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# Determination of the Structure of a Novel Glycolipid from *Thermus aquaticus* 15004 and Demonstration that Hydroxy Fatty Acids Are Amide Linked to Glycolipids in *Thermus* spp.

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**The compositions of the major glycolipids (GL-1) of five strains of *Thermus aquaticus*, the type strain of *T. filiformis*, *T. oshimai* SPS-11, and *Thermus* sp. strain CG-2 were examined by gas chromatography, gas chromatography-mass spectroscopy, fast atom bombardment-mass spectroscopy, and chemical methods. The results showed that, with the exception of *T. aquaticus* 15004, the organisms each have a major glycolipid whose structure was established as diglycosyl-(*N*-acyl)glycosaminyl-glycosyl diacylglycerol. Glucosamine was present in GL-1 of *T. oshimai* SPS-11 and *Thermus* sp. strain CG-2, while galactosamine was present in the GL-1 of *T. aquaticus* and *T. filiformis*. The novel major glycolipid of *T. aquaticus* 15004 was identified as galactofuranosyl-(*N*-acetyl)galactosaminyl-(*N*-acyl)galactosaminyl-glucosyl diacylglycerol. The hydroxy fatty acids found in the *T. aquaticus* strains and in the type strain of *T. filiformis* were exclusively amide linked to the galactosamine of the major glycolipid. Ester-linked hydroxy fatty acids were not detected in the diacylglycerol moiety of GL-1 of these organisms. Hydroxy fatty acids were detected neither in the major glycolipid of *T. oshimai* SPS-11 and *Thermus* sp. strain CG-2, in which glucosamine is present, nor in the major phospholipid of any of the strains examined.**

Strains of the thermophilic eubacterial genus *Thermus*, with an optimum growth temperature of about 70°C, including *T. aquaticus*, *T. filiformis*, *T. thermophilus*, *T. scotoductus*, *T. oshimai*, and other undescribed species of this genus, have polar lipids composed almost exclusively of a major phospholipid, designated PL-2, constituting between 77 and 93% of the polar lipid phosphorus, and a major glycolipid, designated GL-1, constituting between 70 and 96% of the polar lipid carbohydrate. A minor phospholipid (PL-1) and a minor glycolipid (GL-2) are also found in all strains examined (8, 21, 22, 24).

The major glycolipid of the *Thermus* spp. examined contains, depending on the strain, three glucose, two glucose and one galactose, or one glucose and two galactose residues and an N-acylated glucosamine or galactosamine (21, 22, 24). The major glycolipid from *T. thermophilus* HB-8 has been investigated in detail and contains a subterminal galactopyranose and a terminal galactofuranose. The innermost hexose bound to the diacylglycerol is glucose, and glucosamine is amide linked to iso-17:0 (21). The structure of this glycolipid was established as digalactosyl-(*N*-acyl)glucosaminyl-glucosyl diacylglycerol, and the GL-1 of the other strains examined is presumed to be similar.

The fatty acids of *Thermus* spp. are predominantly iso- and anteisobranched, with low relative proportions of straight-chain fatty acids (8, 9, 20). In the strains assigned to the species *T. aquaticus*, 3-hydroxy fatty acids, primarily isobranched, comprise 8 to 10% of the total cellular fatty acids. The type strain of *T. filiformis* contains 3-hydroxy anteisobranched fatty acids, but hydroxy fatty acids are absent in the other strains assigned to this species (9).

To elucidate the structure of the major glycolipid in the hydroxy fatty acid-containing strains and to locate the binding position of these fatty acids to specific polar lipid components, we examined several strains of the genus *Thermus* that contained hydroxy fatty acids and two strains that did not contain them. We show that the hydroxy fatty acids, found in all the *T. aquaticus* strains and in *T. filiformis*, are exclusively amide linked to galactosamine of the major glycolipid, and we identify a novel major glycolipid, containing one N-acetylated galactosamine and one N-acylated galactosamine, in *T. aquaticus* 15004.

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## MATERIALS AND METHODS

**Strains and culture conditions.** *T. aquaticus* YT-1$^T$ (ATCC 25104$^T$) and *T. filiformis* Wai33 A1$^T$ (ATCC 43280$^T$), were obtained from the American Type Culture Collection, Rockville, Md.; *T. oshimai* SPS-11 and *Thermus* sp. strain CG-2 were isolated in Portugal as previously described (27); and *T. aquaticus* 15004, 15025, 15031, and 15052 (33) were kindly donated by Richard J. Sharp (CAMR, Salisbury, United Kingdom).

The cultures were grown in 1-liter metal-capped Erlenmeyer flasks, containing 200 ml of *Thermus* medium (32), in a water bath shaker at 70°C until the late exponential phase of growth.

**Extraction and purification of polar lipids and sugar and fatty acid composition.** The cultures were harvested by centrifugation and washed twice, and lipids were extracted by a modification of the Bligh and Dyer method as described previously by Prado et al. (24).

Separation of the polar lipids from the neutral lipids, thin-layer chromatography, purification of the lipids, and hydrolysis of the polar lipid samples were performed by the methods of Prado et al. (24). Thin-layer chromatography of the aqueous acid hydrolysates of the glycolipids was performed on cellulose plates (MN-300 [10 by 20 cm; 0.1-mm thickness]; Macherey-Nagel) and developed with a solvent system composed of pyridine-ethyl acetate-acetic acid-water (36:36:7:21, vol/vol/vol/vol). Hexoses and hexosamines were visualized on the thin-layer chromatography plates with alkaline silver nitrate (17). The presence of glucose, galactose, and glycerol was determined by the glucose oxidase method (Sigma), with β-galactose dehydrogenase (Boehringer Mannheim), and by the glycerol kinase method (Boehringer Mannheim), respectively. The presence of hexos-



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amines was determined by high-performance liquid chromatography with a Gilson (Middleton, Wis.) ASTED system, fitted with a Spherisorb octyldecyl silane column (particle size, 5 mm; length, 150 mm; inner diameter, 4.6 mm), after precolumn derivatization with *O*-phthaldialdehyde–2-mercaptoethanol as recommended by the manufacturer (Gilson). The amino sugars were monitored with a Gilson model 121 fluorescence detector (excitation and emission wavelengths, 340 and 440 nm, respectively). The presence of hexosamines was also determined by the colorimetric method of Dittmer and Wells (6). Nonhydrolyzed sugars and sugars hydrolyzed under the same conditions as the glycolipids were used as controls.

Fatty acid methyl esters of the polar lipid fraction and the individual polar lipids were obtained by acid hydrolysis for 30 min at 100°C in 6.0 N HCl–methanol (32.5:27.5, vol/vol) followed by extraction with hexane–methyl-*tert*-butyl ether (1.0:1.0, vol/vol). The fatty acid methyl esters were separated with a Hewlett-Packard model 5980 gas chromatograph with a flame ionization detector fitted with a 5% phenyl–methyl silicone capillary column (0.2 mm by 25 m; Hewlett-Packard). The carrier gas was high-purity $H_2$, the column head pressure was 60 kPa, the septum purge rate was 5 ml/min, the column split ratio was 55:1, and the injection port temperature was 300°C. The temperature of the oven was programmed for a gradient from 170 to 270°C at 5°C/min. The fatty acid methyl esters were identified and quantified by comparison of their retention times with those of known standards by using the standard MIS Library Generation Software (Microbial ID Inc., Newark, Del.) and confirmed by gas chromatography-mass spectrometry (GC-MS).

**MS.** GC-MS was performed with a Kratos MS80 RFA spectrometer (Kratos Ltd., Manchester, United Kingdom) directly interfaced to a Carlo Erba 5160 chromatograph. Helium (0.7 ml/min) was used as the carrier gas, and samples were introduced by splitless injection (splitless time, 30 s) into a BPX-5 fused silica column (25 m by 0.2 mm; SGE Ltd., Milton Keynes, United Kingdom). The injector and interface ovens were maintained at 250°C. At 1 min after injection, the column oven temperature was programmed to increase from 60 to 200°C at 40°C/min, then at a rate of 3°C/min to 230°C, and finally at 8°C/min to 265°C; the final temperature was maintained for 10 min.

Electron ionization spectra were recorded at an ionization energy of 70 eV, a trap current of 100 mA, and a source temperature of 220°C. Chemical ionization spectra were obtained with isobutane reagent gas and an emission current of 250 mA. The magnet was scanned at 0.6 s per decade of mass over the range 550 to 40.

Fast atom bombardment (FAB) spectra were obtained with the same instrument, with an Ion Tech FAB apparatus and xenon as the bombardment gas. Spectra of the underivatized materials were recorded in both the positive- and negative-ion modes with a 1:1 (vol/vol) mixture of glycerol and dithiothreitol-dithioerythritol (5:1, wt/wt) as the liquid matrix. Approximately 10 μg of the polar lipids, dissolved in chloroform-methanol (1:1, vol/vol), was loaded onto the probe. The instrument was operated at an accelerating voltage of 4 kV and a resolution of 1,000 (10% valley), and the magnet was scanned at 10 s per decade of mass over the range 2,000 to 200. Peracetyl derivatives (1 to 5 μg) were analyzed with 3-nitrobenzyl alcohol matrix. In order to promote sodium cationization, 1 ml of 0.05 M sodium iodide was mixed, on the target, with the sample and the matrix. The instrument was tuned to a resolution of 3,000 (10% valley) for these experiments and scanned over the range 3,000 to 200 at 10 s per decade of mass. Sugar fragment ions are described according to the nomenclature introduced by Domon and Costello (7).

Terminal residues of peracetylated glycolipids were identified by collision-induced dissociation in the field-free region between the ion source and the electrostatic analyzer. Daughter-ion spectra were recorded by scanning the magnetic field and the electrostatic analyzer voltage while a constant field strength-to-analyzer voltage ratio was maintained. The helium collision gas pressure was adjusted to give 50% attenuation of the ion beam. Reference spectra were obtained by collisional activation of $B_1$ fragments from peracetylated and perdeuterioacetylated hexoses (19, 26). The galactofuranose reference spectrum was obtained by collisional activation of lipopeptidophosphoglycan, a glycoinositol phospholipid containing two terminal β-galactofuranoses, from strain Y of *Trypanosoma cruzi* (4, 25).

**Derivatization.** Samples for GC-MS were trimethylsilylated with 100 μl of *bis*-(trimethylsilyl)-trifluoroacetamide for 30 min at 60°C. The reagent was removed by vacuum centrifugation, and the derivatives were dissolved in hexane. Peracetyl derivatives were prepared by treatment with a 2:1 (vol/vol) mixture of trifluoroacetic anhydride and acetic acid for 10 min at room temperature as described by Dell (5). Perdeuterioacetates were prepared identically, except that acetic acid was replaced by $CD_3CO_2D$.

## RESULTS

**Polar lipid composition.** The polar lipid fraction of the strains examined consisted of a major phospholipid (PL-2) containing between 77 and 91% of the total phosphorus and a major glycolipid (GL-1) containing between 73 and 95% of the total carbohydrate. All strains contained minor amounts of GL-2 (2.1 to 7.7% of the total glycolipids) and PL-1 (3.6 to 19.4% of the total phospholipids); strains 15025 and 15052 also had an additional minor glycolipid that constituted about 10% of the polar lipid carbohydrate (results not shown).

TABLE 1. Composition of the polar head group of the major glycolipid of strains of the genus *Thermus*

| Strain | Composition (ratio)[a] | | | | |
|---|---|---|---|---|---|
| | Glc | Gal | GlcN | GalN | Gro |
| *T. oshimai* SPS-11 | 2.9 | — | 1.0 | — | 1.0 |
| *Thermus* sp. strain CG-2 | 1.8 | 0.8 | 1.0 | — | 0.8 |
| *T. filiformis* Wai33 A1[T] | 1.0 | 1.9 | — | 1.0 | 0.8 |
| *T. aquaticus* | | | | | |
| YT-1[T] | 3.0 | — | — | 1.0 | 0.8 |
| 15004 | 1.0 | 0.9 | — | 2.0 | 1.0 |
| 15025 | 1.0 | 2.0 | — | 1.0 | 0.7 |
| 15031 | 1.0 | 1.9 | — | 1.0 | 0.8 |
| 15052 | 1.0 | 1.9 | — | 1.0 | 1.0 |

[a] The values for glucose, galactose, and glycerol were compared with those for the hexosamines. Abbreviations: Glc, glucose; Gal, galactose; GlcN, glucosamine; GalN, galactosamine; Gro, glycerol; —, not detected.

**Sugar composition of the major glycolipid.** Compositional analysis of the acid hydrolysates showed that glucose, galactose, glucosamine, and galactosamine were the constituents of the polar head group of the major glycolipid (Table 1). Glucosamine was detected only in strains SPS-11 and CG-2, whereas galactosamine was found in the remaining strains. With the exception of *T. aquaticus* 15004, all strains had a polar head composed of three hexose residues and one hexosamine residue. The compositional analysis of GL-1 of *T. aquaticus* 15004 showed that two galactosamine residues instead of one, as in the other strains, were present.

**Fatty acid analysis of polar lipids of *Thermus* strains.** Iso- and anteisobranched fatty acids were the predominant fatty acids of the polar lipid fractions of the *Thermus* strains (Table 2). Straight-chain fatty acids were minor components in all the strains examined, ranging between about 1 and 9% of the total fatty acids. The polar lipid fraction of the strains assigned to the species *T. aquaticus* also possessed moderate levels of 3-OH isobranched fatty acids, whereas the hydroxy fatty acids of *T. filiformis* Wai33 A1 were dominated by anteisobranched isomers.

The fatty acid composition of purified GL-1 and PL-2 in *T. oshimai* SPS-11 was similar to that in *Thermus* sp. strain CG-2. On the other hand, the major glycolipid of the *T. aquaticus* strains and of *T. filiformis* Wai33 A1 contained large relative proportions of hydroxy fatty acids that ranged between 25 and 28% of the total glycolipid fatty acid, indicating that about one-third of the fatty acids were hydroxylated. Hydroxy fatty acids were not detected in the major phospholipid, which, in contrast to the major glycolipid, had high relative proportions of iso-17:0.

**MS of GL-1 from *T. oshimai* SPS-11 and *Thermus* sp. strain CG-2.** The FAB spectrum of the peracetylated GL-1 from *Thermus* strain SPS-11 had an abundant peak, diagnostic of a terminal hexose at a mass-to-charge ratio of 331 (*m/z* 331), and the type B carbenium ions at *m/z* 619, 1,116, and 1,404 established the sequence of the polar head group as hexose-hexose-(*N*-acyl)hexosamine-hexose (Fig. 1). The $B_3$ fragments contain an N-acylated hexose residue (glucosamine by chemical analysis), and their masses were, therefore, diagnostic of the *N*-acyl substituents. In addition to the major species at *m/z* 1,116, corresponding to substitution with a $C_{17}$ (iso-17:0, anteiso-

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TABLE 2. Fatty acid composition of the total polar lipids, major glycolipid, and major phospholipid of *Thermus* strains grown at 70°C[a]

| Fatty acid | Fatty acid composition (% of total) | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *T. oshimai* *SPS-11* | | | *Thermus* sp. strain CG-2 | | | *T. filiformis* Wai33 A1$^T$ | | | *T. aquaticus* YT-1$^T$ | | | *T. aquaticus* 15004 | | | *T. aquaticus* 15025 | | | *T. aquaticus* 15031 | | | *T. aquaticus* 15052 | | |
| | TPL | GL-1 | PL-2 | TPL | GL-1 | PL-2 | TPL | GL-1 | PL-2 | TPL | GL-1 | PL-2 | TPL | GL-1 | PL-2 | TPL | GL-1 | PL-2 | TPL | GL-1 | PL-2 | TPL | GL-1 | PL-2 |
| Straight chain | | | | | | | | | | | | | | | | | | | | | | | | |
| 14:0 | 0.2 | 0.5 | — | 0.2 | 0.2 | — | 0.2 | — | — | 0.7 | 0.8 | 0.8 | 0.2 | 0.3 | — | 0.2 | 0.3 | — | — | — | — | 0.1 | — | — |
| 15:0 | 2.0 | 2.2 | 1.2 | 0.9 | 0.8 | 0.6 | 0.2 | — | — | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | — | 0.3 | 0.4 | 0.3 | 0.2 | — | — | 0.2 | 0.2 | — |
| 16:0 | 1.8 | 1.5 | 3.0 | 1.7 | 1.3 | 1.4 | 1.1 | 0.9 | 1.3 | 7.1 | 6.6 | 7.5 | 2.0 | 1.8 | 2.8 | 1.5 | 1.2 | 2.4 | 0.9 | 0.6 | 2.0 | 1.0 | 0.8 | 2.0 |
| 17:0 | 0.9 | 1.0 | 0.6 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| 18:0 | 0.4 | 0.4 | 0.7 | 0.1 | 0.1 | — | 0.1 | — | — | 0.5 | 0.2 | 0.8 | 0.2 | 0.4 | 0.3 | 0.2 | 0.3 | 0.3 | — | — | — | 0.1 | — | — |
| Isobranched | | | | | | | | | | | | | | | | | | | | | | | | |
| 13:0 | 1.0 | 0.9 | — | 0.6 | 0.9 | 0.3 | 0.1 | — | — | 0.2 | 0.3 | 0.2 | 0.3 | 0.4 | — | 0.2 | 0.3 | — | 0.2 | 0.3 | — | 0.3 | 0.3 | — |
| 14:0 | 0.6 | 0.7 | 0.6 | 1.8 | 2.1 | 1.4 | 0.9 | 1.2 | 0.7 | 0.5 | 0.6 | 0.5 | 0.6 | 0.7 | 0.5 | 0.4 | 0.5 | 0.3 | 0.3 | 0.4 | — | 0.5 | 0.6 | 0.3 |
| 15:0 | 42.1 | 43.2 | 42.5 | 34.8 | 34.7 | 34.1 | 7.4 | 6.9 | 7.6 | 25.9 | 25.4 | 26.2 | 28.8 | 27.0 | 29.6 | 25.2 | 24.0 | 26.2 | 27.2 | 25.9 | 27.4 | 30.5 | 30.4 | 30.5 |
| 16:0 | 3.5 | 3.6 | 3.2 | 12.2 | 13.1 | 11.3 | 6.1 | 5.6 | 7.1 | 6.0 | 5.4 | 6.9 | 4.9 | 4.1 | 5.3 | 4.8 | 4.0 | 5.8 | 4.1 | 3.6 | 4.6 | 4.8 | 4.4 | 5.5 |
| 17:0 | 37.8 | 35.8 | 41.1 | 31.7 | 28.9 | 35.8 | 9.4 | 7.5 | 14.0 | 33.3 | 24.8 | 46.5 | 34.7 | 24.5 | 47.7 | 40.8 | 28.7 | 57.9 | 41.3 | 30.6 | 57.5 | 36.6 | 27.4 | 54.1 |
| 18:0 | — | — | — | 0.6 | 0.6 | 0.7 | 0.5 | 0.3 | 0.8 | 0.2 | — | 0.4 | 0.2 | — | — | 0.2 | — | — | 0.2 | — | — | 0.2 | — | — |
| 19:0 | 0.2 | — | — | 0.2 | 0.2 | 0.2 | — | — | — | 0.2 | — | 0.2 | 0.2 | — | — | 0.3 | — | — | 0.2 | — | — | 0.2 | — | — |
| Anteisobranched | | | | | | | | | | | | | | | | | | | | | | | | |
| 15:0 | 3.5 | 3.8 | 3.2 | 7.7 | 8.4 | 6.7 | 22.5 | 23.1 | 20.2 | 3.4 | 3.4 | 3.1 | 6.3 | 6.7 | 5.5 | 2.5 | 2.7 | 2.1 | 2.8 | 2.9 | 2.7 | 3.2 | 3.4 | 2.8 |
| 17:0 | 3.0 | 2.9 | 3.2 | 5.5 | 5.0 | 6.2 | 31.5 | 25.4 | 46.5 | 3.7 | 2.7 | 5.1 | 6.3 | 4.8 | 8.3 | 3.5 | 2.7 | 4.7 | 3.7 | 2.8 | 5.1 | 3.2 | 2.5 | 4.8 |
| 19:0 | — | — | — | — | — | — | 0.9 | 0.6 | 1.2 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| Hydroxylated | | | | | | | | | | | | | | | | | | | | | | | | |
| i15:0-3OH | 0.3 | 0.3 | — | 0.3 | 0.5 | 0.2 | 1.1 | 1.5 | — | 3.7 | 6.2 | — | 3.3 | 6.6 | — | 2.9 | 5.5 | — | 3.3 | 5.8 | — | 3.8 | 6.1 | — |
| a15:0-3OH | — | — | — | — | 0.1 | — | 1.4 | 2.2 | — | 0.2 | 0.4 | — | 0.4 | 0.8 | — | 0.2 | 0.4 | — | 0.3 | 0.4 | — | 0.2 | 0.4 | — |
| i16:0-3OH | — | — | — | — | 0.1 | — | 0.9 | 1.6 | — | 1.1 | 1.8 | — | 0.7 | 1.3 | — | 0.8 | 1.6 | — | 0.8 | 1.5 | — | 1.0 | 1.5 | — |
| n16:0-3OH | — | — | — | — | — | — | — | — | — | 0.8 | — | — | 0.2 | 0.4 | — | — | — | — | — | — | — | — | — | — |
| i17:0-3OH | 0.3 | 0.4 | — | 0.2 | 0.3 | 0.2 | 3.6 | 5.6 | — | 9.2 | 14.9 | 0.2 | 7.3 | 14.8 | — | 9.3 | 18.4 | — | 10.5 | 19.0 | — | 11.3 | 18.4 | — |
| a17:0-3OH | — | — | — | — | 0.1 | — | 8.9 | 14.0 | — | 0.9 | 1.4 | — | 1.1 | 2.1 | — | 0.7 | 1.2 | — | 0.8 | 1.4 | — | 0.9 | 1.4 | — |
| n18:0-3OH | — | — | — | — | — | — | 1.5 | 2.2 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| Unknown | | | | | | | | | | | | | | | | | | | | | | | | |
| ECL19.060 | 2.3 | 2.6 | 0.7 | 1.2 | 2.0 | 0.5 | 1.1 | 1.4 | — | 2.3 | 2.5 | 1.0 | 1.8 | 2.7 | — | 4.5 | 6.1 | — | 3.2 | 4.5 | 0.7 | 1.5 | 2.1 | — |

[a] Abbreviations: TPL, total polar lipids; i, isobranched; a, anteisobranched; n, straight chain; ECL, equivalent chain length; —, not detected.

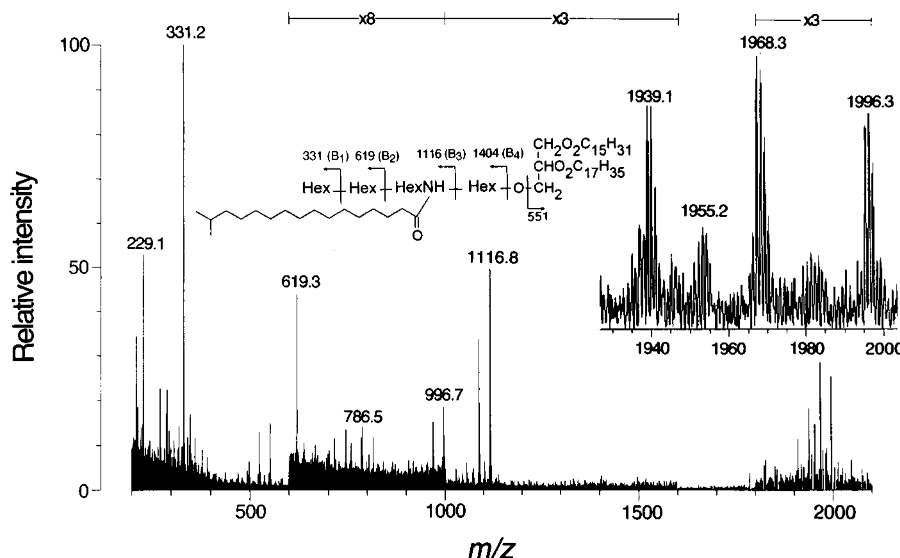


FIG. 1. Positive-ion FAB mass spectrum of peracetylated GL-1 from *T. oshimai* SPS-11. The inset shows an expansion of the molecular ion region and identifies the major fragments.

17:0, or 17:0) fatty acid, $B_3$ ions observed at *m/z* 1,088 and 1,102 indicated that a proportion of the glycolipid was N acylated with $C_{15}$ and $C_{16}$ fatty acids. Ions at *m/z* 523 and 551 originating by cleavage of the sugar-to-glycerol bond identified the ester-linked substituents; *m/z* 523 corresponded to acylation with two $C_{15}$ fatty acids, whereas *m/z* 551 represented substitutions with both $C_{15}$ and $C_{17}$ fatty acids. Comparison of the relative intensities of the N-acylated $B_3$ fragments and the acylglycerol ions enables an approximate estimate of the distributions of fatty acids at these locations. The ratio of the abundances of *m/z* 1,102 and *m/z* 1,088 indicated that approximately 54% of the fatty acids amide linked to glucosamine were $C_{17}$ fatty acids and about 40% were $C_{15}$ fatty acids, the balance being $C_{16}$ fatty acids. The ratio of the relative intensities of the acylglycerol fragments at *m/z* 551 and 523 was about 1.0/0.9, suggesting that of the total O-linked fatty acids, slightly more than 25% were $C_{17}$ fatty acids, while about 70% were $C_{15}$ fatty acids (Fig. 1). The results of the GC (Table 2) indicated that the $C_{15}$, $C_{16}$, and $C_{17}$ fatty acids were predominantly isobranched, since anteisobranched and straight-chain fatty acids were minor components.

Similar spectra were obtained from strain CG-2, except that a more complex distribution of molecular ions was observed because of the higher proportion of iso-16:0 substitutions (results not shown). The greater complexity of the distribution of molecular ions was also reflected in additional acylglycerol fragments at *m/z* 537 and 565, which were attributable to species with $C_{15}$ and $C_{16}$ and with $C_{16}$ and $C_{17}$ fatty acid substitutions, respectively. The $B_3$ ions at *m/z* 1,116, 1,102, and 1,088 suggested that the glucosamine was replaced by $C_{17}$, $C_{16}$, and $C_{15}$ fatty acids in the approximate ratio of 1.0/0.5/1.0. It was impossible to assign unique fatty acid compositions to some of the minor acylglycerol fragments from the GL-1 of strain CG-2 because of the presence of significant levels of $C_{16}$, but it was clear from the ratio of the intensities of the fragments at *m/z* 523 and 551 that $C_{15}$ fatty acids were present in at least twofold excess over $C_{17}$ fatty acids. The negative-ion spectra also provided evidence of a preferential distribution of fatty acids between ester and amide linkages, because abundant fatty acid anions were observed at *m/z* 241 ($C_{14}H_{29}CO_2^-$) and 269 ($C_{16}H_{33}CO_2^-$) in the ratio 1.0/0.45. Carboxylate anions are characteristic fragmentation products of ester-linked acids in negative-ion FAB spectra (11), whereas amide-linked acids are not eliminated in this way (12). Therefore, the observed fatty acid anions must be derived from the glycerol-linked substituents rather than those N linked to hexosamine.

**MS of GL-1 from *T. aquaticus* strains and *T. filiformis* WAI33 A1.** The deprotonated molecules ($[M - H]^-$) of the underivatized GL-1 and the $[M + Na]^+$ ions of the peracetyl derivatives of the *T. aquaticus* and *T. filiformis* strains were observed at *m/z* values 16 and 58 higher than those of the corresponding ions of the SPS-11 and CG-2 glycolipids, suggesting the presence of an additional free hydroxyl group (Table 3; Fig. 2). The $B_3$ ions at *m/z* 1,174, 1,160, and 1,146

TABLE 3. Assignments of the major deprotonated molecules observed in the negative ion FAB spectrum of underivatized GL-1 from *T. aquaticus* YT-1

| $[M - H]^-$ (% RI)[a] | Composition[b] | Calculated $[M - H]^-$ |
|---|---|---|
| 1,426.9 (38) | (Hex)$_3$Hex*N*(acyl)Gro(15:0)$_3$ OH | 1,426.90376 |
| 1,440.7 (39) | (Hex)$_3$Hex*N*(acyl)Gro(15:0)$_2$ (16:0) OH | 1,440.91941 |
| 1,454.8 (88) | (Hex)$_3$Hex*N*(acyl)Gro(15:0)$_2$ (17:0) OH | 1,454.93506 |
| 1,468.8 (65) | (Hex)$_3$Hex*N*(acyl)Gro(15:0)(16:0) (17:0) OH | 1,468.95071 |
| 1,482.9 (100) | (Hex)$_3$Hex*N*(acyl)Gro(15:0) (17:0)$_2$ OH | 1,482.96636 |
| 1,496.9 (41) | (Hex)$_3$Hex*N*(acyl)Gro(16:0) (17:0)$_2$ OH | 1,496.98201 |

[a] $[M - H]^-$ is the *m/z* value of the deprotonated molecule. The value in parentheses is the relative intensity normalized to *m/z* 1,482.9 (100%).
[b] Three fatty acids, one of which is hydroxylated. Hex, hexose; Gro, glycerol.

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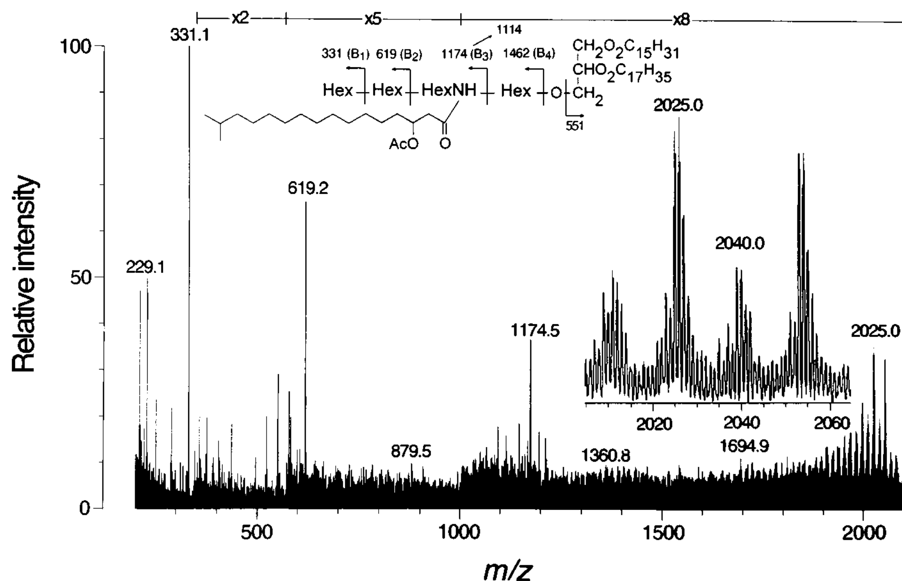


FIG. 2. Positive-ion FAB mass spectrum of peracetylated GL-1 from *T. aquaticus* 15025. The inset shows an expansion of the molecular ion region and identifies the major fragments.

indicated that the galactosamine was linked to hydroxylated $C_{17}$, $C_{16}$, and $C_{15}$ fatty acids. The absence of $B_3$ fragments at *m/z* 1,116, 1,102, and 1,088 showed that nonhydroxylated amide-linked substituents were not present (the ions at *m/z* 1,114 and 1,086 are attributable to the elimination of acetic acid from *m/z* 1,174 and 1,146). Similar patterns of galactosamine acylation were observed in all the *T. aquaticus* strains and in *T. filiformis*, in which the relative proportions of hydroxylated fatty acids were remarkably constant (Table 4). In the *T. aquaticus* strains the 3-hydroxy fatty acids were predominantly isobranched, while in *T. filiformis*, the 3-hydroxy fatty acids were predominantly anteisobranched. The glycerol moiety, by contrast, appeared to be exclusively linked to nonhydroxylated acids, since no hydroxy acid-containing acyl glycerol fragments are observed. The signals at *m/z* 523, 537, 551, 565, and 579 indicate $C_{15}$ and $C_{15}$, $C_{15}$ and $C_{16}$, $C_{15}$ and $C_{17}$, $C_{16}$ and $C_{17}$, and $C_{17}$ and $C_{17}$ fatty acid substitutions, respectively, in relative proportions consistent with the results shown in Table 2. In the negative-ion spectra of the underivatized glycolipids, carboxylate anions were observed at *m/z* 241 ($C_{14}H_{29}CO_2^-$), 255 ($C_{15}H_{31}CO_2^-$), and 269 ($C_{16}H_{33}CO_2^-$) but not at *m/z* 285 (the carboxylate anion of OH-17), which is likewise consistent with the hydroxy fatty acids being exclusively amide linked (Fig. 2).

The two major molecular ions in the negative-ion spectrum of *T. aquaticus* 15004 were observed at *m/z* 1,496.0 and 1,523.8, that is, at *m/z* values 41 higher than those of the corresponding ions in the other strains. On peracetylation, these ions shifted to *m/z* 2,024.4 and 2,052.4, respectively, suggesting the replacement of a hexose by an *N*-acetylhexosamine (Fig. 3a). The $B_1$ carbenium ion at *m/z* 331 indicated that the terminal residue was a hexose, but the $B_2$ fragment, observed at *m/z* 618 rather than at 619, showed that the penultimate residue was hexosamine rather than hexose. This sequence of the terminal portion of the polar head group of GL-1 of strain 15004 was supported by the spectrum of the perdeuterioacetyl derivative in which the major $[M + Na]^+$ ions were shifted to *m/z* 2,060.2 and 2,088.2, indicating the addition of 12 deuterioacetyl groups, compared with 13 in the other strains examined (Fig. 3b). The $B_2$ ion was shifted to *m/z* 636 in the deuterioacetyl derivative, showing that only six acetate groups are added on derivatization, which would be expected if the residue were already N acetylated. The inner hexosamine was determined to be acylated with hydroxy $C_{15}$ and hydroxy $C_{17}$ fatty acids as in the other strains, because the $B_3$ ions were observed at *m/z* 1,145.7, 1,159.7, and 1,173.7. No $B_2$ ions, other than *m/z* 618, are present, indicating that the outer galactosamine was exclusively N acetylated. Acylglycerol fragments were observed only at *m/z* 523, 537, 551, and 579, showing that the glycerol was linked to nonhydroxylated fatty acids only.

TABLE 4. Relative intensities of $B_3$ fragment ions in the major glycolipid of hydroxy fatty acid-containing *Thermus* strains

| Strain | Relative intensity[a] (% total hydroxy acids) | | |
|---|---|---|---|
| | $B_3$ (OH 15:0) *m/z* 1,146 | $B_3$ (OH 16:0) *m/z* 1,160 | $B_3$ (OH 17:0) *m/z* 1,174 |
| *T. aquaticus* YT-1 | 0.476 (28) | 0.24 (13) | 1.0 (58) |
| *T. aquaticus* 15004[b] | 0.59 (33) | 0.16 (9) | 1.0 (57) |
| *T. aquaticus* 15025 | 0.41 (27) | 0.099 (7) | 1.0 (66) |
| *T. aquaticus* 15031 | 0.38 (25) | 0.12 (8) | 1.0 (67) |
| *T. aquaticus* 15052 | 0.36 (25) | 0.1 (7) | 1.0 (68) |
| *T. filiformis* Wai33 A1 | 0.25 (18) | 0.14 (10) | 1.0 (72) |

[a] Relative intensity of $B_3$ ions normalized to *m/z* 1,174 [$B_3$ (OH 17:0)].
[b] $B_3$ fragments are observed at *m/z* 1,145, 1,159, and 1,173.

**Identification of nonreducing terminal residues of major glycolipids.** Although FAB-MS does not usually enable the identification of individual monosaccharide residues, terminal residues can be identified by collisional activation of the $B_1$ carbenium fragment in the spectra of acetate and deuterioacetate derivatives (19, 29). The daughter ion spectra obtained from the $B_1$ ions from the GL-1 of *T. oshimai* SPS-11, *Thermus* sp. strain CG-2, and *T. aquaticus* YT-1 were similar to each other and to the glucopyranose standards (Table 5). The *m/z* 172-to-*m/z* 173 ratio in the daughter ion spectra of the deuterioacetate derivatives ranged from 2.17 to 2.6, being consis-

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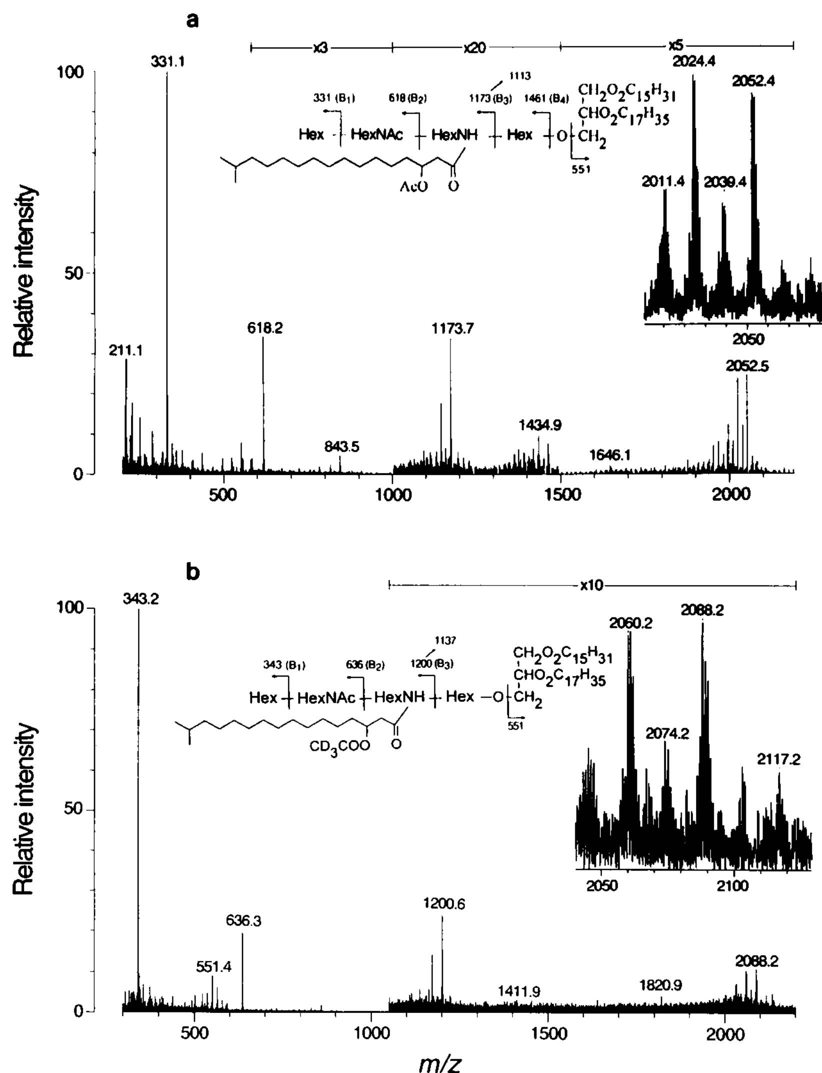


FIG. 3. Positive-ion FAB mass spectra of peracetylated (a) and perdeuterioacetylated (b) GL-1 from *T. aquaticus* 15004. The insets show an expansion of the molecular ion region and identify the major fragments.

tent with the assignment of the terminal residue as glucopyranose.

The spectra of the $B_1$ ions of acetylated GL-1 from the other *T. aquaticus* strains and from *T. filiformis* were very similar to each other, suggesting that the same terminal residue was present in each of these organisms (data not shown). The spectra were galactose-like, but the *m/z* 271 fragment was less abundant than that found in galactopyranose and the *m/z* 211 fragment was more abundant (between 30 and 49% of that found in the *m/z* 271 fragment), as observed in the galactofuranose reference spectrum. The spectra of the perdeuteroacetylated derivatives were also consistent with galactofuranose but not galactopyranose, since the *m/z* 172-to-*m/z* 173 ratio ranged between 0.65 and 0.75, and the *m/z* 217 fragment was consistently more abundant (about 70% of *m/z* 173) than in galactopyranose. The terminal residue of the major glycolipid from these strains was thus identified as galactofuranose.

## DISCUSSION

The sugar composition of the major glycolipid has been examined in a few strains of the species of *Thermus*, including *T. aquaticus* YT-1 and *T. oshimai* SPS-11, which were used in the present study as reference organisms. Previous results showed that, except for GL-1 of *T. aquaticus* YT-1, in which galactosamine was present, each of the other *Thermus* strains had a major glycolipid that contained glucosamine (21, 22, 24). In the present study, we found that galactosamine was present in all *T. aquaticus* strains examined and in the type strain of *T. filiformis*, while glucosamine was present only in strains CG-2 and SPS-11.

The MS analysis after peracetylation permitted the monosaccharide sequence of the glycolipid of most strains to be readily deduced, because these derivatives undergo simple and predictable fragmentation, via pathways producing predominantly nonreducing terminus-containing carbenium ions (5). The major glycolipids of *T. oshimai* SPS-11 and *T. aquaticus* YT-1 contain only glucose and N-acylated hexosamine. The structure of GL-1 of strain SPS-11 can, therefore, be established as glucopyranosyl-glucosyl-(*N*-acyl)glucosaminyl-glucosyl diacylglycerol, and the GL-1 of strain YT-1 can be established as glucopyranosyl-glucosyl-(*N*-acyl)galactosaminyl-glucosyl diacylglycerol. The MS results also show that the terminal

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TABLE 5. Collisional activation of the $B_1$ carbenium ion at *m/z* 343 from peracetylated hexose standards and *Thermus* glycolipids

| Sample[a] | Collision-induced ions of *m/z* 343 (% RI) | | | | | | *m/z* 172/*m/z* 173[b] | Residue |
|---|---|---|---|---|---|---|---|---|
| | *m/z* 109 | *m/z* 172 | *m/z* 173 | *m/z* 217 | *m/z* 235 | *m/z* 280 | | |
| Standards | | | | | | | | |
| Glc*p* | 5 | 100 | 39 | 16 | 4 | 70 | 2.56 | |
| Gal*p* | 5 | 88 | 100 | 55 | 13 | 11 | 0.88 | |
| LPPG | 2 | 74 | 100 | 64 | 7 | 1 | 0.74 | Gal*f* |
| Man*p* | 4 | 100 | 52 | 20 | 5 | 30 | 1.92 | |
| Glycolipids | | | | | | | | |
| *T. oshimai* SPS-11 | 2 | 100 | 42 | 28 | 4 | 73 | 2.38 | Glc*p* |
| *Thermus* sp. strain CG2 | 2 | 100 | 45 | 25 | 4 | 59 | 2.22 | Glc*p* |
| *T. aquaticus* YT-1 | 4 | 100 | 46 | 26 | 4 | 56 | 2.17 | Glc*p* |
| *T. aquaticus* 15052 | 4 | 68 | 100 | 70 | 9 | 2 | 0.68 | Gal*f* |
| *T. aquaticus* 15004 | 3 | 59 | 100 | 87 | 10 | 1 | 0.59 | Gal*f* |
| *T. aquaticus* 15031 | 6 | 75 | 100 | 73 | 11 | <1 | 0.75 | Gal*f* |
| *T. aquaticus* 15025 | 4 | 67 | 100 | 70 | 8 | 1 | 0.67 | Gal*f* |
| *T. filiformis* Wai 33 A1 | 4 | 65 | 100 | 70 | 9 | 1 | 0.65 | Gal*f* |

[a] Abbreviations: Glc*p*, glucopyranose; Gal*p*, galactopyranose; LPPG, lipopeptidophosphoglycan; Gal*f*, galactofuranose; Man*p*, mannopyranose.
[b] Ratio of the relative intensity (RI) of *m/z* 172 to the RI of *m/z* 173.

hexose of *T. aquaticus* 15004 is galactofuranose, the subterminal component is *N*-acetylgalactosamine, and the third component is *N*-acylgalactosamine. These results lead us to establish the structure of this novel glycolipid as galactofuranosyl-(*N*-acetyl)galactosaminyl-(*N*-acyl)galactosaminyl-glucosyl diacylglycerol.

The current data do not permit the determination of the full sequence of sugars in the polar head group of the other *Thermus* strains since it was not possible to determine whether the glucose residue is in the subterminal or the inner position. Nevertheless, all *Thermus* glycolipids examined, such as those of *T. thermophilus* HB-8 and *T. aquaticus* 15004, among others, have at least one glucose residue that is linked to the acylglycerol, and this is assumed to be the case for all *Thermus* strains (22, 24). The results also show very clearly that the composition of the polar head group of GL-1 is variable and is not related to the taxonomic status of the strains examined.

It is interesting to note that the terminal galactoses in GL-1 of the *T. aquaticus* strains and of *T. filiformis* Wai33 A1, as in *T. thermophilus* HB-8, are in the furanose configuration. Although relatively rare, galactofuranose residues have been detected in complex carbohydrates of members of the domains *Archaea* (23) and *Bacteria*, namely, in the capsular polysaccharide of *Streptococcus pneumoniae* serotype 10A and the O antigens of *Klebsiella pneumoniae* O1 and O8 (13, 15). Galactofuranose has also been detected in galactosyl diglycerides of *Bifidobacterium bifidum* and in the unusual phospholipids of a *Butyrivibrio* sp. strain (3, 28).

Our results show that there is a differential distribution of fatty acids in the major phospholipid and the major glycolipid of *T. filiformis* and the *T. aquaticus* strains. The MS results demonstrate that hydroxy fatty acids are exclusively amide linked to the galactosamine in these strains, while nonhydroxy fatty acids are exclusively ester linked to the diacylglycerol. On the other hand, *T. oshimai* SPS-11 and *Thermus* sp. strain CG-2, which contain glucosamine in the major glycolipid, contain only nonhydroxylated fatty acids. A differential distribution of substituents was also observed in the nonhydroxy acid-containing strains SPS-11 and CG-2. Here the glycerol was N acylated with an excess of $C_{15}$ fatty acids, whereas glucosamine was N acylated with a higher proportion of $C_{17}$ than $C_{16}$ fatty acids.

Hydroxy fatty acids are characteristically found in the lipid A moiety of lipopolysaccharides, but they also occur in extractable lipids of some bacteria (31). Unbranched 3-OH fatty acids are among the O-linked substituents of phosphatidylethanolamine in *Mycobacterium tuberculosis*, *Mycobacterium fortuitum* (1), and other gram-positive bacteria (16, 34) and are also encountered in rhamnolipids of *Pseudomonas aeruginosa* (10). Amide-linked OH fatty acids occur in lipoconjugates (2) and ornithine amide lipids of *Pseudomonas* spp. (30); 2-OH 14:0 is also amide linked to glycosphingolipids of *Sphingomonas paucimobilis* (14), and amide linked branched-chain 3-OH fatty acids have also been detected in free ceramides of *Bacteroides fragilis* (18). However, to our knowledge N acylation of hexosamine by hydroxy fatty acids in extractable lipids from bacteria has not been previously reported.

The role of hydroxy fatty acids in strains of *T. aquaticus* and the type strain of *T. filiformis* is not known, but these fatty acids were not detected in 52 high-temperature strains belonging to other genomic species of the genus *Thermus* (9, 20), some of which are known to contain glucosamine in the major glycolipid. These results lead us to conclude that, thus far, the presence of hydroxy fatty acids in *Thermus* spp. is associated with galactosamine-containing glycolipids.

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