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The Viable but Nonculturable State in Bacteria

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It had long been assumed that a bacterial cell was dead when it was no longer able to grow on routine culture media. We now know that this assumption is simplistic, and that there are many situations where a cell loses culturability but remains viable and potentially able to regrow. This mini-review defines what the "viable but nonculturable" (VBNC) state is, and illustrates the methods that can be used to show that a bacterial cell is in this physiological state. The diverse environmental factors which induce this state, and the variety of bacteria which have been shown to enter into the VBNC state, are listed. In recent years, a great amount of research has revealed what occurs in cells as they enter and exist in this state, and these studies are also detailed. The ability of cells to resuscitate from the VBNC state and return to an actively metabolizing and culturable form is described, as well as the ability of these cells to retain virulence. Finally, the question of why cells become nonculturable is addressed. It is hoped that this mini-review will encourage researchers to consider this survival state in their studies as an alternative to the conclusion that a lack of culturability indicates the cells they are examining are dead.

Key words: viability, culturability, virulence, resuscitation, survival

Since the original 1982 paper from the laboratory of Rita Colwell (Xu *et al.*, 1982), over 400 papers have appeared which describe various aspects of the phenomenon most commonly referred to as the "viable but nonculturable (VBNC) state". A great many pathogens, as well as non-pathogens, are now known to enter this dormancy state, and its significance in medicine, bioremediation, the use of bacteria as fecal indicators, and indeed in most microbiological studies where culturability is employed as the (often sole) indicator of viability, is becoming increasingly evident.

What is the VBNC State?

Bacteria in the VBNC state fail to grow on the routine bacteriological media on which they would normally grow and develop into colonies, but are alive and capable of renewed metabolic activity (Oliver, 2000b). Cells in the VBNC state typically demonstrate very low levels of metabolic activity, but on resuscitation are again culturable. The reader is referred to the more extensive reviews by Kell *et al.* (1998) and Oliver (2000a, 2000b, 2000c, 2005) for detailed coverage of the VBNC state, the bacteria described to enter this condition, and the concerns this physiological state raises in public health and bioremediation.

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A typical VBNC response is shown in Fig. 1. As shown by the "culturable" curve, exposure to one or more environmental stresses results in a regular decline in colony forming units. However during this period of decline, "total cell counts" generally remain fairly constant. The key test that determines whether such cells are dead, or alive but in a VBNC state, is the "viability count". Several such assays can be used to demonstrate this trait (see below), but in all cases these characterize some aspect of metabolic activity or of cellular integrity which indicates

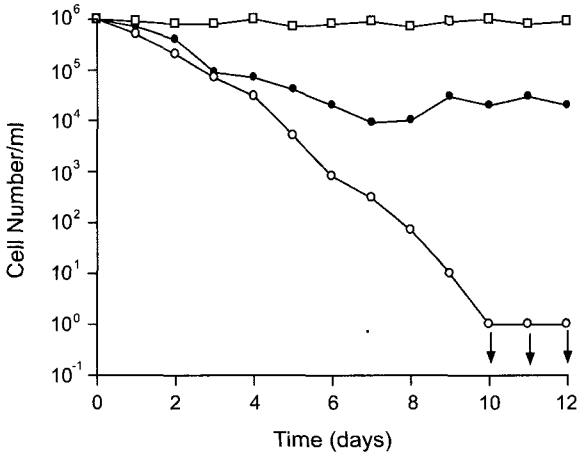

Fig. 1. Entry of *V. vulnificus* into the VBNC state on incubation at 5°C. Shown are total cell counts (□), culturable counts (○), and viable counts (●).

that the cells are alive, even if they are unable to develop into colonies on culture media.

The VBNC state differs significantly from the "starvation survival" state, wherein cells also undergo dramatic decreases in metabolism, but otherwise remain fully culturable (Oliver *et al.*, 1991). A recent proteome study on *Enterococcus faecalis*, for example, has clearly demonstrated that the protein profiles of cells entering the VBNC state are markedly different from those of exponentially growing or starved cells (Heim *et al.*, 2002).

What Induces this State in Bacteria?

Cells enter the VBNC state as a response to some form of natural stress, such as starvation, incubation outside the temperature range of growth, elevated osmotic concentrations (e.g. seawater), oxygen concentration, or exposure to white light (Oliver, 2000c). These typically are environmental stresses that might be lethal if the cells did not enter this dormancy state. In addition, a number of studies have found that processes which are normally assumed to be bactericidal for bacteria may instead result in cells which reside in the VBNC state. These include such treatments as pasteurization of milk (e.g. Gunasekera *et al.*, 2002) and chlorination of wastewater (Oliver *et al.*, 2005).

How Is the Viability of Nonculturable Cells Determined?

Whereas a determination of the total number of cells present in a population may be obtained using DAPI or acridine orange staining, viable cell counts are typically determined using the substrate responsive assay of Kogure *et al.* (1979), by examining intracellular hydrolysis of CTC or reduction of INT as an indication of metabolic activity (Zimmerman *et al.*, 1978; Rodriguez *et al.*, 1992), or by establishing the presence of an intact cytoplasmic membrane (*Bac*Light® or propidium iodide). These methods are described in several reviews (Oliver, 1993, 2000b; McFeters *et al.*, 1995; Breeuwer and Abee, 2000; Créach *et al.*, 2003). Using multi-parameter flow cytometry, Porter *et al.* (1995) found that such measurements of membrane potential, membrane integrity, and intracellular enzymatic activity "... provided extensive evidence for the validity of the methods for monitoring cell viability during adoption of the viable-but-non-culturable state in starved *E. coli*".

What Bacteria Enter the VBNC State?

The number of species described to enter the VBNC state constantly increases, with approximately 60 now reported to demonstrate this physiological response (Table 1). Included are a large number of human pathogens, including *Campylobacter* spp., *E. coli* (including EHEC strains), *Francisella tularensis, Helicobacter pylori, Legionella pneumophila, Listeria monocytogenes, Mycobacterium tuberculosis, Pseudomonas aeruginosa,* several *Salmo-*

Table 1. Bacteria Described to Enter the VBNC State

Aeromonas salmonicida	*Lactobacillus plantarum*	*Serratia marcescens*
Agrobacterium tumefaciens	*Lactococcus lactis*	*Shigella dysenteriae*
Alcaligenes eutrophus	*Legionella pneumophila*	*S. flexneri*
Aquaspirillum sp.	*Listeria monocytogenes*	*S. sonnei*
Burkholderia cepacia	*Micrococcus flavus*	*Sinorhizobium meliloti*
B. pseudomallei	*M. luteus*	*Streptococcus faecalis*
Campylobacter coli	*M. varians*	*Tenacibaculum sp.*
C. jejuni	*Mycobacterium tuberculosis*	*Vibrio anguillarum*
	M. smegmatis	
C. lari	*Pasteurella piscida*	*V. campbellii*
Cytophaga allerginae	*Pseudomonas aeruginosa*	*V. cholerae*
Enterobacter aerogenes	*P. fluorescens*	*V. fischeri*
E. cloacae	*P. putida*	*V. harveyi*
Enterococcus faecalis	*P. syringae*	*V. mimicus*
E. hirae	*Ralstonia solanacearum*	*V. natriegens*
E. faecium	*Rhizobium leguminosarum*	*V. parahaemolyticus*
Escherichia coli (including EHEC)	*R. meliloti*	*V. proteolytica*
Francisella tularensis	*Rhodococcus rhodochrous*	*V. shiloi*
Helicobacter pylori	*Salmonella enteritidis*	*V. vulnificus (types 1&2)*
Klebsiella aerogenes	*S. typhi*	*Xanthomonas campestris*
K. pneumoniae	*S. typhimurium*	
K. planticola		

nella and *Shigella* spp. and *Vibrio cholerae, V. parahaemolyticus, and V. vulnificus*. The latter has been the most studied as regards the VBNC state, and much of what is described in this review is taken from the *V. vulnificus* literature.

What Occurs in a Cell Entering the VBNC State?

Cells entering the VBNC state often exhibit dwarfing, and during this period a number of major metabolic changes occur, including reductions in nutrient transport, respiration rates, and macromolecular synthesis (Porter *et al.*, 1995; Oliver, 2000a). Biosynthesis does not cease, however, and during this period novel starvation and cold shock proteins are formed (Morton and Oliver, 1994; McGovern and Oliver, 1995). ATP levels, which decline rapidly in dead and moribund cells, have been found to remain high in VBNC cells (Beumer *et al.*, 1992; Federighi *et al.*, 1998). Further, recent studies have demonstrated continued gene expression by cells in the VBNC state (Lleò *et al.*, 2000, 2001; Yaron and Matthews, 2002). Yaron and Matthews (2002) found that a variety of genes, including *mobA, rfbE, stx1* and those for 16S rRNA synthesis, were expressed in nonculturable cells of *E. coli* O157:H7. Similarly, Barrett (1998) and Saux *et al.* (2002) have reported continued production of message for several genes after cells of *V. vulnificus* were in the VBNC state for as long as 4.5 months. Such findings are strong indications of viability but whether or not the genes reported to be active are actually involved in the VBNC process is not known. Similarly, Rahman *et al.* (1994), studying *Shigella dysenteriae* cells that had been in the VBNC state for 4 - 8 weeks, found that such cells were capable of active uptake of methionine and its incorporation into protein. Extensive modifications in cytoplasmic membrane fatty acid composition that appear to be essential for entry into this state have been described (Day and Oliver, 2004), and these are likely necessary for the continued membrane potential which has been reported (Porter *et al.*, 1995; Tholozan *et al.*, 1999). Significant, and what might be characteristic biochemical changes in the cell walls of VBNC cells, have also been documented. Signoretto *et al.* (2002), in studying the cell wall peptidoglycan of *E. coli* entering the VBNC state, reported an increase in cross-linking, a 3-fold increase in unusual DAP-DAP cross-linking, an increase in muropeptides bearing covalently bound lipoprotein, and a shortening of the average length of glycan strands in comparison to exponentially growing cells. VBNC cells were also found to have an autolytic capability far higher than that of exponentially growing cells. Similar findings were reported by Signoretto *et al.* (2000) for *Enterococcus faecalis*. Plasmids appear to be retained (Porter *et al.*; 1995), and their presence or absence has been reported in several *Pseudomonas* species to have dramatic consequences on temperature induction of the cells into the VBNC state (McDougald *et al.*, 1995). Genomic changes have also been suggested in studies reported by Bej *et al.* (1997) and Warner and Oliver (1998).

Can VBNC Return to the Fully Culturable State?

While we have found that *Pseudomonas fluorescens* cells can remain in the VBNC state in soil for over a year (Bunker *et al.*, 2004), the VBNC state can only be a significant means of survival if they are able to again become metabolically active. A secondary consequence of this process of "resuscitation" is that they

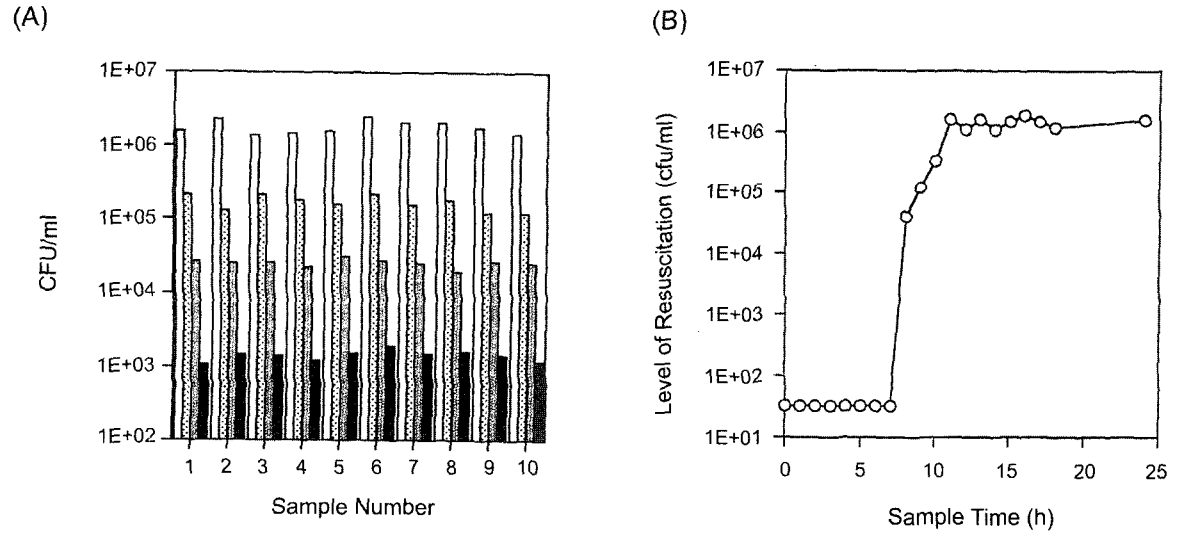

Fig. 2. Resuscitation of *V. vulnificus* from the VBNC state. Fig 2A. Resuscitation as observed in 10 individual 1 ml aliquots of an undiluted sample (□), and of samples diluted 10^{-1} (▦), 10^{-2} (▩), and 10^{-3} (■). Fig 2B. Time required for resuscitation of VBNC *V. vulnificus* cells in nutrient-free artificial seawater. Cells were removed to room temperature and aliquots plated at hourly intervals onto HI agar. Taken from Whitesides and Oliver, 1997.

again become culturable. Resuscitation has been most studied in *V. vulnificus*, a marine bacterium induced into the VBNC state by incubation at temperatures below 10°C (Wolf and Oliver, 1992). A simple temperature upshift results in resuscitation of these cells. This has been demonstrated *in vivo* (Oliver and Bockian, 1995), *in situ* (Oliver *et al.*, 1995) as well as *in vitro* (Oliver *et al.*, 1991; Wolf and Oliver, 1992).

A major and long-standing problem regarding resuscitation has been the difficulty in showing conclusively that the culturable cells that result after resuscitation of a VBNC population are a result of true resuscitation of the dormant cells, as opposed to re-growth of a few culturable cells which were not detected in the otherwise totally VBNC population. This problem developed largely from the lack of stringent controls in many early studies on resuscitation (see Kell *et al.*, 1998, for a discussion of this point). However, studies have now demonstrated conclusively that true resuscitation does occur, at least in some bacteria. One of the earliest was that of Whitesides and Oliver (1997), who studied resuscitation of *V. vulnificus*. The key to their study was the dilution, up to a thousand-fold, of the nonculturable (<0.1 cfu/ml) population. This resulted in samples (n=10 of each dilution) which contained as few as 10^3 total (VBNC) cells, but <0.0001 culturable cell/ml. Following incubation at room temperature, cells in each of the 10 samples were observed to resuscitate to the original cell number (Fig. 2A). They also determined that, during the resuscitation process, over 10 generations would have had to occur during the 1-hour period between 7 and 8 h of temperature upshift if the observed increase in culturability to $>10^6$ CFU/ml had been due to the presence of culturable cells (Fig. 2B). Such an increase would have required a generation time of 6 min, a rate clearly impossible in the absence of exogenous nutrient, the lack of aeration, and incubation at the sub-optimal temperature of 22°C. Further, resuscitation appears to be a genetically controlled even, with the addition of translational or transcriptional inhibitors preventing resuscitation (Kong *et al.*, 2004).

While many bacteria have been reported to enter the VBNC state, few have been conclusively demonstrated to resuscitate to the culturable state. The difficulty in demonstrating resuscitation is exemplified by a study reported by Steinert *et al.* (1997). They examined nonculturability and resuscitation in *Legionella pneumophila*, and while entry into this state was easily induced by nutrient starvation, resuscitation could only be demonstrated following co-incubation of the VBNC cells with the amoeba, *Acanthamoeba castellani.*

Do VBNC Cells Retain Virulence?

Whether or not cells in the VBNC state are capable of causing human infection is an extremely important aspect of this physiological state. Because of the near dormancy exhibited by such cells, it would not be expected that they are capable of initiating infection while in this state (although cells of *V. shiloi*, a coral pathogen, appear able to cause coral death while remaining fully nonculturable; ref. Banin *et al.*, 2000; Rosenberg and Ben-Haim, 2002). The key question is, "can cells in the VBNC state resuscitate and initiate infection". While there is a conflicting literature on this point, the answer is clearly yes, at least for several pathogens.

Colwell *et al.* (1985) inoculated VBNC cells of *V. cholerae* into ligated rabbit ileal loops, and observed positive responses (i.e. enteropathogenicity) in all samples. Intestinal fluid was also reported to contain culturable *V. cholerae* cells. In a subsequent study (Colwell *et al.*, 1996), a human volunteer had culturable *V. cholerae* cells in his stool 48h after ingesting VBNC cholera, while at 5d a second volunteer was stool-positive for this pathogen. As a second example, Oliver and Bockian (1995) made *V. vulnificus* cells VBNC (<0.1 cfu/ml) by incubation at 5°C. These cells were diluted such that, while the preparation contained 10^5 cells in the VBNC state, there were <0.04 cfu/ml. Mice inoculated with this preparation died, and culturable *V. vulnificus* cells were recovered from both blood and the peritoneal cavity.

Oliver (2005) has recently conducted an extensive review on the potential public health hazards of VBNC cells present in foods. A few relevant examples are Jones *et al.* (1991), who reported two of four strains of *Campylobacter jejuni* made VBNC in water caused death in suckling mice. The ability of *E. faecalis* cells in the VBNC state to adhere to cultured heart and urinary tract epithelial cells was shown by Pruzzo *et al.* (2002). While they observed a 50-70% decrease in the adherence capability of VBNC cells compared to actively growing cells, adherence values were similar to growing cells following resuscitation. Makino *et al.* (2000), studying an *E. coli* O157:H7 outbreak in Japan in 1998, found that ca. 0.75-1.5 culturable cells appeared to have been responsible for this outbreak, a number considered too low for infection. However, they provided evidence that a larger number of cells likely existed in the VBNC state in the implicated sushi, and that these were the source of the outbreak. Rahman *et al.* (1994) found *S. dysenteriae* Type 1 retained cytopathogenicity for cultured HeLa cells, and in a follow-up study (Rahman *et al.*, 1996) reported such cells not only retained their ability to produce active shiga toxin, but also the ability to adhere to intestinal epithelial cells.

We have recently been studying the human pathogen, *H. pylori*, which is estimated to infect 50% or more of the worlds population (Lambert *et al.*, 1995) and is associated with the development of chronic human gastritis, peptic ulcers, and gastric adenocarcinoma (Catrenich and Makin, 1991; Peterson, 1991; Asaka *et al.*, 2001). Molecular methods have detected the presence of *H. pylori* DNA in

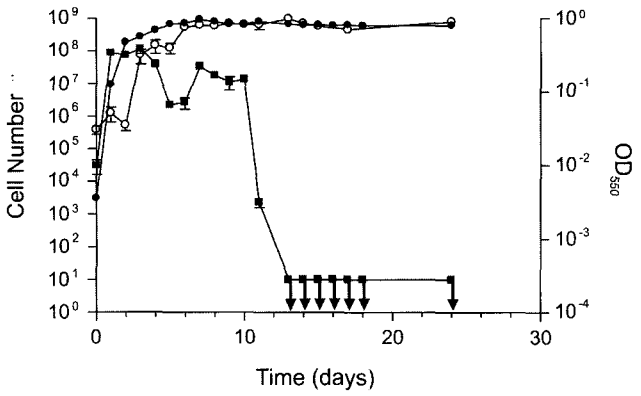

Fig. 3. The viability (cells/mL) (●), optical density (○) and culturability (cfu/mL) (■) of *H. pylori* cells aged in laboratory culture. Error bars represent the SEM of duplicate samples and ↓ indicate culturability below the limit of detection. Taken from Adams *et al.*, 2003.

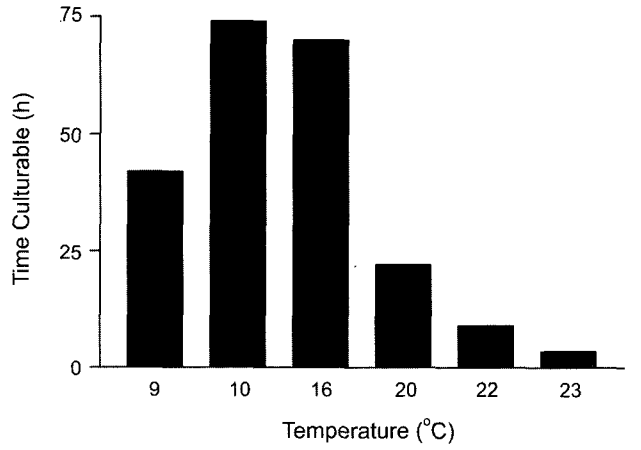

Fig. 4. The time required for *H. pylori* cells to lose culturability at various water temperatures. Taken from Adams *et al.*, 2003.

river water, well water, wastewater, as well as surface and shallow groundwater, suggesting that this organism is waterborne and may be transmitted by the fecal/oral route (Hulten *et al.*, 1996; Hegarty *et al.*, 1999; Moreno *et al.*, 2003). However, despite its high incidence of infection, the bacterium's reservoir and mode of transmission remain undetermined. We have recently demonstrated (Adams *et al.*, 2003) that *H. pylori* is able to enter the VBNC state as cells are exposed to a natural, freshwater environment (Fig. 3), and that this entry is dependent on the ambient temperature (Fig. 4). Cells underwent a transition from culturable rods to predominately nonculturable cocci as they entered the VBNC state. Further, cells could be demonstrated to be producing DNA transcripts for at least 26 hours after they had completely entered the VBNC state (Adams and Oliver, unpublished). Finally, we have observed that cells of *H. pylori* in the VBNC state are resistant to the antibiotics commonly employed for the treatment of ulcers, suggesting that recurrences of these lesions may be initiated by resuscitation of VBNC cells after cessation of antibiotic treatment (Bates *et al.*, 2003). These studies suggest that *H. pylori* is able to persist in the environment in this dormant state until it enters a suitable host, at which time a new round of infection might be initiated.

Why Do Cells Become Nonculturable?

First to suggest that the plating medium itself may be a factor in the nonculturability associated with the VBNC state was Whitesides and Oliver (1997). In their study on resuscitation of *V. vulnificus* from the VBNC state, they stated that ". . elevated nutrient might be toxic in some manner to cells in this [the VBNC] state". Subsequently, Bloomfield *et al.* (1998) suggested that non-growing cells might produce free radicals on exposure to high nutrient, which might prevent colony development on media. Shortly thereafter, Mizunoe *et al.* (1999) determined that some *E. coli* cells which were nonculturable on solid media could, in fact, be cultured to a high level when plated on the same media containing either catalase, sodium pyruvate or α-ketoglutarate. Similar findings were subsequently reported by Mizunoe *et al.* (2000) and Begosian *et al.* (2000), suggesting that the toxic metabolic by-product, H_2O_2, might be involved in the VBNC state.

To test this hypothesis, we constructed an *oxyR* mutation in *V. vulnificus* that is incapable of producing catalase (Kong *et al.*, 2004). These cells are nonculturable on solid media even when at room temperature. This observation suggests that, at least for some cells, one aspect of the VBNC state may involve their production of peroxide when plated onto solid media, or the natural presence of peroxide in solid media, coupled with the inability of the cells to detoxify this lethal metabolite.

The value of such ROS scavengers is demonstrated in Fig. 5, which shows the effect of incorporation of catalase or pyruvate in plated media onto which cells of *V. vulnificus* are placed as they enter the VBNC state. We also found that low temperature, the inducer of the loss of culturability in this bacterium, prevents both catalase activity and its *de novo* synthesis (Fig. 6), rendering the cells highly sensitive to the peroxide present in culture media. Thus, low temperature incubation resulted in cells which, due to this cold shock response, are nonculturable yet remain viable. We also determined that the stress sigma factor, RpoS, plays a major role in survival to peroxide by *V. vulnificus*, as well as to a variety of other environmental stresses (Hülsmann *et al.*, 2003), and may be a major regulatory factor in the VBNC response of this organism.

It must be stressed, however, that as is evident from Fig. 5, the presence of H_2O_2, and the ability of a cell to detoxify it, is only one factor in the VBNC response, as all cells ultimately enter this state even in the presence of catalase.

Is the Phrase "Viable but Nonculturable an Oxymoron?

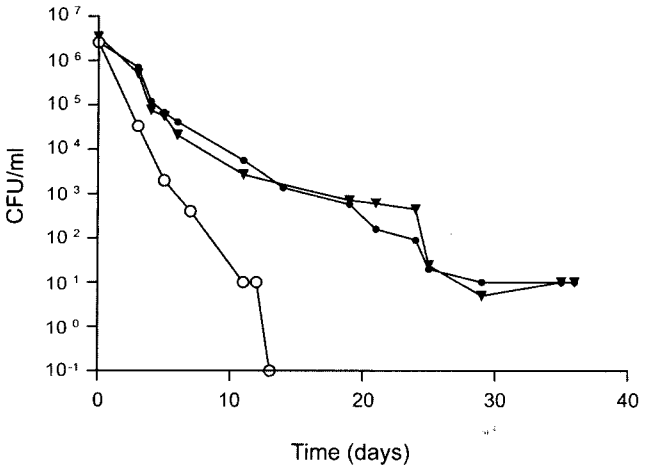

Fig. 5. Effect of ROS scavengers on culturability of *V. vulnificus* C7184/o following low temperature incubation. Cells were incubated at 5°C, then plated onto HI agar (-○-) or HI agar supplemented with catalase (-■-) or pyruvate (▼). Taken from Kong *et al.*, 2004.

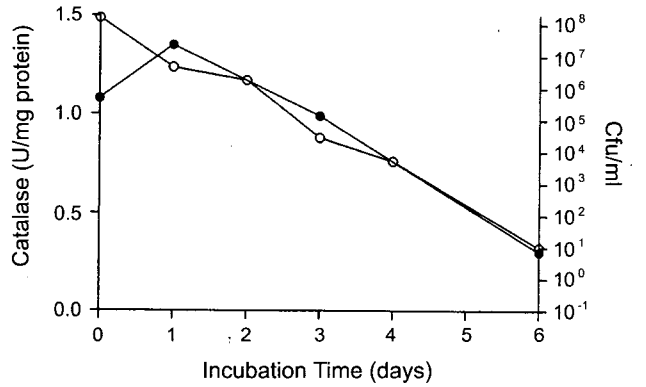

Fig. 6. Catalase activity and culturability on HI agar of *V. vulnificus* incubated at 5°C. A concomitant loss of catalase activity (●) and culturability (○) occurs. Taken from Kong *et al.*, 2004.

A few investigators (e.g. Barer *et al.*, 1998; Kell *et al.*, 1998; Barer and Harwood, 1999; Begosian and Bourneuf, 2001) have argued against the existence of a VBNC state in bacteria. However, much of their concern is a result of terminology. Barer (1997), for example, has stated that viability should only be indicated by colony development, and thus the phrase "viable but nonculturable" is an oxymoron. In fact, given the finding that some cells when present in the VBNC state may be at least partially culturable under certain conditions (e.g. the presence of ROS-scavengers), this phrase is indeed not fully accurate (Oliver, 2000b). However, "viable but nonculturable" has become adopted by the majority of researchers investigating this phenomenon, and the fact remains that certain conditions induce these cells to lose their ability to develop colonies on routine media, and to thus become "nonculturable" by routinely employed methods. Further, the overwhelming body of literature from numerous investigators and covering a wide variety of bacterial species argues that this is a novel physiological state induced by a variety of environmental factors. Indeed, several recent papers from investigators that have previously argued against the VBNC state now offer evidence for it (Mukamolova *et al.*, 1998a, 1998b, 1999) and/or have confirmed what has been previously shown by others (e.g. Begosian *et al.*, 2000).

Conclusion

It is now abundantly evident that numerous bacteria, both gram-positive and negative, both pathogens and non-pathogens, are capable of entering into the VBNC state. There is much yet to learn of the physiology, biochemistry, and genetics of cells as they enter and resuscitate from this physiological state, but such studies are underway by investigators in numerous countries around the world. While the importance of VBNC cells in the initiation of human infection is not yet fully clear, it appears that cells in this state retain virulence, and should be considered by those investigators and government regulators involved in the public health.

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