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Effect of Growth Rate and Starvation-Survival on the Viability and Stability of a Psychrophilic Marine Bacterium†

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Cell populations of the marine bacterium ANT-300, from either batch or continuous culture with dilution rates ranging from $D = 0.015\ h^{-1}$ to $D = 0.200\ h^{-1}$, were monitored for viability, direct counts, and optical density for 98 days under starvation conditions. Three stages of starvation survival were observed for each of the cell populations. Although direct counts remained at 2×10^7 to 3×10^7 cells ml^{-1} throughout the starvation period, large fluctuations occurred in cell viability during stage 1 (0 to 14 days) of starvation survival. Stage 2 (14 to 70 days) involved an overall decrease in viability for each of the cell populations; the rate of viability loss was dependent upon the growth rate. Cell viability stabilized at approximately 0.3% of the direct count in stage 3 (70 to 98 days). Long-term starvation corresponded to the prolongation of stage 3 starvation survival. Cell volumes for each of the cell populations decreased with the length of the starvation period. However, the cell volume of starved cells was also dependent more on growth rate than on the length of the time starved. We hypothesize that the cell population with the slowest growth rate is most closely representative of cells found in the oligotrophic marine environment.

The survival of heterotrophic marine bacteria is dependent upon the ability to withstand long periods of nutrient deprivation. Below the photic zone, the ocean becomes extremely deficient in nutrients, resulting in a low average level of organic carbon in the open ocean. The dissolved organic carbon (DOC) concentration ranges from 0.35 to 0.70 mg $liter^{-1}$, and the particulate organic carbon (POC) concentration ranges from 3 to 10 $\mu g\ liter^{-1}$ (20). Generally, the POC concentration is equal to 10% of the DOC concentration in the open ocean. Both DOC and POC have been found to be at least partially refractory to bacterial degradation. Barber (3) observed no significant change in the levels of deep ocean DOC incubated for over 2 months with viable bacteria. However, it has been demonstrated that deep ocean POC hydrolyzed either enzymatically (12) or chemically (29) can then be used for growth by certain marine bacteria. Tranvik and Höfle (31) found the fraction of the POC consumed by heterotrophic bacteria ranged from 15 to 22% of the total DOC pool from various clear and humic freshwater sources. Geller (11) observed 12 to 22% decomposition of lake dissolved organic matter original macromolecules attributed to bacterial degradation. It is therefore unclear how much DOC and POC is bioavailable. Craig (7) calculated the total deep-ocean oxygen consumption to be 0.004 ml $liter^{-1}$ $year^{-1}$, indicating a low metabolic activity for all organisms. Morita (21) stated that low concentrations of nutrients become a factor that must be reconciled with residence time for deep ocean waters. Residence time for ocean waters can be on the order of 1,000 years (5). The turnover time for DOM has been estimated by Menzel (19) to be 3,000 years. More recently, Williams and Druffel (33) estimated DOC residence times of about 6,000 years. These factors suggest that the deep ocean is indeed low in utilizable nutrients and that starvation conditions do, in fact, exist.

The marine environment is a complex and dynamic ecosystem that is difficult to simulate under laboratory conditions. To control the types of variation that occur naturally and still attempt to approach in situ growth rates, continuous culture technology may be utilized. Bacteria can be grown under well-defined, reproducible conditions where all environmental parameters are kept constant, resulting in a steady-state population with a uniform growth rate. These populations can be used to study the effects of starvation survival on the sizes of cells grown at various rates. The cell populations can be compared entirely on the basis of the growth rate from which the respective population originated before starvation. Growth rates of natural assemblages of thymidine-incorporating marine bacteria have been estimated at 0.075 to 0.036 h^{-1} (8). Growth rates of natural assemblages, similarly estimated for freshwater bacteria, ranged from 0.25 to $<0.005\ h^{-1}$ (30). Specific growth rates as low as $0.005\ h^{-1}$ have also been estimated by Jannasch (15) and by Carlucci and Williams (6) for pelagic marine bacteria. However, it should be remembered that when chemostats are utilized to regulate the specific growth rate, the system will undergo washout when in situ values of organic matter are used, making continuous culture impossible (14, 16).

In this paper we address the question of how growth rate affects the survivability and size of a marine psychrophilic bacterium during starvation survival.

MATERIALS AND METHODS

Organism, media, culturing, and starvation. ANT-300, a heterotrophic, psychrophilic marine bacterium that has been tentatively identified as a *Vibrio* sp. (4), was employed in this study.

Lib-X medium (4) was modified by the addition of ferric EDTA as the iron source to increase the solubility of Fe for chemostat use. This modified medium contained 2.3 g of Trypticase-peptone (BBL Microbiology Systems), 1.2 g of yeast extract (Difco Laboratories), 0.3 g of L-glutamic acid, 0.05 g of $NaNO_3$, 0.007 g of ferric EDTA, 38.0 g of Rila Marine Mix, and 3.2 g of Tris buffer in 1 liter of distilled water. SLX medium differed from Lib-X only in that it

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contained 1/10 of the amount of organic material. A buffered salt mixture for starvation of the organism consisted of 26.0 g of NaCl, 0.8 g of KCl, 5.6 g of $MgCl_2 \cdot 6H_2O$, 7.6 g of $MgSO_4 \cdot 7H_2O$, and 3.2 g of Tris buffer in 1 liter of distilled water. The addition of Tris buffer allowed for the maintenance of a stable pH within the actively growing continuous culture or starvation menstruum. All three media were adjusted to pH 7.8 with 10 N HCl and 10 N NaOH. All solutions were autoclaved (121°C, 15 min), and the salt mixture was additionally filter sterilized twice by passage through 0.45-μm-pore-size membrane filters (Millipore Corp.) to minimize the interference of debris with total cell counts. All materials were held at 5°C before use, including pipette tips, centrifuge bottles, and media. Cells were also grown at 5°C (batch or continuous cultures).

Continuous cultures of ANT-300 were grown in SLX pumped into chemostats, each containing a total volume of 345 ml. Lib-X medium was used only for cells grown in batch culture. The growth medium in the chemostat came in contact with only silicone or borosilicate glass to minimize contamination of the system. The dilution rate in each chemostat was set and maintained constant by employing a Crouzet mini-peristaltic pump. Constant air flow (filter sterilized) was used to maintain aerobic conditions and to achieve uniform mixing within the chemostat. The populations in each chemostat were brought to equilibrium (10 to 12 generations) at each dilution rate and then harvested by centrifugation (4,080 × g, 15 min, 3°C). Batch cell cultures were cultivated in Lib-X in 2.8-liter Fernbach flasks, shaken at 100 rpm on a rotatory shaker at 5°C, until an optical density at 600 nm (OD_{600}) of 0.8 was attained (log growth phase). These were then harvested by centrifugation, washed twice with cold salt mixture, and suspended in sterile 4-liter aspirator bottles containing a Teflon stirring bar to an initial cell density of 1.5×10^7 to 3.5×10^7 ml^{-1}. These cultures were then starved, and at various periods subsamples were taken for analysis after slow stirring for 1 min to insure uniform cell density.

ANT-300 cell populations were monitored during growth and starvation spectrophotometrically (600 nm).

Viability determinations. CFU were determined by the spread plate technique with SLX plus 1.2% agar. Plates were incubated for 7 to 10 days, after which cells were considered viable if they produced a visible colony. Quadruplicate determinations were made and averaged in all cases. SLX medium was chosen for plate counts due to the high recovery rate for ANT-300 after starvation as shown by J. A. Novitsky (Ph.D. thesis, Oregon State University, 1977).

Direct counts. Direct counts were obtained by acridine orange staining and epifluorescent microscopy (AODC). This method was essentially developed by Francisco et al. (9) and modified by the use of Nuclepore filters as described by Zimmermann and Meyer-Reil (34). The procedure was further modified by the use of Irgalan-black-darkened filters (13, 32). K. A. Hoff (Ph.D. thesis, University of Bergen, Bergen, Norway, 1984) improved the method for marine bacteria that exhibited cellular lysis with the use of earlier procedures by minimizing the osmotic shock before fixing. Samples were fixed with buffered Formalin (final concentration, 1%) and then filtered onto Nuclepore filters (0.2-μm pore size) and stained. Silver membrane prefilters (Selas Flotronics, 0.8-μm pore size) were used as supports for the Nuclepore filters to ensure uniform cell distribution. After staining, filters were air dried and then mounted in oil on glass slides with cover slips. Preparations were viewed with a Zeiss epifluorescence microscope. For each sample, 10 to 20 fields were counted and averaged. The total number of cells per milliliter was calculated by using corresponding dilution factors and a microscopic factor (area conversion and magnification).

TABLE 1. ANT-300 dilution rates, with corresponding growth rates and doubling times

Dilution rate[a] D (h^{-1})	Growth rate μ (h^{-1})	Doubling time t_d (h)
0.015	0.015	46.2
0.057	0.057	12.2
0.170	0.170	4.1
0.200	0.200	3.5
Batch culture[b]	0.144	4.8

[a] Cells were grown in continuous culture with SLX medium.
[b] Cells were grown with Lib-X medium.

Cell volume determination. ANT-300 cell volumes were determined by sizing acridine orange-stained cells from the various cell populations. Because of the significant cell shrinkage (up to 37%) that occurs in the preparation of cells for the electron microscopy, epifluorescence microscopy was used to determine cell sizes (10). Cells were stained by using the procedure described above for AODC. Measurements were made from projected photographic images of cells and substage micrometer. Cell sizes were calculated against a substage micrometer, averaging 20 cells from each population. The formulas $V = 4/3\pi r^3$ and $V = \pi r^2 h$, were used to calculate the volumes of starved (spherical) and unstarved (cylindrical) cells, respectively.

RESULTS

ANT-300 cell populations with dilution rates ranging from $D = 0.015$ h^{-1} to $D = 0.200$ h^{-1} are listed in Table 1 along with the corresponding doubling times and growth rates. These cell populations were monitored for total and viable cells as well as turbidity during starvation. At the beginning of the starvation period, the concentrations of viable and total cells were approximately 3×10^7 cells ml^{-1} in all cell populations examined.

The viability of cells from $D = 0.015$ h^{-1} (Fig. 1) began to significantly decrease only after 56 days of starvation, when it then dropped nearly 2 orders of magnitude within 1 week. After 70 days of starvation, viability stabilized at approximately 10^5 viable cells (CFU) ml^{-1}. This corresponds to

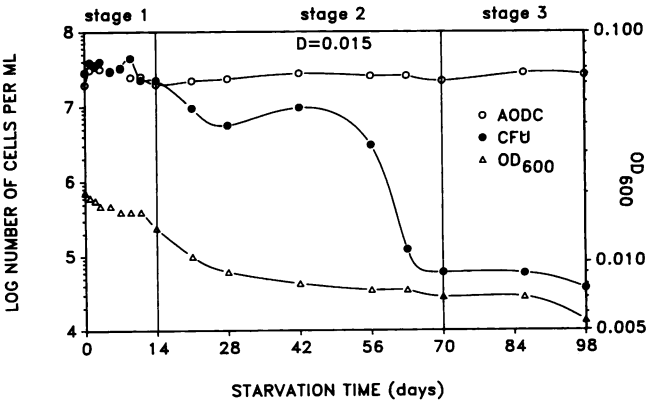

FIG. 1. Total cells, viable cells, and OD_{600} with starvation time for cells from $D = 0.015$ h^{-1}.

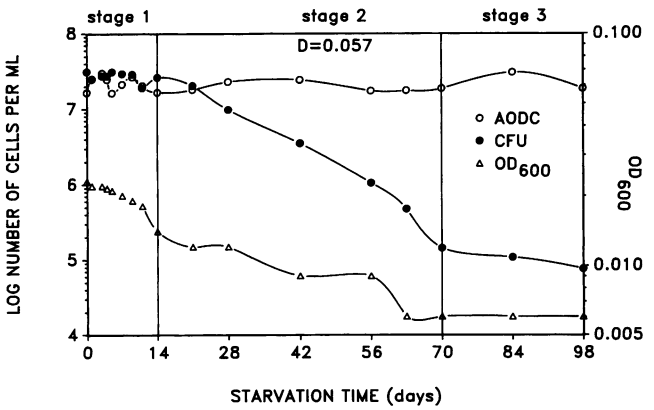

FIG. 2. Total cells, viable cells, and OD_{600} with starvation time for cells from $D = 0.057\ h^{-1}$.

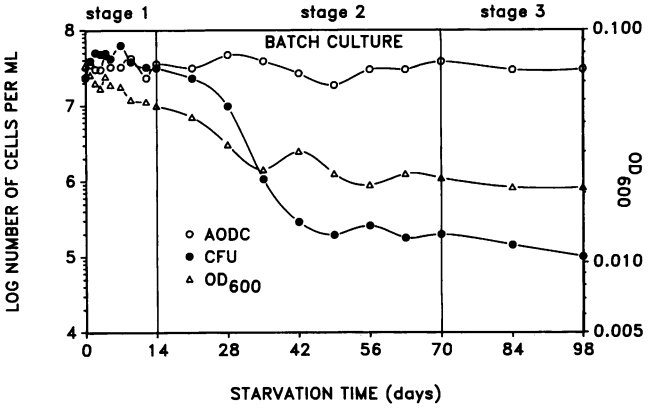

FIG. 4. Total cells, viable cells, and OD_{600} with starvation time for cells from batch culture.

0.3% viability of the total cell number, which remained at 2×10^7 to 3×10^7 cells (CFU) ml^{-1}. Cells from $D = 0.057\ h^{-1}$ (Fig. 2) began dropping in viability after 21 days at a more gradual but constant rate. Again, after 70 days of starvation, viability stabilized at nearly the same level as the $D = 0.015\ h^{-1}$ cell population and maintained at 0.3% of the total cell number. $D = 0.170\ h^{-1}$ cells also lost their viability at a gradual and constant rate (Fig. 3). This began somewhat earlier (7 to 14 days) than for the cells at $D = 0.015\ h^{-1}$ and $D = 0.057\ h^{-1}$. After 70 days of starvation, viability stabilized at approximately 0.3% of the total cell number, and cultures maintained viable cells at the same levels as those of the cell populations from $D = 0.015\ h^{-1}$ and $D = 0.057\ h^{-1}$. Batch culture cells (Fig. 4) began to lose viability after 21 days but showed a significant drop (2 orders of magnitude) in the next 2 weeks. This was much faster than any of the other continuous cultured cell populations tested. Again, after 70 days of starvation, viability stabilized at 0.3% of the total cell numbers or at a cell density of approximately 10^5 cells (CFU) ml^{-1} and was maintained throughout the remainder of the 98-day starvation period, similar to all of the other ANT-300 cell populations examined.

For all cell populations, total cell numbers contrasted with those of viable cell counts in that total cell numbers remained at a nearly constant cell density of 2×10^7 to 3×10^7 cells ml^{-1}, showing no significant changes during the entire starvation period. Likewise for all cell populations turbidity measurements (OD_{600}) indicated steady decreases in biomass, although the faster-growing ($D = 0.170\ h^{-1}$ and batch culture) cells were able to maintain higher levels of biomass throughout the starvation period. This was most likely due to the accumulation of residual cellular material at the faster growth rates or when nutrient rich medium was used in cultivation, respectively.

Long-term starvation of a cell population grown at $D = 0.200\ h^{-1}$ (Fig. 5) showed a pattern similar to that of cells grown at $D = 0.170\ h^{-1}$ (Fig. 3). This included an analogous loss with respect to viable cell counts during stage 2. However, after the initial decrease in viability ending at 70 days, a stabilization or exceptionally slow rate of decreasing viability continued throughout stage 3. This extended starvation period, which was monitored for 175 days, still ended with viable cells equalling approximately 0.3% that of the total cells. Once more, total cell numbers remained nearly constant throughout, indicating minimal cell lysis.

Cell volumes from each cell population were determined for unstarved and starved cells (Fig. 6 and 7) and are summarized in Table 2. Unstarved log-phase cells (Fig. 6) were much larger than any other cell population at $5.94 \pm 0.456\ \mu m^3$ (mean ± SEM). During the microscopic viewing of unstarved $D = 0.170$, 0.057, and $0.015\ h^{-1}$ cells, no cells as large as the unstarved log-phase cells were observed.

The cell volumes for starved cells from each of the ANT-300 cell populations examined are shown in Fig. 7.

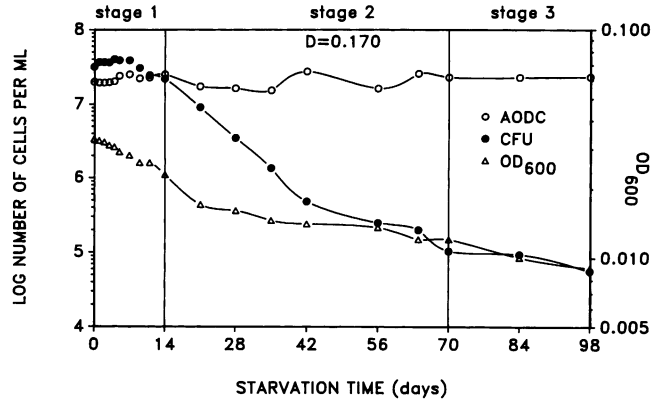

FIG. 3. Total cells, viable cells, and OD_{600} with starvation time for cells from $D = 0.170\ h^{-1}$.

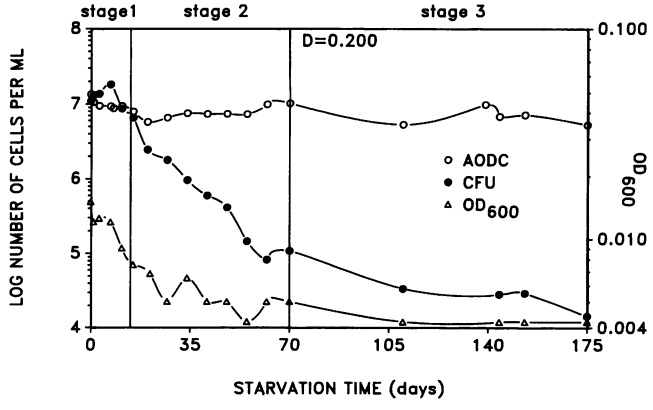

FIG. 5. Total cells, viable cells, and OD_{600} with starvation time for cells from $D = 0.200\ h^{-1}$. Long-term study.

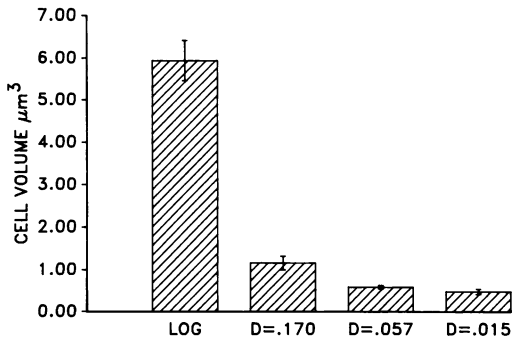

FIG. 6. Average cell volume of unstarved cells. Cell populations sampled were as follows: Batch culture (labeled LOG), $D = 0.170$ h^{-1}, $D = 0.057$ h^{-1}, and $D = 0.015$ h^{-1}. The hatched bars represent the means of 20 cell volume determinations from each population; the vertical bars represent the standard errors of the means.

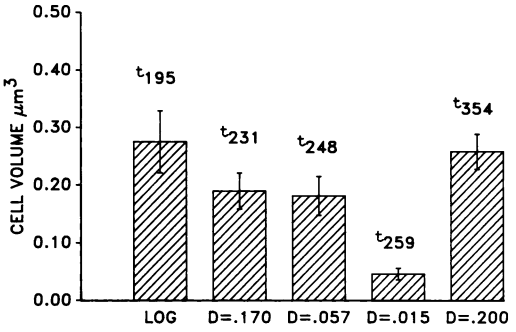

FIG. 7. Average cell volume of starved cells. Cell populations sampled were as follows: Batch culture (labeled LOG) starved for 195 days, $D = 0.170$ h^{-1} starved for 231 days, $D = 0.057$ h^{-1} starved for 248 days, $D = 0.015$ h^{-1} starved for 259 days, and $D = 0.200$ h^{-1} starved for 354 days. The hatched bars represent the means of 20 cell volume determinations from each population; the vertical bars represent the standard errors of the means.

Cells from the $D = 0.015$ h^{-1} population were substantially smaller in volume at 0.046 ± 0.010 μm^3 than any other cell population. However, these cells were starved for 95 days less than cells from the $D = 0.200$ h^{-1} population, which maintained an average cell volume of 0.258 ± 0.030 μm^3 after nearly 1 year of starvation. This indicates that the cells from $D = 0.015$ h^{-1} populations remained at a reduced average cell volume when compared with all other cell populations throughout the starvation period. The percent reduction in cell volume as indicated in Table 2 demonstrates that the $D = 0.015$ h^{-1} and batch culture cells were nearly equal in capability for size reduction at 90.4 and 95.4%, respectively. However, the $D = 0.015$ h^{-1} cells were considerably smaller overall by approximately 1 order of magnitude. The intermediate growth rate populations had a lesser ability for volume reduction at 83.7% for $D = 0.170$ h^{-1} and 69.1% for $D = 0.057$ h^{-1}.

DISCUSSION

The overall pattern from all of these data indicates three separate stages of physiological change occurring with respect to viable counts: (i) large fluctuations with moderate overall decreases within the first 14 days; (ii) a 99.7% decrease between days 14 and 70, with the rate of viability loss depending upon the growth rate of the original cell population; and (iii) the stabilization of the viable cell subpopulation after 70 days to approximately 10^5 cells (CFU) ml^{-1} or 0.3% viability of total cell numbers. The difference between viable cell counts and total cell numbers may be attributed to the survival of viable but nonreplicating cells (28). This has been demonstrated with ANT-300 by utilizing 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl-tetrazolium-formazan production, which indicated that up to 45 to 60% of the cells remained viable after 6 weeks (1). It should also be noted that population effects such as cryptic growth or growth from contaminating nutrients can be ruled out for ANT-300 (27).

TABLE 2. Average cell volume measurements for starved and unstarved ANT-300 cells

Dilution rate[a] D (h^{-1})	Cell volume (μm^3) ± SEM		% Reduction in volume
	Unstarved	Starved[b]	
Batch culture[c]	5.94 ± 0.465	0.275 ± 0.053	95.4
0.170	1.16 ± 0.156	0.189 ± 0.030	83.7
0.057	0.585 ± 0.038	0.181 ± 0.033	69.1
0.015	0.478 ± 0.060	0.046 ± 0.010	90.4
0.200		0.258 ± 0.030	

[a] Cells were grown in continuous culture with SLX medium.
[b] Cells from stage 3 starvation survival.
[c] Cells were grown with Lib-X medium.

The duration of the three stages of starvation survival need not be identical for other marine heterotrophic bacteria. However, the existence of the three stages has been demonstrated in other marine heterotrophic species. The viability of a marine *Pseudomonas* sp. was found by Kurath and Morita (17) to have an initial increase lasting for 5 days, corresponding to the duration of stage 1. After this, viability dropped to 0.1% of that of the total population after just 25 days, corresponding to stage 2. Finally, viability stabilized for the remainder of the 40-day starvation period, corresponding to stage 3. This pattern of starvation survival is most likely indicative only of heterotrophic bacteria adapted to survival for extensive periods of time without significant nutrient inputs and should not be considered exclusive to all bacterial forms. Presently, there are four acknowledged patterns of starvation survival (23). The pattern of starvation survival in this study is most likely indicative of cells undergoing starvation, in which the population increases initially but decreasing during starvation (23). This pattern has been studied the most in terms of starvation survival.

The rate of viability loss during stage 2 indicates that survival is dependent on the growth rate of the cell source population. Cells grown at $D = 0.015$ h^{-1} showed greater survival than the other cell populations. The viable cell counts began to drop only after 56 days of starvation survival. At this time an accelerated drop in viability of over 2 orders of magnitude to 0.3% of the total cell level occurred before the culmination of stage 2. This is markedly different from the viability pattern seen for ANT-300 cell populations, which originally showed faster growth rates during the starvation period. Cell populations originally grown at $D = 0.057$ h^{-1} and $D = 0.170$ h^{-1} were similar; both demonstrated continuous rates of viability decrease from the onset to the end of stage 2. However, they did differ in that cells grown at $D = 0.057$ h^{-1} were able to sustain their survival 7 to 14 days longer before the viability counts began to decrease at slightly slower rate. Cells from both growth rates eventually dropped in viability to approximately 0.3% of the total cell level. Cells originating from logarithmic growth in

batch culture showed a pattern that was the opposite of that found in $D = 0.015\ h^{-1}$ cells. These cells showed a significant decrease in viability early in stage 2, dropping to nearly the 0.3% level of viability within 28 days and stabilizing at that level for the remainder of the starvation period. Therefore, both extreme cases in growth rate ($D = 0.015\ h^{-1}$ and batch culture) demonstrated decreases in viability which were substantially different from the intermediate growth rates; the loss of viability did not occur at a constant rate, even though the eventual cell densities were basically the same.

Once 70 days of starvation survival has been reached (stage 3), the stabilization of viability for each of the cell populations, relative to total cell counts (Fig. 1 through 5), indicates that the stabilization of certain cellular processes is common to all cell populations during starvation survival regardless of the original growth rate. This stabilization is presumed to be due to the metabolic arrest, hypothesized by Morita (25), that occurs during long-term starvation. The importance of long-term starvation studies has also been demonstrated by the survival of a strain of *Alteromonas denitrificans*, which survived in unsupplemented seawater for up to 7 years and had 0.9% viability of the total cell population even after 7.5 months (26).

Throughout the starvation survival period a general decrease in biomass as determined by OD_{600} measurements (Fig. 1 through 5) was observed. Cell volume measurements also indicated a decrease in average cell volume during the starvation of each of the ANT-300 cell populations examined (Fig. 6 and 7). This substantial decrease in volume during starvation increases the surface/volume ratio for each of the cell populations. An increased surface/volume ratio is advantageous for cells because of the increased ability to scavenge energy-yielding substrates from nutrient-limited environments as reviewed by Morita (22). An overall decrease in the average cell volume or size of a cell also allows it to escape predation pressure as found by Andersson et al. (2).

Regardless of starvation time, the average cell volume for each population was dependent upon the original growth rate over the length of starvation time. This factor must be considered by those studying the starvation survival of bacteria from any environment. Morita (24) has stated that ultramicrocells are the dominant or "normal" state of bacterial cells in the marine, aquatic, and terrestrial environments (25). The ANT-300 cells from the slowest-grown cell population ($D = 0.015\ h^{-1}$) showed the smallest average cell volume at $0.046 \pm 0.010\ \mu m^3$ after being starved for 251 days. This contrasts with cells grown at $D = 0.200\ h^{-1}$ and starved for 352 days; these cells had a average cell volume of $0.258 \pm 0.030\ \mu m^3$ (starved for 95 days longer). These data indicate that cells with a greater surface/volume ratio at the beginning of starvation-survival are able to maintain a greater surface/volume ratio throughout the entire starvation survival period. However, once again the extreme growth rates of $D = 0.015$ and batch culture demonstrated the greatest capability to reduce cell volume, even though the slow-growth-rate cells were almost 1 order of magnitude smaller. This suggests that the $D = 0.015\ h^{-1}$ cells would be better adapted to the survival of long-term starvation than would other cell populations. This study has also shown that cells are not required to become large (i.e., log-phase cell size, Fig. 6) before they divide. Maeda and Taga (18) showed that at one station in the Pacific Ocean, 2.2% of the cells were less than 0.4 μm, 73.7% were between 0.4 and 0.8 μm, 22.6% were between 0.8 and 1.2 μm, 1.6% were between 1.2 and 1.6 μm, and none was larger than 1.6 μm. This brings up the question as to how large a cell must be before division can take place under starvation or nutrient-poor conditions.

We have concluded from these data that the ANT-300 cells grown at $D = 0.015\ h^{-1}$ most closely represent cells that would be found in situ because of the increased survivability and the maintenance of a greater surface/volume ratio associated with this cell population during starvation survival. Therefore, to accurately study the effects of starvation, cells must be grown as close to in situ growth rates as possible and without the copious amounts of nutrients that are normally used to culture bacteria. In addition, the period of starvation should be sustained long enough for the advance of stage 3 starvation survival to achieve stabilized metabolic arrest.

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