Research article

Analysis of the Resuscitation-Availability of Viable-But-Nonculturable Cells of *Vibrio parahaemolyticus* upon Exposure to the Refrigerator Temperature

Running title: Determining the resuscitation-availability of VBNC *V. parahaemolyticus*

Jae-Hyun Yoon^a, Young-Min Bae^a, Buom-Young Rye^b, Chang-Sun Choi^a, Sung-Gwon Moon^a, and Sun-Young Lee^{a*}

Department of Food Science and Technology, Chung-Ang University, 72-1 Nae-ri, Daedeok-myeon, Anseong-si, Gyeonggi-do 456-756, Republic of Korea^{a*}, Department of Animal Science and Technology, Chung-Ang University, 72-1 Nae-ri, Daedeok-myeon, Anseong-si, Gyeonggi-do 456-756, Republic of Korea^b

*Corresponding author. Mailing address: Department of Food Science and Technology, Chung-Ang University, 72-1 Nae-ri, Daedeok-myeon, Anseong-si, Gyeonggi-do 456-756, Republic of Korea. Phone: +82 31-676-8741. E-mail: nina6026@cau.ac.kr.

ABSTRACT

Major pathogenic strains of *Vibrio parahaemolyticus* can enter into the viable-but-nonculturable (VBNC) state when subjected to environmental conditions commonly encountered during food processing. Especially, VBNC cells can be recovered to the culturable state reversibly by removing the causative stress, expressing higher levels of virulence factors. Therefore, the aim of this study was to determine if VBNC *V. parahaemolyticus* strains retain the resuscitation-availability upon eliminating the adverse condition, followed by the enrichment in developed resuscitation-facilitating buffers. Bacterial cells were shown to enter into the VBNC state in artificial sea water (ASW, pH 6) microcosms at 4°C within 70 days. VBNC cells were harvested, inoculated in formulated resuscitation-buffers, and then incubated at 25°C for several days. TSB (pH 8) supplemented with 3% NaCl (TSB_A) exhibited the higher resuscitation-availability of VBNC cells. It was also shown that TSB_A containing 10,000 U/mg/protein catalase, 2% sodium pyruvate, 20 mM MgSO₄, 5 mM ethylenediaminetetraacetic acid (EDTA), and cell free supernatants extracted from the pure cultures of *V. parahaemolyticus* was more effective in resuscitating VBNC cells of *V. parahaemolyticus*, showing by 7.69-8.91 log₁₀ CFU/ml.

IMPORTANCE

Generally, higher concentrations ($\leq 40\%$) of NaCl are used for preserving different sorts of food products from bacterial contaminations. However, it was shown from the present study that strains of *V. parahaemolyticus* were able to persist in maintaining the cellular viability, thereby entering into the VBNC state upon exposure to the refrigerator temperature for 80

days. Hence, the ability of VBNC *V. parahaemolyticus* to re-enter into the culturable state was examined, using various resuscitation buffers that were formulated in this study. VBNC cells re-gained the culturability successfully when transferred onto the resuscitation-buffer D, and then incubated at 25°C for several days. Resuscitation-facilitating agent D is consisting of antioxidizing agents, mineral, an emulsifier, and cell free supernatants from the actively growing cells of *V. parahaemolyticus*. It appeared that such a reversible conversion of VBNC cells to the culturable state would depend on multiple resuscitation-related channels.

KEYWORDS cell free supernatant, pathogen, resuscitation, ROS-detoxifying, viable-but-nonculturable

INTRODUCTION

Major food-borne pathogens, including *Vibrio parahaemolyticus*, *Vibrio vulnificus*, *Camphylobacter jejuni*, *Escherichia coli* O157:H7, *Salmonella enterica* serovar Enteritidis, and *Shigella dysenteriae*, are known to become the viable-but-nonculturable (VBNC) state when challenged by various environmental stresses such as low temperatures ($\leq 15^{\circ}\text{C}$), starvation, copper, and CO_2 (1-3). It should be noted that VBNC cells of these pathogens are incapable of producing their own colonies on culture media on which these organisms can grow routinely, thereby escaping from the cultivation-based surveillances and diagnosis tools. Once bacterial cells were induced to the dormant and nonculturable state upon exposure to adverse environmental stresses (nutrient-deprivation and cold temperature) VBNC cells exerted some metabolic activities, including hydrolysis of energy sources, adenosine triphosphate (ATP) synthesis, and maintenance of the membrane integrity, displaying better resistances to environmental conditions commonly encountered during food processing (4-6). Of much importance, it has been well-reported that VBNC *V. parahaemolyticus* can be recovered back to the culturable state by eliminating the causative environmental conditions. 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 (4, 7-8). In particular, it was demonstrated that pathogenic bacteria, including *V. parahaemolyticus* and *Shig. dysenteriae*, remained constant in possessing potential virulence factors even after entering into the VBNC state, retaining the serious infectivity to animal cell lines (9-10). Thus, VBNC pathogens should be closely implicated with causing the food-borne disease outbreaks. Until now, many studies have been conducted to determine

a way of restoring stressed cells of bacteria from the VBNC state. In a study conducted by Zhao et al. (3), VBNC *E. coli* O157:H7 was transferred into a nutrient-rich culture broth such as tryptic soy broth, and then incubated at 37°C for ≤24 hrs, thereby re-gaining the colony-forming capability. Coutard et al. (11) also showed that VBNC cells of *V. parahaemolyticus* VP5 were resuscitated reversibly when further incubated in artificial sea water (ASW) microcosms at 25°C for several days. In contrast, some VBNC bacteria could not be restored from the VBNC state under controlled favorable conditions where these organisms prefer to grow primarily (12-13). Such a failure to recover VBNC bacteria back to the culturable state did not indicate that the environmental challenges used in these studies deprived bacterial cells of the resuscitation-availability completely. It seemed plausible that these resuscitation approaches would not be effective for recovering the culturability of VBNC cells. Bacteria in such a dormant state will be resuscitated opportunely under a favorable environmental condition for their survivals. Considering that bacterial cells in the VBNC state are capable of evading from conventional cultivation-based techniques the incidence of VBNC pathogens on food products could threaten public health concerns potentially. Until now, a preliminary research establishing an optimal resuscitation method of VBNC cells is still unsubstantial. Therefore, the present study aimed at examining the resuscitation-availability of VBNC *V. parahaemolyticus* using by developed resuscitation-facilitating buffers.

RESULTS AND DISCUSSION

Formation of the viable-but-nonculturable cells

It appeared that strains of *V. parahaemolyticus* ATCC 17082, *V. parahaemolyticus* ATCC 33844, and *V. parahaemolyticus* ATCC 27969 were divested of their own culturable

capability within 70 days when incubated in ASW microcosms (pH 6) at 4°C, regardless of the excessive amounts of NaCl (Fig. 1). Populations of *V. parahaemolyticus* ATCC 17082 in ASW microcosms containing 0.75%, 5%, 10%, and 30% NaCl declined remarkably below the detection limits ($<1.0 \log_{10}$ CFU/ml) as being measured at 4°C for 50, 24, 20, and 12 days, respectively. Especially, such a cold-starvation environment enabled cells of *V. parahaemolyticus* ATCC 33844 and *V. parahaemolyticus* ATCC 27969 to be converted into the nonculturable state in ASW microcosms amended with 30% NaCl for ≤ 24 days. In addition, these organisms became nonculturable when incubated in ASW microcosms added with less than 10% NaCl at 4°C for 70 days. Clearly, there were minor modifications in the duration of cold-starvation periods required for these pathogens to lose the 100% culturability. Nevertheless, it seemed likely clear that *V. parahaemolyticus* were converted to the nonculturable state more rapidly with increasing NaCl concentrations. Furthermore, it was shown that viable numbers of *V. parahaemolyticus* ATCC 17082, *V. parahaemolyticus* ATCC 33844, and *V. parahaemolyticus* ATCC 27969 ranged from 4.3 to 6.5 $\log_{10}$ CFU per a slide after incubated at 4°C for 80 days with the fluorescence microscopic assay. Apart from the culturable populations of these bacteria, strains of *V. parahaemolyticus* persisted in surviving under the cold-starvation condition for at least 80 days. In order to determine whether the nonculturable cells were truly dead or still alive, it is inevitable to evaluate the degree to which these organisms were sincerely damaged. Then, the utilization of fluorescent probes such as SYTO9 and propidium iodide (PI) can reflect the levels of cellular integrity quantitatively. In general, SYTO9 penetrates bacteria with the intact cell membrane, interacts with the cell nucleic acid, and then displays green colors for the live cells with the fluorescence microscopy. Propidium iodide can penetrate damaged membranes, labeling only the dead cells as red-coloured fluorescence. Hence, staining bacterial cells with SYTO9 combined with PI can distinguish between live and dead cells effectively. Herein, these

results indicated that cells of *V. parahaemolyticus* were inducted into the VBNC state, still maintaining the cellular integrity of bacterial membranes even after a long term of cold-starvation

As well-known previously, *V. parahaemolyticus* strains are very susceptible to various environmental conditions. Probably, it would be attributable to the low levels of detectability of these bacteria on food products. It was shown that bacterial cells of *V. parahaemolyticus* were derived from the colony-forming capability on culture media after the entry into the VBNC state (19). In general, pathogenic bacteria such as *V. parahaemolyticus*, *V. vulnificus*, and *V. cholerae* can enter into the VBNC state at low temperatures of less than $\leq 10^{\circ}\text{C}$ within a wide range of incubation periods. As shown in Table 1, it was demonstrated that these pathogens can be converted into the VBNC state by various environmental conditions. Baffone et al. (14) reported that a cold-starvation challenge enabled cells of *V. parahaemolyticus* and *V. vulnificus* to enter into the VBNC state within 30 days. Similarly, strains of *V. parahaemolyticus* and *V. vulnificus* were converted into the VBNC state when incubated in ASW microcosms at 4°C within 35 days (11, 18). Moreover, these bacteria became viable-but-nonculturable successfully in various microcosms such as ASW, deionized water (DW), natural sea water, and a mixture of ASW and a Luria-Bertani culture medium. Interestingly, it appeared that strains of *V. parahaemolyticus*, *V. vulnificus*, and *V. cholerae* required very different incubation-periods to enter into the VBNC state under the conditions that are almost the same as proposed in previous studies. The duration of cold-starvation stress ranged from 4 days to a maximum of several months to produce VBNC cells. Although the underlying mechanisms governing the entrance of *V. parahaemolyticus* into such a dormant state has not been understood yet, the formation of VBNC cells would proceed by a multiple mode of actions and complex interactions either directly or indirectly. Clearly, the formation of VBNC cells should be recognized as one of the adaptation-surviving strategies

in response to adverse environments.

Evaluation of the resuscitation-availability of VBNC cells

After forming VBNC cells of *V. parahaemolyticus* strains, these cells were transferred to various nutrient-rich culture fluids, including ASW, TSB, BHI, and APW, and then further incubated to examine if VBNC cells would retain the resuscitation-availability on these media at an ambient temperature (Table 2-5). It was shown that VBNC cells failed to re-gain the culturability when re-suspended in a formal solution of ASW for several days. However, once resuscitated in nutrient-rich media such as TSB and APW, VBNC cells of *V. parahaemolyticus* ATCC 17082 were turned back to the culturable state, showing by 3.45–8.00 log₁₀ CFU/ml as being enumerated on a nonselective medium (TSA). Strains of *V. parahaemolyticus* ATCC 33844 and *V. parahaemolyticus* ATCC 27969 also resuscitated positively when bacterial cells in the VBNC state were incubated in TSB and APW, except for these organisms that had been induced into the VBNC state in ASW microcosms added with 30% NaCl at 4°C for 80 days. Resuscitation-provoking efficiencies of these buffers such as TSB and APW were in the levels of ≥6.0 log₁₀ CFU/ml, whereas the selection of BHI was less effective for the resuscitation of VBNC *V. parahaemolyticus*. Therefore, TSB as a resuscitation-buffer facilitated the recovery of VBNC cells of these pathogens, indicating that certain levels of a minimum nutritional base should be required for VBNC *V. parahaemolyticus* to be recovered to the culturable state. As far as it is controversial to determine the effects of CFS extracted from major food-borne pathogens and a mixture of antioxidizing agents, CSP, on the resuscitation of VBNC *V. parahaemolyticus* VBNC cells resuscitated in CFS-VP showed the colony-forming capability, ranging from 7.50 to 8.38 log₁₀ CFU/ml. The use of CFS-VV was also attributable to the moderate recovery of VBNC

cells, but showed lower levels of the resuscitation-availability than that of CFS-VP. VBNC cells of *V. parahaemolyticus* ATCC 33844, which were challenged by cold-starvation in ASW microcosms added with 5% NaCl for 80 days previously, were not awakened to the culturable state, followed by the resuscitation process to CFS-EC, CFS-ST, and CFS-SA, respectively. These results were in an accordance with a study conducted by Pinto et al. (24). Ayrapetyan et al. (27) also showed that VBNC cells of *V. vulnificus* were able to be awakened from such a dormant state when resuscitated on culture media supplemented with the CFS extracted from the pure cultures of *V. vulnificus*. Preliminarily, it was revealed out that autoinducer-2 (AI-2) could be strongly involved in the resuscitation of VBNC *V. vulnificus*, whereas filtered CFSs from AI-2 mutant strains of *V. vulnificus* failed to restore VBNC cells. These results implied that interspecific quorum sensing modules would play a key role as an important regulator in switching on the resuscitation-availability of VBNC bacteria. In our preliminary studies, several intrinsic parameters such as pH and NaCl% in TSB were adjusted to establish an optimal condition in an attempt to initiate the resuscitation of VBNC *V. parahaemolyticus* and VBNC *V. vulnificus* (data not shown). Consequently, TSB (pH 8) supplemented with 3% NaCl (TSB_A) exhibited the higher resuscitation-availability of VBNC cells of these pathogens. Unexpectedly, APW (pH 7-8) solutions amended with 1%-3% NaCl were not effective in awakening the restoration of VBNC cells. Thus, 100-days-stressed cells of *V. parahaemolyticus* in ASW microcosms at 4°C were transferred to either TSB_A+CFS or TSB_A+CSP, showing that VBNC cells of *V. parahaemolyticus* ATCC 17082 were not converted to the culturable state while strains of *V. parahaemolyticus* ATCC 33844 and *V. parahaemolyticus* ATCC 27969 in ASW microcosms added with ≤10% NaCl re-gained the culturability, corresponding to 7.69-8.91 log₁₀ CFU/g (Table 2). Especially, TSB_A+CFS-VP was more effective in resuscitating VBNC *V. parahaemolyticus* than TSB_A combined with CFS-VV, CFS-EC, CFS-ST or CFS-SA. Based on these results, buffers A-F were prepared to

determine the optimal resuscitation-buffer (Table 4). Above all things, 250-days-stressed cells of *V. parahaemolyticus* ATCC 33844 in ASW microcosms added with 30% NaCl were not awakened from such a dormant state with resuscitation-buffers A-F. However, resuscitation-buffer D facilitated VBNC cells of *V. parahaemolyticus* ATCC 17082 and *V. parahaemolyticus* ATCC 27969 to re-gain the colony-forming capability effectively. It was shown that a mixture of CFS-VP, CSP, MgSO₄, and EDTA was highly effective in resuscitating VBNC cells.

To data, it was believed that the intracellular accumulation of reactive oxygen species (ROS) has a significant influence on the formation of VBNC cells of organisms. Accordingly, the incidence of ROS-detoxifying proteins in bacterial cells could be closely associated with the resuscitation-availability, probably preventing the bacteria from entering into the VBNC state. In our preliminary studies, it was shown that there were no significant differences in the amounts of antioxidizing proteins such as catalase and glutathione-S-transferase between the bacterial cells of *V. parahaemolyticus* in the stationary-phase and in the VBNC state (*data not shown*). Once VBNC cells of *V. parahaemolyticus* in ASW microcosms at 4°C for 90 days were harvested by centrifugation at 1,3000 X g for 3 min, washed twice, and then re-suspended in 5 ml of TSA (pH 7) either containing 1,000 U/mg/protein or 10,000 U/mg/protein none of bacterial cells were resuscitated in TSB added with 1,000 U catalase, while these cells re-suspended in TSB+10,000 U catalase were converted back to the culturable state, showing by $\geq 7.0 \log_{10}$ CFU/ml on media (*data not shown*). It seemed plausible that if accumulated amounts of ROS exceed over an acceptable coverage of ROS-detoxifying enzyme's activity bacteria begin to proceed towards the VBNC stage (Fig. 1). It was suggested that ROS accumulations would play an important role for understanding related mechanisms governing the entrance of *V. parahaemolyticus* into the VBNC state. After VBNC cells of *Ralstonia solanacearum* were incubated in DW amended with 1,000

U/mg catalase at 30°C for 3 days, this bacterium re-gained the culturability on media, showing by $>8.0 \log_{10}$ CFU/ml (28). Mizunoe et al. (15) reported that VBNC cells of *V. parahaemolyticus* was plating-counted on TSA (pH 7) added with 1,000 U/mg protein catalase, thereby resulting in a mild restoration of the resuscitation-availability. Interestingly, Abe et al. (29) revealed out that GST activities of *V. vulnificus* remained constant during cold-starvation for 24 hrs, showing by 3.07-3.83 μ M/mg/protein uniformly. Under the same condition, nonculturable suppression mutant strains of *V. vulnificus* persisted in showing the colony-forming capability in the levels of $\geq 5.0 \log_{10}$ CFU/ml, and then exerted enhanced GST activities more than 10 times as massive as the pure cultures. In a study conducted by Santander et al. (30), mutant strains of *Erwini amylovora* deleting *katAG*⁻ entered into the VBNC state in ASW microcosms at 4°C more rapidly than did the wild cells. Each of *katA* and *katG* is responsible for producing a monofunction catalase and a bifunctional peroxidase, respectively. Therefore, these publications suggested that the activities of ROS-scavenging agents in bacterial cells could be strongly associated with the loss of culturability. These results, along with our findings, would represent the hypothesis that bacterial cells detoxify intracellularly generated ROS materials by synthesizing catalase/GST-like enzymes at the beginning stage of cold-starvation (Fig. 1). After at least several weeks, bacteria would not synthesize enough amounts of the antioxidizing enzymes to hydrolyze the accumulated ROS, eventually resulting in the loss of culturability. Once bacterial cells became the nonculturable state, VBNC cells were further transferred into nutrient-rich media added with different concentrations (ex. 1,000 or 10,000 U/mg/protein) of catalase, and then incubated at ambient temperatures for several days the resuscitation-buffer reinforced with 10,000 U/mg/protein catalase would provide VBNC cells with a sufficient quantity of the ROS-detoxifying agent successfully to neutralize intracellular ROS materials, allowing bacterial cells to be recovered from the culturable state again despite that VBNC bacteria resuscitated in liquid culture broth

containing 1,000 U/mg/protein catalase were incapable of gaining the re-culturability as the existing accumulated amounts of ROS would exceed over those of added Exo-catalase proteins.

MATERIALS AND METHODS

Preparation of bacterial inoculums Strains 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 these cells were maintained at -75°C and further activated in tryptic soy broth (Difco, Detroit, MI, USA) supplemented with 3% NaCl (TSB) at 37°C for 24 hrs before use. Stationary phase cells of *V. parahaemolyticus* were harvested by centrifugation at 10,000 X g for 3 min, washed with artificial sea water (ASW, Sigma-Aldrich, St. Louis, MO, USA), and then final pellets of these organisms were re-suspended in 1 ml of ASW solutions (pH 6), corresponding to the bacterial population of approximately 10^{8-9} CFU/ml. To adjust the pH level in ASW microcosms, filtered 1 N NaOH solution (Kanto chemical, Tokyo, Japan) was used.

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. Then, bacterial cells were inoculated

in 100 ml of ASW (pH 6) microcosms added with 0.75%, 5%, 10%, and 30% NaCl, respectively. Bacterial suspensions were kept at 4°C until the culturable numbers of *V. parahaemolyticus* arrived below the detectable limits ($<1.0 \log_{10}$ CFU/ml). ASW microcosms were withdrawn from the incubator at regular time-intervals to enumerate the bacterial population either directly by the cultivation-based method or indirectly by measuring the viable cell numbers of *V. parahaemolyticus*.

Enumeration of the bacterial population 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 the bacterial cell were prepared in alkaline peptone water (APW, Difco) consisting 10 g of peptone and 10 g of NaCl per 1 liter of DW. Then, 100 µl of these aliquots was spread on TSA. Each of these plates were incubated at 37°C for 24 hrs and colonies developed on media were further enumerated.

Fluorescence dye staining and microscopic assay Numbers of total and viable cells of *V. parahaemolyticus* were determined as being measured with the Live/Dead® BacLight™ Bacterial Viability Kit (Invitrogen, Mount Waverley, Victoria, Australia) combining two nucleic acid stains, SYTO9 and propidium iodide. It has been well-documented that SYTO9 has a high affinity for deoxyribonucleic acid (DNA) and chromosome of bacterial cells, labelling all of the bacteria with intact and compromised membranes, whereas propidium iodide penetrates selectively the bacterial cell with damaged membranes. Briefly, equal volumes (1:1) of SYTO9 and propidium iodide were combined and 3 µl of this mixture was added to each 1 ml of the bacterial cell. After a short period (ca. 15 min) of incubation at an

ambient temperature in the dark, 5-8 μ l of this aliquot was attached on a glass slide and a coverslip was placed on this specimen carefully. Then, bacterial images were demonstrated using an electron-fluorescent microscope (TE 2000-U, Nikon, Japan).

Optimization of the resuscitation-facilitating buffers In the present study, various resuscitation-facilitating strategies were employed to examine the resuscitation-availability of VBNC cells to the culturable state on media. (I) VBNC cells of *V. parahaemolyticus* were centrifuged at 13,000 X g for 3 min, washed twice, and then re-suspended in 5 ml of ASW (pH 7) solutions containing 0.75% NaCl. Immediately, these cells were incubated at 25°C for up to 7 days. (II) Several nutrient-rich media, including APW, TSB, and brain heart infusion (BHI, Difco) broth, were prepared according to instructions provided by suppliers. These media were added with excessive amounts of NaCl, corresponding to either 1% or 3% NaCl, and pH levels of these media were adjusted to either pH 7 or pH 8, using a membrane-filtered 1N NaOH solution. VBNC cells were harvested by centrifugation at 13,000 X g for 3 min, washed twice, re-suspended in 5 ml of these culture media, respectively, and then further were incubated at 25°C for up to 7 days. (III) As shown in Table 4, each of supplementations, including 10,000 U/mg protein catalase (Sigma), 2% sodium pyruvate (Sigma), 20 mM MgSO₄ (Sigma), 5 mM ethylenediaminetetraacetic acid (EDTA, Sigma), and cell free supernatant (CFS), were individually added to TSB (pH 8) containing 3% NaCl to alter various resuscitation-facilitating buffers. When it comes to CFS, these fluids were extracted from the wild cells of *V. parahaemolyticus* ATCC 17082, *V. vulnificus* ATCC 27562, *Escherichia coli* O157:H7, *Salmonella enterica* serovar Typhimurium, and *Staphylococcus aureus*, respectively. Briefly, each of these pathogens grown in TSB at 37°C for 24 hrs were harvested by centrifugation at 13,000 X g for 3 min to collect bacterial pellets. These

supernatants were separately collected, filtered through a 0.2- μ m-size a polycarbonate membrane (ADVANTEC, Tokyo, Japan), and then added in resuscitation-buffers D-F at a ratio of 10% (v/v). It was further confirmed that all the CFSs did not have an influence on the controlled intrinsic pH level in TSB. VBNC cells were centrifugated at 13,000 X g for 3 min, washed twice, and then re-suspended in 5 ml of resuscitation-buffers A-F, respectively. At the end, bacterial cells were incubated at 25°C for up to 7 days.

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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 80 days.

Fig. 2. A hypothesis diagram for elucidating the successful recovery of VBNC cells back to the re-culturable state by the addition of a high degree of (>10,000 U/mg/protein) of catalase proteins (Exo-catalase) in the resuscitation buffer.

(A)

(B)

(C)

(D)

(E)

(F)

TABLE 1 Effects of environmental conditions on the entry of *V. parahaemolyticus*, *V. vulnificus*, and *V. cholerae* into the viable-but-nonculturable state

Microorganism	VBNC-inducing conditions					Resuscitation	Reference
	Major stress	Microcosm	Temperature	Period	Media ²		
<i>Vibrio parahaemolyticus</i>	Temperature/Starvation	ASW	5°C	30	BHI	ND	(14)
<i>Vibrio parahaemolyticus</i>	Temperature/Starvation	ASW	4°C	50	TSA	○	(8)
<i>Vibrio parahaemolyticus</i>	Temperature/Starvation	ASW	4°C	12	LB	— ³	(15)
<i>Vibrio parahaemolyticus</i>	Temperature/Starvation	ASW	5°C	4	HIA	○	(25)
<i>Vibrio parahaemolyticus</i>	Temperature/Starvation	MMS	3.5°C	50-70	TCBS	-	(26)
<i>Vibrio parahaemolyticus</i>	Temperature/Starvation	ASW	10°C	<30	HIA	○	(11)
<i>Vibrio parahaemolyticus</i>	Temperature/Starvation	MMS	4°C	35	TSA	○	(9)
<i>Vibrio vulnificus</i>	Temperature/Starvation	ASW	4°C	14	Tn	○	(21)
<i>Vibrio vulnificus</i>	Temperature/Starvation	ASW	5°C	4	HIA	○	(16)
<i>Vibrio vulnificus</i>	Temperature/Starvation	ASW	5°C	10	HIA	○	(17)
<i>Vibrio vulnificus</i>	Temperature/Starvation	ASW	5°C	<30	LBA	ND	(18)
<i>Vibrio cholerae</i>	Temperature/Starvation	ND	4°C	70	ND	ND	(20)
<i>Vibrio cholerae</i>	Temperature/Starvation	ASW	4°C	20-30	TSA	ND	(22)
<i>Vibrio cholerae</i>	Temperature	ASW+LB	4°C	30-40	TSA	ND	(22)
<i>Vibrio cholerae</i>	Anaerobic atmosphere	ASW	4°C	40	TSA	ND	(22)
<i>Vibrio cholerae</i>	Starvation	Sea water	ND	≤125	BHI	○	(23)

¹ BHI, brain heart infusion agar; TSA, tryptic soy agar; LBA, Luria-Bertani agar; HIA, heart infusion agar; NA, nutrient agar; 2216E, marine agar; TCBS, thiosulphate-citrate-bile salt-sucrose agar.

² ND, not determined.

TABLE 2 Resuscitation ($\log_{10}$ CFU/ml) of *V. parahaemolyticus* from the VBNC state with the temperature upshift at 25°C for 3 days

Pathogen	ATCC	Microcosm	Cold-starvation period (days) ¹	Resuscitation-buffer	
				ASW	TSB
<i>V. parahaemolyticus</i>	17082	ASW	50	- ²	7.44
	17082	ASW+5% NaCl	25	-	-
	17082	ASW+10% NaCl	21	-	-
	17082	ASW+30% NaCl	12	-	-
<i>V. parahaemolyticus</i>	33844	ASW	50	-	7.06
	33844	ASW+5% NaCl	50	-	5.70
	33844	ASW+10% NaCl	50	-	-
	33844	ASW+30% NaCl	25	-	-
<i>V. parahaemolyticus</i>	27969	ASW	72	-	7.44
	27969	ASW+5% NaCl	72	-	-
	27969	ASW+10% NaCl	72	-	-
	27969	ASW+30% NaCl	25	-	-

¹*V. parahaemolyticus* and *V. vulnificus* were incubated in ASW (pH 6) microcosms supplemented with varying levels of NaCl at 4°C until they became the VBNC state. In particular, these pathogens in the VBNC state for the first day were transferred onto these resuscitation-buffers such as ASW and TSB, respectively, and then they were incubated at 25°C for 72 hrs.

² -, No growth.

TABLE 3 Evaluation of the ability ($\log_{10}$ CFU/ml) of *V. parahaemolyticus* pre-incubated in ASW microcosms (pH 6) at 4°C for 60 days to be recovered from the VBNC state

Pathogen	Microcosm	Period (days)	Temperature upshift in resuscitation-buffers ¹ at 25°C for consecutive 7 days									
			ASW	TSB	BHI	APW	CFS-VP	CFS-VV	CFS-ST	CFS-EC	CFS-SA	CSP
<i>V. parahaemolyticus</i> ATCC 17082	ASW	50	- ²	7.43	6.83	7.96	8.07	8.25	7.88	8.14	7.99	8.37
	ASW+5% NaCl	25	-	7.87	-	7.84	8.00	8.18	7.73	8.24	8.04	8.86
	ASW+10% NaCl	21	-	3.45	5.63	4.48	7.50	7.26	7.41	4.49	7.35	8.12
	ASW+30% NaCl	12	-	>8.00	>8.00	3.03	7.60	8.11	≥4.00	8.08	8.18	8.33
<i>V. parahaemolyticus</i> ATCC 33844	ASW	50	-	8.02	-	7.78	8.30	7.76	7.26	8.07	7.73	7.40
	ASW+5% NaCl	50	-	7.75	-	8.56	8.38	7.32	-	-	-	7.37
	ASW+10% NaCl	50	-	≥8.30	-	7.99	8.02	7.58	7.91	7.96	7.83	7.50
	ASW+30% NaCl	25	-	-	-	-	-	-	-	-	-	-
<i>V. parahaemolyticus</i> ATCC 27969	ASW	72	-	7.69	-	>7.00	8.78	9.53	8.72	8.91	8.52	7.79
	ASW+5% NaCl	72	-	8.91	-	>7.00	7.72	7.46	8.36	9.00	7.61	8.26
	ASW+10% NaCl	72	-	7.93	-	-	8.45	-!	6.93	7.90	8.36	-
	ASW+30% NaCl	25	-	-	-	-	-	-	-	-	-	>6.00

¹ ASW, artificial sea water (pH 7); TSB, tryptic soy broth (pH 7) supplemented with 3% NaCl; BHI, brain heart infusion broth supplemented with 3% NaCl; APW, alkaline peptone water (pH 8); CFS, cell free supernatant; VP, *V. parahaemolyticus* ATCC 17082; VV, *V. vulnificus* ATCC 33815; ST, *Salmonella enterica* serovar Typhimurium ATCC 19585; EC, *Escherichia coli* O157:H7 ATCC 35150; SA, *Staphylococcus aureus* ATCC 27994; CSP, TSB supplemented with 10,000 U/mg catalase and 2% sodium pyruvate.

² -, No growth.

³ ND, not determined.

6

TABLE 4 Effects of NaCl contents (1-3%) combined with alkaline pH levels (pH 8-9) on the resuscitation of *V. parahaemolyticus* in the VBNC state at 4°C for 80 days

Pathogen	Microcosm	Temperature upshift in the following resuscitation-buffers ¹ at 25°C for consecutive 7 days							
		TSB	TSB 1	TSB 2	TSB 3	APW	APW 1	APW 2	APW 3
<i>V. parahaemolyticus</i> ATCC 17082	ASW	○	○	○	-	-	-	-	-
	ASW+5% NaCl	- ²	-	-	-	-	-	-	-
	ASW+10% NaCl	-	-	-	-	-	-	-	-
	ASW+30% NaCl	-	-	-	-	-	-	-	-
<i>V. parahaemolyticus</i> ATCC 33844	ASW	○	○	○	○	-	-	○	-
	ASW+5% NaCl	○	○	○	○	-	-	○	-
	ASW+10% NaCl	-	○	○	○	-	-	-	-
	ASW+30% NaCl	-	-	-	-	-	-	-	-
<i>V. parahaemolyticus</i> ATCC 27969	ASW	○	○	○	○	○	○	○	○
	ASW+5% NaCl	○	○	○	-	○	○	○	○
	ASW+10% NaCl	○	-	○	-	○	○	○	○
	ASW+30% NaCl	-	-	-	-	-	-	-	-

¹ TSB, tryptic soy broth (pH 7) supplemented with 3% NaCl; TSB 1, TSB (pH8) supplemented with 3% NaCl; TSB 2, TSB (pH 7) supplemented with 1% NaCl; TSB 3, TSB (pH 8) supplemented with 1% NaCl; APW, Alkaline Peptone Water (pH 8) supplemented with 3% NaCl; APW 1, APW (pH 8) supplemented with 3% NaCl; APW 2, APW (pH 7) supplemented with 1% NaCl; APW 3, APW (pH 8) supplemented with 1% NaCl.

² -, No growth.

7

TABLE 5 Assessment of the ability ($\log_{10}$ CFU/g) of *V. parahaemolyticus* pre-incubated in ASW microcosms (pH 6) at 4°C for 110 days to be recovered from the VBNC state by using optimized resuscitation-buffers

Pathogen	Microcosm	VBNC-induced period (days) ²	Temperature upshift in the following resuscitation-buffers ¹ at 25°C for consecutive 7 days						
			TSB	CFS-VP	CFS-VV	CFS-ST	CFS-EC	CFS-SA	CSP
<i>V. parahaemolyticus</i> ATCC 17082	ASW	50	-	5.60	4.70	7.78	8.00	4.48	6.08
	ASW+5% NaCl	25	-	8.75	5.80	-	5.99	-	6.76
	ASW+10% NaCl	21	8.48	5.08	5.17	-	-	-	5.00
	ASW+30% NaCl	12	-	-	-	-	-	-	-
<i>V. parahaemolyticus</i> ATCC 27969	ASW	50	8.08	-	-	-	-	-	9.15
	ASW+5% NaCl	50	-	8.83	8.91	-	-	-	8.79
	ASW+10% NaCl	50	-	-	-	-	8.81	5.94	-
	ASW+30% NaCl	25	-	-	-	-	-	-	-
<i>V. parahaemolyticus</i> ATCC 33844	ASW	72	7.69	8.78	9.53	8.72	8.91	8.52	7.79
	ASW+5% NaCl	72	8.91	7.72	7.46	8.36	9.00	7.61	8.26
	ASW+10% NaCl	72	7.93	8.45	-	6.93	7.90	8.36	-
	ASW+30% NaCl	25	-	-	8.88	8.28	-	-	6.70

¹ These pathogens in the VBNC state for 110 days were transferred into TSB (pH 8.1) supplemented with 3% NaCl, and then incubated at 25°C for 7 days.

² Days for which *V. parahaemolyticus* and *V. vulnificus* were required for the entry into the VBNC state at 4°C.

TABLE 6 Components of developed resuscitation-buffers

Buffer	Component categories ¹			
	TSB	CSP	MgTA	CFS
A	•	-	-	-
B	•	•	-	-
C	•	•	•	-
D	•	•	•	•
E	•	-	-	•
F	•	-	•	•

¹ TSB, tryptic soy broth (pH 8) supplemented with 3% NaCl.

² CSP, 10,000 U/mg catalase and 2% sodium pyruvate.

³ MgTA, 20 mM MgSO₄ + 5 mM EDTA.

⁴ CFS, the cell free supernatant of *V. parahaemolyticus* ATCC 17082 grown overnight in each of appropriate resuscitation buffers at 37°C.

12

TABLE 7 Effects of developed recovery buffers on the resuscitation of 250-days-nonculturable *V. parahaemolyticus*, followed by temperature upshift method at 25°C for 5-7 days

Strain	ATCC	Microcosm	Resuscitation buffers					
			A	B	C	D	E	F
<i>V. parahaemolyticus</i>	17082	ASW	-	-	-	-	-	-
	17082	ASW+5% NaCl	7.72	8.48	-	7.85	-	7.57
	17082	ASW+10% NaCl	8.48	-	-	8.48	-	-
	17082	ASW+30% NaCl	-	-	-	9.24	-	-
<i>V. parahaemolyticus</i>	33844	ASW	-	-	-	-	-	-
	33844	ASW+5% NaCl	4.00	-	-	-	-	-
	33844	ASW+10% NaCl	-	-	-	-	-	-
	33844	ASW+30% NaCl	-	-	-	-	-	-
<i>V. parahaemolyticus</i>	27969	ASW	7.58	8.89	8.96	9.05	8.91	8.93
	27969	ASW+5% NaCl	9.09	8.72	9.31	9.16	8.86	8.83
	27969	ASW+10% NaCl	8.81	9.09	8.48	9.09	9.68	8.88
	27969	ASW+30% NaCl	-	-	-	-	-	-

¹ -, no growth.

13

14