Structural elucidation of phosphoglycolipids from strains of the bacterial thermophiles *Thermus* and *Meiothermus*

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Abstract The structures of two major phosphoglycolipids from the thermophilic bacteria *Thermus oshimai* NTU-063, *Thermus thermophilus* NTU-077, *Meiothermus ruber* NTU-124, and *Meiothermus taiwanensis* NTU-220 were determined using spectroscopic and chemical analyses to be 2′-*O*-(1,2-diacyl-*sn*-glycero-3-phospho) -3′-*O*-(α-*N*-acetyl-glucosaminyl)-*N*-glyceroyl alkylamine [PGL1 (1)] and the novel structure 2′-*O*-(2-acylalkyldio-1-*O*-phospho)-3′-*O*-(α-*N*-acetylglucosaminyl)-*N*-glyceroyl alkylamine [PGL2 (2)]. PGL2 (2) is the first phosphoglycolipid identified with a 2-acylalkyldio-1-*O*-phosphate moiety. The fatty acids of the phosphoglycolipids are mainly *iso*-$C_{15:0}$, -$C_{16:0}$, and -$C_{17:0}$ and *anteiso*-$C_{15:0}$ and -$C_{17:0}$. The ratios of PGL2 (2) to PGL1 (1) are significantly altered when grown at different temperatures for three strains, *T. thermophilus* NTU-077, *M. ruber* NTU-124, and *M. taiwanensis* NTU-220, but not for *T. oshimai* NTU-063. Accordingly, the ratios of *iso*- to *anteiso*-branched fatty acids increase when grown at the higher temperature.—Yang, Y-L., F-L. Yang, S-C. Jao, M-Y. Chen, S-S. Tsay, W. Zou, and S-H. Wu. **Structural elucidation of phosphoglycolipids from strains of the bacterial thermophiles *Thermus* and *Meiothermus*. *J. Lipid Res.* 2006.** 47: **1823–1832.**

Supplementary key words nuclear magnetic resonance spectroscopy • matrix-assisted laser desorption ionization mass spectroscopy • capillary electrophoresis-mass spectroscopy • tandem mass spectrometry • *Thermus oshimai* NTU-063 • *Thermus thermophilus* NTU-077 • *Meiothermus ruber* NTU-124 • *Meiothermus taiwanensis* NTU-220

Bacteria have evolved interesting thermoadaptive mechanisms, including changes in their membranes (1). For example, when *Escherichia coli*, *Thermus aquaticus*, *Candida* species, thermophilic *Bacillus* species, and *Staphylococcus aureus* are grown at high temperatures, the proportion of branched-chain fatty acids increases and the proportions of monoenoic and heptanoic fatty acids decrease (2). Temperature affects both the chain length and the degree of saturation of fatty acid components in *Synechococcus* species (3, 4). Polar lipid fatty acids can be used as a biochemical marker because many of the lipids of thermophilic bacteria isolated from microbial mats in hot springs have unique diol, plasmalogen, monoether, and diether structures (5).

Thermus and *Meiothermus* species are Gram-negative thermophilic rods isolated from thermal hot springs, industrial and domestic water traps, and hydrothermal vents with neutral to alkaline pH (6). In these species, polar lipids occupy a large proportion of the cellular membrane fractions and usually include a major phospholipid, a major glycolipid, and minor phospholipids and glycolipids (7, 8). The glycolipids usually contain three hexoses, one *N*-hexosamine, and one glycerol (6, 9–13). The hydrophobic parts are predominantly *iso*- and *anteiso*-branched fatty acids; straight-chain fatty acids are minor components. The high proportion of glycolipids in the cell membranes could possibly contribute to the ability of the bacteria to grow at high temperatures, because the relative proportions of the major glycolipids increase concomitantly with the growth temperature (14, 15).

Thermus and *Meiothermus* species have been reported to have phosphoglycolipids (16), although the structures have not yet been elucidated. The highly radiation-resistant bacterium *Deinococcus radiodurans*, which is phylogenetically closely related to *Thermus* and *Meiothermus* (17), contains the phosphoglycolipids 2′-*O*-(1,2-diacyl-*sn*-glycero-3-phospho)-3′-*O*-(α-galactosyl)-*N*-glyceroyl alkylamine (18)

Manuscript received 20 January 2006 and in revised form 7 April 2006 and in re-revised form 3 May 2006.

Published, JLR Papers in Press, May 4, 2006.
DOI 10.1194/jlr.M600034-JLR200

Abbreviations: CE-MS, capillary electrophoresis-mass spectroscopy; DQF-COSY, double quantum filtered-correlation spectroscopy; FAME, fatty acid methyl ester; gHMQC, gradient heteronuclear multiple quantum coherence; HMBC, heteronuclear multiple bond coherence; HPAEC-PAD, high-performance anion-exchange chromatography with pulsed amperometric detection; MALDI, matrix-assisted laser desorption ionization; PGL1, 2′-*O*-(1,2-diacyl-*sn*-glycero-3-phospho)-3′-*O*-(α-*N*-acetyl-glucosaminyl)-*N*-glyceroyl alkylamine; PGL2, 2′-*O*-(2-acylalkyldio-1-*O*-phospho)-3′-*O*-(α-*N*-acetylglucosaminyl)-*N*-glyceroyl alkylamine; ROESY, rotational frame nuclear Overhauser effect spectroscopy; TOCSY, total correlation spectroscopy; 2D, two-dimensional.

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and 2′-*O*-(1,2-diacyl-*sn*-glycero-3-phospho)-3′-*O*-(α-*N*-acetylglucosaminyl)-*N*-glyceroyl alkylamine (19). Thermophilic and hyperthermophilic organisms contain phospho-sugar-related solutes, such as cyclic-2,3-bisphosphoglycerate (20), di-myo-inositol phosphate (21, 22), mannosylglycerate and mannosylglyceramide (23, 24), di-myo-inositol phosphate (21), diglycerol phosphate (1,1′-diglyceryl-phosphate) (1, 25), and galactosyl-5-hydroxylysine (26). These solutes might be adaptive features of these organisms to high temperatures (27) and might protect enzymes against heat inactivation (5, 28, 29).

The chemical structures of two major phosphoglycolipids isolated from four species of *Thermus/Meiothermus* are determined completely in this article. Furthermore, the variations of phosphoglycolipids and their fatty acids at different culture temperatures are also investigated to clarify their roles in physiological adaptation.

MATERIALS AND METHODS

NMR

The conditions used for NMR analysis of phosphoglycolipids were reported previously (4). NMR spectra of samples in $CDCl_3$/CD_3OD (99:1, v/v) were recorded on Bruker Avance 400 and 600 spectrometers (equipped with BBOZ gradient probes) at 300 K, with standard pulse sequences provided by Bruker, except for two-dimensional (2D) ^{1}H-^{31}P gradient heteronuclear multiple quantum coherence (gHMQC), which was modified from the pulse program hmqcgpqf with gradient strength calculated from the gyromagnetic ratios of ^{1}H and ^{31}P so that $G_1 = G_2 = (\gamma_H/2\gamma_P) \times G_3$. The gradient strength values for G_1, G_2, and G_3 were 30, 30, and 24.2% of the maximum gradient strength, respectively. One-dimensional and 2D total correlation spectroscopy (TOCSY) spectra were recorded with mixing times of 80–250 ms, which allows proton chemical shifts of carbohydrates to be assigned. 2D double quantum filtered-correlation spectroscopy (DQF-COSY) spectra were recorded in the analysis of carbohydrate coupling constants. 2D ^{1}H-^{13}C gradient heteronuclear multiple bond correlation spectra were recorded with ^{2}J or ^{3}JH-C coupling constants at 8 and 5 Hz, 2D ^{1}H-^{13}C gHMQC spectra were recorded with ^{1}JH-C coupling constants at 145 Hz, and 2D ^{1}H-^{31}P gHMQC spectra were recorded with ^{3}JH-P coupling constants at 10 Hz. 2D rotational frame nuclear Overhauser effect spectroscopy (ROESY) spectra were recorded with mixing times of 200 to 500 ms for conformation analysis.

GC-MS

GC-MS was carried out on a Hewlett-Packard HP6890 gas chromatograph connected to an HP5973 mass selective detector. An HP-5MS fused silica capillary column [30 m × 0.25 mm inner diameter (ID); Hewlett Packard] at 60°C was used. The fatty acid methyl ester (FAME) analysis program was 60°C for 1 min, 60–140°C at 25°C/min, 140–200°C at 5°C/min, and 200–300°C at 10°C/min.

Matrix-assisted laser desorption ionization mass spectroscopy

Purified phosphoglycolipids were dissolved in CH_3OH and analyzed on a matrix-assisted laser desorption ionization (MALDI)-time of flight mass spectrometer (Micromass, Manchester, UK) with a pulsed nitrogen laser (337 nm). The negative model was used. Cyano-4-hydroxycinnamic acid was used as the matrix.

Capillary electrophoresis-mass spectroscopy and tandem MS

A crystal model 310 capillary electrophoresis (CE) instrument (ATI Unicam, Boston, MA) was coupled to an API 3000 mass spectrometer (MDS/Sciex, Concord, Ontario, Canada) via a micro-ion-spray interface. A sheath solution (isopropanol-methanol, 2:1, v/v) was delivered at a flow rate of 1 μl/min to a low dead-volume tee (250 μm ID; Chromatographic Specialties, Brockville, Canada). All aqueous solutions were filtered through a 0.45 μm filter (Millipore, Bedford, MA) before use. An electrospray stainless-steel needle (27 gauge) butted against the low dead-volume tee enabled the delivery of the sheath solution to the end of the capillary column. Separation was obtained on ~90 cm of bare fused-silica capillary using 10 mM NH_4OAc/NH_4OH in deionized water, pH 9.0, containing 5% CH_3OH. A voltage of 20 kV was typically applied at the injection. The outlet of the capillary was tapered to ~15 μm ID using a laser puller (Sutter Instruments, Novato, CA). The negative model was used. Mass spectra were acquired with dwell times of 3.0 ms per step in 1 *m/z* increments in the full mass scan mode. For CE-electrospray-

TABLE 1. ^{1}H (400 MHz), ^{13}C (100 MHz), and ^{31}P (162 MHz) NMR data for **1** and **2**

Position	δ_H (J in Hz)	δ_C ($J_{C\text{-}P}$ in Hz)	δ_P
Fatty acid			
iso-CH_3	0.77, d (6.8)	22.6	
anteiso-CH_3	0.77, d (6.8)	11.3, 19.1	
normal-CH_3	0.75, m	13.9	
iso-CH	1.43, m	27.9	
anteiso-CH	1.24, m	34.4	
-$(CH_2)_n$-	1.12–1.25, m	28.7–29.6	
-CH_2CO-	2.21, q (7.6)	34.0, 34.2	
-CH_2CH_2CO-	1.50, m	24.8, 24.9	
-CO-		173.5, 173.8	
Alkylamine			
-NH-	7.45, brt		
-CH_2NH-	3.70, m	39.4	
-CH_2CH_2NH-	1.43, m	27.4	
Glycerate			
-OCH_2-	3.67, m	68.1	
-POCH-	4.64, m	75.3 (5.9)	
-CO-		169.4	
α-*N*-Acetylglucosamine			
1-CH	4.75, d (2.8)	97.2	
2-CH	3.80, ddd (11.0, 8.4, 2.8)	53.5	
3-CH	3.62, dd (11.0, 9.3)	71.7	
4-CH	3.39, t (9.3)	70.4	
5-CH	3.55, m	72.0	
6-CH_2	3.64, m	61.1	
-NH-	7.95, d (8.4)		
-$COCH_3$	1.95, s		
-CO-		173.1	
Glycerol			
-$POCH_2$-	3.89, m	63.8 (5.5)	
-CHO-	5.12, m	70.3 (7.9)	
-CH_2O-	4.03, dd (12.0, 6.8)	62.6	
	4.27, dd (12.0, 2.8)		
-$POCH_2$-			−1.80
1,2-Alkyldiol			
-$POCH_2$-	3.82, m	66.8 (5.2)	
-OCH-	4.93, m	72.8 (7.8)	
-$POCH_2$-			−1.71

1, 2′-*O*-(1,2-diacyl-*sn*-glycero-3-phospho)-3′-*O*-(α-*N*-acetyl-glucosaminyl)-*N*-glyceroyl alkylamine (PGL1); **2**, 2′-*O*-(2-acylalkyldio-1-*O*-phospho)-3′-*O*-(α-*N*-acetylglucosaminyl)-*N*-glyceroyl alkylamine (PGL2).

MS experiments, ~30 nL of sample was introduced by applying 300 mbar for 0.1 min. The tandem MS data were acquired with dwell times of 3.0 ms per step in 1 *m/z* increments. Fragment ions formed by collision activation of selected precursor ions with nitrogen in the RF-only quadrupole collision cell were analyzed by scanning the third quadrupole.

Chiral HPLC

Chiral HPLC was carried out using an Agilent 1100 HPLC apparatus and a Chiralpak WH column (0.46 × 25 cm; Daicel Chemical Industries, Osaka, Japan) at a flow rate of 1 ml/min with UV detection at 205 nm. The solvent was 0.25 mM $CuSO_4$ in water. L- and D-forms of glyceric acid standards were purchased from Fluka (Buchs, Switzerland).

High-performance anion-exchange chromatography with pulsed amperometric detection

High-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) was used to determine the carbohydrate composition. The phosphoglycolipid hydrolysates were analyzed on a DX-500 BioLC system (Dionex), which included a GP40 gradient pump, an ED40 electrochemical detector (PAD detection) with a working gold electrode, an LC30 column oven, and an AS3500 autosampler. A Dionex eluant degas module was used to purge and pressurize the eluants with helium. Monosaccharides were separated on a Carbopac PA10 analytical column (4 × 250 mm) with a Carbopac PA10 guard column (4 × 50 mm) at 30°C with a flow rate of 1 ml/min and detected by following pulse potentials and durations: E_1 = 0.05 V (0.4 ms), E_2 = 0.75 V (0.2 ms), and E_3 = −0.15 V (0.4 ms). The integration was recorded from 0.2 to 0.4 ms during the E_1 application. The glucosamine standard was purchased from Sigma.

Materials

Thermus oshimai NTU-063, *Thermus thermophilus* NTU-077, *Meiothermus ruber* NTU-124, and *Meiothermus taiwanensis* NTU-220 were obtained from hot springs in Taiwan (12, 13, 30). All biochemical tests and identification procedures followed previously described methods (6, 30). The voucher specimens (*Thermus* NTU-063, *Thermus* NTU-077, *Meiothermus* NTU-124, and *Meiothermus* NTU-220) were deposited at the Institute of Biological Chemistry, Academia Sinica (Taipei, Taiwan).

Isolation and purification of phosphoglycolipids

Thermus species were grown aerobically in *Thermus*-modified medium at 55, 65, and 75°C and *Meiothermus* species were incubated at 45, 55, and 65°C to the late exponential phase (optical density at 660 nm = 1.4), then harvested by centrifugation (6, 30). A 10-fold volume of absolute ethanol (RDH) relative to the bacterial wet weight was added, and the mixtures were shaken at room temperature for 2 h. After centrifugation, the supernatant was collected, concentrated, and chromatographed on a Sephadex LH20 column (Amersham Pharmacia) with $CHCl_3/CH_3OH$ (4:1, v/v) to separate phosphoglycolipids from pigments and other small molecules. The phosphoglycolipids were then chromatographed on a silica G-60 column (Merck, Darmstadt, Germany) with a $CHCl_3/CH_3OH$ gradient (5:1–0:1, v/v). The carbohydrate-containing fractions were detected by high-performance TLC stained with molybdate solution [0.02 M ammonium cerium sulfate dehydrate/ammonium molybdate tetrahydrate in aqueous 10% (w/v) H_2SO_4]. The phosphoglycolipid fractions were detected by ^{31}P-NMR. Their purities were confirmed by high-performance TLC with $CHCl_3/CH_3OH$ at 3:1.

Chemical modification

Methanolysis. The fatty acid composition was determined by comparing the GC-MS retention times of FAMEs of the phosphoglycolipids with those of standards. FAMEs were prepared by incubating phosphoglycolipids with 0.5 M CH_3OH-HCl at 80°C for 1 h. The solvent was removed under vacuum, and the residue was mixed with $CHCl_3$ and water. FAMEs partitioned into the organic phase.

Acidic hydrolysis. Phosphoglycolipids were acid-hydrolyzed in 5 M HCl at 110°C for 20 h. The hydrolysis product was neutralized (pH 7) with 2 N NaOH and then extracted with $CHCl_3$. The aqueous extract was used to determine the absolute configuration of glyceric acid by chiral HPLC. The released monosaccharides were analyzed by HPAEC-PAD.

Basic hydrolysis. Phosphoglycolipids dissolved in CH_3OH were incubated with 0.2 N aqueous KOH for 20 h. The final product was extracted with $CHCl_3$. The organic extract was applied to a silica gel column and eluted with $CHCl_3$ to obtain 3-*O*-α-*N*-acetylglucosaminyl-*N*-glyceroyl alkylamine (**3**).

TABLE 2. Fatty acid compositions of phosphoglycolipids from four species of *Thermus* and *Meiothermus*

	T. oshimai NTU-063			*T. thermophilus* NTU-077			*M. ruber* NTU-124			*M. taiwanensis* NTU-220	
	55°C	65°C	75°C	55°C	65°C	75°C	45°C	55°C	65°C	45°C	55°C
iso-15:0	17.17	38.86	29.61	3.39	5.41	5.22	—	31.28	2.54	25.38	27.23
anteiso-15:0	5.24	8.13	3.06	3.36	3.06	—	—	7.09	—	10.22	4.16
normal-15:0	0.60	0.52	—	—	—	—	—	1.20	—	1.20	1.65
iso-16:0	6.72	9.68	11.92	20.79	13.43	13.70	7.43	10.35	7.81	11.52	9.79
normal-16:0	—	—	—	—	—	—	3.06	1.81	1.86	2.66	1.39
iso-17:0	50.02	33.87	50.65	32.74	45.00	59.35	47.13	28.29	61.53	25.76	34.07
anteiso-17:0	14.56	6.30	4.46	29.64	29.34	21.73	18.61	10.99	13.49	11.40	10.28
normal-17:0	1.68	—	—	—	—	—	10.25	2.71	5.39	2.93	4.49
iso-18:0	1.36	0.53	—	8.11	3.76	—	7.43	1.85	4.75	1.91	2.31
anteiso-18:0	—	—	—	—	—	—	1.09	—	—	—	—
normal-18:0	—	—	—	—	—	—	2.10	0.70	—	0.54	—
iso-19:0	—	—	—	0.37	—	—	1.54	1.19	2.64	0.45	1.53
anteiso-19:0	—	—	—	—	—	—	—	0.46	—	—	0.96
Others	2.87	2.11	—	0.77	—	—	1.37	2.10	—	6.03	2.15
Ratio *iso-anteiso*	3.80	5.75	12.26	1.98	2.09	3.60	3.22	3.94	5.88	3.01	4.87
Average acyl chain carbon number	16.47	15.94	16.23	16.74	16.73	16.76	17.03	16.13	16.95	16.10	16.29

Values shown are percentages. —, trace or not detectable.

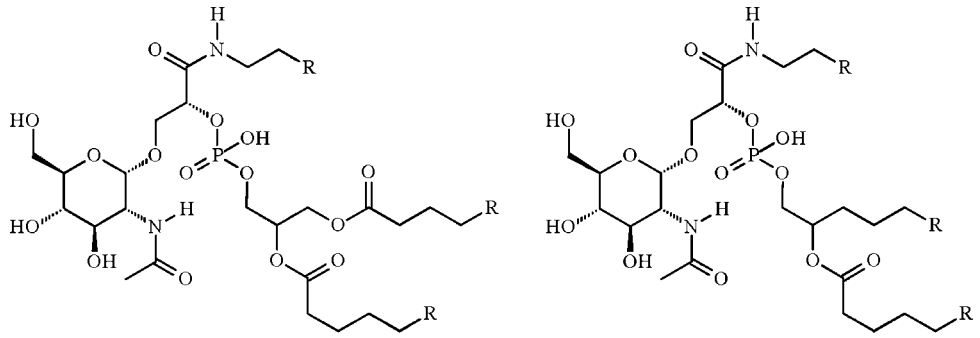


Fig. 1. Phosphoglycolipids 2′-*O*-(1,2-diacyl-*sn*-glycero-3-phospho)-3′-*O*-(α-*N*-acetyl-glucosaminyl)-*N*-glyceroyl alkylamine [PGL1 (**1**)] and 2′-*O*-(2-acylalkyldio-1-*O*-phospho)-3′-*O*-(α-*N*-acetylglucosaminyl)-*N*-glyceroyl alkylamine [PGL2 (**2**)] from strains of *Thermus* and *Meiothermus*. *R* = alkyl chain.

Saponification. Phosphoglycolipids were incubated with 0.2 N sodium methoxide in anhydrous CH_3OH for 2 h. The final product was extracted with $CHCl_3$. The organic extract was applied to a silica gel column and eluted with $CHCl_3/CH_3OH$ (5:1, v/v) to obtain PGL2a (**4**).

RESULTS

To investigate the variation of different phosphoglycolipids among *Thermus/Meiothermus* species as grown at different temperatures, each bacterial strain was individually grown at three different temperatures, varied up and down 10°C of their optimal growth temperatures (for *Thermus* species, 65 ± 10°C; for *Meiothermus* species, 55 ± 10°C). The polar lipid fractions were isolated and purified by ethanol extraction, gel filtration, and silica gel chromatography. Their purities were confirmed by high-performance TLC with $CHCl_3/CH_3OH$ at 3:1. ^{31}P-NMR analysis was also a useful tool to distinguish phosphoglycolipids from other bacterial polar lipids.

^{1}H-NMR of phosphoglycolipids revealed signals of the fatty acids in the upfield area (δ 0–3 ppm; **Table 1**). The *iso*- or *anteiso*-branched fatty acids were identified by the doublet methyl group signals at δ 0.70 and multiplet signals at δ 1.43. Detailed fatty acid compositions of phosphoglycolipids were obtained by GC-MS analysis of FAMEs. On the basis of data shown in **Table 2**, *iso*- and *anteiso*-branched fatty acids were predominant in the composition of fatty acids. Moreover, the ratios of *iso*- to *anteiso*-branched fatty acids increased with higher culture temperatures. The straight-chain fatty acids represented a minor constituent of fatty acids in the *Meiothermus* strains and <3% in the *Thermus* strains. The major fatty acids of phosphoglycolipids in *T. oshimai* NTU-063 and *M. taiwanensis* NTU-220 were *iso*-$C_{15:0}$, *iso*-$C_{17:0}$, *anteiso*-$C_{15:0}$, and *anteiso*-$C_{17:0}$. However, *T. thermophilus* NTU-077 phosphoglycolipids had a different variation, with a relatively lower ratio of *iso*-$C_{15:0}$ but a higher ratio of *iso*-$C_{16:0}$ compared with other strains. Interestingly, both *iso*-$C_{17:0}$ and *anteiso*-$C_{17:0}$ were the major components at all culture temperatures and constituted 30–60% and 20–30% of fatty acids independently, which was different from other strains. This phenomenon was also found in *M. ruber* NTU-124 grown at 45°C and 65°C. Only here, *iso*-$C_{15:0}$ and *iso*-$C_{17:0}$ were the major components of fatty acids at 55°C culture temperature.

The NMR spectra also indicated the presence of other chemical groups in addition to the fatty acids in the

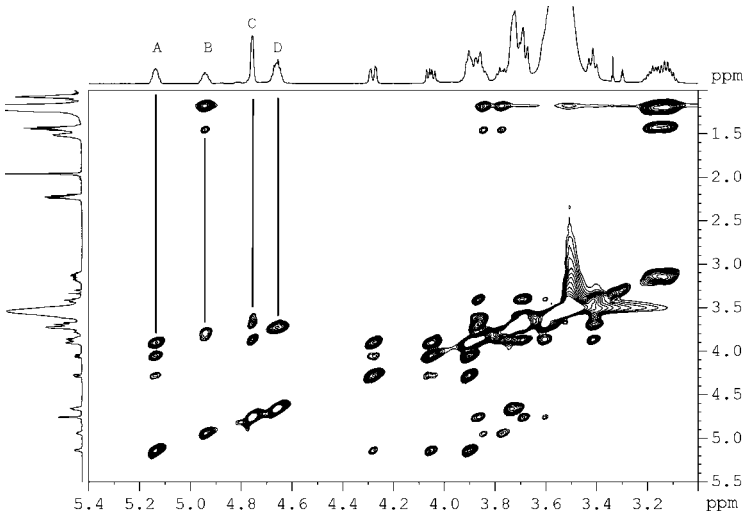


Fig. 2. Two-dimensional (2D) total correlation spectroscopy (TOCSY) spectrum of phosphoglycolipids. The correlations were assigned as follows: A, glycerol; B, 1,2-alkyldiol; C, α-*N*-acetylglucosamine; D, glycerate.

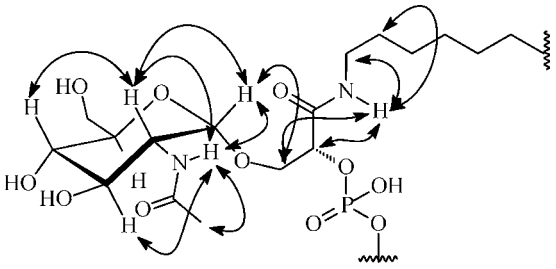


Fig. 3. Nuclear Overhauser effect correlations of the phosphoglycolipids.

phosphoglycolipids: one *N*-acetyl methyl group, one anomeric proton of an α-monosaccharide, one aminomethylene group, two amide protons, five oxymethylene groups, and seven oxymethine groups (Table 1). The results of further spectral analyses, reported below, led to the identification of two phosphoglycolipids, referred to as 2′-*O*-(1,2-diacyl-*sn*-glycero-3-phospho)-3′-*O*-(α-*N*-acetylglucosaminyl)-*N*-glyceroyl alkylamine [PGL1 (**1**)] and 2′-*O*-(2-acylalkyldio-1-*O*-phospho)-3′-*O*-(α-*N*-acetylglucosaminyl)-*N*-glyceroyl alkylamine [PGL2 (**2**)] (**Fig. 1**).

In the one-dimensional and 2D TOCSY spectra, one set of glycerol signals and one set of monosaccharide signals were detected (**Fig. 2**). The coupling constants revealed by the DQF-COSY spectra, the nuclear Overhauser effect correlations in ROESY spectra (**Fig. 3**), the ^{13}C-NMR results, and HPAEC-PAD analysis indicated that the monosaccharide is an α-*N*-acetylglucosamine (α-GlcNAc). In the 2D TOCSY spectra, the moiety -O-CH_2CH-O- was determined. In heteronuclear multiple bond coherence (HMBC) spectra (**Fig. 4**), one amide carbonyl carbon showed ^2J and ^3J correlations with oxymethine and oxymethylene, respectively, of this moiety. This amide carbonyl carbon also provided ^2J and ^3J correlations with the amide proton and the aminomethylene, respectively. These results indicated that a glycerate is connected to an alkylamine. The ^3J correlations between oxymethylene of glycerate and the anomeric proton of α-GlcNAc provided evidence that glycerate and α-GlcNAc were linked together by glycosidic linkage. On the other hand, the multiplet proton signal and the doublet carbon signal (J = 5.9 Hz) of oxymethine in glycerate suggested that oxymethine might be attached to the phosphate group. The complete assignment was also obtained by correlations in ^{1}H-^{31}P HMQC (**Figs.** 4, **5**).

Phosphoglycolipids were hydrolyzed with 0.2 N KOH in water to remove the phosphate group. The organic extract of the basic hydrolytic product was purified and shown to be 3-*O*-α-*N*-acetylglucosaminyl-*N*-glyceroyl alkylamine (**3**), as determined by NMR. This identification was in keeping with the assignments mentioned above. MALDI mass analysis of 3-*O*-α-*N*-acetylglucosaminyl-*N*-glyceroyl alkylamine (**3**) (*m/z* 1118, 1132, 1146, 1160, 1174, 1188, 2[M − 1]$^{2-}$) revealed the alkylamine to be C_{17}–C_{22}, with C_{19} as the major component.

Glycerate was determined to be in the D-configuration by chiral HPLC analysis of the acid hydrolysis product of the phosphoglycolipids and comparison with the L- and D-forms of glyceric acid standards.

Two sets of -O-CH_2CH-O- moieties of the ^{1}H- and ^{13}C-NMR split signals suggest the presence of a phosphate group in the phosphoglycolipids. One of the -O-CH_2CH-O- moieties is glycerol. The other moiety is 1,2-alkyldiol, as shown by lipid signals from TOCSY correlations (Fig. 2). The ^2J and ^3J ^{13}C-^{31}P coupling constants (Table 1) and the ^{1}H-^{31}P HMQC spectra (Fig. 5) allowed the assignment of two oxymethylene groups of these moieties, each connected to two phosphate groups. Both phosphate groups show correlations with oxymethine groups of glycerate. Glycerol and 1,2-alkyldiol are linked with fatty acids by ester bonds, as shown by long-rang correlations in the

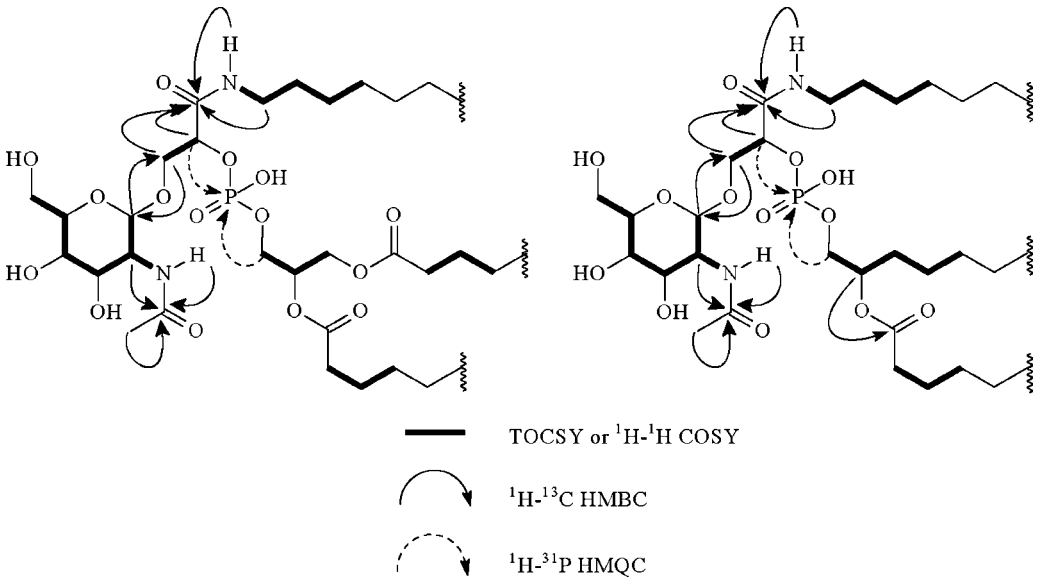


Fig. 4. Major 2D NMR correlations of **1** and **2**. COSY, correlation spectroscopy; HMQC, heteronuclear multiple quantum coherence.

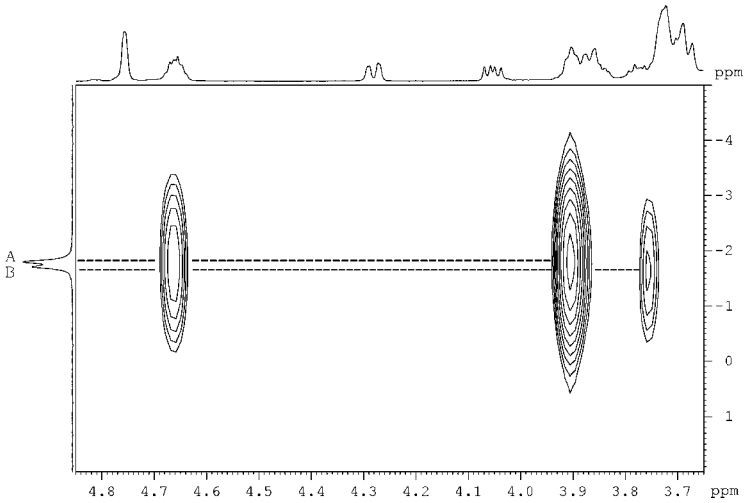
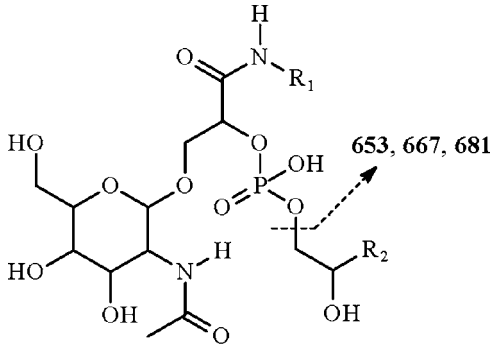


Fig. 5. ^{1}H-^{31}P HMQC spectrum of the phosphoglycolipids. A, correlation between the phosphate group and oxymethylene of glycerol in **1**; B, correlation between the phosphate group and oxymethylene of 1,2-alkyldiol in **2**. Both phosphate groups show correlations with oxymethine groups of glycerate in **1** and **2**.

HMBC spectra (Fig. 4). The results described above provided evidence for two phosphoglycolipids: PGL1 (**1**), which contains glycerol, and PGL2 (**2**), which contains 1,2-alkydiol (Fig. 1).

The remaining part of **2** was characterized by analyzing the saponification product, referred to as PGL2a (**4**) (**Fig. 6**). In the MALDI mass spectrum of **4**, a pseudomolecular ion peak at *m/z* 893 $[M - 1]^-$ and minor peaks with an *m/z* difference of 14 were observed. Together with fragment peaks at *m/z* 653, 667, and 681, the carbon numbers of alkylamine and 1,2-alkydiol were determined to be C_{18}–C_{20} and C_{13}–C_{16}, respectively.

The two major peaks at *m/z* 1176 and 1118 and the minor peaks with an *m/z* difference of 14 in the MALDI mass spectrum (**Fig. 7**) indicated that the aliphatic chains on glycerol, 1,2-alkyldiol, and alkylamine were of different lengths. The peaks at *m/z* 1176 and 1118 in the CE-electrospray tandem mass spectroscopy spectra displayed reasonable fragmentations (**Figs. 8, 9**) of **1** and **2**, respectively. The major fragmentations (**Table 3**) indicated that **1** and **2** have similar skeletons except for the lipid moieties. The FA ion at *m/z* 241 together with the *y* ion suggested that the fatty acid moieties and alkylamines of both phosphoglycolipids are $C_{15:0}$ and $C_{19:0}$, respectively. The other fragmentations, such as *m/z* 905 $[a - f - H]^+$ and 386 $[y - d + H]^+$ of **1** and *m/z* 875 $[M - FA - H_2O]^+$



$R_1 = C_{18}H_{37}, C_{19}H_{39}, C_{20}H_{41}$
$R_2 = C_{11}H_{23}, C_{12}H_{25}, C_{13}H_{27}, C_{14}H_{29}$

Fig. 6. Matrix-assisted laser desorption ionization (MALDI) mass fragments of PGL2a (**4**).

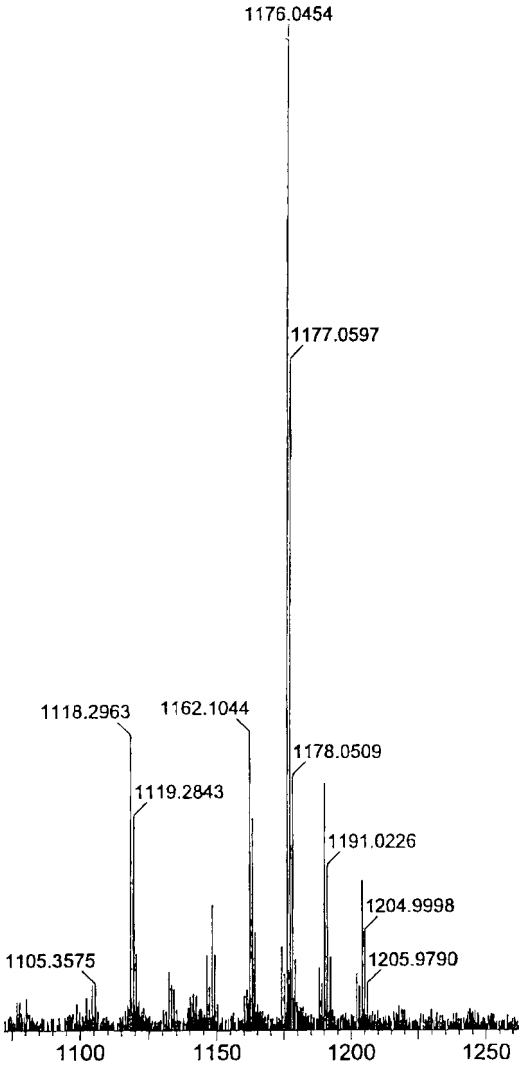


Fig. 7. MALDI mass spectrum of the phosphoglycolipids.

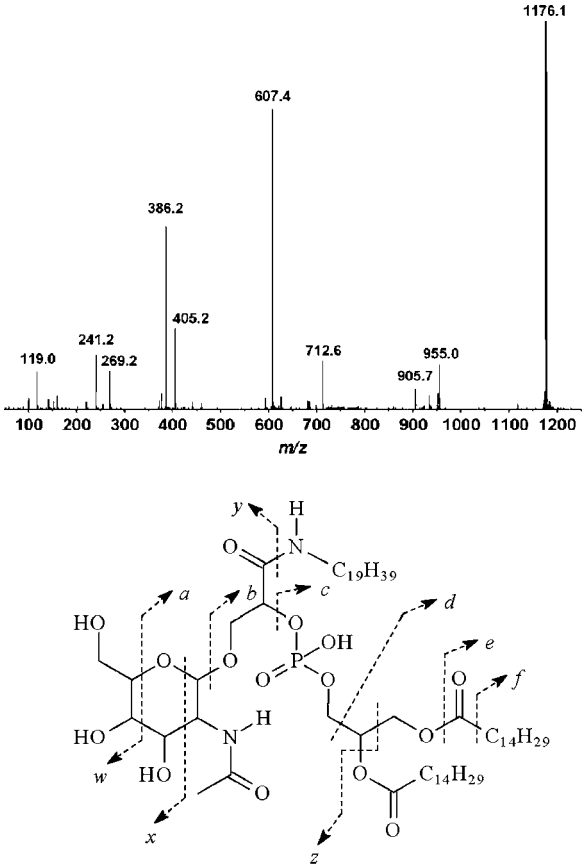


Fig. 8. Tandem MS spectrum and fragment assignments of **1** (precursor ion *m/z* 1176). All of the fragments are listed and interpreted in Table 3.

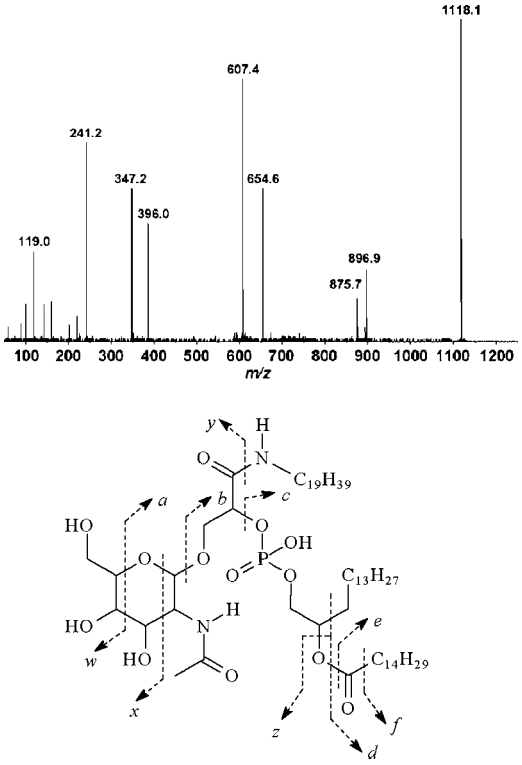


Fig. 9. Tandem MS spectrum and fragment assignments of **2** (precursor ion *m/z* 1118). All of the fragments are listed and interpreted in Table 3.

and 396 $[y - d - H_2O]^+$ of **2**, are also in agreement with the structural assignments mentioned above.

The integration of oxymethine groups of 1,2-alkyldiol in **2** and of glycerol in **1** in NMR analysis could provide us the quantitative information of a **2**:**1** ratio. As shown in **Figs. 10, 11**, at higher culture temperatures, the **2**:**1** ratio was increased significantly, except in *T. oshimai* NTU-063. For *T. oshimai* NTU-063, the **2**:**1** ratio was always maintained at 1:10–2:10 at various culture temperatures (Fig. 10, left). However, in the case of *T. thermophilus* NTU-077, **2** was greater than **1** as grown at 75°C (Fig. 10, right).

DISCUSSION

The structures of the glycolipids of *Thermus* and *Meiothermus* species have been well studied, but the phosphoglycolipids, the other important component of the polar lipids, have not been investigated. This report describes the first detailed structures of the major phosphoglycolipids from four thermophilic species of *Thermus* and *Meiothermus* isolated in Taiwan: *T. oshimai* NTU-063, *T. thermophilus* NTU-077, *M. ruber* NTU-124, and *M. taiwanensis* NTU-220.

Spectroscopic and chemical analyses revealed two novel structures. **1** is similar to a phosphoglycolipid from *D. radiodurans* (19) except for the fatty acid composition. The fatty acids of the phosphoglycolipids of *D. radiodurans* are mostly straight-chained, and C_{16} fatty acids constitute nearly 50% of the total fatty acids (19). In contrast, the fatty acids of the phosphoglycolipids from the *Thermus* and *Meiothermus* species were mainly *iso*-branched $C_{15:0}$, $C_{16:0}$, and $C_{17:0}$ and *anteiso*-branched $C_{15:0}$ and $C_{17:0}$ fatty acids (Table 2). The lack of hydroxy fatty acids in all of the strains examined confirms the results of an earlier study on strains of *Thermus* by Donato, Seleiro, and da Costa (10). The 16S rRNA gene sequences and some distinctive protein signatures provide an index for grouping the genera *Deinococcus*, *Thermus*, and *Meiothermus* together in

TABLE 3. Major fragment ions of phosphoglycolipids PGL1 (**1**) and PGL2 (**2**) in capillary electrophoresis-tandem mass spectroscopy spectra

Fragmentations	Fragment Ions (*m/z*)	
	PGL1 (**1**)	PGL2 (**2**)
$b - H_2O$	955	897
$a - f - H$	905	—
$M - FA - H_2O$	—	875
$b - e - 2H_2O$	712	654
$z - w$	607	607
$c - f - H_2O$	405	347
$y - d + H$	386	—
$y - d - H_2O$	—	396
FA	241	241
$x - 1$	119	119

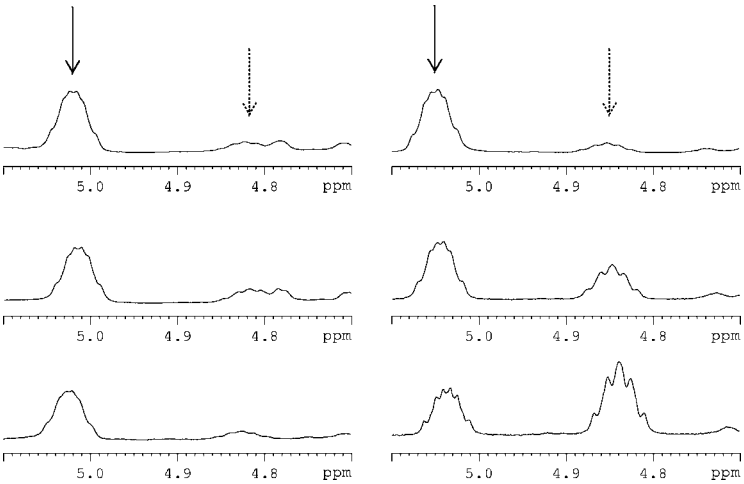


Fig. 10. ^{1}H-NMR spectra of phosphoglycolipids from *T. oshimai* NTU-063 (left) and *T. thermophilus* NTU-077 (right) incubated at 55, 65, and 75°C (from top to bottom). Arrows indicate the oxymethine group of PGL1 (**1**) glycerol, and dashed arrows indicate the oxymethine group of PGL2 (**2**) 1,2-alkyldiol.

one phylum (17, 31, 32). The results show that **1** could be a chemical marker suitable for the taxonomic differentiation of these three genera.

The other identified PGL, **2**, has a novel phosphoglycolipid structure linked with a long-chain diol. The long-chain diols were identified from *Thermomicrobium roseum* (33), *Chloroflexus aurantiacus* (32), *Roseiflexus castenholzii* (34), and some *Thermus* strains (35). According to the previous report, *T. oshimai* SPS-11 had only a minor amount of long-chain diol-linked glycolipid, whereas the glycerol-linked glycolipid was the major component (35). In this study, among the four *Thermus/Meiothermus* strains, *T. oshimai* NTU-063 is the only one to keep the amount of **2** in a constant and relatively low proportion at various culture temperatures. Probably the long-chain diol biosynthesis in *T. oshimai* is not affected by culture temperature. Based on the NMR analysis, in the three other strains, the quantitative **2**:**1** ratio increased significantly at higher culture temperatures. It was suggested in the previous report that long-chain 1,2-diols might not have the major role in thermal adaptation but as a phylogenetic component between *Thermus* and *T. roseum* (35). However, that report did not mention the quantitative ratio between long-chain diol-linked and glycerol-linked glycolipids from *Thermus* strains grown at various temperatures. Many unusual lipids, such as diphytanyl glycerol diethers, dibiphytanyl diglycerol tetraethers, and internal cyclization in dibiphytanyl diglycerol tetraether fatty acids, have been noted for their membrane stabilization in the some archaea and thermophilic bacteria (36). This suggests that 2-acylalkyldio-1-*O*-phosphate would form a more stable structure than 1,2-diacyl-*sn*-glycero-3-phosphate for bacteria to adapt at higher temperatures. Furthermore, **2** also could be used as a chemical marker for the identification of *Thermus* and *Meiothermus* species.

For survival in harsh environments, microorganisms should develop their membrane structures to adapt to the surroundings and ensure the constant stability and permeability of nutritional flow. In thermophilic bacteria and archaea, this adaptation is achieved by adjusting the chemical composition of lipids, such as the average acyl chain carbon number, the degree of saturation, and the ratio of *iso*- to *anteiso*-branched fatty acids. However, in our case, there was no significant difference in the average acyl chain carbon numbers of phosphoglycolipids among *Thermus/Meiothermus* species (Table 2). In our previous and present studies, the major fatty acids of glycolipids and phosphoglycolipids of *Thermus/Meiothermus* were *iso*-branched $C_{15:0}$, $C_{16:0}$, and $C_{17:0}$ and *anteiso*-branched $C_{15:0}$ and $C_{17:0}$. This indirectly indicated that the biosynthesis of fatty acids

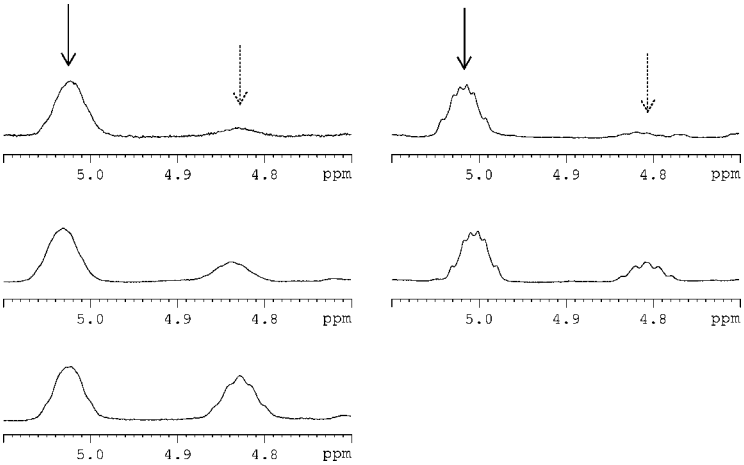


Fig. 11. ^{1}H-NMR spectra of phosphoglycolipids from *M. ruber* NTU-124 (left; incubated at 45, 55, and 65°C, from top to bottom) and *M. taiwanensis* NTU-220 (right; incubated at 45°C and 55°C, from top to bottom). Arrows indicate the oxymethine group of PGL1 (**1**) glycerol, and dashed arrows indicate the oxymethine group of PGL2 (**2**) 1,2-alkyldiol.

in both lipids might be correlated. Otherwise, 2- and 3-hydroxyacyl groups linked on hexosamine of *Meiothermus* or *Thermus* glycolipids showed their differences in biosynthesis (37). In phosphoglycolipids, the ratios of *iso*- to *anteiso*-branched fatty acids increased significantly with higher temperatures. A similar result has been reported for *Bacillus subtilis* (38). The membrane lipids provide the appropriate fluidity by the appropriate fatty acid composition at a given growth temperature. Consequently, the fluidity of membrane lipids is strongly related to the average melting points and phase transition temperatures of their respective fatty acids. In our study, the ratio of *iso*- to *anteiso*-branched fatty acids increased when temperature was increased, mainly as a result of the *iso*-branched fatty acids possessing relatively higher melting points and phase transition temperatures.

One of the possible biological roles of phosphoglycolipids in thermophilic bacteria is to ensure the thermal stability of the cellular membrane. Bulky head groups would enhance steric protection (39), possibly by stabilizing the membrane against environmental stress (e.g., osmotic stress and temperature changes) through hydrogen bonding via glycosyl head groups (40). This study identified bulky head groups in the phosphoglycolipids of *Thermus* and *Meiothermus* strains. The compatible solute diglycerol phosphate protects proteins of *Desulfovibrio gigas* and *Clostridium pasteurianum*; for example, the half-life of rubredoxin is increased 4-fold (1). In *Acholeplasma laidlawii*, the amount of phosphatidylglycerol, the only phosphatide present, relative to the amount of neutral glycolipids reflects the relative balance of uncharged and charged lipids in the membrane (41).

This study is the first to determine the full structure of the phosphoglycolipids in thermophilic *Thermus* and *Meiothermus* strains. This information and the labeling of the lipid precursors with [^{14}C]acetate will be very useful in further investigations of the mechanisms of phosphoglycolipid biosynthesis in vivo and the determination of the physiological roles of the phosphoglycolipids.

The work was supported in part by the National Science Council of Taiwan.

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