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Starvation-Survival Physiological Studies of a Marine *Pseudomonas* sp.†

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Starved cultures of a marine *Pseudomonas* sp. showed a 99.9% decrease in viable cell count during the first 25 days of starvation, yet the culture maintained 10^5 viable cells per ml for over 1 year. The physiological responses of populations of a marine *Pseudomonas* sp. to nutrient starvation were observed for periods of up to 40 days. At various intervals during starvation, the numbers of total, viable, and respiring cells were determined within the cultures. The ATP content, endogenous respiration rate, uptake rates, and percent respiration for exogenous glucose and glutamate were determined throughout the starvation period to characterize the physiological changes in the cells. It was observed that, after initial adjustment periods, all parameters tested reached stabilized states after 18 to 25 days of starvation. The results indicate that the actively respiring subpopulation, rather than the viable or total cell numbers, is the most appropriate denominator for interpretation of observed activities on an individual cell basis.

Bacteria which inhabit marine environments are likely to encounter periods of extremely low nutrient availability as they move with the water masses in routes of global circulation. The total organic content in seawater ranges from an average of 1.0 mg of carbon per liter in surface waters to 0.5 mg of carbon per liter of deep sea water (18). Much of the deep sea organic matter is resistant to microbial decomposition (1). Therefore, the ability of marine bacteria to adapt to and withstand starvation conditions for indefinite periods of time is essential for continuation of each marine species. The exceptional ability of marine organisms to survive starvation has been demonstrated by the marine psychrophilic vibrio ANT-300 (20, 21), which was found to maintain high viability in starvation cultures for periods of up to 4.5 years, at which time an electrical power failure terminated the study (R. Y. Morita, unpublished data).

The majority of literature on starvation involves short-term experiments of less than 48 h and attempts to define the detrimental effects of starvation rather than looking for adaptations of the cells. In addition, most researchers have interpreted observed responses to starvation as functions of entire homogeneous populations. The recently developed INT [2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride] method (24) determines the fraction of a population which is actively respiring without the addition of substrates. This allows for the assessment of changes in activity as a function of an active subpopulation rather than of the entire number of cells.

In this report, the term starvation refers to cells which have been washed and resuspended in minimal salts solution with no exogenous source of organic carbon or nitrogen. Physiological characteristics of the starving cells are described as "stabilized" once the cells maintain relatively constant levels of activity with time, after any initial changes in response to starvation. Cells are referred to as "viable" if they demonstrate the ability to reproduce on agar plates with nutrients. In like manner, cells which lose the ability to reproduce on plates are not necessarily dead or inactive, but they are by definition nonviable.

The entire 40-day experiment for all parameters described was repeated three complete times, with each data point run in at least duplicate or triplicate. Thus, each final data point is the average of at least six to nine separate determinations.

MATERIALS AND METHODS

Organism and media. The species used in this study was a mesophilic marine organism tentatively identified as a *Pseudomonas* sp. (J. Baross, personal communication). The taxonomic characteristics of this organism have been described (G. Kurath, M.S. Thesis, Oregon State University, Corvallis, 1980).

The minimal salts (MS) solution for starvation of the organism consisted of 26.0 g of NaCl, 0.8 g of KCl, 7.6 g of $MgCl_2 \cdot 6H_2O$, 5.6 g of $MgSO_4 \cdot 7H_2O$, 5.0 g of Tris

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buffer, 0.1 g of $CaCl_2$, and 0.1 g of Na_2HPO_4 in 1 liter of distilled water. Calcium chloride and dibasic sodium phosphate were prepared separately as sterile 1% solutions and added to the salts solution after cooling to avoid precipitation. Medium for growth of the organism was prepared by adding sterile glucose and ammonium sulfate to a final concentration of 0.1% each in sterile MS. Marine medium 2216 (Difco Laboratories, Detroit, Mich.) with 1.2% agar was used for viable cell counts.

Growth and starvation conditions. The *Pseudomonas* sp. was grown in glucose-ammonia medium at 22.5°C on a rotary shaker (model G2; New Brunswick Scientific Co., New Brunswick, N.J.) at 100 rpm. The standard inoculum for growth cultures was 0.1 ml of a stock culture which had been starving for longer than 30 days and which maintained a viable cell count of 4 $\times 10^5$ to 6 $\times 10^5$ cells per ml throughout the research. Cells for starvation were harvested in late log phase by centrifugation for 10 min in a Sorvall RC 5 centrifuge. The cell pellet was washed twice in sterile MS and resuspended to an optical density at 560 nm of 0.2 in sterile MS (approximately 3 $\times 10^8$ cells per ml). Starvation cultures were maintained with shaking at 22.5°C.

Cell counts. The number of viable cells in the cultures was determined with duplicate spread plates using 2216 agar (Difco). Cells were considered viable if they produced a colony within 3 days of incubation at room temperature (ca. 20°C). Further incubation produced no new colonies. Direct counts were obtained by acridine orange staining and visualized with a Zeiss epifluorescence microscope (12, 25). The fraction of the total number of cells actively involved in respiration was determined with minor modifications by the INT method (24). For each sample, 15 to 20 fields of 80 to 100 cells each were counted and averaged.

Estimation of ATP concentration. Adenylates were extracted in boiling Tris buffer (0.2 M Tris [pH 7.5]) from 2-ml portions of the starving suspension (13). ATP standards were extracted and frozen along with the samples until assayed. The luciferase assay was carried out with firefly lantern extracts (Sigma Chemical Co., St. Louis, Mo.) (16). Light emission was measured as peak emission data on an Aminco Chem-Glow photometer. Triplicate samples were extracted for each data point in each of three separate 40-day starvation experiments.

Respiration of exogenous glucose and glutamic acid. A modification of the heterotrophic activity procedure (10, 11, 23) was employed to determine the uptake and subsequent respiration of single concentrations of radioactive glucose and glutamic acid by starved cells (6).

For glucose uptake, 50 μl of D-[*U*-^{14}C]glucose (specific activity, 284 mCi/mmol; 1 μCi/ml) was added to 5 ml of a 1:100 dilution of the starving cell suspension. Each sample was run in triplicate, with a fourth sample as a control acidified at time zero. Samples were incubated for 2 to 8 h and processed to determine the counts per minute incorporated and respired as described previously (2, 10–12).

The uptake rate and percent respiration for glutamate were determined by the same procedures with 50 μl of L-[*U*-^{14}C]glutamic acid (specific activity, 285 mCi/mmol; 1 μCi/ml) and incubation times of 0.5 to 2.0 h.

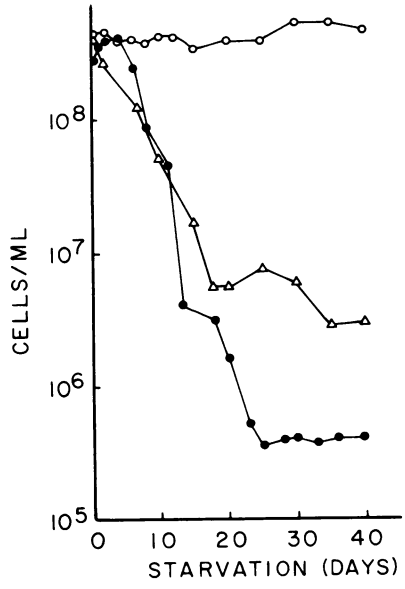

FIG. 1. Viable, direct, and respiring cell counts after various periods of starvation. All counts were carried out on the same starvation culture. Viability (●) was determined by spread plates, direct counts (○) were determined by epifluorescence microscopy, and respiring cell counts (△) were determined by the INT method (24).

Determination of the endogenous respiration rate. The endogenous respiration rate during starvation was determined from the rate of evolution of $^{14}CO_2$ from a starving suspension of ^{14}C-labeled cells. To prepare ^{14}C-labeled cells, a growth culture was harvested in mid-log phase, washed twice with sterile MS, and suspended in 50 ml of a labeled glucose medium for at least three generations of growth. The labeled medium was identical to the growth medium, with the substitution of 0.5 μCi of L-[*U*-^{14}C]glucose (specific activity, 284 mCi/mmol) per ml for the carbon source. Unlabeled glucose was included to make a total concentration of 0.001% glucose. After 14 h in the labeled medium, the cells were harvested as previously described and suspended for starvation in MS. At designated times during the starvation period, 30-ml portions were removed and used to determine the endogenous respiration rate of the cells (20). The rate of endogenous respiration was calculated as the quantity of $^{14}CO_2$ evolved per hour and expressed as a percentage of the total cellular carbon.

Utilization of leached nutrients. After 30 days of starvation, a 125-ml portion of the ^{14}C-labeled culture used to follow the endogenous respiration rate was removed and centrifuged at 5,000 $\times$ *g* for 10 min. The cell pellet was washed twice, resuspended in 125 ml of fresh sterile MS, and incubated at 22.5°C on a rotary shaker. At 4 and 14 h after washing, the endogenous respiration rate of the cells was determined and compared to the respiration rate of the unwashed cells in the original starvation culture. If the respiration of those cells which remained viable and/or respiring during starvation was supported by nutrients leached

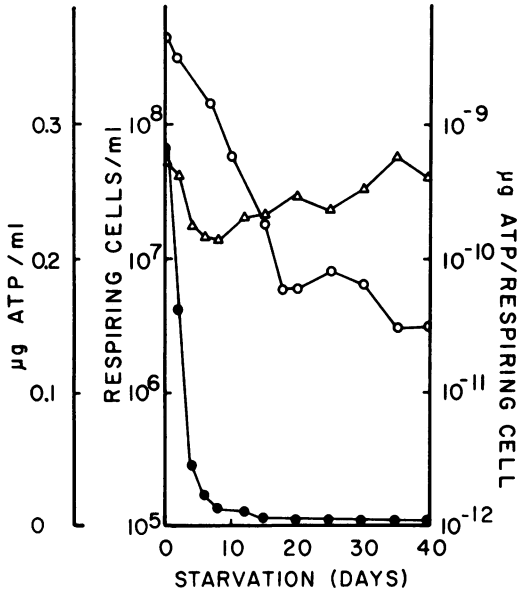

FIG. 2. ATP content after various periods of starvation. △, ATP per milliliter of culture; ●, ATP per respiring cell; ○, respiring cell count.

into the medium by nonviable or nonrespiring cells, then a decrease in endogenous respiration would be expected when the cells were placed in fresh medium.

RESULTS AND DISCUSSION

The *Pseudomonas* sp., under the starvation-survival conditions described above, routinely demonstrated the viability pattern shown in Fig. 1. Populations were starved at an initial density of approximately 3×10^8 viable cells per ml and increased to 4.2×10^8 viable cells per ml in the first 4 days of starvation. Cultures then entered a stage in which the viability dropped at a constant rate of 4.8% per day. After 21 days, the viability was stable at approximately 5×10^5 viable cells per ml for the remainder of the 40-day experimental period and on for at least 1 year (longest period tested). The total number of cells, as determined by direct counts (Fig. 1), started slightly higher than the viable counts at the onset of starvation and showed no significant variation during the entire experimental period. This indicated that no cell lysis occurred and is in agreement with previous reports on starving cell populations (3, 4, 7, 9). The fraction of the total population which was actively respiring during starvation, as determined by the INT method, is also shown in Fig. 1. At the onset of starvation, 97% of the cells in the suspension were actively respiring, as demonstrated by their deposition of large, red, intracellular INT-formazan granules. As the cells starved, the number endogenously respiring decreased rapidly to 27% in 6 days. After 18 days of starvation, the number of respiring cells stabilized at approximately 1.0% of the total number of bacteria present, corresponding to 4.5×10^6 active cells per ml. The difference between the viable and INT counts suggests the existence within the starving population of a subpopulation of nonviable but actively respiring cells which are 10-fold more numerous than the viable cells. This phenomenon has been observed in natural populations by autoradiography (14, 15).

Log phase cells contained 6.5×10^{-10} μg of ATP per cell. This is within the range of 0.5×10^{-9} to 6.5×10^{-9} μg of ATP per cell reported by Hamilton and Holm-Hansen (8) for marine bacteria. In agreement with the same report, the ATP content of our cultures decreased by 80% in the first 5 days of starvation. The cultures dropped from 248 to 45 ng of ATP per ml in 4 days and stabilized after 20 days at 2 to 3 ng of ATP per ml (Fig. 2). To interpret this change in ATP content on an individual cell basis, the data were converted to values of ATP per cell. The question of the proper cell count to use for these calculations depends on which cells contained ATP.

Since the majority of cells lost the ability to reproduce and respire during starvation (Fig. 1), it is obvious that not all cells in the culture maintained equal amounts of ATP. Therefore,

TABLE 1. Uptake rates for exogenous glucose and glutamate after various periods of starvation

Starvation time (days)	[^{14}C]glucose uptake[a]		[^{14}C]glutamate uptake[b]	
	per ml	per 10^7 respiring cells	per ml	per 10^7 respiring cells
0	558.2	13.1	323.3	7.6
4	191.6	4.9	103.4	3.2
8	101.8	13.3	35.5	4.7
13	39.5	11.1	15.9	4.4
17	15.0	26.3	6.4	11.2
25	18.5	24.0	3.9	5.1
32	21.3	40.6	1.6	7.6
40	12.1	36.4	3.3	10.00

[a] Rate of D-[*U*-^{14}C]glucose uptake in nanomoles ($\times 10^{-6}$) per hour.
[b] Rate of D-[*U*-^{14}C]glutamate uptake in nanomoles ($\times 10^{-4}$) per hour.

the total cell count would be an erroneous denominator for calculating the changes in ATP per cell. In like manner, the viable cell count would be an improper denominator since, at a minimum, the larger subpopulation of actively respiring cells would be able to generate and maintain pools of ATP. For these reasons, the INT data were used to calculate changes in ATP per cell during starvation (Fig. 2). There was a decrease in the amount of ATP per respiring cell for the first 8 days of starvation, followed by gradual increase back to the original level of 6.5×10^{-10} μg of ATP per respiring cell by 40 days of starvation.

Thus, on this basis, cells initially lose ATP when starved, but then certain cells adapt and regenerate ATP pools comparable to those before starvation. The question of how certain cells are able to maintain ATP pools without exogenous substrates is not clear from this study, but it would be most advantageous for starving cells to do so. In actively growing cells, 15 to 20% of the ATP is used for transport (22). In oligotrophic waters, it would therefore be a high priority for cells to maintain their ATP reserves. This would ensure that active transport mechanisms are functional if and when nutrient substrate(s) requiring active transport become available for energy.

To investigate the extent to which the active transport systems of the starving cells are maintained, uptake experiments with exogenous [^{14}C]glucose and [^{14}C]glutamic acid were carried out. At the beginning of starvation, the culture was able to take up [^{14}C]glucose and [^{14}C]glutamic acid at rates of 5.58×10^{-5} and 3.23×10^{-2} nmol/ml per h, respectively (Table 1). The 60-fold difference in uptake of these two substrates has been observed for natural mixed marine populations (5). Despite the difference in absolute rates, changes in the uptake rates due to starvation occurred with identical kinetics and relative magnitudes for both substrates tested (data not shown). Glucose and glutamic acid uptake rates were both reduced by 80% in the first 6 days of starvation. This is similar to the rapid drop in oxygen quotient values observed previously for glucose uptake by starving cells (4, 7, 17, 19). On day 17 of starvation, the rates stabilized at 1.5×10^{-5} nmol of [^{14}C]glucose per ml per h and 3.8×10^{-4} nmol of [^{14}C]glutamate per ml per h for the duration of the 40-day test period. This corresponds to 2.7 and 1.2% of the original rates, respectively.

Since not all cells in the starving culture are physiologically the same, they are most likely not equally active in uptake. Therefore, the actual changes in uptake rates for an individual cell were calculated as a function of the respiring cell population (Table 1). There was an initial

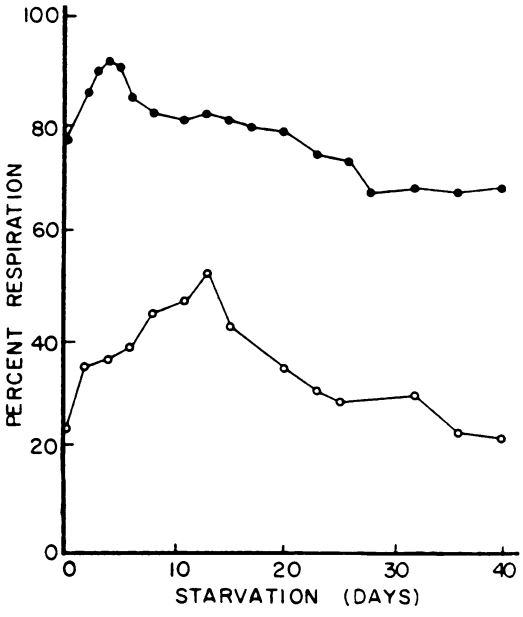

FIG. 3. Percent respiration of exogenous [^{14}C]glucose (○) and exogenous [^{14}C]glutamic acid (●) by cells starved for various periods of time. The percent respiration was calculated by dividing the quantity given off as $^{14}CO_2$ by the total quantity taken up.

decrease in the uptake per 10^7 respiring cells per hour for both substrates tested. After day 4 of starvation, there was a gradual, irregular increase, such that by day 40 the glutamate uptake had returned to approximately its original value and glucose uptake to approximately three times its original rate. Thus, both the ATP content and uptake abilities of those cells which remain active show an initial decrease followed by recovery in response to starvation.

Cells placed under the stress of starvation would be expected to shift their metabolic balance away from biosynthesis and reproduction and toward the acquisition of energy for existing biological functions. This was demonstrated by the increase in the percent respiration of both glucose and glutamic acid in response to starvation (Fig. 3). Log phase *Pseudomonas* sp. cells showed that 23.4% of the [^{14}C]glucose and 67.3% of the [^{14}C]glutamic acid which was taken up was subsequently respired and given off as $^{14}CO_2$. As the cells starved, the respiration of [^{14}C]glutamic acid increased to 92% in 4 days and then declined gradually. At 28 days, the percent respiration stabilized at 70%, close to the log phase value. The respiration of [^{14}C]glucose increased to a maximum of 50% at 13 days of starvation and also subsequently declined. The level stabilized after 36 days at 22%, again quite close to the log phase value. The return to

log phase values for the percent respiration of exogenous substrates could represent certain cells which adapt to the conditions and regain their original balance of respiration and biosynthesis. Those cells which fail to adapt may die, and they would no longer influence the percent respiration measurements.

It is generally reported that the ability of bacteria to survive during starvation is correlated with the ability to quickly establish a low rate of endogenous respiration (3, 4, 19). At the onset of starvation, 0.24% of the total cellular carbon in the culture was respired per h. This rate was maintained for the first 12 h and then dropped rapidly to 0.06% at 3 days of starvation. Thereafter, the rate declined more gradually and stabilized at 0.002 to 0.007% for 20 to 40 days of starvation. This stabilized rate is similar to the 0.007% reported for starved cultures of the marine vibrio ANT-300 (21).

To interpret this total amount of endogenous respiration activity on an individual cell basis, we must identify that portion of the population which is responsible for the activity detected. Since the INT assay quantitates the number of cells active in endogenous respiration, it can be used to calculate the quantity of cellular carbon attributable to those cells which are respiring. This value is then a more suitable denominator for the actual rate of endogenous respiration by only those cells which are producing the $^{14}CO_2$ observed. This rate shows an initial decrease for the first 5 days of starvation from 0.24 to 0.08% of the respiring cell carbon respired per h. However, this is followed by an increase from 6 to 40 days of starvation to values as high as 0.58%. Although this should theoretically be the most accurate interpretation of the individual cell's endogenous respiration, an increase of the rate with starvation seems, at our present level of understanding, to be self-destructive rather than advantageous. A better understanding of the starvation state of bacteria is necessary before this pattern can be explained.

To investigate the role of nutrients possibly leached from nonviable cells, cells from the endogenous respiration culture were removed, washed thoroughly, and resuspended in fresh medium. If the active cells were indeed utilizing leached nutrients in the medium to support their respiration, resuspension in fresh medium would lead to a decrease in endogenous respiration. However, at both 4 and 14 h after washing, the cells respired at a rate of 0.006% of their cellular carbon per h. This was comparable to the 0.004% respired by the unwashed cells in the ongoing culture. Since a drop in respiration rate did not occur, we conclude that the leaching of organic material from the nonviable cells is not a major factor in the survival of the other cells during starvation survival.

TABLE 2. Cell counts and levels of activity within a starving population of cells of a *Pseudomonas* sp. after the responses to starvation stabilized

Parameter	Stabilized level[a] (%)	Time of stabilization (days)
Direct cell counts	100.0	Constant
Respiring cell counts	1.0	18–20
Viable cell counts	0.1	25
ATP (μg/ml)	1.0	15–20
Glucose uptake rate per ml	2.7	17–20
Glutamate uptake rate per ml	1.2	17–20
Endogenous respiration rate per ml	2.9	15–20

[a] Percent of the original level for the total population.

The stabilized levels of activity and the time required for each stabilization within the starving *Pseudomonas* sp. populations are summarized in Table 2. One striking feature is that the content of ATP, uptake rates of glucose and glutamic acid, and endogenous respiration rate of the starving cells all stabilized at approximately the same time as the number of respiring cells. In addition to the coincidence of timing, the magnitudes of the stabilized activity levels most closely match the relative size of the respiring cell population (all approximately 1% of the original value). We interpret these facts as indications that the respiring cell subpopulation is most likely the fraction of the starving population responsible for the activities observed. The individual cell to cell differences which determine survival versus death during starvation are not elucidated in this study but are shown to exist. Whether the determining factors are genotypic or phenotypic remains to be seen.

The ability of marine organisms to withstand nutrient deprivation is important to the survival of each bacterial species in the ocean. The viability pattern exhibited during starvation of the marine *Pseudomonas* sp. in this study is a demonstration of that ability. Although the majority of the cells lost their viability when starved, the fraction which did remain viable was more than sufficient to replace the population if more favorable environmental conditions were encountered.

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