



Optimization of resuscitation-promoting broths for the revival of *Vibrio parahaemolyticus* from a viable but nonculturable state

Jae-Hyun Yoon¹ · Young-Min Bae¹ · Suyoung Jo¹ · Sung-Kwon Moon¹ ·
Se-Wook Oh² · Sun-Young Lee¹

Received: 15 June 2020/Revised: 19 August 2020/Accepted: 23 October 2020/Published online: 18 November 2020
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Abstract This study was conducted to examine the effect of formulated resuscitation-promoting broths on the revival of viable but nonculturable *Vibrio parahaemolyticus* induced by cold and starvation stresses. *Vibrio parahaemolyticus* was incubated in artificial sea water at 4 °C for more than 8 months until this bacterium became undetectable, while retaining its intact cell count of more than 10⁵ CFU/field over time. On day 250, *V. parahaemolyticus* was collected and enriched in tryptic soy broth supplemented with 3% NaCl, 10,000 U/mg catalase, 2% sodium pyruvate, 20 mM MgSO₄, 5 mM EDTA, and a cell-free supernatant taken from *V. parahaemolyticus* ATCC 17802 in the stationary phase (pH 8). *V. parahaemolyticus* returned partially to a culturable state with a maximal cell density of 7.91 log CFU/mL in this formulated medium following 7 days of enrichment at 25 °C. In

contrast, no *V. parahaemolyticus* was resuscitated when enriched in alkaline peptone water and tryptic soy broth.

Keywords Food safety · Pathogen · Resuscitation · Viable but nonculturable · *Vibrio parahaemolyticus*

Introduction

Vibrio parahaemolyticus is a leading pathogenic bacterium of significant concern involved in food-borne diseases and illnesses globally since the consumption of seafood products contaminated with this organism can result in various clinical manifestations, such as abdominal pain, diarrhea, headache, nausea, vomiting, and septicemia (Korean Centers for Disease Control and Prevention, 2019). Generally, clam, eel, mackerel, mussel, oyster, scallop, and shrimp have been considered as the most predominant vehicles for *V. parahaemolyticus* as reflected in many food-borne outbreaks and recalls. Estuarine ecosystems, such as coastal area, seawater, and sediment, and even raw vegetables also have been identified to harbor large numbers of *V. parahaemolyticus* (Tunung et al., 2010).

Especially, *V. parahaemolyticus* was demonstrated to be capable of entering into a viable but nonculturable (VBNC) state when subjected to a variety of environmental stresses, including cold temperature, nutrient-deprivation, osmotic shock, and others (Dong et al., 2019; Falcioni et al., 2008; Yoon et al., 2017; Yoon et al., 2019; Yoon and Lee, 2019). *V. parahaemolyticus* remains viable but no longer grow on culture media that routinely support its growth and presents reduced metabolic activities, including ATP synthesis and expression of gene, RNA, and transcript, while maintaining its cellular integrity (Chaiyanan et al., 2007; Tholozan et al., 1999; Trinh et al., 2015).

✉ Sun-Young Lee
nina6026@cau.ac.kr

Jae-Hyun Yoon
nevbge999@gmail.com

Young-Min Bae
only1617@hanmail.net

Suyoung Jo
suyoung3223@naver.com

Sung-Kwon Moon
sumoon66@cau.ac.kr

Se-Wook Oh
swoh@kookmin.ac.kr

¹ Department of Food and Nutrition, Chung-Ang University, 4726, Seodong-daero, Anseong-si, Gyeonggi-do, Republic of Korea

² Department of Food and Nutrition, Kookmin University, 77, Jeongneung-ro, Seongbuk-gu, Seoul, Republic of Korea

Interestingly, *V. parahaemolyticus* would be resuscitated from a VBNC state reversibly by eliminating a causative environmental stress primarily responsible for its evolution into a VBNC state. Falcioni et al. (2008) determined that VBNC *V. parahaemolyticus* was capable of regaining its colony-forming ability by a temperature upshift method, thereby leading to the restoration of its cell morphology from coccal and helical forms to typical rods. Preliminarily, we observed that several *V. parahaemolyticus* strains had the ability to resuscitate from a VBNC state upon 2 days of enrichment in tryptic soy broth (TSB) supplemented with 3% NaCl (TSB₇₋₃) at 25 °C, and then the resuscitated cells exhibited high levels of cytotoxicity equal to that of their normal cultures against Caco-2 and HEP-2 (data not shown). As standard bacteriological methods can neither accurately estimate the viable cell count of VBNC bacteria nor identify the formation of a VBNC state most likely due to significantly decreased culturability and metabolism (Dong et al., 2019; Oliver, 2016), pathogenic microorganisms may present a potential food safety hazard during the evolution to a VBNC state.

So far, numerous studies have been undertaken to establish an effective procedure that may be attributable to the resuscitation of microorganisms from a VBNC state. Indeed, Coutard et al. (2007) demonstrated that VBNC *V. parahaemolyticus* VP5 regained its colony-forming ability following a subsequent enrichment step in artificial sea water (ASW) at 25 °C for 4 days. When VBNC *V. parahaemolyticus* was enriched in TSB₇₋₃ at 25 °C for 48 h, a maximum platable count of approximately 9.0 log CFU/mL was resuscitated (Wong et al., 2004). Furthermore, previous studies suggested that the presence of resuscitation-promoting stimuli, such as amino acids, antioxidants, cell-free cultures of *V. vulnificus*, and other biological additives, played an important role in the resuscitation of pathogenic bacteria from a VBNC state (Ayrapetyan et al., 2014; Gupte, de Rezende, and Joseph, 2003; Panutdaporn et al., 2006; Pinto et al., 2011). Despite the fact that *V. parahaemolyticus* can be recovered from a VBNC state in a favorable environment, which provides sufficient energy sources to encourage its biological function and growth, little is known regarding the combined effect of resuscitation-promoting stimuli on the persistence and resuscitation of VBNC *V. parahaemolyticus* induced under multiple environmental conditions, including acidification, high salinity, starvation, and refrigerated temperature. Importantly, there is no standardized protocol for efficient resuscitation-provoking methods involved in the restoration of *V. parahaemolyticus* from a VBNC state. Therefore, this study was undertaken to examine the combined effect of resuscitation-promoting stimuli, such as catalase, cell-free supernatant taken from *V. parahaemolyticus* ATCC 17802, sodium pyruvate, EDTA, and MgSO₄, on the

revival of VBNC *V. parahaemolyticus* induced in ASW microcosms (pH 6) supplemented with less than 30% NaCl under prolonged cold and starvation conditions. Alternatively, formulated resuscitation-promoting buffers may trigger the revival of *V. parahaemolyticus* persisted in cold and starvation conditions for an extended period of time, thereby contributing to the establishment of improved microbial risk surveillance programs.

Materials and methods

Preparation of microcosms

ASW microcosms were prepared according to a study conducted by Yoon et al. (2017). Considering that *V. parahaemolyticus* can grow or persist under osmotic environments at $\leq 7\%$ NaCl (Cheng et al., 2004), we assumed that high levels of NaCl could be a key contributor influencing the induction of a VBNC state in *V. parahaemolyticus* strains. To determine the effect of different NaCl contents on the emergence of a VBNC state, ASW was modified by the addition of excessive NaCl amounts. Then, microcosms containing 5%, 10%, and 30% NaCl were designated as ASW_{five}, ASW_{ten}, and ASW_{thry}, respectively, and adjusted to pH 6 using membrane-filtered 1 N lactic acid (Kanto chemical, Tokyo, Japan). Prepared ASW microcosms (500 mL) were autoclaved at 121 °C for 20 min.

Bacterial inoculums

V. parahaemolyticus ATCC 17802, *V. parahaemolyticus* ATCC 33844, and *V. parahaemolyticus* ATCC 27969 were purchased from the Korean Culture Center of Microorganisms (KCCM, Seoul-si, Republic of Korea). Each stock was maintained at -75 °C and activated in tryptic soy broth supplemented with 3% NaCl (TSB₇₋₃; Difco, Detroit, MI, USA) at 37 °C for 24 h. Each of *V. parahaemolyticus* strains was cultured in 50 mL of TSB₇₋₃ at 37 °C for 24 h. Individually, overnight cultures of *V. parahaemolyticus* were harvested by centrifugation at $10,000 \times g$ for 3 min at 4 °C, washed two times in 1 mL of ASW, and final pellets were re-suspended in 1 mL of ASW (pH 6). Each of ASW microcosms with varying concentrations of NaCl was inoculated by *V. parahaemolyticus* ATCC 17802, *V. parahaemolyticus* ATCC 33844 or *V. parahaemolyticus* ATCC 27969, corresponding to 10^{7-8} log CFU/mL, and these microcosms were stored at 4 °C.

Enumeration and fluorescence microscopic assay

The ASW microcosms were withdrawn from a low temperature incubator at a regular interval. To minimize the residual NaCl effects, 1 mL of the bacterial solutions was centrifuged at $10,000 \times g$ for 3 min at 4 °C, washed in 0.1 M PBS, and re-suspended in 0.1 M PBS. Each culture was serially diluted in alkaline peptone water (APW; Difco), and 100 μ L of the diluents was spread onto tryptic soy agar (TSA; Difco) supplemented with 3% NaCl. All agar plates were incubated at 37 °C for 24–48 h.

Total and viable cell counts of *V. parahaemolyticus* were measured using the LIVE/DEAD BacLight® Bacterial Viability Kit (Invitrogen, Mount Waverley, VIC, Australia). The ASW microcosms were withdrawn from a low temperature incubator on day 0, 80, 100, 110 or 250. Approximately, 1 mL of the bacterial solutions was centrifuged at $10,000 \times g$ for 3 min at 4 °C and re-suspended in 1 mL of 0.1 M PBS. Two different nucleic acid stains, SYTO₉ and propidium iodide (PI) were combined at an equal volume (1:1) in a sterile microtube, and 3 μ L of this mixture was added to 1 mL of the bacterial solutions. After 15 min of incubation in the dark, 5 μ L of the stained aliquots was placed onto a glass slide and covered with a coverslip. All images were obtained using an electron-fluorescence microscope (TE 2000-U, Nikon, Tokyo, Japan) at a magnification of $\times 40$.

Resuscitation process

Resuscitation-promoting broths (RPBs) and modified APW or TSB were prepared according to a method described in Table 1. Once *V. parahaemolyticus* was incubated in ASW microcosms at 4 °C for 80 days (Table 2), 1 mL of the bacterial solutions was centrifuged at $13,000 \times g$ for 3 min at 4 °C, washed twice with 0.1 M PBS, and re-suspended in 5 mL of ASW, TSB₇₋₃ or brain heart infusion broth containing 3% NaCl (BHI; Difco). After 3 days of enrichment at 25 °C, the number of the resuscitated cells was quantified followed by direct spreading on TSA containing 3% NaCl.

As stated above, the NaCl concentration of TSB₇₋₃ and APW was modified, corresponding to 1–3%, and these media were adjusted to pH 7–8 by adding membrane-filtered 1 N NaOH (Kanto chemical). Upon exposure to cold and starvation stresses for 100 days (Table 3), all ASW microcosms were withdrawn, and 1 mL of the bacterial solutions was centrifuged at $10,000 \times g$ for 3 min at 4 °C, washed in 0.1 M PBS, and re-suspended in 5 mL of the modified TSB (TSB₇₋₁, TSB₈₋₃ or TSB₈₋₁) and APW (APW₇₋₁, APW₈₋₃ or APW₇₋₃), respectively. Each of the cultures were enriched at 25 °C for up to 7 days.

Individually, halophilic bacteria, such as *V. parahaemolyticus* ATCC 17802 and *V. vulnificus* ATCC 27562, were cultured in TSB₇₋₃ at 37 °C for 24 h, whereas *Escherichia coli* O157:H7 ATCC 35150, *Salmonella enterica* serovar Typhimurium ATCC 43971, and *Staphylococcus aureus* ATCC 12598 were grown in TSB at 37 °C for 24 h. The overnight cultures of each bacteria were harvested by centrifugation at $13,000 \times g$ for 3 min at 4 °C. The supernatant was collected in a sterile 50-ml conical tube and filtered using a 0.2- μ m polycarbonate membrane (ADVANTEC, Tokyo, Japan) to prepare independent cell-free supernatant (CFS) fluids. TSB₈₋₃ added with each CFS taken from *V. parahaemolyticus* ATCC 17802, *V. vulnificus* ATCC 27562, *E. coli* O157:H7 ATCC 35150, *S. Typhimurium* ATCC 43971 and *Staph. aureus* ATCC 12598 at a ratio of 9:1 (v/v) was designated as CFS_{vp}, CFS_{vv}, CFS_{st}, CFS_{ec}, and CFS_{sa}, respectively. Catalase (Sigma-Aldrich, St. Louis, MO, USA) or sodium pyruvate (Sigma-Aldrich) was diluted in TSB₈₋₃, and the diluents were membrane-filtered. Both of the two antioxidants were added to TSB₈₋₃, corresponding to 10,000 U/mg protein catalase and 2% sodium pyruvate (CSP). Then, CFS_{vp}, CFS_{vv}, CFS_{st}, CFS_{ec}, CFS_{sa} or CSP were further used to determine if these broths would stimulate the resuscitation of VBNC *V. parahaemolyticus* which persisted in ASW microcosms at 4 °C for 110 days (Table 4). After 3 days of enrichment at 25 °C, the number of the resuscitated cells was quantified followed by direct spreading on TSA containing 3% NaCl.

Individual respective resuscitation-promoting stimuli, such as 10,000 U/mg catalase, 2% sodium pyruvate, 20 mM MgSO₄ (Sigma-Aldrich), 5 mM EDTA (Sigma-Aldrich), and a CFS taken from the overnight culture of *V. parahaemolyticus* ATCC 17802, were combined with the optimized TSB₈₋₃ to formulate the resuscitation-promoting broths (RPBs) used in this study (Table 5). On day 250 under cold and starvation conditions, 1 mL of the bacterial solutions was centrifuged at $13,000 \times g$ for 3 min at 4 °C, washed twice with 0.1 M PBS, and re-suspended in 5 mL of TSB₈₋₃, RPB_a, RPB_b, RPB_c, RPB_d or RPB_e. After 7 days of enrichment at 25 °C, each solution was serially diluted in APW, and 100 μ L of the diluents was spread onto TSA containing 3% NaCl. All agar plates were incubated at 37 °C for 24–48 h.

Results and discussion

Induction of a VBNC state

Changes in the colony-forming ability of *V. parahaemolyticus* ATCC 17802, *V. parahaemolyticus* ATCC 33844, and *V. parahaemolyticus* ATCC 27969 subjected to

Table 1 Component of formulated resuscitation-promoting media used in this study

Media ^a	pH	Component ^b					
		NaCl	Catalase	Pyruvate	MgSO ₄	EDTA	Supernatant
APW	8	1.0%	– ^c	–	–	–	–
APW ₇₋₁	7	1.0%	–	–	–	–	–
APW ₈₋₃	8	3.0%	–	–	–	–	–
APW ₇₋₃	7	3.0%	–	–	–	–	–
TSB	7	0.5%	–	–	–	–	–
TSB ₇₋₃	7	3.0%	–	–	–	–	–
TSB ₇₋₁	7	1.0%	–	–	–	–	–
TSB ₈₋₃	8	3.0%	–	–	–	–	–
TSB ₈₋₁	8	1.0%	–	–	–	–	–
RPB _a	8	3.0%	+	+	+	+	+
RPB _b	8	3.0%	+	+	–	–	–
RPB _c	8	3.0%	+	+	+	–	–
RPB _d	8	3.0%	+	+	+	–	+
RPB _e	8	3.0%	–	–	–	–	+

^aAPW, alkaline peptone water; TSB, tryptic soy broth; RPB, resuscitation-promoting buffer. ^bTo formulate each of RPBs, 10,000 U/mg protein catalase, 2% sodium pyruvate, 20 mM MgSO₄, 5 mM EDTA, and/or a cell-free supernatant from *V. parahaemolyticus* ATCC 17802 in the stationary phase were combined in this study. ^c + Addition; –, Non-addition

Table 2 Reacquisition of the colony-forming ability (log CFU/mL) of VBNC *V. parahaemolyticus* strains persisted under cold and starvation conditions for 80 days followed by 3 days of enrichment in commercial culture broths at 25 °C

Bacterium	Strain	Microcosm	Culture broth ^a		
			ASW	BHI	TSB ₇₋₃
<i>V. parahaemolyticus</i>	ATCC 17802	ASW	NG ^b	6.83	7.43
<i>V. parahaemolyticus</i>	ATCC 17802	ASW _{five}	NG	NG	7.87
<i>V. parahaemolyticus</i>	ATCC 17802	ASW _{ten}	NG	5.63	3.45
<i>V. parahaemolyticus</i>	ATCC 17802	ASW _{thry}	NG	8.00	8.00
<i>V. parahaemolyticus</i>	ATCC 33844	ASW	NG	NG	8.02
<i>V. parahaemolyticus</i>	ATCC 33844	ASW _{five}	NG	NG	7.75
<i>V. parahaemolyticus</i>	ATCC 33844	ASW _{ten}	NG	NG	8.30
<i>V. parahaemolyticus</i>	ATCC 33844	ASW _{thry}	NG	NG	NG
<i>V. parahaemolyticus</i>	ATCC 27969	ASW	NG	NG	7.69
<i>V. parahaemolyticus</i>	ATCC 27969	ASW _{five}	NG	NG	8.91
<i>V. parahaemolyticus</i>	ATCC 27969	ASW _{ten}	NG	NG	7.93
<i>V. parahaemolyticus</i>	ATCC 27969	ASW _{thry}	NG	NG	NG

^aAs described previously in the Materials and Methods, individual respective strains of *V. parahaemolyticus* were incubated in ASW microcosms supplemented with less than 30% NaCl at 4 °C. After 80 days, each *V. parahaemolyticus* strain was collected, re-suspended in ASW, BHI or TSB₇₋₃, and enriched at 25C for 3 days. ^bNG, no growth

prolonged cold and starvation stresses are characterized in Fig. 1. The initial loads of *V. parahaemolyticus* ranged from 7.0 to 7.8 log CFU/mL. After 21 days of cold and starvation stresses, *V. parahaemolyticus* ATCC 17802 was reduced to approximately 3.6, ≥ 0.95 , ≥ 0.95 , and ≥ 0.95 log CFU/mL in ASW, ASW_{five}, ASW_{ten}, and ASW_{thry}, respectively. *V. parahaemolyticus* ATCC 17802 survived at levels of less than 2.2 log CFU/mL in ASW on day 30. Similarly, *V. parahaemolyticus* ATCC 33844 was capable

of surviving at levels of 2.6–3.1 log CFU/mL in ASW microcosms added with $\leq 10\%$ NaCl at 4 °C for 40 days, while reduced by 7.2 log CFU/ml in ASW_{thry}. *V. parahaemolyticus* ATCC 17802 and *V. parahaemolyticus* ATCC 33844 were reduced to below the detection limits (< 1.0 log CFU/ml) in all microcosms within 50 days. Approximately 21, 70, 70, and 70 days were required for *V. parahaemolyticus* ATCC 27969 to lose its colony-forming ability when incubated in ASW, ASW_{five}, ASW_{ten},

Table 3 Optimization of formulated culture broths containing 1–3% NaCl (pH 8–9) for resuscitating VBNC *V. parahaemolyticus* strains persisted under cold and starvation conditions for 100 days

Bacterium	Strain	Microcosm	Formulate culture broth ^a							
			TSB _{7-3bhi}	TSB ₇₋₁	TSB ₈₋₃	TSB ₈₋₁	APW	APW ₇₋₁	APW ₈₋₃	APW ₇₋₃
<i>V. parahaemolyticus</i>	ATCC 17802	ASW	Growth	Growth	Growth	NG	NG	NG	NG	NG
<i>V. parahaemolyticus</i>	ATCC 17802	ASW _{five}	NG ^b	NG	NG	NG	NG	NG	NG	NG
<i>V. parahaemolyticus</i>	ATCC 17802	ASW _{ten}	NG	NG	NG	NG	NG	NG	NG	NG
<i>V. parahaemolyticus</i>	ATCC 17802	ASW _{thry}	NG	NG	NG	NG	NG	NG	NG	NG
<i>V. parahaemolyticus</i>	ATCC 33844	ASW	Growth	Growth	Growth	Growth	NG	NG	Growth	NG
<i>V. parahaemolyticus</i>	ATCC 33844	ASW _{five}	Growth	Growth	Growth	Growth	NG	NG	Growth	NG
<i>V. parahaemolyticus</i>	ATCC 33844	ASW _{ten}	NG	Growth	Growth	Growth	NG	NG	NG	NG
<i>V. parahaemolyticus</i>	ATCC 33844	ASW _{thry}	NG	NG	NG	NG	NG	NG	NG	NG
<i>V. parahaemolyticus</i>	ATCC 27969	ASW	Growth	Growth	Growth	Growth	Growth	Growth	Growth	Growth
<i>V. parahaemolyticus</i>	ATCC 27969	ASW _{five}	Growth	Growth	Growth	NG	Growth	Growth	Growth	Growth
<i>V. parahaemolyticus</i>	ATCC 27969	ASW _{ten}	Growth	NG	Growth	NG	Growth	Growth	Growth	Growth
<i>V. parahaemolyticus</i>	ATCC 27969	ASW _{thry}	NG	NG	NG	NG	NG	NG	NG	NG
Total positive samples	6	6	7	4	3	5	3			

^aAs described previously in the Materials and Methods, individual respective strains of *V. parahaemolyticus* were incubated in ASW microcosms supplemented with less than 30% NaCl at 4 °C. After 100 days, each *V. parahaemolyticus* strain was collected, re-suspended in formulated culture broths and enriched at 25C for 7 days. Detail information about the formulated culture media was shown in Table 1. ^bNG, no growth

Table 4 Optimization of the formulated culture medium TSB₈₋₃ by adding a cell-free culture of pathogenic bacteria or a mixture of catalase and sodium pyruvate for resuscitating (log CFU/mL) VBNC *V. parahaemolyticus* strains persisted under cold and starvation conditions for 110 days

Bacterium	Strain	Microcosm	TSB ₈₋₃ added with a cell-free culture of bacteria or a mixture of catalase and sodium pyruvate ^a						
			None	CFS _{vp}	CFS _{vv}	CFS _{st}	CFS _{ec}	CFS _{sa}	CSP
<i>V. parahaemolyticus</i>	ATCC 17802	ASW	7.48	4.60	3.70	6.78	7.00	3.48	5.08
<i>V. parahaemolyticus</i>	ATCC 17802	ASW _{five}	NG ^b	7.75	4.80	NG	4.99	NG	5.76
<i>V. parahaemolyticus</i>	ATCC 17802	ASW _{ten}	NG	4.08	4.17	NG	NG	NG	4.00
<i>V. parahaemolyticus</i>	ATCC 17802	ASW _{thry}	NG	NG	NG	NG	NG	NG	NG
<i>V. parahaemolyticus</i>	ATCC 33844	ASW	NG	7.83	7.91	NG	NG	NG	8.15
<i>V. parahaemolyticus</i>	ATCC 33844	ASW _{five}	NG	NG	NG	NG	NG	NG	7.79
<i>V. parahaemolyticus</i>	ATCC 33844	ASW _{ten}	NG	NG	NG	NG	NG	NG	NG
<i>V. parahaemolyticus</i>	ATCC 33844	ASW _{thry}	NG	NG	NG	NG	NG	NG	NG
<i>V. parahaemolyticus</i>	ATCC 27969	ASW	6.69	7.78	8.53	7.72	7.91	7.52	6.79
<i>V. parahaemolyticus</i>	ATCC 27969	ASW _{five}	7.91	6.72	6.46	7.36	8.00	6.61	7.26
<i>V. parahaemolyticus</i>	ATCC 27969	ASW _{ten}	6.93	7.45	6.00	5.93	6.90	7.36	5.70
<i>V. parahaemolyticus</i>	ATCC 27969	ASW _{thry}	NG	NG	NG	NG	NG	NG	NG

^aAs described previously in the Materials and Methods, individual respective strains of *V. parahaemolyticus* were incubated in ASW microcosms supplemented with less than 30% NaCl at 4 °C. After 110 days, each *V. parahaemolyticus* strain was collected, re-suspended in TSB₈₋₃ added with a cell-free culture of bacteria or a mixture of catalase and sodium pyruvate and enriched at 25C for 3 days. TSB₈₋₃ added with a CFS taken from *V. parahaemolyticus* ATCC 17802, *V. vulnificus* ATCC 27562, *E. coli* O157:H7 ATCC 35150, *S. Typhimurium* ATCC 43971 and *Staph. aureus* ATCC 12598 in the stationary phase at a ratio of 9:1 (v/v) was designated as CFS_{vp}, CFS_{vv}, CFS_{st}, CFS_{ec}, and CFS_{sa}, respectively. Detail information about the formulated culture media was shown in Table 1. ^bNG, no growth

and ASW_{thry}, respectively. Despite that each of *V. parahaemolyticus* strains became unculturable under cold and starvation conditions within 70 days, the viable cell

number of these bacteria was between 5.5 and 6.8 log CFU/field over time as evidenced by SYTO₉ and PI uptake analysis. The LIVE/DEAD BacLight assay constituting

Table 5 Resuscitation of VBNC *V. parahaemolyticus* strains persisted under cold and starvation conditions for 250 days followed by 7 days of enrichment in formulated resuscitation-promoting broths at 25 °C

Bacterium	Strain	Microcosm	Formulated RPBs ^a					
			TSB ₈₋₃	RPB _a	RPB _b	RPB _c	RPB _d	RPB _e
<i>V. parahaemolyticus</i>	ATCC 17802	ASW	Growth	Growth	NG	NG	Growth	Growth
<i>V. parahaemolyticus</i>	ATCC 17802	ASW _{five}	NG ^b	Growth	NG	NG	NG	NG
<i>V. parahaemolyticus</i>	ATCC 17802	ASW _{ten}	NG	Growth	NG	NG	NG	NG
<i>V. parahaemolyticus</i>	ATCC 17802	ASW _{thry}	NG	NG	NG	NG	NG	NG
<i>V. parahaemolyticus</i>	ATCC 27969	ASW	Growth	Growth	Growth	Growth	Growth	Growth
<i>V. parahaemolyticus</i>	ATCC 27969	ASW _{five}	Growth	Growth	Growth	Growth	Growth	Growth
<i>V. parahaemolyticus</i>	ATCC 27969	ASW _{ten}	Growth	Growth	Growth	Growth	Growth	Growth
<i>V. parahaemolyticus</i>	ATCC 27969	ASW _{thry}	NG	NG	NG	NG	NG	NG
Total positive samples			4	6	3	3	4	4

^aAs described previously in the Materials and Methods, individual respective strains of *V. parahaemolyticus* were incubated in ASW microcosms supplemented with less than 30% NaCl at 4 °C. After 250 days, each *V. parahaemolyticus* strain was collected, re-suspended in TSB₈₋₃ or RPB_{a-e} and enriched at 25°C for 7 days. Detail information about the formulated culture media was shown in Table 1. ^bNG, no growth

SYTO₉ and PI stains has been widely used to measure the cytoplasmic membrane integrity, so called ‘viability’, of microorganisms (Dong et al., 2019; Yoon and Lee, 2019). SYTO₉ is known to penetrate across intact and damaged cell membranes and interact with nucleic acid, displaying a green colored fluorescence at 480 nm excitation and 500 nm emission. In contrast, PI permeates into cytoplasm through a compromised membrane, causing a reduction of SYTO₉. The loss of the permeability barrier in the stained cells presents any irreparable cellular damage, and PI exhibits a red color at 490 nm excitation and 635 nm emission. Hence, we observed the induction of a VBNC state in three *V. parahaemolyticus* strains persisted under cold and starvation conditions for at least 100 days, thereby indicating that an increase in the NaCl contents of the menstruum can accelerate the entry of *V. parahaemolyticus* into a VBNC state.

The VBNC state is definitively distinct from typical starvation responses that enable microorganisms to persist in hostile environments for longer periods of time, instead of death because *V. parahaemolyticus* and *Enterococcus faecalis* in a VBNC state were found to have a specifically altered overexpression of some proteins involved in central carbohydrate metabolism, general stress response, and protein biosynthesis (Heim et al., 2002; Meng et al., 2015). A microarray-based transcriptional study also revealed that *Vibrio cholerae* O139, *V. parahaemolyticus*, and *V. vulnificus* continued to express their own toxin—(*ctxAB*, *rtxA*, *hlyA*, *tl*, *tdh*, and *vvhA*) and virulence-related (*tcpA* and TTSS) attributes after transited to a VBNC state by nutrient-deficiency coupled with cold temperature (Vora et al., 2005). Furthermore, Cappelier et al. (2007) observed that *Listeria monocytogenes* became avirulent in a VBNC state

but regained the ability to form plaques in HT-29 monolayer cells and colonize mouse spleens after its revival to a culturable state through an embryonated egg passage. The VBNC state is a kind of survival strategy for which microorganisms adapt to withstand hostile environmental stresses, while maintaining minimal vitality requirements involved in potential infectivity or resuscitation capability.

Resuscitation from a VBNC state using conventional culture broths

Effect of conventional culture media, such as APW, ASW, BHI, and TSB, on the resuscitation of VBNC *V. parahaemolyticus* lasted in ASW microcosms at 4 °C for 80 days is shown in Table 2. *V. parahaemolyticus* failed to not only exit from a VBNC state, but also regain its colony-forming ability followed by an enrichment step in ASW (pH 7) at 25 °C. In contrast, *V. parahaemolyticus* returned to a culturable state with its platable count of ≥ 8.91 log CFU/mL in TSB₇₋₃ after 3 days of temperature upshift. In part, *V. parahaemolyticus* ATCC 33844 and *V. parahaemolyticus* ATCC 27969 cells persisted in ASW_{thry} remained nonculturable followed by an enrichment process in ASW and BHI at 25 °C for 3 days. Therefore, TSB₇₋₃ was found to be effective in resuscitating VBNC *V. parahaemolyticus* more than APW.

Resuscitation may result from the re-growth of a very few undetectable cells remaining viable in stressful environments (Coutard et al., 2007; Falcioni et al., 2008). Accordingly, VBNC *V. parahaemolyticus* used in this study should be verified whether its conversion to a culturable state resulted from a true resuscitation, rather than underwent a reductive cell division. To avoid such a

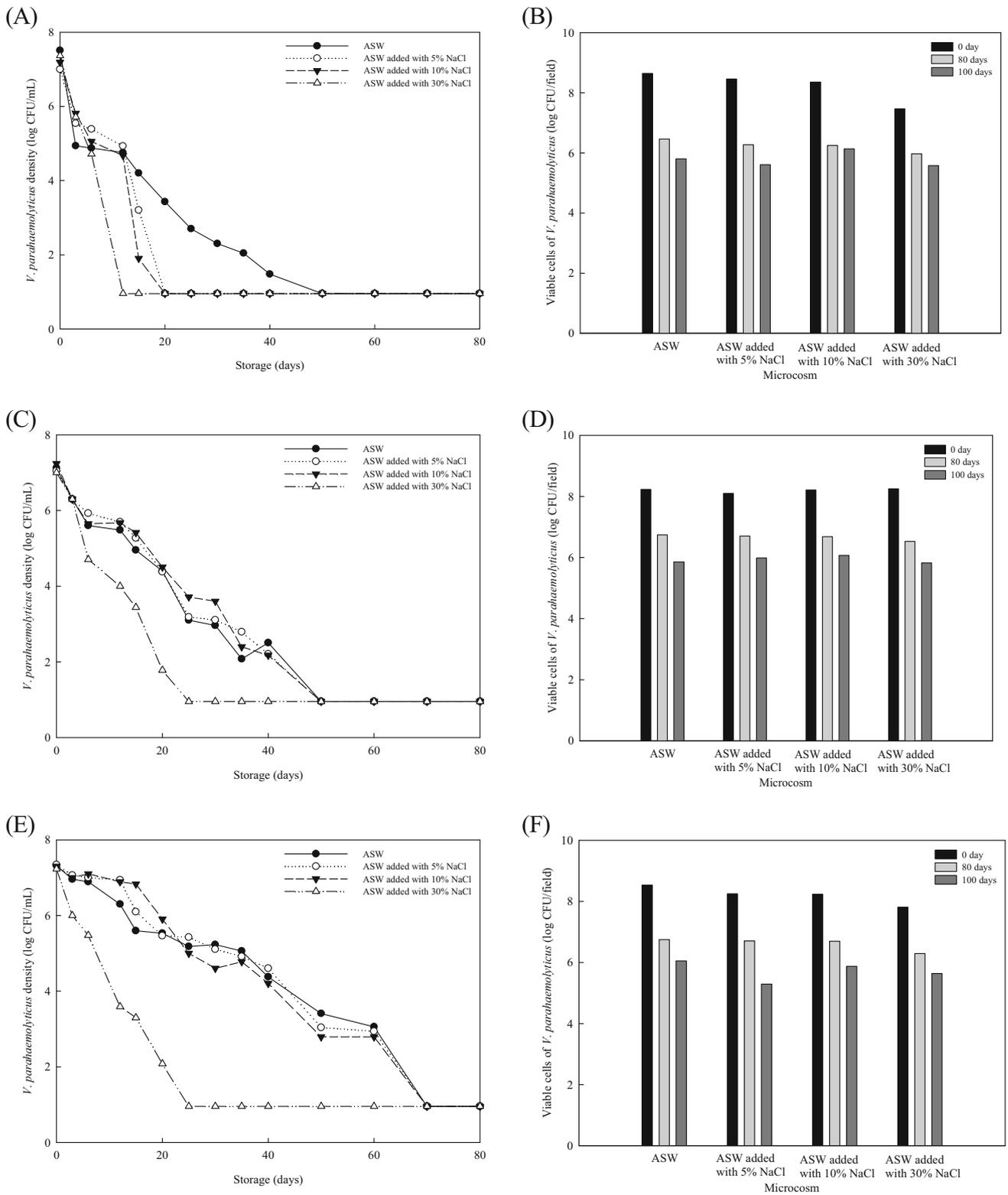


Fig. 1 Changes in (A, C, and E) the colony-forming ability and (B, D, and F) membrane integrity of (A–B) *V. parahaemolyticus* ATCC 17082, (C–D) *V. parahaemolyticus* ATCC 33844, and (E–F) *V. parahaemolyticus* ATCC 27969 inoculated in ASW (pH 6) at 4 °C

debate, *V. parahaemolyticus* ATCC 17802 lasted in a VBNC state for 50 days was collected, washed twice in APW, and diluted, ranging from 10^0 to 10^{-3} , in APW serially. The diluents were further incubated at 25 °C for 3 days. If there are a few residual or undetected sub-culturable cells among the laboratory microcosms, the resuscitated cells continue to grow, exceeding the initial cell inoculum levels (10^5 , 10^6 , 10^7 or 10^8 CFU/ml) of each serial diluent during all the stages of a resuscitation process (Whitesides and Oliver, 1997). After 3 days, *V. parahaemolyticus* was resuscitated to 10^5 , 10^6 , 10^7 or 10^8 CFU/ml in the 10^{-3} , 10^{-2} , 10^{-1} or 10^0 diluted APW solutions (data not shown).

Formulation and optimization of RPBs

Effect of formulated culture broths containing 1–3% NaCl (pH 8–9) for resuscitating VBNC *V. parahaemolyticus* strains subjected to cold and starvation conditions for 100 days is shown in Table 3. Partially, 6, 6, 7 or 4 *V. parahaemolyticus* cultures were reverted to a culturable state after 7 days of enrichment in TSB₇₋₃, TSB₇₋₁, TSB₈₋₃ or TSB₈₋₁, irrespective of the microcosms added with varying levels of NaCl. The modified APWs were shown to be less effective for the restoration of VBNC *V. parahaemolyticus*. Overall, TSB₈₋₃ exhibited the highest resuscitation capacity for VBNC *V. parahaemolyticus* induced by cold and starvation stresses.

The formulated culture medium TSB₈₋₃ was further optimized by adding a cell-free culture of pathogenic bacteria or a mixture of catalase and sodium pyruvate, and its effect on the reacquisition of the colony-forming ability of VBNC *V. parahaemolyticus* exposed to cold and starvation stresses for 110 days is shown in Table 4. *V. parahaemolyticus* ATCC 17802 in ASW was resuscitated at levels of 4.60, 3.70, 6.78, 7.00, and 3.48 log CFU/mL upon enrichment in CFS_{vp}, CFS_{vv}, CFS_{st}, CFS_{ec}, and CFS_{sa}, respectively. VBNC *V. parahaemolyticus* ATCC 33844 induced in ASW, ASW_{five}, and ASW_{ten} returned to culturable at levels of 7.83–8.15 log CFU/mL in CFS_{vp}, CFS_{vv}, and CSP once resuscitated at 25 °C for 7 days. VBNC *V. parahaemolyticus* ATCC 27969 lasted in microcosms containing less than 10% NaCl at 4 °C for 110 days were revived to 6.72–7.78, 6.00–8.53, 5.93–7.36, 6.90–8.00, 6.61–7.52, and 5.70–7.26 log CFU/mL in CFS_{vp}, CFS_{vv}, CFS_{st}, CFS_{ec}, CFS_{sa}, and CSP, respectively. CFS_{vp} exhibited the highest recovery rate (averagely 3.85 log CFU/ml) for resuscitating three *V. parahaemolyticus* strains in a VBNC state.

Resuscitation from a VBNC state using formulated RPBs

Table 5 shows the resuscitation of VBNC *V. parahaemolyticus* strains persisted under cold and starvation conditions for 250 days followed by 7 days of enrichment in formulated resuscitation-promoting broths at 25 °C. *V. parahaemolyticus* ATCC 17802 in ASW regained its culturability when resuscitated in TSB₈₋₃, RPB_a, RPB_c, and RPB_e. While VBNC *V. parahaemolyticus* ATCC 27969 persisted in ASW, ASW_{five}, and ASW_{ten} was returned to a culturable state after 7 days of enrichment in TSB₈₋₃ and RPB_{a-e}. *V. parahaemolyticus* ATCC 33844 failed to revive from a VBNC state. Overall, 6, 3, 3, 4 and 4 *V. parahaemolyticus* cultures were capable of reviving from a VBNC state once resuscitated in RPB_a, RPB_b, RPB_c, RPB_d, and RPB_e, respectively. RPB_a was the most effective in recovering *V. parahaemolyticus* from a VBNC state.

This study showed that the resuscitation-capability of VBNC *V. parahaemolyticus* was highly strain-dependent as influenced by the length of time spent under cold and starvation conditions. Indeed, when *V. parahaemolyticus* ATCC 17802 was induced into a VBNC state at 4 °C for 110 days, VBNC cells could be reverted to a culturable state following enrichment in TSB₈₋₃ with or without the addition of resuscitation-promoting stimuli. In contrast, *V. parahaemolyticus* ATCC 17802 lasted in ASW_{five}, ASW_{ten}, and ASW_{thry} at 4 °C for more than 110 days remained unculturable after 2 days of resuscitation in TSB₈₋₃. Only *V. parahaemolyticus* ATCC 33844 was incapable of returning to a culturable state when incubated in microcosms at 4 °C for 250 days. As well-documented in a study of Pinto et al. (2013), VBNC cells have their own different resuscitation window periods. Behind this point, VBNC cells may lose the resuscitation capability permanently, thereby leading to death.

Previous studies showed that the phase-shift of VBNC cells to a culturable state differed significantly, depending on various physico-chemical factors, including heat shock (Chaiyanan et al., 2007), nutrient-availability (Gupte et al., 2003), and the presence of resuscitation-promoting factors (RPFs). Particularly, RPFs are characterized as muralytic enzymes, which stimulate bacterial growth and resuscitation, and known to be closely involved in cell wall remodeling (Hett et al., 2007; Kana and Mizrahi, 2009; Mukamolova et al., 2006). In the same context, it was demonstrated that amino acid, antioxidizing agent, autoinducer (AI), and CFS played an important role for the resuscitation of *Vibrio* spp. from a VBNC state (Ayrpetyan et al., 2014; Pinto et al., 2011). In this study, the optimized TSB₈₋₃ supplemented with 10,000 U/mg catalase and 2% sodium pyruvate partially led to a revival of VBNC *V. parahaemolyticus* induced by cold and starvation

stresses for more than 100 days. Interestingly, the resuscitation capacity of catalase and sodium pyruvate may be concentration-dependent. In our preliminary studies, VBNC *V. parahaemolyticus* still remained unculturable even after a subsequent resuscitation process with APW and TSA containing either 1000–2000 U/mg catalase or 0.1–1.0% sodium pyruvate. At the same time, VBNC cells of *V. parahaemolyticus*, however, was resuscitated to a culturable state, following enrichment in APW and TSA supplemented with 10,000 U/mg catalase and/or 2.0% sodium pyruvate (data not shown). In a study conducted by Mizunoe et al. (2000), dormant but viable *V. parahaemolyticus* VP190 was resuscitated to levels of 10^{2-3} CFU/mL once further cultured on Luria–Bertani agar plates (LB) supplemented with either 2000 U catalase or 0.1% sodium pyruvate (in contrast, no colony was observed when VBNC *V. parahaemolyticus* VP190 was spread on a routine LB medium). Ramamurthy et al. (2014) determined that sodium pyruvate was responsible for the biosynthesis of some macromolecules involved in a revival of VBNC cells. Given aerobic organisms have evolved complex defense and repair systems to reduce or eliminate any cellular damage caused by reactive oxygen species (ROS) compounds, ROS might be profoundly associated with loss of culturability and induction of a VBNC state. Moreover, Morishige et al., (2013) observed that sodium pyruvate and its analogue, α -ketobutyrate presented a positive resuscitation effect on the revival of VBNC *Salmonella* Enteritidis induced by 0.3 mM H_2O_2 , and there was a marked increase in the synthesis of DNA and protein when VBNC *S. Enteritidis* was enriched in M9 added with 30 mM sodium pyruvate at 37 °C for at least 60 min. Although α -ketobutyrate is known to be oxidized by ferredoxin and 2-oxobutyrate synthase, and finally integrated into the tricarboxylic acid (TCA) cycle, the authors demonstrated that the activation of TCA cycle may not be necessarily responsible for the resuscitation of VBNC *S. Enteritidis* most likely due to the failure of a cofactor, thiamine, which stimulates the production of acetyl-CoA, to repair VBNC cells in the presence of sodium pyruvate. Nevertheless, the mechanism of sodium pyruvate and α -ketobutyrate to drive the resuscitation of VBNC cells to a culturable state remains unclarified.

Interestingly, Ayrapetyan et al. (2014) showed that VBNC *V. vulnificus* C7184 returned to a culturable state when enriched in a formulated culture broth containing a CFS harvested from its wild counterpart. The presence of a chemically synthesized autoinducer-2 (AI-2) led to a revival of VBNC *V. vulnificus*, whereas some CFSs taken from AI-2 mutant *V. vulnificus* strains failed to do so. AIs are defined as small molecules that regulate specific gene expressions in response to fluctuation in cell population density and have a significant impact on the resuscitation of

V. cholerae and *E. coli* from a VBNC state (Bari et al., 2013; Pinto et al., 2011). Considering that mutant strains of *Mycobacterium tuberculosis* with a deletion in RPF-related genes failed to revive from a VBNC state (Kana et al., 2008; Kana and Mizrahi, 2009), and VBNC bacteria, such as enterohemorrhagic *E. coli*, *Salmonella enterica* serovar Infantis, and *V. parahaemolyticus*, returned to a culturable state following co-culture with various eukaryotic cell lines (Senoh et al., 2014; Senoh et al., 2015), CFS may include either AI or RPF, which implies that bacteria in an inactive but viable state might be resuscitated in the presence of some resuscitation-promoting stimuli secreted by either metabolically active resident microbiota or intentionally added fermentation organisms. Reissbrodt et al. (2002) observed the resuscitation of VBNC *E. coli* strains to a culturable state upon exposure to the AI molecules produced by *Yersinia ruckeri*, strengthening this point of views.

Conclusion

To date, little is known about the effect of high NaCl contents combined with cold and starvation stresses on the entry of *V. parahaemolyticus* into a VBNC state or resuscitation of VBNC cells to a culturable state. In this study, we observed the induction of a VBNC state in *V. parahaemolyticus* exposed to ASW microcosms containing less than 30% NaCl (pH 6) at 4 °C. Formation of VBNC cells was accelerated rapidly with an increase in the NaCl concentrations. NaCl levels in a laboratory menstruum may play a critical role in the induction of a VBNC state in *V. parahaemolyticus*. Particularly, combined resuscitation-promoting stimuli facilitated the restoration of *V. parahaemolyticus* cells from a VBNC state followed by a temperature upshift. The modified TSB₈₋₃ supplemented with different types of stimuli may lead to the resuscitation of VBNC *V. parahaemolyticus* strains induced in ASW microcosms at 4 °C for at least 100 days. Especially, VBNC *V. parahaemolyticus* returned to a culturable state following 7 days of enrichment in RPB_a at 25 °C. The resuscitation capability of VBNC *V. parahaemolyticus* can greatly differ depending on the length of time spent under cold and starvation stresses and strain-variability. Taken together, *V. parahaemolyticus* can activate a preferred survival mechanism, referred to as the VBNC state, in response to unfavorable environmental conditions, concomitantly with its maintenance of minimal vitality requirements involved in the opportunistic resuscitative capability, and therefore the revival of *V. parahaemolyticus* from a VBNC state may pose a serious food safety hazard due to the resulting restoration of its infectivity and pathogenicity. RPBs containing various resuscitation-

promoting stimuli may facilitate the revival of *V. parahaemolyticus* subjected to prolonged cold and starvation stresses from a VBNC state, thereby providing useful insights in establishing an effective monitoring strategy for the detection of VBNC *V. parahaemolyticus*.

Acknowledgements This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF; Grant No. NRF-2016R1A6A3A 11932794) and Chung-Ang University Graduate Research Scholarship in 2020.

Compliance with ethical standards

Conflict of interest No conflict of interests was declared.

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