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Modification of membrane lipids of a psychrophilic marine bacterium, *Vibrio* sp. strain ABE-1 and its effect on the respiration-dependent H^+ and Na^+ pumps

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Fatty acid composition of total phospholipids in the cells of a psychrophilic marine bacterium, *Vibrio* sp. strain ABE-1, was markedly changed by cultivating this bacterium in a medium containing a fatty acid, oleic or *n*-undecanoic acid ($C_{11:0}$), but not *n*-capric acid, without any change in the growth rate. Nevertheless, respiration-dependent primary Na^+ and H^+ pumps of such cells exhibited very high activities at 5°C. On the other hand, the H^+ pump activity at 25°C was not detected at pH 8.5 in cells grown in the presence of oleic acid, while those of cells grown in the presence or absence of $C_{11:0}$ were detectable under the same conditions. Since no characteristic changes were found in the membrane fluidity of the intact cells grown in the presence of oleic acid, it was suggested that alteration in physical properties of the whole membranes was not responsible for the specific loss of the H^+ pump activity at 25°C.

Many organisms use H^+ as a coupling ion in the energy transduction system. We have found that, in addition to an ordinary respiration-dependent H^+ pump, a respiration-dependent primary Na^+ pump functions both at acid and alkaline pHs in a psychrophilic marine bacterium *Vibrio* sp. strain ABE-1 (*Vibrio* ABE-1), and that these primary H^+ and Na^+ pumps are very active at low temperatures such as 5°C (TAKADA *et al.*, 1988 and 1989). These facts suggest that Na^+ also should play important roles in energy metabolism of this bacterium besides H^+.

Judging from the functions of these H^+ and Na^+ pumps, they must be proteins embedded in or spanning membrane. The functions of many membrane-bound proteins have been known to be influenced by physical states and constituents of the membranes, particularly by the compositions of

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Abbreviations: MES, 2-(*N*-morpholino)ethanesulfonic acid; Tricine, *N*-tris(hydroxymethyl) methylglycine; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; TPP, tetraphenyl phosphonium; *trans*-parinaric acid, 9, 11, 13, 15-*all*-*trans*-octadecatetraenoic acid.

fatty acids and phospholipids (COLEMAN, 1973; SANDERMANN, 1978). Fatty acid composition of membrane lipids could be changed by changing growth temperature in many bacteria, but remarkable qualitative differences in fatty acid composition exist between psychrophilic and other bacteria (KOGUT, 1980; RUSSELL, 1984; RUSSELL and FUKUNAGA, 1990). Therefore, it is presumed that the pump activities are influenced by alteration in fatty acid composition of phospholipids, and the high pump activities of *Vibrio* ABE-1 at low temperatures are attributable to interaction between the pump proteins and particular molecular species of phospholipids and/or fatty acids.

As a first step to confirm these possibilities, we attempted to alter the native fatty acid composition of total phospholipids in *Vibrio* ABE-1 by cultivating this bacterium in media supplemented with various fatty acids, and investigated the effect of such alteration on the pump activities in the present study.

Materials and Methods

Bacterial strain and growth conditions

Unless otherwise stated, *Vibrio* ABE-1 (TAKADA *et al.*, 1979) was cultivated for 24 and 48 hr with vigorous shaking at 15 and 5°C, respectively, in a Tris-salts medium (pH 7.5; HAKEDA and FUKUNAGA, 1983) with or without some supplements. Cell growth was monitored as described previously (TAKADA *et al.*, 1988).

Assay of respiration-dependent H^+ and Na^+ pump activities

The bacterial cells were washed and suspended in MGN buffer consisting of 0.5 mM MES-NaOH, 10% glycerol and 0.5 M NaCl (pH 6.5) or TGN buffer (pH 8.5) containing 0.5 mM Tricine-NaOH instead of MES-NaOH in MGN buffer under anaerobic conditions. Respiration-dependent H^+ and Na^+ pump activities were assayed as described previously (TAKADA *et al.*, 1988). For the assay of the H^+ and Na^+ pump activities, final concentrations of 2.5 mM TPP and 62.5 μM CCCP were added to the cell suspensions before the O_2 -pulse, respectively. Protein concentration was determined by the method of LOWRY *et al.* (1951) with bovine serum albumin as a standard.

Phospholipid extraction and fatty acid analysis

Total lipids of *Vibrio* ABE-1 were extracted as described by OKUYAMA and SASAKI (1986) and stored as a chloroform solution under nitrogen at −20°C until use. Phospholipids were separated from the total lipids by thin-layer chromatography on glass plates coated with 0.5 mm-thick silica gel (Merck, Art 7736). The plates were developed with either of the following solvent mixtures: chloroform/methanol/water (65 : 25 : 4, v/v) and hexane/

diethyl ether/acetic acid (90 : 30 : 1, v/v). All lipids and phospholipids on the plate were detected by iodine vapor and with spraying molybdenum blue reagent (DITTMER and LESTER, 1964), respectively. All phospholipid spots were scraped off and the silica gel was boiled for 3 hr in 5% methanolic HCl or 10% (v/v) acetyl chloride in methanol. Resultant fatty acid methyl esters were then extracted with hexane and analyzed by gas-liquid chromatography on a Shimadzu gas chromatograph GC-8APF. After injection of sample, column (OKUYAMA *et al.*, 1984) was heated from 100 to 190°C at 4°C/min. The fatty acids were identified by comparing the retention times with methyl ester standards. Fatty acid composition was calculated from the area of each peak on the chromatogram.

Measurement of fluorescence anisotropy

Fluorescent anisotropy of the intact cells was measured as reported by OKUYAMA *et al.* (1986) with the following modifications. *trans*-Parinaric acid (SKLAR *et al.*, 1977) was used as a fluorescent probe. The harvested cells were washed three times with 20 mM MES-NaOH (pH 6.5) or Tricine -NaOH (pH 8.5) containing 10% (v/v) glycerol and 0.5 M NaCl, which was previously bubbled with nitrogen gas for 5 min, and suspended in the same buffer at a protein concentration of about 60 μg/ml (Absorbance at 600 nm of the cell suspension was 0.1625). One μl of *trans*-parinaric acid in ethanol (1.5 mg/ml) was added to 3 ml of the cell suspension. The mixture was incubated for 30 min at 15°C before the measurement of fluorescence intensity.

Chemicals

n-Capric acid, *n*-undecanoic acid and oleic acid were obtained from Nacalai Tesque, Inc. TPP bromide and CCCP were purchased from Tokyo Kasei Kogyo Co., Ltd. and Sigma Chemical Co., respectively. *trans*-Parinaric acid was a product of Molecular Probes, Inc. All other reagents used were of analytical grade.

Results

Fatty acid composition of total phospholipids of the cells grown with fatty acid supplements

At first, the fatty acid composition of total phospholipids of *Vibrio* ABE -1 was analyzed for the cells grown in the medium supplemented with various amounts of oleic acid at 15°C (Table 1). As OKUYAMA *et al.* (1984) previously described for fatty acid composition of the outer and inner membrane fractions of this bacterium, fatty acid composition of the extracted total phospholipids was simple, and major components, hexadecenoic acid ($C_{16:1}$) and

Table 1. Fatty acid composition of total phospholipids from the cells grown with various amounts of oleic acid at 15°C

Fatty acid	No additions	0.4% Brij58	0.4% Brij58 +20mg $C_{18:1}$	0.4% Brij58 +50mg $C_{18:1}$	0.4% Brij58 +100mg $C_{18:1}$
10:0	3.3±0.4	3.6±0.3	3.3±0.4	2.8±0.3	2.7±0.3
12:0	1.1±0.3	1.3±0.2	1.1±0.2	0.7±0.3	0.7±0.1
N.I.	0.1±0.1	0.1±0.1	0±0.05	0	0
14:0	0.7±0.1	0.8±0.1	1.1±0.1	1.1±0.2	1.1±0.3
14:1	1.3±0.3	1.4±0.2	0.8±0.1	0.5±0.2	0.4±0.3
15:0	0±0.06	0	0±0.05	0	0
15:1	0±0.07	0±0.05	0±0.05	0	0
16:0	20.1±0.9	20.3±0.7	19.9±0.8	17.4±0.7	15.3±1.0
16:1	68.7±2.0	67.6±1.2	61.1±2.8	60.7±3.1	58.4±1.0
17:1	1.4±0.4	1.5±0.1	1.5±0.3	1.1±0.2	1.0±0.2
18:1	3.2±0.5	3.2±0.4	10.9±2.6	15.1±2.1	19.7±0.9
19:0	0.1±0.1	0.2±0.2	0.3±0.1	0.6±0.2	0.7±0.1

All values are expressed as weight percentage of total fatty acids and show mean± standard deviation of six or nine determinations with three independent preparations. The amounts of oleic acid shown in the table represent those added into 100ml of the growth medium.

N.I., Not identified.

$C_{16:0}$, comprised about 70% and 20% of total fatty acids, respectively. The supplementation of only a detergent (0.4% Brij 58) into the growth medium, which was used to disperse the added fatty acids, had no effect on the fatty acid composition. On the other hand, in the cells grown with the supplementation of oleic acid, contents of the both C_{16} fatty acids were decreased with concomitant increase in the amount of $C_{18:1}$. The extent of this compositional change was dependent on the amount of added oleic acid. However, no significant change was observed in the contents of other fatty acids.

The fatty acid composition changed markedly in the cells grown in the medium supplemented with 0.2 mg/ml *n*-undecanoic acid ($C_{11:0}$) at 15°C (Table 2). In the phospholipid from these cells, the content of fatty acids with odd numbered carbon chain increased to about 75% of total fatty acids, while the amount of these fatty acids was very small (less than 1% of total fatty acids) in the cells grown without supplementation of $C_{11:0}$. Furthermore, the content of C_{16} fatty acids, particularly $C_{16:1}$, were considerably decreased in these cells. Similar fatty acid composition was obtained from the cells grown with the same concentration of $C_{11:0}$ at 5°C (Table 3). On the other hand, irrespective of the growth temperatures, the supplementation of the growth

Table 2. Fatty acid composition of total phospholipids of the cells grown with different fatty acids at 15°C

Fatty acid	No additions	0.4% Brij58	0.4% Brij58 +20mg $C_{10:0}$	0.48% Brij58 +20mg $C_{11:0}$
9:0	0	0	0	0.3±0.1
10:0	2.8±0.3	3.1±0.5	2.8±0.3	0.5±0.3
11:0	0±0.03	0	0	3.5±0.8
12:0	1.1±0.3	1.0±0.3	0.6±0.2	0.2±0.1
12:1	0±0.1	0	0	0
13:1	0	0±0.12	0.2±0.3	0.8±0.8
14:0	0.6±0.3	0.7±0.1	0.8±0.1	0.2±0.2
14:1	1.5±0.3	1.6±0.2	1.3±0.1	0.2±0.2
15:0	0±0.08	0.1±0.1	0±0.03	11.4±1.7
15:1	0.1±0.1	0.1±0.1	0	13.7±1.8
16:0	19.6±0.9	19.6±1.2	22.1±1.1	5.2±2.2
16:1	69.4±1.8	69.1±1.5	67.0±1.9	17.0±7.5
17.0	0	0	0	6.7±2.4
17:1	0.5±0.7	0.5±0.8	0.7±0.9	39.2±5.6
N.I.	1.1±0.9	0.8±0.8	0.8±1.2	0
18:0	0.2±0.3	0.2±0.2	0.2±0.3	0.2±0.3
18:1	2.8±0.6	2.9±0.8	3.2±0.5	0.8±0.4
19:0	0.3±0.3	0.3±0.3	0.3±0.3	0.1±0.1
19:1	0±0.12	0±0.17	0±0.03	0±0.08

All values are expressed as weight percentage of total fatty acids and show mean± standard deviation of seven to thirteen determinations from three or four independent preparations. The amounts of the fatty acids show in the table represent those added into 100ml of the growth medium.

N. I., Not identified.

medium with 0.2 mg/ml *n*-capric acid ($C_{10:0}$) scarcely influenced the fatty acid composition (Tables 2 and 3).

Growth of Vibrio *ABE-1 in the medium supplemented with fatty acids*

The addition of various amounts of oleic acid into the growth medium had practically no effect on the bacterial growth at 5 and 15°C (Fig. 1) and 10°C (data not shown). Although remarkable increase of absorbance was observed at 5°C at early stage of the growth in the medium containing 0.5 mg/ml oleic acid (Fig. 1A), it seems to be attributed to the overestimation of the absorbance due to insolubility of oleic acid under low temperature because this phenomenon was not found on the growth at higher temperature, 15°C (Fig. 1B). Similar results were obtained on the growth with $C_{10:0}$ or $C_{11:0}$ at 5 and 15°C except a short lag of the growth observed at 5°C (Fig. 2).

Respiration-dependent Na^+ and H^+ pump activities of the cells with the

Table 3. Fatty acid composition of total phospholipids of the cells grown with different fatty acids at 5°C

Fatty acid	No additions	0.4% Brij58	0.4% Brij58 +20mg $C_{10:0}$	0.4% Brij58 +20mg $C_{11:0}$
9:0	0	0	0	0.3±0.04
10:0	3.4±0.5	3.8±0.4	5.3±0.3	0.8±0.4
11:0	0	0	0	3.1±0.3
12:0	1.3±0.3	1.2±0.4	0.4±0.3	0.2±0.2
12:1	0	0	0	0
13:1	0	0.1±0.2	0	0.3±0.4
14:0	1.0±0.1	1.0±0.2	0.7±0.3	0.4±0.2
14:1	3.6±0.2	3.4±0.5	2.3±0.3	0.8±0.4
15:0	0	0.1±0.1	0±0.05	9.6±1.6
15:1	0.2±0.2	0.1±0.1	0.1±0.1	20.6±2.3
16:0	16.3±0.6	16.6±0.9	18.2±2.0	6.5±0.7
16:1	71.2±0.9	69.9±2.0	69.5±1.9	25.4±6.1
17:0	0	0	0	3.7±1.3
17:1	0.7±0.8	0.5±0.7	0.7±0.9	27.3±3.0
N.I.	0.4±0.6	0.9±0.6	0.5±0.5	0
18:0	0	0.1±0.1	0±0.08	0.1±0.2
18:1	1.7±0.2	2.1±0.8	2.2±0.3	0.8±0.3
19:0	0.2±0.2	0.2±0.2	0.2±0.2	0.1±0.1
19:1	0	0	0	0±0.04

All values are expressed as weight percentage of total fatty acids and show mean± standard deviation of seven to thirteen determinations with three or four independent preparations. The amounts of the fatty acids shown in the table represent those added into 100ml of the growth medium.

N.I., Not identified.

modified fatty acid composition in total phospholipids

The respiration-dependent primary Na^+ and H^+ pump activities of the cells with the modified fatty acid composition were assayed at various temperatures. The cells grown at 15°C in the presence of 0.4% Brij 58 showed essentially the same temperature-dependence of both pump activities assayed at pH 6.5 and 8.5 (Figs. 3 and 4) as those of the cells grown in the Tris-salts medium without any supplementation, and the pump activities were very high even at 5°C as reported previously (TAKADA *et al.*, 1988). The striking compositional change of fatty acid, resulted from the growth with the supplementation of 0.2 mg/ml $C_{11:0}$, also scarcely affected the temperature-dependence of the both pump activities at pH 6.5 and 8.5. Similar results were obtained with the cells grown at 5°C in the medium supplemented with Brij 58 alone or plus $C_{11:0}$ (data not shown). These results indicate that

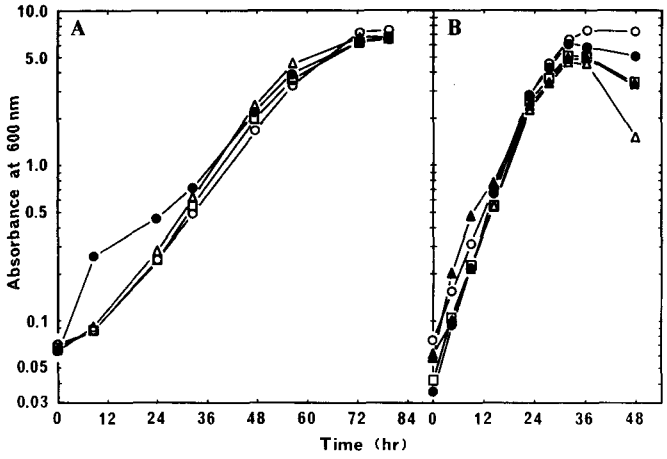

Fig. 1 Effects of various amounts of oleic acid on the growth of *Vibrio* ABE-1 at 5 and 15°C.
The growth of *Vibrio* ABE-1 on the Tris-salts medium containing 0.4% Brij 58 and various amounts of oleic acid was monitored at 5 (**A**) and 15°C (**B**). 0.4% Brij 58 was used to disperse oleic acid. 0% (○), 0.4% Brij 58 (△), or 0.4% Brij 58 and 20 mg (□), 50 mg (●) or 100 mg oleic acid (▲) was added to 100 ml of the medium.

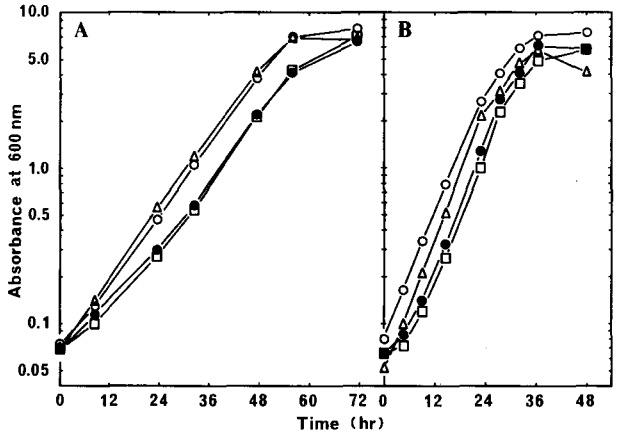

Fig. 2 Effects of *n*-capric acid and *n*-undecanoic acid on the growth of *Vibrio* ABE-1 at 5 and 15°C.
The bacterial growth was monitored at 5 (**A**) and 15°C (**B**) as described in the legend to Fig. 1. 0% (○), 0.4% Brij 58 (△), or 0.4% Brij 58 and 20 mg *n*-capric acid (□) or *n*-undecanoic acid (●) was added to the 100 ml of the medium.

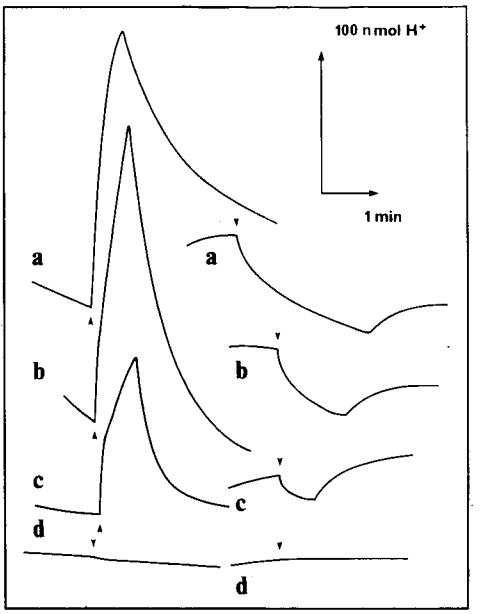

Fig. 3 Effect of temperature on the H^+ and Na^+ pump activities at pH 6.5 of the cells grown at 15°C with Brij 58.
The bacterial cells grown at 15°C in the Tris-salts medium containing 0.4% Brij 58 were washed and suspended in MGN buffer (pH 6.5) under anaerobic conditions. At the time points indicated by arrowheads, 50 μl of O_2-saturated 0.5 M NaCl was injected into the suspension. TPP and CCCP was added to the suspension at final concentrations of 2.5 mM and 62.5 μM in the left and right series of the assays, respectively. pH was monitored at 5 (a), 15 (b), 25 (c), or 40°C (d). An upward change represents acidification of the suspension.

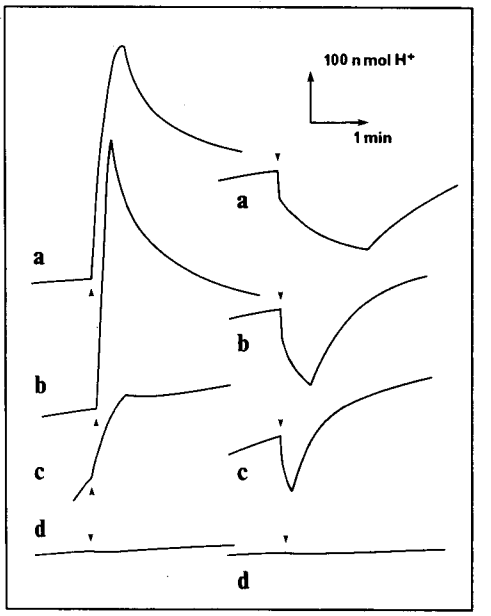

Fig. 4 Effect of temperature on the H^+ and Na^+ pump activities at pH 8.5 of the cells grown at 15°C with Brij 58.
The bacterial cells grown at 15°C in the Tris-salts medium containing 0.4% Brij 58 were washed and suspended in TGN buffer (pH 8.5) under anaerobic conditions. As described in the legend to Fig. 3, the both pump activities were assayed at pH 8.5 and the same symbols were used.

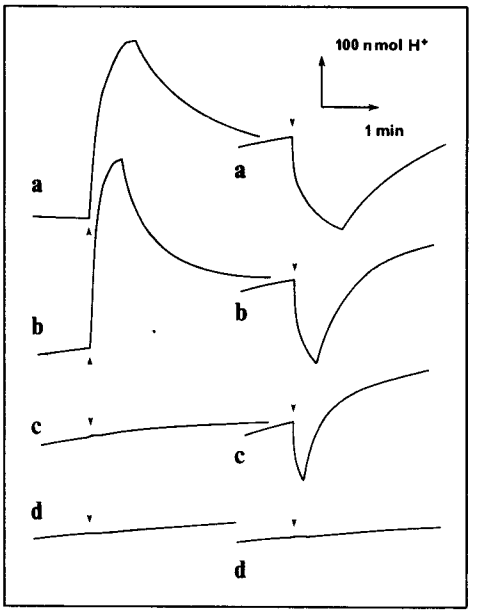

Fig. 5 Effect of temperature on the H^+ and Na^+ pump activities at pH 8.5 of the cells grown at 15°C with oleic acid.
As described in the legend to Fig. 4, the both pump activities of the cells grown at 15°C in the Tris-salts medium containing 0.4% Brij 58 and 0.5 mg/ml oleic acid were assayed at pH 8.5 and the same symbols were used.

lowering the growth temperature had no effect on the temperature-dependence of the both pump activities.

On the other hand, the cells cultivated in the medium supplemented with oleic acid were completely defect in the H^+ pump activity at 25°C at pH 8.5 (Fig. 5), while no change was observed in the temperature-dependence of Na^+ pump activity at pH 8.5 and both pump activities at pH 6.5 (data not shown).

Membrane fluidity of the intact cells

It was speculated that the defect in H^+ pump activity at 25°C was attributed to the change of membrane fluidity caused by the alteration of the fatty acid composition of total phospholipids. Therefore, a parameter for the membrane fluidity, fluorescence anisotropy, of the intact cells grown at 5 or 15°C in the medium containing several supplements was determined at various temperatures with *trans*-parinaric acid as a fluorescent probe (Table

Table 4. Fluorescnce anisotropy of the intact cells grown with different fatty acids

〈Cells prepared at pH 6.5〉

Growth temperature (°C)	Growth media	Fluorescence anisotropy at 5°C	15°C	25°C	40°C
5	0.4% Brij58	0.113	0.113	0.047	0.065
	0.4% Brij58 +20mg $C_{11:0}$	0.122	0.133	0.053	0.082
15	0.4% Brij58	0.094	0.088	0.040	0.047
	0.4% Brij58 +20mg $C_{11:0}$	0.100	0.093	0.056	0.066
	0.4% Brij58 +50mg $C_{18:1}$	0.141	0.151	0.096	0.118

〈Cells prepared at pH8.5〉

Growth temperature (°C)	Growth media	Fluorescence anisotropy at 5°C	15°C	25°C	40°C
5	0.4% Brij58	0.091	0.091	0.052	0.035
	0.4% Brij58 +20mg $C_{11:0}$	0.097	0.093	0.052	0.037
15	0.4% Brij58	0.100	0.102	0.054	0.049
	0.4% Brij58 +20mg $C_{11:0}$	0.101	0.108	0.054	0.048
	0.4% Brij58 +50mg $C_{18:1}$	0.124	0.127	0.057	0.052

The fluorescent anisotropy, r, was calculated by the follwing equation (SHINITZKY and INBAR, 1974):

$$r=(I_{//}-I_{\perp})/(I_{//}+2I_{\perp}),$$

where $I_{//}$ and $I_{\perp}$ was the emission intensity parallel and perpendicular to the excitation polarizer, respectively.

4). In all cells tested, large gaps of fluorescence anisotropies were observed between 15 and 25°C, indicating that alteration in physical state of the membrane occurred between these temperatures. However, in connection with the loss of H^+ pump activity at 25°C, no specific change in the membrane fluidity of the cells grown in the presence of oleic acid was detected at pH 8.5.

Discussion

The modification of membrane lipids in the intact bacterial cells for studying the effect of lipid components on the functions of the membrane -bound proteins has been generally achieved by the cultivation of lipid

auxotrophic mutants in the growth media supplemented lipids (BALDASSARE *et al.*, 1977; COTTAM *et al.*, 1986; EZE and MCELHANEY, 1987). However, in this study, we could readily modify the fatty acid composition of phospholipids in *Vibrio* ABE-1 by the similar supplemental technique using wild type but not the auxotrophic mutants. *Vibrio* ABE-1 grown in a fatty acid -free synthetic medium shows a typical fatty acid composition as seen in other members of *Vibrionaceae* (WILKINSON, 1988). When this bacterium was cultivated in the medium supplemented with $C_{11:0}$, the composition was remarkably changed and major constituents ($C_{16:0}$ and $C_{16:1}$) were replaced by fatty acids with odd numbered carbon chain (Tables 2 and 3). Such composition, comprising fatty acids with odd numbered carbon chain as a dominant, has not been found in any other members of *Vibrionaceae* so far studied (WILKINSON, 1988). In spite of such a striking change in the composition, the growth profile was almost normal in the temperature range between 5 and 15°C (Fig. 2). Fluorescence anisotropies compared between the cells grown without supplements and with $C_{11:0}$ showed almost the same values irrespective of growth and assay temperatures (Table 4), indicating that the both types of cells maintain similar membrane fluidities under these conditions. The maintenance of the membrane fluidity, which is known to be necessary for functioning of the membrane-bound proteins (HERBERT, 1981; WHITE and SOMERO, 1982), may contribute to the normal growth of the cells in the pressence of $C_{11:0}$. In the viewpoint of the mechanism for unsaturated fatty acid synthesis, the remarkable compositional change in fatty acid of phospholipids resulted from the cultivation with the supplement of $C_{11:0}$ may provide a useful resource for these studies.

The striking compositional change of fatty acid in the cells grown in the presence of $C_{11:0}$ had no effect on the activities of the Na^+ and H^+ pumps as well as its growth. Although the complete loss of the H^+ pump activity at pH 8.5 was observed at 25°C with the cells grown in the presence of oleic acid, it should not be ascribed to the change in physical properties of the whole membrane because no specific change was detected on the membrane fluidity (Fig. 5 and Table 4). However, these results does not exclude some effects of membrane lipids on the pump activities. Many membrane-bound enzymes require specific lipid(s) for the activities (COLEMAN, 1973; SANDERMANN, 1978), and therefore the lipid(s) bound to and/or close to these exzyme proteins should have critical effects on their activities. In fact, the activity of NADH dehydrogenase purified from an extremely thermophilic *Bacillus caldotenax* showed a linear Arrhenius plot, whereas those of the membrane -bound exzyme and the purified exzyme mixed with the extracted membrane

lipids showed two discontinuities in the plots (KAWADA and NOSOH, 1981). Temperatures of the break points are substantially consistent with those in a fluorescence anisotropy of the membrane and the extracted membrane lipids of the bacteria. The discontinuity and break point in the plots are altered by mixing the enzyme with various lipids. Since the loss of H^+ pump activity at 25°C was observed only in the cells grown with oleic acid (Fig. 5), it might be attributed to the change of acyl chain(s) of local phospholipids around the pump protein by the addition of oleic acid. Further study is required to clarify this possibility.

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