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Survival of *Vibrio parahaemolyticus* at Low Temperatures under Starvation Conditions and Subsequent Resuscitation of Viable, Nonculturable Cells

XIUPING JIANG AND TUU-JYI CHAI*

Seafood Science Program, Horn Point Environmental Laboratory, Center for Environmental and Estuarine Studies, The University of Maryland System, Cambridge, Maryland 21613

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Morphological changes of *Vibrio parahaemolyticus* from rods to spheres took place after a culture was subjected to starvation at a wide range of temperatures. Scanning electron micrographs revealed that starved spherical cells gradually developed a rippled cell surface with blebs and an extracellular filamentous substance adhesive to the cell surface. Cells starved at a low temperature for certain intervals were counted by various bacterial enumeration methods, including plate count, direct viable count, and total cell count for both Kanagawa-positive and -negative strains. The results indicated that this species could reach the nonculturable stage in 50 to ~80 days during starvation at 3.5°C. Kanagawa-negative strain 38C6 lost culturability more slowly than Kanagawa-positive strain 38C1 at low temperature. As detected by thiosulfate-citrate-bile salts-sucrose plate count, a high percentage of the surviving cells at 3.5°C in starvation medium were possibly injured by the low temperature rather than by starvation. Both addition of nalidixic acid to the starved cultures and the most-probable-number method demonstrated that the cells recovered after a temperature upshift probably represented the regrowth of a few surviving cells. These surviving cells were capable of growth and multiplication with limited nutrients at an extraordinary rate when the temperature was upshifted.

Nutrient insufficiency is the most common environmental stress which microorganisms routinely encounter in natural ecosystems. As nature exerts strong selective pressures in the course of evolution, nondifferentiating bacteria can adapt to nutrient starvation by a variety of genotypic and phenotypic mechanisms (2, 7, 11, 23, 34). Many marine bacteria, especially *Vibrio* spp., can survive for a long time during starvation by sequential changes in cell physiology and gradual changes in morphology (1, 9, 13, 17, 20, 23, 25, 28). However, some pathogenic vibrios, such as *Vibrio cholerae* and *V. vulnificus*, along with other human pathogens, including *Escherichia coli*, *Salmonella enteritidis*, *Shigella sonnei*, *Aeromonas salmonicida*, and *Campylobacter jejuni*, have been found to enter into a viable but nonculturable (VNC) stage rather than die when exposed to a low-nutrient environment (8, 19, 22, 24, 27, 33, 35, 39). A comprehensive review of this subject has been made by Oliver (26).

V. parahaemolyticus, an estuarine bacterium widely distributed in natural aquatic environments around the world, is a well-known food-borne pathogen causing gastrointestinal disease (4, 6). While over 95% of the strains of *V. parahaemolyticus* from clinical samples are Kanagawa positive (K^+), producing hemolysis on Wagatsuma blood agar, the majority of isolates from environmental samples are Kanagawa negative (K^-) (21). Like those of both *V. cholerae* and *V. vulnificus*, the frequency and level of this bacterium are much lower during winter than summer months (5, 6).

The stresses of nutrient deprivation and low temperature have been shown to be the main causes of the induction of VNC stages in some pathogenic bacteria (7, 8, 19, 24, 26, 29, 37–39). Therefore, it is important to understand how *V. parahaemolyticus* survives under these stresses. In this work, both K^+ and K^- strains were studied with regard to cell morphological changes, survival profiles, and cell enumeration characteristics. The possible resuscitation of nonculturable cells during temperature upshift was studied.

MATERIALS AND METHODS

Cultures and media. Both pathogenic strain 38C1 (K^+) and nonpathogenic strain 38C6 (K^-) of *V. parahaemolyticus* were used in this study. Overnight cultures were inoculated 1 to 1,000 into fresh Proteose Peptone-Beef Extract (PPBE; Difco Laboratories, Detroit, Mich.) broth containing 1% NaCl (3). The cultures were grown to the mid-log phase (optical density at 660 nm of 0.5) at 35°C with shaking and harvested by centrifugation at 5,000 × *g* for 15 min. The pellets were washed once with starvation medium. The final pellets were suspended uniformly in starvation medium.

The starvation medium, modified Morita mineral salts solution (MMS) (25), was composed of 26.0 g of NaCl, 0.8 g of KCl, 5.6 g of $MgCl_2 \cdot 6H_2O$, 7.6 g of $MgSO_4 \cdot 7H_2O$, 0.0005 g of $FeSO_4$, 1.54 g of $CaCl_2 \cdot 2H_2O$, 0.1 g of Na_2HPO_4, and 1.21 g of Tris buffer (pH 7.8) per liter of deionized water.

Acid-washed 1-liter screw-cap bottles contained the microcosm used for the experiments. Cultures in MMS were maintained at both 3.5°C and room temperature in a static state.

Cell enumeration. Cell culturability was determined by using both nonselective PPBE agar plates and highly selective thiosulfate-citrate-bile salts-sucrose (TCBS) agar plates. The starvation medium, MMS, was used to dilute microcosms. During sampling, all samples exposed to low temperatures were kept in ice to avoid any temperature increase. The plates were incubated at 35°C for 2 days.

Total cell counts were determined by the acridine orange staining method under an epifluorescence microscope. Direct viable counts (DVC) were determined as described by Kogure et al. (18). Briefly, samples were incubated at 35°C with 0.05% filtered sterile yeast extract and 0.002% nalidixic acid (NA) for 6 h. Only elongated and swollen cells were counted as viable cells. For determination of total cell counts and DVC, a total of at least 10 fields in the range of 30 to 300 cells were counted for each sample.

Temperature upshift. A 5-ml total culture volume was aseptically removed from the entire microcosm at low temperature and placed in a 10-ml test tube. The tube was left at room temperature for 3 days without shaking. The temperature-upshifted cultures were diluted with fresh MMS and spread onto PPBE plates. Most-probable-number (MPN) determination was performed by the three-tube series test, with each tube containing 10 ml of PPBE broth. Prior to room temperature upshift (RTU), 1 ml of the microcosm was aseptically diluted with PPBE broth and placed in MPN tubes.

* Corresponding author. Phone: (410) 228-8200. Fax: (410) 221-8490.

Scanning electron microscopy. Both log-phase young cells in PPBE broth and cells exposed to 3.5°C at various intervals in starvation medium were harvested by centrifugation. The cell pellets were washed three times with sterile Millonig buffer (374 mosM/kg), pH 7.4 (12). Bacterial cells were fixed with 2% (vol/vol) glutaraldehyde in Millonig buffer at room temperature for 1 h. The samples were passed through 13-mm-diameter, 0.2-μm-pore-size Nuclepore polycarbonate filters and then postfixed in 1% osmium tetroxide at room temperature for 1 h, dehydrated in a graded ethanol series (75, 95, and 100%), critical point dried in CO_2 in a DCP1-CPD apparatus, and coated with AuPd in a Denton DV503 vacuum evaporator coating apparatus. The coated samples were examined in an Amray 1820D scanning electron microscope.

RESULTS AND DISCUSSION

Cell morphology. Morphological changes occurred after the cells were suspended in starvation medium. Before starvation, log-phase cells of *V. parahaemolyticus* were normally rod shaped with average dimensions of ca. 0.6 to ~0.9 by 1.6 to ~3.1 μm. Within a week in starvation medium, rod-shaped cells appeared as irregular shapes and finally became coccoid or spheroid forms. The rate of cell morphological changes was dependent on the strains and the temperatures at which the cells were maintained. Cells became spheroid quicker when they were maintained at a lower temperature, and spheroid cells were larger than those at room temperature (25°C). It took about 1 to 2 weeks for strain 38C1 (K^+) to become spheroid at 3.5°C, whereas strain 38C6 (K^-) needed at least 3 weeks under the same conditions. Spheroid cells of both strains had an average diameter of 0.83 to ~1.28 μm at 3.5°C, compared with an average diameter of 0.6 to ~0.7 μm at 25°C. Most microorganisms in soil and aquatic ecosystems are between 0.4 and 0.8 μm in diameter (23). *V. vulnificus* cells were reduced to 0.8 to ~1.0 μm in diameter during the VNC stage (27). It has been proposed that cell size reduction during starvation is a survival strategy for minimizing cell maintenance requirements and enhancing substrate uptake due to a high surface-to-volume ratio (23, 34).

Scanning electron micrographs (Fig. 1A and B) showed that young cells were typically rod like with a single polar flagellum and a relatively smooth surface, and cells starved for 1 week were predominately spheroid without a flagellum. Interestingly, many polymer-like filaments formed in association with cells starved for 1 week. It was reported that *Vibrio* sp. strain DW1 cells formed a bridging polymer after 5 h of starvation in MMS (9). These polymerlike filaments did not look like collapsed flagella and actually appeared to be thinner than flagella with certain orientation patterns. Some investigators suggested that these polymers may be exopolysaccharides dehydrated by ethanol during the scanning electron microscopy preparation process (10, 31). We are currently investigating the functions of these polymers related to cell adhesion. Another important feature of starved cells was the formation of blebs, which appeared in cells starved for 1 week but increased as cells were starved longer at low temperatures (Fig. 1B and C). Similar membrane vesicles were observed in other marine organisms, such as *Vibrio* sp. strain Ant-300 and *V. cholerae*, during the early stage of starvation (13, 20). The blebs could be formed by pieces of cell envelope. After cell size reduction during starvation, excess pieces of cell envelope material may be formed and, in fact, may allow cell volume adjustment via bleb formation. Cells starved for 6 weeks did not appear as smooth spheroid shapes (Fig. 1C). Despite a majority of completely intact cells, some cell envelopes appeared to have collapsed and adhered together inwardly. Thin-section micrographs of *V. cholerae* showed that some parts of the outer membrane were separated and a gap was formed between it and the inner membrane (2, 13). This was supported by scanning electron micrographs of *V. parahaemolyticus* in this study (Fig. 1C). In the study of Baker et al., those cells with a detached cell membrane were viable and responded to nutrient addition as quickly as did young healthy cells (2).

Survival curves during starvation. Figure 2 shows typical survival curves for both strains 38C1 and 38C6 (mid-log-phase cells) when the cultures were maintained at 3.5°C in MMS. The survival curves fell into three stages. At the beginning of starvation, cells lost culturability rapidly within 2 weeks, which corresponded to the stage of spheroid cell formation. This was followed by a slow decline in culturability and then a drop of plate counts to less than 1 cell per ml. The nonculturability tested on agar plates was further confirmed by failure to grow in PPBE broth.

Several reports have discussed methods for recovery of VNC cells. *Legionella pneumophila*, *V. cholerae*, *E. coli*, and *Campylobacter* spp. (8, 14, 15, 33) have been recovered only through animal passage when they failed to form colonies on agar plates or to gain turbidity in rich- or diluted-nutrient MPN tubes. For *V. vulnificus*, which became VNC at low temperature, resuscitation at room temperature for 3 days led to recovery of almost all of the VNC cells (24). In our study, following an RTU for 3 days from the same sampling points as shown in the survival curves in Fig. 2, both 38C1 and 38C6 cultures starved at 3.5°C could be resuscitated to up to 10 to ~100% of the original inoculum size. After full regrowth at room temperature, both rod-shaped and spheroid cells were observed under a microscope. Within 24 h of RTU, some rod-shaped cells were seen in divided forms; however, after 24 h no cell division was detected. Under a microscope, the total cell counts were almost constant during RTU. The plate counts after RTU remained relatively constant throughout the entire experiment. However, when PPBE plate counts dropped to less than 1 cell per ml, the RTU process failed to allow recovery of those nonculturable cells immediately for strain 38C1 or thereafter for strain 38C6 (Fig. 2). Eight other K^+ and K^- strains were tested with similar results (data not shown). The repeated experiments revealed consistent failure of RTU resuscitation but some slight variation at the onset of nonculturability at 3.5°C. In general, no culturable cells were detected after RTU when cells of strain 38C1 were starved for more than 50 days (3.5×10^7 cells per ml [plate count after RTU for cells starved for 42 days]) and when strain 38C6 cells were starved for 80 days (2.1×10^7 cells per ml [plate count after RTU for cells starved for 70 days]) at 3.5°C in MMS (Fig. 2). Wolf and Oliver (38) did not find the nonculturable stage of *V. parahaemolyticus* during starvation at 5°C within 30 days. As a matter of fact, in our study this species reached the nonculturable stage very quickly only after 30 to 40 days in the microcosm.

The selective medium, TCBS, was compared with the nonselective medium, PPBE, for enumeration of culturable cells after storage in MMS at both 3.5°C and room temperature (Fig. 3). The difference between TCBS and PPBE counts of strain 38C1 was about 1 log at the initial time point, but thereafter, with increased time in MMS at 3.5°C, the difference became greater. When the TCBS count was below 10 cells per ml, the PPBE plate count was still 10^6 cells per ml. A similar trend was observed for strain 38C6, except that it survived slightly longer, as measured by both types of plate counts (data not shown). Theoretically, the plate count difference between nonselective and selective media represents the number of injured cells which have been exposed to a sublethal physical or chemical environment, such as heat, refrigeration, freezing, and so on (32). At room temperature, it appeared that starvation did not cause cell injury since the difference between PPBE and TCBS counts was constant regardless of how long the cells were starved. In contrast, more cells were injured at

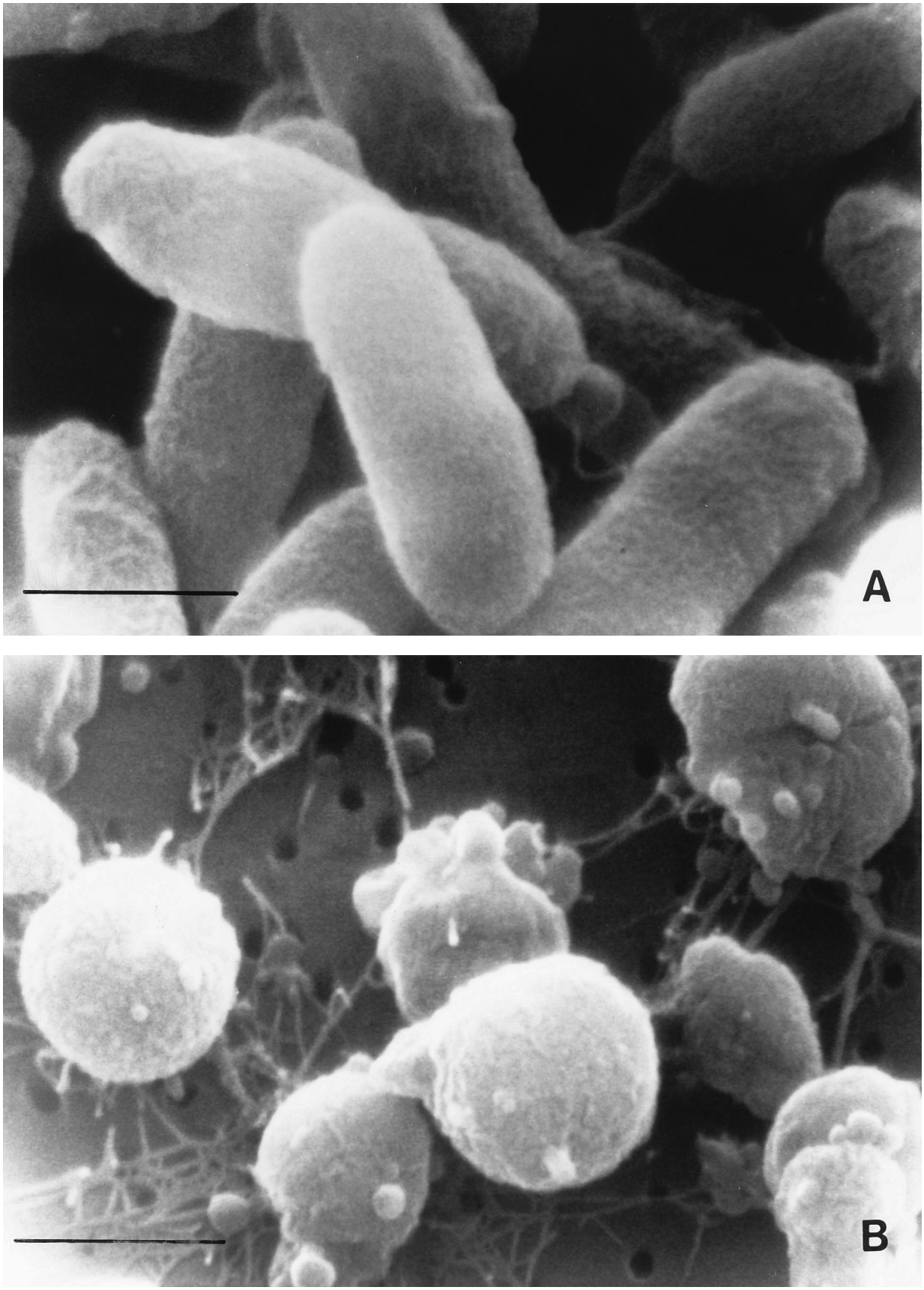

FIG. 1. Scanning electron micrographs of *V. parahaemolyticus* 38C1. A, log-phase young cells; B, cells starved for 1 week at 3.5°C; C, cells starved for 6 weeks at 3.5°C. Bar, 1 μm.

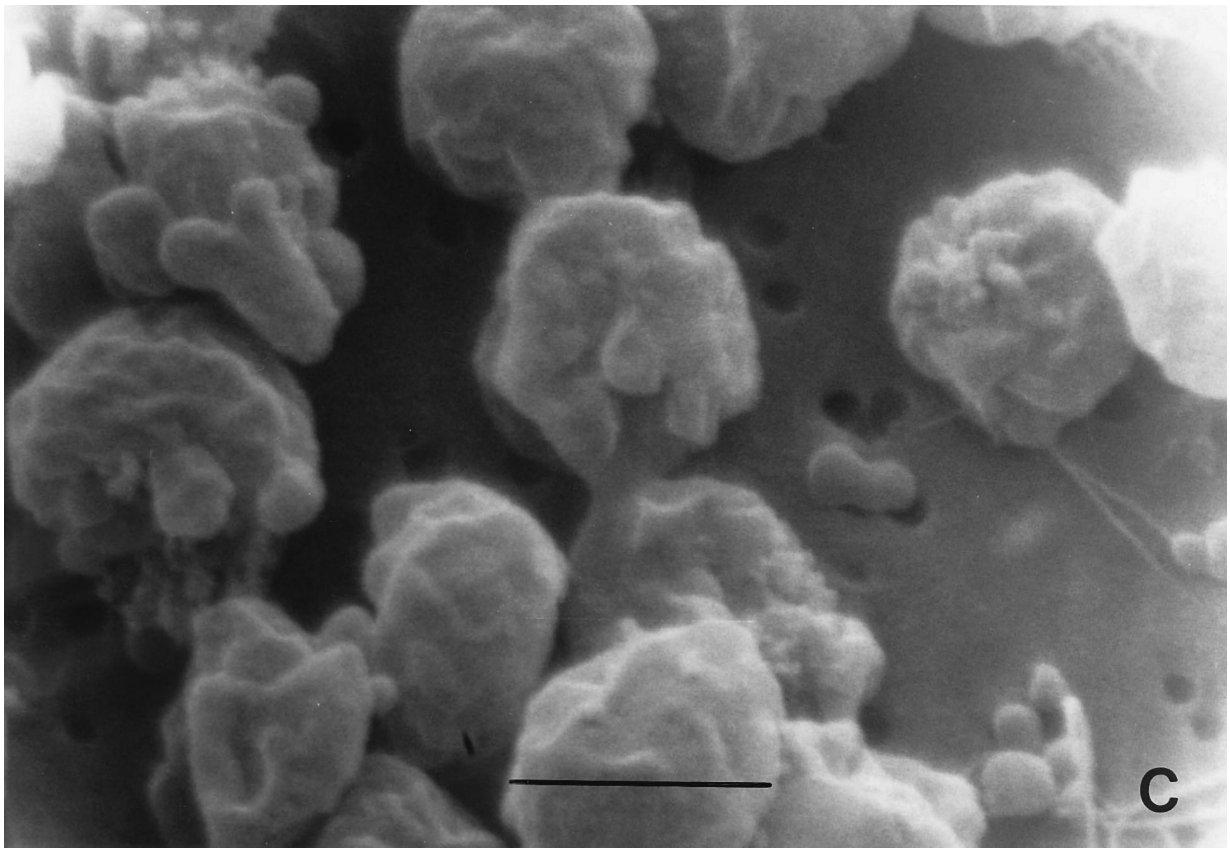

FIG. 1—*Continued.*

3.5°C, as evidenced by an increasing difference between PPBE and TCBS plate counts. It is obvious that low temperature plays an important role in the formation of nonculturable *V. parahaemolyticus* cells.

Under an epifluorescence microscope, the total counts of cells stained by acridine orange dye remained at the initial levels for both strains 38C1 and 38C6 during starvation at low temperature (Fig. 4). The difference between total cell counts and plate counts was about 3 log cycles, while DVC were always at least 1 log cycle higher than plate counts. A significant portion of the elongated cells detected by the DVC method may have been in the VNC stage, since fewer than 10% of these elongated cells were culturable by the plate count method. However, after a few more weeks of starvation at low

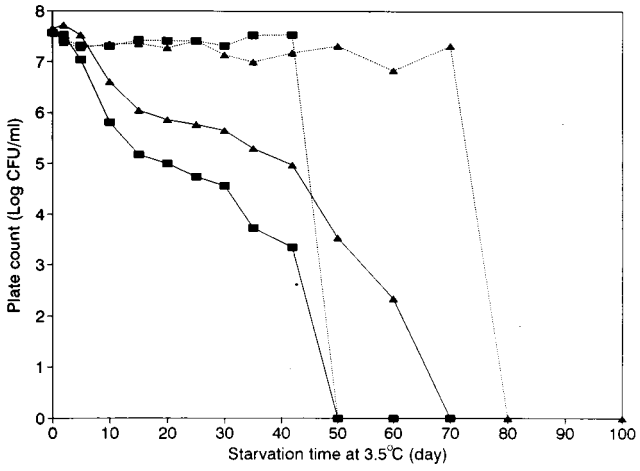

FIG. 2. Survival curves of *V. parahaemolyticus* 38C1 and 38C6 during starvation at 3.5°C and an RTU. Symbols: ■, strain 38C1; ▲, strain 38C6; solid line, 3.5°C; dashed line, RTU for 3 days. Datum points are mean values from two separate experiments.

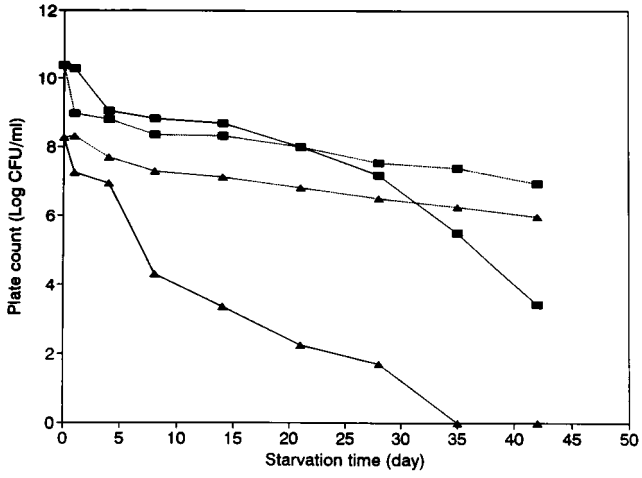

FIG. 3. Survival curves of *V. parahaemolyticus* 38C1 during starvation at 3.5°C and room temperature. Symbols: ■, PPBE plate counts; ▲, TCBS plate counts; solid line, 3.5°C; dashed line, room temperature. Datum points are mean values from two separate experiments.

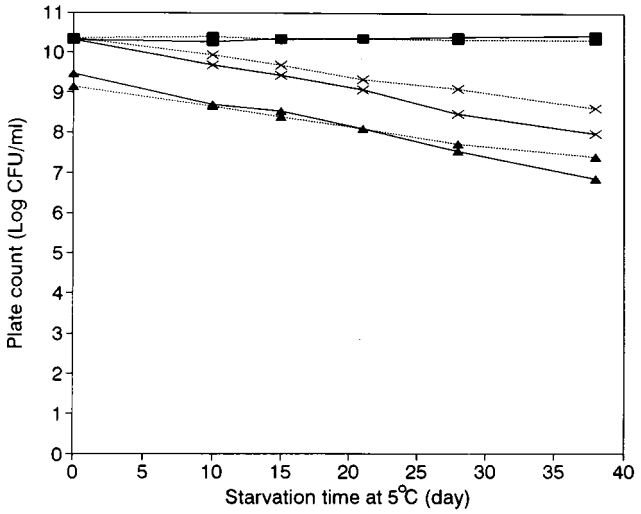

FIG. 4. Survival curves of *V. parahaemolyticus* 38C1 and 38C6 during starvation at 5°C. Symbols: ■, total count; ×, DVC; ▲, plate count; solid line, strain 38C1; dashed line, strain 38C6. Datum points are mean values from two separate experiments.

temperature, depending on the cell physiological ages, *V. parahaemolyticus* did not form filamentous cells when DVC were determined. On the contrary, the responsive cells became bigger spheres and the cell envelope became less distinguished. It was reported that *V. cholerae* cells appeared only slightly swollen but not clearly elongated when enumerated by the DVC method (30). In this study, although nonculturable *V. parahaemolyticus* cells could not be confirmed absolutely as viable by the DVC method, these cells were still intact and stained with acridine orange dye under an epifluorescence microscope. Some problems associated with the DVC method have been reviewed recently (26).

Resuscitation of nonculturable cells. Some controversial reports on the resuscitation of nonculturable cells have been published (16, 24, 29, 36, 37). Ravel et al. (29) have reported that recovery of VNC cells of *V. cholerae* from starvation at 4°C in nutrient-limited artificial seawater after a temperature upshift to 30°C was due to regrowth of surviving cells but not resuscitation of all VNC cells, and the same finding was reported for surviving *V. vulnificus* cells (37).

NA, a DNA synthesis inhibitor, inhibits the multiplication of cells without interfering with protein synthesis. After 24 h at room temperature, divided rods could be seen under a microscope in both control cells and those treated with NA at 20 μg/ml but not in cells treated with NA at 100 and 200 μg/ml. However, after 3 days of incubation, cells treated with NA at 20 or 100 μg/ml had the same number of rod-shaped cells as the control, while NA at 200 μg/ml inhibited rod-shaped cell formation (Table 1). These results indicated that cell multiplication did occur during a 3-day RTU in control cells and those treated with NA at 20 or 100 μg/ml. Although the concentrations of NA in this study were higher than the 20 μg/ml recommended by Kogure et al. (18), NA at up to 250 μg/ml did not inhibit the elongation of viable cells in either young or starved cultures of strain 38C6 during DVC determination at room temperature for 3 days. Apparently, NA concentrations lower than 100 μg/ml were insufficient to inhibit the multiplication of a few cells in this resuscitation test.

Furthermore, the MPN results demonstrated that cell multiplication could indeed occur during RTU. Low-temperature starved cultures were diluted and placed into MPN tubes right before RTU. The MPN results of these cultures in PPBE medium incubated at room temperature for 3 days correlated well with direct plate counts obtained before rather than after RTU (Table 2). The results of MPN determination in MMS even indicated slightly lower bacterial counts than direct plate counts before RTU (data not shown). Logically, in the most highly diluted tube, any growth could result from a single cell multiplication. If no multiplication occurred during RTU, these MPNs should be close to the regrown plate counts after RTU rather than direct plate counts before RTU.

Cells were washed before being resuspended in a nutrient-free starvation medium, MMS. Theoretically, the culturable cells remaining after low-temperature starvation in pure MMS could not multiply during RTU. Table 3 shows that cultures with initially higher cell densities always regrew to higher cell numbers after RTU, and the regrown cell numbers after RTU were constant throughout the entire experiment. As seen in Fig. 1, bleb released from the cell surface could be a mechanism for nutrient supply for cell regrowth when the temperature was upshifted. It was reported that moribund cells of *V. vulnificus* in starvation could release some substances during a temperature upshift (37). Apparently, the amount of released nutrients was determined by initial cell densities and strain differences in this study. Therefore, the amount of these nutrients in the microcosm was a key factor in the determination of how many cells would result after RTU regardless of the number of surviving cells at the onset of regrowth after RTU.

TABLE 1. Effect of NA on resuscitation of *V. parahaemolyticus* 38C6[a]

NA concn (μg/ml)	% Rod-shaped cells by AODC[b]	
	Before resuscitation[c]	After resuscitation
0	0	12.6
20	0	13.2
100	0	12.2
200	0	0

[a] A strain 38C6 culture was stored at 3.5°C for 52 days in starvation medium. The plate count was 4.6×10^3 before an RTU. After a 3-day RTU, the plate count reached 2.2×10^7/ml, which accounted for about 11% of the original inoculum size (2.1×10^8/ml).
[b] AODC, acridine orange direct count.
[c] A total of about 2,000 cells for each sample before and after resuscitation was observed.

TABLE 2. Comparison of culturable cell numbers determined by the MPN and plate count methods

Strain	Starvation time (days)	Plate count before temp upshift	After temp upshift	
			MPN (PPBE)[a]	Plate count
38C1	0	1.8×10^8	2.4×10^8	ND[b]
38C6	0	2.5×10^8	4.6×10^8	ND
38C1	40	2.4×10^2	7.5×10^2	3.8×10^7
38C6	40	2.2×10^4	2.4×10^4	2.5×10^7
38C1	45	1.0×10^1	9.3×10^1	4.9×10^7
38C6	45	6.1×10^3	1.1×10^4	2.5×10^7
38C1	50	<1	<1	<1
38C6	50	2.8×10^3	4.3×10^3	2.8×10^7

[a] MPN tubes were made right before an RTU, and the results were read after a 3-day RTU. Both MPN and plate count results are in numbers of cells per milliliter.
[b] ND, not determined.

TABLE 3. Effect of initial cell density on the regrowth of strains 38C1 and 38C6 in MMS after RTU

Strain (dilution)[a]	Cell count after starvation time (days) of:							
	0	15	21	26	31	45	50	80
38C1	5.2×10^7	4.0×10^7	5.9×10^7	4.0×10^7	5.2×10^7	9.5×10^5	<1	<1
38C1 (10×)	3.8×10^6	4.4×10^6	3.8×10^6	1.2×10^6	<1	<1	<1	<1
38C6	6.1×10^7	2.3×10^7	2.2×10^7	3.0×10^7	4.1×10^7	4.0×10^7	3.8×10^7	<1
38C6 (10×)	8.9×10^6	1.2×10^6	2.6×10^6	2.2×10^6	1.9×10^6	<1	<1	<1

[a] The microcosm was diluted 10-fold compared with the original culture, with fresh MMS at day 0.

However, Nilsson et al. (24) reported that cryptic growth is impossible during a temperature upshift because about 50 dead cells are needed to support the growth of 1 cell. Their calculations were probably based on the growth requirement for log-phase young cells in rich medium rather than starved cells in starvation medium. *V. cholerae* could undergo cell division in a very low-nutrient medium to reach a 1,000-fold increase in cell numbers (30). In this study, the number of cells regrown from a few surviving starved cells reached 10 to 100% of the original inoculum size without any changes in total cell counts during a 3-day RTU. It is possible that the starved cells in a stressed condition are capable of highly efficient nutrient uptake metabolism and respiration. We suggest that cells formed during starvation at a low temperature can grow and multiply with limited nutrients at an extraordinary rate when the temperature is upshifted.

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