*J. Gen. Appl. Microbiol.*, **32**, 483–489 (1986)

# TWO GROUPS OF PHOSPHATIDYLETHANOLAMINE IN *VIBRIO* SP. STRAIN ABE-1 SEPARATED BY SILICA GEL THIN-LAYER CHROMATOGRAPHY[1]

HIDETOSHI OKUYAMA AND SHOJI SASAKI

*Department of Botany, Faculty of Science, Hokkaido University, Kitaku, Sapporo 060, Japan*

(Received September 6, 1986)

We analyzed a previously unknown phospholipid (PL-2) in the psychrophilic bacterium, *Vibrio* ABE-1. In thin-layer chromatography, it moved close to phosphatidylethanolamine but had a different *Rf* value. The polar head group of PL-2 was ethanolamine, and the *sn*-glycerol carbon atoms. This demonstrates that PL-2 is a species of phosphatidyl-bond/ethanolamine was 1 : 1.1 : 2.0 : 1.1. The chloroform- and water-soluble fractions after treatment with phospholipase C were 1,2-diacylglycerol and phosphoryl ethanolamine, respectively. The phosphatidylethanolamine of this bacterium has mainly C16 fatty acids especially hexadecenoic acid (16 : 1). PL-2 has higher amounts of saturated fatty acids such as hexadecanoic acid (16 : 0) and short chain fatty acids with less than 15 carbon atoms. This demonstrates that PL-2 is a species of phosphatidylethanolamine which has fatty acids that are different in average chain length and degree of unsaturation. The possible role of PL-2 in the phase separation of membrane phospholipid is discussed.

Recently, it has been pointed out that the cold-sensitivity of higher plants is related to the content of the disaturated molecular species of PG in the thylakoid membranes. That is, cold-sensitive plants have higher amounts of 16 : 0/16 : 0- and 16 : 0/16 : 1(3-*trans*)-PG in the thylakoid membrane (*1, 2*), and these plants have relatively high phase transition temperature. Thus, the fatty acid composition of individual phospholipids, glycolipids, and sulfolipids have been examined in the thylakoid membrane of the plants (*1–3*).

The fatty acids of individual phospholipids have not been sufficiently analyzed in most bacteria, except *Escherichia coli*. As reported by KITO et al. (*4*), the phospholipids (PE, PG, and CL) in *E. coli* are similar to each other in their fatty

[1] Abbreviations used in this text are as follows: TLC, thin-layer chromatography; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin; C16, fatty acids having 16 carbon atoms; 16 : 0/16 : 0-PG, phosphatidylglycerol (dipalmitoyl).

Address reprint requests to: Dr. H. Okuyama, Department of Botany, Faculty of Science, Hokkaido University, Kita-ku, Sapporo 060, Japan.

acid composition. Heretofore, no molecular species corresponding to disaturated PG in the thylakoid membrane of higher plants has been found in bacteria. In this study, phospholipid molecular species have been separated by TLC on silica gel plates impregnated with silver nitrate as modified neutral compounds such as monoacetyldiglycerol (*2, 5*). This method is based on the number of C=C bonds in the fatty acid moiety of the phospholipids.

The phospholipids of *Vibrio* ABE-1 have large amounts of unsaturated fatty acids, especially 16:1 (*6*). This was observed in the fatty acid composition of the individual phospholipids PE, PG, and CL (unpublished data). However, PL-2 which is located very close to PE on TLC plates, was esterified with a relatively high amount of saturated fatty acids and short chain fatty acids. In this study, PL-2 was purified by repeated ordinary thin-layer chromatography and identified as another group of phosphatidylethanolamine.

## MATERIALS AND METHODS

*Growth of bacteria.* *Vibrio* sp. strain ABE-1 (*Vibrio* ABE-1), a psychrophilic bacterium isolated in our laboratory (*7*), was used throughout this study. *Vibrio* ABE-1 was grown aerobically at 10°C in a liquid medium as described by HAKEDA and FUKUNAGA (*8*).

*Lipid extraction and chromatography.* Lipids were extracted from *Vibrio* ABE-1 cells with chloroform/methanol/water (1:1:0.9, by volume) by the method of BLIGH and DYER (*9*), and stored as a chloroform solution at −20°C. TLC was carried out on commercial plates coated with silica gel (Merck Art 5721). TLC plates coated with Wako gel B-5 containing 5% (w/w) boric acid (0.3 mm depth) were used to detect 1,2-diacylglycerol. The following solvent systems were used (composition in volume ratio): solvent A, chloroform/methanol/water (65:25:4); solvent B, chloroform/methanol/acetic acid (65:25:10); solvent C, chloroform/methanol/28% ammonium hydroxide (65:35:5); solvent D, ethanol/acetic acid/water (90:5:5); solvent E, hexane/ether/acetic acid (90:30:1); solvent F, chloroform/acetone (96:4); and solvent G, *n*-butanol/acetone/acetic acid/28% ammonium hydroxide/water (35:25:15:3:22).

The lipids on TLC plates were nonspecifically visualized with iodine vapor or by charring after spraying with a 30% aqueous solution of sulfonic acid. The functional groups of PL-2 were detected specifically on TLC plates with the following spray reagents: ninhydrin for the amino group, molybdate for organic phosphate, hydroxylamine-ferric chloride for the ester bond, and α-naphthol for sugar.

Ethanolamine and phosphoryl ethanolamine were chromatographed on TLC plates (Merck Art 5721) with solvent D and on Toyo #51A paper with solvent G, respectively. Fatty acid methyl esters were analyzed by gas-liquid chromatography as described previously (*6*).

*General methods.* PL-2 was hydrolyzed by heating the lipid with 1 N HCl

overnight at 100°C. Phosphorus was determined by the method of BARTLETT (*10*). The ester bond was assayed as reported previously (*11*). The fatty acid methyl esters were prepared by boiling the lipids in 5% methanolic HCl solution. The glycerol in PL-2 was determined by the method of LAMBERT and NEISH (*12*) after hydrolysis with 2 N HCl at 125°C for 48 hr. Ethanolamine was assayed by the method of NOJIMA and UTSUGI (*13*).

*Enzymatic method.* PL-2 was incubated with phospholipase C according to HANAHAN and VERCAMER (*14*). *sn*-1,2-Diacylglycerol was prepared from authentic phosphatidylethanolamine (dimyristoyl) by treatment with phospholipase C.

*Reagents.* All authentic phosphatidylethanolamines (dilauroyl, dimyristoyl, dipalmitoyl, and distearoyl) and phospholipase C (EC 3.1.4.3 *Bacillus cereus*) are products of Sigma. All other reagents are of analytical grade.

## RESULTS AND DISCUSSION

*Vibrio* ABE-1 has five major phospholipids in the membrane: PE, PG, CL, PL-

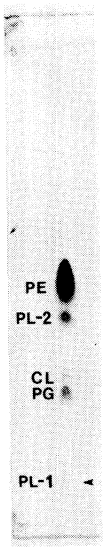


Fig. 1.
Thin-layer chromatogram of total phospholipids of *Vibrio* ABE-1.
Solvent A was used for development. Spots were visualized by exposure to iodine vapor and by spraying molybdate reagent. Arrowhead indicates origin.

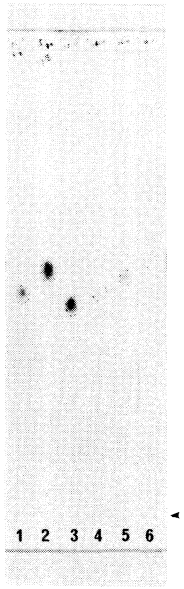


Fig. 2.
Thin-layer chromatogram of PL-2 (lane 1) and PE (lane 2) purified from *Vibrio* ABE-1, and of authentic 12:0/12:0-PE (lane 3), 14:0/14:0-PE (lane 4), 16:0/16:0-PE (lane 5), and 18:0/18:0-PE (lane 6).
Solvent A was used for development. Spots were visualized by exposure to iodine vapor and by spraying molybdate reagent. Arrowhead indicates origin.

1, and PL-2. The first three were identified previously (*6*), but the other two were not.

Phospholipids prepared from *Vibrio* ABE-1 cells grown at 10°C were applied to silica gel TLC plate. As shown in Fig. 1, PL-2 appeared very close to PE, and its position relative to PE was not changed by either acidic (solvent B) or alkaline (solvent C) development systems. The ratios of PE and PL-2 calculated from the phosphorus content on two-dimension TLC plates were about 7 : 1 for the outer membrane and 9 : 1 for the inner membrane (*6*).

To purify PL-2, the spot area was carefully scraped off the TLC plate, avoiding contamination with PE, and extracted with chloroform/methanol/water (1 : 1 : 0.9). After repeating the extraction three or four times, a purified PL-2 that gave a single spot on TLC plate was obtained (Fig. 2, lanes 1 and 2).

On TLC plates, PL-2, like PE, reacted positively with molybdate, ninhydrin, and hydroxylamine-ferric chloride spray reagents, but not with α-naphthol. The water-soluble product obtained by HCl hydrolysis of PL-2 had the same *Rf* value as that of authentic ethanolamine with solvent D (Fig. 3), indicating that the polar head group of PL-2 contains ethanolamine. The chloroform-soluble product

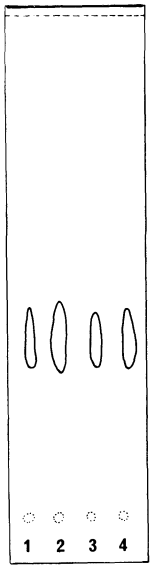


Fig. 3.

Thin-layer chromatogram of PE (lane 1), PL-2 (lane 2), and total phospholipids (lane 4) of *Vibrio* ABE-1 hydrolized with 1 N HCl as described in MATERIALS AND METHODS.

Lane 3 is authentic ethanolamine treated as above. Solvent D was used for development. Spots were visualized by spraying ninhydrin reagent and heating for 5 min at 100°C.

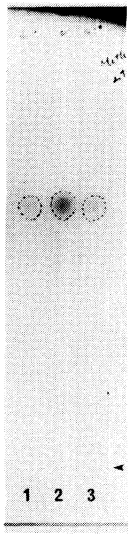


Fig. 4.

Thin-layer chromatogram of methanolysis products of 16 : 0/16 : 0-PE (lane 1), PL-2 (lane 2), and 14 : 0/14 : 0-PE (lane 3).

Methanolysis and development were carried out as described in MATERIALS AND METHODS. Spots were visualized by charring at 180°C. Arrowhead indicates origin.

Table 1. Fatty acid composition of PL-2 and PE in *Vibrio* ABE-1.

| Fatty acid | PL-2 (%) | PE (%) |
|---|---|---|
| 10:0 | 18.3 | 0 |
| 12:0 | 2.7 | 0 |
| 12:1 | 0.5 | 0 |
| 14:0 | 3.2 | 0.6 |
| 14:1 | 0 | 1.5 |
| 15:0 | 0 | T[a] |
| 15:1 | 0 | T |
| 16:0 | 64.0 | 16.1 |
| 16:1 | 7.7 | 78.3 |
| 17:0 | 0.4 | 0 |
| 17:1 | 0 | T |
| 18:0 | 1.3 | T |
| 18:1 | 1.8 | 3.0 |
| U/S[b]+U[c] | 9.5% | 82.8% |
| <15:1[d] | 24.7% | 2.1% |
| Average chain length[e] | 14.70 | 15.97 |

[a] Less than 0.3%.
[b, c] Saturated and unsaturated fatty acids, respectively.
[d] Short chain fatty acids with less than 15 carbon atoms.
[e] Defined as:

$$\frac{\Sigma(\text{number of carbon atoms} \times \text{amount of fatty acid})}{\Sigma(\text{amount of fatty acid})}.$$

Table 2. Molar ratio of glycerol, phosphate, ester bond, and ethanolamine in the PL-2 of *Vibrio* ABE-1.

| | Content (nmol/100 μl of PL-2[a]) | Molar ratio |
|---|---|---|
| Glycerol | 61.9 | 1 |
| Phosphate | 69.6 | 1.1 |
| Ester bond | 125.9 | 2.0 |
| Ethanolamine | 65.0 | 1.1 |

[a] One hundred μl portions of PL-2 dissolved in chloroform were withdrawn and subjected to determination of each group and bonding as described in MATERIALS AND METHODS.

obtained after methanolysis of PL-2 with 5% methanolic HCl consisted only of fatty acid methyl esters, as indicated by TLC with solvent system D (Fig. 4) and gas-liquid chromatography (Table 1). Thus, the fatty acids were proved to be covalently linked. The molar ratio of glycerol/phosphate/ester bond/ethanolamine was 1 : 1.1 : 2.0 : 1.1 (Table 2).

The chloroform- and water-soluble products after treatment with phospho-

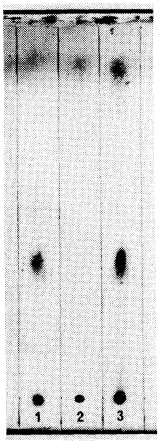


Fig. 5.
Thin-layer chromatogram of chloroform-soluble products of 14:0/14:0-PE (lane 1) and PL-2 (lane 3) after treatment with phospholipase C.
The chloroform-soluble fraction of PL-2 without treatment of phospholipase C is shown in lane 2. Solvent F was used for development. Spots were visualized by charring at 180°C.

Fig. 6.
Paper chromatograms of water-soluble products of PL-2 (right) and authentic phosphoryl ethanolamine (left) after treatment with phospholipase C.
Solvent G was used as the developmental system. Spots were visualized by spraying ninhydrin and heating for 5 min at 100°C. Arrowhead indicates origin.

lipase C were analyzed by TLC and paper chromatography, respectively. As shown in Figs. 5 and 6, the chloroform- and water-soluble products of PL-2 hydrolized by phospholipase C had the same *Rf* value as those of 1,2-diacylglycerol and phosphoryl ethanolamine, respectively. Similarly, as indicated by thin-layer chromatogram, the chloroform- and water-soluble products of PL-2 treated with phospholipase D were phosphatidic acid and ethanolamine, respectively (data not shown). These results indicate that PL-2 is a distinct group of phosphatidylethanolamines. This is why the *Rf* value of PL-2 on TLC plates is different from PE.

As shown in Fig. 2, authentic 12:0/12:0-, 14:0/14:0-, 16:0/16:0-, and 18:0/18:0-PE migrated on the TLC plate with mutually distinct *Rf* values. Apparently this is caused by the difference in the chain length of the fatty acids. Table 1 shows that PL-2 contains much more short chain fatty acids and saturated fatty acids than PE. The average chain length of fatty acids in PL-2 and PE, tentatively calculated from their fatty acid composition (Table 1), were 14.70 and 15.97, respectively.

On the other hand, about 80% of the total fatty acids of PE are unsaturated acids, more than 90% of which is 16:1. In contrast to PE, the unsaturated fatty acids of PL-2 amount to less than 10%. These results indicate that the different *Rf* values in PL-2 and PE come from the difference in the chain length and the degree of unsaturation of their fatty acids. Thus, it is apparent that the two groups of

phosphatidylethanolamine are distinct in the composition of their molecular species. The analysis of the molecular species of PE and PL-2 is in progress.

Recently, it was suggested that the disaturated molecular species of PG may determine the phase separation temperature in chloroplast membrane lipids (*1*). As the most effective phospholipid, 16:0/16:0-PG and 16:0/16:1(3-*trans*)-PG were noted (*1*, *2*). The physiological role of PL-2 in the *Vibrio* ABE-1 membrane is not yet clear, but this phospholipid may play a role similar to the disaturated molecular species of PG in chloroplasts. Indeed, our preliminary experiments indicate that the phase separation temperature of the mixture in *Vibrio* ABE-1 is markedly affected by the content of PL-2.

The authors are grateful to Dr. Akira Hase, Biological Laboratory, Hakodate College, Hokkaido University of Education, for his many stimulating discussions and to Mr. Toshiaki Tokumitsu in our laboratory for his helpful assistance. This work was supported in part by Grant-in-Aid for Scientific Research (61740382) from the Ministry of Education, Science and Culture of Japan.

## REFERENCES

*1*) N. Murata and J. Yamaya, *Plant Physiol.*, **74**, 1016 (1984).
*2*) P. G. Goughan, *Plant Physiol.*, **77**, 740 (1985).
*3*) L. Vigh, I. Horvath, P. R. van Hasselt, and P. J. C. Kuiper, *Plant Physiol.*, **79**, 756 (1985).
*4*) M. Kito, S. Aibara, M. Kato, and T. Hata, *Biochim. Biophys. Acta*, **260**, 475 (1976).
*5*) M. Nishihara, M. Kimura, K. Izui, M. Ishinaga, and M. Kito, *Biochim. Biophys. Acta*, **409**, 212 (1976).
*6*) H. Okuyama, H. Itoh, N. Fukunaga, and S. Sasaki, *Plant Cell Physiol.*, **25**, 1255 (1984).
*7*) Y. Takada, T. Ochiai, H. Okuyama, K. Nishi, and S. Sasaki, *J. Gen. Appl. Microbiol.*, **25**, 11 (1979).
*8*) Y. Hakeda and N. Fukunaga, *Plant Cell Physiol.*, **24**, 849 (1983).
*9*) E. G. Bligh and W. J. Dyer, *Can. J. Biochem. Physiol.*, **37**, 911 (1959).
*10*) E. R. Bartlett, *J. Biol. Chem.*, **234**, 466 (1959).
*11*) F. Snyder and N. Stephens, *Biochim. Biophys. Acta*, **34**, 244 (1959).
*12*) M. Lambert and A. C. Neish, *Can. J. Biochem.*, Sect. B, **28**, 83 (1956).
*13*) S. Nojima and N. Utsugi, *J. Biochem.*, **44**, 565 (1957).
*14*) D. H. Hanahan and R. Vercamer, *J. Am. Chem. Soc.*, **76**, 1804 (1954).