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Effects of Nutrient Deprivation on *Vibrio cholerae*

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Environmental and clinical strains of *Vibrio cholerae* were exposed to nutrient-free artificial seawater and filtered natural seawater microcosms for selected time intervals and examined for changes in cell morphology and number. Cells observed by transmission electron and epifluorescence microscopy were found to undergo gross alterations in cell morphology with time of exposure. The vibrioid cells decreased in volume by 85% and developed into small coccoid forms surrounded by remnant cell walls. The initial number of cells inoculated into nutrient-free microcosms (culturable count and direct viable count) increased 2.5 $\log_{10}$ within 3 days, and even after 75 days the number of viable cells was still 1 to 2 $\log_{10}$ higher than the initial inoculum size. Nutrient-depleted coccoid-shaped cells were restored to normal size and assumed a bacillary shape within 2 h and began to divide within 5 h after nutrient supplementation. The increase in cell number and decrease in cell volume under nutrient-depleted conditions, as well as the rapid growth response after nutrient supplementation, may describe some of the survival mechanisms of *V. cholerae* in the aquatic environment.

Vibrio cholerae has been isolated from a number of brackish-water environments such as river basins and estuaries of the U.S. Gulf Coast and Chesapeake Bay (7, 9, 10). The frequency of isolation, the lack of correlation to fecal contamination, and the growth of organisms at salinities typical of estuarine waters have led to the hypothesis that *V. cholerae* is an autochthonous member of the estuarine ecosystem (7, 10).

The hypothesis that *V. cholerae* occupies an ecological niche in the estuarine environment requires that this organism be able to survive the dynamics of various physiochemical changes, including variations in nutrient concentrations. Organisms such as *V. cholerae* may, in the planktonic state, encounter low nutrient concentrations in the estuary which are insufficient to support a copiotrophic existence. It has been proposed that members of the *Vibrionaceae* and *Pseudomonadaceae*, under conditions of limited exogenous nutrients, can undergo a number of morphological and physiological changes (14, 18–20, 26, 27). Novitsky and Morita (18) demonstrated that, upon starvation in natural and artificial seawater, the marine psychrophilic *Vibrio* sp. ANT-300 increased in cell number, decreased in cell volume, and changed from the typical bacillus shape to a coccoid-shaped cell. Such coccoid cells exhibited more than a 99% decrease in endogenous respiration as well as a 40% decrease in cellular DNA. It was concluded that ANT-300, in response to starvation conditions, had reduced endogenous respiration and cell volume while increasing cell numbers. Reichardt and Morita (22) showed that an aquatic strain of *Cytophaga johnsonae* was also capable of increasing cell number, producing short rods, coccoid cells, and elongated cells under nutrient starvation. It was concluded that these characteristics conveyed a selective advantage since such mechanisms would allow prolonged survival when insufficient nutrient concentrations were encountered in the aquatic environment.

It was the purpose of this study to determine viability and morphological responses of two strains of *V. cholerae*, one of clinical origin and another isolated from the estuarine environment, during prolonged exposure to nutrient-free conditions and subsequent nutrient supplementation.

MATERIALS AND METHODS

Bacterial strains. Two strains of *V. cholerae*, WF110, a non-O1 serotype environmental isolate, and CA401, a toxigenic O1 serotype clinical isolate, were employed. Culture stocks of the CA401 strain were obtained from R. R. Colwell, Department of Microbiology, University of Maryland, College Park. The WF110 strain was isolated from Gulf Coast shellfish. Stocks were maintained at −70°C in tryptic soy broth (Difco Laboratories, Detroit, Mich.) amended with 10% (vol/vol, final concentration) glycerol (Fisher Scientific Co., Fairlawn, N.J.). Fresh cultures of each strain were prepared on seawater complete medium (11) containing (per liter of distilled water) peptone

(Difco), 5 g; yeast extract (Difco), 0.5 g; Instant Ocean marine salts (Aquarium Systems, Mentor, Ohio), 20 g; and agar (Difco), 15 g (pH 7.4).

Inoculum preparation. Cells of each strain were cultivated in nutrient-supplemented 20‰ basal salts containing (per liter distilled water) NaCl, 13.67 g; Na_2SO_4, 2.29 g; KCl, 0.38 g; $NaHCO_3$, 0.112 g; KBr, 0.056 g; H_3BO_3, 0.015 g; NaF, 0.0016 g; $MgCl_2 \cdot 6H_2O$ (Fisher), 0.023 g; peptone (Difco), 5 g; yeast extract (Difco), 0.5 g (pH 7.4). Freshly cultivated (i.e., nutrient-conditioned) cells were grown 18 h at 30°C, harvested by centrifugation (4,234 × *g*, 15 min), washed twice in 20‰ basal salts, and employed as the nutrient-conditioned inoculum in subsequent studies. Approximately 5×10^3 cells per ml (by culturable count) were added to the microcosms.

Microcosm preparation. Erlenmeyer flasks (125 ml) were acid washed for 24 h with potassium dichromate followed by three rinses with 50% (vol/vol) nitric acid and three rinses with 50% (vol/vol) hydrochloric acid. The flasks were then rinsed five times with particle-free, distilled, deionized water. The flasks were filled with 50 ml of 20‰ nutrient-free basal salts (pH 7.4, 560 mOsm/liter) and sterilized by autoclaving (121°C, 15 lb/in^2, 15 min). Microcosms were also prepared with natural seawater (salinity of 24‰) collected from Sabine Island, Fla., and were filter sterilized by passage through polycarbonate membrane filter (Nucleopore Corp., Pleasanton, Calif.; pore size, 0.08 μm).

Bacterial enumeration. Standard plate counts were conducted with osmotically balanced dilution blanks (20‰ basal salts at 560 mOsm/liter) and seawater complete medium plates (560 mOsm/liter) to prevent osmotic shock. Culture plates were incubated at 30°C for 72 h before counting colonies.

Total numbers of cells were determined by direct microscopic counting, in which 1-ml samples of cell suspensions were stained with 4′,6-diamindino-2-phenylinodole (DAPI) (final concentration, 4 μg/ml) for 20 min. The stained cell suspension was added to a Micro Filtration System (Millipore Corp., Bedfore, Mass.) containing a Nucleopore polycarbonate membrane filter (diameter, 25 μm; pore size, 0.2 μm) which had been previously stained for 24 h in an Irgalan Black solution containing 0.2% (wt/vol) Irgalan Black (Ciba-Geigy Corp., Greensboro, N.C.) in 2% (vol/vol) acetic acid and rinsed twice with sterile 20‰ basal salts. The sample was filtered at a negative pressure of 0.8 atm (81 kPa), and the moist filter was placed on a microscope slide with 1 drop of sterile 20‰ basal salts and covered with a cover slip. The fluorescent cells per field at a magnification of ×800 were counted on a Zeiss standard 18 microscope equipped with an epifluorescent illuminator and a 100-W HBO mercury light source. The appropriate filter set (no. 47 77 01, Carl Zeiss, Inc., New York, N.Y.), consisting of a BP 365/10 exciter filter, an FT 390 chromatic beam splitter, and an LP 395 barrier filter, allowed visualization of blue-white bacteria against a dark membrane background (21). The average number of cells per field was multiplied by the appropriate microscope factor to determine number of cells per milliliter.

A modification of the procedure by Kogure et al. (12) provided a direct microscopic method for enumerating viable bacteria. Before staining and counting, a 1-ml sample of the bacterial suspension was amended with 0.25 mg of yeast extract (Difco) per ml and 0.02 mg of nalidixic acid (Sigma Chemical Co., St. Louis, Mo.) per ml and was incubated for at least 6 h at 30°C. The elongated cells were counted, and the number per milliliter was calculated by multiplying by the appropriate microscope factor.

Electron microscopy of gross morphology. Nutrient-conditioned cells and cells exposed for various time intervals in natural and artificial seawater were harvested by centrifugation at 4,234 × *g* for 15 min. This was followed by one wash in sterile Millonig buffer (pH 7.4, 560 mOsm/liter) prepared without glucose (15). The cell button was then suspended in 0.5 ml of buffer, and 1 drop was placed on a Formvar-coated copper grid. The suspension was allowed to remain on the grid for 2 h, and excess fluid was withdrawn with no. 1 filter paper strips (W. R. Balston, Ltd., London, England). Primary fixation was performed with 1% (vol/vol) glutaraldehyde in Millonig buffer (pH 7.4, 560 mOsm/liter) placed dropwise on the grid for 2 h, followed by 2 rinses with filtered (0.2 μm) deionized water. Grids were negatively stained with 2% (wt/vol) ammonium molybdate (J. T. Baker Chemical Co., Phillipsburg, N.J.) in deionized water for 1 min. After two additional rinses in filtered (0.2 μm) distilled water, excess fluid was withdrawn with filter paper, and the grids were dehydrated in an ethanol atmosphere (6).

Thin sections of cells. Cells were harvested and washed as in the whole mount technique. After the cell button was fixed in 1% glutaraldehyde in Millonig buffer for 2 h, it was washed twice in Millonig buffer, enrobed in 2% Noble agar (Difco) at 42°C, allowed to solidify, and cut into cubes (1 by 1 mm) with an ethanol-cleaned razor blade. The cubes were postfixed in 1% (wt/vol) OsO_4 in Millonig buffer (pH 7.4, 560 mOsm/liter) for 2 h. Fixed cubes were serially dehydrated in increasing concentrations of ethanol and were embedded in Spurr medium (25). Sections were cut with a LKB Ultrotome I with glass knives, placed on copper grids, and stained 10 min with 2% uranyl acetate and lead citrate (23). All grids were examined at 60 kV on a Phillips 201 EM transmission electron microscope.

Nutrient supplementation assay. Starved cells (75 days old) were placed on Formvar-coated grids, and a drop of nutrient solution peptone (1 mg/ml) and yeast extract (0.1 mg/ml) in 20‰ basal salts was placed on each coated grid. At timed intervals from 10 min to 2 h, the nutrient was withdrawn with filter paper, and the grid was rinsed and prepared for electron microscopy. Effects of nutrient supplementation of starved cells were also evaluated by employing Irgalan Black-stained polycarbonate membranes (stained 24 h and rinsed three times in sterile 20‰ basal salts) which were incubated for 24 h at 21°C in nutrient-free microcosms with cells previously starved for 20 days. The polycarbonate membranes with adhering starved cells were aseptically removed from the microcosm and washed three times in 20‰ basal salts, followed by a final rinse with a forceful stream of 20‰ basal salts from a 250-ml wash bottle. The membranes were placed in Micro Filtration Systems, and a nutrient solution (1 mg of peptone per ml, 0.1 mg of yeast extract per ml) was added. After 2 and 24 h, a filter was fixed with 1% (vol/vol) glutaraldehyde in 20‰ basal salts and stained with DAPI for 20 min. Changes in cell morphology were recorded by photomicrogra-

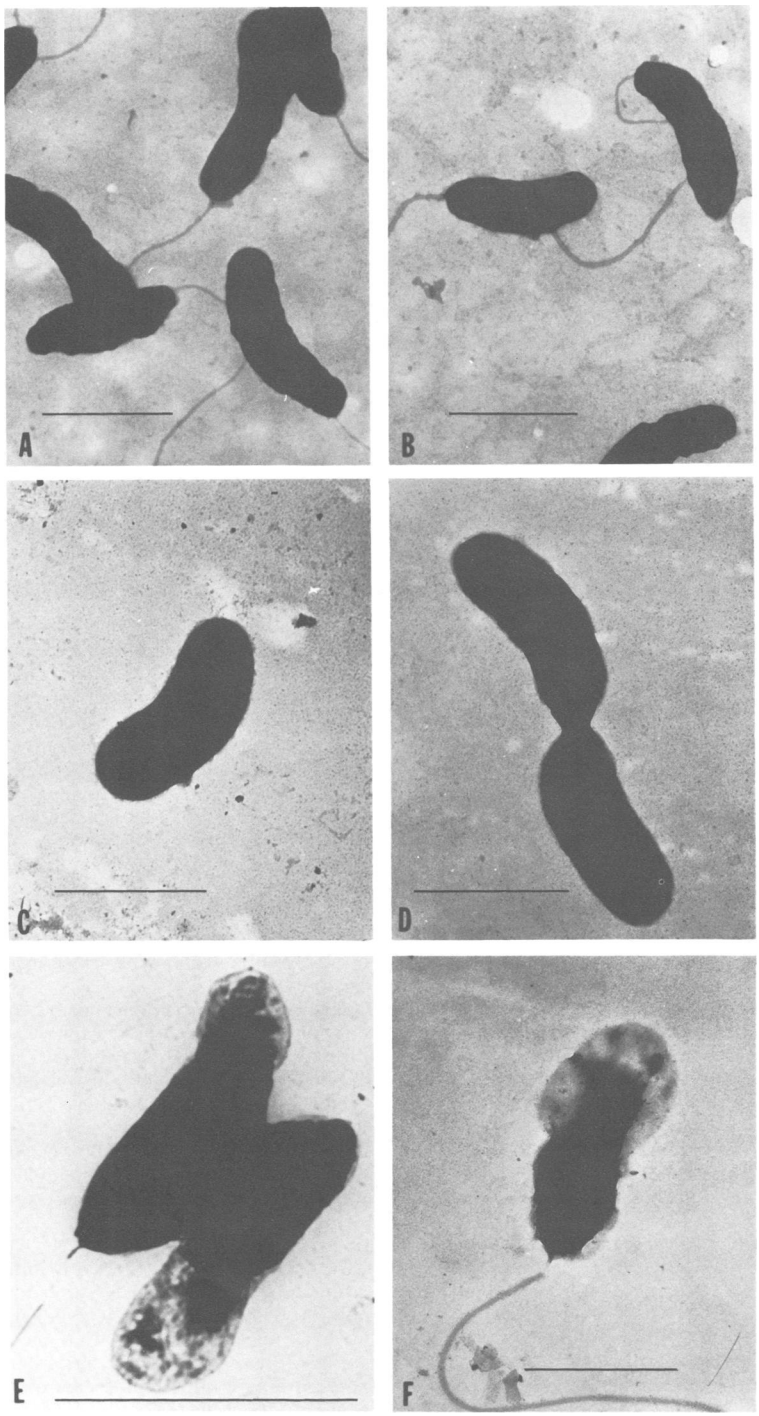

FIG. 1. Transmission electron micrographs of nutrient-conditioned cells (strain WF110) harvested from nutrient-basal salts solution, (A) without washing; (B) with 1× nutrient-free basal salts wash; (C) with 1× sterile Millonig buffer wash. Cells (strain CA401) starved for (D) 24 h, (E) 5 days, and (F) 12 days exhibit increasing polar electron transparency. Ammonium molybdate. Bar, 1 μm.

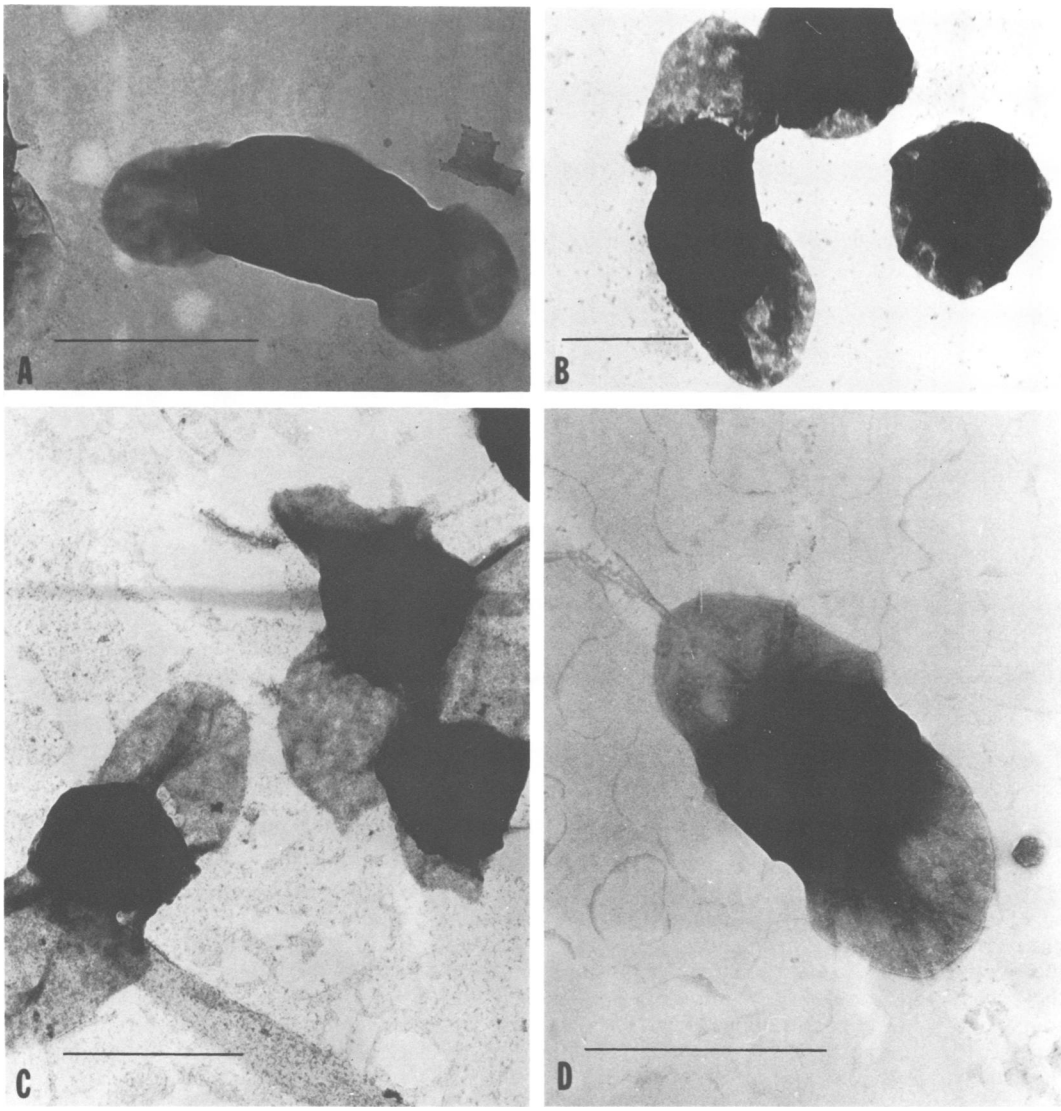

FIG. 2. Transmission electron micrographs of cells (WF110) starved for (A) 15 days, (B) 18 days, (C) 75 days, and (D) 240 days. All exhibit bipolar electron transparency. Ammonium molybdate. Bar, 0.5 μm.

phy with Ectachrome 400 film in a Yashica FR 35-mm SLR camera mounted on the Zeiss standard 18 epifluorescence microscope.

The initiation of cell division and the growth response of starved and nutrient-conditioned cells (strain CA401) was determined as follows. Cells starved for 330 days in artificial seawater, and cells grown at 21°C for 18 h in 1 g of peptone per liter–0.1 g of yeast extract per liter in 20‰ basal salts were inoculated into microcosms also containing 1 g of peptone per liter–0.1 g of yeast extract per liter in 20‰ basal salts. The microcosms were incubated at 21°C, and at selected intervals, plate counts, direct total counts, and direct viable counts were performed as previously described.

RESULTS

Effect of nutrient deprivation. The effect of the washing procedure on the integrity of *V. cholerae* cells was determined by examining cells directly harvested from nutrient broth (without washing) and cells washed in both Millonig buffer and the nutrient-free 20‰ basal salts solution. All preparations (Fig. 1A, B, and C) yielded cells which were rod-shaped, curved, and uniformly electron dense with a mean length and width of 1.6 and 0.4 μm, suggesting that washing had no apparent effect on the gross morphology of the cells.

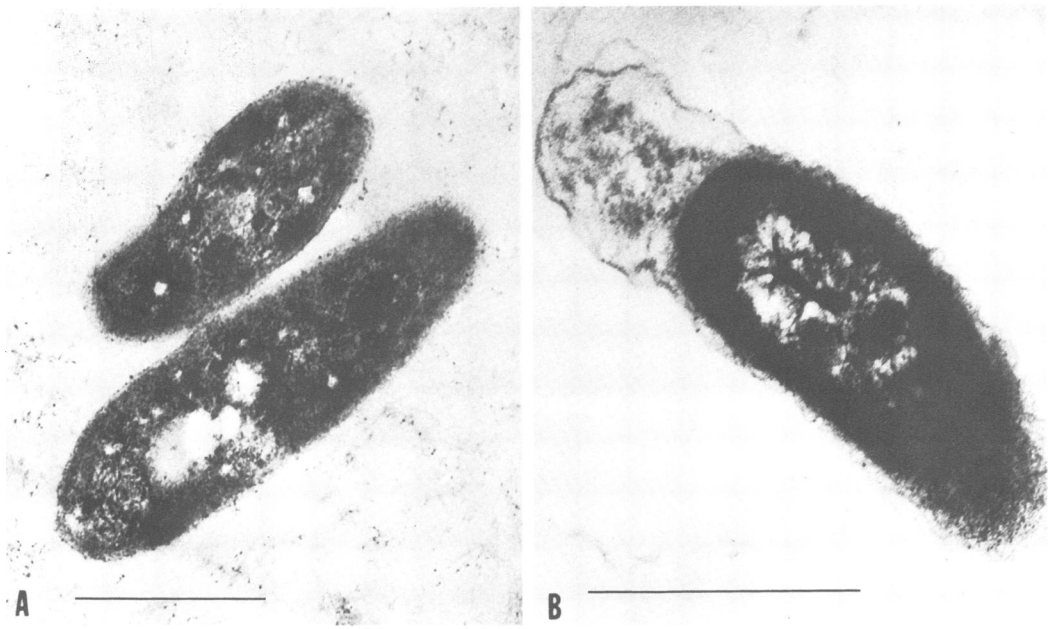

FIG. 3. Transmission electron micrographs of thin sections of cells (strain CA401) comparing (A) nutrient-conditioned cells (18 h old) and (B) cells starved 75 days. Uranyl acetate. Bar, 0.5 μm.

After exposure of the nutrient-conditioned cells to nutrient-free basal salts for 24 h, the cells of both strain WF110 and strain CA401 were still uniformly electron dense, with a mean length of 1.5 μm and a width of 0.38 μm (Fig. 1D). However, after 48 h, the cells began to exhibit small regions of electron transparency at the periphery, but there was no significant change in the overall cell size. After 5 days of exposure, there was an increase in the amount of electron transparency at the polar regions (Fig. 1E). The area of electron transparency appeared opposite the flagellar pole, and the mean length of the electron-dense portion of the cell had decreased to 0.8 μm. Little further change was observed after 12 days (Fig. 1F), but by the 15th day, electron transparency was observed at both ends of the cells, and the electron-dense region of the cell had decreased to 0.6 by 0.3 μm (Fig. 2A).

By 18 days, the cell appeared as a spherical electron-dense central region with a diameter of 0.56 μm surrounded by a remnant cell wall (Fig. 2B). Cells collected after 75 and 240 days exhibited a similar shape (Fig. 2C and D), with the size of the electron-dense body having decreased to 0.33 μm after 240 days. The remnant cell wall appeared as a folded (Fig. 2D) or unfolded (Fig. 2C) structure, with the folded configuration being the most prevalent of the shapes observed after 75 days.

When the two strains of *V. cholerae* were examined in filter-sterilized natural seawater, the changes in morphology were the same as those observed in the chemically defined basal salt solutions. Cells exposed for 55 days to natural seawater exhibited a central electron-dense body whose diameter was 0.53 μm.

Thin sections of the *V. cholerae* cells from nutrient broth before inoculation into the various microcosms revealed the typical rod-shaped cells with a definitive cell wall, the underlying cytoplasmic membrane, and a uniform distribution of electron-dense particles throughout the cytoplasm (Fig. 3A). However, cells harvested after 75 days from nutrient-free basal salts demonstrated a separation of the cell wall and cytoplasmic membrane (Fig. 3B). The central portion of the cell appeared as an electron-dense body surrounded by a three-layered membrane (probably the cytoplasmic membrane), and within the electron-dense body there were regions of complete electron transparency. Although the remnant cell wall appeared convoluted, it retained its three-layered structure.

The response of cell numbers of the two strains in basal salts and filtered natural seawater (Fig. 4) revealed after 2 days an increase in culturable, direct total, and direct viable counts of approximately 2.5 $\log_{10}$. After 20 days of exposure to nutrient-free conditions, culturable counts were 1.5 to 2.0 $\log_{10}$ lower than direct total counts, whereas direct viable counts were approximately 1.0 $\log_{10}$ lower than direct total

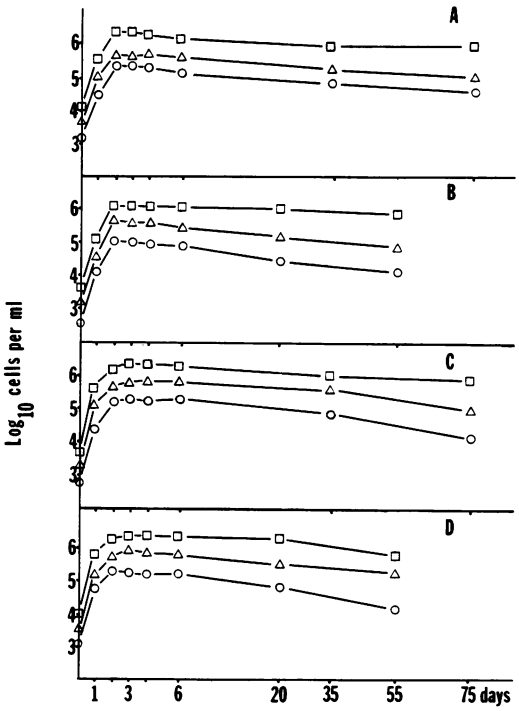

FIG. 4. Direct total counts (□), direct viable counts (△), and plate counts (○) of: (A) WF110 cells in basal salts; (B) WF110 cells in filtered natural seawater; (C) CA401 cells in basal salts; and (D) CA401 cells in filtered natural seawater.

counts for strain WF110. For strain CA401, the difference in the direct viable and direct total counts was only 0.5 to 0.75 $\log_{10}$.

Effect of nutrient supplementation. Upon the addition of nutrients to starved cells, the shapes of the cells changed very rapidly. Within 10 min, the cells began to revert back to the typical comma-shaped cells (Fig. 5A). The electron-dense portions of the cells increased from 0.4 to 0.6 μm, and after 20 min, electron-dense regions were as large as 1 by 0.4 μm (Fig. 5B). By 30 min, the electron-dense region had enlarged to 1.3 by 0.45 μm (Fig. 5C). After 1 h, only a small portion of the electron-transparent remnant cell wall remained at the polar end opposite the flagellum (Fig. 5D). Within 2 h of nutrient supplementation, the cells had attained a size and appearance similar to those of nutrient-conditioned cells, except that the reconditioned cells appeared to have small remnants of the original cell wall material present (Fig. 5E and F).

The effects of nutrient supplementation on starved cells were also examined by epifluorescence microscopy. Starved cells (strain CA401, 20 days old) stained with DAPI appeared as small spherical structures with blue-white fluorescence (Fig. 6A). Two hours after nutrient addition, the cells were larger and vibrioid, and they fluoresced more diffusely (Fig. 6B). After 24 h, the cells were similar in appearance to nutrient-conditioned cells (Fig. 6C).

Initiation of cell division by both starved and nutrient-conditioned cells occurred at approximately the same time, i.e., within 4 to 5 h after nutrient addition (Fig. 7). In both starved and nutrient-conditioned cells, the relationships among direct total, direct viable, and plate counts remained relatively constant over the growth period. However, the exponential phase of starved cells was considerably different from that of nutrient-conditioned cells. Theoretically, the generation time of any bacterial population remains constant during exponential growth. When generation times were calculated for both starved and nutrient-conditioned cells during selected intervals throughout the exponential phase (Table 1), starved cells showed longer generation times than nutrient-conditioned cells, and they showed a significantly longer generation time during phase II than during phase I. Whether this reflects a secondary adaptation phase (perhaps analogous to a lag phase) or more complex molecular events or both is unknown, but the results suggest that starved cells require more time to divide.

DISCUSSION

For a microorganism to survive in an aquatic environment, it must have the ability to cope with transient nutrient deprivation, fluctuations in physical and chemical parameters, and antagonistic microbial factors (5). It has been shown that the exposure of certain gram-negative bacteria to nutrient deprivation results in (i) continued cell division and increases in cell numbers (18, 26) and (ii) the formation of coccoid cells (2, 8, 11, 17, 21). It has been proposed that these responses represent strategies by which the survival of an organism is enhanced when confronted with exogenous nutrient deprivation (21).

From the results of the studies reported here, it is evident that *V. cholerae* reacts to nutrient deprivation in a similar manner, i.e., it undergoes cell division, increases cell numbers, and subsequently forms coccoid cells when exposed to nutrient deprivation. Such an increase in cell numbers in the initial starvation period was demonstrated with a psychrophilic marine vibrio as well as with other filterable marine bacteria (17, 18, 26). Novitsky and Morita (18) also noted that the optical density of a starved suspension of a marine vibrio (strain ANT-300) decreased whereas viable cell counts increased, suggesting a decrease in individual cell volume. This process in which cell numbers increase with constant

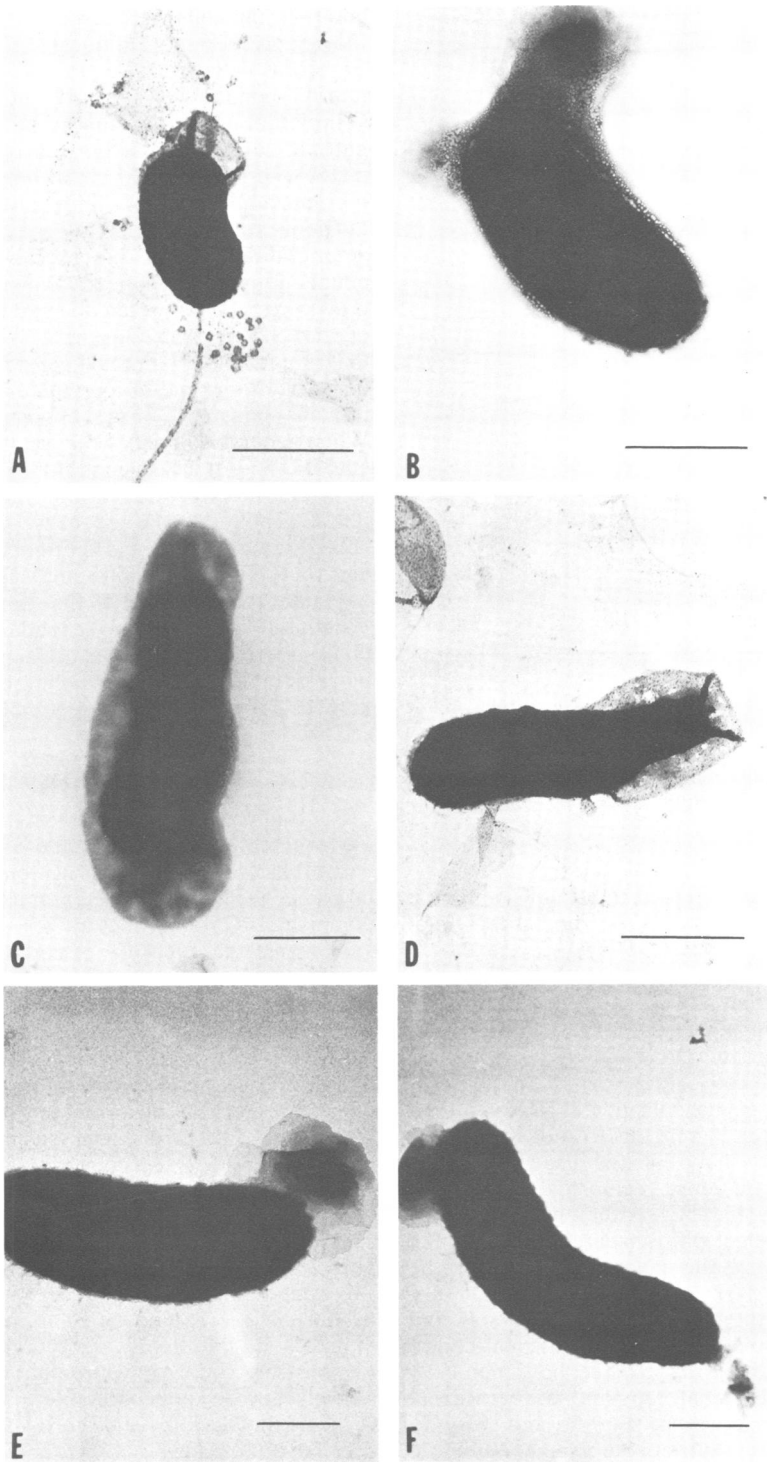

FIG. 5. Transmission electron micrographs of cells (CA401) starved for 75 days at different times after nutrient addition: (A) 10 min; (B) 20 min; (C) 30 min; (D) 1 h; (E) 2 h; (F) 2 h. Ammonium molybdate. Bar, 0.5 μm.

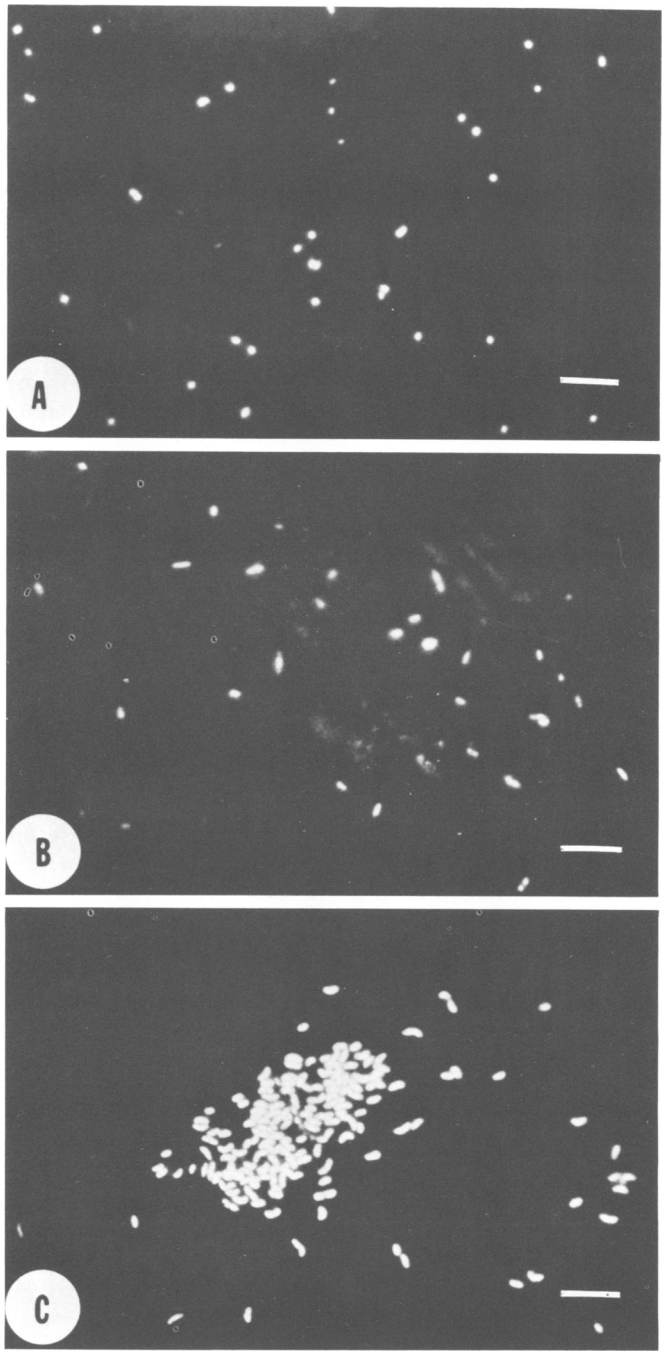

FIG. 6. Photomicrographs of DAPI-stained cells (strain WF110) starved 20 days. (A) Before nutrient addition; (B) 2 h after nutrient addition; (C) 24 h after nutrient addition. Bar, 10 μm

total biomass (reductive division) has been suggested as a survival mechanism (13, 19, 26). For an increase in cell numbers to occur in the initial starvation period, the cells should be able to derive energy from endogenous sources if such cells are nutrient conditioned before starvation. In addition, cells in the initial starvation period should be able to employ previously initiated multiple replication forks and continue DNA synthesis in the absence of exogenous nutrients.

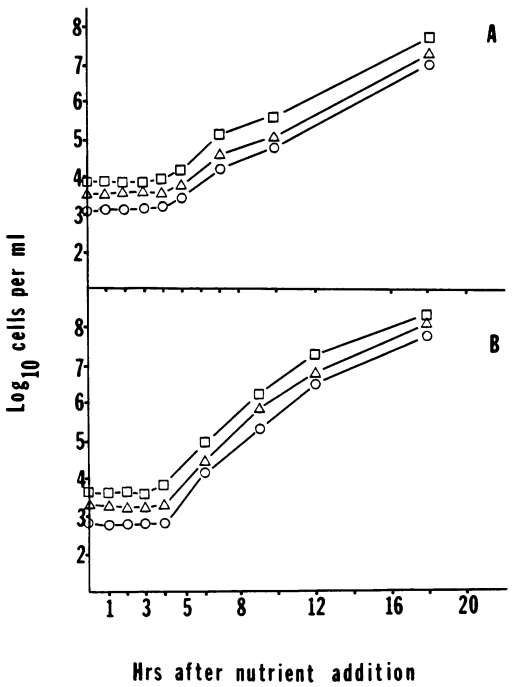

FIG. 7. Direct total counts (□), direct viable counts (△), and plate counts (○) of (A) starved cells (330 days) and (B) nutrient-conditioned cells (strain CA401) monitored for 24 h after nutrient supplementation. Note that both starved and conditioned cells initiate division within 5 h.

Bird and Lark (4) demonstrated that several replication forks exist in *Escherichia coli* upon amino acid starvation. Schaechter (24) demonstrated that *Salmonella typhimurium*, when tryptophan starved, continued DNA synthesis at near-exponential levels for more than 1 h after starvation began. Reliance on endogenous nutrients and the presence of multiple replication forks as well as the continuation of DNA synthesis may account for the significant increase of *V. cholerae* cells in the initial starvation period.

The possibility of scavenging nutrient contaminants was kept to a minimum in our studies by repeated washing of the *V. cholerae* inoculum as well as by employing high-purity constituents in the basal salts solution. If exogenous nutrient scavenging was the primary reason for the cell number increase in the initial starvation period (2 days), then nutrient-grown cells washed repeatedly and inoculated into spent basal salts solution (inoculated 48 h earlier and centrifuged to remove original *V. cholerae* inoculum) should not exhibit the previously dramatic increase in cell numbers. When this experiment was performed, the results were an increase in cell number equal to that shown with a fresh inoculum and fresh basal salts solution (R. M. Baker and M. A. Hood, unpublished results). These results would suggest that the cells are using endogenous nutrients as the energy source for the cell number increase. However, exogenous nutrient scavenging cannot be ruled out completely since cells may leak nutrients into the basal salts solution.

The formation of coccoid cells seems to be a process in concert with but separate from the initial increase in cell numbers while keeping biomass constant. The initial phenomenon of rapid division in the first 48 h is followed by a gradual reduction in cellular volume, i.e., decreasing size of the electron-dense area of the cell while the electron-transparent areas at the cellular poles increase until a coccoid cell surrounded by its electron-transparent envelope is evident after approximately 15 days. The survival advantage of these coccoid cells may be explained by the fact that the increasing ratio of surface area to volume evidenced with spheres of decreasing size would allow the coccoid cell to have the greatest surface area for nutrient uptake while maintaining the least amount of cell mass. Although these coccoid cells were found to be more osmotically sensitive than freshly grown cells (3), preliminary studies suggest that such coccoid cells may be more resistant to cold temperatures (unpublished data) than healthy vibrioid cells.

Several other observations, including the rapid uptake of nutrients, resumption of the larger vibrioid shape, and subsequent cell division, may also confer a survival advantage to a cell that encounters a nutrient source after a period of nutrient deprivation. Amy et al. (1) demonstrated the recovery of a starved coccoid marine vibrio and the resumption of the vibrioid shape after nutrient supplementation. Experiments reported here demonstrate that coccoid starved *V. cholerae* cells revert back to typically healthy vibrioid forms rapidly (within 2 h at 21°C) after nutrient supplementation and begin cell division

TABLE 1. Generation time of nutrient-conditioned and starved cells at 21°C in peptone-yeast extract-basal salts

Phase[a]	Generation time (h)	
	Nutrient-conditioned cells	Starved cells
I	0.52	1.0
II	0.67	1.5
III	0.86	1.2
IV	1.5	1.1

[a] Phase I, 4 to 6 h (nutrient-conditioned cells), 4 to 5 h (starved cells); phase II, 6 to 9 h (nutrient-conditioned cells), 5 to 7 h (starved cells); phase III, 9 to 12 h (nutrient-conditioned cells), 7 to 10 h (starved cells); phase IV, 12 to 18 h (nutrient-conditioned cells), 10 to 18 h (starved cells).

at approximately the same time as their nutrient-conditioned counterparts. The rapid response to nutrient addition demonstrated by the coccoid-to-vibrioid reversion and subsequent cell division of *V. cholerae* would require that macromolecular constituents (RNA, DNA) be sufficiently available for such cells to take advantage of available nutrients immediately (16). It has been shown that endogenous respiration decreases with increasing starvation time (19). Amy et al. (1) demonstrated that in the initial starvation period, the levels of RNA, DNA and protein decrease as viable cell numbers increase. After further starvation, this was followed by a gradual increase in RNA, DNA, and protein synthesis machinery in cells of strain ANT-300. The rapid response of starved *V. cholerae* cells to nutrient supplementation may be explained by a similar macromolecular readiness to take advantage of transient nutrients encountered in the aquatic environment.

When plate, direct viable, and direct total counts were performed on nutrient-supplemented starved cells and nutrient-conditioned cells in our studies, both starved and healthy cells initiated division at approximately the same time. The initiation of division after nutrient supplementation occurred between 4 and 5 h, though subsequent generation times for healthy and starved cells differed.

In the nutrient supplementation studies of healthy and starved cells, the difference of approximately 0.5 log between direct viable and direct total counts is not surprising for the starved cell inoculum as the viable population of starved cells is commonly 1/10 of the total cell number (16). Yet the difference of approximately 0.5 log between the direct viable and direct total counts for the nutrient-conditioned cell inoculum cannot be readily accounted for beyond a twofold difference (nonviable to viable ratio of 1:1 at the end of the log phase). The significance of the comparison of nutrient supplementation of starved versus healthy cells lies in the similar lengths of the lag periods and the longer generation time of the starved cells.

In this study, *V. cholerae* cells have been shown to increase in number and decrease in volume in a defined basal salts solution and filtered natural seawater as a result of nutrient deprivation. Additionally, the sustained viability and rapid reversion of coccoid starved cells to the vibrioid shape and subsequent cell division upon nutrient addition may confer a survival advantage to aquatic microorganism which encounter transient nutrient deprivation.

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