Stepwise changes in the viable but nonculturable *Vibrio cholerae* cells

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List of Abbreviations

CFU, colony-forming units; DMEM, Dulbecco's modified Eagle medium; *E. coli*, *Escherichia coli*; FBS, fetal bovine serum; FCVC, factor converting VBNC *V. cholerae*; MEM, Minimum Essential medium; PBS, phosphate-buffered saline; TCBS, thiosulfate citrate bile salts sucrose agar; *V. cholerae*, *Vibrio cholerae*; VBNC, viable but nonculturable.

ABSTRACT

Many bacterial species are known to become viable but nonculturable (VBNC)-state under conditions that are unsuitable for growth. In this study, we found that the requirements for resuscitation of VBNC-state *Vibrio cholerae* cells change over time. Initially, VBNC cells could be converted to culturable by treatment with catalase or HT-29 cell extract. Cells subsequently entered into a state that was not convertible to culturable by these factors. However, fluorescence microscopy revealed the presence of live cells under these conditions, and these VBNC-state cells were resuscitated by co-cultivation with HT-29 human colon adenocarcinoma cells. Ultimately, all cells entered into a state in which they could not be resuscitated even by co-cultivation with HT-29. These characteristic changes in VBNC-state cells were a common feature of strains in both *V. cholerae* O1 and O139 serogroups. Thus, we conclude that the VBNC state of *V. cholerae* is not a single property but continues to change over time.

Keywords: Resuscitation, VBNC, *Vibrio cholerae*

INTRODUCTION

Viable but nonculturable (VBNC) is defined as a state in which bacteria remain alive, but fail to grow, on standard bacteriological media that normally support their growth (1). Significance of the VBNC state is unclear and controversial. It could be an adaptive response favoring long-term survival under unsuitable condition for growth (2, 3) or the consequence of cellular damage, which maintains some features of viable cells (4-6). Ducret *et al*. named these two views of physiological significance of VBNC state as adaptive-VBNC or damaged-VBNC, respectively (7). Nevertheless, physiological significance of VBNC state-cells is not clear, it is observed in many bacterial species and also some yeasts (1, 8, 9). The VBNC state was first described in *Escherichia coli* and *Vibrio cholerae* more than 30 years ago (10) and has since been confirmed in wide variety of bacterial species, including a large number of human pathogens, such as enterohemorrhagic *E. coli*, *Helicobacter pylori*, *Mycobacterium tuberculosis*, *Salmonella enterica* serovar Enteritidis, and *Shigella* species (1, 11).

The VBNC state may represent a strategy utilized by wide variety of bacteria to survive under conditions that are unsuitable for growth. Therefore, bacteria in the VBNC state constitute an important reservoir of environmental pathogens (12), and as such are a threat to public health and food safety (13, 14). Several disease outbreaks have been reported in which VBNC-state bacterial cells were implicated as the causative agent (15). However, formation and resuscitation mechanisms of VBNC-state cells are poorly understood.

Since VBNC cells are alive, they are capable of subsequent propagation in certain condition. Resuscitation of *V. cholerae* VBNC cells was first achieved by inoculation of

VBNC cells into rabbit ileal loops (16). Subsequent experiments showed that VBNC *V. cholerae* cells could be resuscitated by ingestion by a human volunteer (17). Numerous investigations have explored the identity of resuscitation factors (18). For instance, a simple temperature upshift (19) and H_2O_2-degrading agents, such as pyruvate and catalase (1, 20) were found to resuscitate VBNC cells. Recently, Ayrapetyan *et al*. found that quorum sensing mediates the resuscitation of VBNC-state *V. vulnificus* cells (21). Senoh *et al*. reported the resuscitation of VBNC-state *V. cholerae* cells to a culturable state by an extract of eukaryotic cell lines including HT-29 (22, 23).

In this study, we found that the requirements for resuscitation of VBNC state in *V. cholerae* changes over time. At first, cells were convertible to culturable by addition of catalase or HT-29 extract (23), but this state was transient. Next, cells were not resuscitated by catalase or HT-29 extract, but were converted to culturable by co-cultivation with HT-29 human colon adenocarcinoma cells. Ultimately, all cells died or entered the other VBNC state, for which resuscitation factors are unknown. These observations suggest a more intricate properties of VBNC-state cells than was previously appreciated.

MATERIALS AND METHODS

Bacterial strains and culture media. *V. cholerae* N16961 (O1 serogroup) and *V. cholerae* VC-280/pG13 (O139 serogroup) (22) were used in this study. Alkaline peptone water (pH 8.8) was used for liquid cultivation. HT-29 cell extracts were prepared as described previously (23). Briefly, confluent HT-29 cells in 10-cm-diameter petri dish were collected and disrupted by shaking with 0.1-mm glass beads in phosphate-buffered saline (pH 7.0) (PBS). After centrifugation, the supernatant was passed through a 0.2-µm membrane filter, yielding approximately 0.5 ml of HT-29 extract. Where indicated, NA was supplemented with 1/10 volume of HT-29 extract, indicated concentrations of catalase from bovine liver (Sigma, C9322) and/or pyruvate. Thiosulfate citrate bile salts sucrose agar (TCBS) was used to grow resuscitated *V. cholerae*.

Measurement of colony-forming units (CFU). Nutrient Agar (NA; Difco, Franklin Lakes, NJ) supplemented with 1% NaCl was used for determining CFUs. *V. cholerae* cultures induced into the VBNC state were diluted with sterilized PBS if necessary. 100 µl cultures were spread on NA plates with or without supplementation and then incubated at 37 ºC for overnight.

Preparation of VBNC-state *V. cholerae.* A VBNC state was induced in *V. cholerae* N16961 and VC-280/pG13 cells as described previously (22). Briefly, *V. cholerae* were inoculated into alkaline peptone water and incubated at 37ºC for 16 hours. Cells were washed with a sterile solution of 1% artificial seawater (Instant Ocean [IO]; Aquarium Systems, Mentor, OH), suspended in 200 ml 1% IO in a 1-liter flask to give a final concentration of approximately 10^8 cells/ml, and then incubated at 4ºC in the dark without shaking.

LIVE/DEAD staining and fluorescence microscopy. Following induction of a VBNC state, 1-ml aliquots of *V. cholerae* N16961 cells were washed with sterile saline solution (0.9%), and pellets were resusupended in 1 ml of saline solution. LIVE/DEAD BacLight dye (3 µl), a 1:1 mixture of SYTO 9 and propidium iodide, was added and incubated at room temperature in the dark for 15 minutes. Thereafter, cells were transferred to microscope slides. The fluorescence of SYTO 9 and propidium iodide were observed using an EVOS FL fluorescence microscope with a Plan Fluor 40× objective lens. Images of SYTO 9 and propidium iodide fluorescence were captured using exposure times of 1 and 1.5 seconds, respectively.

Incubation and co-cultivation of HT-29 cells. HT-29 cells were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco) supplemented with 1.5 g/l $NaHCO_3$ (Sigma), 3.56 g/l 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Sigma), 10% fetal bovine serum (FBS; Gibco), 100 µg/ml streptomycin, and 100 U/ml penicillin (Pen Strep; Gibco) at 37ºC in 5% CO_2. For co-cultivation with VBNC *V. cholerae* cells, confluent HT-29 cells in a 10-cm-diameter petri dish was washed twice with 5 ml PBS and 10 ml Minimum Essential medium (MEM; Gibco Life Science) supplemented with 10% FBS and 100 U/ml catalase without antibiotics was added. 100 µl of VBNC culture was inoculated into the medium. 100 µl of VBNC culture was also inoculated into same medium without HT-29 cells as a negative control. Then incubated at 37ºC in 5% CO_2. The cultures were observed and streaked on TCBS plate by inoculation loop every day to check the resuscitation of VBNC *V. cholerae*.

RESULTS

Persistence of VBNC-state *V. cholerae* cells. We first addressed how long VBNC cells are capable of persisting in a state that is convertible to culturable by converting factors. We induced the VBNC state in *V. cholerae* N16961 (O1 serogroup) by keeping cells in artificial seawater at 4ºC, as described previously (22), and monitored the persistence of cells by measuring CFUs (Table 1, left). Colonies were formed until 10 weeks after induction of the VBNC state, but no culturable cells were detected after 11 weeks (Table 1, left). However, addition of catalase supported colony formation until 13 weeks indicating that catalase resuscitated the VBNC state cells.

In addition, time course changes of CFUs with and without catalase were shown in Fig. S1 in the supporting information. CFUs started to decrease just after the induction into VBNC state (Fig. S1) suggesting that cells in the population were not coordinated and progresses to become VBNC cell is highly diverse. Thus some cells became VBNC just after the induction but some cells remained culturable for 10 weeks. However, all cells became non-culturable after 11 weeks and catalase resuscitated the VBNC cells at all time points until 13 weeks (Fig. S1 and Table 1).

In order to determine the efficiencies of resuscitation by anti-reactive oxygen species, CFU of VBNC culture at 5 weeks was determined with various concentrations of catalase and pyruvate (Table S1). Middle point of VBNC formation (5 weeks) instead of 11 weeks was subjected to the measurements to compare CFUs with and without anti-reactive oxygen species, because colonies are not detected after 11 weeks without resuscitation factor (Table 1). Both catalase and pyruvate resuscitated the VBNC cells and increased CFU, consistent with previous work (20, 24). Increased concentration of catalase from

0.01 U/ml to 100 U/ml or pyruvate from 0.001 % to 0.1 % had greater CFUs. However, 100 U/ml and 1000 U/ml catalase and 0.1% pyruvate had equal level of resuscitation activity of VBNC cells. Therefore 100 U/ml catalase was used as excess amount of anti-reactive oxygen species in this study.

Similar results were obtained by addition of HT-29 extract (Table 1). These results indicate that HT-29 extract is able to resuscitate VBNC *V. cholerae*, consistent with previous reports (23). Supplementation of HT-29 extract in addition to catalase did not increase CFU, suggesting that 100 U/ml catalase is sufficient for resuscitation of convertible VBNC cells to culturable by these factors (Supplemental Table S1). However, no colonies were formed even with these resuscitation factors 14 weeks after inducing the VBNC state (Table 1). These results indicate that the convertibility of the VBNC state to culturable by catalase or HT-29 extract is transient.

To address whether these cells had died or entered into a different type of VBNC state that is not convertible to a culturable state by these factors, we first determined the viability of VBNC-state cells using a LIVE/DEAD BacLight Kit (Invitrogen), which stains living cells (intact membrane) green and dead cells (defective membrane) red. A substantial percentage of cells at 11 weeks (39.7%) stained green (Fig. S2a, arrows). Since lysed cells are not observed in this method, actual ratio of living cells compared to the initial population should be lower than this percentage. In addition, some green cells may not be VBNC state and might be damaged cells before dying. However, these results suggest that at least this culture contains some living cells consistent with the fact that this time is within the period in which the VBNC state is convertible to culturable by catalase or HT-29 extract (Table 1). At 15 and 19 weeks, when cells could not be resuscitated by catalase or HT-29 extract (Table 1), 4.0% and 8.4% of cells, respectively, stained green,

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indicating that some cells in these cultures were still alive (Fig. S2b and c). Even at 23 weeks, a small percentage of cells (1.6%) remained alive (Fig. S2d). These results indicate that after 14 weeks, cells have not completely died out, suggesting that some cells have entered into a type of VBNC state that cannot be converted to culturable by catalase or HT-29 extract. To distinguish the two kinds of VBNC state in this study, we referred to hereafter the VBNC state that is convertible to culturable by catalase or HT-29 extract as first phase and the state that is inconvertible using these factors as second phase. Thus, first phase cells are convertible to culturable, whereas second phase cells are inconvertible but can be detected as alive under a fluorescence microscope.

Co-cultivation of second phase VBNC cells with HT-29 cells. If a population of second phase VBNC cells indeed remains alive, as suggested in Figure S2, they may resume growth when they sense a signal of entry into the human intestine. Therefore, we next co-cultivated second phase VBNC cells and HT-29 human colon adenocarcinoma cells. Since HT-29 cells produce catalase, the medium was supplemented with excess amount of catalase (100 U/ml) to distinguish resuscitation of first phase VBNC cells by HT-29 cells from that produced by catalase. Second phase, 14-week VBNC cells did not grow even after incubating for 2 weeks in MEM medium with excess amount of catalase without HT-29 cells. This result indicates that the culture conditions such as catalase, MEM medium or temperature upshift did not resuscitate VBNC cells consistent with the results in Table 1. However, under co-culture conditions, the media became turbid and HT-29 cells detached from the bottom of the dish after incubating for 4 days (data not shown). These cultures were streaked onto plates of TCBS selective medium to determine whether the growing bacterium represented a contamination or resuscitated *V. cholerae* N16961. Typical *V. cholerae*-like yellow colonies formed from the co-culture, but not from the culture lacking HT-29 cells (Fig. S3 panel a in Supporting information). This result was further confirmed by PCR amplification of the *ctxA* gene, a gene for cholera toxin and specific for *V. cholerae* that cause cholera (25). Genomic DNA from single colony of resuscitated *V. cholerae* on the TCBS plate was purified and used as a template for PCR. As shown in Fig. S3 (panel b, lane 4), the *ctxA* gene was present, indicating that the bacterium is not a contamination introduced during the experiment but is resuscitated second phase VBNC *V. cholerae*. These same experiments were repeated at different time points. Resuscitation was observed from 14 to 21 weeks (supplemental table S2, left). In all cases, 3 to 5 days of co-cultivation was required for resuscitation. These results demonstrate that second phase VBNC cells are viable and are convertible to culturable by co-cultivation for up to 5 days with HT-29 cells. However, second phase VBNC also appears to be a transient phase because attempts to resuscitate failed after 22 weeks (supplemental table S2, left). Taken together, these results suggest that *V. cholerae* N16961 transitions through multiple VBNC phases over time, as summarized in Figure S4. *V. cholerae* N16961 was culturable for up to 10 weeks under our experimental conditions. Immediately after induction of the VBNC state, cells were convertible to culturable by catalase or HT-29 extract (first phase). Next, VBNC cells become inconvertible to culturable by catalase or HT-29 extract, but could be resuscitated by co-cultivation with the HT-29 cell line (second phase). Eventually, all cells either died or entered into a VBNC phase that was inconvertible to culturable by these factors (third phase).

VBNC phases of *V. cholerae* O139. It is conceivable that the observed time-course of changes in the VBNC state is a specific behavior of the *V. cholerae* N16961 (O1 serogroup) strain. To determine whether the different phases of the VBNC state are common among *V. cholerae* strains that cause cholera, we carried out the same experiments using *V. cholerae* VC-280/pG13 (O139 serogroup) (26). VBNC formation progressed faster for *V. cholerae* VC-280/pG13 than *V. cholerae* N16961 (Table 1, right). *V. cholerae* VC-280/pG13 did not form colonies 8 weeks after induction of VBNC, but many colonies still formed following addition of catalase or HT-29 extract. However, no colonies were detected after 10 weeks, even in the presence of catalase or HT-29 extract (Table 1, right). Therefore, we tested whether the VBNC cells that could not be resuscitated by catalase (second phase VBNC) were convertible to culturable by co-cultivation with HT-29 cells. Second phase VBNC cells at 15 and 16 weeks resumed growth upon co-cultivation with HT-29 cells (Table S2, right). Using PCR amplification of *ctxA* gene, we confirmed that these strains were resuscitated VBNC *V. cholerae* VC-280/pG13 and not a contamination (Fig. S3 panel b in supporting information). However, VBNC cells were not resuscitated after 17 weeks even by co-cultivation with the HT-29 cell line (Table S2, right). These stepwise changes of VBNC cells are the same to those observed in *V. cholerae* N16961.

DISCUSSION

The results of this study suggest that *V. cholerae* cells transition through multiple VBNC phases over time (Fig. S4). These changes are characterized by the following features: 1) All *V. cholerae* cells become nonculturable from 8 to 11 weeks after induction of the VBNC state under the conditions used in this study; 2) the early phase of VBNC (first phase) is convertible to culturable by catalase or HT-29 extract, but this phase is transient; 3) VBNC cells that were inconvertible to culturable by catalase or HT-29 extract are resuscitated by co-cultivation with HT-29 cells (second phase); and 4) cells eventually die or enter into a VBNC state that is inconvertible to culturable even by co-cultivation with HT-29 (third phase). Thus, conversion factors for a culturable state were demonstrated for first and second phases, but no factors have yet been found for third phase (Fig. S4). These behaviors were common features, at least in two strains of *V. cholerae* in O1 and O139 serogroups that cause cholera. In this study, we examined two strains of *V. cholerae* and one of the strains *V. cholerae* VC-280/pG13 is a genetically engineered strain to check morphology of cells (22). Therefore, examination with more strains including other strains without genetic engineering in same serogroups such as clinically isolated strains are necessary to conclude whether it is a common behavior among *V. cholerae* strains causing cholera. It will be important to determine how wide a range of bacteria has these features; it might be specific for *V. cholerae* or it could common among most VBNC-state bacterial cells. If it were a common characteristic of enteric bacteria, it could represent a strategy for resuscitation in the intestinal tract and not in other environments.

Second phase VBNC cells could be resuscitated by co-cultivation with HT-29 cells

(Table S2). This observation support the hypothesis that *V. cholerae* persist in a VBNC state in the environment and resume growth upon entry into the human intestine to cause cholera. Because resuscitation of second phase VBNC cells by co-cultivation with HT-29 cells required 3 to 5 days in all cases (Table S2), it process seems to be very slow. The resuscitation by co-cultivation may involve complex interactions between VBNC cells and HT-29 cells. Determining what kinds of mammalian cell lines are capable of resuscitating second phase VBNC cells may shed light on resuscitation mechanisms. Our co-culture experiment is not able to measure frequency of resuscitation of VBNC cells because once a cell restarted propagation, population increases rapidly and thus resuscitation of other VBNC cells is not detected. Comprehensive observation of individual VBNC cells or enormous experiment with statistical analysis is necessary to study in more detail.

The second phase of VBNC state was also transient; eventually all cells died or entered into the third phase VBNC state (Fig. S4). Although live cell counts of third phase VBNC cultures were significantly lower than those of first and second phase VBNC state cultures, a few cells appeared green under a fluorescence microscope with the LIVE/DEAD stain (Fig. S2). Bunker and co-workers reported that *Pseudomonas fluorescens* cells remained in a VBNC state in soil for more than a year (27). These observations suggest the possibility that VBNC-state cells are capable of persisting for at least several months without nutrition. Colwell and co-workers reported that VBNC-state *V. cholerae* revert to a cultivable state in the human intestine (17). Third phase VBNC *V. cholerae* cells might also be resuscitated in the human intestine. If third phase VBNC cells of *V. cholerae* are alive and capable of being resuscitated by unknown factors, they may be involved in the transmission of cholera through environmental water in particular areas, including Kolkata, India, where there is a long history of regular cholera outbreaks.

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DISCLOSURE

All authors declare that there are no conflicts of interest.

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Supporting information

Table S1. CFUs with various concentrations of resuscitation factors.

CFUs of 5 weeks VBNC *V. cholerae* N16961 were measured as described in Materials and Methods. Diluted cultures were spread on NA plates with indicated concentrations of supplementation. Representative results of three experiments were presented.

Fig. S1. Time course CFUs with and without catalase.

V. cholerae N16961 was induced into VBNC and CFUs were measured as described in the Materials and Methods. Cultures were plated on NA plates with (closed circle) or without (open circle) 100 U/ml catalase. The average of three independent experiments were shown. Error bars indicate standard deviation.

Fig. S2. LIVE/DEAD-stained VBNC-state *V. cholerae*.

V. cholerae N16961 cells at 11 (a), 15 (b), 19 (c), and 23 (d) weeks after induction of the VBNC state were stained with a LIVE/DEAD BacLight bacterial viability kit (Invitrogen) and observed by fluorescence microscopy. Representative results of three independent experiments were shown. Arrows indicate instances of live (green) cells. Scale bars = 20 μm.

Fig. S3. Confirmation of resuscitated VBNC-state *V. cholerae*.

a; Cultures of 14-week-old, VBNC-state *V. cholerae* cultured with (right) or without (left) HT-29 cells were streaked onto TCBS plates and incubated at 37ºC overnight.

b; An internal fragment of the *ctxA* gene was amplified by PCR using genomic DNA from the following as a template: *V. cholerae* O1 N16961 (lane 2); *V. vulnificus* (lane 3); resuscitated VBNC *V. cholerae* N16961 at 14 (lane 4), 15 (lane 5), 17 (lane 6), 19 (lane 7) or 21 (lane 8) weeks; resuscitated VBNC *V. cholerae* VC-280/pG13 at 15 (lane 9) and 16 (lane 10) weeks. Lane 1: molecular weight marker.

Table S2. Resuscitation of VBNC *V. cholerae* by co-cultivation with HT-29.

Representative results of three co-cultivation experiments were presented. In all cases, incubation of VBNC cells in same condition without HT-29 cell exhibited no growth. Growth of resuscitated VBNC *V. cholerae* were observed (+) after indicated days or not observed (-) after 14 days.

Fig. S4. Schematic representation of multiple VBNC phases in *V. cholerae* N16961. Numbers on the left side of the arrow indicate weeks after induction of the VBNC state. * and × indicate resuscitation and failure of resuscitation, respectively, following co-cultivation with the HT-29 cell line (see also Table S2 left). Factors supporting conversion to a culturable state at each phase are indicated in parenthesis.

Table 1. *V. cholerae* CFUs with/without resuscitation factors at different time points.

Weeks	*V. cholerae* N16961			*V. cholerae* VC-280/pG13		
	Free	Catalase	HT-29 extract	Free	Catalase	HT-29 extract
4				>3000	>3000	
6	>3000	>3000		512	>3000	
7				1	>3000	
8	233	>3000		0	2632	233
9				0	72	
10	1	2440	388	0	0	0
11	0	1124				
12	0	141	20			
13	0	1				
14	0	0	0			

Representative results of three experiments were presented in this table.

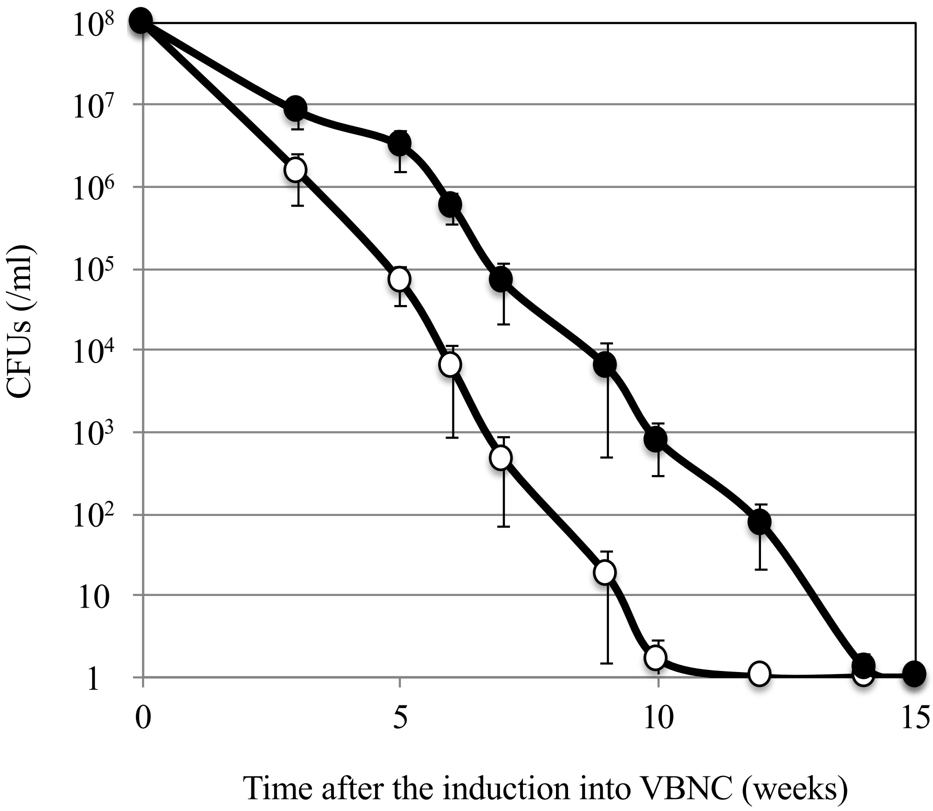

Fig. S1. Time course CFUs with and without catalase.

V. cholerae N16961 was induced into VBNC and CFUs were measured as described in the Materials and Methods. Cultures were plated on NA plates with (closed circle) or without (open circle) 100 U/ml catalase. The average of three independent experiments were shown. Error bars indicate standard deviation.

Table S1. CFUs with various concentrations of resuscitation factors.

Supplement	Concentration	CFU (/ml)
Free		5.0×10^4
Catalase	1000 U/ml	1.5×10^6
Catalase	100 U/ml	1.8×10^6
Catalase	10 U/ml	1.8×10^5
Catalase	1 U/ml	8.2×10^4
Catalase	0.1 U/ml	6.1×10^4
Catalase	0.01 U/ml	2.0×10^4
Pyruvate	0.1%	1.8×10^6
Pyruvate	0.01%	3.2×10^5
Pyruvate	0.001%	7.6×10^4
HT-29 extract	10%	2.2×10^5
Catalase+HT-29 extract	100 U/ml+10%	1.7×10^6

CFUs of 5 weeks VBNC *V. cholerae* N16961 were measured as described in Materials and Methods. Diluted cultures were spread on NA plates with indicated concentrations of supplementation. Representative results of three experiments were presented.

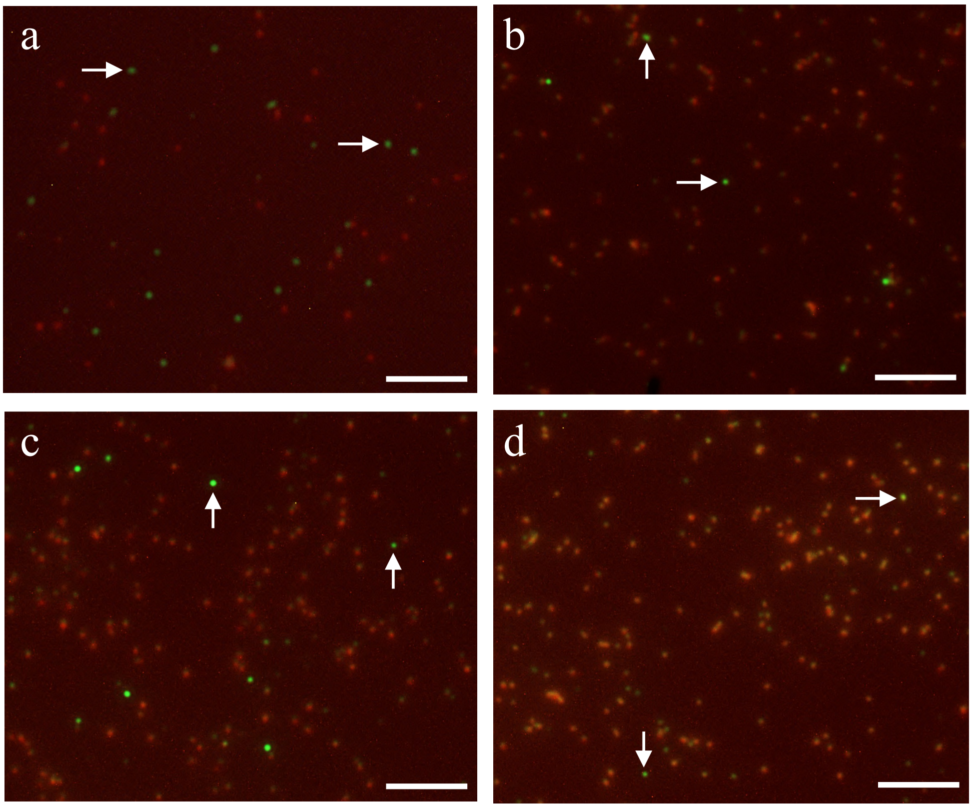

Fig. S2. LIVE/DEAD-stained VBNC-state *V. cholerae*.

V. cholerae N16961 cells at 11 (a), 15 (b), 19 (c), and 23 (d) weeks after induction of the VBNC state were stained with a LIVE/DEAD BacLight bacterial viability kit (Invitrogen) and observed by fluorescence microscopy. Representative results of three independent experiments were presented. Arrows indicate instances of live (green) cells. Scale bars = 20 μm.

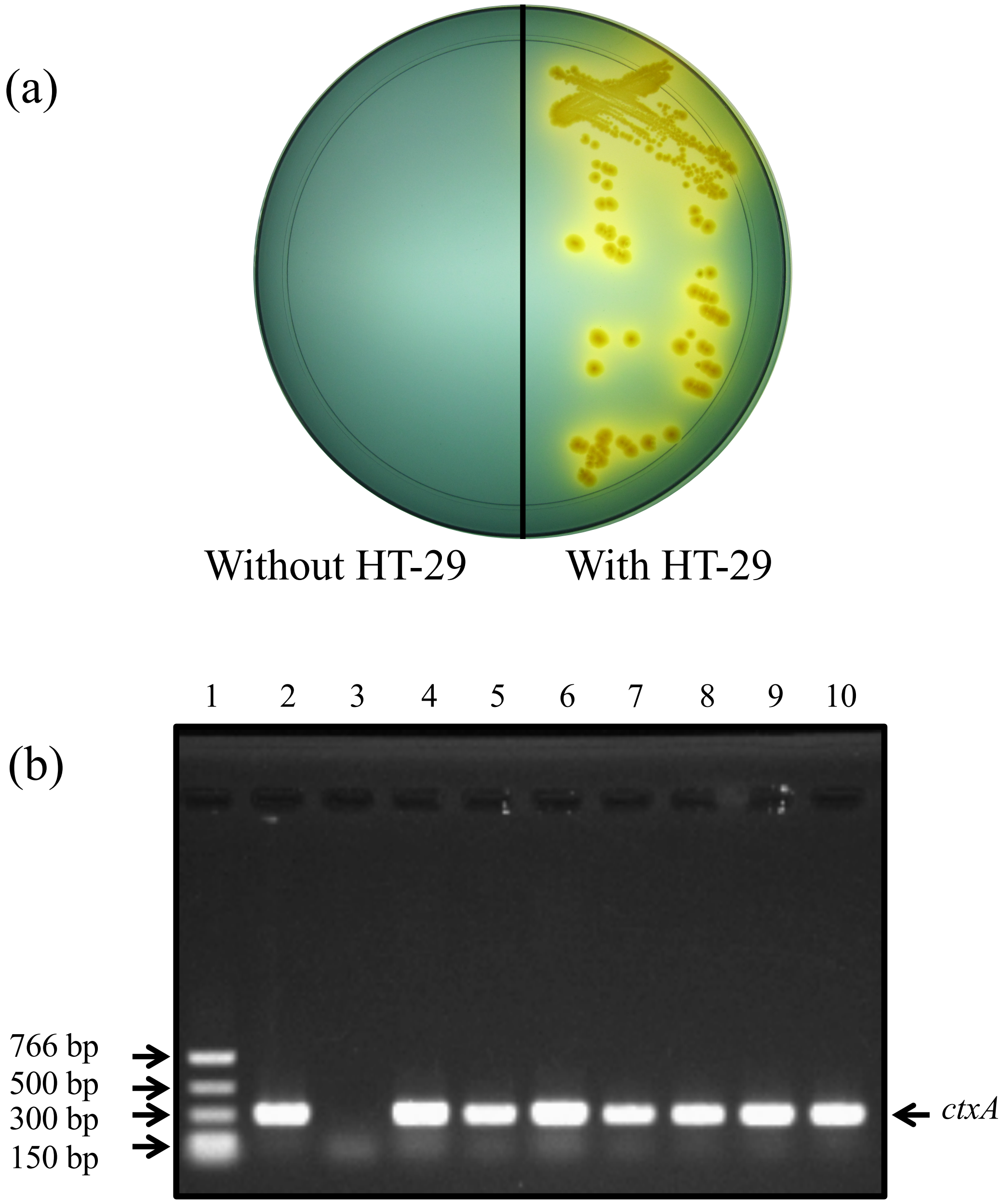

Fig. S3. Confirmation of resuscitated VBNC-state *V. cholerae*.

a; Cultures of 14-week-old, VBNC-state *V. cholerae* cultured with (right) or without (left) HT-29 cells were streaked onto TCBS plates and incubated at 37ºC overnight.

b; An internal fragment of the *ctxA* gene was amplified by PCR using genomic DNA from the following as a template: *V. cholerae* O1 N16961 (lane 2); *V. vulnificus* (lane 3); resuscitated VBNC *V. cholerae* N16961 at 14 (lane 4), 15 (lane 5), 17 (lane 6), 19 (lane 7) or 21 (lane 8) weeks; resuscitated VBNC *V. cholerae* VC-280/pG13 at 15 (lane 9) and 16 (lane 10) weeks. Lane 1: molecular weight marker.

Table S2. Resuscitation of VBNC *V. cholerae* by co-cultivation with HT-29

Weeks	*V. cholerae* N16961		*V. cholerae* VC-280/pG13	
	Growth	Days to grow	Growth	Days to grow
14	+	4		
15	+	5	+	3
16			+	3
17	+	4	-	
19	+	3	-	
21	+	5	-	
22	-			
23	-			
25	-			

Representative results of three co-cultivation experiments were presented. In all cases, incubation of VBNC cells in same condition without HT-29 cell exhibited no growth. Growth of resuscitated VBNC *V. cholerae* were observed (+) after indicated days or not observed (-) after 14 days.

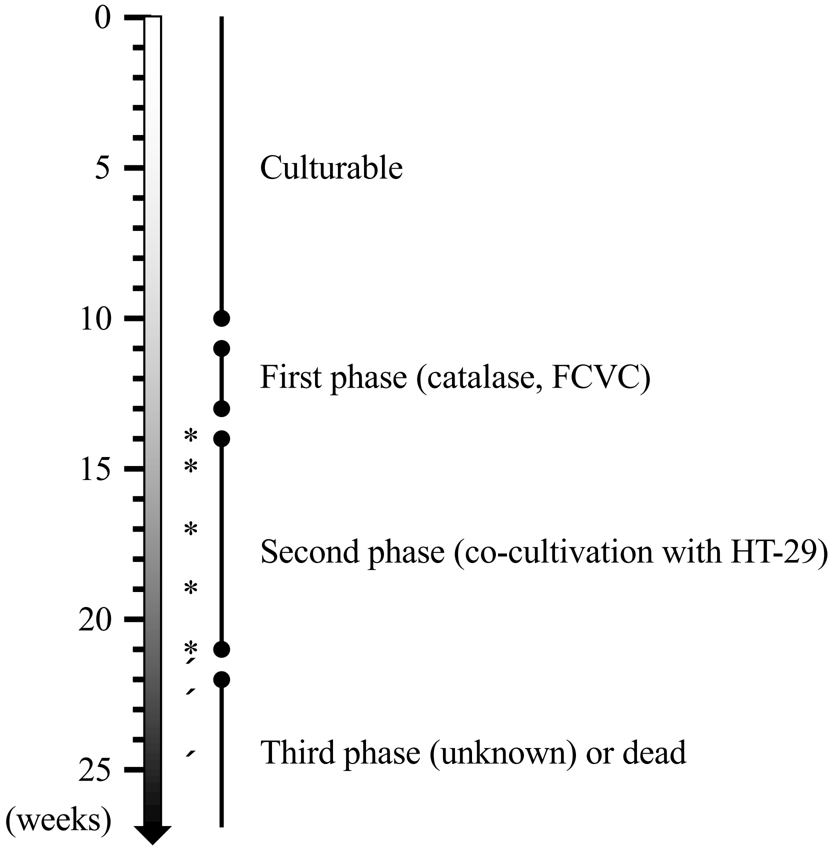

Fig. S4. Schematic representation of multiple VBNC phases in *V. cholerae* N16961.

Numbers on the left side of the arrow indicate weeks after induction of the VBNC state. * and ´ indicate resuscitation and failure of resuscitation, respectively, following co-cultivation with the HT-29 cell line (see also Table S2). Factors supporting conversion to a culturable state at each phase are indicated in parenthesis.