Structural Analyses of the Novel Phosphoglycolipids Containing the Unusual very Long Bifunctional Acyl Chain, α,ω-13,16-Dimethyloctacosanedioate in *Thermoanaerobacter ethanolicus*

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Novel membrane lipids containing the unusual very long chain fatty acid, α,ω-13,16-dimethyloctacosanedioate dimethyl ester (DME C30) was isolated and purified from thermophilic anaerobic eubacterium, *Thermoanaerobacter ethanolicus*. Structures of the lipids containing the bifunctional fatty acyl components were proposed by various analyses such as ^{1}H, ^{13}C, ^{31}P nuclear magnetic resonance (NMR). Fourier transform infrared (FTIR) spectroscopy, gas chromatography/mass spectrometry (GC/MS) and fast atom bombardment mass spectrometry (FAB/MS). Combined with the GC/MS, ^{1}H and ^{13}C NMR data, we confirmed that the head groups of the lipids contained the glycerol and/or glucosamine molecules. ^{31}P NMR spectrum also showed that the lipids contained phosphate in a phosphodiester linkage. The proposed structures of these novel lipid components were the ones in which two head groups were linked by the membrane spanning fatty acyl component (DME C30) and regular chain fatty acids on glycerol moiety of each head group.

Key Words : *Thermoanaerobacter ethanolicus*, Thermophile, Phosphoglycolipids, α,ω-13,16-Dimethyloctacosanedioate dimethyl ester (DME C30), Structural analyses

Introduction

Bacterial membranes are extremely dynamic complex systems that interface directly with the environment and carry out many important functions.[1,2] The membranes serve as a control center for the regulation of the motional dynamics of membrane components. The term "homeoviscous adaptability" has been used to describe the process whereby, after a perturbation that changes membrane viscosity, the membrane chemistry of an organism is altered to restore the original viscosity or fluidity.[3] Membrane lipids are especially known to play various important roles in the physiological functions of bacteria.[4,5] From the standpoint of signal transduction, structural studies of membrane lipids and the induction of the synthesis of unusual structures have a special importance.[11,17]

Thermoanaerobacter ethanolicus is a thermophilic and anaerobic eubacterium whose optimum temperature for growth is 65-68 °C.[16,18] High temperature induces high degrees of motion of lipids, through which the dynamics or fluidity of the membrane is critically affected. The membrane of the bacterium has been known to contain a family of unusual very long chains, α,ω-dicarboxylic fatty acyl components similar to those formed in *Sarcina ventriculi*, a strict anaerobic and facultative acidophilic eubacterium.[7,13,15] In *S. ventriculi*, these unusual lipid species appear to be formed by the tail-to-tail coupling of alkyl chains across the two leaflets of the membrane bilayer as a general response to the external shocks such as temperature and pH.[1,8,14] *T. ethanolicus* contains the very long chain, DME C30, as the most abundant transmembrane fatty acid component in its membrane.[13] Although their exact conformations of physiological functions inside the membrane still remain to be unknown, it is believed to be responsible for the maintenance of membrane integrity against the changes of external temperature.[10] A possible physiological role of the DME C30 as a major fatty acid component has been also reported through the work using a model membrane made of phosphatidylcholine (PC) and the very long chain fatty acid.[10] However, no available information on the structures of the lipid species containing the DME C30 has ever been proposed. Their structural information will be critical for understanding the architecture of the thermophilic membrane as well as the biochemical functions in the cellular physiology.

Here, we propose the partial structures of a family of membrane lipids containing the membrane spanning fatty acyl component, DME C30, in the membrane of *T. ethanolicus*.

Experimental Section

Bacterial Cultures. *T. ethanolicus* was obtained from Korea Collection for Culture Type as KCTC 3184 (ATCC 31938). The bacterium was cultivated as we described previously.[10,13] The cells were grown on complex media that contained 0.2% (w/v) yeast extract, salts, and vitamin with 0.8% (w/v) glucose under a strictly anaerobic condition at

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65 °C.

Extraction of Membrane Lipids. The cells were harvested by centrifugation at 10,000 *g* for 10 min. The cells were then extracted at 40 °C with 40 mL of a mixture of chloroform/methanol/water (15 : 3 : 2, by vol.) for 2 h, and extraction was performed with intermittent sonication over 2 h. After centrifugation at 10,000 × *g*, the supernatant was taken to dryness on a rotary evaporator, dissolved in 10 mL of chloroform/methanol (5 : 1, v/v), and then shaken with water. The lower phase containing the lipids was taken to dryness, and the residue was dissolved in 1 mL of chloroform.

Isolation of the Membrane Lipids Containing the DME C30. Separation of the lipids extracted from the cells was performed on flash column chromatography (Sigma, St. Louis, U.S.A) packed with silica-gel 60 (Merck, Germany). The lipids were sequentially eluted with chloroform, acetone and chloroform/methanol/water (65 : 25 : 0.5, by vol.) at a flow rate of 60 mL/min. Very polar lipids containing the DME C30 were then eluted with methanol.

Analyses of the Lipids. The lipids containing the DME C30 were separated by using thin layer chromatography (TLC) (Merck, Germany) with silica-gel 60 using chloroform/methanol/water (7 : 1.6/0.2, by vol.) as a solvent system. Spots were visualized either by spraying with 50% ethanolicsulfuric acid and heating at 250 °C to char the organic components or by spraying with a 0.1% solution of 2',7-dichlorofluorescein in aqueous ethanol (1 : 1, v/v).[8,15] Standard phospholipids were used as standards in addition to free fatty acids. Spraying agents for the detection of each lipid components were used as we described previously.[15] Each lipid band was scraped from the plate onto a column fitted with a sintered disc and the material was eluted from the silica gel using mixtures of methanol and chloroform. Each fraction was concentrated by evaporation and redissolved in chloroform or a chloroform/methanol (5/1, v/v) mixture for further analysis.

Analyses of Lipid Headgroups. The isolated lipids (2-3 mg) were hydrolyzed with 1 mL of 2 M trifluoroacetic acid (TFA) (Sigma, St. Louis, U.S.A) at 120 °C for 3 h.[9] The hydrolysate was concentrated to dryness, then 1 mL of water was added and the solution was concentrated to dryness to remove any trace amounts of TFA. The hydrolysate was further analyzed by ^{1}H, ^{13}C and ^{31}P NMR spectroscopy or by the conversion to alditiol acetate derivatives for a GC/MS analysis. For the GC/MS analysis with the alditol acetate derivatives, the hydrolysate was extracted with 1 mL of chloroform (2 vol.). The chloroform layer of the extracts was discarded and the aqueous layer was concentrated to dryness under nitrogen. The aqueous residue was dissolved in methanol and reduced with sodium borohydride (Sigma, St. Louis, U.S.A) for 1 h. Three molar HCl was then added to decompose the excess borohydride. The solution was repeatedly concentrated to dryness several times from methanol. Both 0.1 mL of pyridine and 0.1 mL of acetic anhydride were added to the residue. The solution was briefly sonicated and left at room temperature for 16-20 h. The mixture was concentrated to dryness and then 1 mL of chloroform and 1 mL of water were added to the residue. The chloroform layer was washed once with 1 mL of 0.5 M NaCl, concentrated, and subjected to GC or GC/MS analyses on a J&w Scientific DB1 capillary column (Folsom, U.S.A.) with an initial temperature of 100 °C, hold time 5.0 min and a rate of 1.0 °C/min to temperature of 150 °C. A second lamp of a rate of 1.0 °C/min was then immediately started until the final temperature of 250 °C was obtained. This temperature was held for 30 min. The retention times of the alditol acetated derivatives were compared with those of a number of alditol acetate standards. GC/MS analyses were then performed with a JEOL JMS-AX505WA mass spectrometer (Peabody, U.S.A.) interfaced with a Hewlett-Packard 5890A gas chromatograph.

Analyses of Fatty Acyl Components. The fatty acid analyses were performed on the isolated lipids by treatment with a 5% methanolic-HCl solution. A 3 mL of chloroform was added to 1 mL of the isolated lipids suspension followed by 15 mL of a 5% methanolic-HCl solution. The flask was sealed and heated in an oven at 72 °C for 24 h. A 3 mL of chloroform was added every 8 h followed by mild sonication for 5 min. The mixture was then concentrated on a rotary evaporator to dryness and extracted with chloroform. The combined organic fraction was redissolved in 1 mL of hexane. The fatty acid methyl esters were subjected to gas chromatographic analysis on a DB1 capillary column using helium as a carrier gas and with an initial temperature of 150 °C, hold time 0.0 min and a rate of 3.0 °C/min to temperature of 300 °C. This temperature was held for 40 min. The relative proportions of the lipid components were calculated from the integrated peak area. The identification of fatty acids and the determination of molecular weights were accomplished by the GC/MS analysis.

FAB Mass Spectrometry. FAB/MS was performed using a JEOL HX100 (Peabody, U.S.A.) double-focusing mass spectrometer (EB configuration) equipped with a high-field magnet operated in the negative ion mode. The ions were produced by bombardment with a beam of Xe atoms (6 kV). The accelerating voltage was 10 kV, and the resolution was set at 1,000 or 3,000 according to the mass range of interest. The samples were dissolved in a chloroform solution. Generally, 1-1.5 μL of the sample was mixed with 1 μL of *m*-nitrobenzyl alcohol (MBA) on the FAB-MS stainless steel probe tip.[1] The calibration was performed using Ultramark (443 or 6121) or $(CsI)_nI^-$ cluster ions, depending on the mass range of interest. A JEOL DA-5000 data system was used for recording the spectra. The spectrum was scanned over 2 min from *m/z* 0 to 3,000.

^{1}H, ^{13}C and ^{31}P NMR Spectroscopic Analysis. NMR spectroscopic analysis was performed on a Bruker Avance (AMX, Germany) 500 NMR spectrometer operated at 500 MHz for ^{1}H, 125 MHz for ^{13}C and 202.5 MHz for ^{31}P, respectively. All measurements were performed with 0.6 mL samples in 5 mm NMR tubes using $CDCl_3$ or D_2O as a solvent. For the analysis of the isolated lipids, 15 mg of the lipids was dissolved in $CDCl_3$. For the analysis of the head

groups of the lipids, the hydrolysates were dissolved in D_2O. Chemical shifts were quoted relative to the chloroform resonances (δ 7.24 for ^1H and δ 77.00 for ^{13}C), external trimethylsilane (TMS, δ 0.00) and 85% H_3PO_4 (δ 0.00) for ^{31}P NMR measurement, respectively.

FTIR Spectroscopic Analysis. Spectra were obtained on a JASCO FTIR-300E spectrometer (REV. U.S.A.) over the wave number of 4000-300 cm^{-1}. The 5-6 mg of purified lipids was mixed with a KBr pellet.

Results and Discussion

NMR Spectroscopic Analysis of the Isolated Lipids. The ^1H, ^{13}C and ^{31}P NMR spectra of the unusual lipids containing bifunctional acyl chains were obtained. In the ^1H NMR spectra of the isolated total lipids (Figure 1A) and purified lipids (Figure 1B), the signals of the intense resonance at δ 0.79 and δ 1.18 were assigned to the methyl and methylene groups of the long chain and the regular chain fatty acyl components, respectively. The characteristic resonance at δ 0.79 contains the signal of the vicinal methyl groups of the ω-linked bifunctional acyl chains. The signals of the resonance at δ 2.23, δ 1.97 and δ 1.50 corresponded to the methylene protons α related to the carbonyl function, the protons of the methylene groups next to the vinyl carbons, and the methylene protons β related to the carbonyl group, respectively. The small signal at δ 5.28 assigned to the olefinic protons was present only in the regular fatty acyl chains of the lipids, not in the bifunctional very long fatty acyl chains as we described previously.[13] The peaks within the range of δ 3.00-5.18 strongly indicate the presence of glycerol and carbohydrate moieties in both the total and the purified lipids. The ^{13}C NMR spectrum for the isolated total lipids (Figure 2) showed the ester carbonyl carbon at δ 172.4, methylene next to *iso*-branch at δ 38.1, methylene next to *anteiso*-branch at δ 35.6, methyne next to *anteiso*-branch at δ 33.4, methyne next to *iso*-branch at δ 26.1, methyl *iso*-branch at δ 23.4, methyl *anteiso*-branch at δ 18.2, vicinal methyl group at δ 13.0, and methyl *anteiso*-branch at

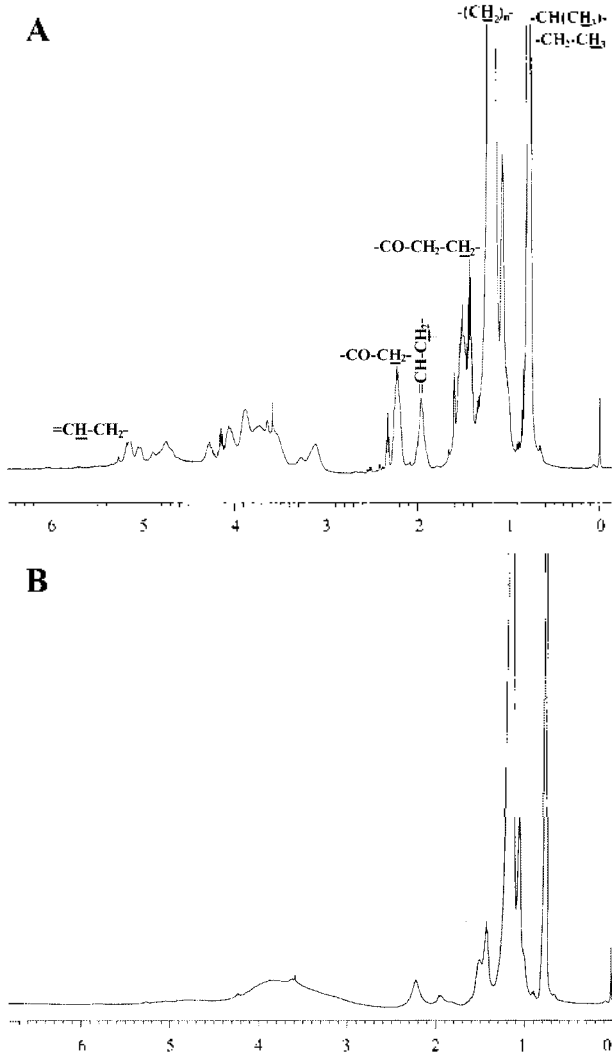

Figure 1. ^1H NMR spectra of the membrane lipids containing the DME C30. (A) Spectrum for the isolated total lipids. (B) Spectrum for the isolated lipids of which DME C30 is one of the fatty acyl components.

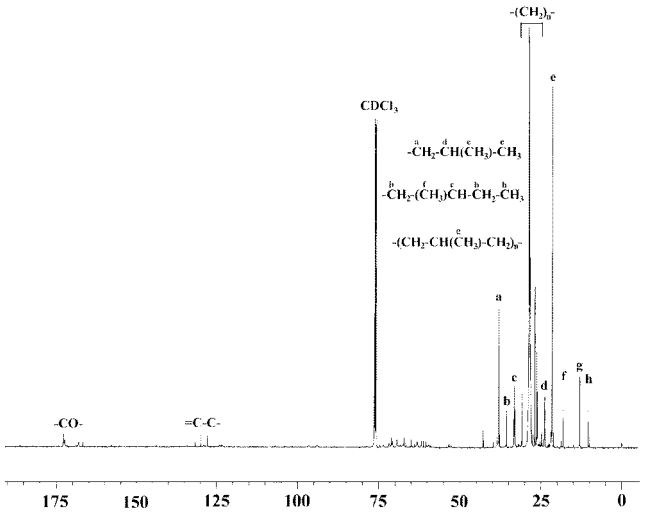

Figure 2. ^{13}C NMR spectrum of the isolated membrane total lipids.

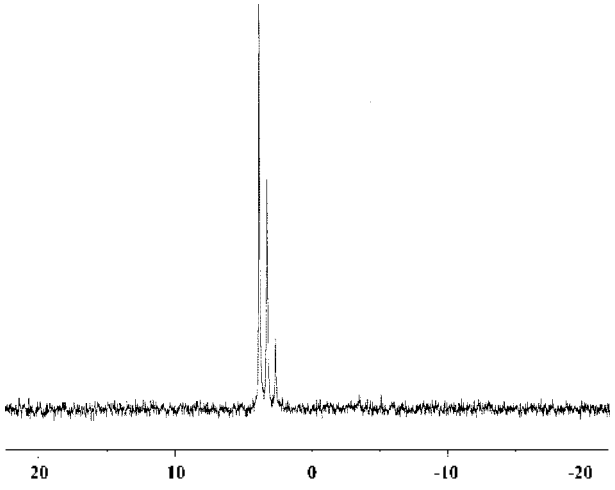

Figure 3. ^{31}P NMR specrum of the isolated membrane lipids containing the DME C30.

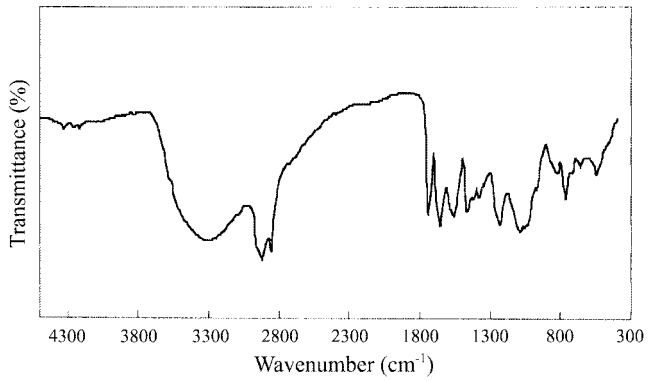

Figure 4. FTIR spectrum of the the isolated membrane lipids containing the DME C30.

δ 10.3, respectively. The peaks between δ 30.9 and δ 26.2 corresponded to the methylene groups of long chain acyl components. The signals between δ 131.5 and δ 124.0 also indicate the presence of glycerol and carbohydrate residues in the headgroups of the lipids. Spectrum for the more purified lipids also showed each resonance for the carbons observed in the total lipids (data not shown). ^{31}P NMR spectrum (Figure 3) showed the presence of phosphorus esters, indicating that the isolated lipids were composed of the mixture of phosphoglycolipids.

FTIR Analysis of the Lipids. The infrared spectrum (Figure 4) showed a strong aliphatic C-H asymmetric stretching absorption at 2921 cm^{-1} and symmetric stretching at 2850 cm^{-1}. The characteristic C=O absorption band of the aliphatic ester group appeared at 1739 cm^{-1}. The C=O overtone absorption at 4323 cm^{-1} was also observed. A strong asymmetric C-O-C stretching band appeared at 1088 cm^{-1}. Band due to P-O-C stretching was observed at 780 cm^{-1}. A broad OH strectching band (3600-3000 cm^{-1}) was also observed. The C-O stretching vibration in the alcohol produced a strong band at 1231 cm^{-1}. The FTIR data thus supported that the purified lipids were in the family of phosphoglycolipids as shown in the NMR spectra.

Analyses of Headgroups of the Lipids. The gas chromatographic profile of the alditol acetates was obtained from the hydrolysate of the lipid fractions by treatment with 2 M TFA (Figure 5). The three major components were observed. The presence of this alditol acetate was confirmed by electron impact (EI) mass spectrometry. The EI mass spectrum of peak A in Figure 5 is shown in Figure 6A. Typically, alditol acetate derivatives did not produce molecular ions. However, [M-CH_3CO_2]$^+$ ion at *m/z* 159 was found in low abundance by the elimination of an acetoxyl group, or by the cleavage of the alditol chains. The base ion peak at *m/z* 145 corresponded to [M-CH_2OCOCH_3]$^+$. The ion at *m/z* 103 was assigned to the loss of ketene [-CH_2CO] from *m/z* 145. These mass fragmentations clearly indicated the presence of glycerol triacetate. Figure 6B shows the EI mass spectrum of peak C in Figure 5. The major ions appeared at *m/z* 390, 374 and 360. These corresponded to [M-$COCH_3$]$^+$, [M-CH_3CO_2]$^-$ and [M-$CH_2CH_3CO_2$]$^+$, respectively. The ion at *m/z* 144 also suggests the typical mass fragmentation of glucosamine in glycolipids. In addition to the major ions, other primary fragments were formed from glucosamitol hexaacetates (Figure 6B). Peak B in Figure 5 was assigned to the glucosamitol pentaacetate that the carbon in position-1 remained aldehyde forms due to incompletely reduced glucosamine during the acetylation. These results were further confirmed by the analysis of the ^{1}H NMR (Figure 7A) and ^{13}C NMR

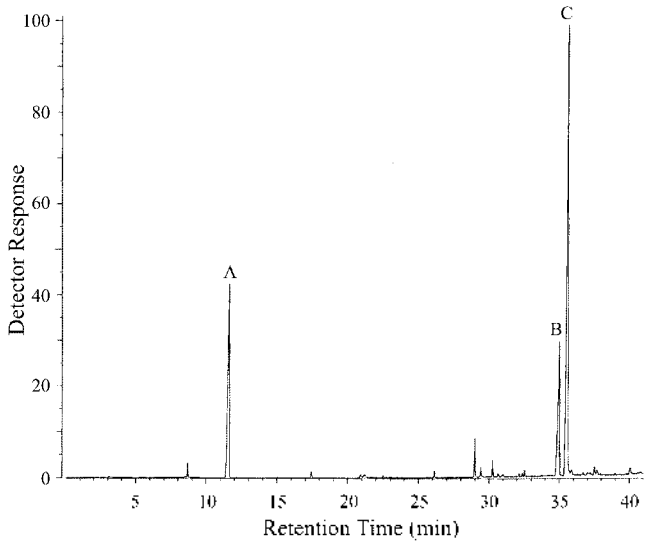

Figure 5. Gas chromatographic profile of the alditol acetates of the headgroup hydrolysates of the lipids. (A) Glycerol triacetate. (B) Glucosamitol pentaacetate. (C) Glucosamitol hexaacetate.

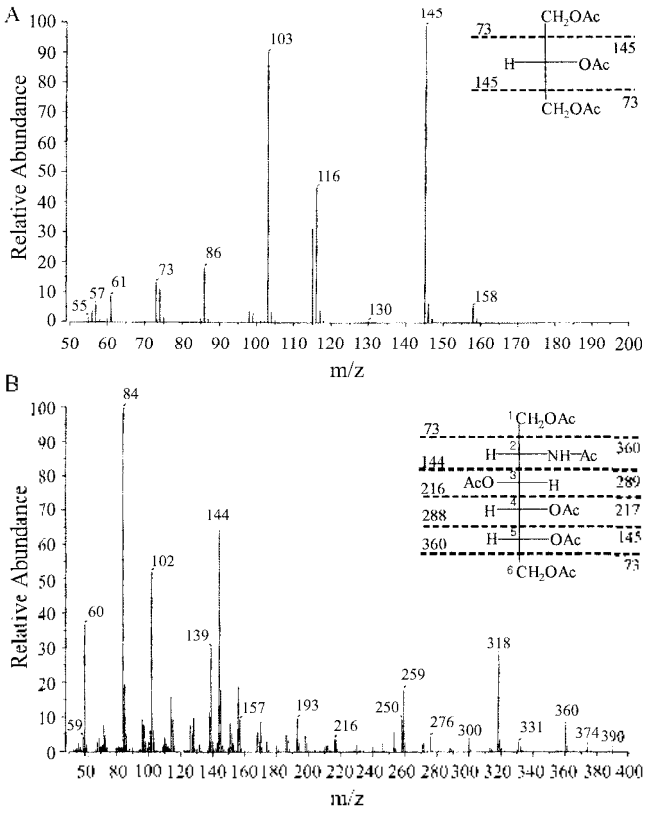

Figure 6. EI mass spectra of (A) Glycerol triacetate and (B) Glucosamitol hexaacetate.

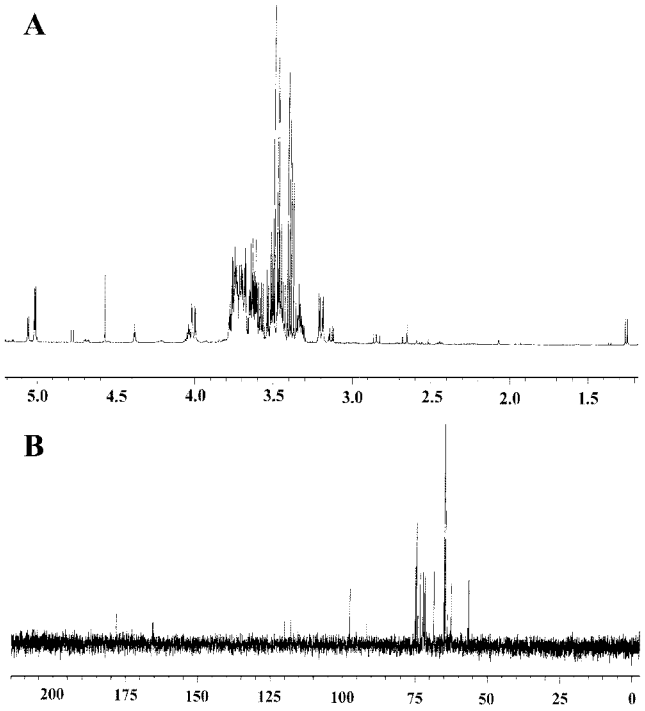

Figure 7. NMR spectroscopic analysis of the headgroup hydrolysates of the lipids. (A) ^{1}H NMR spectrum of the hydrolysates. (B) ^{13}C NMR spectrum of the hydrolysates.

spectra (Figure 7B) of the hydrolysates obtained by the TFA hydrolysis for the purified lipids. The anomeric mixtures of glucosamine were confirmed by the characteristic H-1 protons at δ 4.53 and δ 5.05. Characteristic signals of glycerol appeared at δ 3.51 and δ 3.41 (Figure 7A). The anomeric carbon (C-1) of glucosamine was observed at δ 97.5. Peaks at δ 55.5, δ 63.9, δ 64.7, δ 65.1, and δ 71.5 were also assigned to the carbons in position-2, -6, -4, -3, and -5, respectively (Figure 7B). Peaks at δ 68.5 and δ 62.6 were confirmed as typical glycerol carbons. Peak at δ 178.1 indicated the aldehyde carbon of the glucosamine molecule hydrolyzed by the TFA treatment. Through the ^{13}C NMR analysis, we clearly confirmed that a component of the head groups of the purified lipids was not *N*-acetylglucosamine but glucosamine, due to the absence of signal corresponding to -CH_3 of acetyl group. Combined with the GC/MS, ^{1}H and ^{13}C NMR data, we confirmed that glycerol and glucosamine were the components of the head groups of the purified lipids.

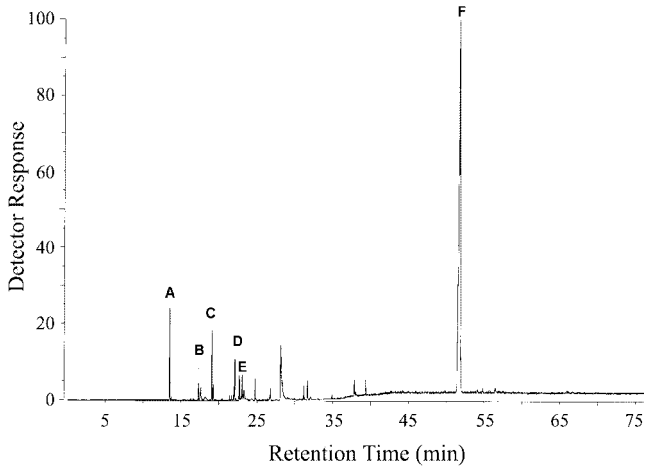

Figure 8. Gas chromatographic analysis of fatty acyl components in the purified lipids. Total fatty acids within the lipids were analyzed as the methyl ester derivatives after methanolysis. (A) $C_{15:0}$ carboxylic acid methyl ester. (B) $C_{16:0}$ carboxylic methyl ester. (C) $C_{17:0}$ carboxylic acid methyl ester. (D) $C_{18:1}$ carboxylic acid methyl ester. (E) $C_{18:0}$ carboxylic acid methyl ester. (F) $C_{30:0}$ dicarboxylic 13,16-dimethyl ester.

Compositional Analyses of Fatty Acyl Components of the Lipids. Total fatty acyl components in the purified lipids were analyzed as fatty acid methyl ester derivatives obtained after methanolysis with 5% methanolic-HCl. These fatty acid methyl esters were then subjected to gas chromatographic analysis. GC analysis of fatty acid methyl esters is shown in Figure 8. Exact structures and masses of the fatty acid methyl esters were determined by a GC/MS analysis as our previous report.[13] The GC profile showed that the peaks from A to E were the typical membrane fatty acyl components ranging from 15 to 18 carbon chains. Major regular fatty acids were *iso*-pentadecanoic acid (peak A, *iso*-$C_{15:0}$) and *iso*-heptadecanoic acid (peak C, *iso*-$C_{17:0}$). The later eluting peak was assigned to the DME C30. The relative proportion of the fatty acid methyl esters was also measured from the gas chromatogram (Table 1). The relative proportion of the unusual DME C30 was more than 75% relative to the typical fatty acyl components in the purified lipids.

FAB/MS Analysis of the Lipids. In this study, data obtained by FAB/MS provide the structural information of the purified transmembrane lipids. The negative FAB mass spectra of the purified lipids were obtained to determine the ranges of the molecular weights (Figure 9). The spectra showed a mixture of phosphoglycolipids. The general structure proposed could be also predicted from the mass data obtained in Figure 9. The molecular weight of the tail-to-tail joined lipid between the two diacyl glycerol (DAG) containing four *iso*-$C_{15:0}$ is 1076, which was observed at *m/z* 1058 as $[M\text{-}H_2O]^-$ (Figure 9A). The ion at *m/z* 1182 could be assigned to the pseudomolecular ion $[M\text{-}H]^-$ of the tail-to-tail joined lipid between the lyso-phosphatidylglycerol (PG) and PG containing two *iso*-$C_{15:0}$, and one *anteiso*-$C_{15:0}$ (Figure 9B). Figure 9C shows the proposed structures of the tail-to-tail coupled lipids between the *sn*-1-glyceryl-*O*-DAG (GDAG) and PG molecules, the phosphatidylglucosamine (PGA) and

Table 1. The composition of fatty acid methyl ester in the purified lipid

	Fatty acid methyl ester	Relative abundance (%)
A	$C_{15:0}$	4.81
B	$C_{16:0}$	4.41
C	$C_{17:0}$	6.95
D	$C_{18:1}$	5.96
E	$C_{18:0}$	2.73
F	$C_{30:0}$	75.14

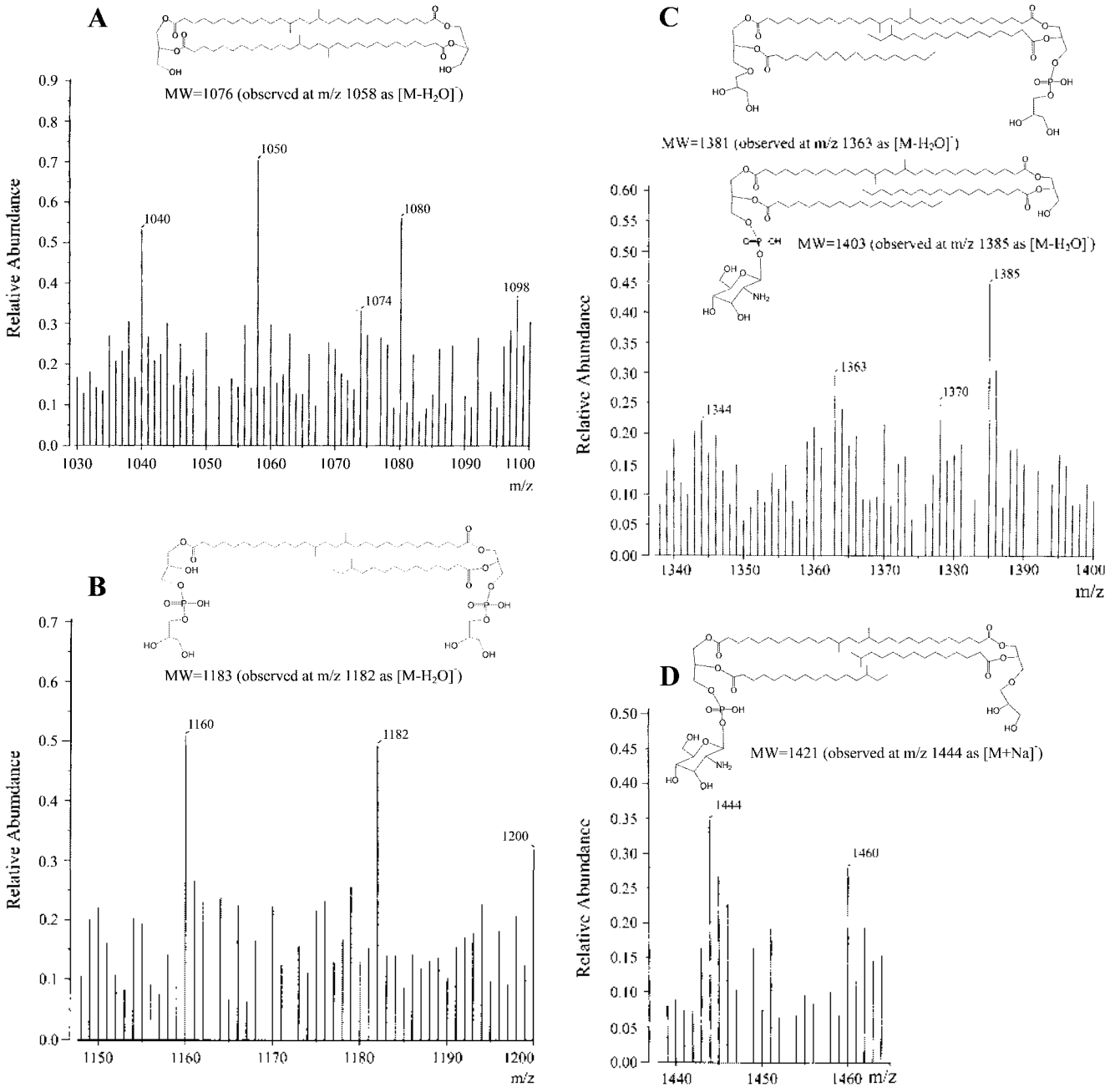

Figure 9. Proposed novel lipid structures and the negative ion FAB mass spectra of the lipids containing the DME C30 in *T. ethanolicus*. (A) Negative ion FAB mass spectrum and the structure of tail-to-tail coupled lipids of DAG molecules. (B) Negative ion FAB/MS spectrum and the structure of tail-to-tail coupled lipids of PG molecule and lyso-PG molecules. (C) Negative ion spectrum and the structures of tail-to-tail coupled lipids of GDAG/PG and PGA/DAG. (D) Negative ion spectrum and the structures tail-to-tail coupled lipids of PGA/GDAG.

DAG molecules. The FAB/MS spectrum obtained in the negative mode gave *m/z* 1363 and 1385 as the pseudomolecular ions $[M\text{-}H_2O]^-$, respectively. Additionally, the mass spectra also confirmed the tail-to-tail coupled lipids between the PGA and GDAG molecules containing three *iso*-$C_{15:0}$ and one *anteiso*-$C_{17:0}$ (Figure 9D). The ions at *m/z* 1444 were observed as $[M+Na]^-$.

Studies on the fatty acyl chains in the membrane lipids in *T. ethanolicus* has shown that the membrane lipids of the bacterium contain the α,ω-dicarboxylic fatty acids as components similar to those formed in *S. ventriculi*.[6,10,13] However, among the α,ω-dicarboxylic fatty acyl components, the DME C30 have been known to be only found in the membrane lipids of *T. ethanolicus*. In the case of *S. ventriculi*, the acidophilic bacterium has been known to contain the membrane lipids including MGDG, DAG and PG. In the bacterium, a mechanism has been also proposed by the hydrocarbon tail-to-tail coupling between the bilayer leaflets and the lipid molecules partially cross-linked between the head groups during the environmental adaptation.[1] On the contrary, *T. ethanolicus*, contains a mixture of phosphoglycolipids containing PG, DAG, GDAG and PGA in its

membrane components as we here described. In this study, we also confirmed that the carbohydrate moiety of the lipids were composed of the glucosamine molecule whereas the moiety as the glucose molecule in *S. ventriculi*. The unusual structure of the DME C30 in *T. ethanolicus* imply that the mechanism for the formation of the long chain fatty acid is due to the tail-to-tail ω coupling of the regular *iso*-branched fatty acids across opposite sides of the membrane to survive against the extreme environmental condition.[13] The synthesis of the DME C30 explains the result that the percentage of the transmembrane fatty acid is raised in response to increasing levels of ethanol as recently reported.[12] The couplings of lipid chains have a very high significance because it represents a new and unusual mechanism for maintaining membrane integrity during adaptation to extreme condition in extremophilic bacteria. This type of response explains the remarkable structural complexity and high dynamic fidelity of membrane systems.

Here, we reported the proposed molecular structures of the novel lipid components of which DME C30 is a major fatty acyl component in the membrane of *T. ethanolicus*. One of the proposed physiological roles of these novel membrane components may be the enhancement of membrane thermostability which would be endowed by the increased van der Waals interactions due to the presence of these characteristic, unusually very long chain fatty acyl components of the lipids. Further research will be continued in this viewpoint.

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