Enhanced Resistance to Several Abiotic Stresses in *Vibrio cholerae* during Starvation

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**SUMMARY:** In this study, we investigated whether *Vibrio cholerae* cells exposed to nutrient-limited conditions developed resistance to abiotic stresses because of which most, if not all, starved cells turn coccoid in shape and exhibited marked resistance to freezing, low pH, and chlorine, but not against bile.

*Vibrio cholerae* is a rod-shaped gastrointestinal pathogen that causes cholera, one of the most notorious enteric diseases with serious morbidity and mortality in developing countries. Infection occurs through oral ingestion of food or water contaminated with this pathogen. Cholera epidemics have often been reported in developing countries with poor sanitation practices (1). However, people in developed countries have also suffered from sporadic cholera outbreaks transmitted through travelers and seafood imported from endemic areas (2,3).

Recently, Nowakowska et al. (4) reported that *V. vulnificus* cells in a dormant state (commonly referred to as the "viable but nonculturable" [VBNC] state) induced by low temperature and nutrient deprivation exhibited more marked resistance to environmental stresses such as high temperature, ethanol, and antibiotics than did those in the logarithmic phase of growth. Waturangi et al. (5,6) also reported that *V. cholerae* was frequently isolated from edible ice cubes commercially available in Jakarta. Meanwhile, Krebs and Taylor (7) showed that *V. cholerae* cells changed their shape from rod to coccoid under starvation stress, to survive better in aquatic environments. Circumstantial evidence suggests that *V. cholerae* cells isolated from ice cubes were exposed to nutrient-limited conditions and became resistant to freezing stress, thereby remaining viable in the ice cubes for a prolonged period. We evaluated the above-described hypothesis by exposing artificially created starved *V. cholerae* cells to several major abiotic stresses, namely, freezing, chloride, low pH, and bile acid, which are most likely to be experienced by the pathogen in vitro or in vivo.

A total of 6 strains of *V. cholerae* O1 Ogawa were used (Table 1). Their taxonomic identity as *V. cholerae* and biotype, production of cholera toxin (CT), and CT genotype were determined by a PCR assay targeting the 16S-23S rRNA intergenic spacer regions specific to *V. cholerae* (8), a latex agglutination kit (VET-RPL; Denka Seiken, Tokyo, Japan), and a mismatch amplification mutation PCR assay (9), respectively, while their serotypes were determined by agglutination tests using commercial polyclonal antisera specific to *V. cholerae* O1 polysaccharide (Denka Seiken). Among them, KVC88008, KVC88017, and KVC95029, which were isolated from river water in Japan, were found to not produce CT, while 18H24, 5H332, and VF192, which were isolated from patients, did produce CT. They were maintained on Heart Infusion Agar (Becton Dickinson, Sparks, MD, USA) slants with 1% salt until use.

Krebs and Taylor (7) reported that ≥90% of *V. cholerae* O1 Classical O395 cells cultured in a nutrient-limited condition changed from the rod form to the coccoid form, and that nutrient deprivation might be the main factor causing this morphological transition. Starved *V. cholerae* cells were prepared according to the method described by them. Briefly, *V. cholerae* cells were grown in LB broth (Becton Dickinson) at 30°C for 16 h with shaking (LB 16 h). Cultures were then diluted 1:1 with fresh LB broth and further incubated at 30°C for 24 h without shaking in this 50% conditioned medium (CM50). In the present study, cells grown in CM50 and LB 16 h were defined as "starved" and "normal" cells, respectively. Starved and normal cells were harvested, washed twice with sterile phosphate-buffered saline (PBS; 8 mM Na2HPO4, 1.5 mM KH2PO4, pH 7.2, 137 mM NaCl, 2.7 mM KCl), and suspended in 1 ml of sterile PBS. The nuclei of these cells were stained using a DAPI kit (NucBlue® Fixed Cell ReadyProbes™ Reagent, Life Technologies, Carlsbad, CA, USA), according to the manufacturer’s instruction. Then, a drop of each sample was fixed with a layer of 1% agarose on a glass slide with a cover glass. Cell images were captured and viewed using BIORHOBO® ZF-9000 fluorescence microscope (Keyence, Osaka, Japan). KVC88017 cells form the LB 16 h group exhibited rod-shaped morphology, whereas the cells from the CM50 group were frequently observed to be coccoid in shape, both of which contained intact DNAs (Fig. 1). Such a morphological transition in starved cells was also observed in the other 5 strains (data not shown). Further, the coc- coid KVC88017 cells grown on LB soft agar plate exhibited morphological reversion to the rod-shape after several rounds of division (Fig. 2).

Subsequently, starved and normal *V. cholerae* cells

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Table 1. *Vibrio cholerae* strains used

<table>
<thead>
<tr>
<th>Strain No.</th>
<th>Taxonomic identification</th>
<th>Serogroup</th>
<th>Serotype</th>
<th>Biotype</th>
<th>CT production</th>
<th>Yr isolated</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>VF-192</td>
<td><em>V. cholerae</em></td>
<td>O1</td>
<td>Ogawa</td>
<td>El Tor</td>
<td>+</td>
<td>2009</td>
<td>Human patient in Indonesia</td>
</tr>
<tr>
<td>5H332</td>
<td><em>V. cholerae</em></td>
<td>O1</td>
<td>Ogawa</td>
<td>El Tor</td>
<td>+</td>
<td>1994</td>
<td>Human patient in Japan</td>
</tr>
<tr>
<td>18H24</td>
<td><em>V. cholerae</em></td>
<td>O1</td>
<td>Ogawa</td>
<td>El Tor variant</td>
<td>+</td>
<td>2006</td>
<td>Human patient in Japan</td>
</tr>
<tr>
<td>KVC88008</td>
<td><em>V. cholerae</em></td>
<td>O1</td>
<td>Ogawa</td>
<td>El Tor</td>
<td>-</td>
<td>1988</td>
<td>River water</td>
</tr>
<tr>
<td>KVC88017</td>
<td><em>V. cholerae</em></td>
<td>O1</td>
<td>Ogawa</td>
<td>El Tor</td>
<td>-</td>
<td>1988</td>
<td>River water</td>
</tr>
<tr>
<td>KVC95029</td>
<td><em>V. cholerae</em></td>
<td>O1</td>
<td>Ogawa</td>
<td>El Tor</td>
<td>-</td>
<td>1995</td>
<td>River water</td>
</tr>
</tbody>
</table>

1): following the methodology of PCR (8).
2): determined by commercial serotyping kits of slide and latex agglutination test.
3): determined by a mismatch amplification mutation PCR assay (9).
4): determined by commercial cholera toxin detection kit.

Fig. 1. *V. cholerae* O1 El Tor KVC88017 cells transitioning from rod to coccoidal shape. (A) KVC88017 cells grown in LB 16 h. (B) KVC88017 cells grown in CM50. Bars, 5 μm.

were harvested, washed twice with PBS, and suspended in sterile distilled water to simulate the actual condition of *V. cholerae* in ice cubes. Starved and normal microcosms were suspended in sterile distilled water to reach optical densities at 660 nm (O.D. 660) of 0.3 and 0.4, respectively. One milliliter each of the starved and normal cell suspension was then dispensed into 8 Eppendorf Safe-Lock Tubes™ (1.5-ml capacity; Eppendorf Japan Co. Ltd., Tokyo, Japan) and frozen for approximately 10 min in a freezer (Sanyo MDF-135; Sanyo Electric Co. Ltd., Osaka, Japan), which was set at −30°C. Each tube was withdrawn from the freezer daily for 7 days and allowed to thaw for 1 h at room temperature. Serial 10-fold dilutions of the thawed sample were then plated on LB agar, and the plates were incubated at 37°C for 24 h to enumerate viable cells. The susceptibility of cells from the LB 16 h and CM50 groups to freezing stress is shown in Fig. 3. The viable cell counts of the normal and starved cells immediately before being frozen were comparable (7–8 log CFU/ml); however, cell viability decreased with increase in the duration of the frozen state; compared to the starved cells, normal cells were, in most cases, 10~50-fold more susceptible to the freezing challenge (in terms of viable cell counts; P<0.01). Some of the starved cells endured the week-long freezing stress, which proved lethal to normal cells. Because sporadic outbreaks of cholera attributed to frozen foods imported from endemic areas have been reported in developed countries (3,4), it may be worth investigating the possible involvement of starved *V. cholerae* cells in future outbreaks attributed to consumption of contaminated frozen foods.

Starved and normal cells were harvested, washed twice, and diluted with PBS to obtain an O.D. 660 of 0.3. Prepared cells were harvested and re-suspended in LB broth (pH 3), 2 ppm NaOCl (Wako, Osaka, Japan), or PBS supplemented with 10% bile salts (a mixture of sodium cholate and sodium deoxycholate; Sigma, St. Louis, MO, USA). It should be noted that, in our preliminary experiments, we evaluated different pH values and various concentrations of chlorine and bile salts across a wide range of exposure periods, and found that the above-described experimental conditions, which were close to the actual conditions, were the most appropriate to highlight the differences in the survival of starved and normal cells.

The cells exposed to low pH for 9 min were harvested by centrifugation at 12,000 rpm for 1 min and re-suspended in PBS. The survivors were enumerated by the plate count method using LB agar with incubation at 37°C for 24 h. After chlorine challenge for 5 min or bile acid challenge for 30 min, the survivors were enumerated by the same method. The susceptibility of *V. cholerae* cells grown in LB 16 h (normal cells) and CM50
Vibrio cholerae in a Starved State

Fig. 2. Morphological reversion of KVC88017 starved cell while being divided during incubation on LB soft agar. (A) a coccoid cell at the start of incubation. (B) incubation for 60 min. (C) incubation for 75 min. (D) rod-shaped cells after incubation for 90 min. Bars, 5 μm.

Table 2. Susceptibility of V. cholerae strains exposed to chlorine, low pH, and bile acid challenges

<table>
<thead>
<tr>
<th>Strain No.</th>
<th>Cell state</th>
<th>Viable cell counts (CFU/ml) before and after being treated with</th>
<th>Low pH</th>
<th>Bile acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Before treatment</td>
<td>after treatment</td>
<td>Before treatment</td>
</tr>
<tr>
<td>KVC88008</td>
<td>normal</td>
<td>6.0 ± 2.6 × 10^8</td>
<td>n.d.</td>
<td>3.3 ± 1.5 × 10^8</td>
</tr>
<tr>
<td></td>
<td>starved</td>
<td>4.3 ± 1.2 × 10^8</td>
<td>4.3 ± 0.4 × 10^4**</td>
<td>1.7 ± 0.6 × 10^8</td>
</tr>
<tr>
<td>KVC88017</td>
<td>normal</td>
<td>5.0 ± 1.7 × 10^8</td>
<td>n.d.</td>
<td>6.0 ± 2.6 × 10^8</td>
</tr>
<tr>
<td></td>
<td>starved</td>
<td>7.3 ± 0.6 × 10^8</td>
<td>2.6 ± 0.4 × 10^4**</td>
<td>6.7 ± 4.0 × 10^8</td>
</tr>
<tr>
<td>KVC95029</td>
<td>normal</td>
<td>5.7 ± 2.1 × 10^8</td>
<td>n.d.</td>
<td>9.7 ± 3.1 × 10^8</td>
</tr>
<tr>
<td></td>
<td>starved</td>
<td>5.3 ± 2.9 × 10^8</td>
<td>5.3 ± 1.5 × 10^4**</td>
<td>8.7 ± 1.2 × 10^8</td>
</tr>
<tr>
<td>VF192</td>
<td>normal</td>
<td>7.0 ± 1.0 × 10^8</td>
<td>9.7 ± 1.5 × 10^1</td>
<td>9.3 ± 2.1 × 10^8</td>
</tr>
<tr>
<td></td>
<td>starved</td>
<td>8.3 ± 2.1 × 10^8</td>
<td>7.3 ± 2.9 × 10^4**</td>
<td>9.7 ± 3.1 × 10^8</td>
</tr>
<tr>
<td>5H332</td>
<td>normal</td>
<td>7.3 ± 2.5 × 10^8</td>
<td>4.7 ± 3.0 × 10^1</td>
<td>6.7 ± 1.2 × 10^8</td>
</tr>
<tr>
<td></td>
<td>starved</td>
<td>9.0 ± 2.0 × 10^8</td>
<td>2.2 ± 0.4 × 10^4**</td>
<td>8.0 ± 1.0 × 10^8</td>
</tr>
<tr>
<td>18H24</td>
<td>normal</td>
<td>8.7 ± 1.2 × 10^8</td>
<td>n.d.</td>
<td>7.0 ± 2.6 × 10^8</td>
</tr>
<tr>
<td></td>
<td>starved</td>
<td>8.7 ± 2.9 × 10^8</td>
<td>6.7 ± 1.5 × 10^4**</td>
<td>5.3 ± 1.5 × 10^8</td>
</tr>
</tbody>
</table>

n.d., plate count below the limit of detection (<10 CFU/ml).
*, significantly (P<0.05) greater numbers of survived cells in the starved cell than the normal cells after treatment.
**, significantly (P<0.01) greater numbers of survived cells in the starved cell than the normal cells after treatment.

(starved cells) to stress induced by low pH, chlorine, and bile acid is shown in Table 2. The viable counts of the normal and starved cells immediately before being challenged were comparable (7–8 log CFU/ml).

Normal V. cholerae strains lost their culturability completely or almost completely on exposure to stress induced by low pH chlorine (Table 2). Chlorine has been the principal means of preventing waterborne infectious diseases, including cholera, since the first decade of this century (10). Although we used NaOCl at concentrations approximately 20-fold higher than those of free chlorine (typically present in municipal water supplies), the starved cells were able to withstand high concentrations of chlorine (Table 2). Yildiz and Schoolnik (11) reported that the rugose colony variant of V. cholerae grown from a starved culture produced an exopolysaccharide (EPS), which conferred chlorine resistance and biofilm-forming capacity. The starved CM50 cells might have thus acquired chlorine tolerance by producing EPS; this aspect needs to be investigated further.

The HCl present in gastric fluid is responsible for
lowering the pH of the stomach, which presents a lethal environment for most microorganisms, thereby preventing bacterial infections (12). The culturability of starved cells also decreased dramatically following exposure to stress induced by low pH (2–5 log CFU/ml); however, it was significantly higher than that of normal cells \( (P < 0.01, \text{Table 2}) \). Compared to solids, liquids are exuded more easily from the stomach. The gastric half-emptying time of solids ranged from 40 to 160 min, while that of liquids ranged from 8 to 107 min (13–15). The starved \emph{V. cholerae} cells orally ingested via liquids are thus more likely to survive the acidic conditions of the stomach and pass into the intestinal tract where they can cause gastroenteritis.

Bile is composed of salts of primary bile acids (e.g., cholic acid and Chenodeoxycholic acid) and it can disrupt the membrane permeability of bacterial cells (16). We, therefore, initially expected that bile would act differently on \emph{V. cholerae} cells depending on their physiological and morphological status. The viable cell counts of normal and starved cells after exposure to bile acid stress were, however, almost comparable (4–7 log CFU/ml) for all strains, although 2 strains exhibited marginally higher level of culturability (Table 2). Available evidence suggests that starved cells cannot prevent the abiotic effects of bile.

Bacterial cells that enter the VBNC state in response to many challenges, including nutrient deprivation, exhibit morphological and metabolic modifications (16), including alterations to cell membrane composition (17).
and decreased levels of macromolecule synthesis, nutrient transport, and respiration rate (18). Starved cells of the CM50 group, which is independent of the VBNC state, might exhibit not only a morphological transition from rod to coccoid shape, but also similar metabolic modifications. These changes may cause *V. cholerae* cells to adapt to, as well as resist several physical and chemical stresses, which, in turn, may present a critical issue for our public health system, with specific reference to possible contamination of food and water by pathogenic *V. cholerae*.

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**Conflict of interest**  None to declare.

**REFERENCES**