REVIEW ARTICLE

Recent findings on the viable but nonculturable state in pathogenic bacteria

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Abstract

Many bacteria, including a variety of important human pathogens, are known to respond to various environmental stresses by entry into a novel physiological state, where the cells remain viable, but are no longer culturable on standard laboratory media. On resuscitation from this 'viable but nonculturable' (VBNC) state, the cells regain culturability and the renewed ability to cause infection. It is likely that the VBNC state is a survival strategy, although several interesting alternative explanations have been suggested. This review describes the VBNC state, the various chemical and physical factors known to induce cells into this state, the cellular traits and gene expression exhibited by VBNC cells, their antibiotic resistance, retention of virulence and ability to attach and persist in the environment, and factors that have been found to allow resuscitation of VBNC cells. Along with simple reversal of the inducing stresses, a variety of interesting chemical and biological factors have been shown to allow resuscitation, including extracellular resuscitation-promoting proteins, a novel quorum-sensing system (AI-3) and interactions with amoeba. Finally, the central role of catalase in the VBNC response of some bacteria, including its genetic regulation, is described.

Introduction

Bacteria in the viable but nonculturable (VBNC) state fail to grow on the routine bacteriological media on which they would normally grow and develop into colonies, but are still alive (Oliver, 2000). Despite their typically low levels of metabolic activity, they are again culturable upon resuscitation. Since the pioneering study by Xu *et al.* (1982) over 25 years ago, a large body of literature has evolved from researchers worldwide documenting the existence of a VBNC state in a wide variety of bacteria. Most investigators believe it to be a survival strategy in response to harsh environmental conditions, and it is now clear that the VBNC state constitutes an important reservoir of pathogens in the environment (Lleò *et al.*, 2007a). The medical implications of this fact are numerous (Sardessai, 2005). For example, it appears that the 'latent' or the 'dormant' phase of *Mycobacterium tuberculosis* infections represents the VBNC state in this pathogen (Shleeva *et al.*, 2004; Young *et al.*, 2009), and that the recurrence of tuberculosis years after a person was thought to be tuberculosis free is due to resuscitation of this pathogen from the VBNC state (Pai *et al.*, 2000). The list of pathogenic bacteria that have adopted this lifestyle as a means of survival includes not only those that infect humans but also those that infect such diverse animals as fish (Magariños *et al.*, 1994; Biosca *et al.*, 1996; Rahman *et al.*, 2001), corals (Banin *et al.*, 2000; Israely *et al.*, 2001) and sea urchins (Masuda *et al.*, 2004). Many plant pathogenic bacteria entering this state have also been described (e.g. Grey & Steck, 2001; Imazaki & Nakaho, 2008; del Campo *et al.*, 2009; Ordax *et al.*, 2009). Indeed, the list of pathogens is ever increasing, and includes *Campylobacter* spp., *Escherichia coli* (including EHEC strains), *Francisella tularensis*, *Helicobacter pylori*, *Legionella pneumophila*, *Listeria monocytogenes*, *M. tuberculosis*, *Pseudomonas aeruginosa*, several *Salmonella* and *Shigella* spp. and numerous pathogenic *Vibrio* spp. A list (likely incomplete) of these is provided in Table 1; a more complete list, including nonpathogens, can be found in Oliver (2005a).

The existence of a VBNC state has been debated for many years (Bogosian & Bourneuf, 2001; Nyström, 2001; Oliver, 2005a, b; Barcina & Arana, 2009). At least some of the

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Table 1. Pathogens known to enter the VBNC state

Aeromonas hydrophila	*Helicobacter pylori*	*Serratia marcescens*
A. salmonicida	*Klebsiella aerogenes*	*Shigella dysenteriae*
Agrobacterium tumefaciens	*K. pneumoniae*	*S. flexneri*
Burkholderia cepacia	*K. planticola*	*S. sonnei*
B. pseudomallei	*Legionella pneumophila*	*Streptococcus faecalis*
Campylobacter coli	*Listeria monocytogenes*	*Vibrio alginolyticus*
C. jejuni	*Mycobacterium tuberculosis*	*V. anguillarum*
C. lari	*M. smegmatis*	*V. campbellii*
Cytophaga allerginae	*Pasteurella piscicida*	*V. cholerae*
Enterobacter aerogenes	*Pseudomonas aeruginosa*	*V. harveyi*
E. cloacae	*P. syringae*	*V. mimicus*
Enterococcus faecalis	*Ralstonia solanacearum*	*V. parahaemolyticus*
E. hirae	*Rhizobium leguminosarum*	*V. shiloi*
E. faecium	*R. meliloti*	*V. vulnificus* (types 1 & 2)
Erwinia amylovora	*Salmonella enterica*	*Xanthomonas campestris*
Escherichia coli (including EHEC)	*S. typhi*	*X. axonopodis* pv. *citri*
Francisella tularensis	*S. typhimurium*	

disagreement revolved around the phrase 'viable but nonculturable', which is most commonly used to describe this phenomenon (Barer & Harwood, 1997; Kell *et al.*, 1998; Colwell & Grimes, 2000). However, the debate over whether a VBNC state truly exists has largely been put to rest, largely as a result of numerous molecular studies reported in recent years (discussed below). Several reviews on the VBNC state have also appeared in recent years, mostly in its support (McDougald *et al.*, 1998; Colwell & Grimes, 2000; Edwards, 2000; Yamamoto, 2000; Rowan, 2004; Oliver, 2005a, b, 2006; Sardessai, 2005; Barcina & Arana, 2009) but a few opposed (Nyström, 2005; Sinton, 2006). An extensive review of the potential public health hazards specifically of food-borne pathogens has also been presented (Oliver, 2005a). The reader is referred to these reviews for discussions on various aspects of the VBNC state in general; more recent findings on the VBNC state in human pathogens are presented here.

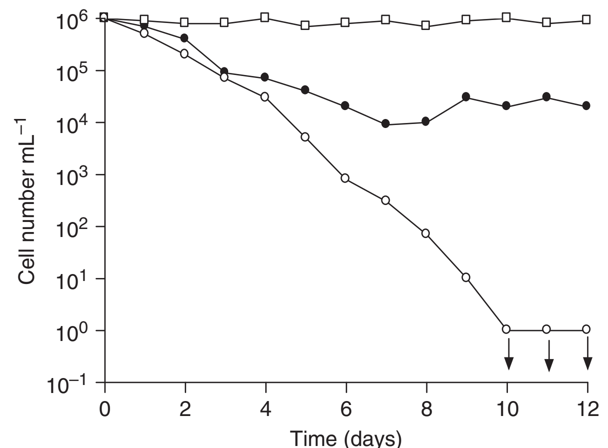

Fig. 1. Entry of *Vibrio vulnificus* into the VBNC state on incubation at 5 °C. The total cell counts (□), culturable counts (○) and viable counts (●) are shown (from Oliver, 2005b).

Inducers of the VBNC state

A list of factors, both chemical and environmental, which have been reported to induce the VBNC state, are varied and numerous. It includes nutrient starvation (e.g. Cook & Bolster, 2007), incubation outside the normal temperature range of growth (e.g. Besnard *et al.*, 2002; Maalej *et al.*, 2004; Wong & Wang, 2004), elevated or lowered osmotic concentrations (e.g. Asakura *et al.*, 2008; Wong & Liu, 2008), oxygen concentrations (Kana *et al.*, 2008), commonly used food preservatives (Cunningham *et al.*, 2009; Quirós *et al.*, 2009), heavy metals (Ghezzi & Steck, 1999) and even exposure to white light (Gourmelon *et al.*, 1994). A common response to such stresses by bacterial cells is their ultimate inability to develop into colonies on routine culture media (Fig. 1), even though the cells may remain viable for long periods of time.

Detection of VBNC cells

As cells in the VBNC state are no longer culturable, alternate nonculture methods must be used to demonstrate that cells in this state are alive. Commonly used are reagents (e.g. the *Bac*Light® Live/Dead assay) designed to demonstrate, through direct microscopic examination, the presence of an intact cytoplasmic membrane. An increasingly popular molecular method is reverse transcriptase (RT)-PCR, which detects gene expression (see Gene expression by VBNC cells). Because the half-life of bacterial mRNA is typically only 3–5 min (Conway & Schoolnik, 2003), continued gene expression by nonculturable cells is considered an excellent indicator of bacterial cell viability. Other less commonly used methods are described in Oliver (2005b).

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Cellular traits

Cells entering the VBNC state typically exhibit significant dwarfing (e.g. cells of *Vibrio vulnificus* are *c.* 2 μm long in the log phase, but 0.6 μm in diameter in the VBNC state). Such cells generally undergo major decreases in macromolecular synthesis and rates of respiration, but plasmids are retained and ATP levels and membrane potential remain high. Continued amino acid uptake and incorporation have been reported (Rahman *et al*., 1994), and a study of several probiotic *Bifidobacterium* spp. by Lahtinen *et al*. (2008) found all to retain high levels of rRNA, despite losing all culturability in fermented food products. Membrane fatty acids also change (Day & Oliver, 2004), as would be expected for cells undergoing environmental stress. Using proteomics, Muela *et al*. (2008) recently studied modifications in the outer membrane of *E. coli* cells as they entered the VBNC state. Cells were induced into this state by starvation, by incubation in seawater and/or by exposure to visible light. They observed a drastic rearrangement of the outer membrane subproteome with the appearance of over 100 new spots. Ten of these remained in the VBNC cells, although none were found to be exclusive to this state. In contrast, a proteome study on *Enterococcus faecalis* clearly demonstrated that the total cell protein profiles of cells entering the VBNC state are markedly different from those of exponentially growing or starved cells (Heim *et al*., 2002). Similarly, protein profiling of *Vibrio parahaemolyticus* revealed a number of upregulated proteins that remained at high levels for several weeks into the VBNC state (Lai *et al*., 2009).

Significant, and what might be characteristic biochemical changes in the cell walls of VBNC cells, have been well studied by Signoretto and colleagues. For example, Signoretto *et al*. (2002), in studying the cell wall peptidoglycan of *E. coli* entering the VBNC state, reported a threefold increase in unusual DAP–DAP cross-linking, an increase in mucopeptides bearing a covalently bound lipoprotein, and a shortening of the average length of glycan strands in comparison with 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 *E. faecalis*. del Mar Lleo *et al*. (2007) examined the effects of several antibiotics acting on peptidoglycan or protein synthesis in *E. faecalis* and found several β-lactams to block resuscitation of VBNC cells. Surprisingly, vancomycin, even when used at 100 times its minimum inhibitory concentration (MIC), was totally ineffective in this regard. The authors suggested that this insensitivity is due to the lack of synthesis of D-ala-D-ala, the specific target of this antibiotic, by the metabolically relatively inactive (VBNC) cells. In that peptidoglycan rearrangements have been observed in both gram-positive and -negative cells as they enter the VBNC state (Costa *et al*., 1999; Signoretto *et al*., 2002), such events may be hallmarks of the VBNC state.

Virulence of cells in the VBNC state

While not all investigators have reported pathogens to be capable of initiating infection when in the VBNC state, many have done so. In some cases, the same investigators have reported an organism to be avirulent while in the VBNC state, for example Cappelier *et al*. (2005) studying *L. monocytogenes*, and have subsequently reported that certain conditions (e.g. incubation of an embryo in egg yolk) are required to observe virulence (Cappelier *et al*., 2007). In fact, it seems most likely that pathogens are not generally able to initiate disease when present in the VBNC state, but that virulence is retained and infection can be initiated following their resuscitation to the actively metabolizing state. Sun *et al*. (2008), for example, found that VBNC cells of *Vibrio harveyi* ceased to express the hemolysin gene and did not cause death when inoculated into zebra fish. However, resuscitated cells were lethal, indicating that VBNC *V. harveyi* cells retained pathogenic potential. Similarly, Oliver & Bockian (1995) reported *V. vulnificus* to lose virulence for mice in proportion to the length of time that the cells were in the VBNC state. The cells retained virulence, however, and even when fully nonculturable, were able to cause fatal infections, with resuscitation occurring within the mouse. Continued virulence for a variety of pathogenic vibrios has also been demonstrated by Baffone *et al*. (2003).

Antibiotic resistance of VBNC cells

One of the interesting and significant consequences of entry of pathogens into the VBNC state includes its effects on antibiotic resistance when pathogens are in this state (often in biofilms). As noted by del Mar Lleo *et al*. (2007), '. . . antibiotics which are highly active on growing cells do not necessarily act on VBNC (i.e. quiescent) cells'. It seems likely that, because VBNC cells demonstrate such low metabolic activity, they effectively become resistant to antibiotics, and yet are able to resuscitate and reinitiate infections. For example, Ehrlich *et al*. (2002) reported that antibiotic-resistant VBNC cells of *Haemophilus influenzae* present in biofilms are able to initiate chronic ear infections. In some reports, antibiotic resistance is exceptional. Vancomycin was reported to be effective against VBNC cells of *E. faecalis* only when at 500 times the MIC (Lleò *et al*., 2007a), and Anuchin *et al*. (2009) observed drastic increases in resistance to hydromycin and doxycyclin in dormant cells of *Mycobacterium smegmatis* compared with 48-h cultures. Another example is *H. pylori*, which produces gastric and duodenal ulcers, which are among the most widespread and common syndromes in the world (Kusters *et al*., 2006), and was shown by Adams *et al*. (2003) to rapidly enter the VBNC

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state when present in natural waters. Bates *et al.* (2003) subsequently reported that VBNC *H. pylori* cells are antibiotic resistant, which likely accounts for the frequent reinfections suffered by persons who undergo remission despite antibiotic treatment. Similarly, studies by Rivers & Steck (2001), following the work of previous investigators (Dominque *et al.*, 1995; Mulvey *et al.*, 2001), provided evidence suggesting that uropathogenic *E. coli* cells, typically not detected by standard methods, were not eliminated by antibiotic treatment. The recurrent urinary tract infections suffered by many individuals are thus likely a result of cells in this temporarily dormant state, which are able to resist antibiotic treatment and resuscitate back to the metabolically active state. Subsequent studies by Anderson *et al.* (2004) documented that VBNC cells were present not only in mouse but also in human urine specimens.

As a possible alternate strategy for becoming antibiotic resistant, Mason *et al.* (1995) reported a study in which over 90% of culturable *E. coli* cells were treated with 10 or 100 times the MIC of ciprofloxacin, and yet retained membrane potential and protein synthesis. The authors suggested that the results demonstrated a 'quinolone-induced VBNC state' in which the cells might be capable of continued pathogenesis.

Attachment and persistence of VBNC cells in the environment

Whether or not VBNC cells remain capable of attaching to surfaces appears to be species or strain dependent. Duffy & Dykes (2009) reported continued attachment by *Campylobacter jejuni*, and Cappelier *et al.* (1999b) observed this species to adhere to HeLa cells after resuscitation. In contrast, Lleò *et al.* (2007b) reported that while VBNC cells of several medically important *Enterococcus* spp. do not form biofilms on medical device materials, they continue to synthesize exopolysaccharides for a limited time, supporting their contention that such cells retain a public health risk. Signoretto *et al.* (2004) did report, however, that adhesion of nonculturable *E. faecalis* cells to plankton is an important mechanism for its persistence in aquatic environments, and Islam *et al.* (1994) found *Vibrio cholerae* O1 strains associated with freshwater cyanobacteria in Bangladesh. Rahman *et al.* (2001) concluded that VBNC cells of *Aeromonas hydrophila* can persist in aquatic environments for prolonged periods of time, although they gradually lose virulence to fish. Indeed, although data have not been reported on many species, it appears that cells may remain in the VBNC state for long periods. We found that *Pseudomonas fluorescens* cells can remain in this state in soil for over a year (Bunker *et al.*, 2004), and Amel *et al.* (2008) showed that *Vibrio fluvialis* could be resuscitated 6 years after becoming VBNC in marine sediment. Unfortunately, how resistant VBNC cells are to environmental stresses has not been well studied. Wong & Wang (2004), studying *V. parahaemolyticus*, reported that VBNC cells of this human pathogen were 'highly resistant to thermal (42 °C, 27 °C), low-salinity (0% NaCl), or acid (pH 4.0) inactivation'. Weichart & Kjelleberg (1996) reported that VBNC *V. vulnificus* cells exhibited an initial sonication sensitivity similar to that of growing cells, but that resistance increased with increased cold incubation, with final resistance equaling that of starved cells. Rowe *et al.* (1998) reported that VBNC cells of *C. jejuni* were significantly more sensitive to a variety of the quaternary ammonium compounds commonly used in food-processing operations compared with culturable cells, but that the VBNC cells were more resistant to chlorine than were culturable cells. Finally, Anuchin *et al.* (2009) reported dormant forms of *M. smegmatis* to show elevated resistance to heat (up to 80 °C).

Gene expression by VBNC cells

Some of the most exciting studies in the desire to understand the biology of cells in the VBNC state have been elucidated by investigations into various molecular aspects of these cells. This is exemplified by the use of RT-PCR to demonstrate continued gene expression in VBNC cells (e.g. Lleò *et al.*, 2000, 2001). In addition to providing an essential insight into factors regulating the VBNC state, such studies have offered definitive proof that such cells remain metabolically active and are not dead. A few examples of such RT-PCR studies are described here. Yaron & Matthews (2002) found that a variety of genes, including *mobA*, *rfbE*, *stx1* and those for 16S rRNA synthesis, were expressed in nonculturable *E. coli* O157:H7 cells. Similarly, Barrett (1998) and Saux *et al.* (2002) 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. Pai *et al.* (2000) found continued expression of antigen 85B in *M. tuberculosis*, and Gunasekera *et al.* (2002) reported the expression of *gfp* in VBNC cells of *E. coli* and *Pseudomonas putida* following pasteurization. Such findings are strong indications of viability, but whether or not the genes reported to be active are essential to the VBNC process is not known.

In a highly significant study, Vora *et al.* (2005) combined RT-PCR and a 90-plex PCR amplification scheme with microarray hybridization to analyze VBNC cells of *V. cholerae* O1, *V. parahaemolyticus* O3:K6 and *V. vulnificus* that had been incubated in artificial seawater (ASW) at 4 °C to induce the VBNC state. They reported that the '... unambiguous detection of mRNA species in each of the three nonculturable ASW cultures confirmed that (1) these bacteria were indeed VBNC, (2) bacteria in the VBNC state could be detected in this manner and (3) although VBNC, these strains continued to express known toxin (*ctxAB*, *rtxA*, *hlyA*, *tl*, *tdh* and *vvhA*) and virulence (*tcpA* and TTSS) genes, thus retaining their pathogenic potential'. Similarly,

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Pommepuy *et al.* (1996) showed retention of enteropathogenicity by VBNC *E. coli* cells through their continued production of enterotoxin. Thus, even when not actively multiplying, if a pathogen in the VBNC state is producing toxin, a potential public health concern exists. Virulence of pathogens in the VBNC state is reviewed in considerable detail in Oliver (2005a).

Using the membrane diffusion chambers pioneered by McFeters & Stuart (1972), we have been able to study *in situ* gene expression by various pathogens incubated in their natural aquatic environments. For example, we reported *H. pylori* cells to become nonculturable in a freshwater stream (Adams *et al.*, 2003), and subsequently that they maintained the expression of *murG*, a glycosyltransferase (B. Adams & J.D. Oliver, unpublished data). This enzyme has been shown by Signoretto *et al.* (2002) to be required in the late stages of peptidoglycan assembly in *E. faecalis* cells entering the VBNC state, and may explain continued production of this enzyme in nonculturable cells. We also observed peptidoglycan synthesis and expression of the virulence factors CagA, VacA and UreA by *H. pylori* for at least 32 h while in this state (B. Adams & J.D. Oliver, unpublished data). These studies are in agreement with Nilsson *et al.* (2002), who showed the expression of these same virulence factors *in vitro* using nonculturable *H. pylori* cells. Such data provide further evidence that not only does this pathogen enter into and persist in the VBNC state in the environment, but that it likely remains infectious.

In subsequent *in situ* studies on the human pathogen, *V. vulnificus* (Jones & Oliver, 2009), we found continued expression of the various genes examined for as long as 4.5 days when the cells were in warm coastal waters (Smith & Oliver, 2006a; Jones *et al.*, 2008). In contrast, when cells in three separate studies were incubated *in situ* in coastal waters with temperatures from 8 to 11 °C, several genes, including the hemolysin/cytolysin gene (*vvhA*) and two capsule-related genes (*wza* and *wzb*), ceased to be expressed in some strains as the cells entered the VBNC state (Smith & Oliver, 2006b). Regardless of whether the cells remained culturable or entered the VBNC state, continued expression of the major stress σ factor, RpoS, was observed (for as long as 14 days). This is consistent with the findings of Boaretti *et al.* (2003), who reported RpoS to be involved in the persistence of *E. coli* in the VBNC state.

Especially interesting in our *in situ* studies was our finding on expression of the *katG* gene (regulator of catalase production) and its role in resuscitation (see Why do cells enter the VBNC State?).

Resuscitation of cells from the VBNC state

The VBNC state can only be a significant means of survival if the cells are able to increase metabolic activity and again become culturable. Proving that true resuscitation of cells from the VBNC state occurs (as opposed to simple regrowth of a few undetected and culturable cells present in the VBNC population) has been problematic and a source of much of the disagreement concerning the validity of a VBNC state among bacteria (Barcina & Arana, 2009; Sachidanandham & Gin, 2009). While numerous investigations have reported on this aspect of the VBNC state, resuscitation has been most extensively studied in *V. vulnificus* (first reviewed by Oliver, 1995). The most common VBNC-inducing factor for many vibrios (as well as many other genera) is a simple temperature downshift. For example, *V. vulnificus* enters this state in response to temperatures of *c.* 10 °C. Numerous studies have found that a simple reversal of this stress (e.g. a temperature upshift) is sufficient to allow their resuscitation from the VBNC state (Gupte *et al.*, 2003; Wong *et al.*, 2004; Du *et al.*, 2007a, b). Indeed, in *V. vulnificus*, this has been demonstrated *in vivo* in clams (Birbari *et al.*, 2000) and in mice (Oliver & Bockian, 1995), *in situ* in estuarine waters (Oliver *et al.*, 1995), as well as *in vitro* (Nilsson *et al.*, 1991; Wolf & Oliver, 1992). Similarly, Bates & Oliver (2004) found that temperature down- and upshifts controlled the VBNC state in *V. parahaemolyticus*, with neither the presence nor the absence of the Kanagawa hemolysin genes (*tdh1* and *tdh2*) having any effect on entry into or resuscitation from this state.

That a temperature upshift resulted in true resuscitation, as opposed to regrowth of undetected culturable cells, was shown conclusively by Whitesides & Oliver (1997). Recently, Abe *et al.* (2007) described a mutant of *V. vulnificus* that remained culturable at a low temperature. Suppression subtractive hybridization studies revealed that glutathione *S*-transferase was the responsive gene, which was highly expressed in the mutant at a low temperature.

Interestingly, several higher organisms may be biological mediators of resuscitation from the VBNC state. For example, *L. pneumophila* has been reported to enter the VBNC state following both starvation and hypochlorite treatment, but to *resuscitate* in the protozoans *Acanthamoeba polyphaga* (Garcia *et al.*, 2007) and *Acanthamoeba castellanii* (Steinert *et al.*, 1997). In another study, *A. castellanii* was found to *induce* the VBNC state in *A. hydrophila* (Rahman *et al.*, 2008). In a different system, Sussman *et al.* (2003) reported the association of VBNC cells of the coral pathogen, *Vibrio shiloi*, with a marine fireworm. Other conditions found to allow resuscitation of pathogens include inoculation into yolk sacs of embryonated eggs (Cappelier *et al.*, 1999b, 2007), into mice (Cappelier *et al.*, 1999a) and into human volunteers (Colwell *et al.*, 1996). Thus, a wide variety of bacterial–host associations may have special value in influencing the survival of bacteria, including those in the VBNC state.

Another exciting development in the reactivation of dormant cells is the role of a group of extracellular bacterial proteins, known as 'resuscitation-promoting factors' (Rpfs), which have been shown to induce resuscitation in

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M. tuberculosis and *M. smegmatis* (Mukamolova *et al.*, 1998a, 2002; Shleeva *et al.*, 2004). Studies by Mukamolova *et al.* (2006) indicate that at least some Rpfs are peptidoglycan hydrolases, involved in cell wall digestion and thus cell division (Hett *et al.*, 2007). Thus, again, peptidoglycan rearrangement (c.f. Signoretto *et al.*, 2000, 2002) appears to be prominent in the VBNC story. Rpf-like compounds have been identified in several other genera (Mukamolova *et al.*, 1998b; Hett *et al.*, 2007), and although not yet widely studied as an aspect of resuscitation, such activity may be common in the resuscitation of other genera. Most recently, Kana *et al.* (2008) showed that the Rpfs of *M. tuberculosis* are required not only for resuscitation but also for virulence. Interestingly, these authors also reported that the Rpfs were not required for *in vitro* growth.

Another 'class' of resuscitation factors was reported by Reissbrodt *et al.* (2002). This was described as a heat-stable 'autoinducer of growth', which was secreted by a variety of gram-positive and (primarily) gram-negative bacterial species when incubated in media containing the human catecholamine hormone, norepinephrine (Freestone *et al.*, 1999). Norepinephrine is produced in large amounts in humans following severe tissue injury, and is thus considered to be a stress-related hormone. The bacterial growth stimulation observed in the presence of this hormone appeared to be due to non-nutritional factors (Lyte *et al.*, 1996). These showed a high degree of cross-species activity, and appeared to be a family of signaling molecules (Freestone *et al.*, 1999). Subsequently, Sperandio *et al.* (2003) identified the factors to represent a novel quorum-sensing system, which they termed AI-3. Because both epinephrine and norepinephrine could substitute for AI-3 in activating enterohemorrhagic *E. coli* virulence gene expression, and the effects of AI-3 and epinephrine/norepinephrine could be blocked by adrenergic receptor antagonists, they suggested that these compounds have a similar structure (Sperandio *et al.*, 2003). Reissbrodt *et al.* (2002) found that the autoinducers present in the spent media of various bacteria resulted in resuscitation from the VBNC state of several strains of *Salmonella enterica* serovar Typhimurium and of two *E. coli* O157:H7 strains. The findings of Reissbrodt *et al.* (2002) and of Sperandio *et al.* (2003) would appear to have major implications for the resuscitation of enteropathogens from the VBNC state, especially those occurring in the human intestinal tract, at a time (e.g. tissue damage) when the host may be under significant physiological stress.

Why do cells enter the VBNC state?

It has been recognized for many years that H_2O_2 might play a significant role in inducing the VBNC state in a variety of bacteria, including *E. coli* (Mizunoe *et al.*, 1999). We produced a catalase (*katG*)-negative mutant of *V. vulnificus* through inactivation of the *katG* regulator *oxyR*, which was thus incapable of degrading potentially fatal H_2O_2 (Kong *et al.*, 2004). Whereas *V. vulnificus* enters the nonculturable state when exposed to temperatures $< 13\,^{\circ}C$, the mutant cells were nonculturable on solid media *even at room temperature*. This observation suggested that one aspect of the VBNC state in this pathogen likely involves H_2O_2, either following its production by the cells when plated onto solid media, or its natural presence in solid media, coupled with an inability of the cells to detoxify this lethal metabolite. However, if cells entering into the VBNC state were plated onto conventional laboratory media (e.g. heart infusion agar) supplemented with peroxide-neutralizing agents (e.g. catalase), considerably enhanced culturability was seen (Fig. 2). We subsequently found that low temperature (the VBNC-inducer in this bacterium) prevents both catalase activity and its *de novo* synthesis (Fig. 3), rendering the cells highly sensitive (and nonculturable) to the peroxide present in culture media. Thus, low-temperature incubation resulted in cells that, due to this cold shock response, are nonculturable and yet remain viable.

Using the membrane diffusion chambers described above, we extended these studies by examining the effects of low temperature on culturability and the expression of the catalase gene (*katG*) in *V. vulnificus* when the cells were incubated in natural estuarine waters. Under these *in situ* conditions, we observed that *katG* underwent a demonstrable decrease in expression within as little as 30 min at $11\,^{\circ}C$ (Fig. 4). During these cold-temperature studies, the cells of all three *V. vulnificus* strains examined entered the VBNC state ($< 0.1\,CFU\,mL^{-1}$) within 14 days, while the

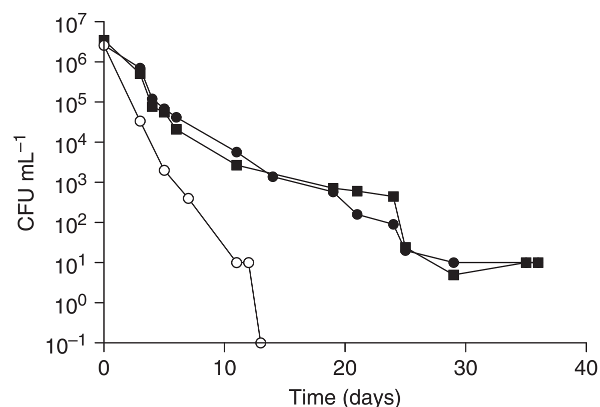

Fig. 2. Effect of anti-ROS agents on the culturability of *Vibrio vulnificus* following low-temperature incubation. Cells were incubated in ASW at $5\,^{\circ}C$ for periods up to 36 days, with plating onto heart infusion (HI) agar (-○-) or HI agar supplemented with catalase (-●-) or pyruvate (-■-). Whereas cells entered the VBNC state by 12 days, as indicated by culture on unsupplemented HI agar, continued culturability was observed under the same conditions when either of the H_2O_2-degrading agents was present in the medium. Reproduced from Kong *et al.* (2004).

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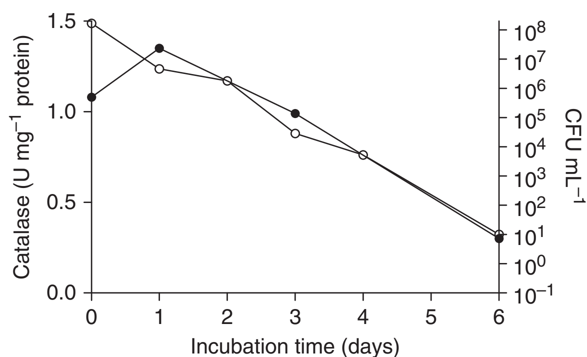

Fig. 3. Catalase activity and culturability of *Vibrio vulnificus* incubated at 5 °C. Cells were incubated in ASW at 5 °C, and examined for culturability on heart infusion agar (-○-) and for catalase activity (-●-) over time. A concomitant loss of catalase activity was observed as culturability decreased. Reproduced from Kong *et al*. (2004).

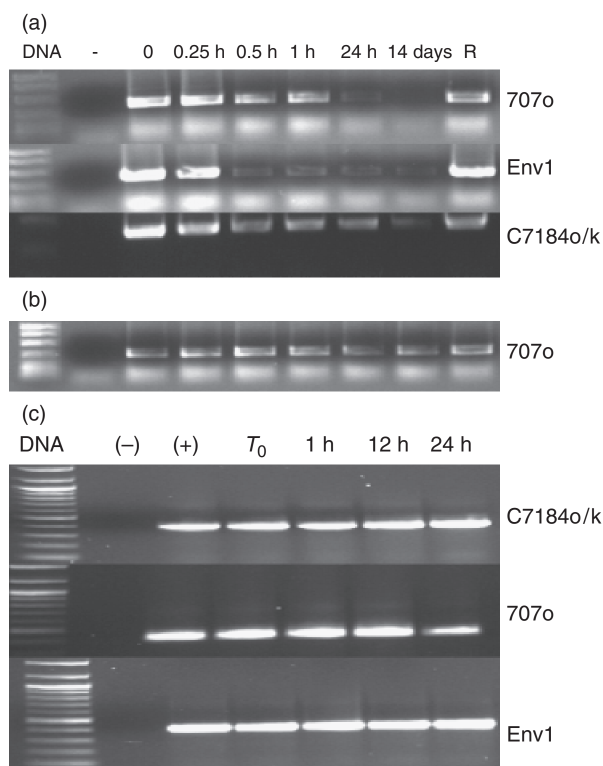

Fig. 4. Detection of *katG* mRNA in three *Vibrio vulnificus* strains during *in situ* incubation in estuarine waters during cold and warm months. (a) Incubation for up to 14 days (time of study indicated in hours or days) at a water temperature of 11 °C, and following resuscitation ('R'; 24-h room temperature upshift of the 14-day VBNC cells). (b) *In situ* expression of *tuf*A in *V. vulnificus* under the same conditions (11 °C). (c) Incubation for up to 24 h in estuarine water at 21 °C. Reproduced from Smith & Oliver (2006a, b). Copyright © American Society for Microbiology.

total and viable (*Bac*Light® Live/Dead) direct microscopic counts remained elevated. Following a temperature upshift to *c.* 22 °C for 24 h, all three strains resuscitated to levels above 10^4 CFU mL^{-1}. Resuscitation also resulted in renewed and full expression of *katG*. That peroxide is critical to the VBNC response in *V. vulnificus* has also been documented recently by Abe *et al.* (2007). This appears, to date, to be the only bacterium for which a molecular basis of the VBNC state has been described.

Concluding remarks

The exact role of the VBNC state in bacteria is yet to be elucidated. It is likely that its role and significance differ from bacterium to bacterium. Most investigators believe it to be a response to certain environmental stresses that allows the cell's survival. In some, for example *V. vulnificus*, entry into this dormancy state appears to be simply one aspect of the much-studied and complex 'cold shock response' that bacteria exhibit (Phadtare, 2004), with nonculturability being an 'artifact' of the sensitivity of such cells to the toxic peroxide present in laboratory media. Cells of *V. vulnificus* may be quite 'content' in their natural estuarine environment, albeit with decreased metabolic rates, as long as they are not subjected to laboratory culture! Or, as proposed by Barcina & Arana (2009), the VBNC state may be an intermediate in an altruistic death process that is part of a survival strategy. Or, as recently suggested by Epstein (2009), dormancy, and 'waking up' from this state, could be a method analogous to 'sending out scouts' to 'test the environment' for its suitability for growth of the entire population. In this scenario, if the resuscitating cells 'detect' that the previously stressful/adverse environment is now growth-permissive, they would signal the remaining cells to resuscitate.

Regardless of the role that the VBNC state plays, it is clear that a large number of non-spore-forming bacteria, most notably a large number of human pathogens, are capable of entering this state, maintaining cellular structure and biology and continuing significant gene expression while otherwise nonculturable by 'standard' laboratory methods. That they can exit from this state, and become culturable again, is also undeniable. Finally, it can no longer be questioned that the VBNC state plays a critical role in the survival of important human (and other) pathogens, and possibly in their ability to produce disease.

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