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Survival of a Psychrophilic Marine Vibrio Under Long-Term Nutrient Starvation[1]

JAMES A. NOVITSKY AND RICHARD Y. MORITA*

Department of Microbiology and School of Oceanography, Oregon State University, Corvallis, Oregon 97331

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Ant-300, a psychrophilic marine vibrio isolated from the surface water of the Antarctic convergence, was starved for periods of more than 1 year. During the first week of starvation, cell numbers increased from 100 to 800% of the initial number of cells. Fifty percent of the starved cells remained viable for 6 to 7 weeks while a portion of the population remained viable for more than 1 year. During the first 2 days of starvation, the endogenous respiration of the cells decreased over 80%. After 7 days, respiration had been reduced to 0.0071% total carbon respired per hour and remained constant thereafter. After 6 weeks of starvation, 46% of the cellular deoxyribonucleic acid had been degraded. Observation of the cellular deoxyribonucleic acid with Feulgen staining before starvation showed the average number of nuclear bodies per cell varied from 1.44 to 4.02 depending on the age of the culture. A linear relationship was found between the number of nuclear bodies per cell and the increase in cell numbers upon starvation. Our data suggest that Ant-300 is capable of surviving long periods of time with little or no nutrients and is therefore well adapted for the sparse nutrient conditions of the colder portions of the open ocean.

In comparison to other facets of microbiology, very little is known about the starvation and death of microbes since most microbiological studies have been conducted using rich media containing several grams of nutrients per liter. Compared with laboratory conditions and other natural environments, the nutrient content of the ocean is very low. Although estimates vary, the amount of dissolved organic matter in the sea is usually less than 1 mg of C/liter for surface water and 0.5 mg of C/liter for deep water (22). In addition to low temperature and hydrostatic pressure, nutrient availability has been suggested as an important factor in the distribution of bacteria in the oceanic environment (33). Thus, more bacteria are generally found near river outfalls, in sediments, and in surface waters. Due to the constant circulation and mixing of the oceans, a high concentration of cells in a productive area such as an estuary will eventually be diluted and dispersed. Moreover, it has been estimated that converging surface waters that mix and subsequently sink will not appear at the surface again for 1,100 years. How then can the bacteria in these waters survive such great periods of time with little or no nutrients between the nutritive areas of the ocean?

Morita (23) first questioned whether some marine bacteria are transients or cells not actually metabolizing in the oceanic environment. This point was reiterated by Sieburth et al. (27) when they suggested that marine bacteria "free" in the water column may be nothing more than transients starving to death, even though there have been no reports of starvation survival for any marine bacteria. On the other hand, the starvation survival of various terrestrial bacteria has been investigated but it has been found that most strains are not well suited for starvation survival. Boylen and Ensign (1) have reviewed the reported half-life starvation times for various bacteria and found that most are under 5 days. The exceptions have been *Arthrobacter* sp. The half-life starvation time for *Arthrobacter crystallopoietes* has been estimated at 100 days (1). The purpose of the present study, therefore, was to investigate the survival capabilities and mechanisms of a marine bacterium subjected to long-term nutrient starvation.

MATERIALS AND METHODS

Organism, media, and reagents. Ant-300, a psychrophilic marine vibrio isolated from the Antarctic convergence during the 1972 Cruise 46 of the R/V Eltanin, was used for this study. Ant-300 has been tentatively identified as a *Vibrio* sp. (John Baross, personal communication). Cultures were grown and maintained on a glucose medium (GM) and starved

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in a phosphate-buffered salt mixture (SM) as previously described (24).

A stock solution of nalidixic acid (Calbiochem) containing 10 mg/ml in 0.1 N NaOH was prepared and filter sterilized immediately before use.

Growth and starvation conditions. Ant-300 was grown and starved at 5°C on a reciprocating shaker (New Brunswick Psychrotherm). Cells for starvation were harvested during the exponential phase of growth (80 h after inoculation unless otherwise noted) and prepared for starvation as previously described (24). Growth and starvation were monitored spectrophotometrically using a Beckman DB spectrophotometer operating at 600 nm with a light path of 1 cm.

Viability determination. Viability was determined by the spread-plate technique. After the appropriate dilution of the starving suspension in cold SM, 0.1 ml was spread on the surface of a GM agar plate. Duplicate determinations were made in all cases and cells were considered viable if they could produce a visible colony after 1 week of incubation at 5°C. Direct counts were obtained using epifluorescence microscopy. A portion of the starving-cell suspension was fixed with glutaraldehyde (final concentration 1.0%) at 5°C for 1 h. The cells were then filtered onto a 0.2-μm nuclepore filter, stained with acridine orange according to the method of Zimmermann and Meyer-Reil (32), and observed with a Zeiss epifluorescent microscope. The cells in at least 50 fields from the center to the edge of the filter were counted and the number of cells of the original suspension per milliliter was calculated using an area conversion factor between the microscopic field and the working surface of the filter.

Preparation of ^{14}C-labeled cells. Before starvation, cells were grown for at least two generations in GM containing [U-^{14}C]glucose (New England Nuclear; specific activity, 4.06 mCi/mmol; final concentration 0.2 Ci/ml). Unlabeled glucose was omitted from the medium.

Determination of endogenous respiration. At various times during starvation, 5-ml portions of a ^{14}C-labeled starving-cell suspension were placed into four 50-ml serum bottles which were capped with a rubber stopper fitted with a cup and filter paper assembly. Two of the bottles were placed on a shaker in an incubator at 5°C. After an incubation period sufficient to produce a significant quantity of $^{14}CO_2$ (3 to 24 h), 1 ml of 3.0 N HCl was injected through the cap into the sample and 0.2 ml of β-phenethylamine was injected onto the filter paper to collect the evolved $^{14}CO_2$. After shaking for 20 min at room temperature, the filter paper was removed and placed into a scintillation vial. The remaining two bottles which served as blanks were injected with acid and treated as described above immediately after collection of the sample. Also at the time of sample collection the total labeled cellular carbon was determined as follows: two 5-ml samples of the starving suspension were filtered through 0.45-μm filters (Millipore, type HA) and washed twice with 5-ml portions of cold SM. The filters were dried and placed into scintillation vials. The radioactivity of the filters and filter papers was determined using a Nuclear Chicago Mark I liquid scintillation counter. Omnifluor (4 g of Omnifluor [New England Nuclear] per liter of toluene; 5 ml/vial) was used as a scintillation cocktail. Endogenous respiration was calculated as the net amount of $^{14}CO_2$ evolved per hour expressed as a percentage of the total labeled cellular carbon.

DNA determination. The deoxyribonucleic acid (DNA) content of both the starving cells and the starvation menstruum was determined. At various times during starvation, 5-ml portions of a ^{14}C-labeled starving-cell suspension were removed and centrifuged at 4,100 $\times$ g for 15 min at 4°C. The supernatant was decanted and the pellet was resuspended in 1 ml of 1.0 N NaOH. Both the supernatant and the resuspended pellet were then frozen until assayed. Duplicate samples were prepared for each assay. Before the assay all samples were thawed at room temperature and the tubes containing the cellular fractions were placed in a boiling water bath for 15 min to assure cellular lysis. All samples were then cooled on ice and the DNA content was determined according to the procedure of Kennell (18). The isolated DNA fragments were assayed for radioactivity as described above after mixing 1 ml of the preparation with 10 ml of Triton X-100 fluor (8 g of Omnifluor per liter of toluene plus 500 ml of Triton X-100 [Sigma]).

Observation of cellular DNA. Cellular DNA was observed after Feulgen staining of the cells. The technique of Feulgen and Rossenbeck (10) was modified to produce the best microscopic image for a psychrophile as follows. Ten milliliters of a culture was fixed with glutaraldehyde (final concentration 5%) for 2 h at 5°C, centrifuged at 4,100 $\times$ g for 15 min at 4°C, washed twice, and resuspended in sodium phosphate buffer (pH 7.1, 0.1 M). A drop of the suspension was then spread on a microscope slide, air dried, fixed in 70% ethanol for 5 min, and washed with distilled water. The slide was then immersed in 1.0 N HCl for 15 min at 60°C. After a distilled-water wash the smear was covered with a basic fuchsin dye solution (Schiff reagent) for 1 h. The slide was then washed with sulfite solution three times (5 min each) followed by distilled water. After air drying the cells were viewed with a phase-contrast microscope. The Schiff reageant was prepared by dissolving 5 g of basic fuchsin (BBL) in 1 liter of boiling distilled water. After cooling to 50°C, 10 g of Na_2SO_3 and 100 ml of 1.0 N HCl were added and the solution was stored overnight in the dark. Five grams of Norite was then added and the solution was filtered until clear. The resulting filtrate was stored at 1°C in the dark until used. The sulfite solution was prepared by combining 10 ml of 1.0 N HCl, 10 ml of a 5% solution of Na_2SO_3, and 180 ml of distilled water. When viewed under phase contrast, dark bands were observed within the cells which we designated as nuclear bodies. The nuclear bodies in at least 100 cells were counted and the average number of nuclear bodies per cell was calculated.

RESULTS

When placed into a starvation menstruum Ant-300 increased in numbers during the first

week of starvation (Fig. 1). After the first week of starvation the total number of cells continued to increase slightly while the viable cells decreased in number. After 2 weeks, 50% of the total number of cells were still viable while 50% of the initial number of cells remained viable for 5 to 7 weeks. Extended experiments showed that 10^3 cells/ml were still viable after 1 year of starvation. Extensive cellular lysis was not noted during starvation as judged by the absence of cellular debris during microscopic observation of the starving suspension and by the absence of a significant decrease in the total number of cells.

Also during the first week of starvation the optical density of the starving suspension decreased 62% (Fig. 1). After 7 weeks of starvation the optical density decreased an additional 21%.

The endogenous respiration of the starving cells is shown in Fig. 2. During the first 2 days of starvation, endogenous respiration decreased over 80%. After 7 days, respiration had been reduced to 0.0071% per h (greater than 99%) and remained constant thereafter.

The DNA content of the starving cell population and the starvation menstruum is shown in Fig. 3. Cellular DNA decreased rapidly during the first 14 days of starvation. Thereafter, it continued to decrease slightly. During 6 weeks of starvation, the cellular DNA decreased 46%. The DNA detected in the menstruum could account for only 5% of this decrease.

The increase in cell numbers upon starvation was found to be dependent on the age of the culture before starvation as shown in Fig. 4. Generally the older the culture, the greater the increase. Increases from 100 to 800% of the initial number of cells were noted for cultures starved from logarithmic growth (65 to 95 h after inoculation). Irregardless of the initial increase in cell numbers, 100 to 200% of the initial

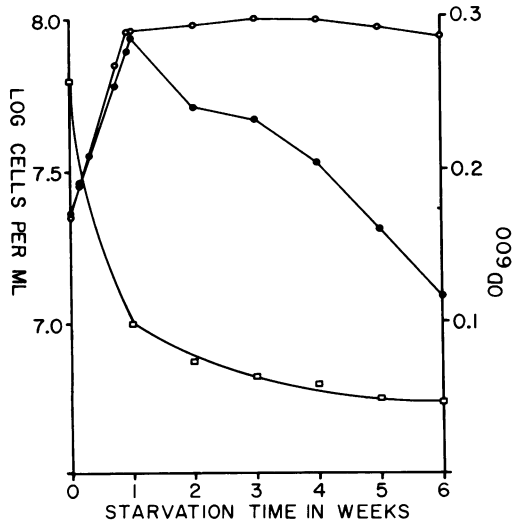

FIG. 1. *Starvation survival of Ant-300. Cells harvested from exponential growth were starved in a buffered-salt mixture. Total direct counts, ○; plate counts, ●; and optical density, □.*

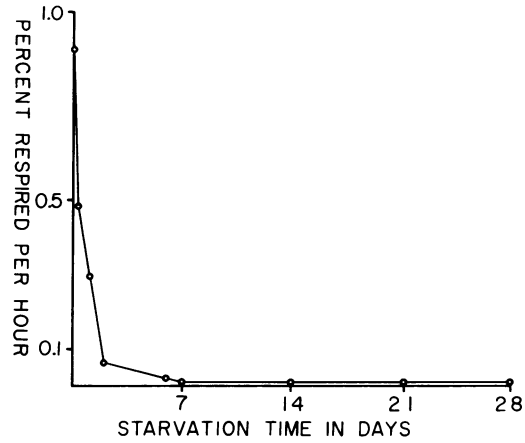

FIG. 2. *Endogenous respiration of starving Ant-300 cells. Respiration was determined at various times during starvation by the amount of $^{14}CO_2$ evolved from a previously labeled starving culture. Values were expressed as a percentage of the total cellular carbon respired per hour.*

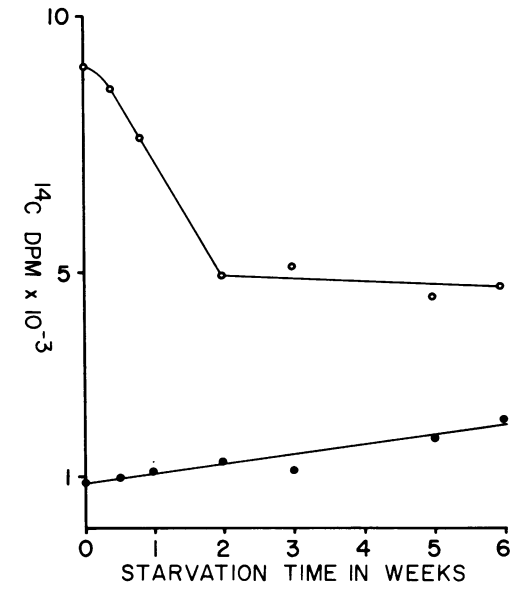

FIG. 3. *DNA content of starving Ant-300 cells. DNA was determined at various times during starvation for both the cells (○) and the starvation menstruum (●).*

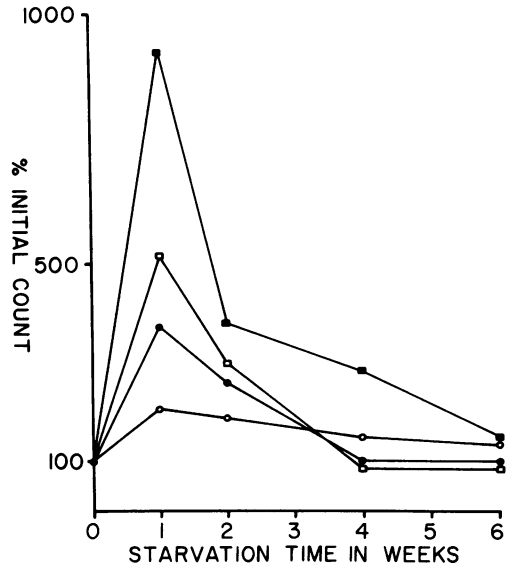

FIG. 4. *Starvation viability of Ant-300 starved from various times during exponential growth. Viability was determined by plate counts and expressed as a percentage of the initial count. The cells were starved after 65 h (○), 80 h (●), 95 h (□), and 115 h (■) of growth.*

number of cells were still viable after 6 weeks of starvation.

The average number of nuclear bodies per cell of an exponentially growing culture varied from 1.44 to 4.02, depending on the age of the culture, as shown in Table 1. To determine the significance of these multiple nuclear bodies during starvation, a culture of Ant-300 was grown, and at various times portions were removed and prepared for starvation. At the same time, the average number of nuclear bodies per cell of each culture portion was determined. As can be seen in Fig. 5, a linear relationship was found between the average number of nuclear bodies per cell and the percent increase in cell numbers during starvation. No relationship was found between the number of nuclear bodies per cell and the age of the culture.

Nalidixic acid added to an early-exponential-phase culture (final concentration, 20 μg/ml) caused growth to cease 1 h after the addition and only permitted a 20% increase in cell numbers. However, nalidixic acid added to a suspension of cells at the onset of starvation had no effect on the initial increase in cell numbers.

DISCUSSION

The starvation survival of Ant-300 is characterized by an increase in cell numbers during the first week of starvation followed by a decrease such that after 6 to 7 weeks of starvation, 50% of the initial number of cells are still viable. Compared with other bacteria cited in the literature, this starvation survival is exceeded only by *Arthrobacter* sp. (1, 20) when compared on a half-life basis. The initial increase in numbers appears to be unique since no such increase in cell numbers has ever been reported for any other starving bacteria. However, many of the studies relied upon slide culture techniques, made popular for determining the viability of starving-cell suspensions by Postgate and Hunter (25), which have a disadvantage heretofore unrecognized: if a starving population such as Ant-300 increases in cell numbers, it cannot be monitored by slide culture since the viability so determined cannot exceed 100%.

The initial increase in cell numbers is related to the age of the culture before starvation. Generally the older the culture the greater the

TABLE 1. *Average number of nuclear bodies per cell in an exponentially growing Ant-300 culture*

Time after inoculation (h)	Avg no. of nuclear bodies per cell
63	1.44
71	3.17
78	2.86
82	3.13
95	4.02

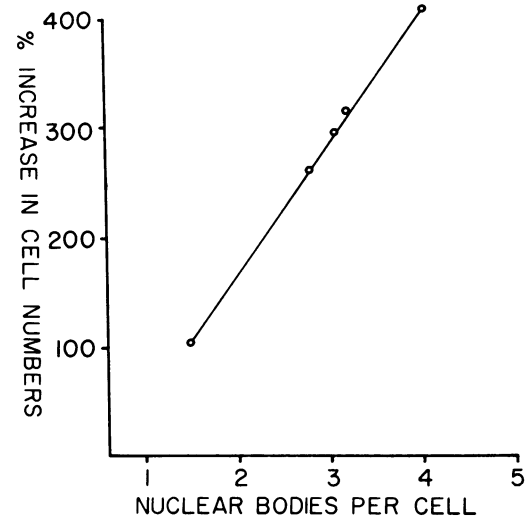

FIG. 5. *Effect of multiple nuclear bodies on cell number increases during Ant-300 starvation. The average number of nuclear bodies per cell was determined for five cultures before starvation by Feulgen staining. Cell number increases during starvation were monitored by plate counts.*

increase. Irregardless of the initial increase, 50% of the population in all cultures remains viable for approximately the same amount of time. It appears therefore, that the age of the culture is not important in determining its survival potential as was reported by Thomas and Batt (30) for *Streptococcus lactis*. However, the initial increase is dependent upon the age of the culture, and the magnitude of this increase can be an important factor in population survival if the culture were to be dispersed after the increase. In order for the cutures to have similar numbers of viable cells after 6 weeks of starvation, the cultures with the greatest increase in numbers must also have the greatest death rate afterwards. The reason for this increased death rate is unclear. At high population densities, Harrison (13) noted an increased death rate for *Bacterium lactis aerogenes* which he attributed to anoxia. To determine if this was also true for Ant-300, a culture was diluted after the initial increase but the death rates for both cultures were identical. It appears that this phenomenon therefore is characteristic of the cells rather than of the population. If this is the case, then older cultures die more rapidly than younger ones (also observed for *Arthrobacter crystallopoietes* (1) and *Sphaerotilus discophorus* [28]) and their survival depends solely on the large initial increase in cell numbers.

The degradation of DNA, generally considered a stable cellular component, by starving cells without a loss in viability is unprecedented; however, during the first week of starvation, Ant-300 increases in numbers while degrading its DNA. DNA has not been found to be degraded by starving bacteria (4, 7, 8, 17, 31) or it has been found to increase slightly (2, 3, 5, 29). There has been only one report of DNA degradation during starvation. Harrison and Lawrence (14) reported that *Aerobacter aerogenes* degraded 48% of its DNA in 19 h; however, at this time less than 1% of the population was viable. This contradicts the data of Postgate and Hunter (25) who reported no significant reduction in DNA for the same organism. The reason for DNA degradation by starving Ant-300 cells is not clear at this time. The DNA loss, however, is biphasic: during the first 14 days the degradation is relatively rapid followed by continued degradation but at a much slower rate. This may indicate that during the first 14 days the cells are degrading extraneous DNA such as extrachromosomal DNA or partially replicated chromosome copies.

On first observation, the increase in viable cells during the first week of starvation with a concomitant decrease in DNA appears paradoxical; cells increasing in number would be expected to increase in DNA as well. One possible explanation of the observed results is a fragmentation process similar to that observed for *Arthrobacter* sp. which undergo an increase in viable cells without an increase in cell mass (9). Further support for this hypothesis was provided by Novitsky and Morita (24) who found that Ant-300 decreases in size during starvation and that size reduction is most rapid during the first week of starvation. Feulgen-stained Ant-300 cells show distinct dark bands which we designated as nuclear bodies. The number of nuclear bodies per cell so observed does not remain constant but generally increases with the age of the culture. Cells starved from various times during the exponential growth phase increase in numbers in proportion to the average number of nuclear bodies per cell observed before starvation. The nuclear bodies therefore appear to be complete nuclei, several of which are produced by a cell during growth conditions and subsequently packaged into functional cells upon starvation. Functioning in this manner, the additional nuclei produced serve as reserve material and permit a population to increase in numbers upon starvation with a minimum expenditure of energy. If this hypothesis is true, once a cell enters starvation conditions, no synthesis of DNA need occur. Nalidixic acid, an inhibitor of DNA synthesis (11, 15), when added to a culture of Ant-300 caused growth to stop but had no effect on starving cells, indicating that DNA synthesis is not required for the starvation-induced fragmentation process. The mechanism for the production of multiple nuclei and this altered type of cell division is not known but may be similar to the recovery of *Escherichia coli* from the effects of penicillin. Hirokawa (16) observed that spheroplasts induced by penicillin treatment increased fourfold in DNA before giving rise to four rod-shaped cells. He also observed three to four ultraviolet light-absorbing areas within each spheroplast.

Under starvation conditions, Ant-300 reduces its endogenous respiration over 99% within the first week of starvation. At this rate, if respiration were the only loss of carbon, it would take approximately 58 weeks to reduce the cellular carbon by one-half. Actual measurements indicate that one-half of the cellular carbon is lost after only 39 days, a value close to the time when the viability of Ant-300 has been reduced to 50% of the initial number of cells. This apparent relationship may only be coincidental, however, since total cellular carbon was measured for both viable and dead cells. A sharp decrease

in endogenous respiration may be a factor in long-term survival since the bacterial strains with the three longest survival times reported, *Nocardia corallina* (20 days) (26), *Arthrobacter globiformis* (56 days) (20), and *A. crystallopoietes* (100 days) (1), all step-down their endogenous respiration over 90%. Evidence that a decrease in endogenous respiration is not coupled to a long survival time has been reported (4, 6, 25), but with rapidly dying populations it is difficult to determine with certainty whether the decrease in respiration is actually a survival mechanism or as a result of, or previous to, cell death. Halvorson (12) showed that agents that inhibit respiration such as 2,4-dinitrophenol, sodium azide, and arsenate also suppress protein and nucleic acid degradation in yeast cells. Similar results were reported by Mandelstam (21) for protein degradation in *E. coli*. It is possible that starvation induced respiration reduction acts in the same manner to prevent macromolecular degradation leading to increased survival.

Lamanna and Mallette (19) have stated that evolution should have selected for starvation-resistant microbes but this may only be true for microbes normally subjected to starvation stress. Boylen and Ensign (1) have pointed out that the long starvation survival of *A. crystallopoietes* is consistent with this hypothesis. Apparently, Ant-300 is also well adapted for starvation survival. Whether or not this holds true for other marine bacteria remains to be seen.

The present study has relied upon plate counts for the determination of viability which were expressed in reference to the original number of cells rather than to the total number of cells. Reporting data in this manner allows the determination of the survival capabilities of the cells in relation to the initial population. This relationship must be considered when hypothesizing the survival potential of any organism in the natural environment. Since the gene pool of a species can be conserved by the survival of only one organism, the concern in the literature over half-life survival times and the ratios of living to dead cells is therefore a moot point in considering the ultimate survival of a species.

ACKNOWLEDGMENTS

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