection of culturable and nonculturable *Vibrio vulnificus* cells. Appl Environ Microbiol 57:2651-2655
4. Dodd CER, Sharman RL, Bloomfield SF, Booth IR, Stewart GSAB (1997) Inimical processes: bacterial self-destruction and sub-lethal injury. Trends Food Sci Technol 8:239-241
5. Kell DB, Kaprelyants AS, Weichart DH, Harwood CL, Barer MR (1998) Viability and activity in readily culturable bacteria: a review and discussion of the practical issues. Ant. van Leeuvenhoek 73:169-187.
6. Koch, AL (1996) What size should a bacterium be? A question of scale. Ann Rev Microbiol 50:317-348
7. Linder K, Oliver JD (1989) Membrane fatty acid and virulence changes in the viable but nonculturable state of *Vibrio vulnificus*. Appl Environ Microbiol 55:2837-2842
8. McGovern VP, Oliver JD (1995) Induction of cold responsive proteins in *Vibrio vulnificus*. J Bacteriol 177:4131-4133
9. Nilsson L, Oliver JD, Kjelleberg S (1991) Resuscitation of *Vibrio vulnificus* from the viable but nonculturable state. J Bacteriol 173:5054-5059
10. Oliver JD, Nilsson L, Kjelleberg S (1991) Formation of nonculturable *Vibrio vulnificus* cells and its relationship to the starvation state. Appl Environ Microbiol 57:2640-2644
11. Oliver JD (1993) Formation of viable but nonculturable cells. In: Kjelleberg S (ed) Starvation in Bacteria, Plenum Press, New York, pp 239-272
12. Oliver JD (1995) The viable but nonculturable state in the human pathogen *Vibrio vulnificus*. FEMS Microbiol Lett 133:203-208

13. Oliver JD, Nilsson L, Kjelleberg S (1991) Formation of nonculturable cells of *Vibrio vulnificus* and its relationship to the starvation state. Appl Environ Microbiol 57:2640-2644
14. Oliver JD, Hite MF, McDougald D, Andon NL, Simpson LM (1995) Entry into, and resuscitation from, the viable but nonculturable state by *Vibrio vulnificus* in an estuarine environment. Appl Environ Microbiol 61:2624-2630
15. Steinert M, Emody L, Amann R, Hacher J (1997) Resuscitation of viable but nonculturable *Legionella pneumophila* Philadelphia JR32 by *Acanthamoeba castellanii*. Appl Environ Microbiol 63:2047-2053
16. Warner JM, Oliver JD (1998) Randomly amplified DNA (RAPD) analysis of starved and viable but nonculturable (VBNC) cells of Vibrio vulnificus. Appl Environ Microbiol 64:3025-3028
17. Whitesides MD, Oliver JD (1997) Resuscitation of *Vibrio vulnificus* from the viable but nonculturable state. Appl Environ Microbiol 63:1002-1005
18. Wolf PW, Oliver JD (1992) Temperature effects on the viable but nonculturable state of *Vibrio vulnificus.* FEMS Microbiol Ecol 101:33-39