Effects of varying concentrations of sodium chloride and acidic conditions on the behavior of *Vibrio parahaemolyticus* and *Vibrio vulnificus* cold-starved in artificial sea water microcosms

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Abstract There has been limited information available on the behavior of *Vibrio parahaemolyticus* and *Vibrio vulnificus* as a function of higher levels of NaCl in combination with acidic pH. In the present study, bacterial suspensions were transferred into artificial seawater (pH 4–7) microcosms containing 0.75% NaCl and supplemented with 5, 10, and 30% NaCl, respectively. Each of *V. parahaemolyticus* and *V. vulnificus* was inoculated in these microcosms and fermented seafood, and then stored at 4 °C until the microbial populations reached below the detectable levels on agar plates (thiosulphate-citrate-bile salts-sucrose agar and tryptic soy agar amended with 3% NaCl). Consequently, *V. parahaemolyticus* ATCC 27969, *V. parahaemolyticus* ATCC 33844, and *V. vulnificus* ATCC 33815 rapidly reached the viable-but-nonculturable (VBNC) state with increasing levels ($\leq 30\%$) of NaCl at 4 °C. Within seven days, these pathogens in seafood appeared to enter the VBNC state at 4 °C, as shown by the fluorescence microscopic assay.

Keywords Fermented seafood · Morphology · Pathogen · Sodium chloride · Viable-but-nonculturable state

Introduction

Amongst a number of pathogenic bacteria isolated from seafood, *Vibrio parahaemolyticus* and *Vibrio vulnificus* are known to be closely associated with food-borne outbreaks

caused by seafood [1, 2]. *Vibrio parahaemolyticus* and *V. vulnificus* are major food-borne pathogens and are commonly found in natural aquatic environments [3–5]. Consumption of contaminated seafood containing these bacteria may cause serious human infections ranging from acute gastrointestinal disease, vomiting, and abdominal pain to septicemia [6, 7]. Importantly, previous studies have revealed that *V. parahaemolyticus* and *V. vulnificus* can enter into the viable-but-nonculturable (VBNC) state, wherein bacteria do not develop any colonies on typical culture media on which they can produce an aggregate of colonies under various environmental conditions [8–10]. The loss of bacterial culturability could be induced by exposing *V. parahaemolyticus* and *V. vulnificus* to low temperatures (<15 °C), a nutrient-limited environment, and osmotic stress. It has been revealed that the entry of some pathogenic bacteria, including *Listeria monocytogenes*, *Salmonella enterica* serovar Typhimurium, *Vibrio harveyi*, *V. parahaemolyticus*, and *V. vulnificus*, into the VBNC state took a long period of storage ranging from several days to a whole year under unfavorable stresses [1, 3, 11–14]. Bacteria in the VBNC state may express potentially higher levels of virulence within several days after the removal of environmental stress, indicating that *V. parahaemolyticus* and *V. vulnificus* in VBNC would still remain pathogenic and pose a risk of human infections during the storage period [15]. Furthermore, one of the major considerations regarding the relationship between seafood consumption and microbial contamination is that a few food-borne outbreaks caused by *V. parahaemolyticus* and *V. vulnificus* have been linked with fermented seafood products [16, 17]. As a non-thermally processed product, fermented seafood can be susceptible to microbial contamination through a variety of channels [18]. Previous studies have identified the major environmental factors

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mediating the entry of *V. parahaemolyticus* and *V. vulnificus* into the VBNC state using a laboratory culture broth, which resembled the components of seawater [19–21]. However, only a few studies are available on the behavior of *V. parahaemolyticus* and *V. vulnificus* at the refrigeration temperature as a function of varying levels of sodium chloride (NaCl) and acidic pH levels in artificial sea water (ASW) microcosms. Therefore, this study investigates the effects of different concentrations of NaCl (0.75, 5, 10, and 30%) and acidic pH (4, 5, 6, and 7) on the survival of *V. parahaemolyticus* and *V. vulnificus* incubated in ASW microcosms that simulate the general conditions of various fermented seafood at 4 °C. Subsequently, the ability of the cells of *V. parahaemolyticus* and *V. vulnificus* to exit from the nonculturable state was examined by performing a simple reversal of the VBNC-inducing stress. Morphological changes in *V. vulnificus* were also demonstrated using transmission electron microscopy (TEM) before and after the loss of culturability on media.

Methods and materials

Preparation of microcosms

An ASW solution (Sigma-Aldrich, St. Louis, MO, USA) was prepared according to the manufacturer's instruction. Specifically, 100 mL of ASW microcosms were supplemented with excessive amounts of NaCl, matching the content of 5, 10, and 30% NaCl and autoclaved at 120 °C for 20 min. Then, each of the microcosms was adjusted to pH 4–7 using a 10% lactic acid solution (Kanto chemical, Tokyo, Japan) because the acidic condition of fermented seafood results from the accumulation of metabolites such as lactic acid.

Bacterial inoculation and enumeration

Vibrio parahaemolyticus ATCC 27969, *V. parahaemolyticus* ATCC 33844, and *V. vulnificus* ATCC 33815 were purchased from the Korean Collection for Type Cultures (Daejeon, Korea). They were maintained at –75 °C and activated by two successive transfers in tryptic soy broth (TSB, Difco, Detroit, MI, USA) supplemented with 3% NaCl at 37 °C for 24 h. These pathogens in TSB supplemented with 3% NaCl were harvested via centrifugation at 10,000g for 3 min and then washed twice with a 0.1 M phosphate buffer saline (PBS, pH 7.2). The final pellets were resuspended in 1 mL of the ASW solution. Bacterial suspensions were inoculated in 100 mL of ASW microcosms (pH 4–7) amended with different amounts of NaCl and then stored at 4 °C. ASW microcosms were

withdrawn from the incubator, and the microbial populations were enumerated at regular intervals.

V. parahaemolyticus ATCC 27969, *V. parahaemolyticus* ATCC 33844, and *V. vulnificus* ATCC 33815 in ASW were plating-counted on tryptic soy agar supplemented with 3% NaCl (TSA, Difco) and thiosulphate-citrate-bile salts-sucrose (TCBS, Difco). Briefly, decimal dilutions (10^{-1}) of the bacterial suspensions were prepared in alkaline peptone water (pH 8.2) comprising 10 g of peptone and 10 g of NaCl per 1 L of distilled water, and 100 µL of the aliquot was spread on TSA and TCBS. These plates were incubated at 37 °C for 24 h, and then the colonies developed on the media were enumerated. Simultaneously, bacterial viability was measured using the Live/Dead BacLight bacterial viability kit (Life technologies, Eugene, OR, USA). Equal volumes (1:1) of SYTO9 and propidium iodide were combined, and 3 µL of this mixture was added to 1 mL of the bacterial suspension. Each vial was incubated at the ambient temperature for 15 min in the dark. Then, 5–8 µL of the stained bacterial suspension was mounted on a glass slide and covered with a coverslip. Bacterial images were demonstrated using an electron microscope (Eclipse TE 2000-U, Nikon, Tokyo, Japan). For each sample, at least 10 randomly selected fields containing 2 to more than 500 cells per field were counted. The number of bacteria per milliliter was calculated using a conversion factor derived from the number of fields in a bacterial droplet on a slide and the volume of a diluted cell suspension. The number of all bacteria-like particles was designated as the total cell number. The cells stained with green color were counted and designated as viable counts.

Transmission electron microscopy (TEM)

Bacterial cells incubated in ASW microcosms (pH 6) containing 0.75% NaCl or supplemented with 5, 10 or 30% NaCl at 4 °C for seven days were used for TEM, according to the manufacturer's instruction. Briefly, these bacteria were centrifugated at 3000×g for 10 min and the supernatants were removed. They were rinsed three times with 0.1 M PBS (pH 7.2), and then resuspended in 0.1 M PBS in order to collect the pellets. These cells were prefixed in a 0.05 M sodium cacodylate buffer containing 2.5% glutaraldehyde and 4% paraformaldehyde at 4 °C for 2 h. After washing three times with a 0.05 M sodium cacodylate buffer, they were postfixed in 1 mL of 0.1 M sodium cacodylate buffer and 1 mL of 2% osmium tetroxide at 4 °C for 2 h. The resultant cells were washed twice with sterile distilled water and then stained with 0.5% uranyl acetate at 4 °C for 24 h. Continuously, these cells were dehydrated via successive extractions using 30, 50, 70, 80, 90, 100, 100, and 100% ethanol for a period of 10 min and then embedded in 2 mL of epoxy resin. These resins were

stored at 60 °C for 48 h. Then, the resins containing *V. vulnificus* ATCC 33815 were cut using MTX ultramicrotome (section thickness: 5–5000 nm, cutting speed: 0.1–49.9 mm/s, RMC Inc., Tucson, Arizona, USA). These sections were examined and photographed using a JEOL JEM 1010 transmission electron microscope (JEOL USA Inc., Peabody, MA, USA).

Resuscitation

Vibrio parahaemolyticus and *V. vulnificus* were restored from the nonculturable state using the temperature upshift method modified by Wong et al. [3]. To proceed with this approach, the microbial populations of *V. parahaemolyticus* and *V. vulnificus* cold-incubated in ASW microcosms were enumerated using a conventional culture method and fluorescence microscopic assay at regular intervals, i.e., the cells of *V. parahaemolyticus* and *V. vulnificus* exhibited a culturability lower than the undetectable levels ($<0.95 \text{ Log}_{10} \text{ CFU/mL}$), whereas those that maintained a viability higher than $5.0 \text{ Log}_{10} \text{ CFU/mL}$ were recognized as the cells in the VBNC state. The cells of *V. parahaemolyticus* and *V. vulnificus* that had just entered into the VBNC state were harvested via centrifugation at $10,000g$ for 3 min and then washed with 0.1 M PBS (pH 7.2). The final pellets were resuspended in 1 mL of the ASW solution. Bacterial suspensions were inoculated in 100 mL of ASW microcosms (pH 7) and then incubated at 25 °C for three days. These bacteria were plating-counted on TCBS and TSA.

Food application

Fermented seafood products made of shrimp or clam were purchased from the traditional market (Anseong-si, South Korea). Aseptically, these products were squeezed with a sterile cloth to collect the fluids, which were then filtered through a 20- μm membrane. A total of 50 mL of each seafood juice was transferred into a sterile cap tube to prepare natural microcosms. *V. parahaemolyticus* ATCC 33844 and *V. vulnificus* ATCC 33815 grown overnight in TSB at 37 °C were harvested via centrifugation at $10,000g$ for 3 min, washed with 0.1 M PBS (pH 7.2) twice, and then resuspended in PBS individually; moreover, 1 mL of each bacterial suspension was inoculated in 50 mL of fermented shrimp and clam juices. The samples were stored at 4 °C for five days, and then *V. parahaemolyticus* and *V. vulnificus* in seafood were plating-counted on both TSA and TCBS at regular intervals.

Measurement of pH and A_w

The pH values of the fermented seafood products were measured using a Beckman pH meter (Beckman

Instruments Inc., Houston, TX, USA); moreover, 10 g of individual samples were mixed with 20 mL of sterile deionized water followed by vigorous vortexing for 1 min. Two replicates of each sample were tested, and the measurements were repeated twice. The water activities (A_w) of the samples were measured using a Labmaster water activity meter (Novasina, Pfaffikon, Switzerland). Briefly, individual samples weighing 10 g were put in a circled $5 \times 5 \text{ cm}$ tube and then inserted into the A_w meter. Two replicates of each sample were performed, and the measurements were repeated twice.

Results and discussion

Evaluation of the loss of culturability on media

The changes in the culturability of *V. parahaemolyticus* ATCC 27969, *V. parahaemolyticus* ATCC 17082, and *V. parahaemolyticus* ATCC 33844 incubated in ASW microcosms (pH 7.2–7.8) containing 0.75% NaCl at 4 °C have been shown in Fig. 1. On the 12th day, the numbers of *V. parahaemolyticus* ATCC 27969, *V. parahaemolyticus* ATCC 33844, and *V. vulnificus* ATCC 33815 decreased, as

Fig. 1 Loss of culturability in *Vibrio parahaemolyticus* ATCC 27969, *V. parahaemolyticus* ATCC 33844, and *Vibrio vulnificus* ATCC 33815 incubated in artificial sea water microcosms containing 0.75% NaCl at 4 °C. These culturabilities were enumerated on a nonselective medium (tryptic soy agar, TSA) and a selective medium (thiosulphate-citrate-bile salts-sucrose agar, TCBS). The number of viable cells of *V. parahaemolyticus* and *V. vulnificus* were measured via the fluorescent microscopic assay using the Live/Dead BacLight[®] bacterial viability kit. Filled circle, *V. parahaemolyticus* ATCC 27969 (TSA); open circle, *V. parahaemolyticus* ATCC 27969 (TCBS); filled inverted triangle, *V. parahaemolyticus* ATCC 33844 (TSA); open triangle, *V. parahaemolyticus* ATCC 33844 (TCBS); filled square, *V. vulnificus* ATCC 33815 (TSA); open square, *V. vulnificus* ATCC 33815 (TCBS); filled diamond, *V. parahaemolyticus* ATCC 27969 (viability); open diamond, *V. parahaemolyticus* ATCC 33844 (viability); filled triangle, *V. vulnificus* ATCC 33815 (viability)

indicated by the microbial reductions of ca. 1.5, 1.9, and 0.2 Log₁₀ CFU/mL, respectively, in ASW microcosms containing 0.75% NaCl. The culturability of *V. vulnificus* ATCC 33815 on TSA was completely lost within 19 days. In cases of *V. parahaemolyticus* ATCC 27969 and *V. parahaemolyticus* ATCC 33844, it took 32–78 days for these pathogens to enter into the nonculturable state on a nonselective medium at 4 °C. Within 19 days of storage, *V. parahaemolyticus* ATCC 33844 exhibited loss of culturability below the undetectable levels (<0.95 Log₁₀ CFU/mL) on TCBS. In particular, *V. parahaemolyticus* ATCC 27969 and *V. vulnificus* ATCC 33815 did not develop colonies on TCBS at 4 °C for 42 days. The number of days these bacteria required to enter the nonculturable state decreased more in a selective medium when compared with a nonselective medium. Simultaneously, the total number of viable cells of *V. parahaemolyticus* and *V. vulnificus* were measured using the fluorescence microscopic assay with the dyes of SYTO9 and propidium iodide. Consequently, the initial populations of these bacteria were not significantly different ($p < 0.05$) from those counted at the end of the storage period, showing a value ≥ 7.00 Log₁₀ CFU/mL at every interval. Then, it was shown that the loss of culturability might not stem from the death of the cells of *V. parahaemolyticus* and *V. vulnificus*.

Despite the fact that the induction of the bacterial VBNC state was defined for a cell that retains metabolic activities such as energy production and cellular elongation while being incapable of developing colonies on media even after the elimination of unfavorable stresses [22, 23], the viability of these bacteria entering into the VBNC state could be evaluated using various staining methods. One such method, which involves staining bacteria with SYTO9 and propidium iodide, has been widely used to optically determine bacterial viability because this approach can distinguish live and dead cells. SYTO9 penetrates intact membranes, interacting with the cell nucleic acid, and then indicates live cells by displaying a green color. Conversely, propidium iodide penetrates damaged membranes and labels dead cells only. The loss of the permeability barrier in stained cells represents irreparable damages and eventual cell death [24, 25]. Thus, fluorescence microscopic enumeration of the cells in *V. parahaemolyticus* ATCC 27969, *V. parahaemolyticus* ATCC 33844, and *V. vulnificus* ATCC 33815 demonstrates that these pathogens were induced into the VBNC state. This shows that they appeared to be alive in ASW microcosms at 4 °C for more than 114 days. In addition, it was revealed that the losses of culturability in *V. parahaemolyticus* and *V. vulnificus* were significantly different and depended on strain-to-strain variation and the type of culturing media. Similarly, Whitesides and Oliver [10] reported that *V. vulnificus* cultured in the ASW microcosm consumed approximately

four days to reduce the culturability to the undetectable levels on heart infusion agar at 4 °C; this result supports our findings. In the present study, the use of TCBS resulted in a serious underestimation for enumerating the number of *V. parahaemolyticus* and *V. vulnificus*, thereby posing a potential risk of causing food-borne infections. These results were in agreement with the findings reported in several studies [26–28]. As one of the selective media recommended by US Food and Drug Administration (FDA) [29], such an underestimation in counting the actual number of *V. parahaemolyticus* and *V. vulnificus* in seafood would cause serious public health issues in that if *V. parahaemolyticus* and *V. vulnificus* in seafood are subjected to long-term cold-starvation stress, the presence of pathogenic bacteria could be either directly underestimated or indirectly overlooked. Although a polymerase chain reaction can be used for detecting pathogens in seafood, this approach cannot distinguish whether the cells are in the nonculturable state. Hence, developing a rapid and effective strategy for detecting human pathogens in seafood with higher levels of pathogenicity should be of much importance in the food industry.

Effects of NaCl and acidic pH

As shown in Table 1, the levels of the two major intrinsic factors in microcosms, i.e., NaCl and pH, were artificially adjusted to investigate the influence of different amounts of NaCl and acidic pH on the behavior of *V. parahaemolyticus* and *V. vulnificus* at 4 °C (Figs. 2, 3, 4). *V. parahaemolyticus* ATCC 27969 in ASW (pH 7) containing 0.75% NaCl reached the undetectable levels (<0.95 Log₁₀ CFU/mL) within 30 days at 4 °C. At pH 6, this organism always maintained the microbial population at levels ≥ 5.0 Log₁₀ CFU/mL. A microcosm (pH 5) induced *V. parahaemolyticus* ATCC 27969 to enter into the nonculturable state at a low temperature for 21 days. The microbial population of *V. parahaemolyticus* ATCC 27969 was below the detectable levels in the ASW microcosm (pH 4) for three days. With respect to the elevated NaCl concentrations, the culturability of *V. parahaemolyticus* ATCC 27969 reduced rapidly even though it took 14 days for the microbial loads to reach the nonculturable state in ASW microcosms (pH 4–7) supplemented with 10% NaCl. Regardless of the pH levels, bacterial populations taken from the microcosms amended with 30% NaCl were below 1.0 Log₁₀ CFU/mL for three days. These results indicate that ASW microcosms with decreasing pH levels offered an unfavorable environment for the survival of *V. parahaemolyticus* ATCC 27969. The numbers of *V. parahaemolyticus* ATCC 33844 incubated in ASW microcosms (pH 4, 5, 6, and 7) were below the undetectable levels (<0.95 log₁₀ CFU/mL) within 3, 3, 21, and 21 days,

Table 1 Evaluation (Log_{10} CFU/mL) of the ability of *Vibrio parahaemolyticus* ATCC 27969, *V. parahaemolyticus* ATCC 33844, and *Vibrio vulnificus* ATCC 33815 to be recovered from the nonculturable state using temperature upshift at 25 °C for five days

Bacteria	VBNC-inducing conditions			Resuscitation (days for which these bacteria were incubated at 25 °C)			
	NaCl (%)	pH	Period ^b	0	1	3	5
<i>Vibrio parahaemolyticus</i> ATCC 27969	0.75	7	28	– ^a	7.44	7.38	>6.00
	0.75	5	21	–	–	3.19	3.30
	0.75	4	3	–	–	5.88	6.11
	5	7	28	–	7.45	7.45	>6.00
	5	5	14	–	7.23	6.00	>6.00
<i>Vibrio parahaemolyticus</i> ATCC 33844	0.75	7	21	–	6.26	7.19	>6.00
	0.75	6	21	–	2.54	4.50	6.56
<i>Vibrio vulnificus</i> ATCC 33815	0.75	7	21	–	–	–	4.81
	5	7	21	–	–	–	7.06

^a Below the detectable levels (<0.95 Log_{10} CFU/mL)^b Days for which these bacteria lost the culturability at 4 °C

respectively, at 4 °C. These numbers were also below the detectable levels when incubated in ASW microcosms (pH 4–7) supplemented with 10% and 30% NaCl within seven days. *V. parahaemolyticus* ATCC 33844 required three days of storage to exit from the culturable state in the ASW microcosm (pH 6) containing 0.75% NaCl or supplemented with 5, 10, and 30% NaCl. Similarly, increasing levels of pH and NaCl in the ASW microcosms resulted in the drastic loss of culturability in *V. parahaemolyticus* ATCC 33844 (Fig. 3).

Vibrio vulnificus is one of the leading pathogenic bacteria of much importance because it is closely linked with food-borne outbreaks caused by the consumption of contaminated seafood. It has been reported that this bacterium is isolated from oysters, clams, mussels, and other sea creatures [30, 31]. Changes in the culturability of *V. vulnificus* ATCC 33815 in ASW microcosms were examined as a function of varying levels of NaCl and pH (Fig. 4). Within 21 days, *V. vulnificus* ATCC 33815 in ASW microcosms (pH 5–7) entered into the nonculturable state at 4 °C. The cells of *V. vulnificus* required 21, 3, and 3 days to attain complete loss of culturability when incubated in ASW microcosms (pH 6–7) fortified with 5, 10, and 30% NaCl, respectively. At pH 5, the bacterial population of *V. vulnificus* declined rapidly, resulting in the attainment of the nonculturable state within three days. Regardless of the amounts of NaCl, *V. vulnificus* under cold-starvation stress were liberated from the culturable state on media within 3–7 days under a highly acidic condition (pH 4). Although the numbers of *V. parahaemolyticus* and *V. vulnificus* reduced gradually, their viabilities were always higher than 6.0 Log_{10} CFU/mL during the entire storage period. Nevertheless, *V. parahaemolyticus* and *V. vulnificus* survived for at least 30 days under acidic-starvation stress at the

refrigerated temperature, suggesting that reliable measures for the control of these bacteria should be further developed to prevent microbial contamination in seafood.

The entry of *V. parahaemolyticus* into the nonculturable state was significantly different whenever they were counted at each interval. Cold starvation for 78 days was required for *V. parahaemolyticus* ATCC 27969 to be derived from the culturable state in the ASW microcosm (pH 7) containing 0.75% NaCl (Fig. 1), whereas such a period decreased by approximately 50 days as a result of the duplicate experiment (data not shown). Several previous studies have reported similar findings. In fact, Bates and Oliver [32] examined the loss of culturability in *V. parahaemolyticus* present in ASW microcosms at 5 °C and determined that a variation of approximately 10 days between the duplicate performances occurred in four different strains of *V. parahaemolyticus*, leading to the attainment of the nonculturable state at 5 °C. It was also reported that the number of days required for the transition of *V. cholera* O139 to the VBNC state was always different when they were incubated in the river microcosm at 4 °C [12]. It seemed likely that the ability of *V. parahaemolyticus* to form colonies on media would be highly susceptible to various indigenous factors found in the culture environment, including the presence of oxygen, the formation of oxidative agents, the amount of NaCl, and acidification. Among these causative agents, the nonculturable state of the bacteria would be closely implicated with the formation of hydrogen peroxide because the use of agar media supplemented with antioxidative materials such as catalase and sodium pyruvate elongates the culturability of *V. parahaemolyticus* and *V. vulnificus* against cold starvation [4, 33]. In our preliminary studies, the culturability of several strains of *V. parahaemolyticus* and *V.*

Fig. 2 Loss of culturability in *Vibrio parahaemolyticus* ATCC 27969 incubated in artificial sea water microcosms (pH 4, 5, 6, and 7) containing (A) 0.75% NaCl or supplemented with (B) 5%, (C) 10%, and (D) 30% NaCl, respectively, at 4 °C for 30 days. These culturabilities were enumerated on a nonselective medium (tryptic soy agar, TSA). The number of viable cells of *V. parahaemolyticus*

ATCC 27969 was measured via the fluorescent microscopic assay using the Live/Dead BacLight[®] bacterial viability kit. Filled circle, culturability at pH 7; open circle, viability at pH 7; filled triangle, culturability at pH 6; open triangle, viability at pH 6; filled square, culturability at pH 5; open square, viability at pH 5; filled diamond, culturability at pH 4; open diamond, viability at pH 4

vulnificus exposed to low-temperature-starvation stress was plating-counted on TSA supplemented with 1000 U/mg catalase and 0.1% sodium pyruvate. Consequently, the bacterial counts enumerated on TSA containing these antioxidants were higher than those enumerated on TSA without catalase and sodium pyruvate at the same time intervals, as indicated by the differences of ≥ 1.5 Log₁₀ CFU/mL before and after (data not shown). Similarly, it was revealed that culturable periods of *V. cholerae* O139 in river water microcosms at low temperatures were largely extended on a medium supplemented with either catalase or sodium pyruvate [12], indicating that such an oxidative stress would play a key role in controlling the bacterial culturability.

Resuscitation

Previous studies on the VBNC state determined the ability of bacteria to exit from the nonculturable state by eliminating a VBNC-inducing environmental stress [3, 10, 34]. Since it has been demonstrated that pathogens recovered from the VBNC state after resuscitation exhibit higher levels of virulence factors to organisms [1, 13], this course of resuscitation could be of much importance in terms of public-health concerns. As shown in Table 1, *V. parahaemolyticus* and *V. vulnificus* in the nonculturable state were transferred to a newly prepared ASW microcosm (pH 7) containing 0.75% NaCl and then incubated at an ambient temperature for five successive days. After three

Fig. 3 Loss of culturability in *Vibrio parahaemolyticus* ATCC 33844 incubated in artificial sea water microcosms (pH 4, 5, 6, and 7) containing (A) 0.75% NaCl or supplemented with (B) 5%, (C) 10%, and (D) 30% NaCl, respectively, at 4 °C for 30 days. These culturabilities were enumerated on a nonselective medium (tryptic soy agar, TSA). The number of viable cells of *V. parahaemolyticus*

ATCC 33844 was measured via the fluorescent microscopic assay using the Live/Dead BacLight[®] bacterial viability kit. Filled circle, culturability at pH 7; open circle, viability at pH 7; filled triangle, culturability at pH 6; open triangle, viability at pH 6; filled square, culturability at pH 5; open square, viability at pH 5; filled diamond, culturability at pH 4; open diamond, viability at pH 4

days, *V. parahaemolyticus* ATCC 27969 regained the culturability, which ranged from 3.19 to 7.45 Log₁₀ CFU/mL on TSA. *V. parahaemolyticus* ATCC 33844 and *V. vulnificus* ATCC 33815 also appeared to exit from the nonculturable state owing to the resuscitation process. Generally, *V. parahaemolyticus* and *V. vulnificus* that were induced into the VBNC state in the microcosms supplemented with less than 10% NaCl at 4 °C before being resuscitated were capable of restoring the ability to develop colonies on media. Although it was demonstrated that the nonculturable states of *V. parahaemolyticus* and *V. vulnificus* can be achieved in microcosms with higher contents of NaCl (10–30%) at 4 °C in this study, these VBNC-induced bacteria did not recover the culturability after

resuscitation (data not shown). It seemed likely that *V. parahaemolyticus* and *V. vulnificus* would be highly injured and were not able to regain the ability to develop colonies on agar plates such as TSA and TCBS under environments containing ≤10% NaCl. In a study conducted by Besnard et al. [11], *L. monocytogenes* in the VBNC state were transferred to the brain heart infusion broth and then incubated at 37 °C for several days. Consequently, no colonies were developed on agar plates. They suggested that this pathogen became the VBNC in that they defined the VBNC state as bacteria that are unable to grow in usual culturing media and those that cannot be resuscitated via the traditionally used temperature upshift method but retain metabolic activities. Several studies took this point of view

Fig. 4 Loss of culturability in *Vibrio vulnificus* ATCC 33815 incubated in artificial sea water microcosms (pH 4, 5, 6, and 7) containing (A) 0.75% NaCl or supplemented with (B) 5%, (C) 10%, and (D) 30% NaCl, respectively, at 4 °C for 30 days. These culturabilities were enumerated on a nonselective medium (tryptic soy agar, TSA). The number of viable cells of *V. vulnificus* ATCC

33815 was measured via the fluorescent microscopic assay using the Live/Dead BacLight[®] bacterial viability kit. Filled circle, culturability at pH 7; open circle, viability at pH 7; filled triangle, culturability at pH 6; open triangle, viability at pH 6; filled square, culturability at pH 5; open square, viability at pH 5; filled diamond, culturability at pH 4; open diamond, viability at pH 4

into the consideration for defining bacteria in the VBNC state [14, 23].

Morphological changes

A number of aggregates of typically rod-shaped cells of *V. parahaemolyticus* and *V. vulnificus* without any damages in cytoplasmic spaces and held on laboratory culture media prior to exposure to environments have been frequently reported in several studies [24, 35]. The present study revealed that cold nutrient-limited stress caused severe morphological changes in *V. vulnificus* ATCC 33815 (Fig. 5). Numerous cocci and rod-shape cells of *V. vulnificus* were observed after being subjected to these stresses

for seven days. It appeared that the numbers of cocci cells grew with increasing amounts of NaCl in the microcosms. In the ASW (pH 6) containing 0.75% NaCl, microbial cells with denser ribosome and nucleic acid throughout the cytoplasm are shown in Fig. 5(B). Particularly, empty spaces between the outer and inner membranes are demonstrated. Along with the elevated concentrations of NaCl, internal staining in the cells of *V. vulnificus* was significantly less probably because of the loss of membrane integrity. In a study conducted by Mizunoe et al. [20], these morphological changes were observed in the cells of *V. parahaemolyticus* VP190 that experienced the starvation stress at 4 °C for 12 h. It was also reported that the intact structure of the outer and inner membranes was enlarged in

Fig. 5 Morphologies of *Vibrio vulnificus* ATCC 33815 incubated in artificial sea water (pH 6) microcosms with varying concentrations of NaCl at 4 °C on the seventh day. (A, B) Bacterial cells in a microcosm containing 0.75% NaCl; (C, D) cells in a microcosm amended with 5% NaCl; (E, F) cells in a microcosm amended with 10% NaCl; and (G, H) cells in a microcosm amended with 30% NaCl. Arrows point out the collapse of cytoplasmic spaces, which resulted in the development of gaps between the cell membrane and the peripheral membrane

the cells of *V. cincinnatiensis* VIB 287 in a nutrient-limited environment at 4 °C for 50 days [15], indicating that the nonculturability of *V. parahaemolyticus* and *V. vulnificus* would result from these morphological changes from rod to cocci microbial shapes and injuries in the peripheral space.

Bacterial behaviors in fermented seafood products

Vibrio parahaemolyticus ATCC 33844 and *V. vulnificus* ATCC 33815 were inoculated in fermented seafood products made from shrimp and clam and were then stored at 4 °C to examine the entry of these pathogens into the nonculturable state (data not shown). Within three days, the plating counts of *V. parahaemolyticus* ATCC 33844 and *V. vulnificus* ATCC 33815 were $<0.95 \text{ Log}_{10} \text{ CFU/g}$ on both TSA and TCBS, respectively, whereas the viability of these pathogens was always higher than $7.0 \text{ Log}_{10} \text{ CFU/g}$ owing to the microscopic inspection. Fermented seafood products maintained A_w in the range of 0.75–0.78 at 4 °C during the whole storage period (data not shown), implying that A_w did not have an effect on the culturability of *V. parahaemolyticus* ATCC 33844 and *V. vulnificus* ATCC 33815. Conversely, the amounts of NaCl ranging from 7% to 13% in the fermented seafood products possibly caused drastic decreases in the number of these bacteria on media. These results indicate that higher concentrations of NaCl in combination with the refrigerator temperature were able to induce *V. parahaemolyticus* and *V. vulnificus* into the nonculturable state, thereby determining the potential presence of pathogens in the VBNC state on seafood.

Since food-borne outbreaks caused by microbial contaminations are reported frequently, achieving higher levels of hygienic practices in the food industry will not only assure food safety but also satisfy the consumer's demand for the consumption of food products free from microbial contamination. To implement these approaches, understanding the behaviors of pathogenic bacteria can be an essential step considering the bacterial characteristics involved in the surviving strategies in response to various environmental stresses. In conclusion, artificial cold-starvation stress induced *V. parahaemolyticus* and *V. vulnificus* to enter the nonculturable state. In this study, culturability decreased rapidly with increasing concentrations of NaCl

and acidic pH in ASW microcosms at 4 °C. It was revealed that *V. parahaemolyticus* and *V. vulnificus* could survive for at least 30 days under these conditions as these bacteria in the nonculturable state were proven via fluorescence microscopy to be still alive. This shows that while most of the bacterial cells emitted green fluorescence, red-colored (dead) cells were in a range of ≤ 10 cells per slide. The morphological changes in the bacterial cells experiencing cold-starvation stress indicated that the intact structures containing small organelles within the peripheral space were injured and empty spaces were then formed between the outer and inner membranes. Since the entry of *V. parahaemolyticus* and *V. vulnificus* into the VBNC state involves increased resistance against various environmental challenges, it is necessary to further study its mode of action in response to the nature of seafood. The results obtained from this study will provide useful implications for developing control management systems for preventing bacterial contaminations in seafood.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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