Trans-Monoenoic and Polyunsaturated Fatty Acids in Phospholipids of a *Vibrio* Species of Bacterium in Relation to Growth Conditions

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A *Vibrio* species of bacterium known to contain the polyunsaturated fatty acid 20:5n-3 was grown in both freshwater and seawater media at 5 and 20°C and examined for adaptive changes in lipid composition. Phosphatidylethanolamine (PE) and phosphatidylglycerol (PG), together with a smaller proportion of nonesterified fatty acids (NEFA), comprised almost all the lipid under all growth conditions examined. Temperature had a more pronounced effect than the salinity of the medium on lipid composition. The proportion of PE in total lipid was always higher at 5 than at 20°C. Conversely, the proportion of NEFA was lower at 5 than 20°C whereas that of PG was not altered. The levels of saturated fatty acids in total lipid, PE and PG were all decreased by growth at 5°C. No differences were observed with respect to growth temperature in the levels of *cis* 16:1n-7, the principal monoenoic fatty acid in both PE and PG. *Trans* 16:1n-7 was found to comprise 12.8–15.2% of fatty acids in PE and PG of bacteria grown at 5°C but only 4.4–8.5% of phospholipid fatty acids in bacteria cultured at 20°C. Regardless of medium composition, a reduction in growth temperature from 20 to 5°C also caused the proportions of 20:5n-3 to increase from around 0.8 to 4.4% in PE and from around 4 to 20% in PG. The simultaneous occurrence of *trans* 16:1n-7 and 20:5n-3 is unique to this *Vibrio* species of bacterium. The increased proportions of both these fatty acids with decreasing temperature suggest that they have a role in retailoring biomembrane phospholipids during temperature acclimation of the bacterium.

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In general, the lipids of bacteria do not contain polyunsaturated fatty acids (1). An exception to this rule is the occurrence of polyunsaturated fatty acids in a few species of marine bacteria (2–5) isolated from diverse sources including seawater, deep sea sediments and the intestines of fish. These bacteria are invariably motile and Gram-negative, with *Vibrio* species being the most common (4,5).

Studies to date on the lipids of these bacteria which contain polyunsaturated fatty acids have examined only their total lipid and have not reported the distribution of individual fatty acids in the component lipid classes. Furthermore, although the biosynthesis of monounsaturated fatty acids has been well studied in bacteria (1), nothing is known of the mechanisms of bacterial synthesis of polyunsaturated fatty acids.

An increase in the level of unsaturated fatty acids in phospholipids of eukaryotic poikilotherms, both invertebrate and vertebrate, has been shown frequently to occur as an adaptation to a decrease in environmental temperature (6–8). In contrast, the effect of changes in environmental temperature on the detailed lipid composition of bacteria has been less well studied. The few studies that have been carried out show that there is no uniformity between different bacterial species in the changes that occur in lipid composition in response to changes in growth temperature. For example, in marine *Vibrios*, increases in the proportion of monounsaturated fatty acids, or increased synthesis of shorter chain fatty acids or no changes at all have all been observed as responses to reduced growth temperature (9). Most recently, the interconversion of *cis* and *trans* isomers of 16:1n-7 has been implicated in the thermal adaptation of a psychrophilic species of *Vibrio* (10,11).

In addition to growth temperature, the salt concentration of the medium may also influence the lipid composition of bacteria. This aspect has been studied in several species of bacteria including species of *Paracoccus* (12), *Staphylococcus* (13,14), *Vibrio* (15) and *Pseudomonas* (16). With Gram-negative bacteria in general, the proportions of phosphatidylglycerol (PG) and phosphatidylethanolamine (PE) increase and decrease, respectively, with increasing salt concentration in the growth medium (12,15, 16). However, the effect is somewhat different with the Gram-positive *Staphylococcus* species in that the PG decreases with increasing salinity while cardiolipin increases (13,14). Changes in fatty acid composition of total lipid are also observed in relation to the salinity of the growth medium. Thus, the level of monounsaturated fatty acids in total lipid has been shown to decrease significantly when marine species of *Vibrio* are grown in medium of reduced salinity (17) whereas with the moderate halophile *Vibrio costicola* the relationship between salt concentration and the proportion of monounsaturated fatty acids is parabolic with a maximum at 1.0 M (18).

To establish whether adaptive changes occur in the lipid composition of bacteria which are capable of producing polyunsaturated fatty acids, the lipids synthesized by a *Vibrio* species known to contain 20:5n-3 were examined in relation to growth conditions.

MATERIALS AND METHODS

Chemicals. Lipid standards were purchased from Sigma Chemical Co. Ltd. (Poole, Dorset, United Kingdom) and Supelchem U.K. Ltd. (Saffron Walden, United Kingdom). Solvents were of glass distilled or high-performance liquid chromatography (HPLC) grade and were supplied by Rathburn Chemicals (Walkerburn, United Kingdom).

Organism and growth conditions. The *Vibrio* species used in the study was originally isolated as described elsewhere (19) from the gut of Arctic char (*Salvelinus alpinus* L.) maintained in freshwater. To remove lipid from growth media, Oxoid general purpose nutrient broth powder (Unipath Ltd., Basingstoke, United Kingdom) was extracted with chloroform/methanol (2:1, vol/vol), filtered and air dried before being used for the preparation of the

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Abbreviations: GC/MS, gas chromatography/mass spectrometry; HPLC, high-performance liquid chromatography; HPTLC, high-performance thin-layer chromatography; NEFA, nonesterified fatty acids; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; R_f, retardation factor; Tris, tris(hydroxymethyl)aminomethane.

media. The defatted nutrient broth was made up at a concentration of 13 g/L either in distilled water or artificial seawater (approximately 0.4M NaCl) buffered with 1.6 mM Tris HCl to produce freshwater and seawater medium, respectively. The pH of both media was 7.2. Cultures were grown in the dark under sterile conditions in glass conical flasks in temperature-controlled shaking incubators at either 5 or 20°C. Growth rates were assessed by measurement of absorbance at 600 nm against non-inoculated medium as the blank. Bacterial cells were harvested from cultures grown at 20 and 5°C after 24 h and 5 d, respectively, by centrifugation at 7,000 × *g* for 10 min. The resulting pellet was resuspended in the washing buffer of Hanna *et al.* (18) and the centrifugation repeated. Pellets of cells were lyophilized and weighed prior to lipid extraction.

Lipid analysis. Lipid was extracted by blending pelleted cells with chloroform/propan-2-ol (2:1, vol/vol) in a glass/Teflon homogenizer. The organic phase was then filtered and washed with one-quarter its volume of 0.88% (wt/vol) KCl. After removal of the solvent by evaporation under nitrogen, the lipid extract was desiccated overnight *in vacuo.* After weighing, the lipid extract was redissolved in a known volume of chloroform/methanol (2:1, vol/vol) and stored at −70°C until analyzed further.

Lipid class analyses were performed by high-performance thin-layer chromatography (HPTLC) using various solvent systems. Separated lipid classes were identified by reference to authentic standards and by the staining of chromatograms with reagents specific for functional groups (20). Two-dimensional HPTLC was performed using methyl acetate/propan-2-ol/chloroform/methanol/0.25% KCl (25:25:25:10:9, by vol) and chloroform/methanol/7M aqueous ammonia (65:35:5, by vol) for development in the first and second dimensions, respectively. For quantitation of lipid classes, plates were developed to two-thirds of their length in chloroform/methanol/water (65:25:4, by vol) followed, after drying of the plate, by development to full distance with hexane/diethyl ether/acetic acid (90:10:1, by vol). Developed chromatograms were sprayed with 3% (wt/vol) copper acetate in 8% (vol/vol) phosphoric acid and the stained classes quantitated by scanning densitometry using a Shimadzu (Kyoto, Japan) CS9000 dual wavelength scanner attached to a Shimadzu DR-13 data recorder as described elsewhere (21).

For analysis of their constituent fatty acids, lipid classes were separated by HPTLC using the double development solvent system described above. Developed chromatograms were sprayed lightly with (0.01% wt/vol) 2′,7′-dichlorofluorescein in methanol and viewed under ultraviolet light. Bands of adsorbent containing the required classes were scraped from the plates and subjected to acid-catalyzed transesterification to produce the methyl ester derivatives of the constituent fatty acids (20). Fatty acid methyl esters were purified by HPTLC on silica gel plates using hexane/diethyl ether/glacial acetic acid (85:15:1, by vol) as developing solvent and recovered from the adsorbent by elution with hexane/diethyl ether (1:1, vol/vol). Aliquots of total lipid were also subjected directly to the acid-catalyzed transesterification procedure.

The analyses of fatty acid methyl esters of total lipid were performed using a Packard (Pangbourne, Berks., United Kingdom) 436 gas chromatograph equipped with a CP Wax 51 fused silica capillary column (50 m × 0.34 mm i.d.) (Chrompack UK Ltd., Milharbour, London, United Kingdom), on-column injection and H_2 as carrier gas. The oven temperature was programmed to increase from 50 to 225°C during the course of an analysis. For the analysis of fatty acid methyl esters of individual phospholipid classes, a fused silica capillary column coated with BPX 70 (25 m × 0.32 mm i.d.) was used, and the oven temperature was programmed to rise from 50 to 180°C. These samples were further analyzed using a chemically bonded CP Sil 5CB fused silica capillary column (50 m × 0.34 mm i.d.) with a single step thermal gradient from 50 to 260°C at 4°/min.

Separated methyl esters were identified by comparison with known standards and were quantitated using a Shimadzu CR-3A recording integrator attached to the gas chromatograph. The unsaturated nature of component methyl esters was confirmed by hydrogenation using PtO_2 followed by re-analysis. Identities were confirmed by gas chromatography/mass spectrometry (GC/MS) of selected samples by Dr. W.W. Christie, Hannah Research Institute, Ayr, United Kingdom, using conditions described elsewhere (22).

To confirm the identity of *trans* 16:1n-7, the fatty acid methyl esters of representative samples were separated on HPTLC plates impregnated with silver nitrate using hexane/diethyl ether/glacial acetic (94:4:2, by vol) as the developing solvent system (23). Components corresponding to saturated, *cis*-monounsaturated and *trans*-monounsaturated fatty acid methyl esters [identified by comparison of retardation factor (R_f) values with those of authentic standards run on the same chromatogram] were recovered from the adsorbent and analyzed by GC using the BPX70 phase as described above. The methyl ester of *trans* 16:1 was isolated as a component having an R_f value between that of saturated and *cis*-monounsaturated fatty acids.

Further confirmation of the identity of *trans* 16:1n-7 was obtained by stereospecific epoxidation with peracetic acid of fatty acid methyl esters separated by argentation chromatography (24). The epoxy derivatives were analyzed by GC using the BPX70 capillary column described above. The oven temperature was programmed to increase from the injection temperature of 50 to 150°C over 2.4 min and then after 18 min to increase to 220°C at 3 min. The separated epoxy derivatives of the methyl esters of the *cis* and *trans* isomers of 16:1n-7 in samples were identified by comparison of retention times of derivatives synthesized using standards of these fatty acids.

Student's *t*-test was used to determine the significance of differences between pairs of particular means (25), and differences were considered significant when $P < 0.05$. For the sake of clarity, values which are significantly different are not denoted in tables but are referred to in the text. The effects of the two factors (temperature and medium composition) were assessed by means of a two-way analysis of variance at a confidence level of 95% ($P < 0.05$) (25).

RESULTS

Growth characteristics. The *Vibrio* was capable of growing in both freshwater and seawater media. Growth rates, as assessed by absorbance measurements, were much higher at 20 than 5°C (Fig. 1) in both types of media. At 20°C the growth rates of bacteria grown in freshwater and

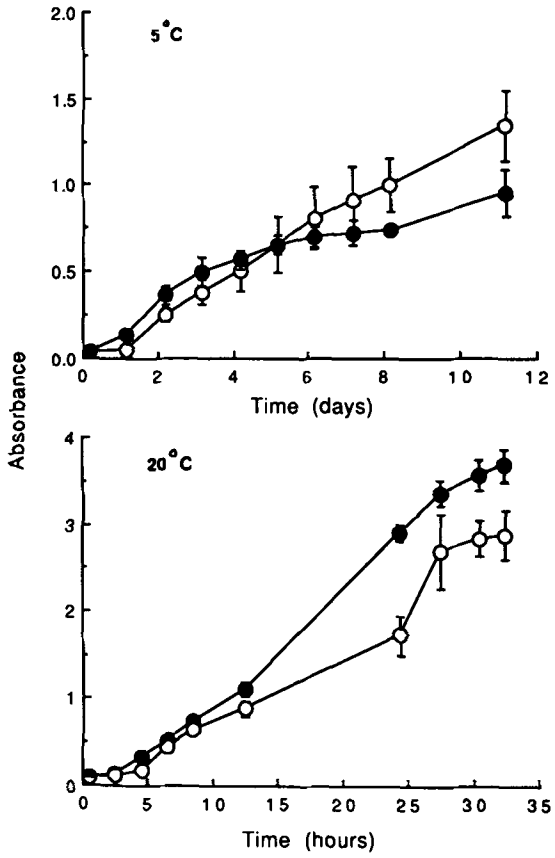

FIG. 1. Growth rates of *Vibrio* sp. cultured at 5 and 20°C in freshwater and seawater media. O, Seawater medium; ●, freshwater medium. Note different scales on x-axis.

seawater media were initially similar but diverged as the culture aged so that cultures grown in seawater medium finally contained fewer cells. The growth rate of bacteria at 5°C was higher in the freshwater medium than seawater medium over the initial 4 d of culture; but thereafter a higher biomass was observed in the seawater medium. Nevertheless, the influence of media composition on bacterial growth in Figure 1 was not marked. In contrast, further experiments showed that the *Vibrio* had poor growth rates in media containing 1M or 1.5M NaCl (data not shown).

Lipid content. Bacteria growth in seawater medium at 20°C contained only 4.5% of their dry weight as lipid whereas this component accounted for 7.4–7.9% of the dry weight of bacteria cultured under the other three conditions examined (Table 1). However, the only significant difference in lipid content was that observed between bacteria grown in seawater medium and bacteria grown in freshwater medium at 20°C. No statistically significant differences in lipid content existed between bacterial cultures in relation to growth temperature.

Lipid class composition. The lipid of the bacteria in all cultures was composed mainly of PE and PG (Table 1). At a given temperature (5 or 20°C) the lipid class composition was very similar for cells grown either in freshwater or seawater media. In bacteria grown at 5°C, more than 50% of the total lipid was PE, with PG and nonesterified fatty acids comprising around 31 and 12%, respectively, while at 20°C in both media the percentage of PE was substantially lower, and that of nonesterified fatty acids much higher, than at 5°C. The proportion of PG was only slightly higher in cells grown at 20°C than in those grown at 5°C in either medium. Overall, temperature affected lipid class composition more than the nature of the medium. LysoPE was present in trace amounts in lipid extracted from all cultures. When, in preliminary studies, chloroform/methanol (2:1, vol/vol) was used as extraction solvent, the levels of nonesterified fatty acids in the total lipid were notably higher, and those of PE and PG correspondingly lower (data not shown), than the values presented in Table 1. No components were observed in the total lipid extract which gave a positive stain for carbohydrate moieties.

Fatty acid composition of total lipid. The fatty acid compositions of total lipid from the *Vibrio* grown at 5 and 20°C in freshwater and seawater media are presented in Table 2. The major fatty acid component of total lipid was always the monounsaturated 16:1, but under the conditions employed complete resolution of the component *cis* and *trans* isomers of 16:1 was not attainable using the CP Wax 51 column. Overall, monounsaturated fatty acids accounted for just over half of the total fatty acids present

TABLE 1

Effect of Growth Temperature and Medium Composition on Lipid Content and Lipid Class Composition of *Vibrio* sp. Showing Significance of Each Factor and Their Interaction[a]

	5°C		20°C		Significance level *(P)*		
	Seawater	Freshwater	Seawater	Freshwater	Temp.	Medium	Interaction[b]
Lipid content (% dry weight)	7.90 ± 2.19	7.36 ± 0.50	4.53 ± 1.45	7.80 ± 0.60	—	—	0.0446
Lipid class composition (% total lipid)							
PE	55.6 ± 3.7	53.0 ± 1.6	38.9 ± 1.7	38.7 ± 4.2	0.0000	—	—
PG	31.1 ± 1.3	34.5 ± 3.1	36.3 ± 0.5	38.8 ± 1.6	0.0024	0.0278	—
NEFA	13.3 ± 3.0	12.3 ± 1.6	24.7 ± 2.0	22.4 ± 3.5	0.0001	—	—

[a]Values are means ± SD obtained with three cultures.

[b]Interaction between temperature and medium composition. PE, phosphatidylethanolamine; PG, phosphatidylglycerol; NEFA, nonesterified fatty acids; —, not significant.

TABLE 2

Effect of Growth Temperature and Medium Composition on Fatty Acid Composition (wt%) of Total Lipid from *Vibrio* sp. Showing Significance of Each Factor and Their Interaction[a]

Fatty acids	5°C		20°C		Significance level *(P)*		
	Seawater	Freshwater	Seawater	Freshwater	Temp.	Medium	Interaction[b]
i-12:0	0.5 ± 0.1	0.4 ± 0.1	0.7 ± 0.2	0.3 ± 0.1	—	—	—
12:0	4.0 ± 0.4	2.0 ± 1.7	4.3 ± 0.3	3.4 ± 0.3	—	0.0103	—
i-14:0	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.0	0.3 ± 0.1	—	—	—
14:0	2.9 ± 0.1	4.3 ± 0.2	2.4 ± 0.2	1.8 ± 0.2	0.0000	0.0324	0.0000
14:1n-5	0.6 ± 0.1	0.5 ± 0.1	0.3 ± 0.0	0.3 ± 0.1	0.0000	—	—
i-15:0	10.9 ± 0.4	12.6 ± 0.4	11.6 ± 0.5	15.4 ± 2.0	0.0200	0.0019	—
a-15:0	0.5 ± 0.1	0.6 ± 0.3	0.3 ± 0.1	0.3 ± 0.3	—	—	—
15:0	3.1 ± 0.2	2.2 ± 0.3	5.2 ± 0.2	5.8 ± 1.5	0.0001	—	—
15:1n-6	2.1 ± 0.5	1.0 ± 0.2	1.9 ± 0.1	3.0 ± 1.4	0.0420	—	0.0230
16:0	10.4 ± 0.3	13.3 ± 0.5	14.5 ± 1.8	13.3 ± 0.3	0.0042	—	0.0051
16:1	38.0 ± 1.0	39.8 ± 1.6	31.7 ± 0.7	28.9 ± 0.6	0.0000	—	0.0182
i-17:0	0.6 ± 0.2	0.6 ± 0.2	1.1 ± 0.1	1.4 ± 0.4	0.0033	—	—
17:0	0.7 ± 0.1	0.4 ± 0.1	2.7 ± 0.8	2.9 ± 0.2	0.0000	—	—
17:1n-8	6.7 ± 0.3	3.1 ± 0.4	10.6 ± 0.8	10.7 ± 1.9	0.0000	0.0052	0.0044
17:1n-6	1.4 ± 0.1	0.5 ± 0.2	1.2 ± 0.2	1.4 ± 0.2	0.0030	0.0030	0.0003
18:0	1.1 ± 0.4	1.4 ± 0.4	1.2 ± 0.5	1.3 ± 0.2	—	—	—
18:1n-9	1.7 ± 0.1	1.7 ± 0.2	3.0 ± 0.4	2.7 ± 0.4	0.0002	—	—
18:1n-7	4.2 ± 0.1	3.8 ± 0.4	3.9 ± 0.3	4.4 ± 0.9	—	—	—
19:1n-8	0.7 ± 0.1	0.4 ± 0.1	0.3 ± 0.2	0.4 ± 0.1	—	—	—
20:5n-3	9.0 ± 0.7	9.0 ± 0.2	2.1 ± 0.6	1.9 ± 0.2	0.0000	—	—
22:0	0.2 ± 0.1	0.2 ± 0.1	ND	ND	—	—	—
Total sat.	35.3 ± 0.3	39.6 ± 0.8	44.3 ± 2.5	46.3 ± 1.6	0.0000	0.0073	—
Total mono.	55.3 ± 1.0	50.8 ± 0.6	52.8 ± 2.0	51.8 ± 2.0	—	0.0137	—
Total PUFA	9.0 ± 0.7	9.0 ± 0.2	2.1 ± 0.6	1.9 ± 0.2	0.0000	—	—
Unidentified	0.4 ± 0.1	0.5 ± 0.2	0.6 ± 0.4	ND			

[a]Values are means ± SD of three cultures.

[b]Interaction between temperature and medium composition. Mono., monoenoic fatty acids; PUFA, polyunsaturated fatty acids; ND, not detected; sat., saturated fatty acids; —, not significant. *i-, iso; a-, anteiso.*

in the bacterial lipid regardless of growth conditions. An increase in the level of 16:1 with the lowering of growth temperature was largely balanced by reductions in the proportions of 17:1n-8 and 18:1n-9, and consequently the overall proportion of monounsaturated fatty acids in total lipid was not greatly affected by growth temperature. The proportions of saturated fatty acids in total lipid were significantly higher in bacteria grown in seawater and freshwater medium at 20°C than in those grown at 5°C. Palmitic acid (16:0) and *iso*-15:0 were the predominant saturated components present although smaller proportions of other saturated fatty acids, both with even and odd numbered chains, were also present. Under the conditions employed for the analysis of total lipid fatty acid methyl esters, any 13:0 and *iso*-13:0 present in the total lipid were obscured by the butylated hydroxytoluene added as antioxidant. The only polyunsaturated fatty acid present in the bacteria was 20:5n-3 which accounted for 9% of the fatty acids of total lipid from cultures grown at 5°C and around 2% of those from cultures grown at 20°C in either medium.

In contrast to temperature, the salt content of the medium had only a small effect on the fatty acid composition of total lipid at both temperatures examined. Small but significant differences in the levels of several fatty acids were notable between bacteria grown at 5°C in seawater and freshwater, with the overall result being higher and lower proportions of monounsaturated and saturated fatty acids, respectively, in the total lipid of bacteria grown in seawater medium. At 20°C, however, there were no overall changes in the levels of total saturated, monounsaturated or polyunsaturated fatty acids in the total lipids in relation to the salinity of the medium. Two-way analysis of variance with pooled means showed that, overall, temperatures had a greater effect on the fatty acid composition of total lipid than medium composition. The levels of 14:0, 15:1, 16:0, 16:1, 17:1n-8 and 17:1n-6 were also influenced significantly by the interaction of temperature and medium composition. That is to say, changing temperature and medium composition simultaneously brought about a significant change in the proportions of these components.

Fatty acid composition of lipid classes. The use of two columns for the analysis of fatty acid methyl esters of phospholipid classes allowed the resolution of more components than were obtained for total lipid samples. Thus 13:0 and *iso*-13:0, as well as the various isomers of 16:1, were discernable.

Although the fatty acid composition of PE (Table 3) showed general similarities with that of total lipid, it was notable that the proportions of polyunsaturated fatty acids in the phospholipid were only half those present in total lipid (Table 2) while those of total saturated fatty acids were higher under all growth conditions examined. The overall levels of monoenoic fatty acids in PE were only slightly lower than those observed in total lipid. *Iso*-15:0 and 16:0 were always the major saturated fatty acids in PE. Although the proportion of *iso*-15:0 in bacteria grown

TABLE 3

Effect of Growth Temperature and Medium Composition on Fatty Acid Composition (wt%) of Phosphatidylethanolamine from *Vibrio* sp. Showing Significance of Each Factor and Their Interaction[a]

Fatty acids	5°C		20°C		Significance level *(P)*		
	Seawater	Freshwater	Seawater	Freshwater	Temp.	Medium	Interaction[b]
i-12:0	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	ND	—	—	—
12:0	3.8 ± 1.2	5.1 ± 0.6	3.7 ± 0.4	3.4 ± 0.3	—	—	—
i-13:0	4.5 ± 0.1	5.2 ± 0.6	3.8 ± 0.3	3.6 ± 0.4	0.0007	—	—
13:0	0.2 ± 0.0	0.1 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	—	—	—
i-14:0	0.2 ± 0.1	0.1 ± 0.1	0.5 ± 0.0	0.5 ± 0.1	0.0041	—	—
14:0	3.1 ± 0.3	4.7 ± 0.2	2.6 ± 0.2	1.7 ± 0.1	0.0000	0.0283	0.0000
14:1n-5	0.4 ± 0.1	0.4 ± 0.0	0.2 ± 0.0	0.3 ± 0.1	0.0008	—	—
i-15:0	14.2 ± 1.1	15.7 ± 0.4	13.5 ± 1.0	19.1 ± 1.8	—	0.0005	0.0149
a-15:0	0.2 ± 0.2	0.5 ± 0.3	0.2 ± 0.1	0.4 ± 0.4	—	—	—
15:0	3.5 ± 0.2	2.2 ± 0.4	5.5 ± 0.3	5.6 ± 1.3	0.0001	—	—
15:1n-6	2.5 ± 0.1	1.7 ± 0.7	1.8 ± 0.1	2.1 ± 0.6	—	0.0197	0.0033
16:0	10.5 ± 0.6	12.4 ± 0.4	14.6 ± 0.6	13.0 ± 0.9	0.0003	—	0.0010
trans 16:1n-7	12.8 ± 0.7	15.4 ± 0.7	4.4 ± 2.2	6.3 ± 0.8	0.0000	0.0420	—
16:1n-9	1.8 ± 0.1	1.9 ± 0.1	2.3 ± 0.1	2.3 ± 0.1	0.0010	—	—
cis 16:1n-7	23.3 ± 0.6	21.1 ± 0.8	23.1 ± 5.5	18.0 ± 3.4	—	0.0187	—
i-17:0	0.3 ± 0.2	0.4 ± 0.2	0.9 ± 0.1	1.4 ± 0.4	0.0020	—	—
17:0	0.7 ± 0.1	0.4 ± 0.1	2.6 ± 0.4	2.6 ± 0.3	0.0000	—	—
17:1n-8	6.4 ± 0.3	2.7 ± 0.4	10.2 ± 0.3	9.4 ± 2.1	0.0000	0.0024	0.0128
17:1n-6	1.4 ± 0.3	0.5 ± 0.2	1.2 ± 0.2	1.4 ± 0.1	0.0065	0.0096	0.0003
18:0	0.4 ± 0.1	0.4 ± 0.2	1.2 ± 0.4	1.0 ± 0.3	0.0015	—	—
18:1n-9	1.1 ± 0.1	1.0 ± 0.2	2.6 ± 0.5	2.1 ± 0.3	0.0000	—	—
18:1n-7	3.9 ± 0.5	3.4 ± 0.5	3.5 ± 0.3	4.3 ± 1.0	—	—	—
19:1n-8	0.5 ± 0.0	0.2 ± 0.1	0.4 ± 0.2	0.4 ± 0.1	—	—	—
20:5n-3	4.2 ± 0.4	4.4 ± 0.2	0.9 ± 0.3	0.8 ± 0.1	0.0000	—	—
Total sat.	41.7 ± 1.7	47.3 ± 0.9	49.4 ± 3.1	52.7 ± 0.2	0.0001	0.0014	—
Total mono.	54.1 ± 1.5	48.3 ± 1.0	49.7 ± 2.7	46.5 ± 0.3	0.0031	0.0006	—
Total PUFA	4.2 ± 0.4	4.4 ± 0.2	0.9 ± 0.3	0.8 ± 0.1	0.0000	—	—

[a]Values are means ± SD of three cultures.

[b]Interaction between temperature and medium composition. Mono., monoenoic fatty acids; PUFA, polyunsaturated fatty acids; ND, not detected; sat., saturated fatty acids; —, not significant. *i-, iso; a-, anteiso.*

in the seawater medium was not affected by temperature, that in freshwater bacteria was significantly higher at 20 than 5°C (19.1 *vs.* 15.7%). A different effect was noted in the proportions of 16:0, in that temperature did not affect its level in bacteria grown in freshwater, but its proportion in seawater bacteria was significantly higher at 20°C (14.6%) than at 5°C (10.5%). Overall, the PE of *Vibrio* grown at 20°C in both seawater and freshwater media had a significantly higher proportion of saturated fatty acids than that of bacteria grown at 5°C. However, only at 5°C was there a significant difference between bacteria grown in seawater and freshwater in terms of the total level of saturated fatty acids in the PE.

The predominant isomer of 16:1 in PE was *cis* 16:1n-7 which accounted for 18–23% of the total PE fatty acids. Growth conditions had no major effect on the level of this fatty acid in PE although its level was slightly, but significantly, lower in freshwater than seawater at 5°C. The only *trans*-monoenoic fatty acid detected was *trans* 16:1n-7 which comprised 12.8 and 15.4% of the total PE fatty acids in *Vibrio* grown at 5°C in seawater and freshwater medium, respectively. The level of this *trans*-monoenoic fatty acid was markedly lower in the corresponding cultures grown at 20°C, *i.e.,* 4.4 and 6.3% in seawater and freshwater, respectively. The proportions of 20:5n-3 in PE in bacteria grown at 5°C in seawater and freshwater were very similar at around 4.3%. Likewise there was no difference between bacteria grown in seawater and freshwater medium at 20°C in terms of the percentage of 20:5n-3 in PE but the percentage (around 0.9%) was significantly lower than were those of the 5°C cultures. As a consequence of temperature-related changes in the contents of monoenoic and polyunsaturated fatty acids, the PE of bacteria grown at 5°C was more unsaturated overall than that of bacteria grown at 20°C. As with total lipid, analysis of variance of the pooled mass for individual fatty acids showed that the effect of temperature was generally more significant than that of medium composition and that significant interaction between these two factors occurred with only a few fatty acids.

The overall fatty acid composition of PG (Table 4) was notably different from that of PE in that it contained higher proportions of unsaturated fatty acids. Conversely, the overall proportion of saturated fatty acids was lower in PG than PE. Bacteria grown in either freshwater or seawater medium at 20°C contained higher proportions of saturated fatty acids in this phospholipid than those cultured at 5°C.

The levels of monoenoic fatty acids in PG were significantly reduced by growth at 5°C regardless of whether the bacteria were cultured in freshwater or seawater media. Thus, for seawater medium, monoenoic fatty acids comprised 60.2 and 66.2% of total PG fatty acids at 5 and

TABLE 4

Effects of Growth Temperature and Medium Composition on Fatty Acid Composition (wt%) of Phosphatidylglycerol from *Vibrio* sp. Showing Significance of Each Factor and Their Interaction[a]

Fatty acids	5°C		20°C		Significance level *(P)*		
	Seawater	Freshwater	Seawater	Freshwater	Temp.	Medium	Interaction[b]
i-12:0	0.1 ± 0.1	0.2 ± 0.0	ND	ND	—	—	—
12:0	0.7 ± 0.6	0.9 ± 0.1	0.6 ± 0.2	0.5 ± 0.2	—	—	—
i-13:0	0.5 ± 0.4	0.6 ± 0.1	0.2 ± 0.1	ND	—	—	—
14:0	1.5 ± 0.4	2.5 ± 0.1	0.9 ± 0.1	0.8 ± 0.0	0.0000	0.0081	0.0029
14:1n-5	0.1 ± 0.1	0.3 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	—	—	—
i-15:0	5.4 ± 0.8	6.1 ± 0.2	4.4 ± 0.3	5.7 ± 0.6	—	0.0154	—
a-15:0	0.4 ± 0.2	0.4 ± 0.2	0.2 ± 0.2	0.1 ± 0.1	—	—	—
15:0	2.7 ± 0.3	1.6 ± 0.3	4.1 ± 0.3	4.0 ± 0.9	0.0001	0.0351	—
15:1n-6	1.5 ± 0.6	0.9 ± 0.1	1.9 ± 0.2	1.5 ± 0.3	0.0204	0.0316	—
16:0	10.3 ± 0.5	11.0 ± 0.6	13.0 ± 1.3	12.8 ± 0.7	0.0017	—	—
trans 16:1n-7	15.2 ± 1.0	15.1 ± 0.4	6.1 ± 3.6	8.5 ± 0.8	0.0007	—	—
16:1n-9	2.1 ± 0.2	1.9 ± 0.2	2.2 ± 0.2	2.6 ± 0.3	0.0053	—	0.0346
cis 16:1n-7	23.4 ± 1.1	26.9 ± 0.3	27.7 ± 3.8	25.2 ± 0.7	—	—	0.0165
i-17:0	0.4 ± 0.2	0.3 ± 0.1	1.3 ± 0.3	1.6 ± 0.5	0.0002	—	—
17:0	0.8 ± 0.1	0.3 ± 0.0	3.5 ± 0.6	2.7 ± 0.1	0.0000	0.0004	—
17:1n-8	8.2 ± 0.3	3.6 ± 0.3	16.0 ± 0.3	{ 16.8 ± 3.1	0.0000	0.0011	0.0047
17:1n-6	1.4 ± 0.2	0.3 ± 0.2	1.7 ± 0.3				
18:0	0.6 ± 0.1	0.3 ± 0.1	2.0 ± 0.2	1.3 ± 0.2	0.0000	0.0005	—
18:1n-9	2.0 ± 0.2	0.8 ± 0.5	4.7 ± 0.8	4.3 ± 0.8	0.0000	—	—
18:1n-7	5.6 ± 0.4	4.8 ± 0.6	5.3 ± 0.4	6.4 ± 0.7	—	—	0.0296
19:1n-8	0.8 ± 0.1	0.4 ± 0.1	0.4 ± 0.2	0.5 ± 0.1	—	—	0.0110
20:5n-3	16.2 ± 1.8	20.2 ± 0.6	3.7 ± 1.6	4.3 ± 0.3	0.0000	0.0499	—
Total sat.	23.4 ± 1.2	24.2 ± 0.7	30.1 ± 2.2	29.8 ± 0.5	0.0000	—	—
Total mono.	60.2 ± 2.7	55.0 ± 0.4	66.2 ± 0.6	65.9 ± 0.7	0.0000	0.0170	0.0345
Total PUFA	16.2 ± 1.8	20.2 ± 0.6	3.7 ± 1.6	4.3 ± 0.3	0.0000	0.0499	—
Unidentified	0.2 ± 0.2	0.6 ± 0.3	ND	ND			

[a]Values are means ± SD of three cultures.

[b]Interaction between temperature and medium composition. Mono., monoenoic fatty acids; PUFA, polyunsaturated fatty acids; ND, not detected; sat., saturated fatty acids; —, not significant. *i-, iso; a-, anteiso.*

20°C respectively, with the corresponding values for freshwater medium being 55.0 and 65.9%. The only monounsaturated fatty acid which did not decrease or stayed constant in proportion between 20 and 5°C was *trans* 16:1n-7, which exhibited a marked increase in proportion at the lower growth temperature with both media. As with total lipid and PE, the only polyunsaturated fatty acid present in PG of the *Vibrio* was 20:5n-3 which accounted for 16.2% of the total fatty acids present in PG of bacteria grown at 5°C in seawater and 20.2% in the same phospholipid of bacteria grown at the same temperature in freshwater medium. The proportion of 20:5n-3 was very notably and significantly ($P < 0.001$) lower in the PG from bacteria grown in either medium at 20°C. As with total lipid and PE, the fatty acid composition of PG was apparently more influenced by the temperature at which the *Vibrio* was grown than whether seawater or freshwater medium was employed. The significant interactions between the effects of temperatures and medium composition on fatty acid composition occurred mainly with monoenoic fatty acids.

DISCUSSION

The lipid class composition of the *Vibrio* species examined in the present study is relatively simple with PE and PG predominating and is typical of Gram-negative bacteria in general (1). However, the bacterium apparently lacks the phosphatidylserine and cardiolipin which have been reported for psychrophilic *Vibrio* isolated from marine sediments (9).

The bacterium possessed two types of fatty acids which are not typical of bacteria in general, namely, polyunsaturated fatty acids and *trans*-monoenoic fatty acids. Although these fatty acids have been reported as occurring independently in *Vibrio* (2,10,11,26), as far as we are aware this is the first report of their simultaneous presence in the lipids of a single bacterial species. Since the nutrient was defatted before its incorporation into the growth media, all the fatty acids extracted from the bacteria must have been synthesized *de novo* and not taken up preformed from the medium. The level of 20:5n-3 in the total lipid of the *Vibrio* examined here, however, is considerably less than that observed for bacteria isolated from deep-sea sources (2) where this polyunsaturated acid can account for up to 36.7% of the total fatty acids. As with *Vibrio* species in general (9,17), 16:1 was a major fatty acid of the *Vibrio* species examined in the present study. However, the levels of *iso*-13:0 and *iso*-15:0, particularly the latter, were much higher than have previously been reported for *Vibrio* and other marine species of bacteria (2,9,17,27). It is notable that the reported fatty acid compositions of *Vibrio* bacteria do vary widely (2,9).

Despite their overall rarity in bacteria, *trans* fatty acids have been found in some strains of marine bacteria including *Pseudomonas atlantica* (28) and an unidentified *Vibrio* sp. grown in seawater (11). The levels of *trans*

16:1n-7 in both the PG and PE of the *Vibrio* employed in this study were notably higher, especially at 5°C, than those observed by Okuyama *et al.* (10) in another *Vibrio* species. Furthermore, in the *Vibrio* studied here, the proportions of the *trans*-monoenoic fatty acid in PE and PG were similar, whereas in the bacteria studied by Okuyama *et al.* (10) the *trans* 16:1n-7 was present in higher concentration in PE than PG. The biological significance of *trans* fatty acids in bacteria remains to be established.

Given the relatively high levels of 18:1n-9 present in the lipids of the *Vibrio*, it seems likely that, in addition to the enzymes of the so-called anaerobic pathway of desaturation (1), the bacterium also possesses the aerobic Δ9 desaturase of eukaryotic cells. The wide spectrum of monounsaturated fatty acids present also indicates that the inferred Δ9 desaturase can act on a wide range of both odd- and even-numbered saturated fatty acids and that the *Vibrio* contains an active system for the elongation of acyl chains. Although the profile of monounsaturated fatty acids can be explained by the existence in the *Vibrio* of a single desaturase, the Δ9 desaturase, the synthesis of 20:5n-3 requires the bacterium to possess Δ12, Δ15, Δ6 and Δ5 desaturases if its formation proceeds *via* conventional pathways found in eukaryotes. It is notable that intermediates between monounsaturated and the pentaenoic fatty acid product are not found, even in trace amounts, in the *Vibrio* lipid. Although uncommon, this situation is similar to that found with the dinoflagellate *Crypthecodinium cohnii* in which 22:6n-3 is a major fatty acid and no other polyunsaturated fatty acid comprises more than 0.1% of the total fatty acids (29). It remains to be established whether the desaturations leading to the formation of 20:5n-3 in the *Vibrio* involve acyl carrier protein derivatives or fatty acids esterified in phospholipids, as substrates.

The effects of temperatures on lipid composition were far more pronounced than those of the salinity of the medium. The increased proportions of PE in lipid at low growth temperature is in keeping with the situation observed with poikilothermic animals (7). It is notable, however, that the increase in the proportion of PE was balanced by reductions in those of NEFA rather than those of PG. This lack of change in the proportion of PG is consistent with the fact that significant alterations in the level of this phospholipid in the halophilic *Vibrio costicola* occur only when the salt concentration of the medium is increased to values far in excess of those employed here (30).

Temperature effects on *Vibrio* fatty acid compositions have been reported previously with the actual effect varying with species (9). The present study showed a definite increase in the proportion of polyunsaturated fatty acids in the bacterial lipid as temperature decreased. This is in keeping with the situation reported for many poikilothermic vertebrate and invertebrate animals (7). The increase in the level of 20:5n-3 in the *Vibrio* by lowering growth temperature from 20 to 5°C was slightly less than the sixfold increase in the proportion of 22:6n-3 which occurs when *Vibrio marinus* is subjected to a decrease in environmental temperature from 20 to 2°C (2).

Also very notable was the increase in the content of *trans* fatty acids as the growth temperature decreased. This is in marked contrast to the report of Okuyama *et al.* (10) who found that the *trans*-monoenoic fatty acid content of the lipid from *Vibrio* sp. strain ABE-1 was higher at 20 than 5°C. Previously, increases in the proportion of *trans* 16:1n-7 with increasing growth temperature have been explained in terms of the *trans* fatty acid increasing the phase transition temperature of the membrane phospholipid as an adaptation to changes in the ambient temperature (10). However, this explanation is not applicable to the situation observed in the present study where the level of *trans* 16:1n-7 was higher at 5 than 20°C. Since the proportions of *trans* 16:1n-7 and 20:5n-3 both increased with decreasing temperature, it is possible that the net effect is a cooperative response between two fatty acids in the same phospholipid molecule which ensures molecular packing compatible with membrane bilayer properties required at lower temperature.

In summary, the present study demonstrates that 20:5n-3 and *trans* 16:1n-7 occur simultaneously in both PG and PE of a *Vibrio* species of bacterium and that a reduction in growth temperature significantly increases the proportions of both fatty acids in these lipids. Studies are continuing with the *Vibrio* to establish the pathways of 20:5n-3 biosynthesis. In addition, the positional distribution of *trans* 16:1n-7 and 20:5n-3 in PG and PE and how this is influenced by growth temperature, is under examination.

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REFERENCES

1. Harwood, J.L., and Russel, J.N. (1984) *Lipids in Plants and Microbes*, pp. 35-93, George Allen and Unwin, London.
2. DeLong, E.F., and Yayanos, A.A. (1986) *Appl. Envir. Microbiol. 51*, 730-737.
3. Johns, R.B., and Perry, G.J. (1977) *Arch. Microbiol. 114*, 267-271.
4. Yazawa, K., Araki, K., Okazaki, N., Watanabe, K., Ishikawa, C., Inoue, A., Numao, N., and Kondo, K. (1988) *J. Biochem. 103*, 5-7.
5. Yazawa, K., Araki, K., Watanabe, K., Ishikawa, C., Inoue, A., Kondo, K., Watabe, S., and Hashimoto, K. (1988) *Nippon Suisan Gak. 54*, 1835-1838.
6. Farkas, T., and Nevenzel, J.C. (1981) *Lipids 16*, 341-346.
7. Hazel, J.R., and Williams, E.E. (1990) *Prog. Lipid Res. 29*, 167-227.
8. Henderson, R.J., and Mackinlay, E.E. (1989) *Phytochemistry 28*, 2943-2948.
9. Bhakoo, M., and Herbert, R. (1979) *Arch. Microbiol. 121*, 121-127.
10. Okuyama, H., Okajima, N., Sasaki, S., Higashi, S., and Murata, N. (1991) *Biochim. Biophys. Acta 1084*, 13-20.
11. Okuyama, H., Sasaki, S., Higashi, S., and Murata, N. (1990) *J. Bacteriol. 172*, 3515-3518.
12. Hiramatsu, T., Yano, I., and Masui, M. (1980) *FEMS Microbiol. Lett. 7*, 289-292.
13. Kanemasa, Y., Yoshioka, T., and Hayashi, H. (1972) *Biochim. Biophys. Acta 280*, 444-450.
14. Komaratat, P., and Kates, M. (1975) *Biochim. Biophys. Acta (1975) 398*, 464-484.
15. Ohno, Y.I., Yano, I., Hiramatsu, T., and Masui, M. (1976) *Biochim. Biophys. Acta 424*, 337-350.
16. Ohno, Y.I., Yano, I., Hiramatsu, T., and Masui, M. (1979) *J. Biochem. (Tokyo) 85*, 413-421.
17. Oliver, J.D., and Colwell, R.R. (1973) *Int. J. System. Bacteriol. 23*, 442-458.
18. Hanna, K., Bengis-Garber, C., Kushner, D.J., Kogut, M., and Kates, M. (1984) *Can. J. Microbiol. 30*, 669-675.

19. Jostensen, J.P., Olsen, R.E., and Ringø, E. (1990) *Fisk. Dir. Skr. Ser. Ernaering 3,* 31–35.
20. Christie, W.W. (1982) *Lipid Analysis,* 2nd edn., pp. 51–122, Pergamon Press, Oxford.
21. Olsen, R.E., and Henderson, R.J. (1989) *J. Exp. Mar. Biol. Ecol. 129,* 189–197.
22. Christie, W.W., Brechany, E.Y., and Stefanov, K. (1988) *Chem. Phys. Lipids 46,* 127–135.
23. Volkman, J.K., Dunstan, G.A., Jeffrey, S.W., and Kearney, P.S. (1991) *Phytochemistry 30,* 1855–1859.
24. Emken, E.A. (1972) *Lipids 7,* 459–465.
25. Zar, J.H. (1984) *Biostatistical Analysis,* pp. 206–215, Prentice-Hall, Englewood Cliffs.
26. Guckert, J.B., Hood, M.A., and White, D.C. (1986) *Appl. Envir. Microbiol. 52,* 794–801.
27. Bhakoo, M., and Herbert, R. (1980) *Arch. Microbiol. 126,* 51–55.
28. Guckert, J.B., Ringelberg, D.B., and White, D.C. (1987) *Can. J. Microbiol. 33,* 748–754.
29. Henderson, R.J., Leftley, J., and Sargent, J.R. (1988) *Phytochemistry 27,* 1679–1683.
30. Kogut, M., and Russell, N.J. (1984) *Curr. Microbiol. 10,* 95–98.

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