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Phospholipid Ester-Linked Fatty Acid Profile Changes during Nutrient Deprivation of *Vibrio cholerae*: Increases in the *trans/cis* Ratio and Proportions of Cyclopropyl Fatty Acids

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The phospholipid ester-linked fatty acids of 0-day-, 7-day-, and 30-day-starved cultures of *Vibrio cholerae* were compared. Statistically significant trends were noted in the fatty acid profiles as the cells starved. The amount of the *cis*-monoenoic fatty acids declined (e.g., 16:1ω7c: 0 day, 39%; 7 day, 18%; 30 day, 11%). In contrast, the saturated fatty acids, the cyclopropyl derivatives of the *cis*-monoenoic fatty acids, and *trans*-monoenoic fatty acids increased during starvation. For instance, the amounts of 16:1ω7t were: 0 day, 1%; 7 day, 13%; 30 day, 17%; which increased the *trans/cis* ratio for 16:1ω7 from 0.02 (0 day) to 0.70 (7 day) to 1.56 (30 day). This may be due to the reported high turnover rates of *cis*-monoenoic fatty acids of membrane phospholipids and the availability of enzymes for the metabolism of these isomers. During starvation-induced phospholipid loss, the *cis*-monoenoic fatty acids would, therefore, be preferentially utilized. The ability to either synthesize *trans*-monoenoic acids (which are not easily metabolized by bacteria) or modify the more volatile *cis*-monoenoic acids to their cyclopropyl derivatives may be a survival mechanism which helps maintain a functional (although structurally altered) membrane during starvation-induced lipid utilization. In addition, a *trans/cis* fatty acid ratio significantly greater than that reported for most bacterial cultures and environmental samples (<0.1) may be used as a starvation or stress lipid index. Such a ratio could help determine the nutritional status of ultramicrobacteria and other reported dormant cells in natural aquatic environments. These organisms may represent a direct source of *trans*-monoenoic fatty acid input into marine and estuarine sediments as distinct from *cis*-to-*trans* isomerization.

Vibrio cholerae is able to survive several months of laboratory-induced starvation (24). These cells decline to the size of ultramicrobacteria (55) (i.e., 0.2- to 0.4-μm filterable cells) (24), but can be restored to normal size and shape within 2 h of nutrient supplementation (1). *Vibrio* spp. are a prominent part of the ultramicrobacteria shown to be abundant year-round in the subtropical estuaries of the Gulf Coast states (34, 35).

Dwarfing of cells to form ultramicrobacteria is a common phenomenon for starvation survival of aquatic bacteria (29, 38, 55). Although the presence of ultramicrobacteria in the oceans seems universal, the direct evidence that these small cells are necessarily starvation forms in a dormant state is lacking (41, 53). Further work is required to describe these as nutrient-deficient, starving, dormant cells. This evidence must be obtained without any metabolic manipulation of the organism.

This study is a companion to a larger investigation of the degradation of cellular constituents in *V. cholerae* during nutrient deprivation (23). The fatty acids ester linked to phospholipids were analyzed for *V. cholerae* cultures at several stages of starvation. Profiles of these lipids have been shown to be reproducible indices of viable aquatic procaryotic communities (20, 60) and have been used in statistical descriptions of microbial cultures (4, 13) and communities (20, 37). Reproducible shifts in these profiles can be interpreted as owing to changes in community structure or physiology or both (20). Procaryotic fatty acids are characterized by the ubiquitous even-chain saturated and *cis*-monounsaturated acids as well as odd-chain acids, branched acids, and hydroxy and cyclopropyl derivatives (22). There are reports of *trans*-monounsaturated acids (18, 19, 36), but no de novo biosynthetic pathway has yet been determined. The *trans/cis* ratios for most bacterial cultures and sediments have been found to be less than 0.1 (17, 20, 46, 58, 59).

Knowledge of changes in microbial lipid biomarkers is also important for food chain and geochemical studies since these changes should be used in conjunction with known lipid degradative pathways for studies of lipid inputs and diagenesis in sediments (6, 57).

Fatty acid profiles have been shown to change during starvation of some bacteria (30, 45, 54). Reproducible changes in the phospholipid ester-linked fatty acid profiles during nutrient deprivation of *V. cholerae* may be a useful starvation index for assessing the nutrient status of in situ populations, particularly the ultramicrobacteria.

MATERIALS AND METHODS

Microbiological starvation systems. All microbiological manipulations were done at the facilities of the University of West Florida and are described in detail by Hood et al. (23). *V. cholerae* CA401, the isolate examined, was grown for 18

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h at 35°C and pH 7.4 in a peptone-yeast extract-seawater complete medium (1). Cells were harvested by centrifugation, and the pellet was washed three times with basal salts. The cells were resuspended in basal salts and inoculated as pseudoreplicates into separate 2-liter flasks, each containing 1 liter of basal salts (organic free). Zero-day-starved cells were immediately harvested by centrifugation and lyophilized. Cells starved for 7 and 30 days were incubated in the dark at 22°C without shaking before harvesting.

Experiment 1. An initial experiment was performed in which two 0-day- and one 7-day-starved cultures were analyzed.

Experiment 2. A test of the hypotheses developed from experiment 1 was done utilizing a completely randomized experimental design. Starvation effects on lipids were tested at three levels: 0 day (n = 5 independent replicates), 7 day (n = 4), and 30 day (n = 5). The peptone-yeast extract in the seawater complete medium was lipid extracted as previously described (20) to minimize exogenous lipid sources. Lyophilized cells were stored in sealed ampoules, blindly coded to ensure no bias, and sent to Florida State University for lipid analysis.

Experiment 3. An examination of 7-day starvation effects was done under the same conditions as experiment 2, except that the cells were incubated in a continuous-flow system. The continuous-flow system consisted of a 2-liter chamber containing 1 liter of basal salts, with a dilution rate of 1 liter/day (1-day retention time) maintained with a peristaltic pump (Harvard Apparatus, Millis, Mass.). Cells were inoculated as in experiment 2 into the chamber. Two independent systems were used for replication.

Experimental and procedural blanks. The experimental blanks consisted of uninoculated basal salts prepared as above. The procedural blanks were glassware identically prepared as that for sample analysis (20) with no sample added. These blanks were analyzed in an identical manner to the samples except that they were injected at greater concentrations on the gas chromatograph (GC) to detect possible interfering peaks.

Lipid analysis. Lipid extraction, fatty acid purification, and quantification by capillary GC were performed as previously described (20). Briefly, a modified Bligh and Dyer chloroform-methanol lipid extraction was used. Total extractable lipids were fractionated by silicic acid column chromatography. The fatty acids ester linked to the phospholipids were methylated by mild alkaline methanolysis of the polar lipid fraction. The resulting fatty acid methyl esters were purified by thin-layer chromatography before GC analysis. Quantification was based on comparison of peak areas to an internal injection standard (19:0). Tentative peak identifications were based on coelution on a nonpolar, cross-linked, methyl silicone fused silica capillary column (50 m by 0.2-mm inner diameter; Hewlett-Packard Co., Palo Alto, Calif.) with standards obtained from either Supelco, Inc. (Bellefonte, Pa.) and Alltech Associates, Inc., Applied Science Div. (State College, Pa.) or previously identified laboratory standards.

Fatty acid structural verification. Identification of fatty acid methyl esters, position of methyl branching, and degree of unsaturation were verified by GC/mass spectrometry (MS) as described previously (20). Mass spectral data collected by GC/MS were processed with a Hewlett Packard RTE-6/VM data system.

Monounsaturation position and geometry were chemically determined by using dimethyl disulfide derivatization and GC/MS (14). Samples in 100 μl of hexane were treated with 100 μl of dimethyl disulfide (Aldrich Chemical Co., Inc., Milwaukee, Wis.; gold label) and 1 drop of iodine solution (60 mg of iodine in 1 ml of diethyl ether). Reactions were performed in 2-ml glass vials sealed with a Teflon-lined septum at 50°C for 48 h, cooled, and diluted with 200 μl of hexane. The iodine was removed by shaking with 300 μl of 5% $Na_2S_2O_3$. The upper organic phase was removed, the aqueous phase was washed with 100 μl of hexane, and the combined hexane fractions were dried under nitrogen gas before GC and GC/MS analysis. The GC/MS was temperature programmed up to 300°C for the dimethyl disulfide adducts. Detection of both the omega and delta fragments verified the original double-bond position of the monounsaturate (14). Geometry was verified by GC separation of the dimethyl disulfide adducts of identical positional isomers analyzed by MS.

The determination of the ring position of cyclopropyl fatty acids was by hydrogenation and GC/MS of the resultant branched fatty acids as previously described (20).

Chemical verification was not possible for the cultures of experiment 3 owing to insufficient amounts of sample. Fatty acid identification for these samples is based on coelution with the above-mentioned standards on a dual-column GC equipped with both a nonpolar column (as before) and one of intermediate polarity (OV-1701; Chemical Research Associates) set up in a parallel configuration from a common injector (52).

Fatty acid nomenclature. Fatty acids are designated as total number of carbon atoms:number of double bonds with the position of the double bond closest to the aliphatic (ω) end of the molecule indicated with the geometry "c" for *cis* and "t" for *trans*. The prefixes "i" and "a" refer to iso and anteiso branching, respectively. Other branching is indicated as the position of the additional methyl group from the carboxyl (Δ) end, e.g., 11Me 19:1. Cyclopropyl fatty acids are designated as "cy" with the ring position in parenthesis relative to the aliphatic end.

Statistical analysis. The test of differential loss of fatty acids with starvation was done by the procedure of Scheffe (49) available within the SPSS programs on the Florida State University Cyber 730 mainframe computer. This conservative test for multiple comparison of means was used on untransformed mole percent fatty acid data. The significant difference map generated by this procedure for experiment 2 data was constructed keeping the within-experiment, family wise error rate set at $\alpha = 0.01$.

RESULTS

Experiment 1. The phospholipid ester-linked fatty acids recovered from 0-day- and 7-day-starved cultures are shown in Table 1. The molar percentage of unsaturated fatty acids decreased, while that of the saturates and branched fatty acids increased with starvation. The *trans/cis* ratio for the major monounsaturated fatty acids increased with starvation (Fig. 1). The ratio 16:1ω7t/16:1ω7c increased from 0.14 to 0.73, and that of 18:1ω7t/18:1ω7c increased from 0.03 to 0.18 (0-day- to 7-day-starved cultures).

Experiment 2. The average fatty acid profiles for the 0-day cells were similar to the profile of experiment 1, and the 7-day-starved culture matched its corresponding profile (Table 1). The standard deviations indicated excellent reproducibility for the independent replicates of each treatment. Before knowledge of the sample coding, and based on the results from experiment 1, these fatty acid profiles were used to successfully predict the days of starvation for each sample

TABLE 1. Phospholipid ester-linked fatty acid profiles for starvation treatments of *V. cholerae* expressed as average mole percent (+ one standard deviation) see text for details of different experiments)

Fatty acid	Expt 1		Expt 2			Expt 3
	0 days	7 days	0 days	7 days	30 days	7 day flowing
Saturates						
14:0	2.9 (<0.1)	3.0	1.8 (0.1)	1.7 (0.2)	2.1 (0.3)	1.4 (0.4)
15:0	0.3 (<0.1)	0.5	0.5 (<0.1)	0.8 (<0.1)	0.9 (0.1)	2.2 (1.7)
16:0	24.2 (0.2)	31.6	27.4 (0.3)	31.5 (1.5)	34.0 (0.7)	24.5 (6.2)
17:0	0.2 (0.1)	1.1	1.1 (<0.1)	1.4 (0.1)	2.0 (0.2)	1.4 (1.1)
18:0	1.8 (<0.1)	3.0	2.8 (0.1)	5.1 (0.9)	5.5 (0.4)	9.0 (5.6)
Branched						
i14:0	Tr[a]		Tr	0.1 (<0.1)	0.1 (0.1)	
i15:0				0.1 (0.1)	0.4 (0.4)	0.4 (0.6)
a15:0	0.1 (<0.1)	0.5	Tr	Tr	0.2 (0.2)	0.6 (0.9)
i16:0	0.4 (0.1)	1.0	1.0 (0.3)	0.9 (0.1)	1.2 (0.2)	0.2 (0.3)
a16:0				Tr	0.2 (0.3)	
i18:0	0.1 (<0.1)		0.3 (0.1)	0.4 (<0.1)	0.3 (0.2)	
11Me 19:1[b]	0.7 (<0.1)	2.4	0.8 (0.3)	0.1 (<0.1)	0.2 (0.2)	0.8 (1.1)
Cyclopropyl						
cy17:0 (ω7,8)		1.7	0.3 (<0.1)	0.5 (0.1)	0.6 (0.1)	1.5 (1.4)
cy19:0 (ω7,8)		1.1		Tr	0.4 (0.5)	2.5 (1.6)
Unsaturates						
14:1ω5c[b]			0.2 (<0.1)	0.1 (<0.1)	0.1 (0.1)	0.2 (0.3)
15:1[b]			0.3 (0.2)	0.2 (<0.1)	0.3 (0.1)	
16:1ω9c	1.0 (0.4)	0.9	1.4 (0.1)	1.2 (0.1)	1.1 (0.1)	1.6 (0.4)
16:1ω7c	37.0 (0.3)	17.7	39.1 (0.3)	18.4 (0.9)	11.0 (0.9)	1.5 (1.3)
16:1ω7t	5.2 (0.1)	13.0	1.0 (0.1)	12.8 (0.8)	17.1 (0.7)	3.4 (2.3)
16:1ω5c[b]	0.3 (0.0)		0.3 (<0.1)	0.3 (<0.1)	0.3 (0.1)	0.4 (0.6)
17:1ω8c	0.5 (<0.1)	0.4	1.2 (0.1)	0.8 (<0.1)	0.7 (<0.1)	
17:1[b]	0.1 (0.0)			Tr	0.1 (0.2)	
18:2ω6,9[b]	0.3 (<0.1)		0.4 (0.3)	0.2 (0.1)	0.1 (0.1)	1.3 (0.4)
18:1ω9c	1.0 (<0.1)	0.9	1.0 (<0.1)	2.0 (0.3)	2.8 (1.4)	9.6 (7.4)
18:1ω7c	22.6 (0.4)	17.9	18.7 (0.5)	18.3 (3.0)	12.7 (1.0)	34.8 (10.5)
18:1ω7t	0.6 (<0.1)	3.3	0.2 (<0.1)	2.8 (0.4)	5.2 (0.4)	1.4 (1.1)
18:1ω9t[b]			0.2 (<0.1)	0.3 (<0.1)	0.3 (<0.1)	
19:1[b]			0.1 (0.1)	0.1 (<0.1)	0.1 (0.1)	
20:1[b]				0.2 (0.1)	Tr	
20:1ω9c[b]	0.2 (0.0)		Tr	0.2 (<0.1)	0.1 (0.1)	1.6 (1.5)
Total saturates	29.3 (0.3)	39.3	33.6 (0.4)	40.0 (2.1)	43.4 (0.8)	38.5 (15.1)
Total unsaturates and cyclopropyl	68.1 (0.1)	53.6	64.8 (0.4)	58.1 (1.8)	51.6 (1.1)	59.9 (17.5)
Total branched	1.7 (<0.1)	4.4	2.2 (0.3)	1.6 (0.1)	2.7 (1.0)	2.0 (2.9)
No. of replicates	2	1	5	4	5	2

[a] Tr, Trace levels (<0.1 mol%) detected; a blank entry indicates below the limits of detection.
[b] Double-bond positions of these acids are based on coelution with verified standards alone, since insufficient sample was available for structural confirmation by GC/MS.

with 100% accuracy. The trends indicated for experiment 1 are also found in experiment 2. In addition, the trends continued from the 7-day- through the 30-day-starved cells. Figure 1 indicates the progressive increase in the *trans/cis* ratio during starvation for both the 16:1ω7 and 18:1ω7 isomers. The progression for the 16:1ω7 isomers was 0.02, 0.70, 1.56 and that for the 18:1ω7 isomers was 0.01, 0.16, 0.41 for 0 day, 7 days, and 30 days, respectively. The variability of these ratios was low, as indicated by the error bars in Fig. 1. The significant difference map (Table 2) indicates the results of the test for differential loss of fatty acids with starvation. Treatments connected by a common line segment were not significantly different for this test. Of the 17 fatty acids which were significant, 14 showed progressive changes correlating (either positively or negatively) with the length of starvation. Nine of these were unsaturated fatty acids. The fatty acids progressively decreasing during starvation included almost all the *cis*-monoenoic acids: 14:1ω5c, 16:1ω9c, 16:1ω7c, 17:1ω8c, and 18:1ω7c. Those acids progressively increasing were generally the saturated, *trans*-monoenoic, and cyclopropyl fatty acids: 15:0, 16:0, 17:0, 18:0, 16:1ω7t, 18:1ω7t, and cy17:0 (ω7,8). In addition, 15:1 and 18:1ω9c showed a progressive increase during starvation. The other three acids, 11Me 19:1, 20:1, and 20:1ω9c, did not change in a progressive manner with respect to the length of starvation, although the amount of 11Me 19:1 was greatest for the 0-day cultures (Table 2).

Experiment 3. The low cell densities of the continuous-flow system after 7 days (1.7×10^6 cells per ml, direct count [23]) resulted in extremely low amounts of recovered lipid,

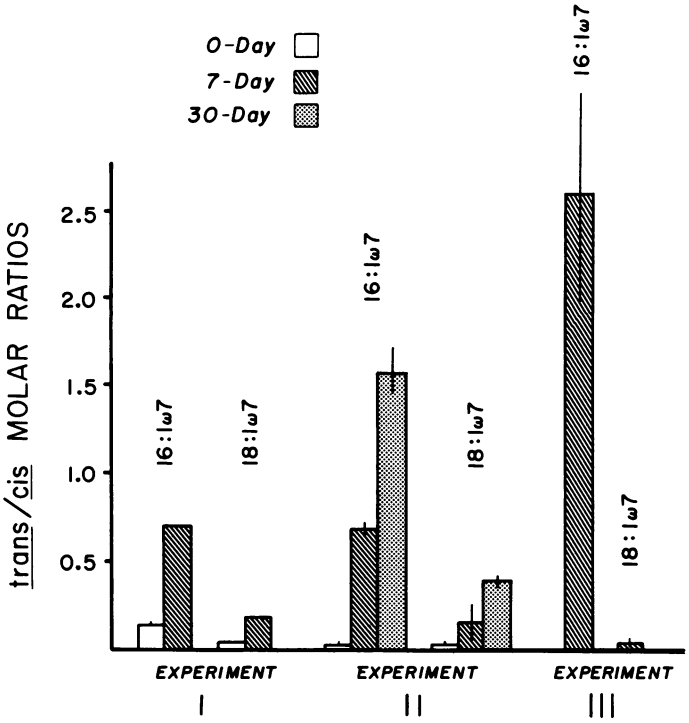

FIG. 1. Histograms indicating the progressive increase in the *trans/cis* ratios for both 16:1ω7 and 18:1ω7 as *V. cholerae* is starved. Experiment 1 (I in figure) was the initial experiment, and experiment 2 (II in figure) was designed to test the hypotheses developed from the results of experiment 1. Experiment 3 (III in figure) was a continuous-flow starvation experiment to test the effects of dilution of leaked cellular constituents which may be used as exogenous nutrient sources. Details of the experimental designs are in the text. Histograms indicate mean (for number of replicates see Table 1), and errors bars indicate ±1 standard deviation.

and the data for the two continuous-flow systems indicated higher variances than for experiment 2 (Table 1). The averages were quite low for both 16:1ω7c and 16:1ω7t while unusually high for 18:1ω7c. Regardless, the averages of the total saturates and unsaturates were in agreement with both the 7-day-starved cells of experiment 1 and the average of those in experiment 2. The *trans/cis* ratio of 16:1ω7 was extremely high (2.6 ± 0.6); however, the ratio for 18:1ω7 (0.04 ± 0.02) was low and closer to the 0-day cultures of both experiments 1 and 2 (Fig. 1).

Experimental and procedural blanks. No fatty acids were recovered from the basal salt experimental blank above those found in the glassware procedural blanks. A possible interfering peak detected in the procedural blank was a slight amount of 18:1ω7c.

DISCUSSION

The data in Table 1 are the most detailed, chemically verified, fatty acid profiles for *V. cholerae* reported to date. The importance of structural verification of all reported lipid analyses for proper interpretation has been emphasized for both cultural and environmental work (18, 20, 42, 56, 58).

The profile for the 0-day-starved cells (Table 1) is in close agreement with a previously published profile for a different strain of *V. cholerae* (44) if their reported values of 16:1 and 18:1 are equivalent to the sum of all the verified isomers reported in Table 1 for each chain length. An earlier study reports a higher proportion of saturated and a lower proportion of unsaturated fatty acids for two other strains of *V. cholerae* (7). The stability of the unsaturated acids during their boiling BCl_3-methanol transesterification (7) compared with the mild (37°C, 15 min) alkaline methanolysis used in this study (20) is unknown, but may be a factor (25). The use of lipid class fractionation, a specific mild methanolysis, higher-resolution fatty acid separation conditions, and chemical verification of fatty acid structure (including position and geometry of monounsaturation) in this report provide a fatty acid profile (Table 1) with sufficient detail and reproducibility to be suitable for inclusion in libraries of biochemical parameters for identification of these organisms from environmental samples (20). Other analytical techniques such as pyrolysis GC (21) have been suggested for the identification of *V. cholerae* and other species of bacteria. Although useful for the differentiation of pure cultures, identification, interpretation, and interlaboratory comparison of the resulting components separated by the GC are inadequate to meet the needs of complex ecological studies (20).

Many of the microbial starvation studies which report degradation of cell constituents do not report lipid data (11, 31), possibly because lipids are not considered to be stored reserve material (12). Two studies which did measure total lipids during starvation of *Escherichia coli* (12) and *Nocardia corallina* (47) both concluded that there was no significant utilization of lipid during endogenous respiration.

In at least three studies, microbial lipids were degraded during starvation. When *Streptococcus lactis* was starved for 55 h, there was a net loss of about 10% of its total lipid per gram of dry weight (54). When *Arthrobacter crystallopoietes* was starved for 2 weeks, there was a loss of 30% of total lipid (30). A greater loss of phospholipid (65% over 21 days) was reported for a starving psychrophilic marine *Vibrio* sp. (45). The 90% decrease in phospholipid fatty acids per gram of dry weight and the 99%+ decrease in fatty acids per cell for the 7-day-starved cultures reported for *V. cholerae* (23), however, are the largest phospholipid losses during starvation reported to our knowledge.

Although all fatty acids decreased in absolute concentration per cell (23), the molar percentages of each acid did not remain constant (Table 1). The general trends are the significant decrease in the *cis*-monoenoic acids and an increase in the saturated, *trans*-monoenoic, and cyclopropyl acids (Table 2). The increase in the *trans/cis* ratio (Fig. 1) has also been shown during the short-term (<24 h) starvation of a marine isolate (37a; G. Odham and A. Tunlid, personal communication). A similar loss of *cis*-monoenoic acids was also reported for *S. lactis* (54). When *S. lactis* is starved for 55 h, the proportion of 18:1ω7c declines and its corresponding cyclopropyl derivative, cy19:0 (ω7,8), increases. The fatty acid changes for a starved psychrophilic marine *Vibrio* sp. (45), however, indicated an increase in the proportion of unsaturated acids (principally 16:1) and a slight decrease in saturated acids during its 21-day starvation.

Changes in the profiles of membrane fatty acids have been generally interpreted with respect to maintenance of membrane fluidity. The physical properties of the *cis*-monoenoic and cyclopropyl fatty acids are similar, while *trans*-monoenoic fatty acids are more stable and have properties much closer to saturated acids (10). Therefore, the conclusion of this work might be that there is an overall decrease in membrane fluidity during starvation of *V. cholerae*. On the

other hand, the methylation of 18:1ω7c to cy19:0 (ω7,8) during the starvation of *S. lactis* (54) would have the effect of maintaining the degree of membrane fluidity found in the unstarved cells. The results for the psychrophilic *Vibrio* sp. are interpreted as an increase in membrane fluidity as a function of starvation (45). There appears to be no consistent trend of membrane fluidity with starvation.

To explore other hypotheses dealing with the reasons for these fatty acid changes, one must consider the source of the *cis*- and *trans*-monoenoic acids and their possible modifications or degradation within the membrane. The *cis*-monoenoic acids are the only reported products of the known bacterial de novo biosynthetic pathways for unsaturated fatty acids (22), and many positional isomers are known (3, 16, 50). The biosynthesis of polyunsaturated fatty acids by bacteria is rare (15, 44). The de novo synthesis of *trans*-monoenoic acids has been suggested based on the recovery of the acids from marine sediments and isolates of those sediments (18), but this has not been conclusively proven.

Exogenous *trans*-monoenoic fatty acids can be incorporated into membrane lipids, and workers with unsaturated fatty acid auxotrophs of *E. coli* have investigated the physiological results of the presence of these acids in the membrane (2, 40, 48). Rumen bacteria are able to produce *trans*-monoenoic acids by hydrogenation of exogenous polyunsaturated fatty acids (28). There are a number of rumen isolates that can hydrogenate linoleic acid (18:2ω6,9) and linolenic acid (18:3ω3,6,9) to the 18:1ω7t product predominantly (26), although neither substrate nor product has been found incorporated into cellular lipids (27). Bacterial isolates which have been reported to have significant levels of *trans*-monoenoic acids have generally been grown on media which contained yeast extract (18, 19), a source of exogenous polyunsaturated acids. However, the problem of incorporation of exogenous lipid is probably minimal in dilute medium concentrations (46). The continuous-flowing culture in experiment 3 was a test for the influence of exogenous lipid, in this case from leaking starving cells. The molar percentages of *trans*-monoenoic acids were lower than in experiments 1 and 2 (Table 1), but the 16:1ω7 *trans/cis* ratio was higher than in the 30-day-starved cells of experiment 2 (Fig. 1). This suggests that the increasing *trans/cis* ratio with starvation is not due to incorporation of unmetabolized exogenous *trans*-monoenoic acids. The low *trans/cis* ratio for 18:1ω7 is probably due to contaminant levels of 18:1ω7c which interfere with these low biomass samples. Methane-utilizing bacteria are able to produce a significant amount of *trans*-monoenoic acids of several positional isomers utilizing methane as their sole carbon source (36, 43). In this study, the cultures of experiment 2 were grown on peptone and yeast extract which had been previously lipid extracted to minimize the source of exogenous lipid. There was a consistent proportion of 18:2ω6,9 recovered from the cultures of experiments 1 and 2, and there was little difference in the *trans*-acid proportions (Table 1), suggesting that the decrease of exogenous lipid in the lipid-extracted medium of experiment 2 had little effect on the fatty acid profiles. Taken together, these examples suggest the likelihood of de novo synthesis of *trans*-monoenoic acids in bacteria.

Regardless of the source of *trans*-monoenoic acids, they are present in significant quantities in *V. cholerae* and increase during starvation (Fig. 1). The *cis*-monoenoic acids of the C-2 position of the phospholipids would be expected to turn over faster than the saturated acids of the C-1 position (61). The phospholipid position of *trans*-monoenoic acids is unknown. Work with an unsaturated fatty acid auxotroph of *E. coli* has shown that although the bacteria can incorporate *trans*-monoenoic acids from the medium, they are unable to modify them stereochemically or positionally (51). Based on these differences of turnover rates and ability to modify the acids, it follows that if there was utilization of membrane fatty acids during starvation, there would be a preferential loss of the *cis*-monoenoic acid as compared with the saturated and *trans*-monoenoic acids as shown for this strain of *V. cholerae* in Table 2.

The preferential loss of the *cis*-monoenoic acids explains the increase in the *trans/cis* ratio shown in Fig. 1 but does not explain how this might be related to starvation survival. One response to nutrient deprivation in *V. cholerae* is loss of membrane phospholipids and fatty acids (23). If membrane integrity is lost during this degradation, survival of long-term starvation is unlikely. However, if the *trans*-monoenoic fatty acids could not be utilized by the microbial degradative enzymes, as suggested by the auxotroph research (51), an organism with a significant proportion of its membrane fatty acids as *trans* isomers might be unable to completely de-

TABLE 2. Significant difference map generated from Scheffe's multiple comparison of means test (SPSS) with the within-experiment familywise error rate set at $\alpha = 0.01$ for the untransformed mole percent data from experiment 2

Fatty acid	Days starved		
	Low[a]		High
Decrease[b]			
14:1ω5c[c]	30	7	0
16:1ω9c	30	7	0
16:1ω7c	30	7	0
17:1ω8c	30	7	0
18:1ω7c	30	7	0
Increase[d]			
15:0	0	7	30
16:0	0	7	30
17:0	0	7	30
18:0	0	7	30
cy17:0 (ω7,8)	0	7	30
15:1[c]	0	7	30
16:1ω7t	0	7	30
18:1ω9c	0	7	30
18:1ω7t	0	7	30
No change[e]			
11Me 19:1[c]	7	30	0
20:1[c]	0	30	7
20:1ω9c[c]	0	30	7

[a] Treatment means for each fatty acid increase from left to right, and those connected by a common line segment are not significantly different for this test.

[b] For this group, there was a progressive, relative decrease during starvation.

[c] Double-bond positions of these acids based on coelution with verified standards alone, since insufficient sample was available for structural confirmation by GC/MS.

[d] For this group, there was a progressive, relative increase during starvation.

[e] For this group, there were no progressive changes during starvation.

grade its membrane. Although the *trans* isomers are in relatively low proportions for the 0-day cultures (1 to 6%; Table 1), after 30 days of nutrient deprivation and the resulting loss of total phospholipid, the *trans* fatty acid proportion is greater than 20% (Table 1). The result is that membrane integrity may be maintained and survival during starvation-induced lipid degradation would be possible.

The above mechanism, however, does not explain the increase in cyclopropyl fatty acids with starvation (Table 2). This same trend occurred when *S. lactis* was starved (54). Cyclopropyl fatty acids are only formed by the transmethylation of a *cis*-monoenoic fatty acid esterified to a phospholipid (32, 39). An increase in cyclopropyl acids with the loss of their *cis*-monoenoic precursors has been shown to occur as cultures age and enter the stationary phase (33) and as a result of decreasing pH of the medium (9), low oxygen tension, high temperature, and high Mg^{2+} ions (32). These conditions suggest that cyclopropyl acids are formed under stressful conditions, generally when growth ceases (54). What is not understood is the reason for this modification, since the transmethylation reaction uses *S*-adenosyl-L-methionine (which requires ATP for its synthesis) as the methyl donor (32). A clue may be the common physical properties of the cyclopropyl and *cis*-monoenoic acids. If there was more stability to turnover and degradation in the cyclopropyl configuration, then the energetic investment might be worthwhile to minimize membrane lipid losses or changes in membrane fluidity owing to cellular degradation during starvation.

This may explain the fatty acid profiles of an atypical *V. cholerae* displaying rugose colony morphology. These organisms contained much higher percentages of both cy17:0 and cy19:0 than the identically grown parental strains (8), which were in agreement with the 0-day cultures in Table 1. Pure cultures of these rugose variants have been reported to exhibit unusual resistance to adverse conditions (8), and the presence of the cyclopropyl acids was postulated to be related to the survival characteristics of this atypical organism.

These same changes in fatty acid profiles have been observed for environmental samples which are complex microbial consortia. In a set of microcosms of estuarine sedimentary microbial communities, it was found that as microcosms were rendered anaerobic, they either responded with an increased proportion of cyclopropyl acids or with an increased *trans/cis* ratio (20). In either case, the molar percentage of the *cis*-monoenoic isomer decreased in the profile of the entire community during the stress of anaerobic conditions.

It may be possible to utilize a *trans/cis* ratio as a starvation index or stress index for microbial communities. This ratio in the past has been used by geochemists as an indication of diagenesis of the *cis*-monoenoic acids, thought to be of direct microbial input (5, 46, 56, 58, 59). An increase in the *trans/cis* ratio for the total lipid fatty acids with depth into sediment (57) has been attributed to preferential degradation of the *cis* isomer or clay-catalyzed isomerization to the *trans* isomer. The data in this report suggest that the *trans/cis* ratio of the phospholipid ester-linked fatty acids may alternatively be interpreted as a relative measure of membrane stress, such as that caused by starvation. On the basis of this work, we would hypothesize that the viable filterable ultramicrobacteria, thought to represent the marine and estuarine in situ examples of starving dwarf bacteria (35), would have *trans/cis* ratios significantly higher than those reported for bacterial cultures (from 0.01 to 0.08 [46, 59]) or marine, mangrove, and estuarine sediment surfaces (from 0.01 to 0.09 [17, 20, 46, 58, 59]).

In conclusion, during the nutrient deprivation of *V. cholerae* monitored at 7 and 30 days, there is a loss of phospholipid ester-linked fatty acids per cell as these cultures increase in number and decrease in cell volume (23). The lipid utilization is preferential for the *cis*-monoenoic fatty acids, probably because of their faster turnover and ease of metabolism. During starvation, the molar percentages of saturated, cyclopropyl, and *trans*-monoenoic acids increased. Rather than a mechanism for the maintenance of membrane fluidity, the ability to modify the *cis*-monoenoic acids to the cyclopropyl acids or synthesize *trans*-monoenoic acids or both may be a survival mechanism for the maintenance of membrane integrity during starvation despite lipid utilization. Finally, the *trans/cis* ratio of monoenoic phospholipid ester-linked fatty acids may be useful as a stress or starvation index for determining the nutritional status of the ultramicrobacteria and as a consequence addressing the question of bacterial dormancy (53) in natural aquatic environments.

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