Induction of viable but nonculturable state in *Vibrio parahaemolyticus* and its susceptibility to environmental stresses

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**ABSTRACT**

H.C. WONG AND P. WANG. 2004. Aims: This work analysed factors that influence the induction of viable but nonculturable (VBNC) state in the common enteric pathogen, *Vibrio parahaemolyticus*. The susceptibility of the VBNC cells to environmental stresses was investigated.

Methods and Results: Bacterium was cultured in tryptic soy broth-3% NaCl medium, shifted to a nutrient-free Morita mineral salt-0.5% NaCl medium (pH 7.8) and further incubated at 4°C in a static state to induce the VBNC state in 28–35 days. The culturability and viability of the cells were monitored by the plate count method and the *Bac* Light viable count method, respectively. Cells grown at the optimum growth temperature and in the exponential phase better induced the VBNC state than those grown at low temperature and in the stationary phase. Low salinity of the medium crucially and markedly shortened the induction period. The VBNC cells were highly resistant to thermal (42, 47°C), low salinity (0% NaCl), or acid (pH 4.0) inactivation.

Conclusions: Optimal conditions for inducing VBNC *V. parahaemolyticus* were reported. The increase in resistance of VBNC *V. parahaemolyticus* to thermal, low salinity and acidic inactivation verified that this state is entered as part of a survival strategy in an adverse environment.

Significance and Impact of the Study: The methods for inducing VBNC *V. parahaemolyticus* in a markedly short time will facilitate further physiological and pathological study. The enhanced stress resistance of the VBNC cells should attract attention to the increased risk presented by this pathogen in food.

**Keywords:** environmental stress, viable but nonculturable state, *Vibrio parahaemolyticus*.

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**INTRODUCTION**

*Vibrio parahaemolyticus*, a halophilic gram-negative bacterium, causes acute gastroenteritis in humans. This bacterium is a prevalent food-borne pathogen in Japan, Taiwan, and other coastal countries (Joseph *et al.* 1983; Chiou *et al.* 1991; Pan *et al.* 1997). Most clinical isolates are haemolytic on Wagatsuma agar (Kanagawa phenomenon-positive, KP+) and produce a major virulence factor, thermostable direct haemolysin (TDH) (Joseph *et al.* 1983).

*Vibrio parahaemolyticus* inhabits warm seawater, and has been frequently isolated from fish, clams and crustaceans (Wong *et al.* 1992). This bacterium is also absorbed onto chitin particles and phytoplanktons (Kaneko and Colwell 1975a), and is commonly isolated from water samples collected in the near sea, and less frequently, in the open sea. When the temperature of the seawater is below 13–15°C, *V. parahaemolyticus* is seldom isolated (Kaneko and Colwell 1974; Kaneko and Colwell 1975b). Like other vibrios that inhabit similar marine environments (Oliver *et al.* 1995), *V. parahaemolyticus* may be present in a viable but nonculturable (VBNC) state during the winter, and could not be cultured on agar medium.

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The VBNC state may represent a dormant form that enhances the survival of nonsporulating bacteria in an adverse environment (Colwell 2000). VBNc bacteria exhibit increased resistance to ethanol and mechanical stresses (Weichart and Kjelleberg 1996). The VBNC state of some pathogenic bacteria maintains virulence (Rahman et al. 1996). Accordingly, VBNC state of pathogenic bacteria in food is potentially harmful to public health and should be considered at the time of food inspection.

*Vibrio parahaemolyticus* in natural and food processing environments often experience cold, low salinity and nutrient deprivation. These conditions are known to induce the VBNC state in several other bacteria (Oliver et al. 1991) and thus could also induce the VBNC state in *V. parahaemolyticus*. VBNC *V. parahaemolyticus* has been developed by starving cells at low temperature (Jiang and Chai 1996), but has not been well characterized. This investigation analyses factors that influence the induction of VBNC *V. parahaemolyticus* and establishes the optimal conditions for further investigation, to provide more information about the VBNC state of this pathogen. The susceptibility of VBNC *V. parahaemolyticus* cells to thermal, low salinity or acidic inactivation is examined.

**MATERIALS AND METHODS**

**Cultures and media**

*Vibrio parahaemolyticus* ST550, a serotype O4 : K13 and KP+ clinical strain isolated in Thailand, was provided by T. Arai (Wong et al. 1998) and used to characterize the induction and resuscitation of VBNC state. Bacterial culture was stocked in culture broth with 10% glycerol at −85°C, and cultured in Tryptic Soy Broth Medium (TSB; Difco Laboratories, Detroit, MI, USA)-3% NaCl or Tryptic Soy Agar (TSA; Difco)-3% NaCl at 25°C.

**Induction of VBNC state**

A 100-μl aliquot of overnight culture was inoculated into 50 ml of fresh TSB-3% NaCl medium in a 250-ml Erlenmeyer flask, and incubated at 25°C with shaking at 110 rev min⁻¹ for 4-5 (mid-exponential phase) or 8 h (stationary phase). Bacterial cells were harvested by centrifugation at 6000 × g for 15 min, washed twice in modified Morita mineral salt solution (MMS-0.5% NaCl) which consisted of 5 g of NaCl, 0.8 g of KCl, 5.6 g of MgCl₂-6H₂O, 7.6 g of MgSO₄·7H₂O, 0.9 mg of FeSO₄·7H₂O, 1.54 g of CaCl₂·2H₂O, 0.1 g of Na₂HPO₄ and 1.21 g of Tris buffer (pH 7.8) per litre of deionized water to prevent the carry-over of nutrients (Novitsky and Morita 1976). The bacterial cells were suspended in the 250-ml Erlenmeyer flask that contained 100 ml of MMS-0.5% NaCl or MMS-3% NaCl medium at a concentration of about 10⁷ cells ml⁻¹, and incubated at 4°C in a static state, to induce the VBNC state. Bacteria were counted every 7 days.

The acidity and salinity of the MMS medium and conditions for preparing the bacterial inoculum were assayed for the induction of the VBNC state in *V. parahaemolyticus*. NaCl was added to make 1, 2 or 3% NaCl supplemented MMS media. The acidity of the MMS-0.5% NaCl medium was adjusted to pH 5.0, 6.0, 7.0 and 7.8 by adding 3 mol l⁻¹ HCl.

**Susceptibility of VBNC cells to stresses**

The VBNC *V. parahaemolyticus* cells were subjected to thermal inactivation at 42 or 47°C, and at different intervals, cells were harvested by centrifugation at 10 000 × g for 15 min, and viable cells were counted. The VBNC cells were harvested by centrifugation and resuspended in 0 or 0.5% NaCl MMS to determine the inactivation caused by low salinity. Also, these cells were harvested by centrifugation and resuspended in MMS-0.5% NaCl, which had been adjusted to pH 4.0 or 7.8 by 3 mol l⁻¹ HCl, to determine inactivation caused by mild acid treatment. The VBNC cells were challenged by various stresses for 0, 10, 20, 40 and 60 min, and viable cells were counted and survivability determined.

**Enumeration of bacteria**

The culturability of bacteria was determined by the plate count method. Decimal dilutions were obtained in MMS-0.5% NaCl at 4°C and plated on TSA-3% NaCl. The plates were incubated at 37°C for 16 h and the number of colonies were counted. Duplicate experiments were performed and the data were obtained as means of three determinations in each experiment.

The total number of viable cells was counted using LIVE/DEAD Bac Light Bacterial Viability Kits (Molecular Probe Inc., Eugene, OR, USA). The kits are used in an epifluorescent method to determine the number of viable cells of, and total bacteria in several bacteria (Naganuma 1996; Decamp et al. 1997; Boulos et al. 1999; Chaiyanan et al. 2001) including *V. parahaemolyticus* (Mizunoe et al. 2000). Two fluorescent dyes, SYTO 9 and propidium iodide, were prepared following the instructions of manufacturer, and equal volumes of both dyes were mixed. A quantity of 3 μl of mixed dyes was diluted into 1 ml of membrane-sterilized 0.5% NaCl solution. Cells were harvested by centrifugation and allowed to react with this mixed dye solution for 30 min in the dark. They were then mounted on a haemocytometric slide and counted using a Nikon Photo Head V-TP epifluorescence microscope (Nikon Corp., Tokyo, Japan). The viable cells were stained green and the dead cells stained red, and the viable cells
were counted. Data were obtained as means of counts determined in five randomly selected microscopic fields.

**Statistical analysis**
The data in each experiment were analysed using ANOVA at \( P < 0.05 \), with the SPSS for Windows Release 6.0 program (SPSS Inc., Chicago, IL, USA).

**RESULTS**

**Induction of VBNC culture**
The effect of bacterial growth phase, growth temperature, starvation of the bacterial cultures, the salinity and the acidity of the medium, on the induction of VBNC *V. parahaemolyticus*, were examined. When the bacterial cells in the mid-exponential phase or stationary phase were shifted to 4\(^\circ\)C in MMS-3% NaCl or MMS-0.5% NaCl, the culturable and viable cells were enumerated. The culturability of the mid-exponential phase culture dropped to less than 1 cell ml\(^{-1}\) after 56 days in MMS-3% NaCl, and this induction period was markedly faster than the stationary phase cells \((P < 0.05)\). After 56 days, about 10\(^5\) cells ml\(^{-1}\) remained culturable in the stationary phase cell (Fig. 1). In the MMS-0.5% NaCl medium, cells rapidly entered the VBNC state after 28–35 days, and the induction period was not significantly different in the mid-exponential phase or stationary phase cultures \((P > 0.05)\) (Fig. 2).

The growth temperature while preparing bacterial culture also influenced the induction of the VBNC state in *V. parahaemolyticus*. When the bacterial cells were cultured at 15, 25 or 37\(^\circ\)C in TSB-3% NaCl, they entered the VBNC state after 49, 35 or 35 days, respectively (Fig. 3).

![Fig. 1](image1.png) *Vibrio parahaemolyticus* cells enter the viable but nonculturable state from different culture phases in Morita mineral salt-3% NaCl medium at 4\(^\circ\)C. Mid-exponential phase cells (○, ◦) and stationary phase cells (■, □) were examined, and viable cell counts (open symbols) and plate counts (solid symbols) were determined. Plate counts are given in CFU ml\(^{-1}\)

![Fig. 2](image2.png) *Vibrio parahaemolyticus* cells enter the viable but nonculturable state from different culture phases in Morita mineral salt-0.5% NaCl medium at 4\(^\circ\)C. Mid-exponential phase cells (○, ◦) and stationary phase cells (■, □) were examined, and viable cell counts (open symbols) and plate counts (solid symbols) were determined. Plate counts are given in CFU ml\(^{-1}\)

![Fig. 3](image3.png) Induction of viable but nonculturable state by *Vibrio parahaemolyticus* grown at different temperatures. Bacterial cells were cultured at 15 (■, □), 25 (○, ◦) or 37\(^\circ\)C (■, □) until they entered mid-exponential phase and moved to Morita mineral salt-0.5% NaCl medium, and then incubated at 4\(^\circ\)C. Viable cell counts (open symbols) and plate counts (solid symbols) were determined. Plate counts are given in CFU ml\(^{-1}\)

Cells in the exponential phase were suspended in MMS-3% NaCl, incubated static at 25°C for 24 h before shifting to 4°C, and bacteria were counted to study the influence of starvation. Both the plate count and viable cell count remained at the original high level and were similar to each other, implying the inhibition of the VBNC state. The induction of the VBNC state also failed when the experiment was repeated in MMS-0Æ5% NaCl medium (data not shown).

Lowering the salinity of the medium markedly accelerated the induction of the VBNC state. When the experiment was performed in 0Æ5, 1Æ0, 2Æ0 or 3Æ0% NaCl-MMS medium, the VBNC state was observed after 28, 42, 42 or 56 days, respectively. The VBNC induction time was significantly shortened in 0Æ5% than in other tested levels of NaCl (P < 0Æ05) (Fig. 4).

The acidity of the medium also influenced the induction of the VBNC state. In the MMS-0Æ5% NaCl medium at pH 5, 6, 7 and 7Æ8, the VBNC state was attained after 21, 42, 42, and 35 days, respectively. At pH 5, approx. 10^3 cells ml^-1 were inactivated in the first week and the viability recovered afterwards, as monitored both by plate count and viability count (Fig. 5). In another experiment, all cells entered VBNC at 28 days in MMS-0Æ5% NaCl medium at pH 7Æ8 (Fig. 4). This experiment was also conducted in MMS-3% NaCl medium at pH 6Æ6 and 7Æ8, and the VBNC state was attained after 42 and 56 days, respectively (data not shown).

**Fig. 4** Induction of viable but nonculturable state by *Vibrio parahaemolyticus* in nutrient-free media with different salinities. Mid-exponential phase cells were resuspended in Morita mineral salt-0Æ5% NaCl medium that contained 0Æ5 (○, □), 1Æ0 (■, △) or 3Æ0% NaCl (▲, ◇) and then incubated at 4°C. Viable cell counts (open symbols) and plate counts (solid symbols) were determined. Plate counts are given in CFU ml^-1.

**Fig. 5** Induction of viable but nonculturable state by *Vibrio parahaemolyticus* in nutrient-free media with different acidities. Mid-exponential phase cells were resuspended in Morita mineral salt-0Æ5% NaCl medium adjusted to pH 5Æ0 (●, ○), 6Æ0 (▲, △), 7Æ0 (■, □) or 7Æ8 (▲, ◇) and then incubated at 4°C. Viable cell counts (open symbols) and plate counts (solid symbols) were determined. Plate counts are given in CFU ml^-1.

### Susceptibility of VBNC cells to stresses

The susceptibility of the mid-exponential phase culture and the VBNC culture of *V. parahaemolyticus* to several environmental stresses was compared. When these cultures were subjected to thermal inactivation at 42 or 47°C for 60 min, around 80% of the VBNC cells remained viable. The mid-exponential phase culture was more rapidly killed by thermal inactivation than the VBNC cells, and was more susceptible to inactivation at 47 than at 42°C (P < 0Æ05) (Fig. 6).

The mid-exponential phase culture of *V. parahaemolyticus* was highly sensitive to low salinity and was quickly inactivated by being suspended in sterile distilled water. Around half survived in the MMS medium without NaCl. The VBNC cells were highly resistant to low salinity and no significant decline in viability was observed. The survival of the VBNC cells in sterile distilled water, MMS medium with and that without 0Æ5% NaCl, was equally high (P > 0Æ05) (Fig. 7).

VBNC cells of *V. parahaemolyticus* were more resistant to acidic inactivation than the mid-exponential phase culture; 80% of the former survived after pH 4 challenge for 1 h,
while only around 50% of the latter survived (Fig. 8). When the acidic challenge was prolonged to 3 days, the survival of the VBNC cells remained almost constant, while all of the exponential phase cells were killed (data not shown).

**DISCUSSION**

Strong arguments concerning the presence of a real VBNC state or just stress-injured cells that become nonculturable in a common growth medium have been presented (Bogosian and Bourneuf 2001). When injury in bacteria is simply defined as an increased sensitivity to constituents of growth media that are not normally inhibitory (Bogosian and Bourneuf 2001), the VBNC state can also be regarded as an injured state induced by mild environmental stress. However, the VBNC state usually accompanies a change of cell morphology from rods to cocci (Nilsson et al. 1991) and an increased resistance to environmental stresses, as demonstrated here. Common cell injury, meanwhile, frequently sensitizes cells to environmental stresses (Kalchayanand et al. 1992; Bogosian et al. 2000) without significant morphological changes (Mizunoe et al. 2000). Although occasionally confused with the nonculturable injured state, the VBNC state is better regarded as a specific phenomenon in which specific factors or regulons are activated, and are responsible for increasing the resistance to stress and morphological changes. As bacterial cells changed from rods to cocci, and the addition of catalase (Mizunoe et al.)

2000) to the plating medium did not significantly increase the culturable count (data not shown), a real VBNC state was induced in *V. parahaemolyticus* in this study.

Inducing the VBNC state in *V. parahaemolyticus* (Wolf and Oliver 1992) is known to be difficult. Nevertheless, incubating *V. parahaemolyticus* cultures in the nutrient-free MMS medium (containing 2.6% NaCl) at 35°C, induces the VBNC state in 50–80 days (Jiang and Chai 1996). Such a long induction time would make further physiological or pathological study of VBNC *V. parahaemolyticus* very time-consuming. In the present study, the period for inducing VBNC *V. parahaemolyticus* was significantly shortened and the optimal induction conditions were to culture the bacteria in TSB-3% NaCl medium at 25°C for 4.5 h until the mid-exponential phase, move the cells into MMS-0.5% NaCl medium (pH 7-8) and then further incubate them at 4°C in a static state for 28–35 days.

The culture history or physiological conditions of the bacterial cells affect the induction of VBNC *V. parahaemolyticus*. In the MMS-3% medium, with optimal salinity for growth, mid-exponential phase cells entered the VBNC state significantly (*P* < 0.05) more quickly than the stationary phase cells (Fig. 1). For *V. vulnificus*, cells in the stationary phase have been reported to require twice as long to become nonculturable than exponential phase cells. The production of stationary-phase-induced stress proteins is believed to protect the cells from entering the VBNC state (Oliver et al. 1992). VBNC *V. parahaemolyticus* was induced much more quickly in the present work than by Jiang and Chai (1996). This was probably attributable to the use of an induction medium with low salinity. Using inducing medium with a higher salinity (1–3%), caused the VBNC state to be induced after a considerably longer time (42–56 days) (Fig. 4) similar to the conditions used in the work of Jiang and Chai (1996). Salinity of 1–3% supports the growth of *V. parahaemolyticus*, and deviation from this range represents an environmental stress on this bacterium (Wong et al. 1998). Salinity also crucially influenced the induction of VBNC in *Escherichia coli*. However, the VBNC state in *E. coli* is induced in artificial seawater, but not in sterile, distilled water (Byrd et al. 1992). Also, the VBNC state in *Pseudomonas fluorescens* is induced by a high concentration of NaCl (1.0 or 1.7 M) but not by a low concentration (Mascher et al. 2000). NaCl is not normally required for the growth of *E. coli* or *P. fluorescens*. Thus, abnormal salinity is then a stress factor that could induce the formation of the VBNC state.

Typically, the VBNC state is efficiently induced in many bacteria by a combination of environmental stresses, frequently by starving cells in a nutrient-free medium while simultaneously incubating them at a low temperature (Oliver et al. 1991). Other VBNC state-inducing factors have also been reported, for example, HOCl stress in *E. coli* (Dukan et al. 1997), copper stress in *Agrobacterium tumefaciens* (Alexander et al. 1999), osmotic stress (Joergensen et al. 1994) and a combination of low redox potential and limited oxygen availability in *P. fluorescens* (Mascher et al. 2000). Duncan et al. (1994) reported extraordinary conditions for inducing VBNC *E. coli*, by starving the cells at 30°C, but not at 4°C. However, not all kinds of stress synergistically facilitate the induction. High hydrostatic pressure inactivates *V. parahaemolyticus* and several other *Vibrio* species, but does not induce the VBNC state (Berlin et al. 1999). Starving the cells before the temperature downshift is known to inhibit the formation of the VBNC state in *V. vulnificus* (Oliver et al. 1991) and some other bacteria (Chmielewski and Frank 1995; Mary et al. 2002). The interaction of various stresses on the induction of the VBNC state is complex and requires more studies.

The acidity of the induction medium may affect the induction of the VBNC state in a complicated way, and this factor has not been considered. The acidity of the medium at pH 7–8 is optimal for growing *V. parahaemolyticus*, and it is also the optimal pH for inducing the VBNC state in this study (Fig. 5). pH 6–9 and 7–0 are sub-optimal acidities for growth, that only slightly affect the survival of *V. parahaemolyticus* (Wong et al. 1998; Hasegawa et al. 2002). The reasons why the induction of the VBNC state took significantly longer at this sub-optimal acidity (mild stress) than at the optimal growth pH is unknown. Incubation at pH 5–0 certainly represents an environmental stress on this pathogen (Wong et al. 1998). The number of culturable and viable cells declined immediately after the pH of *V. parahaemolyticus* moved from 7–8 to 5–0. However, the viability was recovered in the following days (Fig. 5). Transient sublethal injuries may reasonably been caused by such abrupt acid stress, reducing the culturability and viability of the cells. The recovery of the viable count and culturability after 14 days is probably unrelated to the multiplication of cells, because *V. parahaemolyticus* growth is limited by salinity in the MMS-0.5% NaCl medium. As reported earlier (Wong et al. 1998), such acidic stress could trigger the acid adaptation process and activate the stress protection mechanism to repair the injured cells, at least enough to count them as viable cells, even though they cannot form a colony on an agar plate. In further studies should not induce VBNC *V. parahaemolyticus* at pH 5–0, and thus prevent the complicated effects of sharp acid injury.
The VBNC state is entered in a survival strategy of bacteria, especially in nutrient-limited and low temperature environments (Colwell 2000). These VBNC cells reasonably exhibit an increased resistance to various stresses. For *V. vulnificus*, the final resistance of VBNC cells to ethanol and ultrasonic stresses has been reported to equal the markedly increased resistance of starving cells (Colwell 2000; Mary et al. 2002). Berlin et al. (1999) demonstrated that the VBNC states of *V. parahaemolyticus* and several other *Vibrio* species are highly resistant to high hydrostatic pressures. This study demonstrated that the VBNC *V. parahaemolyticus* was more resistant to thermal inactivation (Fig. 6), low salinity (Fig. 7) and mild acid stresses (Fig. 8) than the exponential phase cell. Nearly 100% of VBNC *V. parahaemolyticus* survived after being treated in sterile water for 1 h, while the exponential phase cells were almost all killed (Fig. 7). The maintenance of the virulence of the VBNC *V. parahaemolyticus* was confirmed in our laboratory, although adhesion to the HEp-2 cells and the killing of mice was reduced or delayed by the VBNC cells (unpublished data). Thus, enhancing the resistance to stress and maintaining virulence in the pathogenic bacteria in the VBNC state raised the associated food hygiene risks.

In conclusion, optimal conditions for inducing VBNC *V. parahaemolyticus* were reported, and various parameters were considered. The resistance of VBNC *V. parahaemolyticus* to thermal, low salinity and acidic stress was markedly increased, increasing the risk associated with this pathogen in food.

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