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Effect of Growth Rate and Starvation-Survival on Cellular DNA, RNA, and Protein of a Psychrophilic Marine Bacterium†

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DNA, RNA, and protein concentrations from starved ANT-300 cell populations grown at different growth rates fluctuated corresponding to the three stages of starvation-survival on total and viable cell bases. During stage 1 of starvation-survival, two to three peaks in the concentration levels for all three macromolecules were characteristic. During stage 2, DNA per total cell dropped to between 4.2 and 8.3% of the original amount for all of the cell populations examined, and it stabilized throughout stage 3. The decrease in DNA per cell was also observed in electron micrographs of cellular DNA in unstarved compared with starved cells. The fluctuations of RNA and protein per total cell concentrations observed during stage 2 coincided in all cases, except for the cells from dilution rate (D) = 0.015 h^{-1}. This ANT-300 cell population showed a decrease in RNA per total cell to only 29.2% and an increase in protein to 129.7% of the original amount after 98 days of starvation. During stage 3, DNA, RNA, and protein concentrations per total cell also stabilized to continuous levels. Cells from the faster-growth-rate cell populations of D = 0.170 h^{-1} and batch culture had elevated protein per total cell concentrations, which remained primarily residual during the starvation period. Starved cells from D = 0.015 h^{-1} had estimated nucleoid and cell volumes of 0.018 and 0.05 μm^3, respectively, yielding a nucleoid volume/cell volume ratio of 0.40. We consider these data to indicate that slow-growth-rate cells are better adapted for starvation-survival than their faster-growth-rate counterparts.

The intracellular macromolecule concentrations for various marine bacteria have been previously measured in starved cells (2, 6, 20, 26). Four Feulgen-staining bodies have been found in ANT-300 cells growing in the log phase in batch culture with rich medium (Lib-X). When these cells were starved, they had only one per cell (28). Protein synthesis has been demonstrated to occur when cells are starved (1, 6, 10, 29). De Ley (4) determined the amounts of DNA in *Escherichia coli* in the early, middle, and late logarithmic phases and in late stationary phase to be 9.56, 9.17, 9.70, and 7.35 μg/10^9 cells. However, it must be noted that these experiments were conducted only on cells which were grown in batch culture with nutrient-rich media before they were starved. Moyer and Morita (25) found that there were differences in the starvation patterns and sizes of cells that were grown under various growth rates in a dilute medium, as well as those from batch culture grown on a rich medium. The small size of bacteria which is achieved during starvation is most likely a strategy for the maximizing of nutrient uptake via increased surface area/volume ratio, as well as for escaping predation by bacteriovores. Cell size reduction has been shown for ANT-300 during starvation-survival (25, 27). This cellular reduction is enhanced by the slower-growth-rate population (dilution rate [D] = 0.015 h^{-1}) of ANT-300, which is able to maintain a substantially smaller cell volume of 0.05 μm^3 when starved (25).

We have also estimated the nucleoid volumes of unstarved and starved ANT-300 cells. This is important because the amount of DNA in seawater has been used to extrapolate microbial biomass (3, 31–34). In addition, most bacterial cells in the marine environment are ultramicrocells (9, 16, 17, 36) and are in the starved state (23). This paper addresses the differences between the DNA, RNA, and protein in cells grown under various growth rates in dilute medium as compared to those grown under batch culture in rich medium.

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MATERIALS AND METHODS

Organisms, media, and cultivation. The cells, media, and cultivation of ANT-300, a marine psychrophilic vibrio, were identical to those cited by Moyer and Morita (25).

Nucleic acid assay. Nucleic acids were estimated spectrofluorimetrically by using the intercalating compound ethidium homodimer (Molecular Probes, Eugene, Oreg.), which has been found to be up to 100 times more sensitive to DNA concentrations than ethidium bromide (22). An assay was designed for the detection of nucleic acids in marine bacteria by using RNase on subsamples to estimate for both RNA and DNA. Subsamples were measured in duplicate (C. L. Moyer, M.S. thesis, Oregon State University, Corvallis, 1988).

Protein assay. Total protein was measured by a new, highly sensitive method in which bicinchoninic acid is used (35). Protein concentrations in the range of 0.5 to 10.0 μg/ml were measured by the micro-reagent procedure, also described by Smith et al. (35).

Prior to running the standard micro-reagent procedure, cell samples with 0.1% (vol/vol) of 10% Triton X-100 were treated with sonication (Branson Sonifer Cell Disrupter model 350; Branson Sonic Power Co., Danbury, Conn.) for 30 s. The only alterations made from the standard micro-reagent procedure were that 0.16% (wt/vol) was used in the preparation of micro-reagent A, instead of 1.6% (wt/vol), and the sample size volume in the reaction tube was doubled in order to achieve better reproducibility.

Protein concentrations of samples in quadruplicate were estimated against a standard curve prepared for each sam-

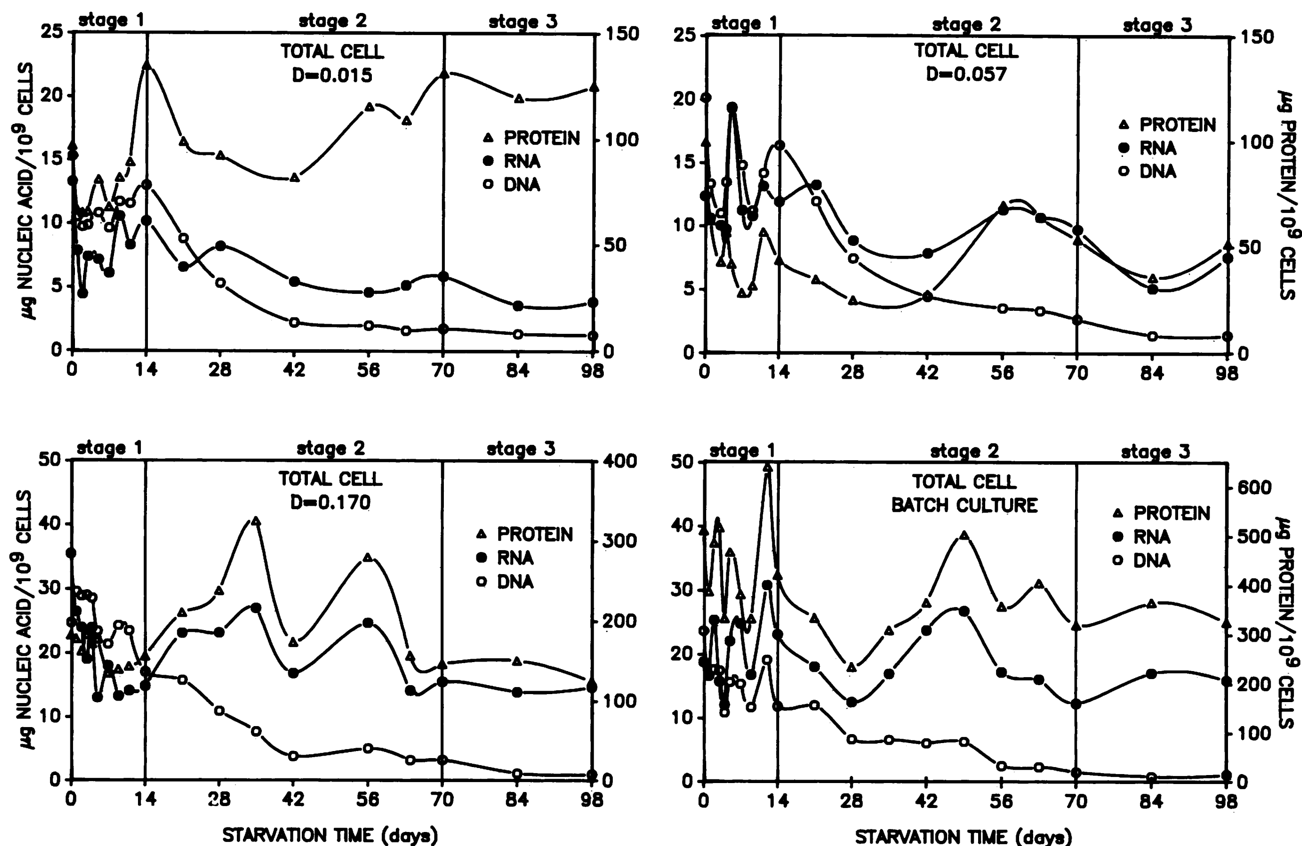

FIG. 1. DNA, RNA, and protein per total cell, with starvation times and stages for cells grown under different growth rate conditions.

pling set by using a spectrophotometer at 562 nm (Spectronic 710; Bausch & Lomb, Inc., Rochester, N.Y.).

DNA electron microscopy. The DNA from ANT-300 cells was extracted and isolated for electron microscopy analysis by using a modified alkaline lysis (19) in order to isolate intact chromosomal DNA from the cells while eliminating as much cellular debris as possible. The DNA was then suspended to approximately 5 μg/ml (optical density at 260 nm ≈ 0.1) in 50 mM NaCl-5 mM EDTA (pH 7.5) and hydrophobically transferred onto Formvar copper-coated grids. DNA samples were chromium shadowed under a vacuum and viewed with a Philips EM 300 microscope.

RESULTS

During the starvation period, the concentrations of the macromolecules (DNA, RNA, and protein) were monitored on a per total cell basis (Fig. 1) and a per viable cell basis (Fig. 2). Stages 1, 2, and 3 represent three distinct stages during the starvation process and correspond to the viable and total cell count data previously presented (25). During the initial starvation-survival period, DNA, RNA, and protein normalized to direct cell counts showed similar patterns of wide fluctuations. This corresponds to stage 1 of the starvation-survival period. All three of the macromolecules within stage 1 showed two to three characteristic peaks in concentration level. During stage 2, each of the populations showed DNA levels which steadily dropped on a per total cell basis and then stabilized at approximately 42 to 56 days; RNA concentrations remained higher than DNA concentrations and, with the exception of the D = 0.015 h^{-1} cells, followed the fluctuations in protein concentration very closely. However, it should be noted that for the faster-growing cells derived from D = 0.170 h^{-1} and batch culture (Fig. 1), the concentration scale for proteins was up to approximately four times higher than that of the total cells for D = 0.015 h^{-1} and D = 0.057 h^{-1} (Fig. 1) during the entire 98-day period of starvation. During stage 2, it was also observed that protein concentrations per total cell (Fig. 1), after initially dropping, increased twice before dropping again for all cell populations except D = 0.015 h^{-1}, in which the concentration remained elevated throughout stage 3. These two peaks in protein concentration which occurred for each of the different cell populations during stage 2 were also characteristic. Finally, stage 3 shows an overall stabilization in DNA, RNA, and protein concentrations for all of the cell populations.

Over the course of starvation-survival, ANT-300 batch culture cells showed significant decreases in DNA per total cell. Only 5.2% of the original DNA per total cell remained after 98 days of starvation. However, RNA and protein per total cell for the batch culture cells decreased minimally to 85.0 and 63.7% of the original amounts per total cell, respectively, at the end of the 98-day starvation period. The cells from D = 0.170 h^{-1} showed overall decreases in DNA to 4.2%, in RNA to 41.4%, and in protein to 68.6%. The cells from D = 0.057 h^{-1} showed overall decreases in DNA to 7.2%, in RNA to 61.1%, and in protein to 51.6%. The D = 0.015 h^{-1} cells also demonstrated a decrease in DNA per total cell to 8.3% after 98 days of starvation. However, the

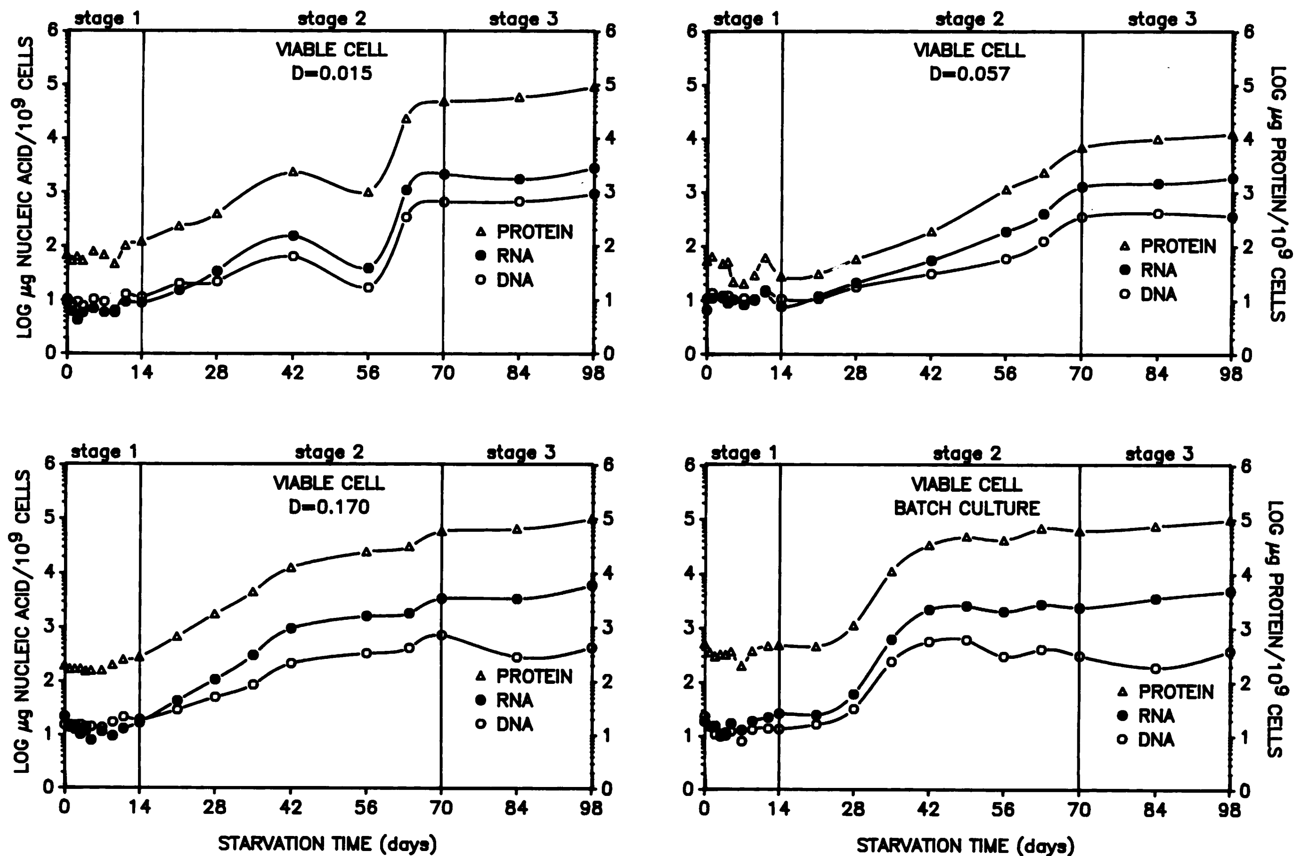

FIG. 2. DNA, RNA, and protein per viable cell, with starvation times and stages for cells grown under different growth rate conditions.

RNA per total cell declined steadily during stage 2 to only 29.2%, and the protein per total cell increased to 129.7% of the original amount. This demonstrates an overall protein production and accumulation on a per total cell basis during the starvation period.

All three stages of starvation-survival showed reproducible patterns of DNA, RNA, and protein levels normalized to viable cells (Fig. 2). Once again, stage 1 showed a high degree of fluctuation, with two to three characteristic peaks in cellular DNA, RNA, and protein for each population. During stage 2, amounts of all macromolecules increased 2 to 3 orders of magnitude. The large overall increases in macromolecule levels reflect a bias due to the magnitude of losses in viable cells, especially during stage 2, for each of the populations examined. Stage 3 showed a stabilization for each of the macromolecule concentrations with respect to viable cells in each of the populations. These results are influenced by the inability to separate the viable cells from dead or dormant cells in each population. However, intact cells have been demonstrated to retain the majority of their macromolecules, including DNA and RNA (2, 8). It should also be recognized that acridine orange is an intercalator of nucleic acids and that the normalization of the data to viable counts would produce a greater error, due to the nucleic acids present in the remainder of the cells measured via direct counts. Therefore, the data for concentrations of macromolecules in total cells are considered to have the highest accuracy. Overall, the cells from each of the populations remained intact throughout the starvation period, helping to support the validity of this assumption, as was also demonstrated by acridine orange staining and epifluorescence microscopy enumeration (25).

In addition to the quantification of cellular DNA levels during starvation-survival, unstarved and starved cellular DNA was qualitatively analyzed by electron microscopy (Fig. 3). The cellular DNA from an unstarved cell logarithmically grown in batch culture is presented in Fig. 3A. The cellular DNA from a single cell grown by the same method, which was starved 98 days, is presented in Fig. 3B. The latter DNA molecule is significantly smaller, demonstrating the loss of DNA which occurs during starvation-survival.

The DNA per total cell was calculated for unstarved and starved cell populations of ANT-300 (Table 1). The DNA levels per total cell decreased during starvation and stabilized in stage 3 at between 1.0 and 1.5 fg of DNA per total cell. This was then related to the corresponding cell volumes for each of the unstarved and starved cell populations (25). If we make the assumption that the DNA packaging efficiency in ANT-300 cells is equal to that of *E. coli* (0.07 μm^3/5.0 fg) (38), then the nucleoid volume for each of the ANT-300 cell populations can be estimated (Table 1). The estimated nucleoid volume for starved cell populations is approximately 1 order of magnitude less than the estimated nucleoid volume for unstarved cell populations. By using this estimation, we calculated the nucleoid volume/cell volume ratio in order to achieve a relative volume percentage of the DNA content. Therefore, the DNA in the unstarved ANT-300 cell populations would be between 30 to 48% for continuous-culture cells, whereas it would be only 6% for batch culture cells. Upon stage 3 starvation, the DNA in the continuous-culture

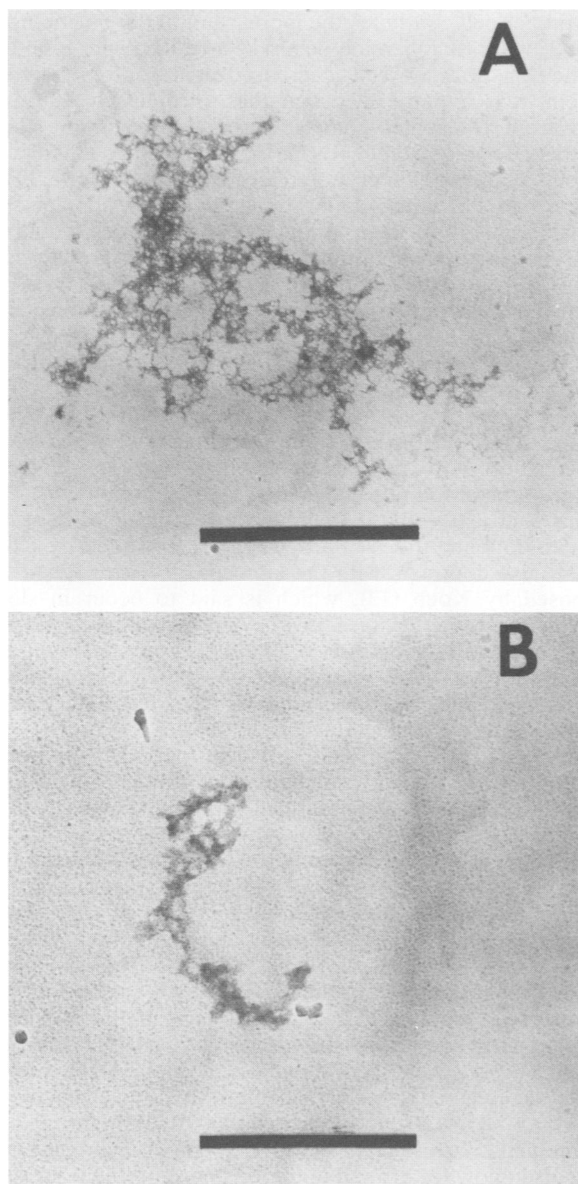

FIG. 3. Electron micrographs of the DNA molecule from a logarithmic-growth unstarved cell (A) and stage 3 starved cell (B). Bars = 1 μm.

TABLE 1. Effect of growth rate on DNA content in relation to average cell volume for unstarved and starved ANT-300 cells

Culture	DNA/cell (fg) ± SEM	Cell vol[a] (μm^3)	Estimated nucleoid vol (μm^3)	Nucleoid vol/cell vol
Unstarved				
Batch	23.66 ± 0.01	5.94	0.33	0.06
$D = 0.170\ h^{-1}$	24.69 ± 0.01	1.16	0.35	0.30
$D = 0.057\ h^{-1}$	20.12 ± 0.06	0.59	0.28	0.48
$D = 0.015\ h^{-1}$	15.33 ± 0.27	0.48	0.21	0.45
Starved[b]				
Batch	1.23 ± 0.17	0.28	0.017	0.06
$D = 0.170\ h^{-1}$	1.03 ± 0.35	0.19	0.014	0.08
$D = 0.057\ h^{-1}$	1.45 ± 0.02	0.18	0.020	0.11
$D = 0.015\ h^{-1}$	1.28 ± 0.02	0.05	0.018	0.40

[a] Data from Moyer and Morita (25).
[b] All cell samples were taken from stage 3 of starvation-survival.

cell populations of $D = 0.057\ h^{-1}$ and $D = 0.170\ h^{-1}$ (11 and 8%, respectively) decreased to approximately the same level as that of the batch culture cells (6%). The $D = 0.015\ h^{-1}$ ANT-300 cell population was able to maintain the highest nucleoid volume/cell volume ratio at 40%, which was nearly the original level of 45%.

DISCUSSION

The cellular levels of DNA, RNA, and protein showed similar fluctuation patterns during stage 1 of starvation-survival. The characteristic two to three major peaks with respect to the concentration of each of the macromolecules were monitored for each of the cell populations examined and were calculated on both total cell (Fig. 1) and viable cell (Fig. 2) bases. The fluctuations which took place during stage 1 occurred with greater magnitude and periodicity than any others seen throughout the rest of starvation-survival. This suggests a redistribution of cellular constituents, primarily during stage 1, which prepares the cells for long-term starvation-survival. Mården et al. (21) demonstrated that a period of metabolic shutdown occurs during which exchanges with the environment are reduced, as displayed by decreased oxygen uptake, but that an initial energy-dependent reorganization involving an increased endogenous respiration occurs in the marine *Vibrio* sp. strain S14. Mården et al. (20) and Nyström et al. (30) have observed bacterial DNA synthesis and protein synthesis, respectively, which occur during the first few hours of starvation for the marine *Vibrio* sp. strains S14 and DW1. Overall, this process represents a metabolic adjustment which leads to the cellular adjustment of the macromolecules DNA, RNA, and protein and, in all probability, other cellular components. During starvation, the cells must rely on internal energy reserves as the sole energy supply. Evidence in support of this is shown by Hood et al. (8), who starved *Vibrio cholerae* and found that its entire reserves of poly-β-hydroxybutyrate were utilized in 7 days of starvation-survival. The poly-β-hydroxybutyrate reserves were utilized faster in marine *Vibrio* sp. strain S14 and could not be detected after only 3 h when S14 was starved under complete energy- and nutrient-depleted conditions (18). Changes in the fatty acid profile of starved *V. cholerae* cell membrane have been demonstrated to occur within 7 days (7). It is possible that the high degree of fluctuation in macromolecule concentrations, especially that seen on a total cell basis, is representative of switching metabolism towards nutrient scavenging prior to preparation for long-term starvation-survival. This phenomenon has also been observed through the production of high-affinity uptake systems (5) and increased chemotaxis (37) in ANT-300 cells.

After the initial transition period, the cells entered stage 2, which was a period in which the fluctuations of intercellular constituents were buffered from any drastic changes due to the decrease in metabolic activity. Endogenous metabolism for ANT-300 cells has been shown to decrease to 0.0071% total carbon respired per h after 7 days and to remain constant for the rest of the experiment (28 days in total) (28). The endogenous metabolism of the marine *Vibrio* sp. strain DW1 has been shown to decrease during initial starvation-survival to 58% after 5 h and to 6% after 5 days, via oxygen uptake measurements (11). It is during this period that the metabolism slows to a point which leaves the cell unable, or

with limited ability, to catabolize macromolecules such as DNA, RNA, and protein. This is most likely due to the lack of usable carbon, which is quickly exhausted. It is also during stage 2 that the viability of cells diminishes to only 0.3% of that of total cell numbers and remains there for the duration of starvation-survival (25).

The final metabolic adjustment, as the cell enters stage 3, is the conservation of the essential cellular molecules to permit survival for long periods of time, as well as to retain the mechanism by which the cell can use energy again when it becomes available. In other words, the cells undergo an overall change so that they can become physiologically fit to withstand the loss of energy in the system and adapt to the starvation-survival mode. The fact that the total cell numbers remain close to original levels indicates that the cells do not lyse with the onset of starvation-survival but remain in a state of metabolic arrest which allows them to survive extremely long periods of time without further nutrient input, i.e., stage 3 starvation-survival (24).

The peak levels in protein and RNA per total cell, as demonstrated by all the populations of ANT-300 during mid-to-late stage 2 of the starvation period as well as the stage 3 elevated protein levels of the $D = 0.015\ h^{-1}$ cell population, probably represent the production of starvation-resistant proteins. Starvation proteins for ANT-300 cells have been demonstrated by Amy and Morita (1) to occur only after a sufficient starvation period has elapsed. The synthesis of starvation proteins has also been observed for *E. coli* (6) and three marine isolates, including the marine *Vibrio* sp. strains DW1 and S14 (10). The de novo synthesis of membrane and periplasmic proteins under starvation conditions for the marine *Vibrio* sp. strain S14 has been demonstrated by Nyström et al. (29), who also showed that the rate of proteolysis during early stage 1 is 16 times greater than during exponential growth. An enlarged periplasmic space in ANT-300 cells has also been shown after 5 weeks of starvation (27).

As stated above, the cellular protein concentrations for the $D = 0.0170\ h^{-1}$ and batch culture populations are up to four times higher than those of the other total cell populations (Fig. 1). These data indicate that the cells from $D = 0.170\ h^{-1}$ and batch culture had to contend with elevated protein concentrations during starvation-survival but had insufficient energy for their utilization. Koch (12, 13) has found that *E. coli* is not capable of degrading certain classes of proteins under similar conditions. One possible reason why these cell populations are unable to degrade these high levels of protein during starvation is the unavailability of sufficient energy for the enzymes responsible for proteolysis during stages 1 and 2 of the starvation process. This could also be due to the disproportional amounts of the macromolecules represented in the cell at the onset of starvation-survival, which skews the ecologically normal metabolic arrest process resulting from an unusually fast growth rate or high levels of nutrients during laboratory culturing. Nevertheless, because these cell populations were apparently unable to degrade the high levels of protein acquired from fast growth and rich media, high residuals of protein were observed throughout all three stages of starvation-survival, even though the production of starvation proteins still occurred.

The concentrations of cellular protein and RNA per total cell were closely coupled through stages 2 and 3 for all cell populations examined, except for $D = 0.015\ h^{-1}$. These uncouple early at the beginning of stage 2, when the RNA per cell decreases much like the DNA per cell, and the protein per cell continues the increase until the beginning of stage 3, when stabilization occurs. Since RNA per total cell for the $D = 0.015\ h^{-1}$ cells decreased significantly relative to protein, it is hypothesized that these proteins were translated from ribosomal complexes (rRNA being the most abundant form of stable RNA) that were more efficient at protein synthesis under a reduced metabolic state. This increase in efficiency could be induced by the lack of available energy for translational systems, which would be especially evident when nutrient-rich or faster-growth conditions do not exist, as in the case during the starvation-survival of cells under natural physiological conditions or as would be demonstrated by continued protein synthesis of $D = 0.015\ h^{-1}$ cells during stage 2 of starvation-survival. Whether this switching over to a higher-efficiency translational system during starvation-survival is due to the modification of RNA or protein or both is still unknown and under investigation; however, RNA sequences from *Tetrahymena* spp. have been found to have enzymatic function (39).

This contrasts with the inefficiency of ribosomes in bacteria with doubling times of less than 2 h, as stated by Koch (14). It also contrasts with the concept of "extra RNA" as proposed by Koch (12), which is said to occur in slow-growing bacteria because of the energetically expensive nature of these molecules (15). The differences involved are most likely due to the use of *E. coli*, which is normally a fast-growing bacterium unsuited for long-term starvation-survival, in these studies.

Throughout the starvation-survival period, concurrent fluctuations of RNA and protein concentrations per cell were observed, and DNA per total cell dropped steadily to 5 to 10% of the original concentration, regardless of the original growth rate of the cell population. As stated above, this corresponds to the amount of DNA stabilizing at ca. 1.0 to 1.5 fg of DNA per total cell for each of the cell populations during stage 3. The actual reduction in DNA content per cell between unstarved and starved ANT-300 cells is visualized by electron microscopy in Fig. 3. The DNA content per cell has also been reported to decline to approximately 30% after 30 days (early stage 2) of starvation for both ANT-300 cells (2) and *V. cholerae* (8). The decrease in cellular DNA may be due to the cells reducing DNA content to a single genome or at least to the DNA required to maintain function throughout the metabolic arrest state of stage 3. This remaining DNA must also allow for growth initiation and reproduction when conditions permit.

The data obtained in this study, coupled with the data of Moyer and Morita (25), provide a means by which the cell volume, nucleoid volume, and the nucleoid volume/cell volume ratio could be estimated (Table 1). Valkenburg and Woldringh (38) estimated the nucleoid volume in relatively slow-growing *E. coli* ($D = 0.400\ h^{-1}$; 1.2 genome equivalents) to be 0.07 μm^3. The entire cell volume of starved ANT-300 cells from $D = 0.015\ h^{-1}$ was only 0.05 μm^3 (Table 1). Therefore, either these marine bacteria are able to subsist with a considerably smaller DNA content, i.e., genome, than their unstarved counterparts or the cells achieve a greater DNA-packaging efficiency. As demonstrated by Valkenburg and Woldringh (38), the decrease in nucleoid volume does not appear to be the result of compaction of the DNA but rather an actual decrease in the amount of DNA. However, since marine bacteria are independently living organisms in a nutritionally complex and dynamic environment, their genome size and complexity cannot decrease completely in proportion to the cell size. This must then represent an extreme physiological optimization between survival and

growth rate in a nutrient-limited environment, especially in view of the concept of metabolic arrest, which has been hypothesized to occur during the starvation process (24).

In addition, we have examined a novel concept of the nucleoid volume/cell volume ratio (N/C) as a relative measure of health during starvation. This is demonstrated by the $D = 0.015$ h^{-1} cell population, which was able to maintain a N/C ratio of 40% during stage 3 starvation-survival. We also consider the low N/C ratio in the batch culture cells of 6% for both unstarved and starved cells to be a demonstration of the lack of metabolic fitness of this cell population for long-term starvation-survival. These values for the N/C ratio also correlate well with the cell viability data during starvation for these cell populations in Moyer and Morita (25). It is hypothesized that the N/C ratio of the ANT-300 cells originally grown at the lowest growth rate ($D = 0.015$ h^{-1}) more accurately represents the conditions for microbes in the ocean and, coupled with the evidence of increased capacity for starvation protein synthesis and optimized bacterial chromosome size, that this population is the most physiologically fit of all cell populations examined to undergo starvation-survival. It is hoped that these concepts will aid in a better estimation of the bacterial biomass and productivity in the marine environment, since most of the ocean is oligotrophic.

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