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Effect of Nutrient Deprivation on Lipid, Carbohydrate, DNA, RNA, and Protein Levels in *Vibrio cholerae*

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The response of *Vibrio cholerae* to low nutrient levels was determined by measuring the concentrations of lipids, carbohydrates, DNA, RNA, and proteins over a 30-day starvation period. Ultrastructural integrity was observed by transmission electron microscopy. Total lipids and carbohydrates declined rapidly within the first 7 days, while DNA and protein exhibited a more constant decline over the 30 days of starvation. In contrast, RNA showed little decrease upon starvation. Although neutral lipids were lost, the percentage of neutral lipids did not decline as rapidly as the phospholipids. Detectable levels of poly-β-hydroxybutyrate disappeared completely by 7 days. Carbohydrate profiles revealed the relative loss of the five-carbon sugar ribose and *N*-acetylglucosamine and a relative increase in the total six-carbon sugars, especially glucose. Morphologically, ribosomes appeared to exhibit no structural change, while inclusion bodies and mesosomelike structures disappeared completely, and cell wall and membrane integrity was lost. The data suggest that *V. cholerae* differs somewhat from other marine vibrios in its response to low nutrients but shares some characteristics in common with them. The data also suggest that certain lipids and carbohydrates may provide the endogenous energy sources needed for dormancy preparation and cell maintenance under nutrient starvation.

Considerable information is available on the survival of bacteria at low nutrient levels (1–5, 13–15, 18–20, 27–29; also, reviews 11, 26, and 35). Although a number of starvation studies have been conducted with many species of bacteria, the most extensive studies have used the marine vibrios ANT-300 (2–4, 28, 31) and DW1 (15, 18–20). In earlier reports (5, 13) we observed that strains of *Vibrio cholerae* were able to survive long periods of low-nutrient stress and that like ANT-300, the strains exhibited a type 1 starvation survival pattern, i.e., upon nutrient deprivation in a closed system, an initial increase in viable cells occurred followed by a decrease in the viable population until a constant level was reached (1). Furthermore, we have inoculated low levels of *V. cholerae* (10^3/ml) into solutions of carbon-free basal salts (15 to 20‰), and after as long as 5 years at ambient temperatures, viable populations were recovered that were as high as the initial inoculum (unpublished data).

In an effort to more fully understand the response of *V. cholerae* to low nutrient levels, we determined the changes in levels of macromolecules, i.e., lipids, carbohydrates, DNA, RNA, and proteins, that occur when the organism is exposed to nutrient starvation. This report presents the results of those studies.

MATERIALS AND METHODS

Microcosms. *V. cholerae* CA401 was grown in seawater complete broth made with basal salts (5) for 18 h at 35°C. The cells were harvested by centrifugation and washed three times in basal salts. Cells were inoculated into sterile 2-liter flasks containing 1 liter of basal salts at an inoculum size of 10^7 cells per ml and incubated at 22°C in the dark. At 0, 7, or 14 and 30 days after inoculation, cells were counted by standard plate counting, acridine orange direct counting (12), and direct viable counting (21) and harvested by centrifugation for chemical analysis. For the second lipid analysis, seawater complete broth was lipid extracted before its use to reduce exogenous fatty acids. Three or five replicas were used for each analysis, and the experiments were repeated twice, giving 6 or 10 replicas.

Carbohydrates. The extraction and analysis of carbohydrates was carried out by the methods of Oades (30) and Shaw and Moss (34) and included the following modifications. After digestion with HCl, samples were blanketed with nitrogen and hydrolyzed for 5 h at 100.5°C. The cooled samples were extracted three times with chloroform-hexane (1:5, vol/vol) and dried with nitrogen concentrator-evaporators at 45°C. After NH_4OH and sodium borohydride treatment and neutralization with glacial acetic acid, the samples were concentrated with nitrogen at 75°C. Methanol-benzene (5:1, vol/vol) with 25 μl of acetic acid was added, heated to 85°C for 5 min, cooled, and evaporated with nitrogen evaporators at 37°C. Three extractions with methanol and nitrogen evaporation at 75°C to remove boron were followed by the addition of acetic anhydride and pyridine and heating at 100°C for 30 min. After nitrogen evaporation at 37°C, samples were diluted with chloroform and water extracted three times. The chloroform layer was evaporated to dryness with nitrogen at 37°C, diluted with chloroform, and used for gas chromatographic injection. Standard pentoses, hexoses, heptoses, and amino and deoxy sugars were obtained (Sigma Chemical Co., St. Louis, Mo.), and gas-liquid chromatography was performed with a Varian model 2100 gas chromatograph equipped with flame ionization detectors and dual differential electrometers.

Total lipids. The extraction and analysis of fatty acids in total lipids of whole cells was determined by the method

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FIG. 1. Transmission electron micrographs of thin sections of *V. cholerae*. (A) Nonstarved *V. cholerae* cell; Om, outer membrane; Cm, cell membrane; (B) starved *V. cholerae* cell; rb, ribosome; (C) starved *V. cholerae* cell. Bar = 0.1 μm.

described by Liebert et al. (22). In addition, a second total lipid determination was performed by silicic acid column chromatography (9). The resulting neutral, glycolipid, and phospholipid fractions were subjected to mild alkaline methanolysis, and the fatty acid methyl esters were quantified by gas chromatography as previously described (9).

PHB. *Poly-β-hydroxybutyrate* (PHB) (poly-β-hydroxyalkanoate) was measured by the procedure described by Findlay and White (7).

RNA. RNA was extracted by a procedure based on the methods of Bolton (6), Kirby (17), and Johnson (16). Cells were lysed with 2% sodium dodecyl sulfate and centrifuged at 17,000 rpm (Beckman JA-20 rotor) for 10 min to pellet high-molecular-weight chromosomal DNA, insoluble proteins, and cell fragments. The supernatant was extracted with 1% sodium dodecyl sulfate–tris-saturated phenol to remove proteins. After centrifugation at 17,000 rpm for 10 min, the supernatant was extracted with chloroform-isoamyl alcohol (4:96, vol/vol) for additional protein removal. Purity of the RNA was determined by the ratio of A_{260} to A_{280}. Absorbance was measured with a Gilford spectrophotometer, and the amount of RNA was calculated by comparing our data with a standard curve.

DNA. DNA was extracted by the method described by Marmur (25) with a phenol extraction as the first step (16). The purity of DNA was determined by the ratio of A_{260} to A_{230} (protein) and A_{260} to A_{280} (RNA). The amount of DNA was calculated by comparing our data with a standard curve.

Protein. Protein was determined by the Lowry method (23).

Electron microscopy. Modifications of the methods described by Baker et al. (5) were used for electron microscopy. Nonstarved and starved cells were harvested by centrifugation, enrobed in 2 or 4% (wt/vol) gelatin, and fixed in gluteraldehyde and osmium tetroxide. In the starved cells, 0.01% $CaCl_2$ was added to the fixatives to prevent cell rupture. Specimens were washed in phosphate buffer and postfixed for 8 h in 2% osmium tetroxide in phosphate buffer at 0°C. The second series of phosphate buffer washes were sequentially graded to distilled water, and cells were dehydrated in an ethanol series ranging from 30 to 100% ethanol. After dehydration, the blocks were embedded in Epon, and thin sections were cut out on an LKB Ultratome microtome with glass knives. Poststaining consisted of 2% lead citrate and 2% uranyl acetate at pH 7.4 and 3.6. Thin sections were examined on a Phillips EM 201 transmission electron microscope.

RESULTS

Upon starvation, *V. cholerae* exhibited the following gross morphological changes (Fig. 1). (i) The cells became coccoid and decreased in volume, losing over 90% of their original volume in 30 days; (ii) all granules and inclusion bodies were

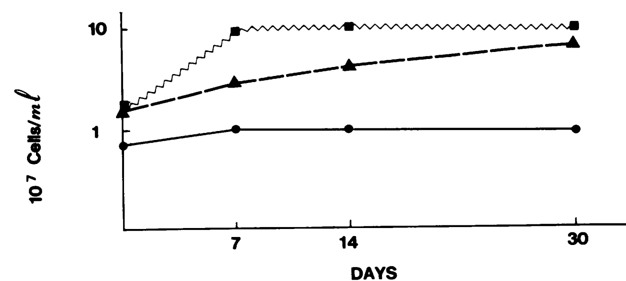

FIG. 2. Effect of nutrient deprivation on cell number. Symbols: ●, standard plate counts; ▲, direct viable counts (Kogure); ■, Acridine orange direct counts.

lost; (iii) the distinct three-layered integrity of the outer membrane and the cell membrane was lost, but remnants of the structures remained; (iv) the nuclear region (electron-clear area) was compressed into the center of the cell surrounded by a denser cytoplasm; and (v) the cell wall formed an extended or convoluted structure which pulled away from the cell membrane. The ribosomal structure, however, appeared to be conserved as it exhibited no apparent change in the starved cells.

In terms of cell numbers, there were nearly ten times more viable cells (direct viable count) after 30 days of starvation (Fig. 2). When lower inoculation sizes were used as reported in an earlier study (5), it was observed that 2 to 2.5 log increases in viable cell numbers occurred. Such increases in viable cell numbers are common among aquatic bacteria (1).

The response of the cells with regard to total carbohydrates and lipids followed a similar pattern. After 7 days of starvation, 88.7% of the carbohydrates (Fig. 3) and 99.8% of the total lipids (Table 1), had disappeared followed by small decreases at 30 days. Phospholipids were the most abundant lipids, making up 99.88% of the total lipids, while neutral lipids made up only 0.12% of the total lipids in nonstarved cells (Table 1). However, with starvation, phospholipids disappeared more rapidly relative to neutral lipids. There were no glycolipids or free fatty acids detected in either nonstarved or starved cells. Similarly, PHB disappeared rapidly, and there were no detectable levels after 7 days of starvation (Table 1). Fatty acid patterns in the total lipid fraction changed with starvation. Saturated fatty acids increased, while unsaturated and hydroxy fatty acids decreased (Table 2). The most dramatic changes occurred with respect to the most abundant fatty acids, i.e., the 16-carbon fatty acids. There was a decline in the unsaturated fatty acid 16:1w7c (from 39.6 to 14.1%) and a concomitant relative increase in the saturated fatty acid 16:0 (from 23.5 to 37.0%) with starvation.

Carbohydrate profiles revealed a relative decline of the three- and five-carbon sugars glyceraldehyde and ribose, as well as *N*-acetylglucosamine, with a concomitant relative increase in the six-carbon sugars mannose, galactose, and especially glucose. Originally, the six-carbon sugar fructose was believed to be the L-sugar fucose, but when standard fucose (alditol acetate derivative) was examined, a difference of 0.08 minute between the retention time of fucose and

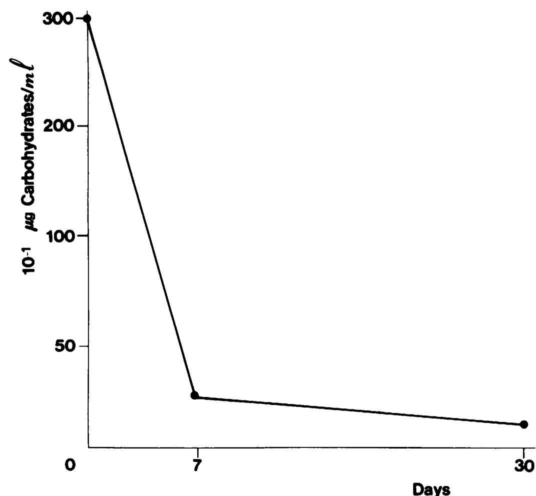

FIG. 3. Effect of nutrient deprivation on total carbohydrates.

TABLE 1. Changes in quantities of *V. cholerae* lipid classes during starvation

Cells	nmol/10^4 cells (% total lipids)					
	Total lipids	Phospholipids	Glycolipids	Neutral lipids	Free fatty acids	PHB
Nonstarved	3,679.18	3,675 (99.88)	0	4.18 (0.12)	0	0.37
Starved						
7 days	7.75	6.95 (89.94)	0	0.80 (10.06)	0	0
30 days	2.43	2.05 (83.55)	0	0.38 (16.45)	0	0

the unknown sugar was observed, suggesting that the sugar is probably fructose in a furanose form. Without mass spectrophotometry, however, the identification of this sugar is unconfirmed (Table 3).

DNA concentrations (Fig. 4) and protein levels (Fig. 5) exhibited a gradual but constant decline over the 30-day starvation period. By 30 days, approximately 75% of the total DNA and 70% of the protein had disappeared. In contrast to DNA and protein, RNA levels changed very little upon starvation (Fig. 6). For example, after 14 days, there was only a 2% decrease in concentration, and by 30 days, only a 20% decline.

DISCUSSION

A number of events have been shown to occur when aquatic bacteria are exposed to nutrient deprivation. Among these are an increase in cell numbers, first described as reductive division or fragmentation (20, 28), and size reduction characterized as dwarfing (15).

The morphological response of *V. cholerae* to starvation shared characteristics both common as well as dissimilar to the marine vibrio ANT-300 (3, 27). Although *V. cholerae* and ANT-300 did not respond in the same way to starvation in terms of total size reduction, intracellular integrity decreased in both organisms, granules disappeared, and the nuclear regions became more compact. *V. cholerae* exhibited considerable convolution of the cell wall, while ANT-300 showed little distortion in the cell wall (27). When three marine strains were short-term starved (48 h), one showed little cell wall distortion, while two strains produced vesicles in 24 h and released these vesicles with time (24). It was concluded that these vesicles were related to the continuous size reduction during starvation. We also noticed some vesicle formation early in the starvation period (within 24 h) with *V. cholerae*, but such vesicles disappeared quickly.

Another feature common to *V. cholerae* and ANT-300 was the apparent conservation of ribosomal structure (3), although the number of ribosomes appeared to decline. The preservation of the ribosome has also been noted in endospores, and it has been suggested that because the protein-synthesizing machinery is so energy expensive, this is a wise survival strategy (8). This argument might similarly be applicable to an aquatic organism that frequently encounters low nutrients and must become dormant (35) to survive.

It has been proposed that cells do not merely go into dormancy passively but rearrange their cellular constituents into different compounds so that survival is enhanced (2), and there have been a number of studies which have examined compositional changes in aquatic bacteria exposed

TABLE 2. Changes in whole-cell hydroxy and normal fatty acids of *V. cholerae*

Fatty acid	Nonstarved cells[a]	Starved cells[a]	
		7 day	30 day
2OH 12:0	3.7	2.1	0.0
14:0	7.3	8.1	9.8
i15:0	0.1	0.2	0.6
15:0	0.3	0.7	1.5
3OH 14:0	1.4	0.3	0.4
i16:0	0.0	0.0	0.0
16:1w7c	39.6	33.3	14.1
16:1w7t	0.0	0.0	12.7
16:0	23.5	31.8	37.0
3OH 15:0	0.0	0.0	0.0
17:0	0.6	0.5	1.4
18:2	2.6	0.3	0.0
3OH 16:0	1.0	0.0	0.0
18:1w9c	0.0	1.3	3.0
18:1w7c	12.2	12.4	10.0
18:0	2.2	3.2	5.4
19:0	2.4	0.6	0.2
20:1	3.1	1.4	0.4
20:0	0.0	0.3	0.6
Saturated	36.3	45.2	55.9
Unsaturated	57.5	48.7	40.2
Hydroxy	6.1	2.4	0.4

[a] Data are expressed as mole percent.

to low nutrients. For example, it was observed that when ANT-300 was starved for 21 days at 5°C, phospholipids decreased by 65% with a large relative increase (57%) in the neutral lipid fraction especially during the first week (31). In contrast, the phospholipids of *V. cholerae* declined 99.8% during the first week of starvation at 22°C. Neutral lipids also declined (from 4.18 to 0.38 nmol/10^4 cells) within 30 days, but the ratio of neutral lipids to phospholipids increased. Neutral lipids were only 0.12% of the total lipids in unstarved cells but were 16.5% of the total lipids in 30-day-starved cells. Changes in the individual fatty acids are discussed in more detail in the companion paper (10). The rapid disappearance of phospholipids in *V. cholerae* with the visible loss of cell wall integrity suggests that cell wall lipids are used as an endogenous energy source to prepare the cell for dormancy. Furthermore, the rapid disappearance of PHB suggests that this compound might similarly serve as an energy source for dormancy preparation.

Total carbohydrates in *V. cholerae* exhibited a rapid and immediate decline, with a relative increase in the six-carbon sugars (from 32 to 58%) and a decrease in the three- and five-carbon sugars glyceraldehyde and ribose and the amino sugar *N*-acetylglucosamine. The cell wall of gram-negative bacteria is typically composed of an outer membrane and a small peptidoglycan layer. The outer membrane is composed of (i) the essential lipid A (hydrophobic in nature), (ii) an R core (in *V. cholerae* there is no 2-keto-3-deoxyoctulosonic acid), and (iii) O-side chains of many tetra- and pentasaccharide units (which are hydrophilic in nature). It is possible that the more hydrophilic molecules of the O-side chains (which could be composed of the three- and five-carbon sugars in *V. cholerae*) are more readily utilized under starvation conditions, while the seven-carbon sugar (mannoheptose) and the six-carbon sugars which probably make up the oligosaccharides of the R core are relatively conserved. This idea is certainly consistent with the findings of Kjelleberg and Hermansson (18) who demonstrated that the outer membranes of certain environmental vibrios became more hydrophobic under starvation.

Although certain carbohydrates apparently decline more rapidly relative to others, why this occurs is really unclear. However, the fact that total carbohydrates and lipids decline so quickly suggests that they are used to prepare the cell for dormancy. It would seem a logical strategy for a cell to use the more energy-efficient available compounds (lipids and carbohydrates) to carry out these activities.

Concentrations of protein, DNA, and RNA in starving *V. cholerae* cells did not demonstrate the exact patterns as in ANT-300. For example, in ANT-300, protein, DNA, and RNA levels declined approximately 43, 63 and 65%, respectively, during the first 4 days of starvation, and after this initial phase, RNA and (to some small extent) DNA were resynthesized, while protein levels remained the same (3). In *V. cholerae* cells, protein and DNA concentrations declined at a constant rate, from 20 to 80% and 30 to 70%, respectively, in 7 to 30 days, while RNA levels declined only 2% in the first 14 days of starvation and only 20% by 30 days. Whether RNA is lost and resynthesized by *V. cholerae* (as it is suggested to occur in ANT-300) within the first several days could not be determined from these experiments.

It might be assumed that the initial reduction in DNA per cell may be related to the increase in cell number which occurs during the fragmentation or reductive division stage. However, after the first week of starvation, there was no increase in the number of cells, but there was a continuing decline in the amount of DNA per cell. Whether this represents a reduction in extra DNA copies, configuration changes in the molecule, or some other process is unknown, but it is an area that warrants further study.

It has been shown that upon starvation, ANT-300 (2) as well as *Escherichia coli* (32) were able to synthesize new proteins. In the case of *E. coli* (32), it was demonstrated that

TABLE 3. Effect of nutrient deprivation on carbohydrates of *V. cholerae*

Carbohydrate	Nonstarved cells[a]	Starved cells[a]	
		7-day	30-day
Glyceraldehyde	4.6	3.6	3.5
2-Deoxyribose	0.0	0.0	0.0
Rhamnose	T[b]	0.0	0.0
Fructose (or a methyl pentose)[c]	1.8	7.5	11.6
Ribose	36.8	13.3	11.5
Arabinose	T	0.0	0.0
Xylose	0.4	1.3	2.0
2-Deoxyglucose	0.0	0.0	0.0
Mannose	1.9	3.3	3.6
Galactose	0.5	6.5	6.6
Glucose	27.6	37.4	36.3
Inositol	0.0	0.0	0.0
L-Glycero-D-mannoheptose	2.8	5.6	4.1
Mannoheptulose	0.0	0.0	0.0
N-Acetylglucosamine	16.6	12.5	11.8
N-Acetylgalactosamine	0.0	0.0	0.0
2-Keto-3-deoxyoctonate	0.0	0.0	0.0
Unknown (C-6) amino sugar[d]	2.3	T	0.0

[a] Data are expressed as mole percent total.
[b] T, Trace amounts detected.
[c] Unconfirmed.
[d] Possibly quinavosamine, unconfirmed.

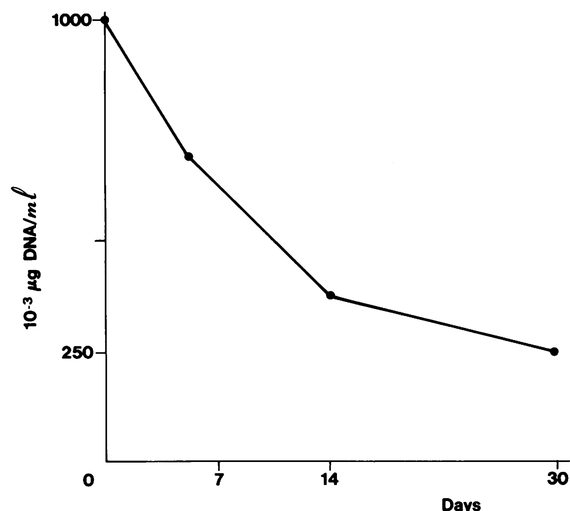

FIG. 4. Effect of nutrient deprivation on total DNA.

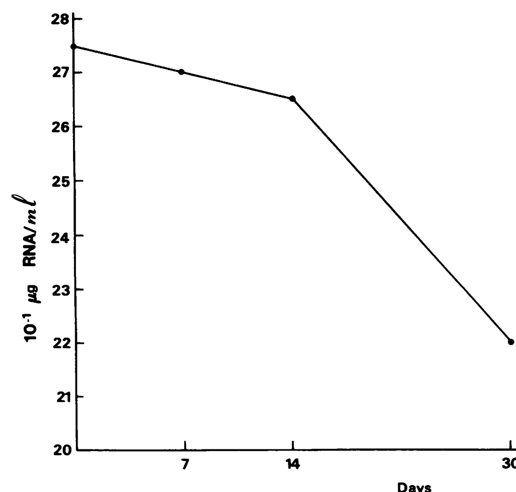

FIG. 6. Effect of nutrient deprivation on total RNA.

survival was enhanced by these new proteins, and it was concluded that these proteins enabled the cell to shift into a starvation-induced maintenance physiology. While we have no definitive data as yet on protein profiles of starving *V. cholerae* cells, the possibility that such proteins are synthesized and that they enhance survival in *V. cholerae* is entirely likely.

A typical endospore-forming organism responds to adverse environmental conditions by producing the dormant endospore. This model of dormancy is probably the most obvious and well understood of the microbial dormant forms. Since desiccation, UV light, and low nutrients are stresses that a typical spore might encounter, the spore-forming organism produces a structure with many tough protective layers such as the exosporium, spore coats, and a cortex. However, an aquatic organism would certainly not experience the lack of water nor probably excessive UV light, but it would experience low-nutrient stresses. Thus, an aquatic bacterium would not necessarily need to produce protective layers, but it may need to carry out some of the same macromolecular changes that occur in spore formation. The conservation of structural ribosomes and a decrease in total lipids, DNA, and protein are responses observed in some species of endospore formers (33). These are responses that also occurred when *V. cholerae* cells were starved. While it is clear that *V. cholerae* and other vibrios do not exhibit the same responses to nutrient deprivation as endospore-forming cells, the bacteria certainly appear to rearrange some of their macromolecules, and to consider such rearrangements in terms of a spore model may not be inappropriate.

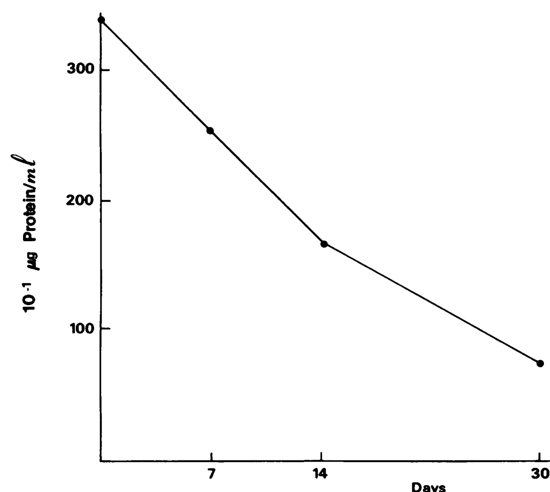

FIG. 5. Effect of nutrient deprivation on total protein.

V. cholerae also does not show the exact macromolecular responses to starvation as ANT-300, but why such differences occur are unknown. *V. cholerae* is a species that inhabits the more nutrient-rich estuarine environment and is rarely found in pelagic waters. It may be that *V. cholerae* has evolved different cellular strategies that allow the organism to survive the estaurine environment with its rapid flux in nutrients rather than the more constant stress of low nutrients in open ocean waters. We are currently attempting to better understand these survival mechanisms by examining compositional changes in lipids (10) and other macromolecules.

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