Starvation Response of the Marine Barophile CNPT-3

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The psychrophilic marine barophile CNPT-3 underwent a starvation-survival response similar to that reported for the marine bacteria Ant-300, DW1, and S-14. The number of culturable cells increased initially and then decreased gradually over a 24-day starvation period, with corresponding decreases in total cell number and direct viability count. A significant reduction in cell size and biovolume accompanied these changes. Starved cells demonstrated a greater tendency to attach at the in situ pressure (400 atm; ca. 40.5 MPa) and temperature (5°C) than at 1 atm (ca. 101 kPa), and the extent of attachment increased with increasing duration of starvation. The membrane fatty acid profile of the marine barophile CNPT-3 was studied as the cells were subjected to starvation conditions. A 37.5% increase in saturated fatty acids was observed during the first 8 days of starvation, with a concomitant decrease in unsaturated fatty acids. There was also an increase in the amount of short-chain (<C₁₅₀₆) fatty acids.

CNPT-3 is a spirillum originally isolated from a decaying amphipod found in the central North Pacific at a depth of 5,700 m (37). This organism is both barophilic and psychrophilic, with an optimum pressure for growth between 3 x 10⁷ and 7 x 10⁴ Pa (296 and 690 atm, respectively) and an optimum temperature of 2 to 4°C (37, 38). The gut of the amphipod contains organic material at a concentration high enough to provide adequate carbon and energy sources for growth of bacteria (10). However, the question arises as to the fate of such organisms once they leave the gut and enter deep open waters, where a dissolved organic carbon level of less than 1 mg/liter (9) would be expected to result in starvation conditions for copiotrophic bacteria. Further, it is likely that bacteria inside a host organism also encounter and respond to starvation conditions when the host organism is not feeding. The starvation responses of the intestinal microflora of several marine animals have been described by Conway et al. (6). Bacteria were observed to reduce in size from predominately rods to cocci and to maintain viability for extended periods of time. This may be salient to the survival of CNPT-3 in association within the gut of its amphipod host.

Numerous studies have concentrated on the starvation response of marine bacteria (2, 3, 11, 22–26, 28; for a recent review on the starvation response, see reference 15). These studies, which have focused on organisms found in relatively shallow open waters, indicate that bacteria may exhibit a 400% increase in cell number with a corresponding decrease in cell size under starvation conditions (23). Kjelleberg and Hermansson (14) described this process as taking place in two phases. The first phase involves reductive division, with a decrease in cell size and a concomitant increase in cell number. The second phase consists of further reduction in cell volume with no corresponding increase in cell number. The resulting cells, termed "dwarf" cells by Novitsky and Morita (23), reduce their endogenous metabolism by over 99% under starvation conditions (24) but remain viable for extended periods of time. At the same time, there may be major changes in cellular components such as membrane lipids (31) and proteins, RNA, and DNA (19, 27). In the case of the marine Vibrio sp. strain Ant-300, survivability has been extrapolated to as long as 20 to 30 years (26). Another marine Vibrio sp., strain S-14, has been demonstrated to maintain 100% viability for 2 weeks in artificial sea water (ASW) (28). Despite the interest in this area, no studies have been reported on the starvation-survival response of marine barophiles.

Attachment may also be a survival response of marine bacteria; this phenomenon may be nutrient related, although there is conflicting evidence as to whether bacteria are more or less likely to attach under low-nutrient conditions or whether surfaces are of value to bacteria. Dawson et al. (7) reported that the marine Vibrio sp. strain DW1 exhibited an increased ability to attach under nutrient limitation. Marshall (20) described the attachment-detachment process as a dynamic process whereby nutrient-depleted cells will attach to a surface, grow, and divide and then detach, reenter the bulk phase, and begin the starvation-survival response anew. Conway et al. (6) indicated that, at least in the intestine of flounder under low-nutrient conditions, the gut microflora increased their ability to attach. In contrast to these studies, Mardén et al. (19) described a system whereby an exopolysaccharide, produced in response to low-nutrient conditions, enhanced the detachment of cells from a solid surface. In their review on the subject of attachment, van Loosdrecht et al. (34) concluded that there was no experimental evidence for the influence of surfaces on bacterial activity. In the only report on the effects of elevated hydrostatic pressure on bacterial attachment, Smith and Oliver (33) recently showed that increased hydrostatic pressure inhibited the attachment of a nonbarophilic bacterium. The same authors previously reported (32) that barophiles may require active metabolism for attachment; the authors postulated that, whereas attachment of nonbarophiles is inhibited by pressure, barophiles require increased pressure for irreversible attachment.

To date, there are no studies that address the starvation response of barophilic bacteria or the effects of nutrient limitation on the attachment of such bacteria at in situ pressure. It was the purpose of this study to examine, under in situ conditions, the response of CNPT-3 to nutrient deprivation, specifically, to examine its starvation response and its attachment to a solid surface.

Because these bacteria demonstrate such dramatic morphological changes, it would be expected that there would be...
accompanying changes in the membrane fatty acid profile. Oliver and Stringer (31), studying the fatty acid composition of the marine bacterium Ant-300 under starvation conditions, found an increase in the percentage of C16:1 and a concomitant decrease in the percentage of C14:0. The overall effect of the fatty acid changes was speculated to increase membrane fluidity, which might facilitate transport of nutrients under starvation conditions. Malmerca-Friberg et al. (18) demonstrated that the marine Vibrio sp. strain S14 increased its percentage of short-chain fatty acids during starvation, which would similarly be expected to increase membrane fluidity. Hood et al. (13) also reported increases in the relative proportion of short-chain fatty acids in Vibrio cholerae under long-term nutrient deprivation. However, these authors reported a decrease in the unsaturated fatty acids with a corresponding increase in the saturated fatty acids of V. cholerae under starvation conditions. Guckert et al. (12) also demonstrated a decrease in unsaturated fatty acids in this species, although their study showed only a slight increase in the short-chain fatty acids.

Although the fatty acid composition of CNPT-3 has been determined (8), there are no published studies on the fatty acid composition of any barophilic bacterium under starvation conditions. Because it is likely that barophiles such as CNPT-3 experience extreme fluctuations in nutrient availability, the present study was undertaken to characterize the changes in membrane fatty acid composition in response to nutrient deprivation.

MATERIALS AND METHODS

Organism and growth conditions. CNPT-3 was supplied by A. A. Yayanos (Scripps Institute of Oceanography, La Jolla, Calif.). Cells to be used for the experimental procedure were grown in Marine Broth 2216 (Difco Laboratories, Detroit, Mich.) at 400 atm (ca. 40.5 MPa) and 5°C. Cells were harvested at the logarithmic phase, as determined by acridine orange direct counts (AODC; see below) with comparison to previously established growth curves. Cells were washed once in 0.2-μm-pore-size filter-sterilized ASW and resuspended to a final concentration of ca. 10^6 cells per ml of ASW in a 60-ml syringe (starvation syringe). Cultures were maintained at 400 atm and 5°C throughout all studies except during sample processing at 1 atm (ca. 101 kPa); Chastain and Yayanos (5) have shown that such short-term exposure to 1 atm is not deleterious to barophilic bacteria as long as they are kept cold. Elevated hydrostatic pressure was maintained as described by Smith and Oliver (33). Briefly, experiments were carried out in model OC-3 pressure reactors (High Pressure Equipment Co., Erie, Pa.). Hydrostatic pressure was applied with a model 11-400 pump (Enerpac, Butler, Wis.) with both pressurization and depressurization at a rate of 100 atm (ca. 10,100 kPa/min).

Cell enumeration. After various times of starvation, starvation syringes were depressurized and total numbers of cells were determined by using AODC essentially as described by Moyer and Morita (22). Formalin (final concentration, 2%) and acridine orange (final concentration, 0.01%) were added to 1 ml of cells from a starvation syringe. After a 2-min incubation, the suspension was filtered onto a 0.2-μm-pore-size black polycarbonate membrane filter (Poretics Co., Livermore, Calif.) and observed by epifluorescence microscopy as previously described (29). Cell concentration was determined based on the average number of cells counted in 15 to 20 fields per sample. Cell size was determined with a stage micrometer. The percentage of viable cells was determined by the direct viable count (DVC) method of Kogure et al. (17); 1 ml of cells was incubated for 72 h at 400 atm and 5°C. The number of cultivable cells was determined by adding ASW dilutions of cells taken from the starvation syringes to sterile culture tubes. A molten mixture (2:1) of Marine Broth 2216–12% gelatin was cooled to 10°C and aseptically added to the tubes containing the cells. The tubes were capped with sterile rubber stoppers; care was taken to eliminate any air bubbles. Tubes were then incubated at 400 atm and 5°C for 21 days, at which time the number of isolated colonies was determined. The results presented here represent typical data from five separate experiments.

Bacterial attachment. Cells (1 ml) were removed from the starvation syringes after various periods of starvation at 400 atm and 5°C and transferred to 5-ml syringes containing ASW and coverslips fixed to a rubber stopper. For attachment of nonstarved cells, CNPT-3 was grown under pressure in Marine Broth 2216 to the logarithmic phase, diluted, and resuspended to ca. 10^6 cells per ml in a syringe containing sterile Marine Broth 2216. Cells (1 ml) were removed from the growth syringe and transferred to a 5-ml syringe as described above. For both starved and unstarved cells, attachment was allowed to proceed at 5°C at both 1 and 400 atm. At intervals of 24 and 72 h, a coverslip from each syringe was removed, fixed with 2% Formalin, and stained with 0.01% acridine orange. The coverslips were then washed with ASW to remove any unattached cells. The coverslips were viewed with epifluorescent microscopy, and the average number of attached cells per square millimeter was determined. These studies were repeated twice.

Extraction and analysis of fatty acids. For fatty acid extraction, 30 ml of cells from a single syringe was centrifuged for 10 min at 10,750 × g at 5°C and resuspended in 5 ml of ASW. Fatty acids were extracted by the method of Bligh and Dyer (4) and methylated with BCl3-methanol by the method of Metcalfe and Schmitz (21). Fatty acid methyl esters were analyzed by gas chromatography (Agrograph series 1400 chromatograph; Varian, Palo Alto, Calif.) with a 10% DC200 column on a 80/100 Chromosorb W-AW-DMCS column (Supelco, Inc., Bellefonte, Pa.) and a flame ionization detector. Operating conditions were as follows: column temperature, 190°C; injector temperature, 220°C; detector temperature, 190°C. The carrier gas was N2, and the flow rate was 20 ml/min. Fatty acids were quantified with a C-R6A Chromatopac (Shimazdu, Kyoto, Japan); values are reported here on a weight percent basis. The fatty acid methyl esters were identified by comparison with authentic fatty acid standards (Supelco, Inc.) and through hydrogenation studies. Hydrogenation was performed in methanol with palladium oxide as the catalyst. The results presented here are the result of two extractions with fatty acid methyl ester samples analyzed two to five times by gas chromatography.

RESULTS AND DISCUSSION

Starvation response. Figure 1 shows a typical response of CNPT-3 to starvation conditions at 5°C and 400 atm. In three of the five replicate experiments, no initial increase in total cell number (AODC) was observed at the onset of starvation. In two studies, small (ca. 0.5-log-unit) increases in the total cell number were seen. Overall, the total cell counts declined gradually over the 24-day study period.

Like the total number of cells, cell viability, as measured by the method of Kogure et al. (17), decreased during the starvation period (Fig. 1) to a level averaging 90% below the
initial value. Typically, log-phase cells of CNPT-3 demonstrated an initial viability of ca. 30 to 50% of the AODC value and then decreased gradually during starvation to ca. 15 to 35% of the AODC value. This is consistent with results reported for the starvation of other marine bacteria, which also show a gradual decrease of viability over time, but with a significant percentage of the initial population remaining viable for extended periods (3, 19, 24, 27).

The number of culturable cells, initially ca. 10⁶ cells per ml, generally rose slightly during the first 2 to 5 days and then decreased gradually during the course of the study (Fig. 1). The ability of these starved cells to respond rapidly to nutrient addition (Marine Broth 2216) indicated that, although the surviving cells may exist in a dormant state, they are capable of resuming rapid growth and division when nutrient conditions are suitable. This is evident from the culturable cell counts, which, even after 24 days of starvation, indicated that the starved cells are ready to respond to the addition of nutrients. Cells that had been starved and then given Marine Broth 2216 responded to the elevated nutrient level with an increase in cell number by ca. 2.5 log units after a lag phase of 24 h (data not shown). This lag phase increased with increasing length of time of starvation.

Along with the change in cell numbers, marked changes in the morphology of the cells and in cell size were observed. Before starvation, log-phase cells of CNPT-3 in Marine Broth 2216 at 5°C and 400 atm were rods measuring ca. 2.5 by 0.58 μm (biovolume, 0.66 μm³). After 24 days of starvation at this temperature and pressure, the cells had become cocci 0.5 to 0.6 μm in diameter with a biovolume (0.08 μm³) that was only 10% of that of unstarved cells.

Our results with CNPT-3 are similar to those reported for the starvation responses of Ant-300 (23, 24), S-14 (19, 27), and DW1 (7) but differ in that no dramatic initial increase in cell numbers of CNPT-3 was observed. However, the overall pattern is similar; a significant portion of the population remained after extended periods of starvation, and a change in cell morphology from rods to small cocci during starvation was also observed. In a study of 16 marine isolates, Amy and Morita (2) described three different response patterns to starvation. One of these was characterized by a gradual decrease in cell number until a constant level was reached and with no large initial increase in cell number at the onset of starvation. This pattern is consistent with our data for CNPT-3. The decrease in cell size is likely due to reductive division, with further decreases in cell size occurring as a result of endogenous metabolism (23). The lack of an initial increase in total cell number may be due to the initial density of cells used (10⁶ cells per ml). Indeed, Novitsky and Morita (24) and Albertson (1) reported that, at progressively higher cell densities, the relative increase in total cell numbers during starvation decreased.

**Attachment.** In the presence of nutrients (Marine Broth 2216) and elevated hydrostatic pressure, the number of cells attaching to a glass substrate in 24 h steadily increased from ca. 500 cells per mm² to a final level of ca. 7,000 cells per mm² after 4 days of growth (Fig. 2a). These levels were significantly higher than those observed for cells incubated at 1 atm (Fig. 2a). When the attachment incubation period was extended from 24 h to 72 h, cells at both pressures continued to attach to the coverslips (Fig. 2b). These results indicate that attachment of CNPT-3 was enhanced by ele-
vated hydrostatic pressure but that pressure is not an absolute requirement for irreversible attachment.

Under starvation conditions, the total number of attached organisms at 400 atm also rose in relation to the duration of starvation. Attachment for 24 h at 400 atm increased from ca. 300 cells per mm² for unstarved cells to ca. 1,350 cells per mm² after 9 days of starvation (Fig. 3). Increasing the time allowed for attachment to 72 h did not markedly increase the number of attached cells (data not shown). Cells starved for as long as 9 days showed relatively little attachment (Fig. 3) at 1 atm. The difference between the number of bacteria attached at 1 atm and the number attached at 400 atm again suggests that elevated hydrostatic pressure strongly enhances attachment but may not be an absolute requirement for CNPT-3. Although bulk-phase cells were quantified as cells per milliliter and attached cells as cells per square millimeter, a comparison of the numbers of attached and bulk-phase populations consistently revealed a greater percentage of the cells attached under pressure (400 atm) compared with the number attached at 1 atm and an increase in numbers of attached cells with increased time of starvation (Fig. 4).

Attached, starved cells under pressure were predominantly rods (2.5 by 0.58 μm; biovolume, 0.66 μm³) with a small percentage of cocci (0.6 μm in diameter; biovolume, 0.08 μm³). Since these attached cells were considerably larger than unattached starved cells, it seems likely that the attached cells scavenge nutrients that are known to accumulate at the solid-liquid interface (20). Indeed, Kjelleberg et al. (16) demonstrated that attached bacteria readily take up nutrients associated with the surface-water interface and that attached bacteria maintain motility much longer than do cells in the bulk phase.

The few studies that exist on the effect of starvation on attachment indicate that starvation increases the ability of an organism to attach (16, 20). The data presented here suggest that, on a percentage basis, long-term (but not short-term) starvation of CNPT-3 may enhance the ability of the organism to attach to solid surfaces. When faced with starvation conditions, possibly induced when the amphipod is no longer able to scavenge nutrients or when CNPT-3 is released to seawater with fecal pellets, CNPT-3 may lose its ability to attach during short-term starvation. This would allow the cell to escape the nutrient-deficient situation, in search of a more favorable environment. Wrangstad et al. (35, 36) described an exopolysaccharide that was responsible for the attachment of *Pseudomonas* sp. strain S9 under starvation conditions. CNPT-3 may display a similar response. During long-term starvation, it appears that CNPT-3 may again develop an enhanced attachment ability.

**Membrane fatty acids.** The profile for the major fatty acids observed for CNPT-3 during starvation is shown in Fig. 5.

![Graph 1: Effect of time of starvation on the number of CNPT-3 cells attaching to a glass substratum at 5°C at 1 atm (■) and 400 atm (■). Attachment was for 24 h.](image1)

![Graph 2: Percentage of the bulk-phase cells, starved for various periods, that attached to the glass substratum within 24 h at 1 atm (■) or 400 atm (■). See the text for a discussion.](image2)

![Graph 3: Fatty acid changes occurring in CNPT-3 during starvation. Only the major fatty acids are shown: □, C₁₄:₀; ◆, C₁₅:₀; □, C₁₆:₀; ■, C₁₆:₁; ◆, C₁₇:₀; □, C₁₈:₁. Data shown are the averages of two separate studies.](image3)
The fatty acids that were identified are typical of gram-negative bacteria (30), but as many as 17 additional, unidentifed acids were observed as minor (<4%) constituents. The profile for the unstarved cells differs from that reported by Delong and Yamanos (8), who reported significantly greater levels of C16:1. However, differences in growth conditions in their study versus ours, such as growth temperature (2°C versus 5°C), pressure (580 atm versus 400 atm), and age of the culture, are known to affect the lipid profile of a bacterium (30) and may account for the differences observed. It is evident from Fig. 5 that changes did occur in the fatty acid profile of CNPT-3 during the starvation response. The most dramatic changes were noted during the first 8 days of starvation, which correspond to the period of greatest morphological and physiological changes in these cells. There was a net 37% increase in the saturated fatty acids, with the most significant change occurring in the C16 species. Saturated short-chain (<C15:0) fatty acids also increased. Among the unsaturated fatty acids, only C16:1 underwent any marked change, decreasing ca. 35% during the first 8 days (Fig. 5). Other unsaturated fatty acids, including C16:1, showed little change. Although there were no major overall changes observed in the monounsaturated fatty acids, there may still be alterations. Guckert et al. (12) demonstrated that there were cis-to-trans shifts in the double bond of monounsaturated fatty acids under starvation conditions, a possibility not addressed in our study. Although polysaturated fatty acids have been reported in marine barophiles, they have not been reported to be normal constituents of CNPT-3 (8) and were not observed in our study.

Based on the concept that increasing monounsaturated fatty acids increases membrane fluidity (12), our data would indicate a decrease in the membrane fluidity of CNPT-3 during starvation. The increase in short-chain fatty acids may, to some extent, counteract this decrease in fluidity. The significance of the changes in fatty acids observed during starvation is difficult to determine, since we conducted no studies of membrane characteristics and we did not address the question of nutrient transport during starvation. The observed changes in membrane fatty acids likely represent a modification of cellular components directed toward enhancing the ability of the organism to survive under starvation conditions. Understanding the decreases in fluidity we observed will require further studies on more barophilic bacteria and on the physiological functions of such cells as they undergo a starvation response.

In conclusion, CNPT-3, a psychrophilic and barophilic marine bacterium, demonstrated a starvation-survival response similar to that reported for other marine bacteria. The ability of CNPT-3 to attach was highest under elevated hydrostatic pressure and, based on relative percentage, was greatest under starvation conditions. This starvation response may be due to adaptations of the organism for survival in an environment very different from that of shallow-water organisms.

REFERENCES
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