Research article

**Physiological Characteristics of Viable-but-nonculturable *Vibrio*
parahaemolyticus upon Prolonged Exposure to the Refrigerator
Temperature**

Running title: Cellular properties of VBNC *V. parahaemolyticus*

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ABSTRACT

Although it has been reported that viable-but-nonculturable (VBNC) cells of *Vibrio parahaemolyticus* can be developed by a prolonged duration of cold-starvation there are restricted cellular characteristics available on understanding the exact mechanisms governing the entry of pathogens into the VBNC state. Therefore, this research was aimed at determining the cellular profile of VBNC cells of *V. parahaemolyticus* upon exposure to the refrigerator temperature. Strains of *V. parahaemolyticus* were incubated in artificial sea water (ASW) microcosms (pH 6) added with different amounts of NaCl at 4°C until these pathogens entered into such a dormant state. At a regular time-interval, both culturability and viability of these bacteria were enumerated, and then cellular profiling were carried out in terms of cellular membrane permeability, enzymatic activity, hydrophobicity, fatty acid composition, and morphological changes after cells of *V. parahaemolyticus* became the VBNC state. Three strains of *V. parahaemolyticus* used in this study showed that VBNC cells retained the strong virulent properties to Vero and CACO-2 cell lines, re-gained the cytotoxicity even after resuscitation, became permeabilized in terms of the outer membrane, showed lower levels of enzymatic (catalase and glutathione-S-transferase) activities, exerted the increasing hydrophobicity, and then exhibited increasing amounts of saturated fatty acids.

IMPORTANCE

To the current best knowledge, there are restricted information available on understanding the physiological characterization of viable-but-nonculturable cells. Most

previous studies are still making a degree of efforts in discovering the causative effector causing microorganisms to be induced into the VBNC state. Herein, the present study showed that pathogenic *V. parahaemolyticus* can enter into the VBNC state when challenged by a certain environmental stress where higher amounts of NaCl combined with acidic pHs was artificially controlled. Importantly, it was indicated that VBNC *V. parahaemolyticus* maintained peculiarly different physiological characteristics. Furthermore, this study proposed a novel approach on the transient/stepwise conversion of the bacteria into the VBNC state. Specific alternative tools for measuring and controlling the incidence of VBNC pathogens on food are not established until now. In this aspect, results obtained from this study will be used to provide an effective insight in determining physiological properties of viable-but-nonculturable *V. parahaemolyticus*.

KEYWORDS fatty acid composition, cytotoxicity, NaCl, pathogen, viable-but-nonculturable

INTRODUCTION

V. parahaemolyticus has been recognized as one of the major food-borne pathogens commonly found in estuarine environments such as seawater, coastal area, and marine sediment (1-2). It has been also reported that human pathogens such as *V. parahaemolyticus*, *Vibrio vulnificus*, and *Vibrio cholerae* can be isolated from a wide variety of raw aquatic products, including clam, mussel, oyster, scallop, and shrimp (3-4). Consumption of marine products contaminated with these pathogens could result in serious clinical symptoms, ranging from acute abdominal pain, vomiting, and nausea to fatal septicemia (5). Especially, it has been determined that cells of *V. parahaemolyticus* are able to enter into a viable-but-nonculturable (VBNC) state in response to a physiological ecology such as higher amounts of NaCl (6) and low temperatures of $\leq 10^{\circ}\text{C}$ (7). Until now, more than 65 species of microorganisms, including *V. parahaemolyticus*, *V. vulnificus*, *V. cholerae*, *Campylobacter jejuni*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella enterica* serovar Typhimurium, *Shigella dysenteriae*, and *Staphylococcus aureus* are proven to be induced into the VBNC state upon exposure to environmental stresses (8-13). It should be noted that VBNC bacteria cannot represent the colony-forming capability on agar plates, on which these organisms can grow routinely, thereby indicating that viable-but-nonculturable *V. parahaemolyticus* might not be detected by means of the standardized cultivation-based methods. Although lots of studies have been conducted to find out the exact mechanisms governing the entrance of bacteria into such a dormant state there are still restricted scientific evidences available on understanding physiological characteristics of VBNC organisms. After producing VBNC *V. parahaemolyticus*, these bacteria showed a significantly higher resistance to ethanol shock, as compared with the actively growing cells cultured in tryptic

soy broth (14). Above all, it was demonstrated that co-culturing VBNC pathogens such as *V. parahaemolyticus* and *Shig. dysenteriae* with clinical cell lines resulted in reducing a magnitude of viable animal cells, disrupting the cellular substructure indiscriminately (13, 15). Thus, it appeared that VBNC pathogens would be associated with the food-borne disease outbreaks, thereby threatening public health concerns. Nevertheless, it might be difficult to construct an effective microbial risk assessment for controlling the pathogenic bacteria due to a significant lack of cellular properties of the VBNC cells. In the present study, cellular properties of viable-but-nonculturable *V. parahaemolyticus* were characterized by measuring cytotoxic effects to human cell lines, the membrane permeabilization with β -galactosidase and β -lactamase assays, intracellular leakages of nucleic acid and protein, enzymatic activities of catalase and glutathione-S-transferase (GST), and the cellular hydrophobicity. Meanwhile, cellular fatty acid composition assays were conducted between pure cultures and VBNC cells of *V. parahaemolyticus* for the purpose of revealing the kinetic of mechanisms mediating the conversion of bacterial cells into the VBNC state. Furthermore, not only the ability of *V. parahaemolyticus* strains to be recovered from the VBNC state, but morphological changes before and after the entry into the VBNC state were examined to develop a well-standardized mean for determining the physiological characterization of *V. parahaemolyticus* in the VBNC state.

RESULTS AND DISCUSSION

Loss of the culturability of *V. parahaemolyticus* strains

In order to induce cells of *V. parahaemolyticus* into the VBNC state, three strains of *V. parahaemolyticus* ATCC 17082, *V. parahaemolyticus* ATCC 33844, and *V. parahaemolyticus*

ATCC 27969 were incubated in ASW microcosms (pH 6) supplemented with different amounts of NaCl at 4°C, respectively. Bacterial populations were enumerated at regular time-intervals by means of the cultivation-based method on agar plates. As shown in Fig. 1, initial densities of *V. parahaemolyticus* ATCC 17082, *V. parahaemolyticus* ATCC 33844, and *V. parahaemolyticus* ATCC 27969 ranged from 6.0 log₁₀ CFU/ml to 8.0 log₁₀ CFU/ml. Populations of *V. parahaemolyticus* ATCC 17082 exceeded approximately 4.0 log₁₀ CFU/ml until incubated in ASW microcosms containing 0.75% NaCl or amended with 5% NaCl at 4°C for 40 days, whereas microbial loads of *V. parahaemolyticus* ATCC 17082 dropped to below the detectable levels (<1.0 log₁₀ CFU/ml) at 4°C within 80 days. Especially, loss of the culturability of *V. parahaemolyticus* ATCC 17082 was accelerated more progressively at increasing NaCl concentrations in ASW solutions, showing that cells of *V. parahaemolyticus* ATCC 17082 in ASW microcosms added with 10% and 30% NaCl were induced into the nonculturable state at 4°C within 21 days. There were no differences in the bacterial reductions between three strains of *V. parahaemolyticus* incubated in ASW microcosms containing 0.75% NaCl or supplemented with 5% NaCl. During the cold-starvation challenge, the culturability of these pathogens declined below the detectable levels within 80 days. Within 21 days, cells of *V. parahaemolyticus* ATCC 33844 entered into the nonculturable state in ASW microcosms added with ≥10% NaCl at 4°C. Cells of *V. parahaemolyticus* ATCC 27969 were also converted into the nonculturable state when incubated in either formal ASW microcosms or those amended with 5%, 10%, and 30% NaCl at 4°C for 7, 21, 80, and 80 days, respectively. These results indicated that higher degrees of NaCl facilitated the loss of bacterial culturability more rapidly at the refrigerator temperature. However, it seemed likely clear that changes of the culturability are not the determination of making a decision on the true bacterial viability. In order to determine if bacterial cells are not dead, but still alive, it is

inevitable to evaluate the degree by which organisms are sincerely damaged. Accordingly, a part of fluorescent probes can reflect the level of cellular integrities quantitatively. Recently, staining dyes of SYTO9 and propidium iodide (PI) are widely applied in a field of the microbiology because these dyes are well-known for the ability to penetrate inside the cellular membrane with increasing cell injuries. SYTO9 is capable of penetrating bacteria with the intact membrane, interacting with the cell nucleic acid, and then displaying green colors for the live cell with the fluorescence microscopy. Propidium iodide can only penetrate bacterial cells through damaged membranes, labeling the dead cell only as red-coloured fluorescence. Thus, staining bacterial cells with SYTO9 and propidium iodide can distinguish between live and dead cells. Loss of the permeability barrier of stained cells represents irreparable membrane damages and thus cell death (16-17). In the present study, three strains of *V. parahaemolyticus* showed the viable numbers in the levels of $>5.5 \log_{10}$ CFU per a slide uniformly until these pathogens were challenged by cold-starvation stress (nutrient-deprivation and cold temperature) for more than 100 days, regardless of the controlled environmental effector such as excessive concentrations of NaCl and acidic pHs, which means that although the culturable count of *V. parahaemolyticus* were below the detectable levels when incubated in ASW microcosms at 4°C for 80 days bacterial cells were proven to be still alive, maintaining the intact cell membrane. Accordingly, it was demonstrated that a prolonged period of cold-starvation challenge enabled three strains of *V. parahaemolyticus* to enter into the VBNC state within 80 days.

Cytotoxic effects of VBNC pathogens

As shown in Fig. 2A, there was no difference in the cellular virulence (cytotoxicity)

between the cells of *V. parahaemolyticus* ATCC 17082 either actively grown overnight in TSB at 37°C or incubated in ASW microcosms at 4°C for ≤80 days. In contrast, VBNC *V. parahaemolyticus* ATCC 17082 exhibited decreased cellular damages in the levels of ≤45% against Vero cells when incubated in ASW microcosms added with 5% NaCl at 4°C for 80 days previously (Fig. 2B). Cells of *V. parahaemolyticus* ATCC 33844 and *V. parahaemolyticus* ATCC 27969 showed gradual decreases in the cytotoxicity during cold-starvation. In particular, less than 40% of the cellular toxicity was achieved when these bacteria were incubated in ASW microcosms at 4°C for 21 days. Clearly, it appeared that the cellular virulence of *V. parahaemolyticus* ATCC 33844 and *V. parahaemolyticus* ATCC 27969 against Vero cells declined in the reverse proportion to a prolonged duration of cold-starvation. Especially, VBNC *V. parahaemolyticus* ATCC 33844 showed no cytotoxic effects on Vero cells as maintained in ASW microcosms supplemented with 30% NaCl at 4°C for 80 days (Fig. 2B). However, it was shown that once challenged by the cold-starvation for 80 days VBNC *V. parahaemolyticus* ATCC 27969 represented the strong cytotoxicity, disrupting the cellular structure of animal cell lines apparently (*data not shown*). Of much importance, strains of *V. parahaemolyticus* which were converted from the nonculturable state recovered the whole cytotoxicity in the levels of ≥100%. It has been well-reported that VBNC *V. parahaemolyticus* can be recovered back to the culturable state by eliminating the causative environmental stress. Several studies showed that strains of *V. parahaemolyticus* and *V. vulnificus* in such a dormant state were converted to the culturable state on solid agar plates, followed by culturing these long-term-stressed cells in liquid nutrient-rich media at ambient temperatures for several days (6, 35). Dwidjosiswojo et al. (18) reported that while VBNC-induced cells of *Pseudomonas aeruginosa* did not show the cytotoxicity these cells re-cultured in a nutrient-rich culture broth (which recovered back to the culturable state) killed

100% Chinese hamster ovary cells within 24 hrs, supporting these findings. According to Wong et al. (2), cells of *V. parahaemolyticus* were induced into the VBNC state when incubated in Morita minimal salt broth at 4°C for 35 days. Consequently, either VBNC or resuscitated cells of *V. parahaemolyticus* maintained higher levels of the cellular virulence, causing 100% cell death of Hep-2 cells within 24 hrs. Findings of Cappelletti et al. (12) were also in an agreement with these results. Thus, it should be noted that VBNC cells of *V. parahaemolyticus* were not dead, but rather maintained peculiar metabolic activities to survive in an unfavorable environment such as cold-starvation. In this study, it was revealed out that three strains of *V. parahaemolyticus* in the VBNC state still remained the cellular toxicity against human cell lines, including Vero and CACO-2. Notably, VBNC *V. parahaemolyticus* strains were able to be resuscitated after temperature upshift, and then resulted in lethal injuries to the human cell lines. Therefore, it could be postulated that VBNC cells of pathogenic bacteria, including *V. parahaemolyticus*, would retain the infectious virulence or re-gain the infectivity after resuscitation process. VBNC-induced pathogen on marine products may present a potential risk of causing public health hazards. Necessarily, it should be further required for determining the environmental stress which activates either the transition of the food-borne pathogens into the VBNC state or the resuscitation-availability of VBNC bacteria to the culturable and infectious state.

Assessment of cell membrane permeabilization

In the present study, inner and outer cell membrane permeabilization assays were carried out by using β -galactosidase and β -lactamase, respectively (Fig. 3). Outer cell membranes of *V. parahaemolyticus* were damaged under cold-starvation challenge at 4°C for 30 days as

being assessed with the β -lactamase activity. Commonly, optical densities at 482 nm of *V. parahaemolyticus* ATCC 17082 grown overnight in TSB and in the VBNC state were increased with the prolonged duration. In contrast, β -galactosidase activities of VBNC *V. parahaemolyticus* were not different from the pure cultures of *V. parahaemolyticus* ATCC 17082. Given that β -galactosidase is localized in cytoplasm and is capable of hydrolyzing ONPG if the inner membrane of bacteria is compromised (19), it seemed likely that such a cold-starvation stress interacted with the outer cell membrane either mainly or preferentially. In addition, β -lactamase, which is present in the periplasmic space, responds to pyridine-2-azo-4'-(N', N'-dimethylaniline) cephalosporin (PADAC) unless permeability of the outer membrane is decreasing (20). From these results, it could be accepted that the release of intracellular materials contributed to the loss of cell membrane permeabilization during a long-term of cold-starvation stress.

The cellular leakage of nucleic acid and protein into microcosm fluids was also measured to evaluate the degree to which cell membrane permeabilization of VBNC *V. parahaemolyticus* was truly damaged. In Table 1, initial densities of nucleic acid ranged from 0.417 to 1.567 when *V. parahaemolyticus* began to be exposed at 4°C in ASW microcosms. After 150-days-incubation at 4°C, higher levels of nucleic acid were released from these cells; leakages of nucleic acid were 2.611, 2.550, 2.315, and 2.683 from VBNC *V. parahaemolyticus* ATCC 33844 in ASW microcosms at controlled NaCl concentrations of 0.75%, 5%, 10%, and 30%, respectively. Similarly, it has been shown that intracellular proteins were permeabilized through the membrane of VBNC cells into bacterial microcosms. After the entry of *V. parahaemolyticus* into the VBNC state, nucleic acid and protein outflowed more than twice over those of the actively growing cells. These results implied that cold-starvation stress could increase the membrane permeability of *V. parahaemolyticus*,

resulting in the cellular leakages of nucleic acid and protein from the bacterial cytoplasm to external fluids. According to Zhang et al. (21), these authors stated that the release of nucleic acid and protein would be strongly involved in persisting the integrity of cell membrane. At this point of view, the temporary accumulation of these cellular components in ASW microcosms was implicated with the loss of membrane integrity (22).

Enzymatic activities of catalase and GST

While culturing aerobically, reactive oxygen species (ROS) such as $O_2^{\cdot -}$, $^{\cdot}OH$, and H_2O_2 are generally accumulated in the cell of bacteria. Especially, hydrogen peroxide (H_2O_2) has been recognized as one of the leading toxic compounds due to its ability to penetrate the cellular wall and membrane, thereby damaging nucleic acids directly (23). During exposure to environmental stressors such as starvation, bacteria might encounter intracellularly accumulated H_2O_2 (24). Hydrogen peroxidase is able to separate ROS into a molecule of H_2O and O_2 . Generally, detoxifying enzymes, including catalase, GST, and peroxidase, prevented microbial cells from being damaged by ROS. It was determined that when *Ralstonia solanacearum* was induced into the VBNC state upon exposure to 200 μM copper, significantly ($p < 0.05$) higher amounts of H_2O_2 were accumulated in the bacterial pellets (25). Such a cellular damage caused by H_2O_2 could have a strong influence on the entry of pathogens into the VBNC state. In particular, it has been reported that cells of *V. vulnificus* incubated in ASW microcosms added with $\leq 500 \mu M$ H_2O_2 at $4^{\circ}C$ were converted to the nonculturable state more rapidly (26). Thus, effects of H_2O_2 to the cellular damage could be examined by measuring the amounts of catalase produced intracellularly. In Fig. 4, *V. parahaemolyticus* ATCC 17082 grown in TSB exerted <20 U/mg/protein catalase, whereas

VBNC-induced cells in ASW microcosms containing 0.75% NaCl and supplemented with 5%, 10%, and 30% NaCl showed <60, ≤100, <30, and <30 U/mg/protein catalase, respectively. When incubated in ASW microcosms amended with ≥10% NaCl at 4°C for 100 days *V. parahaemolyticus* ATCC 27969 exhibited a less decreased catalase activity (<50 U/mg/protein), as compared with that of the stationary-phase cells. While initial catalase activities of *V. parahaemolyticus* ATCC 33844 were <30 U/mg/protein, these organisms suspended in ASW microcosms added with ≥5% NaCl at 4°C for 100 days displayed enhanced catalase activities, ranging from 80 to <140 U/mg/protein. These results indicated that H₂O₂-degrading capability of *V. parahaemolyticus* would be dependent on strain-to-strain variables. GST was also recognized as one of the important agents associated with oxidative stress response of pathogens. Three strains of *V. parahaemolyticus* showed no differences among GST activities before and after a prolonged duration of cold-starvation (Fig. 5). Similarly, GST activities of these organisms ranged from >1.5 to <2.0 U/mg/protein. In the present study, there was no close correlation between the loss of culturability and ROS-detoxifying activities.

Assessment of the Cellular Hydrophobicity

In Table 3, hydrophobicity of *V. parahaemolyticus* in the VBNC state at 4°C for 100 days was measured for determining a hallmark of VBNC-induced bacteria. When subjected to the refrigerator temperature for 100 days, levels of hydrophobicity of *V. parahaemolyticus* ATCC 17082 in ASW microcosms added with 5%, 10%, and 30% NaCl were 37.5%, 46.8%, and 62.2%, respectively. Surface hydrophobicity of VBNC *V. parahaemolyticus* ATCC 33844 and *V. parahaemolyticus* ATCC 27969 increased dramatically more than those of the pure cultures.

Strains of *V. parahaemolyticus* used in this study exhibited the highest hydrophobicity of $\leq 62.2\%$, followed by cold-starvation in ASW microcosms supplemented with 30% NaCl at 4°C for 100 days. Probably, hydrophobicity of bacterial cells was closely related with a change of fatty acid composition, thereafter implying that the increasing amounts of saturated fatty acid (SFA) would result in a rise of the hydrophobicity of bacterial cells. Wong et al. (2) reported that total amounts of saturated fatty acid were largely declined in cells of *V. parahaemolyticus* ST550 during cold-starvation for 40 days, ranging from 60% to <40%. In this point of view, our results shown in Table 4 would indicate that decreases of the content of SFA, followed by a decline of the hydrophobicity, in VBNC cells of *V. parahaemolyticus* would correspond to the increasing resistances to environmental challenges. Several studies reported that nonculturable cells of bacteria, including *V. parahaemolyticus*, *V. vulnificus*, and *Campylobacter jejuni*, exerted notably higher resistances to 20 ppm H₂O₂, antibiotics, and heat (55°C for 3 min), supporting our findings (14, 27-28).

Morphological changes of VBNC cells

In a stationary-phase grown cell of *V. parahaemolyticus* ATCC 17082 (Fig. 6A), the cytoplasmic matrix was filled with granules and the cell membrane became intact without minor damages. On contrast, VBNC cells of *V. parahaemolyticus* ATCC 17082 were shown to have less densed cytoplasmic spaces than those of the stationary-phase cells. In particular, cellular membranes of VBNC cells were largely loosed, resulting in generating empty spaces between the outer and inner membranes (Fig. 6B-C). When it comes to a study conducted by Zhao et al. (29), it was reported that the number of ribosomes was notably reduced in VBNC cells of *Escherichia coli* O157:H7. These authors also pointed out that this change in the

interior structure of cells would be implicated with decreases of both deoxyribonucleic acid (DNA) amplification and protein translation, thereby causing these VBNC cells to minimize cell maintenance requirements (compactness)

Fatty acid compositions of VBNC *V. parahaemolyticus*

Cells of *V. parahaemolyticus* ATCC 17082 grown overnight in TSB accounted for 41.34% of the saturated fatty acid and 58.67% of unsaturated fatty acid out of the whole fatty acid composition. Normal cells exerted the highest amounts of palmitic acid (C_{16:0}, 25.64%) and palmitoleic acid (C_{16:1 w7c}, 26.76%), respectively. When cells of *V. parahaemolyticus* ATCC 17082 were incubated in ASW microcosms added with higher concentrations of NaCl at 4°C for 90 days, contents of palmitic acid were increased slightly, ranged from 23.05% to 23.88%. Among the saturated fatty acid, contents of lauric acid (C_{12:0}), 2-hydroxylauric acid (C_{12:0 2OH}), and myristic acid (C_{14:0}) were increased as *V. parahaemolyticus* ATCC 17082 became the viable-but-nonculturable state. In terms of the unsaturated fatty acid, VBNC cells exhibited increasing concentrations of palmitoleic acid, ranging from 32.98% to 35.77%. In particular, it was shown that unsaturated fatty acids were more synthesized with the increasing concentrations of NaCl. On the other hand, levels of cis-vaccenic acid (C_{18:1 w7c}) declined largely upon exposure of *V. parahaemolyticus* to the low temperature for 90 days in ASW microcosms with the addition of increasing amounts of NaCl, showing by 18.1%, 14.90%, 14.16%, and 13.18%, respectively. In addition, it seemed clear that several saturated fatty acids, including lauric acid, 2-hydroxylauric acid, and myristic acid, were responsible for the increasing amounts of total saturated fatty acid in VBNC cells of *V. parahaemolyticus* ATCC 17082. In a study conducted by Jia et al., (30), four strains of *V. parahaemolyticus* isolated

327 from seafood showed that palmitic acid ($C_{16:0}$) was decreased dramatically, ranging from 47.6%
 328 to 9.9% when these organisms were incubated in TSB at 4°C for 30 days, whereas
 329 palmitoleate ($C_{16:1}$) increased greatly after the entry into the VBNC state. This study was in
 330 an agreement with our findings. It was also reported that palmitic acid contents (%) of *V.*
 331 *vulnificus* C7184 were significantly ($p < 0.05$) lower once this organism was incubated in
 332 ASW microcosms at 5°C for 24 hrs (31). In VBNC cells of *V. vulnificus*, palmitoleic acid
 333 ($C_{16:1}$) and stearic acid ($C_{18:0}$) increased significantly ($p < 0.05$), while margaric acid ($C_{17:0}$)
 334 declined remarkably at the refrigerator temperature within 24 hrs, supporting our findings.
 335 Furthermore, it was demonstrated that VBNC cells of *V. parahaemolyticus* ATCC 17082 were
 336 recovered to the culturable state reversibly as being resuscitated in a nutrient-rich medium
 337 such as TSB (pH 8) and brain heart infusion broth (pH 8) at 25°C for several days (*data not*
 338 *shown*). Thus, resuscitated cells were further used to perform the fatty acid composition
 339 analysis in the present study. As shown in Table 6, the total density of unsaturated fatty acids
 340 was increased more than normal and VBNC cells. Especially, lauric acid ($C_{12:0}$), 2-
 341 Hydroxylauric acid ($C_{12:0} \text{ 2OH}$), and unknown ($C_{14:0} \text{ 3OH}$) were increased comparatively,
 342 whereas palmitic acid ($C_{12:0}$) was decreased remarkably among the total saturated fatty acid
 343 of resuscitated cells. After resuscitation, cis-vaccenic acid ($C_{18:1 \text{ w7c}}$) increased largely,
 344 ranging from 20.65% to 32.17%. Of which on the resuscitated cells of *V. parahaemolyticus*
 345 ATCC 17082, the total amounts of saturated fatty acid declined, but unsaturated fatty acids
 346 increased to that extent. In our preliminary studies, VBNC *V. parahaemolyticus* exhibited
 347 higher resistances through a simulated human gastric digestion (*data not shown*). Along with
 348 our findings in this study, it would be suggested that increasing synthesis of saturated fatty
 349 acids in the VBNC cells correspond to the improved environmental resistance. As saturated
 350 fatty acids display a straight-line structure VBNC cells would acquire more compact cell

membranes, thereby resulting in more decreased cellular permeabilization than that of the normal cells. Consequently, it would administer higher resistances to various environmental challenges for these VBNC cells. Previously, several studies disclosed that there were significantly different fatty acid compositions on the bacterial membranes between actively growing and VBNC cells (2, 31). Interestingly, it has been shown that ratios of saturated fatty acids/unsaturated fatty acids increased significantly after forming VBNC cells. Probably, this change would be closely associated with a distinct physiology of VBNC cells. Given that VBNC cells might be capable of re-gaining the platable-capability on culture media, followed by further resuscitation efforts, there are still restricted information available on understanding cellular properties of VBNC *V. parahaemolyticus*. By comparing the fatty acid composition between VBNC and resuscitated cells, it would provide a meaningful insight in revealing out mechanisms governing the entry of *V. parahaemolyticus* into the VBNC state.

Based on all the findings obtained from the present study, a novel hypothesis explaining the entrance of *V. parahaemolyticus* into the viable-but-nonculturable state would be established (Fig. 7). It would be plausible that the morphological transformation of bacterial cells either from rods to coccus or the dwarfing in the cellular size has to be preceded before the actively growing cells could turn into the VBNC state. Previously, it was determined that 100-days-stressed cells of VBNC *V. parahaemolyticus* displayed the increasing cellular leakages such as nucleic acid and protein as much twice as the pure cultures. Besides, it was also shown that VBNC *V. parahaemolyticus* exhibited the increasing permeabilization through the outer membrane, indicating that the loss of cell membrane integrity caused by the increasing membrane permeabilization could result in the cellular leakage before VBNC cells could be steadily developed. Hence, such a transient conversion of bacterial cells could aim to alter the surface area as compact as possible, minimizing the cellular damage and the consumption of

energy sources under continuous environmental stresses (nutrient-deprivation and cold temperature). One of our findings about the morphological change of VBNC *V. parahaemolyticus* can be in an agreement with this hypothesis strongly. Subsequently, fatty acid composition of these cells will begin to be altered, showing the increasing levels of saturated fatty acid. This procedure might be significantly associated with the complete compactness of the cellular membrane structure, and then could diminish the increased permeabilization of outer membranes into normal ranges, thereby stabilizing VBNC cells at the end. Seriously, as most VBNC cells of bacteria exerted higher resistances to various environmental conditions more than the actively grown cells enhanced survivals of VBNC cells could be elucidated by the alternation of fatty acid compositions after forming VBNC cells. Overall, it can be defined that once challenged by environmental stresses nonculturable bacteria, which show cellular modifications such as the increasing synthesis of saturated fatty acid, causing the cellular dwarfing in sizes to minimize the exposure of bacterial surfaces and to save the energy consumption, and then prevent intracellular periplasms from being seriously damaged by ROS, maintaining the integrity of bacterial membranes, could be called as viable-but-nonculturable cells.

In conclusion, this research was conducted to determine the cellular characteristics of VBNC *V. parahaemolyticus*. As shown in Table 4, three strains of *V. parahaemolyticus* used in this study showed that VBNC cells (I) retained the cellular virulence to Vero and CaCO₂ cell lines, (II) re-gained the 100% cytotoxicity even after resuscitation process, (III) became permeabilized mainly at the binding sites of the outer membrane, (IV) showed lower levels of enzymatic (catalase and GST) activities, (V) exerted the increasing hydrophobicity, (VI) showed increasing amounts of SFA, and then (VII) displayed flappy outer membranes out of

the cytoplasm in the cellular morphology. As pathogens can encounter a number of environmental stresses through a wide variety of channels contaminated food materials are subjected to heat, desiccation, cold temperature, freeze, and others in the middle of the preparation and the processing. As one of the surviving strategies, pathogens on food respond to these challenges, could turn into the VBNC state, and then can pose a risk of causing the food-borne outbreaks potentially. Clearly, it was demonstrated that VBNC cells could be the source of food-borne outbreaks upon eliminating the initiative environmental stress. Therefore, understanding the cellular characterization of nonculturable pathogens is of much importance in an attempt to designing adequate control measures for assuring the food safety. Although these results cannot reveal out all of the properties of VBNC bacteria, it will provide useful basic natures of a bacterial surviving strategy, understanding a mechanism governing the transition into the VBNC state.

MATERIALS AND METHODS

Preparation of microcosm solutions ASW solutions (Sigma-Aldrich, St. Louis, MO, USA) were prepared according to the manufacturer's instruction. Formal ASW microcosms (pH 7.2-7.8) contained 19,290 mg of Cl, 10,780 mg of Na, 2,660 mg of SO₄, 420 mg of K, 400 mg of Ca, 200 mg of CO₃, 8.8 mg of Sr, 5.6 mg of B, 56 mg of Br, 0.24 mg of I, 0.3 mg of Li, 1.0 mg of F, and 1,320 mg of Mg per 1 liter of sterile distilled water. These microcosms were autoclaved at 125°C for 20 min before use. Then, NaCl concentrations of these microcosms were adjusted to 0.75%, 5%, 10%, and 30% (m/v), respectively. Each of these microcosms was adjusted to pH 6.0–6.2, using a membrane-filtered 1N NaOH solution (Kanto chemical, Tokyo, Japan), facilitating the induction of *V. parahaemolyticus* into the VBNC state.

Preparation of bacterial inoculums Cells of *V. parahaemolyticus* ATCC 17082, *V. parahaemolyticus* ATCC 27969, and *V. parahaemolyticus* ATCC 33844 were purchased from the Korean Collection for Type Cultures (KCTC, Daejeon, Korea). Bacterial stocks were maintained at -75°C and further activated by a series of transfers in tryptic soy broth (Difco, Detroit, MI, USA) supplemented with 3% NaCl (TSB) at 37°C for 24 hrs before use. Each strain of *V. parahaemolyticus* ATCC 17082, *V. parahaemolyticus* ATCC 33844, and *V. parahaemolyticus* ATCC 27969 grown overnight in 35 ml of TSB at 37°C for 24 hrs was harvested by the centrifugation at 10,000 X g for 3 min, washed twice, and then bacterial pellets were re-suspended in 1 ml of ASW solutions (pH 6), corresponding to the bacterial density of approximately 10⁸⁻⁹ CFU/ml. Finally, cells of these pathogens were inoculated in 100 ml of ASW microcosms (pH 6) added with 0.75%, 5%, 10% and 30% NaCl, respectively. Bacterial fluids were kept at 4°C and ASW microcosms were withdrawn from the incubator at regular time-intervals to conduct the following process.

Enumeration of the bacterial population In order to count the bacterial number, cells of *V. parahaemolyticus* ATCC 17082, *V. parahaemolyticus* ATCC 33844, and *V. parahaemolyticus* ATCC 27969 were plating-counted on tryptic soy agar (Difco) supplemented with 3% NaCl (TSA). Decimal dilutions (10⁻¹) of these cells were prepared in alkaline peptone water (APW, pH 8) consisting 10 g of peptone and 10 g of NaCl per 1 liter of distilled water and 100 µl of these aliquots was spread on TSA. These media were further incubated at 37°C for 24 hrs. Colonies developed on media were further enumerated. At the same time, the numbers of living and dead cells of *V. parahaemolyticus* were measured using the Live/Dead^(R) BacLight Bacterial Viability kit (Life technologies, Eugene, Oregon, USA). Briefly, equal volumes (1:1)

of SYTO9 and propidium iodide (PI) were combined and 3 µl of this mixture was added to each 1 ml of the bacterial cell. After a short time of incubation (approximately 15 min) at an ambient temperature in the dark, 5-8 µl of this aliquot was attached on a glass slide and a coverslip was placed on it. Then, bacterial images were demonstrated using an electron microscope (TE 2000-U, Nikon, Japan).

Preparation of human cell lines CACO-2 and Vero cells were cultured in 5-10 ml of Dulbecco's modified eagle's medium (DMEM, Corning, NY, USA) supplemented with 5% and 20% fetal bovine serum (FBS, Corning) at 37°C for 1-2 days in 5% CO₂, respectively. The medium was removed in a petri-dish and washed 5 ml of PBS three times, and then 5 ml of trypsin (Corning) was added to facilitate the cell lysis. Each of these cells was incubated at 37°C for 5 min in 5% CO₂. To alleviate the enzymatic activity caused by trypsin, 2-3 ml of the medium was added to all the cell cultures. Cell fluids were further transferred into 15 ml of a sterile cap tube and centrifugated at 15,000 X g for 3 min. The supernatant was eliminated, re-suspended in 5 ml of the medium, corresponding to the cellular density of 10⁴/ml, and then 100 µl of the eukaryotic cell solution was loaded into a 96-well plate containing 100 µl of the DMEM medium. CACO-2 and Vero cells were incubated at 37°C for 24 hrs in 5% CO₂ before use.

Measurement of the cytotoxicity of VBNC *V. parahaemolyticus* against the cell line

Cellular virulence of *V. parahaemolyticus* in the VBNC state against the human cell line was measured according to several studies conducted by Beattie and Williams (32), Jeßberger et al. (33), and Dektas et al. (34). As mentioned above, prepared cell lines, corresponding to 10⁴ CFU/ml, were added into a 96-well plate, respectively. Serial dilutions (0.1 ml/well) of

the filtered cell-free bacterial supernatants were placed into 96-well plates. Or bacterial suspensions, corresponding to 10^8 CFU/ml, was added in each of wells. Animal cell suspensions (0.1 ml/well), corresponding to 10^4 cells per a well for Vero cells and 2×10^4 cells per a well for CACO-2 cells, were supplemented into 96-well plates, respectively. Cell fluids were incubated at 37°C for 24 hrs under the atmospheric environment containing 5% CO₂, replaced with either 100 µl/well of EMEM containing 5 mg/ml 3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyl tetrazolium bromide (MTT, Corning), and then incubated at 37°C for 1 hr.

These mixtures were further removed in each of the wells, 100 µl of dimethyl-sulphoxide (DMSO, Corning) was added into a 96-well plate, and then read on a microtiter plate reader at 570 and 620 nm (Multiskan GO Microplate Spectrophotometer, Thermo Scientific, Vantaa, Finland).

Analysis of the cell membrane permeabilization The release of cytoplasmic β-galactosidase from cells of VBNC *V. parahaemolyticus* was measured to determine the inner membrane permeabilization. Briefly, 10 µl of VBNC *V. parahaemolyticus* cells in ASW microcosms (pH 6) were transferred onto each well of a 96-well plate containing 50 µl of *O*-Nitrophenyl-L-D-galactoside (Sigma) and 40 µl of ASW solutions (pH 6). Once bacterial suspension was incubated at 30°C for 30 min in a dark room the absorbance was read on a microtiter plate reader at 420 nm (Multiskan GO Microplate Spectrophotometer). When it comes to determine the outer membrane permeabilization of VBNC cells, 10 µl of VBNC *V. parahaemolyticus* cells in ASW microcosms were added to each well of a 96-well plate containing 80 µl of ASW solutions and 10 µl of nitrocefin (Sigma), incubated at 30°C for 30 min in a dark room, and then the absorbance was read on a microtiter plate reader at 482 nm

(Thermo Scientific).

Analysis of the cellular leakage in the bacterial suspension Cell membrane integrity was assessed by measuring cellular leakages such as nucleic acid and protein from VBNC cells of *V. parahaemolyticus*. Cell suspensions (1.5 mL) of *V. parahaemolyticus* ATCC 17082, *V. parahaemolyticus* ATCC 33844, and *V. parahaemolyticus* ATCC 27969 which had been incubated in ASW microcosms at 4°C for 100 days were transferred onto a sterile microtube, respectively. Pure cultures of these pathogens grown overnight in TSB at 37°C were centrifuged at 13,000 X g for 3 min, washed twice, and then re-suspended in ASW solutions (pH 8) added with 0.75%, 5%, 10%, and 30% NaCl, respectively. These pathogens were centrifuged at 10,000 X g at 4°C for 15 min to collect bacterial pellets. Each of these supernatants was collected in a sterile microtube and further used to assess the cellular leakage such as nucleic acid and protein by measuring the absorbance at 260 and 280 nm via multi-scan Go spectrophotometer (Thermo Scientific Inc.).

Measurement of enzymatic activities Catalase activity was measured in terms of H₂O₂-degradation with a spectrophotometric assay (CAT100, sigma). This assay was undertaken following by a method introduced in several studies (Santander et al., 2017; Molina-Cruz et al., 2008). Overnight grown cultures of *V. parahaemolyticus* and VBNC cells incubated in ASW (pH 6) microcosms at 4°C for 100 days were re-suspended in 50 mM potassium phosphate buffer (pH 7) containing 1 g of 3-mm-sized glass bead (Sigma), vortexed for 25 min, and then centrifuged at 13,000 X g for 3 min. According to a manual provided by a manufacture, supernatant was separately transferred into a sterile microtube. In a total of 100 µl of volume, 15 µl of supernatant was combined with 5 mM H₂O₂. Such a reaction was

carried out at 25°C for 15 min and ceased with 900 µl of 15 mM sodium azide. Then, absorbance was colorimetrically read at 520 nm via multi-scan Go spectrophotometer (Thermo Scientific Inc.). Glutathione-S-Transferase (GST) activity was measured, using GST assay kit (CS0410, Sigma). In this assay, 1-Chloro-2, 4-DiNitroBenzene (CDNB) was used as a substrate. Twenty µl of bacterial supernatants were added to 180 µl of a substrate solution in each well of a 96-well plate, respectively. Then, absorbance was read at 340 nm via multi-scan Go spectrophotometer.

Microbial adhesion to hydrocarbons (MATHs) assay Cells of *V. parahaemolyticus* grown overnight in TSB and incubated in ASW microcosms at 4°C for 50 days were centrifugated at 8,000 X g for 1 min, washed twice, and then re-suspended in 0.1 M PBS to fit an optical density of 1.0 (A_0) at 600 nm via a UV-Visible Spectrometer (Multiskan GO, Thermo Scientific). One hundred µl of hexadecane was added to 1 ml of these bacterial suspensions and incubated at an ambient temperature for 10 min. Optical density of these bacteria in the aqueous phase was measured at 600 nm (A_1). The degree of hydrophobicity was calculated, following as (Choi, et al., 2013);

$$[1-A_1/A_0] \times 100 (\%).$$

Transmission electron microscopic (TEM) Assay Bacterial cells incubated in ASW microcosms (pH 6) containing 0.75% NaCl or supplemented with 5%, 10%, and 30% NaCl at 4°C for 7 days were centrifugated at 10,000 X g for 3 min, rinsed three times with 0.1M Phosphate Buffered Saline (PBS, pH 7.2), and then re-suspended in 0.1M PBS to collect the pellets. These cells were fixed in 2% paraformaldehyde overnight at 4°C. After washing with

PBS, cells of *V. parahaemolyticus* were post-fixed in 1% osmium tetroxide in 0.1M PBS and dehydrated in a series (30%, 50%, 70%, 95%, and 100%) of ethanol solutions. Thereafter, each of bacterial cells were infiltrated with 2 ml of epoxy resin. Polymerization of the resin was performed at 60°C for 24 hrs. Then, sections, approximately 120 nm thickness, were cut randomly and photographed with a JEOL JEM 1200 EX transmission electron microscope (JEOL USA Inc., Peabody, MA, USA).

Fatty acid composition analysis Whole cell fatty acid analysis was conducted according to a standardized method by Microbial Identification System (MIDI, Microbial ID Inc., Newark, Del., USA). Cells from 5 ml of a stationary-phase culture and 5 ml of VBNC *V. parahaemolyticus* were harvested by centrifugation at 10,000 X g for 5 min. Bacterial cells were subjected to a series of procedures, including saponification, methylation, and washing to extract carboxylic acid derivatives of long-chain aliphatic molecules. Then, extracted lipids were used to analyze the fatty acid composition of *V. parahaemolyticus* in the VBNC state, following several procedures provided by the supplier (Microbial Identification System, Newark, Del.)

Assessment of VBNC bacterial resistances to an acidic environment Ten ml of an exponential-phase culture and 10 ml of VBNC *V. parahaemolyticus* ATCC 17082 were harvested by centrifugation at 10,000 X g for 5 min, washed twice, and then re-suspended in 10 ml of PBS, respectively. A simulated human gastric juice was prepared according a study conducted by Lee et al. (2016). This human digestion model (pH 1.5) consisted of 6.5 ml of HCl (37 g per 1 L of DW), 18 ml of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (37 g per 1 L of DW), 1 g of bovine serum albumin (Corning), 2.5 g of pepsin (Sigma), and 3 g of mucin (Sigma). These chemicals and

enzymes used in the present study were either autoclaved or filtered through a 0.2-um-size a polycarbonate membrane (ADVANTEC) before use. Thus, 10 ml of the bacterial cell was mixed with 10 ml of the gastric juice. Each of *V. parahaemolyticus* ATCC 17082 cells in a simulated stomach digestion model was incubated in a shaking water bath (120 rpm) at 37°C for 60 min. At a regular time-interval, the bacterial population was plating-counted on TSA and the viability of nonculturable *V. parahaemolyticus* ATCC 17082 was also enumerated with the fluorescence microscopic assay.

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Figure legends

FIG 1 Loss of the culturability (A, C, and E) and the viability (B, D, and F) of *V. parahaemolyticus* ATCC 17082 (A-B), *V. parahaemolyticus* ATCC 33844 (C-D), and *V. parahaemolyticus* ATCC 27969 (E-F) incubated in ASW (pH 6) microcosms supplemented with varying concentrations of NaCl at 4°C for 100 days.

FIG 2 Changes of the cytotoxicity of *V. parahaemolyticus* ATCC (A and D) 17082, *V. parahaemolyticus* ATCC (B and E) 33844, and *V. parahaemolyticus* ATCC (C and F) 27969 incubated in ASW (pH 6) microcosms at 4°C for 80 days against (A-C) Vero and (D-F) CACO-2 cells, respectively. (A-C); a, 0 day; b, 7 days; c, 21 days; d, resuscitated cells in TSB after 21 days of cold-starvation; f, 80 days; g, resuscitated cells in TSB after 21 days of cold-starvation; *, not determined. (D-F); a, 0 day; b, 7 days, c, resuscitated cells in TSB after 21 days of cold-starvation; d, 80 days; e, resuscitated cells in TSB after 80 days of cold-starvation; *, not determined. Cells of VBNC *V. parahaemolyticus* suspended at 4°C for 21 and 80 days were transferred onto TSB, and then incubated at 25°C for consecutive 3 days, respectively.

FIG 3 Assessment of the cellular membrane permeability (A, β -lactamase activity; B, β -galactosidase activity) of *Vibrio parahaemolyticus* ATCC 17082 and *V. parahaemolyticus* ATCC 33844 incubated in ASW microcosms at 4°C for 30 days.

FIG 4 Assessment of catalase activity (U/mg protein) of *V. parahaemolyticus* ATCC (A)

17082, *V. parahaemolyticus* ATCC (B) 33844, and *V. parahaemolyticus* ATCC (C) 27969 in the VBNC state at 4°C for 100 days.

FIG 5 Investigation on the morphology of *V. parahaemolyticus* ATCC 33844 (A) before and (B-C) after the entry into the VBNC state. Bacterial cells of *V. parahaemolyticus* ATCC 33844 were incubated in ASW (pH 6.1) microcosms containing (B) 0.75% NaCl or (C) supplemented with 5% NaCl at 4°C for 100 days.

FIG 6 Comparison of fatty acid compositions between *V. parahaemolyticus* ATCC 17082 incubated in ASW (pH 6) microcosms at 4°C for 90 days and resuscitated cells.

FIG 7 A schematic diagram for elucidating the transition process of *V. parahaemolyticus* cells into the viable-but-nonculturable state.

(A)

(B)

(C)

(D)

(E)

(F)

(A)

(B)

(C)

(D)

(E)

(F)

(A)

(B)

(A)

(B)

(C)

(B)

(A)

(C)

Envrionmental stress

Normal cell

Morphological change

VBNC cell

Resistance ▲

1

TABLE 1 Measurement of the cellular leakage (nucleic acid, at OD 260 nm) of *V. parahaemolyticus* cells incubated in ASW (pH 6) microcosms at 4°C for 150 days

Pathogen	ATCC	Before (0 hrs):				After (150 days):			
		ASW	ASW+5% NaCl	ASW+10% NaCl	ASW+30% NaCl	ASW	ASW+5% NaCl	ASW+10% NaCl	ASW+30% NaCl
<i>V. parahaemolyticus</i>	17082	0.433	0.502	0.598	0.974	0.868	1.200	0.999	1.053
<i>V. parahaemolyticus</i>	33844	0.576	0.685	0.783	1.562	2.611	2.550	2.315	2.683
<i>V. parahaemolyticus</i>	27969	0.751	0.778	0.659	1.107	3.255	3.417	3.385	3.755

2

3

TABLE 2 Measurement of the cellular leakage (protein, at OD 280 nm) of *V. parahaemolyticus* cells incubated in ASW (pH 6) microcosms at 4°C for 150 days

Pathogen	ATCC	Before (0 hrs):				After (150 days):			
		ASW	ASW+5% NaCl	ASW+10% NaCl	ASW+30% NaCl	ASW	ASW+5% NaCl	ASW+10% NaCl	ASW+30% NaCl
<i>V. parahaemolyticus</i>	17082	0.390	0.435	0.465	0.797	0.426	0.602	0.600	0.638
<i>V. parahaemolyticus</i>	33844	0.466	0.537	0.630	1.316	1.270	1.218	1.226	1.433
<i>V. parahaemolyticus</i>	27969	0.573	0.597	0.521	0.910	1.588	1.747	1.872	2.351

4

5

TABLE 3 Measurement of hydrophobicity (%) of viable-but-nonculturable *V. parahaemolyticus* at 4°C for 100 days

Pathogen	Microcosm	Incubation period (days)	
		0	100
<i>V. parahaemolyticus</i> ATCC 17082	ASW	37.1±2.7	ND ²
	ASW+5% NaCl	26.3±3.2	37.5±0.2
	ASW+10% NaCl	45.8±3.6	46.8±5.0
	ASW+30% NaCl	27.2±2.5	62.2±2.8
<i>V. parahaemolyticus</i> ATCC 33844	ASW	22.0±1.9	ND
	ASW+5% NaCl	22.8±0.4	20.9±2.5
	ASW+10% NaCl	22.2±2.4	37.1±3.7
	ASW+30% NaCl	29.8±1.8	41.1±0.9
<i>V. parahaemolyticus</i> ATCC 27969	ASW	22.0±0.8	25.2±16.6
	ASW+5% NaCl	23.0±2.0	17.5±1.2
	ASW+10% NaCl	23.1±1.9	23.1±6.4
	ASW+30% NaCl	20.8±15.8	43.7±5.8

¹ Surface hydrophobicity (%) of *V. parahaemolyticus* strains was calculated, following an equation as; $(A_0 - A_1)/A_0 \times 100$. A_0 , the absorbance of the untreated cell suspension at 600 nm; A_1 , the absorbance of the bacterial assay tube at 600 nm.

² ND, not determined.

6

TABLE 4 Comparison on the fatty acid composition (%) of viable-but-nonculturable *V. parahaemolyticus* ATCC 17082 at 4°C for 90 days

Fatty acid	Name	Category	Microcosm				
			Ctrl	ASW	ASW+5% NaCl	ASW+10% NaCl	ASW+30% NaCl
11:0 iso 3OH	3-Hydroxy-9-Methyldecanoic acid	Branched-chain	0.75%	ND ¹	ND	0.30%	0.32%
12:0	Lauric acid	Saturated	2.02%	4.37%	4.38%	4.37%	4.44%
12:0 2OH	2-Hydroxylauric acid	Hydroxy	1.63%	1.94%	3.12%	3.05%	2.92%
14:0	Myristic acid	Saturated	7.25%	8.05%	9.11%	9.19%	8.81%
14:0 3OH	ND	Hydroxy	1.86%	1.50%	1.68%	1.58%	1.84%
16:0	Palmitic acid	Saturated	25.64%	28.65%	23.88%	23.36%	23.05%
16:0 N alcohol	Cetyl alcohol	Saturated	- ²	ND	0.20%	0.40%	0.50%
18:0	Stearic acid	Saturated	2.19%	2.71%	ND	1.23%	1.22%
Total (saturated fatty acid)			41.34%	47.22%	42.37%	43.48%	43.10%
16:1 w7c	Palmitoleic acid	Unsaturated	26.76%	24.52%	32.98%	34.11%	35.77%
16:1 w6c	cis-10-Palmitoleic acid	Unsaturated	3.78%	7.13%	5.11%	4.44%	3.82%
16:1 w5c	cis-11-Palmitoleic acid	Unsaturated	-	-	0.50%	0.42%	0.53%
16:1 w7c alcohol	Palmitoleyl alcohol	Unsaturated	-	-	-	0.37%	-
17:1 anteiso w9c	(7Z)-13-Methyl-7-Hexadecenoic acid	Unsaturated	7.52%	3.12%	2.13%	2.42%	2.84%
17:1 iso	Isomargaric acid	Branched-chain	-	-	0.85%	0.24%	-
18:1 w9c	Oleic acid	Unsaturated	-	-	-	0.38%	-
18:1 w7c	cis-Vaccenic acid	Unsaturated	20.61%	18.01%	14.90%	14.16%	13.18%
18:1 w5c	cis-13-oleic acid	Unsaturated	-	-	-	0.35%	-
18:2 w6c	Linoleic acid	Unsaturated	-	-	-	-	0.37%
Total (unsaturated fatty acid)			58.67%	52.78%	56.47%	56.89%	56.51%

¹ ND, not determined.² -, not found.

TABLE 5 Comparison of fatty acid composition (%) of *V. parahaemolyticus* ATCC 17082 to be recovered from the VBNC state

Fatty acid	Name	Category	Microcosm			
			Ctrl	ASW	ASW+5% NaCl	ASW+10% NaCl
11:0 iso 3OH	3-Hydroxy-9-Methyldecanoic acid	Branched-chain	0.75%	0.39%	- ¹	-
12:0	Lauric acid	Saturated	2.02%	2.71%	2.75%	2.95%
12:0 2OH	2-Hydroxylauric acid	Hydroxy	1.63%	2.48%	2.55%	2.78%
13:0 iso	Isotridecanoic acid	Branched-chain	-	0.65%	0.63%	0.65%
13:0 3OH	ND	Branched-chain	-	-	0.29%	0.28%
14:0	Myristic acid	Saturated	7.25%	4.50%	4.18%	4.70%
14:0 3OH	ND	Hydroxy	1.86%	2.79%	3.09%	2.40%
15:0 anteiso	12-Methylmyristic acid	Saturated	-	-	-	0.38%
16:0	Palmitic acid	Saturated	25.64%	10.40%	15.52%	14.65
16:0 iso	Isopalmitic acid	Branched-chain	-	-	0.33%	0.52%
16:0 N alcohol	Cetyl alcohol	Saturated	-	-	-	-
17:0	Margaric acid	Saturated	-	1.45%	2.11%	1.75%
17:0 anteiso	Anteisomargaric acid	Branched-chain	-	0.82%	0.47%	0.63%
17:0 iso	Isomargaric aid	Branched-chain	-	-	0.34%	0.44%
18:0	Stearic acid	Saturated	2.19%	1.68%	1.57%	0.55%
Total (saturated fatty acid)			41.34%	27.87%	33.83%	32.30%
16:1 w7c	Palmitoleic acid	Unsaturated	26.76%	24.55%	23.14%	26.80%
16:1 w7c alcohol	Palmitoleyl alcohol	Unsaturated	-	-	0.22%	-
16:1 w6c	cis-10-Palmitoleic acid	Unsaturated	3.78%	7.03%	8.22%	10.16%
17:1 anteiso w9c	(7Z)-13-Methyl-7-Hexadecenoic acid	Unsaturated	7.52%	3.83%	1.09%	2.38%
17:1 w8c	cis-margaroleic acid	Unsaturated	-	2.00%	1.90%	1.84%
17:1 w6c	(11Z)-11-Heptadecenoic acid	Unsaturated	-	1.39%	1.22%	1.37%
18:1 w7c	cis-Vaccenic acid	Unsaturated	20.61%	32.17%	30.38%	23.98%
18:2 w6c	Linoleic acid	Unsaturated	-	0.93%	-	0.26%
Total (unsaturated fatty acid)			58.67%	71.90%	66.17%	66.79%

¹ -, not found.

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TABLE 6. Determination of cellular properties of *V. parahaemolyticus* in the VBNC state

Property ¹		Category		
		Normal cells	VBNC cells	Resuscitated cells
Cell size		-	▼	-
Morphology		Rod	Coccus	Rod
Membrane damage		-	Blebbing	-
Cytotoxicity		▲	▼	▲
Hydrophobicity		-	▲	ND ²
Fatty acid composition	SFA	-	▲	▼
	USFA	-	▼	▲
Enzyme activity	Catalase	-	-	ND
	GST	-	-	-
Cellular leakages		-	▲	ND

¹ SFA, saturated fatty acid; USFA, unsaturated fatty acid; GST, glutathione-S-transferase.² ND, not determined.

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