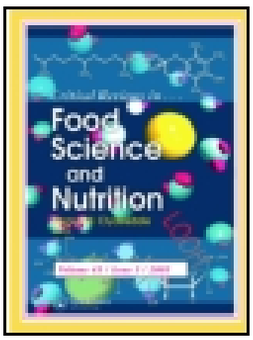

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REVIEW



# Characteristics of viable-but-nonculturable *Vibrio parahaemolyticus* induced by nutrient-deficiency at cold temperature

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**ABSTRACT**

*Vibrio parahaemolyticus* has been consistently found to be involved in the food-borne disease outbreaks every year. Particularly, *V. parahaemolyticus* can be induced into a viable but nonculturable (VBNC) state under cold-starvation conditions. In this physiological state, *V. parahaemolyticus* losses its colony-forming ability and shows reduced metabolic activities. The subsequent failure of its detection may threaten public health-hygiene practices. Until now, evident information on physiological properties of VBNC *V. parahaemolyticus* and its underlying mechanism remains unclear and unorganized. Therefore, this review summarized survival behavior, persistence, and entry of pathogenic microorganisms into a VBNC state in response to various environmental conditions and discussed distinctive characteristics of VBNC cells. To survive under unfavorable environments, VBNC *V. parahaemolyticus* shows marked modifications in cell membrane composition, fatty acid synthesis, morphology, metabolism, gene expression, and capability of adhesion and virulence. These physiological modifications might be closely associated with an imbalance in maintaining essential biological processes within VBNC cells, thereby causing a decrease in cell membrane fluidity. To develop an efficient surveillance method and to prevent the recovery of VBNC cells in food, the induction of a VBNC state needs to be profoundly understood.

**KEYWORDS**

cold-starvation; food safety; viable but nonculturable; *Vibrio parahaemolyticus*

## Introduction

Pathogenic *V. parahaemolyticus* strains have been recognized as one of the major causative agents involved in serious food-borne diseases and illnesses. In particular, clam, eel, mackerel, mussel, oyster, scallop, and shrimp have been considered as the most predominant vehicles for *V. parahaemolyticus*, as reflected in many food-borne outbreaks and recalls (Wong et al. 2004a; Wong et al. 2004b; Xu et al. 2014; Yu et al. 2013; Yue et al. 2010). Estuarine ecosystems such as coastal area (Hara-Kudo et al. 2003), seawater (Cabrera-García, Vázquez-Salinas, and Quiñones-Ramírez 2004) and sediment (Alipour, Issazadeh, and Soleimani 2012), and even raw vegetable (Tunung et al. 2010) have been identified to harbor large numbers of *V. parahaemolyticus*. There are fewer barriers protecting the consumers if contamination occurs in the food chain or during processing and handling because marine food products are frequently eaten raw or undercooked. Consumption of food products contaminated with pathogenic *V. parahaemolyticus* may be linked with various human infections, leading to severe clinical manifestations and symptoms, including acute gastroenteritis and septicemia (Nair et al. 2007). Indeed, consumption of raw or undercooked oysters contaminated with *V. parahaemolyticus* resulted in 104 cases and 6 hospitalizations in the United States in 2012 (Centers for Disease Control and Prevention 2013), as well as this pathogen accounted for 354 patients in the Republic of Korea in 2017 (Ministry of Food Drug and Safety in Republic of Korea 2018). Thus, concerns of hygiene and food processing that prevents or inactivates the growth of pathogenic or spoilage microorganisms are increasing to consumers.

The optimal growth temperature of *V. parahaemolyticus* ranges from 35 °C to 37 °C (Miles et al. 1997). Generally, *V. parahaemolyticus* can grow under osmotic environments at 1%-7% NaCl, with optimal rages of 2.5%-3.5% (Cheng, Juang, and Chen 2004). In phosphate buffered saline (PBS) containing 20% NaCl, *V. parahaemolyticus* was found to survive at 37 °C for more than 200 min, but viable cell numbers markedly decreased to ≤10% (Chiang, Ho, and Chou 2008). At pH levels between 5.5 and 12.5, *V. parahaemolyticus* continued to grow at 37 °C, with optimal growth pH ranges of 8.0-8.5 (Sun et al. 2014). Since *V. parahaemolyticus* is typically prevalent in important sources of food supplies commonly found in the food industry, a well-organized risk assessment program for detecting and identifying the occurrence of food-borne pathogens should be needed for ensuring food quality uniformly (Pinto et al. 2011). According to the laboratory methodologies provided by USFDA (2004) and National Institute of Food and Drug Safety Evaluation (2018), the cultivation-based methods are recommended to estimate the incidence of *V. parahaemolyticus*, using several

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selective media such as thiosulfate-citrate-bile salt-sucrose, and modified colistin-polymyxin B-cellobiose agar. Briefly, the identification approaches include stepwise procedures, including enrichment, isolation and enumeration, screening, and confirmation. Once serial diluents of samples re-suspended in either PBS or alkaline peptone water (APW) are incubated overnight at 35 °C, bacterial aliquots are then inoculated on the selective media, thereby enabling the enumeration of culturable *V. parahaemolyticus* cells. However, *V. parahaemolyticus* becomes nonculturable after several months of incubation in nutrient-depriving environments at ≤10 °C, limiting the conventional culture-based surveillance methods. Previously, formation and persistence of some bacteria into a dormant but viable state have been well-documented in several studies (Oliver 2010; Pinenaar, Singh, and Barnard 2016; Ramamurthy et al. 2014). Although important physiological and morphological changes in microorganisms during phase transition into a state of dormancy have been reported, the underlying mechanisms of the "viable but nonculturable (VBNC)" state remain unclear. Knowledge on the occurrence and potential hazard of the nonculturable form of *V. parahaemolyticus* is essential to better understand its stress response under various environmental conditions, which have critical implications for food safety.

## Entry of *V. parahaemolyticus* into a VBNC state

### *Induction of V. parahaemolyticus into a VBNC state*

A wide variety of microorganisms have been shown to be induced into a VBNC state when they exposed to certain adverse conditions, including *E. coli* O157:H7, *Listeria monocytogenes*, *Salm. enterica* spp., *Shigella dysenteriae*, *Shig. flexneri*, *Staph. aureus*, *V. parahaemolyticus*, and *V. vulnificus* (Highmore et al. 2018; Lin et al. 2017; Pasquaroli et al. 2013; Xu et al. 2018; Yoon, Bae, and Lee 2017). VBNC bacteria cannot be cultured on routine media that normally support their growth, but maintain their cellular integrity and reduced metabolic activities, including ATP synthesis and expression of gene, RNA, and transcript (Asakura et al. 2007; Chaiyanan et al. 2007; Dolezalova and Lukes 2015; Jia et al. 2014; Tholozan et al. 1999; Trinh et al. 2015). Pathogenic microorganisms in the VBNC state may represent a potential food safety hazard because VBNC cells are not detected by the culture-based surveillances. Peculiarly, *V. parahaemolyticus* enters a VBNC state upon exposure to cold-starvation challenge. Incubation at low temperatures between 4 °C and 13 °C induces *V. parahaemolyticus* and *V. vulnificus* into a VBNC state. Mizunoe et al. (2000) investigated the fate of *V. parahaemolyticus* VP190 under cold-starvation condition, showing that this pathogen was induced into a VBNC state in PBS at 4 °C for 12 days. *V. parahaemolyticus* NY477 also readily became VBNC when incubated in artificial sea water (ASW) microcosms at 4-5 °C for 5-7 days (Bates and Oliver 2004). Approximately more than 30 days of incubation were required for *V. parahaemolyticus* strains to enter a VBNC state in ASW microcosms at ≤10 °C (Baffone et al. 2003; Coutard et al. 2007a; Falcioni et al. 2008; Yoon, Bae, and Lee 2017). Furthermore, *V. parahaemolyticus* VP99 and *V. parahaemolyticus* WR1 strains declined slowly during the first 7 days, following prolonged incubation in a diluted ASW at 5 °C, but their colony counts remained culturable until day 20 (Bates and Oliver 2004). In ASW, *V. parahaemolyticus* was unculturable at 4 °C as determined by plate-counting on tryptic soy agar added with 3% NaCl ($TSA_S$) by day 7. When *V. parahaemolyticus* strains were starved in saline solution (SS) and Morita minimal salt broth (MMS) at 4 °C, the bacterial densities declined to below the detection limits within 30-35 days (Jia et al. 2014; Zhong et al. 2018) and 35-70 days (Falcioni et al. 2008; Jiang and Chai 1996; Lai et al. 2009; Wong et al. 2004a), respectively. According to some studies conducted by Yoon, Bae, and Lee (2017) and Chen (2012), the conversion of *V. parahaemolyticus* to a VBNC state was accelerated by the addition of NaCl at a high concentration. When *V. parahaemolyticus* was incubated in ASW microcosms supplemented with 10% NaCl at 4 °C, approximately 10-16 days were needed for the VBNC forms. Instead, cold-starvation in ASW microcosms containing <3% NaCl necessitated more than 30 days to induce a VBNC state. In our preliminary studies, *V. parahaemolyticus* ATCC 17802 dropped to below the detection limits (<1.0 log CFU/ml) in ASW (pH 6) microcosms with or without addition of ≤5% NaCl at 4 °C for 80 days, whereas the organism became undetectable in ASW (pH 6) supplemented with 10%-30% NaCl under cold-starvation conditions within at least 21 days (*unpublished*). In addition, the loss of culturability of *V. parahaemolyticus* was largely different, depending on the strain-to-strain variables. Especially, it was shown that while *V. parahaemolyticus* strains originated from the laboratory culture stocks took approximately 27.7 days to become VBNC, environmental or food-borne isolates were converted to such a stage of dormancy within 40.3 days. Clinical *V. parahaemolyticus* strains, which were shown to be closely implicated with certain infectious potentials (virulence and pathogenicity), entered a VBNC state within approximately 35.2 days at 3-5 °C. Similarly, *Vibrio cholerae* (Chaiyanan et al. 2007; Masmoudi, Denis, and Maalej 2010; Wai et al. 1996), *Vibrio shiloi* (Vattakaven et al. 2006), and *V. vulnificus* (Abe et al. 2007; Day and Oliver 2004; Linder and Oliver 1989; Nowakowska and Oliver 2013) were also shifted to a VBNC state upon exposure to cold-starvation, over a wide range of incubation-times ranging from 10 days to 235 days. Comprehensively, the findings indicated that several factors such as the use of culture media, NaCl concentration, and nutrient-availability may have an important impact on the formation of VBNC *V. parahaemolyticus*. In the most cases, environmental conditions such as nutrient-deprivation (starvation) and cold temperature (less than 10 °C) contributed to the VBNC forms of *V. parahaemolyticus*.

### *Incidence of VBNC microorganisms in food*

As well-documented in a review of Huss, Reilly, and Embarek (2000), seafood products are generally processed by various physico-chemical methods in the food manufacturing lines. Fresh materials have been sealed hermetically,

heated moderately at 70-90 °C for 2-10 min, and chill-stored to ensure food quality and safety. For preparation of ready-to-eat products, raw samples have been held in saline solutions (<10% NaCl) for 10-12 weeks following a frozen step at −20 °C. Nicolò and Guglielmino (2012) determined that phase shift of pathogenic bacteria into the VBNC state can be triggered by the surrounding characteristics of food processing lines and chemo-physical properties of food dimensions, such as pH, redox potential, water activity, major composition, and others. As stated above, certain decontaminating strategies also initiated the conversion of microorganisms to the VBNC state. Supposedly, VBNC pathogens induced in natural habitats by fluctuating environmental stresses or on seafood would cause cross-contamination during harvest, handling, processing, and storage (Ayrapetyan and Oliver 2016; Fakruddin, Mannan, and Andrews 2013). In shrimp, *V. parahaemolyticus* was not detectable with the plate-counting method, but polymerase chain reaction (PCR) in combination with denaturing gradient gel electrophoresis (DGGE) confirmed the abundance of *V. parahaemolyticus*, though the suggested detection tools need to be further verified for its ability to distinguish from live and dead cells of bacteria (Liao et al. 2014). Zhang, Dong, and Ross (2017) also implied the occurrence of VBNC *V. parahaemolyticus* on sand shrimp during cold-storage. Although the formation and persistence of VBNC microorganisms in food during any processing stage is not clearly understood, previous studies indicated that some pathogenic bacteria and fermentation-associated yeasts can be induced into a VBNC state on vegetables and beverages (Table 2), as evidenced by quantitative real-time PCR or flow cytometry assays in combination with permeable fluorescence probes. Capozzi et al. (2016), Millet and Lonvaud-Funel (2000), Serpaggi et al. (2012), and Salma et al. (2013) showed the transient shift of fermentation-associated microorganisms such as acetic acid bacteria (AAB), *Brettanomyces bruxellensis*, and *Saccharomyces cerevisiae* BDY4742 into a VBNC state in synthetic wine products during wine storage. In common, $SO_2$ used for preserving wine quality might be a critical factor for inducing some microorganisms into the VBNC state. Interestingly, *B. bruxellensis* LO2E2 persisted in a VBNC state for several days and was recovered to a culturable state immediately after $SO_2$ removal from the synthetic wine microcosm by nitrogen entrainment (Serpaggi et al., 2012). As VBNC cells are no longer culturable, the persistence of VBNC pathogens on food may threaten public health seriously. According to Jang et al. (2007), *Campylobacter jejuni* W1 cells were transformed to a VBNC state in modified Brucella broth under aerobic condition at 4 °C, 25 °C, and 37 °C for 10, 16, and 26 days, respectively. Then, VBNC cells were strongly attached to chicken skin and persisted for several hours. It was also demonstrated that when *E. coli* was inoculated on vegetables at 8 °C, this organism lost its colony-forming ability on media and was proven to enter a VBNC state within 15 days (Dinu and Bach 2013; Dinu and Bach 2011). Moreover, either thermal inactivation processing such as thermosonication or exposure to sunlight may trigger the formation of VBNC cells in *Helicobacter pylori* and *Salm*. Typhimurium on acidified carrot juice and spinach (Buck and Oliver 2010; Liao et al. 2018). The results indicated that pathogenic or spoilage microorganisms might be present in the dormant but viable state on food products during preparation and processing steps, given that their viable numbers seemed almost unchanged or slightly reduced under stressful environments. Taken together, the persistence of pathogens in the VBNC state either in natural environments or food materials may be a critical public health concern due to the lack of their detectability with

**Table 1.** Entry of *V. parahaemolyticus* cells into a VBNC state.

| **Strain**[1] | Environmental condition | **Microcosm**[2] | Temp | Period | Reference |
|---|---|---|---|---|---|
| AT | Cold-starvation | SW | 4°C | 40 days | Zhong et al. (2018) |
| AT | Cold-starvation | ASW | 4°C | 30 days | Yoon, Bae, and Lee (2017) |
| AT | Cold-starvation + 10% NaCl | ASW | 4°C | 10 days | |
| NC | Cold-starvation + 3% NaCl | ASW | 4°C | 35 days | Chen (2012) |
| NC | Cold-starvation + 5% NaCl | ASW | 4°C | 35 days | |
| NC | Cold-starvation + 10% NaCl | ASW | 4°C | 16 days | |
| CL | Cold-starvation | MMS | 4°C | 35 days | Wong et al. (2004a) |
| CL | Cold-starvation | MMS | 4°C | 42 days | Lai et al. (2009) |
| CL | Cold-starvation | ASW | 4°C | 11-24 days | Coutard et al. (2007a) |
| CL | Cold-starvation | ASW | 4°C | 16 days | Coutard et al. (2007b) |
| CL | Cold-starvation | ASW | 4°C | 32 days | Coutard et al. (2005) |
| CL | Cold-starvation | MMS | 4°C | 35-58 days | Wong and Wang (2004) |
| CL | Cold-starvation | MMS | 4°C | ≤49 days | Wong et al. (2004b) |
| ID | Cold-starvation | MMS | 4°C | ≤35 days | |
| ID | Cold-starvation | ASW | 5°C | 30 days | Baffone et al. (2003) |
| ID | Cold-starvation | ASW | 4°C | 69 days | Falcioni et al. (2008) |
| ID | Cold-starvation | SS | 4°C | <30 days | Jia et al. (2014) |
| ID | Cold-starvation | ASW | 5°C | 7-8 days | Bates and Oliver (2004) |
| CL | Cold-starvation | ASW | 5°C | 5-12 days | |
| ND | Cold-starvation + lower nutrient concentrations | $\frac{1}{5}$ASW[3] | 5°C | 20 days | |
| ND | Cold-starvation | PBS | 4°C | 12 days | Mizunoe et al. (2000) |
| CL | Cold-starvation | MMS | 4°C | 50 days | Jiang and Chai (1996) |
| ID | Cold-starvation | MMS | 4°C | 70 days | Jiang and Chai (1996) |

[1]AT, ATCC strain; CL; clinical strain; ID, isolated strain from environment or seafood samples; NC, NCTC strain; ND, not determined.
[2]APW, alkaline peptone water; ASW, artificial sea water; MMS, Morita minimal salt broth; PBS, phosphate buffered saline; SS, saline solution; SW, sea water.
[3]In this study, cells of *V. parahaemolyticus* were incubated in $\frac{1}{5}$-diluted ASW microcosm at 5 °C.

conventional laboratory culture methods. VBNC cells could retain their infectivity to human beings upon elimination of the putative stress-related agents (some VBNC pathogens reverted to their culturable forms after passing through the digestive intestinal tract of animals), posing a potential health risk (Fakruddin, Mannan, and Andrews 2013).

### Relevance of ROS and the VBNC state

Preliminarily, the accumulation of reactive oxygen species (ROS) has been identified as one of the major factors affecting the biological responses of *C. jejuni*, *E. coli* O157:H7, *Erwinia amylovora*, *Photorhabdus temperata*, *Staph. aureus*, *V. parahaemolyticus*, and *V. vulnificus* to various environmental conditions, showing that ROS was linked to the loss of culturability and formation of VBNC cells (Abe et al. 2007; Jallouli, Zouari, and Jaoua 2010; Klančnik et al. 2009; Kong et al. 2004; Masmoudi, Denis, and Maalej 2010; Munna et al. 2013; Noor, Murata, and Yamada 2009; Santander, Figas, and Biosca 2018; Wong et al. 2004a; Wong et al. 2004b). ROS compounds such as hydrogen peroxide ($H_2O_2$), hydroxyl free radical (ʹOH), and superoxide anion(O2(−)) penetrate across the cell wall and membrane under aerobic conditions, thereby degrading nucleic acids, proteins, and lipids (McDougald et al. 2002; Nakashima et al. 2010). ROS mainly attacks polyunsaturated fatty acids and proteins in cell membrane, causing a decrease in membrane fluidity (Cabiscol, Tamarit, and Ros 2000). Microorganisms encounter various toxic ROS compounds in aerobic environments and evolve specific survival mechanisms to neutralize ROS. In particular, there are several antioxidant defense systems in *V. parahaemolyticus* cells, each of which is used to protect themselves against ROS, including alkyl hydroperoxide reductase (Ahp), catalase, glutathione peroxidase, glutathione-S-transferase, peroxidase, and superoxide dismutase (Lai & Wong 2013; Wang et al. 2013). As shown in Table 3, the progression of the VBNC state was delayed by ROS-detoxifying compounds, including catalase and sodium pyruvate. *V. parahaemolyticus* VP 190 was nonculturable in ASW at 4 °C for 12 days as determined by the plate-counting on Luria-Bertani agar (LBA) (Mizunoe et al. 2000). When the cultivable populations were enumerated using LBA supplemented with ROS-degrading agents, *V. parahaemolyticus* VP 190 did not enter into a VBNC state until day 16. After 21 days of incubation at 4 °C, *E. coli* O157:H E32511/HSC dropped to below the detection limits on LBA; however, its culturability exceeded approximately 3.5 and 4.0 log CFU/ml on LBA supplemented with 2,000 U catalase and 0.1% sodium pyruvate, respectively (Mizunoe et al. 1999). During cold-starvation, the entry of *V. vulnificus* C7184 into a VBNC state was extended to 11 days by the addition of catalase to heart infusion agar (Kong et al. 2004).

Some decontamination strategies such as plasma jet (Dolezalova and Lukes 2015) and thermosonication (Liao, Jiang, and Zhang 2017; Liao et al. 2018) were correlated with VBNC forms of *Salm*. Typhimurium. The physical methods were known to directly generate ROS compounds

**Table 2.** Occurrence of the VBNC forms of microorganisms on food products.

| Microorganism | Major stress | Microcosm | Temp | Period | Reference |
|---|---|---|---|---|---|
| *V. parahaemolyticus* | Low temperature | Shrimp | 4-10°C | ND[1] | Liao et al. (2015) |
| *V. parahaemolyticus* | Low temperature | Sand shrimp | 4°C | <2 weeks | Zhang, Dong, and Ross (2017) |
| Acetic acid bacteria | ≤50 mg/l $SO_2$ | Red wine | ND | 2 days | Millet and Lonvaud-Funel (2000) |
| *B. bruxellensis* | ≤1.2 mg/l $SO_2$ | Synthetic wine (pH 3.5) | 28°C | 11 days | Capozzi et al. (2016) |
| *B. bruxellensis* LO2E2 | 0.8 mg/l $SO_2$ | Synthetic wine (pH 3.5) | 28°C | 2-4 days | Serpaggi et al. (2012) |
| *C. jejuni* W1 | Aerobically, *C. jejuni* W1 cells were transformed to a VBNC state in modified Brucella broth at 4°C, 25°C, and 37°C for 10, 16, and 26 days, respectively. The VBNC cells were strongly attached to chicken skin and persisted. | | | | Jang et al. (2007) |
| *E. coli* O157:H7 ATCC 43895 | Low temperature | Lettuce and spinach | 8°C | 7-9 days | Dinu and Bach (2013) |
| *E. coli* O157:H7 BRMSID | Low temperature | Lettuce | 8°C | 15 days | Dinu and Bach (2011) |
| *H. pylori* ATCC 43504 | Sunlight | Baby spinach | 25°C | <2 days | Buck and Oliver (2010) |
| *Sac. cerevisiae* BDY4742 | 4.5 mg/l $SO_2$ | Synthetic wine (pH 3.5) | 28°C | <30 days | Salma et al. (2013) |
| *Salm.* Typhimurium CMCC 50115 | Thermosonication (380 W) | Carrot juice (pH 4.5) | 53-62°C | 6-10 min | Liao et al. (2018) |

[1]ND, not determined.

or free radicals (carbon-centered and hydroxyl radicals or hydrogen proton) during processing, thereby altering the interior structure and composition in bacterial cells. When *Salm*. Typhimurium CMCC 50115 was inactivated in carrot juice by thermosonication (380 W) at 53 °C for 6 min, this organism was inducted into a VBNC state (Liao et al. 2018). Under the same conditions, *Salm*. Typhimurium cells remained culturable at levels of $10^3$ log CFU/ml in carrot juice added with 100 mM sodium pyruvate. The results were in agreement with the findings of Jallouli, Zouari, and Jaoua (2010), Liao, Jiang, and Zhang (2017), Liao et al. (2018) and Pinto et al. (2011), who showed that the formation of VBNC cells was largely delayed in the presence of various ROS-scavenging agents. It can be suggested that as a cell's defense capacity against active ROS compounds malfunctions or is perturbed, *V. parahaemolyticus* may respond to some membrane injuries caused by ROS through reversible modifications of morphology and physiological properties (Ferro, Amorico, and Deo 2018). Cell membrane might be a major site of ROS-induced degradation under cold-starvation conditions. Lai and Wong (2013) showed that there were no significant differences in the amounts of ROS produced under respective stressful conditions such as low salinity (0.6% NaCl), low temperature (4 °C), and starvation. However, combined sublethal stresses (low salinity + low temperature or low salinity + low temperature + starvation) induced significantly higher ROS productions at levels of 242-352 fluorescent unit (FU) per log CFU in *V. parahaemolyticus* cells. It should be noted that the respective sublethal stresses such as low temperature and starvation did not affect survivals of *V. parahaemolyticus*, whereas this organism was reduced to below the detection limits when treated with their combinations for 24 h. While treatment of *Salm. Enteritidis* with 3-10 mM hydrogen peroxide caused a complete loss of its colony-forming capability but retained its respiration activity, resulting in the formation of VBNC cells at 37 °C within 60 min, the addition of pyruvate and α-ketobutyrate postponed the induction of VBNC forms (Morishige, Fujimori, and Amano 2013). Along with the results of Dolezalova and Lukes (2015) and Zhang et al. (2015), ROS compounds may be one of the most promising VBNC-inducing factors in bacterial cells, and if bacterial cells are unable to defend themselves against toxic ROS compounds generated either intra- or extracellularly under unfavorable environmental conditions, it would facilitate the formation of VBNC cells. It is noteworthy that correlations between cold-starvation and membrane potential in VBNC cells were reported by Tholozan et al. (1999), who determined that VBNC *C. jejuni* strains induced in natural lake water at 4 °C for 30 days showed the dramatically reduced membrane potentials (tetra[$^3$H]phenylphosphonium bromide; TPPB) at 2-14 mV; the membrane potential of the stationary-phase cells ranged from 54 to 79 mV. Similarly, *Micrococcus luteus* became VBNC in lactate (0.01%) minimal medium at 4 °C, and VBNC cells had a decreased membrane potential, as evidenced by quantitative flow cytometry with the Rhodamine 123 probe, which is an indicator of intact and viable cells (Kaprelyants and Kell 1992). Upon exposure to 0.05% $H_2O_2$ in PBS at 15 °C for 2 days, VBNC *E. coli* O157 strain MP37 showed a significantly decreased expression of oxidative stress responsive parameters, including Ahp subunits C and F, dihydrolipoyl-lysine-residue-acetyltransferase subunit F, and a peptidyl-prolylcis-trans isomerase A (Asakura et al. 2007). If bacteria underwent a phase transition to the VBNC state temporarily, membranes became less fluid, with an intracellular leakage of $K^+$ from

**Table 3.** Effects of chemical additives (supplementations) on the entry of microorganisms into a VBNC state.

| Microorganism | Environmental condition | Microcosm[1] | Temp | Agar media | Period (days)[2] Before | Period (days)[2] After | Reference |
|---|---|---|---|---|---|---|---|
| *V. parahaemolyticus* VP190 | Cold-starvation | PBS | 4°C | LBA + catalase | 12 | 16 | Mizunoe et al. (2000) |
| | Cold-starvation | PBS | 4°C | LBA + sodium pyruvate | 12 | 16 | |
| *E. coli* | Oxidative stress | NB + 3 mM $H_2O_2$ | 37°C | NA | Culturable | 2 | Munna et al. (2013) |
| *E. coli* | Cold-starvation | M9 | 4°C | TSA + sodium pyruvate | 4-22 weeks | 9-29 weeks | Pinto et al. (2011) |
| *E. coli* O157 | Oxidative stress | PBS + 0.05% $H_2O_2$ | 4°C | TSA + sodium pyruvate | 6 h | 72 h | Asakura et al. (2008) |
| *E. coli* O157 MP37 | Oxidative stress | PBS + 0.05% $H_2O_2$ | 4°C | TSA + sodium pyruvate | 6 h | 24 h | |
| *E. coli* O157:H E32511/HSC | Cold-starvation | DW | 4°C | LBA + catalase | 21 | ≥27 | Mizunoe et al. (1999) |
| | Cold-starvation | DW | 4°C | LBA + pyruvate | 21 | ≥27 | |
| *Photo. temperata* K122 | ND[3] | OM | 30°C | LBA + catalase | 55 h | 90 h | Jallouli, Zouari, and Jaoua (2010) |
| *Salm*. Typhimurium CMCC 50115 | Thermosonication | BPY + 91 mM sodium pyruvate | 53°C | SSA | 25 min | Cultivable[4] | Liao, Jiang, and Zhang (2017) |
| *Salm*. Typhimurium CMCC 50115 | Thermosonication (380 W) | Carrot juice (pH 4.5) + 100 mM sodium pyruvate | 53°C | SSA | 6 min | Cultivable | Liao et al. (2018) |
| *V. vulnificus* C7184 | Cold-starvation | ASW | 5°C | HIA + catalase | 3 | 11 | Kong et al. (2004) |

[1]ASW, artificial sea water; BPY, beef peptone yeast broth; DW, deionized water; NB, nutrient broth; OM, optimized medium; HIA, heart infusion agar; LBA, Luria-Bertani agar; NA, nutrient agar; PBS, phosphate buffered saline; TSA, tryptic soy agar; SS, Salmonella-Shigella agar.
[2]Periods that microorganisms were required to enter a VBNC state.
[3]ND, not determined.
[4]Under the same conditions, each of bacteria was still culturable at levels of $10^{1\text{-}3}$ log CFU/ml in microcosm added with sodium pyruvate.

the cytoplasm (Ramamurthy et al. 2014; Trevors, van Elsas, and Bej 2013). After 30 days of starvation at 4 °C, VBNC *C. jejuni* cells had at least $10^2$-fold decreases in the internal K + concentrations (Tholozan et al. 1999). As potassium is known be an important biological indicator of the maintenance of cell turgor pressure and intracellular pH homeostasis, its export from the cytoplasm might be interpreted as a sign of fluctuating cell fluidity. VBNC cells had less molecular crowding and leakage of cellular compounds such as amino acid, protein, and $Mg^{2+}$ to the external environments (Trevors, van Elsas, and Bej 2013). Certain fluorescent probes such as N-phenyl-1-naphthylamine (NPN), TPPB, and Rhodamine-123 are used to determine the structure and function of biological membranes (Table 4). In our preliminary studies, *V. parahaemolyticus* cells showed an increase in fluorescence due to the partitioning of NPN uptake into the outer membrane when entering a VBNC state in ASW at 4 °C for 30 days. Interestingly, essential oils (EOs) introduced the formation of VBNC bacteria, instead of pore formation and cell lysis on the membranes. EOs have been proven to inactivate pathogenic bacteria by perturbing membrane potentials, depleting intracellular ATP concentrations, and reducing intracellular pH levels (Bouhdid et al. 2010; Tholozan et al. 1999). According to a study conducted by Bouhdid et al. (2010), cinnamon EO at 1.5X minimal inhibitory concentration allowed *Staph. aureus* ATCC 29213 to enter a VBNC state within 60 min, showing an increase in the intracellular $K^+$ leakage from the cytoplasm or membrane depolarization ranging from 0.89% to 6.50%. This result was in agreement with a study by Trinh et al. (2015), wherein decreasing levels of membrane potential, unsaturated fatty acid, and cell fluidity caused by EOs contributed to the induction of a VBNC state, leading to a temporary increase in cytoplasmic membrane rigidity.

## Characteristics of VBNC *V. parahaemolyticus*

### Morphology

Figure 1 depicts the morphological characteristics of bacteria in the VBNC state. Under prolonged starvation conditions at 4 °C, VBNC *V. parahaemolyticus* ATCC 33844 had less molecular crowding and unorganized cytoplasmic membranes (Figure 1B and C) in comparison with the actively growing cells grown in $TSB_S$ at 37 °C (Figure 1A). In particular, the morphological transition of *V. parahaemolyticus* from a rod shape to a coccoid form progressed rapidly with increasing NaCl concentrations at 4 °C, and there were numerous vesicles in the cytoplasmic matrix and spatial gaps between the outer and inner membranes in VBNC *V. parahaemolyticus* cells. The irregularly-shaped cells appeared when *V. parahaemolyticus* VP190, *E. coli* K12, and *V. cholerae* O139 were induced into a VBNC state (Figure 1H–I). Chen et al. (2009) and Mizunoe et al. (2000) found that the bacterial cells become coccoid with the formation of membrane blebs after the onset of the VBNC forms. Oxidative stress was found to induce not only a VBNC state, but also a morphological transition in *C. jejuni* cells (Oh, McMullen, and Jeon 2015). A majority of VBNC cells were changed from helical rods to rounded forms upon exposure to an aerobic environment in Mueller-Hinton broth for 12 h. Linder and Oliver (1989) observed that VBNC *V. vulnificus* contained significantly reduced densities of ribosome and nucleic acid. The VBNC state was also accompanied by a significant reduction in cell size; the length of VBNC cells was shorter than the exponential-phase cells (Chen et al. 2009; Falcioni et al. 2008; Pienaar, Singh, and Barnard 2016; Yoon, Bae, and Lee 2017). Moreover, the aberrantly-shaped forms of VBNC cells were induced by various environmental stresses. VBNC *V. tasmaniensis* cells changed into the coccal forms with the thicker cell walls and aggregated nucleic acids after 150 days of cold-starvation (Figure 1D) (Vattakaven et al. 2006). Hung, Jane, and Wong (2013) showed the bulged cells of VBNC *V. parahaemolyticus* strain 1137, which were induced in MMS at 4 °C for 12 h. The cell wall was shown to become considerably loosened and flexible (Figure 1E). As shown in Figure 1F, VBNC *Salm.* Enteritidis zSE1 cells were elongated more than twice over the pure cultures upon exposure to 3 mM $H_2O_2$ at 37 °C for 24 h (Xu et al. 2018). In a study conducted by Chen et al. (2009), VBNC *V. parahaemolyticus* cells displayed the enlarged (densely stained) peripheral layer, shrunken (lightly stained) cytoplasm, and indented membrane (Figure 1J). The changes in the interior structure of VBNC cells may be involved in reduced cell volume, which results from the

**Table 4.** Changes in membrane potential of VBNC microorganisms.

| | | | Result | | |
|---|---|---|---|---|---|
| Microorganism | VBNC-inducing condition[1] | Membrane potential[2] | Normal cell | VBNC cell | Reference |
| *V. parahaemolyticus* | Cold-starvation | NPN uptake | 1,737 RFU | 2,284-2,503 RFU | In our preliminary studies (*unpublished*) |
| | | Leakage of protein | 0.521-0.910 | 1.588-2.351 | |
| *C. jejuni* | Cold-starvation | TPPB | 54-79 mV | 2-14 mV | Tholozan et al. (1999) |
| | | Leakage of intracellular $K^+$ | 115.0-170.1 mM/l | 1.5-2.3 mM/l | |
| *E. coli* | Plasma jet | Leakage of DNA | VBNC cells always showed comparatively higher levels of DNA leakage. | | Dolezalova and Lukes (2015) |
| *L. innocua* | EO | Membrane depolarization | VBNC cells always showed comparatively higher levels of membrane depolarization (%). | | Trinh et al. (2015) |
| *M. luteus* | Cold-starvation | Rhodamine-123 uptake | The fluorescence probe was accumulated across the VBNC cells. | | Kaprelyants and Kell (1992) |
| *Staph. aureus* | EO | Leakage of intracellular $K^+$ | ND[3] | 0.493 ppm | Bouhdid et al. (2010) |
| | | Membrane depolarization | 0.89% | 6.50% | |

[1]EO, essential oil.
[2]NPN, $_N$-phenyl-$_1$-napthylamine; TPPB, tetra[$^3$H]phenylphosphonium bromide.
[3]ND, not determined.

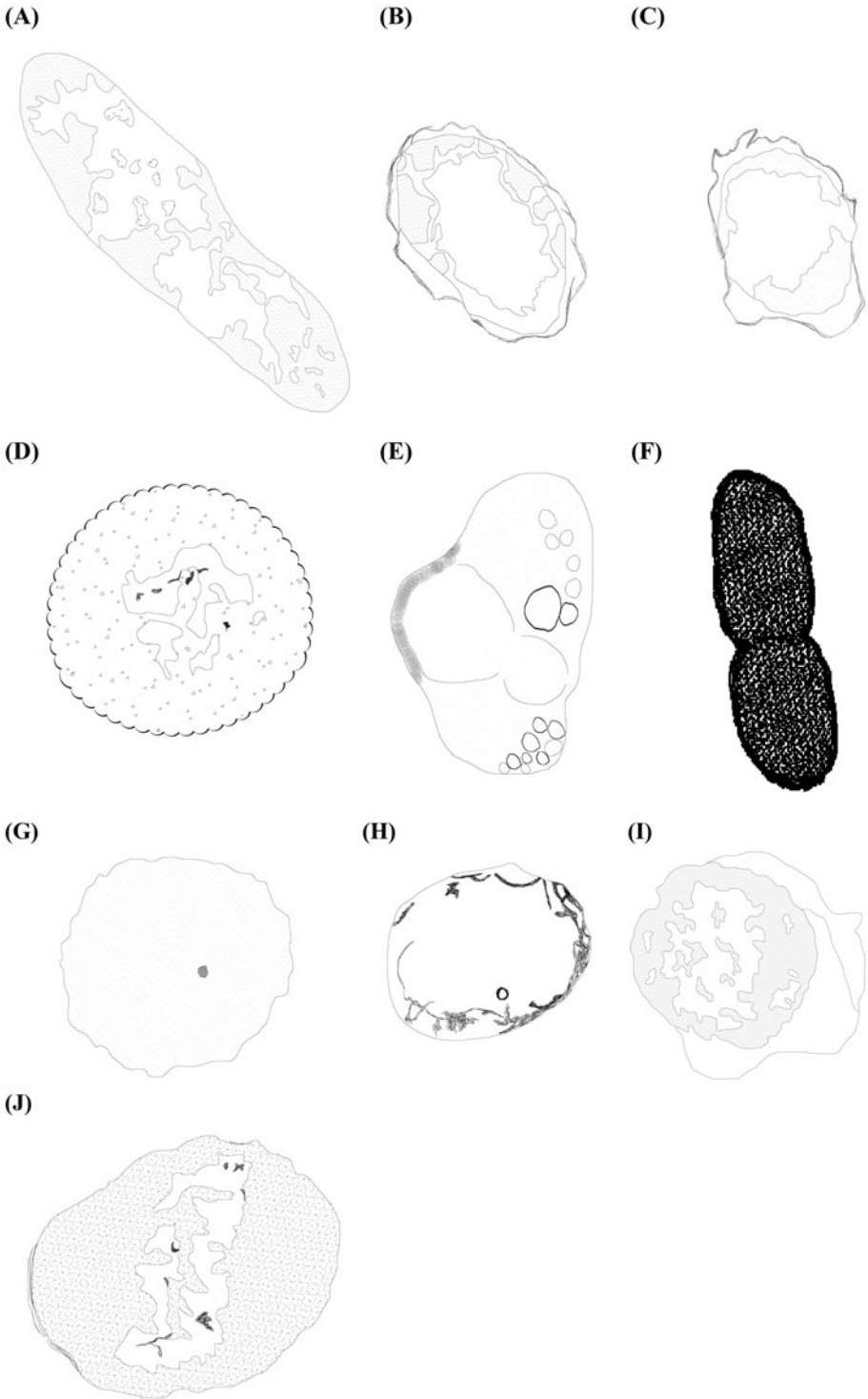


**Figure 1.** Morphological characteristics of VBNC-induced cells. A, *V. parahaemolyticus* ATCC 33844 grown in $TSB_S$ overnight at 37 °C; B, VBNC *V. parahaemolyticus* ATCC 33844 induced in ASW at 4 °C for 30 days; C, VBNC *V. parahaemolyticus* ATCC 33844 induced in ASW supplemented with 5% NaCl at 4 °C for 30 days; D, VBNC *V. tasmaniensis* induced in ASW at 4 °C for 150 days. E, VBNC *V. parahaemolyticus* induced in MMS at 4 °C for 12 h; F, VBNC *Salm.* Enteritidis induced on TSB added with 3 mM $H_2O_2$ at 37 °C for 24 h; G, *E. coli* K12 incubated in a saline solution added with 100 ug/ml rifampicin at 37 °C for 3 h; H, *E. coli* K12 incubated in a saline solution at 37 °C for 35 days; I, VBNC *V. cholerae* O139 induced in ASW at 4 °C for 60 days; J, *V. parahaemolyticus* incubated in 3M at 4 °C for 7 days. All these pictures used in this study were re-designed according to the results presented in previous publications (A-C, our preliminary studies; D, Vattakaven et al. (2006); E, Hung, Jane, and Wong (2013); F, Xu et al. (2018); G-H, Kim et al. 2018; I, Chaiyanan et al. (2007); J, Chen et al. (2009).

phenotypic modulations of fatty acid profile, hydrophobicity, and membrane potential. As shown in Figure 2, all these phenotype traits might correspond to a decrease in cell fluidity, linking to an adaptive strategy for minimizing cell maintenance requirement, including DNA amplification, protein translation, and nutrient transport (Alonso et al. 2002; Chaiyanan et al. 2007; Zhao et al. 2013). The results indicated that VBNC cells show a great variety of their morphological changes under various environmental conditions, but these data obtained from this study never mean that the

aberrantly-shaped cells are not the only traits of VBNC bacteria. Some bacterial cells also can change into the aberrantly-shaped morphologies similar to those of VBNC cells as a consequence of cold-shock process. Gram-negative bacteria respond to nutrient-deficient environments by reducing their cell volume and size, with the conversion of cell morphologies from rod to round and helical forms (Abdallah et al. 2011). Nevertheless, it would be inferred that such a marked modulation in the cell morphology may be caused by the rearrangement and maintenance of cell membrane structure and integrity during the evolution to the VBNC state.

### Fatty acid composition and cell fluidity

Changes in cell physiology and fatty acid composition may be accompanied by modified membrane fluidity, thereby leading to altered cell viability. Bacterial cells in the VBNC state might also be capable of changing their membrane fatty acid compositions in response to adverse environmental conditions, permitting sequential adaptive approaches to control cell functions (Figure 2A). In Table 5, the modification of fatty acid composition in *V. parahaemolyticus* and *V. vulnificus* was evaluated before and after the induction into a VBNC state. In a study conducted by Wong et al. (2004a), myristic acid ($_{C14}$), pentadecylic acid ($_{C15}$), palmitoleic acid ($_{C16:1}$), and oleic acid ($_{C18:1}$) were mostly abundant in VBNC *V. parahaemolyticus* ST550 cells. As compared with the actively growing cells, VBNC *V. parahaemolyticus* ST550 showed that while the concentration of pentadecylic acid was greatly increased to 10%, palmitoleic acid dropped from 40% to <20%. In our previous studies, VBNC *V. parahaemolyticus* ATCC 17802 induced at 4 °C for 80 days gave a rise to the amount of saturated fatty acids such as lauric acid ($_{C12}$), myristic acid, palmitic acid ($_{C16}$), and stearic acid ($_{C18}$), whereas the quantity of unsaturated fatty acids, including palmitoleic acid, $_{(7Z)\text{-}13}$-methyl-$_{7}$-hexadecenoic acid ($_{C17;1\ ante\text{-}iso\ w9c}$), and $_{cis}$-vaccenic acid ($_{C18:1\ w7c}$), were reduced. Jia et al. (2014) isolated two different strains of *V. parahaemolyticus* from marine products and found major differences in the fatty acid profile between the food-isolates after these bacteria entered into a VBNC state. In general, myristic acid, palmitic acid, palmitoleic acid, and oleic acid were the most predominant in VBNC *V. parahaemolyticus* I and II cells. Among the saturated fatty acids, lauric acid ($_{C12}$), pentadecylic acid, and marmaric acid ($_{C17}$) were lower in the former than in the latter; VBNC food isolates exerted dramatic decreases of approximately 11%-12% in the concentrations of palmitic acid. Saturated fatty acids such as pentadecylic acid and palmitic acid might be of much importance for the formation of VBNC *V. parahaemolyticus* in cold-starvation conditions. This finding was in accordance with those for VBNC *V. vulnificus* (Day and Oliver 2004; Linder and Oliver 1989). Palmitic acid has been shown to be involved in enhanced tolerances of *V. parahaemolyticus* to low pH (Chiang, Wu, and Chen 2014), ethanol (Chiang, Ho, and Chou 2008), and NaCl (Chiang, Yu, and Chou 2005). Nevertheless, it was determined that controlling the saturation and chain length of phospholipid affects membrane fluidity in microorganisms, and changes in fatty acid composition might contribute to the formation of VBNC cells (Day and Oliver 2004; Magnuson et al. 1993). Chiang, Wu, and Chen (2014) suggested that acid-adapted *V. parahaemolyticus* strains had significantly higher contents of saturated fatty acids such as lauric acid, myristic acid, pentadecylic acid, palmitic acid, marmaric acid, and stearic acid ($_{C18}$) than those of non-adapted cells, thereby leading to a decrease in cell membrane fluidity. The findings indicated that *V. parahaemolyticus* cells might be though to exhibit an adaptive response to prolonged duration of cold-starvation by changing their intracellular solute contents and/or modifying the cell membrane permeability (Figure 2A).

### Modulation of responsive proteins

Bacteria tend to represent a progressive biological response to a rapid temperature downshift in combination with starvation by regulating the expression of responsive proteins that are differentially identified only during the shift into the VBNC state. As microorganisms enter the VBNC state under various environmental conditions, new proteins are synthesized, or the expression of existing proteins is inhibited. Table 6 represents specific proteins identified during the induction of a VBNC state in pathogenic bacteria. Starved cells of *V. parahaemolyticus* F8-4 at 4 °C for 18 h showed that 69 proteins were significantly up-regulated, of which their functional categories were nucleotide transport, metabolism, transcription, and defense system (Tang et al. 2018). Moreover, the expression of homoserine kinase (HSK), glutamate-$_{5}$-kinase (or gamma-glutamyl kinase; GK), and cytochrome-$_{c}$-oxidase (COX) subunit I were highly increased in cold-shocked cells. HSK was shown to participate in the aspartate pathway involved in amino acid biosynthesis and increasing resistances to cold-shock in *E. coli* (Huo and Viola 1996). GK is known to be involved in the proline synthesis that is one of the major compatible osmoregulation solutes at a moderate saline solution in *B. cereus* (Vashishtha and Dhawal 2015). Previous studies indicated that COX is the terminal electron receptor of the respiratory chain in aerobic microorganisms (Ostermeier, Iwata, and Michel 1996) and controls the oxidative phosphorylation, preventing the accumulation of $H_2O_2$ (Sun et al. 2014). Besides, COX protein may increase the ability of *V. parahaemolyticus* to withstand in extremely harsh environments such as artificial gastric fluids (pH 4.9) (Tang et al. 2018). There were more than five-fold changes in the expression levels of oxidation-responsive factors and outer membrane proteins in *E. coli* O157:H7 cells that were induced into a VBNC state in PBS supplemented with 0.05% $H_2O_2$ at 15 °C for 3 days (Asakura et al. 2007). Under oxidative stress, VBNC *E. coli* O157H7 showed a significant decrease in AhpC, whereas outer membrane protein W (OmpW) was dramatically increased approximately 236-fold, over that for the unstressed control groups after the shift into a VBNC state. As AhpC proteins are a family of peroxidases generally

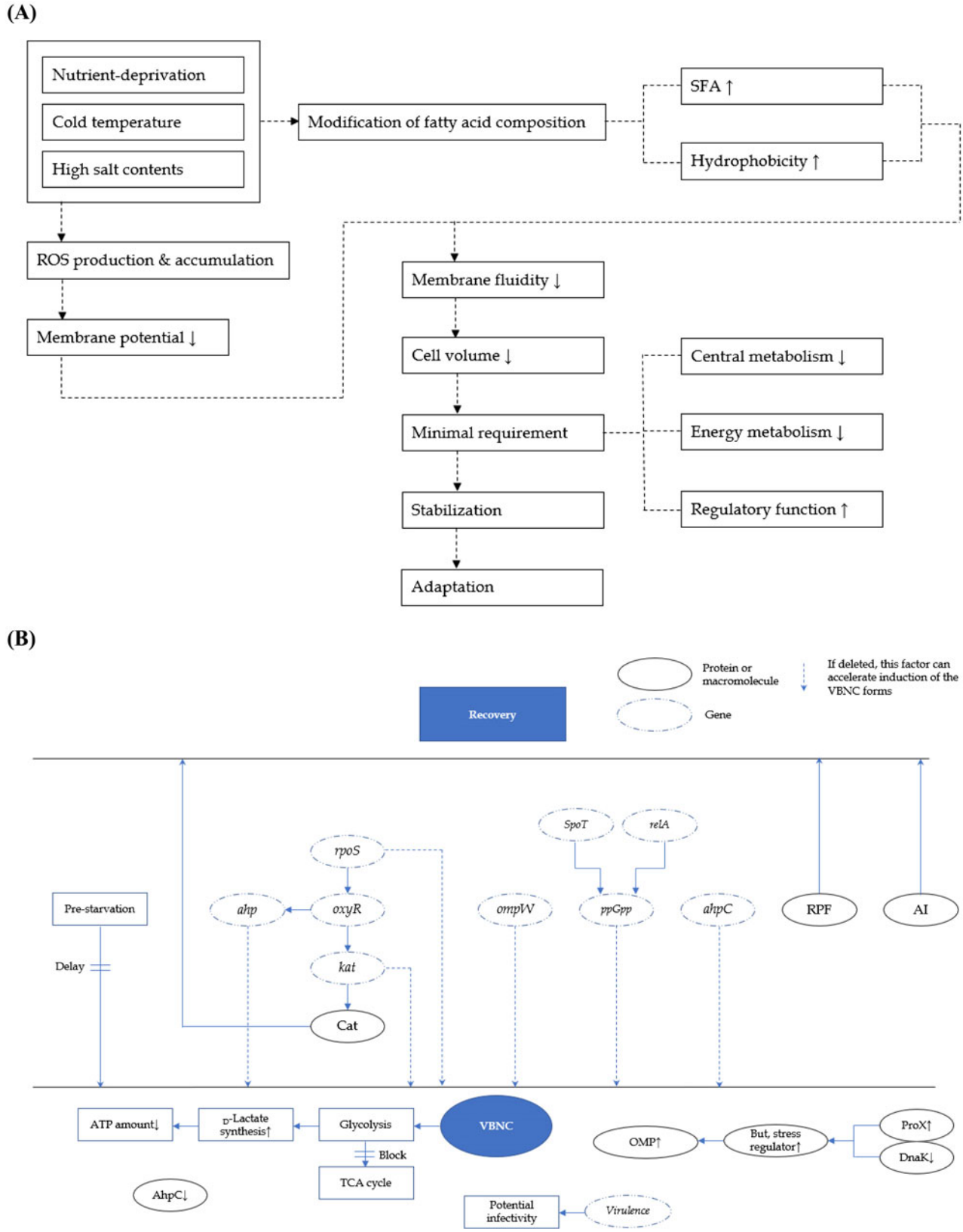


**Figure 2.** Representation of putative mechanistic processes (A) involved in the formation of VBNC *V. parahaemolyticus* and important factors affecting the VBNC forms (B).

regulated by OxyR (Charoenlap et al. 2005), the function of AhpC was identified for the detoxification of ROS compounds and maintenance of colony-forming ability in *V. parahaemolyticus* (Wang et al. 2013). OmpW and glycine betaine-binding protein (ProX) were abundant in VBNC *E. coli* O157 MP3 cells upon induction of oxidative stress (Asakura et al. 2008). Zhong et al. (2018) confirmed that while ProX was overexpressed in VBNC *V. parahaemolyticus*

**Table 5.** Comparison of fatty acid composition in VBNC *V. parahaemolyticus* and VBNC *V. vulnificus* cells induced under cold-starvation conditions.

| | *V. parahaemolyticus* | | | | *V. vulnificus* | |
|---|---|---|---|---|---|---|
| Fatty acid[1] | **ATCC 17802** | ST550 | Food-isolate I | Food-isolate II | C7184 I | C7184 II |
| $C_{10}$ | -[2] | - | △ (+1%) | △ (+1%) | - | - |
| $C_{12}$ | △ (+2%) | △* | ▼ (-1%) | △ (+1%) | △ (+25%) | - |
| $C_{13}$ | - | - | △ (+1%) | △ (+1%) | | - |
| $C_{14}$ | △ (+1%) | △* | △ (+1%) | △ (+2%) | | △ (+25%) |
| $C_{15}$ | - | △ (+10%) | ▼ (-3%) | △ (+2%) | | - |
| $C_{16}$ | △ (+3%) | △ (+4%) | ▼ (-12%) | ▼ (-11%) | ▼ (-22%) | ▼ (-42%) |
| $C_{17}$ | - | - | ▼ (-1%) | △ (+2%) | - | △ (+4%) |
| $C_{18}$ | △ (+1%) | UC[3] | △ (+1%) | ▼ (-1%) | ▼* | △ (+5%) |
| $C_{16:1}$ | ▼ (-2%) | ▼ (-25%) | ▼ (-1%) | ▼ (-3%) | ▼ (-37%) | △ (+12%) |
| $C_{16:1\ w6c}$ | △ (+3%) | - | △ (+4%) | UC | - | - |
| $C_{17:1}$ | - | - | UC | UC | - | - |
| $C_{17:1\ an\ w9c}$ | ▼ (-4%) | - | - | - | - | - |
| $C_{18:1}$ | - | △ (+5%) | △ (+7%) | △ (+5%) | - | ▼ (-4%) |
| $C_{18:1\ w7c}$ | ▼ (-3%) | - | - | - | - | - |

[1]an, ante-iso.
[2]-, not detected.
[3]UC, this value was rarely changed before and after the entry of microorganisms into a VBNC state.
[4]Reference; *V. parahaemolyticus* ATCC 17802, in our previous studies; *V. parahaemolyticus* ST550, Wong et al., 2004a; food isolates of *V. parahaemolyticus*, Jia et al. 2014; *V. vulnificus* C7184 I, Linder and Oliver 1989; *V. vulnificus* C7184 II, Day and Oliver 2004.
*This fatty acid was upregulated or downregulated at very small concentrations.

ATCC 17802 cells that were induced in natural seawater at 4 °C for 40 days, multi-drug efflux pump component (MtrF) was significantly down-regulated by the prolonged temperature downshift. The role of OmpW remains uncharacterized in microorganisms, but OmpW protein triggers a survival mechanism against osmotic stress (3%-4.5% NaCl) in *V. parahaemolyticus* (Xu et al. 2005) and participates in the iron homeostasis in *E. coli* (Lin et al. 2008). ProX is engaged in bacterial osmoregulation mechanisms via ProU transport system (Stirling et al. 1989). This enzyme also is implicated with quorum sensing signals regulated by LuxR in *Vibrio harveyi* (van Kessel et al. 2015). MtrF is a kind of cytoplasmic membrane protein that is needed for the export of hydrophobic compounds such as antibiotics, nonionic detergents, and antibacterial peptides across the cytoplasmic membrane in *Neisseria gonorrhoeae* (Shafer et al. 2001). In a spite of a strong increase in the expression of ProX and COX proteins, the overall ROS-scavenging ability of VBNC cells was notably weaker than that of the stationary-phase cultures. In addition, Muela et al (2008) showed that VBNC *E. coli* STCC 416 had a total of 68 modulated proteins, of which some were identified as outer membranes or cytoplasmic-associated subproteomes. Among them, protease VII, elongation factor, enolase, $_{D\text{-}3}$-phosphoglycerate dehydrogenase, and threonine synthase were specifically increased, whereas antigen 43 $\beta$ chain and outer membrane protein TolC (OmpTolC) were greatly decreased under starvation conditions. VBNC *Enterococcus faecalis* V583 exhibited the reduced quantities of proteins such as ATPS-$\beta$, ATP synthase, $\beta$-chain, DnaK, and enolase when incubated in natural lake water at 4 °C (Heim et al. 2002). After 12 days of incubation in artificial soil, VBNC *Cupriavidus metallidurans* CH34 showed a strong decrease in the expression of 117 proteins involved in cellular process and signaling, storage and processing, base metabolism, protein synthesis, and energy process (Giagnoni et al. 2018).

Meanwhile, it was found that pre-starvation at 25 °C extended the times required to enter a VBNC state under subsequent cold-starvation conditions. In a study conducted by Lai et al. (2009), *V. parahaemolyticus* ST550 was converted to a VBNC state at 4 °C for 42 days. However, pre-starved cells at 25 °C for 24 h were consistently culturable at levels of ≥5.0 log CFU/ml in MMS at 4 °C for 42 days. Pre-adaption at 25 °C for 24 h before being shifted to 4 °C dramatically downregulated or eliminated the expression of several oxidation-associated proteins such as AhpC and phosphoribosylaminoimidazole-carboxamide-formyltransferase/IMP cyclohydrolase (PurH), which were specifically identified during evolution into a VBNC state. ATP synthase F1, α subunit (ATPS-α; 22,223 Da) and DNA-directed RNA polymerase, α subunit (RNAP; 36,450 Da) appeared only in the VBNC cultures in MMS at 4 °C for 42 days. The results demonstrated that protein profiles of VBNC microorganisms were greatly different from those of the actively growing cells, thereby leading to a responsive modulation of physiological characteristics against extrinsic stressful factors.

## *Expression of responsive genes during evolution to a VBNC state*

The VBNC state is thought to be a transient physiological phase in which microbial cells switch off energy-associated metabolism activities that are mainly responsible for cell division without remaining culturable on routine microbiological media (Signoretto et al. 2004). Despite protein profiles in VBNC microorganisms being markedly different, depending on the strain-to-strain variables and VBNC-induced laboratory conditions, proteomic approaches determined that the functional annotations of VBNC *V. parahaemolyticus* cells were sustained by the increasing levels of stress regulators, albeit at the slower rates of biological process and protein synthesis. To demonstrate the findings, exploring specific genes and regulation pathways associated with the development of VBNC cells will clarify physiological characteristics of VBNC bacteria encountered under various environmental stresses (Table 7). A transcriptomic

**Table 6.** Proteomic characterization of significantly up-/down regulated proteins after the formation of VBNC cells.

| Microorganism | Major stress | Protein[2] | Functional group | Expression | Function | Reference |
|---|---|---|---|---|---|---|
| *V. parahaemolyticus* ATCC 17802 | Cold-starvation + potassium sorbate | ProX | Stress regulator | ++ | • Engaged in bacterial osmoregulation mechanisms via ProU transport system.<br>• Involved in quorum sensing signals mediated by LuxR. | Zhong et al. (2018) |
| | | MtrF | Cytoplasm protein | – | • Needed for the export of hydrophobic compounds such as antibiotics, nonionic detergents, and antibacterial peptides across the cytoplasmic membrane. | |
| *V. parahaemolyticus*[1] | Cold-starvation | HSK | Protein synthesis | ++ | • Participates in the aspartate pathway of amino acid biosynthesis and in increasing resistances to a cold temperature. | Tang et al. (2018) |
| | | GK | Protein synthesis | ++ | • Involved in the synthesis of proline that is one of the major compatible osmoregulation solutes at a moderate saline solution. | |
| | | COX | ND[3] | ++ | • Prevents the accumulation of $H_2O_2$.<br>• May increase a resistance to artificial gastric fluid environments (pH 4.9). | |
| *E. coli* O157:H7 | Oxidative stress (0.05% $H_2O_2$) | OmpW | Membrane protein | ++ | • Triggers survival mechanisms upon induction of osmotic and oxidative stress.<br>• Required for the maintenance of intracellular iron levels uniformly. | Asakura et al. (2007) |
| | | AhpC | ND | – | • Detoxifies ROS compounds.<br>• Maintains the ability of *V. parahaemolyticus* to produce its colony on solid media under oxidative stress. | |
| *E. coli* O157 MP3 | Oxidative stress | OmpW | Membrane protein | ++ | ND[3] | Asakura et al. (2008) |
| | | ProX | Stress regulator | ++ | ND | |
| *Ent. faecalis* V583 | Cold-staravatin | DnaK | Stress regulator | - | • Characterized as one of heat shock proteins (HSPs), activating protein folding.<br>• Restores the normal functions of inactivated protein under environmental stresses. | Heim et al. (2002) |
| | | Enolase | Metabolic protein | - | • Involves in central energy metabolism and cell adhesion to plasminogen. | |
| *Salm.* Enteritidis | Oxidative stress (3 mM $H_2O_2$) | PagC | Membrane protein | ++ | • Up-regulates the expression of 40 virulent genes.<br>• Involved in increasing survivals of *Salm.* Enteritidis under an environmental condition of low $Mg^{2+}$ concentrations and increasing tolerances to antimicrobials, bile salts, and acidic pH levels. | Xu et al. (2018) |

[1]This organism was not induced into the VBNC state but was incubated in a 10% saline solution at 4 °C for 18 h.
[2]Ahp, alkyl hydroperoxide reductase subunit C; ATPS-$\alpha$, ATP synthase F1, $\alpha$ subunit; ATPS-$\beta$, ATP synthase, $\beta$-chain; COX, cytochrome-$_c$-oxidase; GK, glutamate-$_5$-kinase (or gamma-glutamyl kinase); HSK, homoserine kinase; MtrF, multi-drug efflux pump component; OmpW, outer membrane protein W; PGD, $_{D}$-$_3$-phosphoglycerate dehydrogenase; ProX, glycine betaine-binding protein; RNAP, DNA-directed RNA polymerase, $\alpha$ subunit.
[3]ND, not determined.

characterization of VBNC *V. parahaemolyticus* RIMD2210633 revealed that 714 differentially expressed genes were upregulated, while 516 genes were downregulated in chromosomes, as compared with the stationary phase cultures (Meng et al. 2015). Of the differentially expressed genes, functional categories related to amino acid synthesis; central intermediary metabolism; energy metabolism; protein synthesis; and purine, pyrimidine, nucleoside, and nucleotide metabolism were significantly downregulated more than four-fold in VBNC cells. VBNC *V. cholerae* included high levels of downregulation in several transcriptional activities, including cell envelope, (central) energy metabolism, and protein synthesis, supporting the above findings (Asakura et al. 2007). In contrast, specifically modulated gene groups related to cellular process; regulatory function; and transport and binding proteins responsible for the transport of iron, magnesium, potassium, and cobalamin were comparatively more highly induced in VBNC *V. cholerae* cells. Zhao et al. (2016) demonstrated formation of VBNC *E. coli* O157:H7 induced by high $CO_2$ pressure,

showing that genetic functions related to bacterial reproduction such as membrane transport, central energy metabolism, DNA replication, and cell division were significantly downregulated in VBNC cells. While carbohydrate activity was greatly repressed by a subsequent downregulation in *ptsI*, *agaD*, *srlA*, and *ulaA* genes related to phosphoenolpyruvate carbohydrate phosphotransferase system, *ldhA*, which is required for conversion of pyruvate to D-lactate, was more abundant in VBNC *E. coli* O157:H7 cells, indicating that reduced ATP levels would occur concomitantly with the entry into a VBNC state. *Pseudomonas syringae* was found to exhibit an upregulation of biological functions such as polyamine metabolism and transport, peptidoglycan and cell wall polymers, and carbohydrate metabolism and transport after induction of a VBNC state (Postnikova et al. 2015). The authors highlighted that selective permeability of nutrients and metabolites by overexpressed ABC transporters and the activation of respiratory complex I- and NADPH-generating systems may induce a minimal energy expenditure in VBNC *P. syringae* cells. A global gene transcriptional analysis of VBNC *Rhodococcus* spp. strain T13 demonstrated that prolonged cold-starvation induced a total of 634 differentially expressed genes, of which included 391 upregulation and 243 downregulation (Su et al. 2016). Among them, the specifically downregulated genes were functionally identified to be involved in co-factor binding and oxidoreductase activity, encoding NADH dehydrogenase subunit and catalase. The result indicated that cold-starvation might interrupt the synthesis of ROS-detoxifying compounds and result in the loss of culturability.

Intracellular RpoS levels declined in the wild-type cultures, ranging from 1.0 to 0.2-0.6 during 7 days of osmo-starvation. The roles of *ahpC* in the induction and formation of a VBNC state in *V. parahaemolyticus* KX-V231 cells were demonstrated by Wang et al. (2013), who showed that the parent strain took 6 weeks to enter the VBNC state upon exposure to starvation at 4 °C; however, the time required by the *ahpC2*-deleted mutant strain to become VBNC was significantly reduced by 4.6 weeks under cold starvation condition. Notably, *ahpC* directly controlled the size of bacterial colonies and the colony-forming ability of *V. parahaemolyticus*. Furthermore, *rpoN*, considered a global regulator and sigma factor, was found to influence the maintenance of

**Table 7.** Responsive genes of VBNC microorganisms under various environmental conditions.

| | | Condition | | | Periods (days) required to enter a VBNC state | | |
|---|---|---|---|---|---|---|---|
| Microorganism | Mutation[1] | Stress | Microcosm[2] | Temp | Before | After | Reference |
| *V. parahaemolyticus* KX-V231 | *ahpC*$^-$ | Cold-starvation | MMS | 4°C | 42 | <30 | Wang et al. (2013) |
| *V. parahaemolyticus* NCTC 10884 | *yeaZ*$^-$ | Cold-starvation | ASW | 4°C | 52 | 130-140 | Chen (2012) |
| *V. vulnificus* ATCC 27562 | *GST*$^+$ | Cold-starvation | ASW | 4°C | 10 | >30 | Abe et al. (2007) |
| *Erwa. amylovora* CFBP 1430 | *KatA*$^-$*G*$^-$ | Starvation | NW | 28°C | Culturable | 33 | Santander, Figas, and Biosca (2018) |
| *E. coli MC4100* | *rpoS*$^-$ | Cold-starvation | AOM | 4°C | 33 | 20 | Boaretti et al. (2003) |
| *E. coli CF1648* | *ppGpp*$^-$ | Cold-starvation | AOM | 4°C | 30 | 22 | |
| *E. coli GC2700* | *ppGpp*$^+$ | Cold-starvation | AOM | 4°C | 31 | 49 | |
| *E. coli* O157 F2 | *ompW*$^-$ | Oxidative stress | PBS | 15°C | Culturable | 3 | Asakura et al. (2008) |
| *Staph. aureus* 8325-4 | *soda*$^-$ | Cold-starvation | NSW | 4°C | 200 | 70 | Masmoudi, Denis, and Maalej (2010) |
| | *katA*$^-$ | Cold-starvation | NSW | 4°C | 200 | 67 | |
| *Salm. enterica* LT2 | *rpoS*$^-$ | Osmotic stress | $SS_{7\%}$ | 37°C | 4 | 2 | Kusumoto, Asakura, and Kawamoto (2012) |

[1]Erwa. amylovora remained culturable when incubated in natural water microcosm added with 1 mM H2O2 at 28 °C for more than 35 days.
[2]AOM, artificial oligotrophic medium; ASW, artificial sea water; MMS Morita mineral salt solution; NSW, natural sea water; NW, natural water; PBS, phosphate buffered saline; SS7%, saline solution (7% NaCl).

**Table 8.** Determination of potential virulence of VBNC bacteria.

| | | Survival rates (%) before and after co-cultured with VBNC microorganisms | | | |
|---|---|---|---|---|---|
| Model | Microorganism | Before | After | Virulence | Reference |
| HEp-2 | *V. parahaemolyticus* | 100 | 0 | ++ | Wong et al. (2004a) |
| Animal cell | *C. jejuni* | VBNC *C. jejuni* cells had the ability to invade the human intestinal epithelial cells, as well as were able to attach to Caco-2. | | | Chaisowwong et al. (2012) |
| *Caen. elegans*[1] | *L. monocytogenes* | 100 | 0 | ++ | Highmore et al. (2018) |
| Human macrophage[2] | *Legionella* spp. | VBNC cells persisted for 263 days in water infected 4.0%-4.5% human macrophages at $MOI_{100}$. | | | Dietersdorfer et al. (2018) |
| Mouse | *Salm.* Typhimurium | 100 (10/10) | 100 | - | Caro et al. (1999) |
| HeLa | *Shig. dysenteriae* | 100 | 25 | + | Rahman et al. (1994) |
| Fish | *Strep. parauberis* | 100 | 45 | + | Currás et al. (2002) |
| Mouse | *V. cholerae* | 100 (0/3) | 0 (3/3) | ++ | Asakura et al. (2007) |
| Mouse | *V. vulnificus* | Injection of only VBNC cells resulted in death of mice. | | | Oliver and Bockian (1995) |

[1]A nematode.
[2]Human peripheral blood monocytes such as GM-CSF and M-CSF; MOI, multiplicities of infection.

**Table 9.** Evaluation of the resuscitation-availability of VBNC bacteria to the culturable state.

| Microorganism[1] | Resuscitation-conditions[2] | | | | Resuscitation | Culturability (log CFU/g or ml) | Reference |
|---|---|---|---|---|---|---|---|
| | Buffer | Medium | Temperature | Period | | | |
| *V. parahaemolyticus* $KP^+$ | ASW | HIA | 22°C | 1-2 days | + | >5.0 | Bates and Oliver (2004) |
| *V. parahaemolyticus* | ASW | $TSA_S$ | 25°C | ≤5 days | - | ND[5] | Yoon, Bae, and Lee (2017) |
| | $TSB_S$ | $TSA_S$ | 25°C | ≤5 days | + | 7.0-8.0 | |
| *V. parahaemolyticus* VP190 | LB | LBA | 37°C | 24 h | - | ND | Mizunoe et al. (2000) |
| | LB | LBA + antioxidant | 37°C | 24 h | + | 2.0-3.0 | |
| *V. parahaemolyticus* | MMS | $TSA_S$ | 25°C | 40 h | + | 7.0-8.0 | Wong et al. (2004b) |
| | $TSB_S$ | $TSA_S$ | 25°C | 40 h | + | 8.0-9.0 | |
| *V. parahaemolyticus* VP5 | ASW | $HIA_S$ | 20°C | <2days | + | 6.0 | Coutard et al. (2007a) |
| | ASW | $HIA_S$ | 37°C | <2days | + | 6.0 | |
| *V. vulnificus* | ASW | HIA | 50°C | 30-60 min | ○ | 5.0-7.0 | Nowakowska and Oliver (2013) |
| *V. vulnificus* | NSW | Chrome | 20°C | 24 h | ○ | 5.1 | Ayrapetyan, Williams, and Oliver (2014) |
| *V. vulnificus* (*luxS*⁻) | NSW | Chrome | 20°C | 24 h | X | - | |
| *V. vulnificus* (heat-shock) | ASW | HIA | 25°C | 24 h | ○ | 8.0-9.0 | Nowakowska and Oliver (2013) |
| *V. cholerae* TSI-4 | L-broth | L-agar | 37°C | 1 day | ○ | ≥3.0 | Wai et al. (1996) |
| *V. cholerae* TSI-4 | L-broth | L-agar | 37°C | 1 day | X (35 days)[3] | - | |
| *V. cholerae* TSI-4 (heat-shock[6]) | L-broth | L-agar | 45°C | 1 min | ○ | ≥3.0 | |
| *V. cholerae* O134 (heat-shock) | LB | LBA | 45°C | 1 min | ○ | ND | Chaiyanan et al. (2007) |
| *V. cholerae* | ND | In the gut of mice[4] | ND | ND | ○ | 3.0 | Asakura et al. (2007) |
| *Strep. parauberis* RA-99.1 | $TSB_S$ | $TSA_S$ | 22°C | 48 h | ○ | 5.0 | Currás et al. (2002) |
| *E. coli* | M9 added with 7% NaCl | TSA | 37°C | 1-15 days | X | - | Pinto et al. (2011) |
| | M9 added with 7% NaCl + amino acid or AI | TSA | 37°C | 1-15 days | ○ | ND | |
| *E. coli* | LB | L-agar | 37°C | 24 h | ○ | ND | Zhang et al. (2015) |
| *E. coli* | LB | LBA | 25°C | 24 h | ○ | 3.0-7.0 | Boaretti et al. (2003) |
| | LB | LBA | 25°C | 24 h | X (42 days)[3] | - | |
| *Salm.* Typhimurium CMCC 50115 | BPY + 20 mM sodium pyruvate | BPYA | 37°C | 3 days | ○ | 8.0-8.5 | Liao et al., (2018) |
| *Salm.* Typhimurium CMCC 50115 | BPY + 24 mM Tween 20 | BPYA | 37°C | 3 days | ○ | 8.0-8.5 | Liao, Jiang, and Zhang (2017) |
| *Salm.* Typhimurium DT104 | BPB | TSA | 56°C | <1 min | ○ | ≥4.0 | Gupte, de Rezende, and Joseph (2003) |
| *Salm.* Oranienburg | BHI | TSA | 30°C | 2 days | X | - | Panutdaporn et al. (2006) |
| | BHI + ≤10 ug/ml RPF protein | TSA | 30°C | 2 days | ○ | ND | |
| *Staph. aureus* | Seawater | BHI | 22°C | 5 days | ○ | <4.5 | Masmoudi, Denis, and Maalej (2010) |
| | Seawater + chloramphenicol | BHI | 22°C | 24 days | X | - | |

[1] $KP^+$, Kanagawa phenomenon (hemolysin) positive strains of *V. parahaemolyticus*.
[2] ASW, artificial sea water; BHI, brain heart infusion broth; BPB, Butterfield phosphate buffer; BPY, beef peptone yeast broth; LB, Luria-Bertani broth; L-broth, nutrient broth; MMS, Morita mineral salt solution (0.5% NaCl); NSW, natural sea water; $TSB_S$, tryptic soy broth (TSB) added with 1%-3% NaCl; BPYA, beef peptone yeast agar; HIA, heart infusion agar; $HIA_S$, heart infusion agar supplemented with 2% NaCl; LBA, Luria-Bertani agar; L-agar, nutrient agar; RPF, resuscitation promoting factor; $TSA_S$, tryptic soy agar (TSA) added with 1%-3% NaCl.
[3] Microorganisms that lasted for more than 35 days after the entry into the VBNC state yielded no further resuscitative effects.
[4] After resuscitation, all the mice (3/3) were infected and dead.
[5] ND, not determined.
[6] VBNC cells were heat-shocked in L-broth at 45 °C for 1 min, and then incubated at 37 °C overnight.

colony-forming ability and colony morphological phenotype in *V. parahaemolyticus* cells (Whitaker, Richards, and Boyd 2014). The *rpoN*-mutant strain of *V. parahaemolyticus* RIMD2210633 colonized streptomycin-treated mouse models 2.5-fold higher than the wild-type. The deletion of *rpoN* produced round and symmetrical cells in *V. parahaemolyticus*, as determined by transmission electron microscopy. Chen (2012) showed that the transition into a VBNC state can also be greatly extended by the deletion of *yeaZ* in *V. parahaemolyticus* NCTC 10884 cells. The mutant strains of *V. parahaemolyticus* with *tdh*1 and *tdh*2 deletions were induced into a VBNC state in ASW microcosms at 5 °C within 15 days, whereas phase shift of the mutant strains complemented with a plasmid vector containing *tdh*1 and *tdh*2 to the VBNC state was extended by 2–3 days (Bates & Oliver 2004). According to Chen et al. (2009), some cytoskeleton genes were greatly affected by the induction of a VBNC state. In this study, the expression levels of *mreB*,

*minE*, and *ftsZ* were significantly downregulated in VBNC *V. parahaemolyticus* O3:K6 1137 cells as determined by real-time quantitative PCR.

Reverse transcription PCR assay showed *V. vulnificus* ATCC 27562 to display *GST* expression until 24 h of incubation in distilled water (DW) at 4 °C, whereas the N-methyl-$N_0$-nitro-nitrosoguanidine mutant strain exhibited increased levels of GST over time during cold starvation (Abe et al. 2007). When GST activity of *V. vulnificus* was measured using 1-chloro-2,4-dinitrobenzene, the wild-type strain showed unchanged GST levels (3.1–3.8 μM/mg) upon induction of a VBNC state. However, GST activity of the mutant strain increased slowly during the first 4 h following cold starvation in DW but showed the highest expression (32.7 μM/mg) after 6 h. In *E. coli* cells, *katG* activated the catalase activity to hydrolyze ROS compounds in response to oxidative stress, and the expression of *kaG* disappeared in the *rpoS* mutant strain (Boaretti et al. 2003). *Erwa. amylovora* CFBP 1430 remained culturable when incubated in a natural water microcosm supplemented with 1 mM $H_2O_2$ at 28 °C for more than 35 days, whereas the mutant ($\triangle KatA^-G^-$) of *Erwa. amylovora* entered a VBNC state within 30 days (Santander, Figas, and Biosca 2018). Notably, complementation of *KatA* significantly increased the VBNC-inducing times wherein *Erwa. amylovora* maintained its culturability under oxidative stress. After 35 days, catalase activities were 15,000, <2,500, 15,000, and zero point in the wild-type, $KatA^-$, $KatG^-$, and $KatA^-G^-$ mutant strains, respectively. Thus, the roles of *KatA* and *KatG* may strongly correlate with the loss of culturability, and *KatA* overexpression can delay the transition into the VBNC state. Furthermore, Boaretti et al. (2003) showed that the *rpoS* mutant strain of *E. coli* retained its cell membrane integrity only for 2 weeks when exposed to cold starvation. Under osmotic stress (7% NaCl) in a saline solution, wild *Salm. enterica* was transformed to a VBNC state after approximately 4–5 days, whereas *rpoS*-deficient strains were undetectable and entered the VBNC state more rapidly, within 2–3 days (Kusumoto, Asakura, and Kawamoto 2012). When *E. coli* O157 F2 was incubated in PBS supplemented with 0.05% $H_2O_2$ at 15 °C, the deletion of *ompW* allowed this strain to enter a VBNC state for 3 days, while the wild-type strain was consistently detectable at levels of ≥2.0 log CFU/ml during oxidative stress (Asakura et al. 2008). *rpoS* is involved in universal stress responses of a wide range of microorganisms to adverse environments. The simultaneous deletion of *relA* and *spot* in *Salm*. Enteritidis increased the amount of *rpoS* expression under cold starvation (Rodrigues et al. 2015). As increased concentrations of relA and spot proteins are known to promote the expression of ppGpp, which contributes to the persistence of *E. coli* in nutrient-limited environments (Gentry and Cashel 1996), *rpoS* might be upregulated by the absence of *ppGpp*, and low or deficient ppGpp levels would result in a faster advent of the VBNC state. After 24 h of starvation at 25 °C, *mreB*, *minE*, and *ftsZ* expression levels were 1.00–0.23, 0.07, and 0.13, respectively. *mreB* is thought to be an essential cell-shape determinant in *E. coli* (Wachi and Matsuhashi 1989). It was shown that the *mreB* mutant became smaller (<5 μm) and rounded at 37 °C, whereas the exponential-phase wild-type *E. coli* displayed typical long rod shapes. Considering that mutant *E. coli* cells lacking *mreB* changed from rod to spherical form, Kruse, Møller-Jensen, A. Løbner-Olesen, and Gerdes (2003) concluded that mreB forms helical filaments and is required to control cell width and linear axis. Furthermore, *minE* and *ftsZ* are known to be associated with cell structure and cell division (Hu and Lutkenhaus 1999; Raskin and de Boer 1997).

### Virulence and resuscitation

Table 8 represents the potential virulence of VBNC bacteria. VBNC *V. parahaemolyticus* displayed the strong cytotoxic effect towards HEp-2, disrupting the entire cell line cultures (Wong et al. 2004a). *Shig. dysenteriae* killed 75% HeLa cells after the shift into a VBNC state (Rahman et al. 1994). Rahman et al. (1996) showed that VBNC *Shig. dysenteriae* maintained expression of Shiga toxin (*stx*) gene during prolonged starvation and had the reduced ability to attach to intestinal epithelial Henle 407 cells. Chaisowwong et al. (2012) reported that starved *C. jejuni* CG8486 possessed virulence-associated genes such as *cdtB, cdtC*, and *flB* even after the formation of a VBNC state at 4 °C. Importantly, VBNC *C. jejuni* cells had the ability to invade human intestinal epithelial cells, as well as were able to attach to Caco-2 cells. Baffone et al. (2003) found that VBNC *V. parahaemolyticus* cells were able to colonize the intestines of mouse models and were isolated from the gut of about half of the mice. When co-cultured with the host (*Caenorhabditis elegans*), VBNC *L. monocytogenes* was highly infectious as its culturable counterpart (Highmore et al. 2018). As a consequence of *in vivo* experiments, *V. cholerae* and *V. vulnificus* were shown to be fatally infectious to mouse models, showing higher levels of mortality upon induction of a VBNC state (Asakura et al. 2007; Oliver and Bockian 1995). Although several studies indicated that the entry of a VBNC state was not always relevant to the maintenance of pathogenicity in *Salm*. Typhimurium (Caro et al. 1999), some VBNC pathogens can be still metabolically active and capable of retaining their *in vivo* pathogenicity (Baffone et al. 2003). Therefore, VBNC bacteria cannot be considered dead, but rather should be recognized as a potential hazard to public health.

As one of the most notable physiological properties, VBNC microorganisms can be recovered to a culturable state by removing the causative stress that originally introduced the VBNC state. Lots of studies have characterized the resuscitation of VBNC bacteria upon restoration in favorable environmental conditions (Table 9). Fundamentally, a temperature upshift was known to be associated with the reversibility of VBNC bacteria (Ayrapetyan, Williams, and Oliver 2014; Bates and Oliver 2004; Masmoudi, Denis, and Maalej 2010; Pinto et al. 2011; Yoon, Bae, and Lee 2017). These studies showed that while *Staph. aureus*, *V. parahaemolyticus* $KP^+$, and *V. vulnificus* were easily revived from a VBNC state after temperature upshift process between 20-22 °C. *E. coli* and *V. parahaemolyticus* in

a VBNC state sometimes failed to regain their culturability at 25-37 °C for more than 5 days. The reactivation of *E. coli* from the dormancy may depend on the exposure times to the cold-starvation challenge (Boaretti et al. 2003). The stressed cells which persisted only for less than 42 days in artificial oligotrophic medium at 4 °C could be resuscitated upon exposure to subsequent incubation in Luria-Bertani broth (LB) at 25 °C. This result was in accordance with a finding by Wai et al. (1996). Meanwhile, VBNC *Staph. aureus* cells were not shifted to the culturable state when incubated in natural seawater supplemented with chloramphenicol, indicating that protective protein synthesis may also be of importance for facilitating an active resuscitation response (Masmoudi, Denis, and Maalej 2010). *Staph. aureus*, *V. parahaemolyticus* $KP^+$, and *V. vulnificus* were resuscitated from a VBNC state at an ambient temperature for 1-5 days (Ayrapetyan, Williams, and Oliver 2014; Bates and Oliver 2004; Masmoudi, Denis, and Maalej 2010). It was shown that the incubation in nutrient-rich culture media induced the recovery of VBNC bacteria, with the culturable counts of $10^3$-$10^7$ CFU/ml (Boaretti et al. 2003; Currás et al. 2002; Wai et al. 1996; Yoon, Bae, and Lee 2017; Zhang et al. 2015). Furthermore, the resuscitation abililty may be greatly influenced by various physicochemical parameters, including anti-oxidizing compounds (Liao et al. 2018; Mizunoe et al. 2000), heat-shock (Chaiyanan et al. 2007; Gupte, de Rezende, and Joseph 2003; Nowakowska and Oliver 2013), nutrient-availability (Currás et al. 2002; Pinto et al. 2011; Yoon, Bae, and Lee 2017; Zhang et al. 2015), and resuscitation-promoting stimuli (Asakura et al. 2007; Panutdaporn et al. 2006). Morishige, Fujimori, and Amano (2013) showed that pyruvate and its analog (α-ketobutyrate) only containing a methyl group showed a resuscitative effect on the recovery of *Salm. Enteritidis* cells from a VBNC state.

## Conclusions

Considerable evidences suggest that VBNC *V. parahaemolyticus* shows markedly modified compositions of cell membrane, fatty acid synthesis, metabolism, expression of genes, resistance, capability of adhesion and virulence to survive under nutrient-deficient environments at cold temperature. In particular, oxidative stress regulators might be mainly responsible for the formation of VBNC cells. The changes in cell physiology and the fatty acid profile might be accompanied by reduced membrane fluidity, concomitant with a significant decrease in cell volume and size to minimize cell maintenance requirement under hostile environments. Considering that the isolation of *V. parahaemolyticus* is extensively reported during summer, when the incidence of food-borne diseases and illnesses is the highest. This phenomenon might be explained by the shift of *V. parahaemolyticus* into the VBNC state in natural habitats at cold temperatures. It can be believed that VBNC *V. parahaemolyticus* might easily be overlooked by culture-based surveillance methods, and subsequent resuscitation of the VBNC cells under favorable environments could be linked to the food-borne outbreaks. Therefore, further studies for unraveling the underlying mechanisms of the VBNC state are crucial.

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