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Morphological Characterization of Small Cells Resulting from Nutrient Starvation of a Pyschrophilic Marine Vibrio[1]

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Upon starvation, Ant-300, a psychrophilic marine vibrio, was observed to decrease in size and change in shape from a rod to a coccus. After 3 weeks of starvation 50% of the starved population was able to pass through a filter with a pore size of 0.4 μm. Electron microscopy of thin sections of the small cells revealed normal cell structure except for an enlarged periplasmic space. When inoculated into a fresh medium, small starved cells grew without a significant lag and regained "normal" size and shape within 48 h.

The occurrence of small bacterial cells in the natural environment has been reported by Casida (7), who observed cells in soil that were approximately 0.5 to 0.8 μm in diameter. Casida (6) also isolated soil organisms that were 0.5 μm and less in diameter, but was unable to pass any of these cells through a cellulose membrane filter with an average pore size of 0.45 μm. Bae et al. (3) reported that 72% of the soil organisms examined were less than 0.3 μm in diameter. Oppenheimer (14) reported the occurrence of up to 12 viable cells per ml of seawater filtered through a membrane with an approximate pore size of 0.4 μm. Martin (12) isolated a filterable *Vibrio* from freshwater, and Anderson and Heffernan (2) found from 12 to greater than 1,000 filterable organisms per 40 ml of seawater. The preliminary observations in this laboratory using the epifluorescent technique of Zimmermann and Meyer-Reil (18) indicate that there are many small cells in the marine environment (Meyer-Reil, Geesey, and Morita, unpublished data). These small spherical cells may represent as yet unisolated species of bacteria or isolated species that have increased in size and changed morphology upon laboratory cultivation. Specialized groups of bacteria are known to change morphology, and this subject has been reviewed by Ensign (8). Changes in morphology of *Vibrio* sp. have been reported by Felter et al. (10) and Baker and Park (4). In all cases the changes in morphology appear to be part of a cellular life cycle. Although the sphere-rod-sphere cycle of *Arthrobacter crystallopoietes* has been nutritionally controlled (9), there have been no reports of changes in cell size or morphology due to nutrient starvation. The present study describes the effect of starvation on the size and morphology of a psychrophilic marine vibrio.

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MATERIALS AND METHODS

Organism and media. Ant-300, a psychrophilic marine vibrio isolated from the Antarctic convergence during 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) containing the following: glucose, 2.5 g; $NaNO_3$, 0.5 g; yeast extract (Difco), 0.2 g; Na_2HPO_4, 0.5 g; $FeSO_4$, 0.0005 g; and 1 liter of a salt mixture (SM) containing: NaCl, 26 g; KCl, 0.8 g; $MgCl_2 \cdot 6H_2O$, 5.6 g; $MgSO_4 \cdot 7H_2O$, 7.6 g; and distilled water, 1 liter. The glucose and Na_2HPO_4 were autoclaved separately as 25 and 5% solutions, respectively. The solid medium contained 1.2% agar as a solidifying agent. The cells were starved in SM buffered with the sterile Na_2HPO_4 (final concentration, 0.05%).

Growth and starvation conditions. Ant-300 was grown in 100 ml of GM at 5°C on a reciprocating shaker (New Brunswick Psychrotherm), harvested during the exponential phase of growth by centrifugation for 15 min at 4,100 × *g* and 4°C, and washed twice with cold SM. Cell concentrations of 1×10^7 to 3×10^7 cells/ml were suspended in 200 ml of buffered SM (5°C) in a 1-liter Erlenmeyer flask that was shaken at 5°C.

Viable cell size determination. At various times 3-ml portions of the starving cell suspension were filtered through polycarbonate filters (Nuclepore) with different pore sizes. Plate counts of the filtrate were expressed as a percentage of the counts obtained from the nonfiltered suspension. Due to the psychrophilic nature of this organism, all filter apparatuses, dilution blanks, and agar plates were cooled to 4°C prior to use. Cells were considered viable if they could produce visible colonies after a 1-week incubation at 5°C.

Light microscopy. Heat-fixed smears were stained with crystal violet for 1 min and observed under oil immersion. Photomicrographs were re-

corded on Ilford FP4 film using a Leitz Orthomat camera and a Leitz Ortholux microscope.

Electron microscopy. Cells starved for 5 weeks and exponentially growing cells were harvested by centrifugation for 30 min at 7,700 × *g* and 4°C. Both pellets were then fixed with 3% (vol/vol) glutaraldehyde in SM buffered with Sorenson phosphate buffer (11) (pH 7.4, 0.125 M) for 3 h at 0°C, embedded in 2% agar, and postfixed in 1% (wt/vol) OsO_4 for 3 h at 4°C. The fixed preparations were stained with uranyl acetate (saturated solution in 70% ethanol) for 3 h during dehydration in a graded ethanol series and embedded in a modified Mollen-Hauer (13) resin containing 53% dodecenyl succinic anhydride, 35% Araldite 6005, and 12% Epon 812. Sections were cut with a diamond knife, stained with lead citrate (15), and observed with a Philips EM300 electron microscope operating at 60 kV.

RESULTS

Ant-300 cells decrease in size during starvation (Fig. 1). After 1 and 6 weeks of starvation the cells appeared as small rods and small cocci, respectively. Filtration of the population through filters of various pore sizes showed the cell size distribution during starvation as presented in Fig. 2. Size reduction is most rapid during the first 2 days of starvation and continues for 3 weeks. After 2 weeks of starvation, 100% of the viable cells were able to pass through 3.0- and 1.0-μm filters. After 3 weeks of starvation, 100% of the viable cells were able to pass through 0.8- and 0.6-μm filters. Initially no cells were capable of passing through a 0.4-μm filter, but 3 weeks of starvation resulted in filterable viable cells (50%). After 4 weeks of starvation, 100% of the initial number of cells were still viable. At no time during starvation were any cells able to pass through a 0.2-μm Nuclepore filter or a 0.45-μm cellulose membrane filter (Millipore Corp., type HA).

An increase in cell numbers occurs when Ant-300 is placed into a starvation menstruum (100 to 600%), which leads then to the formation of small cells. The process of starvation and the increase in cell numbers will be the subject of a subsequent paper.

Electron microscopy of a thin section of nonstarved Ant-300 cells is shown in Fig. 3a. The cells are typical for gram-negative cells and show no unusual structures. The small starved cells (Fig. 3b and c) appear smaller and roughly spherical. The ultrastructure is generally the same as nonstarved cells except for an enlarged periplasmic space containing stainable material observed in all the starved cells examined. No unusual membrane structures were observed.

After 5 weeks of starvation cells were passed through a 0.4-μm filter and inoculated into fresh GM. The cells grew without a significant lag period and had a generation time of 6 h, equal to that for nonstarved cells. When inoculated into a fresh medium, small cells immediately begin to increase in size and regain their rod shape. After 48 h (eight generations) the cells were indistinguishable in size from nonstarved cells. The increase in size was almost synchronous for all the cells in the population; however, even after 60 h of growth, a few small cells were still observed.

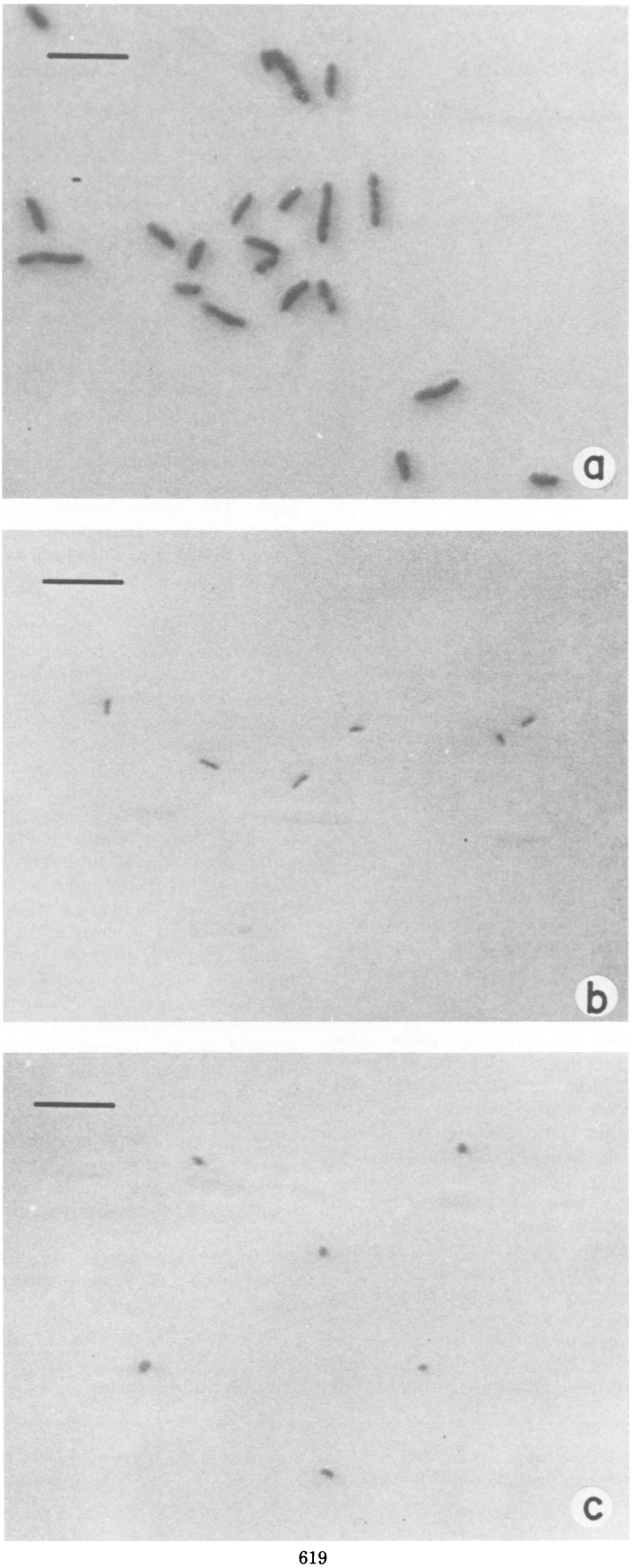
DISCUSSION

The reduction in size of Ant-300 cells is most pronounced during week 1 of starvation. In contrast, the morphology change is a gradual process commencing after week 1 and continuing until week 4 of starvation, at which time no further apparent changes occur. This may indicate that a reduction in size is necessary before a morphological change can take place.

Nuclepore filters were chosen for this study because the average minimum sizes of the particles they retain are similar to the stated pore size of the filter, whereas cellulose membrane filters are able to retain particles much smaller than their stated pore size (16). This is probably the reason why Ant-300 cells that were able to pass through a 0.4-μm polycarbonate filter were retained by a 0.45-μm cellulose filter. This may also be the reason why Casida (6) was unable to filter cells observed to be less than 0.5 μm through a 0.45-μm filter. Using polycarbonate filters, if the sample volume is large or contains a dense suspension of particles, the filter will retain particles smaller than the stated pore size (16). To avoid this problem, samples passed through filters were of minimum volume (3 ml). Taking this into account, the data indicate that after 3 weeks of starvation at least 50% of the population is between 0.2 and 0.4 μm in diameter and no more than 50% of the population is between 0.4 and 0.6 μm in diameter.

The fact that the small cells are able to grow indicates that they are complete cells, unlike the minicells of *Escherichia coli* that contain no deoxyribonucleic acid and are unable to divide (1). The formation of *E. coli* minicells appears to be an alteration of normal cell division, with

FIG. 1. *Light microscopy of Ant-300 under starvation conditions. Bars represent 5 μm. (a) Zero time; (b) 1 week after starvation; (c) 6 weeks after starvation.*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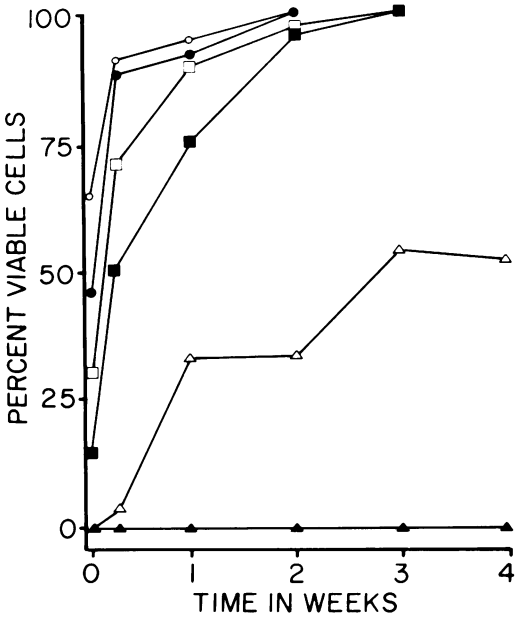

FIG. 2. *Effect of time of starvation on the size distribution of Ant-300 cells. Portions of the starving culture were passed through filters of various pore sizes. Plate counts of the filtrates are expressed as a percentage of the unfiltered counts. Filter pore sizes:* ○, *3.0 μm;* ●, *1.0 μm;* □, *0.8 μm;* ■, *0.6 μm;* △, *0.4 μm; and* ▲, *0.2 μm.*

a population producing approximately one minicell for every two normal cells in the culture (1). In a starving culture of Ant-300, all the cells undergo size reduction, indicating a generalized physiological mechanism. The small cells also appear unlike the round bodies described by Baker and Park (4). These round bodies, formed in the stationary phase of growth of a *Vibrio* sp., are not viable. Also unlike the small cells are the round bodies formed by *Vibrio marinus,* which possess distinct ultrastructures unlike normal cells. The freshwater filterable *Vibrio* isolated by Martin (12) produces filterable particles when grown in tryptone broth that appear to be specialized cells produced by and in addition to normal cells. No round bodies, spherical cells, or filterable particles have ever been observed in growing or stationary cultures of Ant-300.

Electron microscopy of the small starving cells revealed an enlarged periplasmic space similar to that of natural soil "dwarf" organisms examined by Bae et al. (3). Together with the size similarity, this may indicate that cells under starvation conditions more closely resemble cells in the natural environment than do laboratory strains grown on rich media. This may also indicate that relatively large cells are the result of laboratory cultural conditions and are not indicative of cells in the natural environment. Small cells in the natural environment may have arisen through a natural starvation process similar to that used to induce small cell formation in Ant-300. On the other hand, *Arthrobacter crystallopoietes* cells under long-term starvation conditions showed no ultrastructural changes. In addition, cell size and shape remained unaltered (5). The significance of the periplasmic space is not known. This space may be the result of cell shrinkage during starvation or preparation for electron microscopy. The latter seems unlikely since nonstarved cells prepared identically show no internal shrinkage. The electron density suggests the presence of cellular components within the space.

Ant-300 (Fig. 1a) is normally a straight or curved rod. When growing in liquid or on solid media no morphological changes are observed except for an increase in cell length in stationary-phase cultures. The change in morphology upon starvation appears to be a direct effect of nutrient deprivation rather than part of a life cycle. When inoculated into a fresh medium, starving cells increase in size and regain their "normal" shape. Cells recovered from starvation, grown on GM, and starved again show the same size reduction and morphology change. Of the 25 filterable strains isolated by Anderson and Heffernan (2), only eight retained their filterable property after cultivation. They hypothesize the existence of a life cycle or growth on rich media to explain this finding. The existence of a life cycle cannot be ruled out as Tuckett and Moore (17) reported filterable particles produced during the life cycle of *Cellvibrio gilvus* that gave rise to normal size cells. However, our data show that growth on rich media after starvation does produce an increase in cell size. Since most microbiological studies are carried out using rich media unlike most natural habitats, the data obtained must be interpreted carefully. Studying bacteria using dilute media or under starvation conditions may more closely simulate normal natural conditions for bacterial populations.

ACKNOWLEDGMENT

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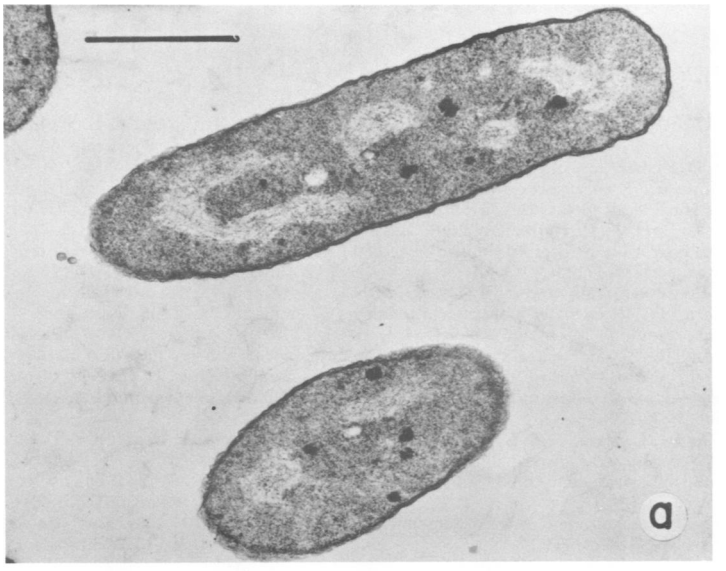
FIG. 3. *Electron micrographs of Ant-300 cells. (a) Nonstarved cells; bar represents 1.0 μm. (b) and (c) Cells starved for 5 weeks; bar represents 0.2 μm.*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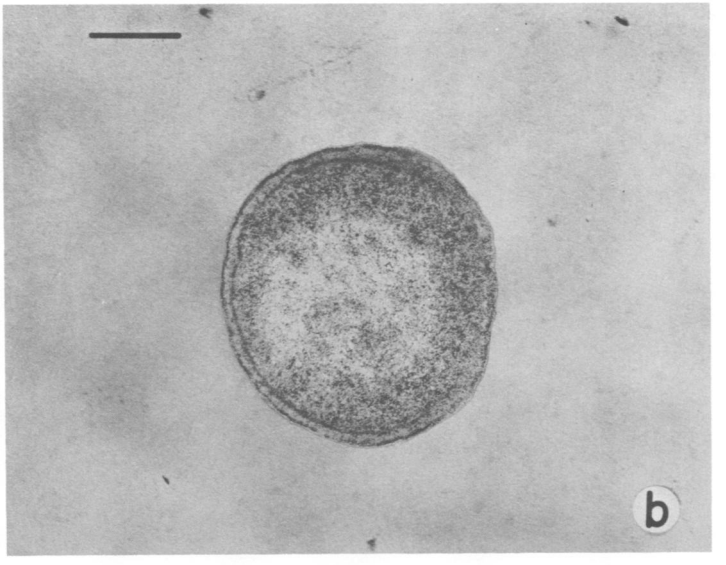
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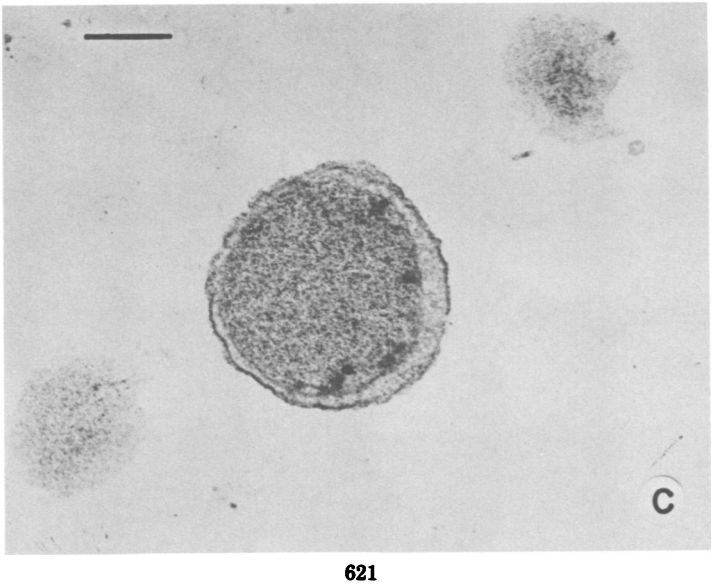
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