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Characterization of Novel Long-Chain 1,2-Diols in *Thermus* Species and Demonstration that *Thermus* Strains Contain Both Glycerol-Linked and Diol-Linked Glycolipids

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In this study, we purified and characterized tetra- and triglycosyl glycolipids (GL-1 and GL-2, respectively) from two different colonial forms of *Thermus scotoductus* X-1, from *T. filiformis* Tok4 A2, and from *T. oshimai* SPS-11. Acid hydrolysis of the purified glycolipids liberated, in addition to the expected long-chain fatty acids, two components which were identified by gas chromatography-mass spectrometry as 16-methylheptadecane-1,2-diol and 15-methylheptadecane-1,2-diol. Fast atom bombardment mass spectrometry of the intact glycolipids indicated that a major proportion consisted of components with glycan head groups linked to long-chain 1,2-diols rather than to glycerol, although in all cases glycerol-linked compounds containing similar glycan head groups were also present. As in other *Thermus* strains, the polar head group of GL-1 from *T. filiformis* Tok4 A2 and from *T. scotoductus* X-1 colony type t2 was a glucosylgalactosyl-(*N*-acyl)glucosaminylglucosyl moiety. However, GL-2 from *T. scotoductus* X-1 colony type t1 and from *T. oshimai* SPS-11 was a truncated analog which lacked the nonreducing terminal glucose. Long-chain 1,2-diols have been previously reported in the polar lipids of *Thermomicrobium roseum* and (possibly) *Chloroflexus aurantiacus*, but to our knowledge, this is the first report of their detection in other bacteria and the first account of the structural determination of long-chain diol-linked glycolipids.

Bacteria of the genus *Thermus* have optimum growth temperatures of about 70°C and maximum growth temperatures of about 78 to 85°C (17, 30), and it has been suggested that the high proportion of glycolipids in their cell membranes contributes to this ability to grow at elevated temperatures, since the relative proportions of the major glycolipid increases concomitantly with growth temperature (20, 24). Previous studies have shown that the polar lipids of these organisms usually comprise a major phospholipid, designated PL-2, a major glycolipid, designated GL-1, and in some strains or under certain culture conditions, a minor phospholipid, designated PL-1, and a minor glycolipid, designated GL-2 (6, 24, 27). The structures of the phospholipids have not yet been elucidated, but the major glycolipid of most strains has been identified as a diglycosyl-(*N*-acyl)glycosaminyl-glycosyldiacylglycerol, which contains three hexose residues and one *N*-acylated hexosamine, giving a hexose/hexosamine/glycerol ratio of approximately 3:1:1 (2, 18, 19, 24). On the other hand, GL-1 from *Thermus aquaticus* 15004 was recently shown to have *N*-acetylgalactosamine in place of the subterminal hexose residue, resulting in a hexose/hexosamine/glycerol ratio of 2:2:1 (2).

Thermus scotoductus X-1 (ATCC 27978) produces two colony types, designated t1 and t2 (27). The polar lipid composition of strain X-1 colony type t2 [X-1(t2)] is typical of most *Thermus* strains, consisting of a major phospholipid (PL-2), a major glycolipid (GL-1), and traces of a minor glycolipid (GL-2), whereas X-1(t1) has GL-2 as its major glycolipid and only trace amounts of GL-1 (27). Whole DNA-DNA hybridization studies indicate that the two colony types are extremely closely related (27), and the GL-1-deficient colony type t1 could therefore constitute a possible model system for investigating the role of glycolipids in the regulation of the structure and function of the membranes of *Thermus* strains at high temperature.

In this study, we purified GL-1 and GL-2 from both colony types of *T. scotoductus* X-1 and characterized them by fast atom bombardment (FAB) mass spectrometry (MS) and by gas chromatography (GC)-MS. We show that, as in other *Thermus* strains, the polar head group of GL-1 is a glucosylgalactosyl-(*N*-acyl)glucosaminylglucosyl moiety, whereas that of GL-2 lacks the terminal glucose. However, in contrast to previously described glycolipids from other *Thermus* species (2, 18, 19, 24), a major proportion of the glycolipids from *T. scotoductus* consists of components in which the glycan head groups are linked to long-chain 1,2-diols rather than to glycerol. The major glycolipid from *Thermus filiformis* Tok4 A2 was likewise shown to be primarily diol rather than glycerol linked. We have also characterized the long-chain diols liberated by hydrolysis of the intact glycolipids and show that the principal component is 16-methylheptadecane-1,2-diol; smaller quantities of its anteiso-branched isomer, as well as C_{17} and C_{19} analogs, are also present.

Long-chain 1,2-diols have been previously reported as constituents of the polar lipids of *Thermomicrobium roseum*, but to our knowledge, this is the first account of the structural determination of long-chain diol-linked glycolipids from other bacteria.

MATERIALS AND METHODS

Strains and culture conditions. Colony types t1 and t2 were obtained from *T. scotoductus* X-1 (ATCC 27978) as previously described (27). *Thermus oshimai* SPS-11 and *Thermus* strain CG-2 were isolated and characterized as described by Santos et al. (26). *T. filiformis* Tok4 A2 was kindly donated by H. Morgan (Hamilton, New Zealand), and *Thermomicrobium roseum* (ATCC 27502^T) was obtained from the American Type Culture Collection, Rockville, Md. Bacteria

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were cultured in 1 liter metal-capped Erlenmeyer flasks, containing 200 ml of *Thermus* medium (30), in a shaking water bath at 70°C until the late exponential phase of growth.

Extraction and purification of polar lipids. The cultures were harvested by centrifugation and washed twice with Tris-HCl (pH 7.9), and lipids were extracted by the Bligh-Dyer method (1). Polar lipids were separated from neutral lipids by thin-layer chromatography (TLC), and the polar lipid fraction was further purified by TLC as described previously (24).

Hydrolysis and analysis of products. The purified glycolipids were hydrolyzed with 2 N HCl for 3 h at 100°C, and nonpolar products were recovered by extracting the hydrolysates twice with hexane. The resulting mixture of fatty acids and diols was separated by TLC on Silica G plates (23) developed with hexane-diethyl ether-acetic acid (70:30:1, vol/vol/vol), and each fraction was eluted from the silica gel by using hexane–*tert*-butyl methyl ether (1:1, vol/vol).

The aqueous phase was neutralized with NaOH and analyzed by TLC on cellulose plates (MN-300; 10 by 20 cm; 0.1-mm thickness; Macherey-Nagel) 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 TLC plates with alkaline silver nitrate (15). The presence of glucose, galactose, and glycerol in the hydrolysates was determined by using glucose oxidase (Sigma), β-galactose dehydrogenase (Boehringer Mannheim), and glycerol kinase (Boehringer Mannheim) test kits, respectively. The presence of hexosamines was determined by high-performance liquid chromatography with a Gilson (Middleton, Wis.) ASTED system fitted with a Spherisorb octadecyl silane column (particle size, 5 μm; 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 410 nm, respectively). Nonhydrolyzed sugars and sugars hydrolyzed under the same conditions as the glycolipids were used as standards.

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

Derivatization. For GC-MS analysis, long-chain diols were converted to *O*-trimethylsilyl (*O*-TMS) ether or acetyl derivatives. Trimethysilylation was achieved by treatment of the dry samples with 100 μl of *bis*(trimethylsilyl)trifluoroacetamide for 30 min at 60°C. The reagent was removed with a vacuum centrifuge, and the derivatives were dissolved in trimethylpentane.

Purified long-chain diols were acetylated with 200 μl of a mixture of acetic anhydride-pyridine (1:1, vol/vol). The samples were allowed to stand at room temperature for 24 h, after which the reagents were removed by vacuum centrifugation. The perdeutero analogs were prepared by use of d_6-acetic anhydride in place of acetic anhydride.

For FAB MS analysis, intact glycolipids (5 to 50 μg) were peracetylated as described by Dell (3), using 100 μl of trifluoroacetic anhydride-acetic acid (2:1, vol/vol) for 10 min at room temperature. The reagent was removed by vacuum centrifugation, and the residue was dissolved in 1 ml of chloroform and desalted by being washed three times with equal volumes of water, the water washes being discarded. The chloroform was evaporated by vacuum centrifugation, and the derivatives were dissolved in 10 μl of chloroform-methanol (1:1, vol/vol) for FAB MS. Perdeuteroacetate derivatives were prepared as described above except that the acetic acid in the reaction mixture was replaced by d_4-acetic acid.

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. One minute after injection, the column oven temperature was programmed 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 (EI) spectra were recorded at an ionization energy of 70 eV, trap current of 100 μA, and 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.

FAB spectra were obtained on the same instrument, using an Ion Tech saddle-field atom gun supplied with high-purity xenon gas. Spectra of the underivatized glycolipids were recorded in both the positive- and negative-ion modes, using a mixture of (1:1, vol/vol) glycerol and dithiothreitol-dithioerythritol (5:1, wt/wt) as the liquid matrix. Approximately 10 μg of each sample was loaded onto the FAB 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 of 2,000 to 200. Peracetyl derivatives (1 to 5 μg) were analyzed with a 3-nitrobenzyl alcohol matrix. To promote sodium cationization of acetylated samples, 1 μl 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 of 3,000 to 200 at 10 s/decade. Sugar fragment ions are described by the nomenclature introduced by Domon and Costello (5).

Terminal residues of peracetylated glycolipids were identified by collision-induced dissociation of their B_1 carbenium ions, as previously described (2). Daughter ion spectra were recorded by scanning the magnetic field (B) and the electrostatic analyzer voltage (E) while maintaining a constant ratio of B to E. 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 perdeuteroacetylated hexoses (16, 25).

TABLE 1. Compositions of the polar head groups of the major glycolipids of strains of the genus *Thermus*

Strain/glycolipid	Composition (ratio)[a]			
	Glc	Gal	GluN	Gro
T. scotoductus				
X-1(t2)/GL-1	1.7	0.8	1.0	0.6
X-1(t1)/GL-2	0.9	0.9	1.0	0.6
T. oshimai				
SPS-11/GL-1	2.9	−	1.0	1.0
SPS-11/GL-2	2.0	−	1.0	1.0
Thermus sp. strain CG-2/GL-1	1.8	0.8	1.0	0.8
T. filiformis Tok4 A2/GL-1	2.1	1.0	1.0	0.6

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

RESULTS

Composition of GL-1 and GL-2. GL-1 constituted between 90 and 96% of the total polar lipid sugar in *T. oshimai* SPS-11, *Thermus* strain CG-2, *T. scotoductus* X-1(t2), and *T. filiformis* Tok4 A2, whereas GL-2 was a minor component ranging in concentration from 2 to 10%. However, in *T. scotoductus*, X-1(t1), GL-2 constituted about 93% of the total polar lipid sugar and GL-1 was present only in trace amounts (<3%).

GL-1 from *T. scotoductus* X-1(t2), *T. filiformis* Tok4 A2, and *Thermus* strain CG-2 contained glucose, galactose, and glucosamine in the ratio of 2:1:1. GL-2 of strains SPS-11 and X-1(t1) had a lower hexose-to-glucosamine ratio, consistent with the presence of one fewer glucose residues compared to GL-1 (Table 1).

The ratio of sugars to glycerol in the glycolipids from *T. oshimai* SPS-11, in *Thermus* strain CG-2 (Table 1), and in previously characterized *Thermus* glycolipids (2) indicated that GL-1 is predominantly glycerol linked. However, compositional analysis of the glycolipids from *T. filiformis* Tok4 A2 and both colony types of *T. scotoductus* X-1 detected less than the stoichiometric amount of glycerol expected for a glycerolipid, suggesting that a proportion of the glycolipids from these strains were not glycerol linked (Table 1).

Characterization of long-chain diols from *Thermus* spp. Acid methanolysis and GC indicated that the whole-cell fatty acid compositions of the organisms examined were typical of those previously reported for *Thermus* strains, comprising predominantly iso- and anteiso-branched 15:0 and 17:0 fatty acids (Table 2). *T. scotoductus* X-1(t1) and X-1(t2), and *T. filiformis* Tok4 A2 contained, in addition, moderate amounts of two late-eluting components with equivalent chain lengths (ECLs) of 19.060 (major peak) and 19.160 (minor peak), which were

TABLE 2. Fatty acid composition of the total polar lipids, glycolipids, and major phospholipids of some *Thermus* strains grown at 70°C

Fatty acid	Fatty acid composition (% of total)															
	T. scotoductus X-1(t1)			*T. scotoductus* X-1(t2)			*T. oshimai* SPS-11				*Thermus* strain CG-2			*T. filiformis* Tok4 A2		
	TPL[a]	GL-2	PL-2	TPL	GL-1	PL-2	TPL	GL-1	GL-2	PL-2	TPL	GL-1	PL-2	TPL	GL-1	PL-2
Straight chain																
14:0	0.2	0.2	0.2	0.2	0.2		0.2	0.5	0.3		0.2	0.2		0.2	0.3	0.4
15:0	1.1	0.7	0.6	1.0	1.3	0.9	2.0	2.2	2.3	1.2	0.9	0.8	0.6	0.4	0.5	0.4
16:0	1.7	0.9	1.5	1.6	1.3	1.7	1.8	1.5	1.5	3.0	1.7	1.3	1.4	2.3	2.0	3.1
17:0	0.9	0.5	0.5	0.8	0.7	0.7	0.9	1.0	0.7	0.6				0.3	0.2	0.3
18:0	0.2	0.1	0.1	0.2			0.4	0.4	0.5	0.7	0.1	0.1		0.6	0.2	0.7
Iso branched																
13:0	0.2	0.4	0.2	0.4	0.5	0.2	1.0	0.9	1.1		0.6	0.9	0.3	0.3	0.5	0.2
14:0	1.3	1.4	1.1	1.1	1.6	1.4	0.6	0.7	0.8	0.6	1.8	2.1	1.4	0.6	0.7	0.4
15:0	25.0	27.1	28.3	27.6	27.2	27.8	42.1	43.2	47.5	42.5	34.8	34.7	34.1	29.5	31.5	27.2
16:0	13.7	10.8	8.9	8.2	14.1	13.1	3.5	3.6	3.0	3.2	12.2	13.1	11.3	5.7	6.1	5.4
17:0	31.5	31.4	36.2	30.7	26.9	33.1	37.8	35.8	31.9	41.1	31.7	28.9	35.8	36.2	31.3	44.2
18:0	0.9	0.5	0.8	0.5	0.7	1.1					0.6	0.6	0.7	0.3	0.2	0.5
19:0	0.3	0.3	0.3	0.4	0.3	0.3	0.2				0.2	0.2	0.2			
Anteiso branched																
15:0	7.3	8.1	8.2	10.4	8.7	7.5	3.5	3.8	4.0	3.2	7.7	8.4	6.7	6.2	7.3	4.9
17:0	8.5	7.4	9.9	10.3	7.2	9.0	3.0	2.9	2.4	3.2	5.5	5.0	6.2	8.0	7.0	9.5
19:0		0.2	0.2													
Unknown																
ECL, 19.060	6.3	8.4	2.7	5.6	8.0	3.1	2.3	2.6	2.2	0.7	1.2	2.0	0.5	7.9	10.2	2.6
ECL, 19.160	0.6	0.8	0.2	0.7	0.8	0.2						0.1		0.5	0.6	

[a] TPL, total polar lipids.

detected only at low levels in strains CG-2 and SPS-11. Methanolysis of purified GL-1, GL-2, and PL-2 indicated that these components were predominantly associated with the glycolipids rather than the phospholipid (Table 2). In contrast to the FAMEs, trimethylsilylation of the two late-eluting components resulted in increased retention times (to ECLs of 19.832 and 19.925, respectively), suggesting the presence of hydroxyl or other derivatizable functional groups.

Abundant protonated molecules were observed at *m/z* 431 in the isobutane chemical ionization spectra of the TMS ethers of both compounds, suggesting that their molecular mass was 430. The EI mass spectra of their TMS ethers were effectively identical to each other (Table 3). No molecular ions were observed at the predicted mass of 430, but ions at *m/z* 415 were consistent with loss of a methyl group from the parent component, a process very characteristic of *O*-TMS derivatives.

TABLE 3. Summary of the mass spectra of the *O*-TMS ethers of long-chain diols from *T. scotoductus* and *Thermomicrobium roseum*

Composition	$[M + H]^{+a}$	M-15[b]	α-Cleavage ion[b]
T. scotoductus			
16-Methyl-1,2-heptadecanediol	431	415 (4)	327 (100)
15-Methyl-1,2-heptadecanediol	431	415 (4)	327 (100)
17-Methyl-1,2-octadecanediol	445	429 (3)	341 (100)
Thermomicrobium roseum			
1,2-Nonadecanediol	445	429 (4)	341 (100)
13-Methyl-1,2-nonadecanediol	459	443 (5)	355 (100)
1,2-Icosanediol	459	443 (3)	355 (100)
13-Methyl-1,2-icosanediol	473	457 (3)	369 (100)
1,2-Henicosanediol	473	457 (5)	369 (100)

[a] Protonated molecule from the chemical ion spectrum.
[b] Normalized relative intensity in parentheses.

The base peak at *m/z* 327 was assigned as an α-cleavage ion, which would be consistent with the presence of an *O*-trimethylsilylated alkyl chain of at least 17 carbon atoms [CH_3-$(CH_2)_{15}$ − $CHOSi(CH_3)_3$ = 327]. The spectra of the underivatized compounds were less informative. No molecular ion was present, the highest mass signal being observed at *m/z* 255; the only other fragments were members of a low-mass alkenyl ion series (C_nH_{2n-1}), which were absent beyond *m/z* 135.

These spectra do not resemble those of hydroxylated fatty acids but are consistent with long-chain diols containing 18 carbons, the masses of which would be 286 underivatized and 430 when trimethylsilylated. The published spectrum of the TMS ether of synthetic octadecane-1,2-diol is extremely similar to those of the two unidentified compounds from these *Thermus* strains (10).

Further purification by TLC resulted in a diol fraction largely free of fatty acids, and examination of this purified fraction by GC-MS indicated that two other minor diols were also present. The earlier-eluting of these minor diols had a base peak at *m/z* 313 (α-cleavage ion) and an M-15 ion at *m/z* 401, whereas the later-eluting component had a base peak at *m/z* 341 and M-15 at *m/z* 429, suggesting that they were C_{17} and C_{19} diols, respectively.

Long-chain diols have not previously been detected in *Thermus* species, but *Thermomicrobium roseum*, a thermophilic member of the green nonsulfur bacteria (GNS) which has an optimum growth temperature of about 70 to 75°C (12, 21), has been shown to contain a mixture of straight-chain and branched long-chain 1,2-diols in its polar lipids (22, 23). The spectra of the TMS ethers of the diols from the glycolipids of *T. scotoductus* X-1 and *T. filiformis* Tok4 A2 were therefore compared with those of authentic 1,2-diols obtained by acid methanolysis of *Thermomicrobium roseum* biomass. Four major late-eluting components were detected in *Thermomicro-*

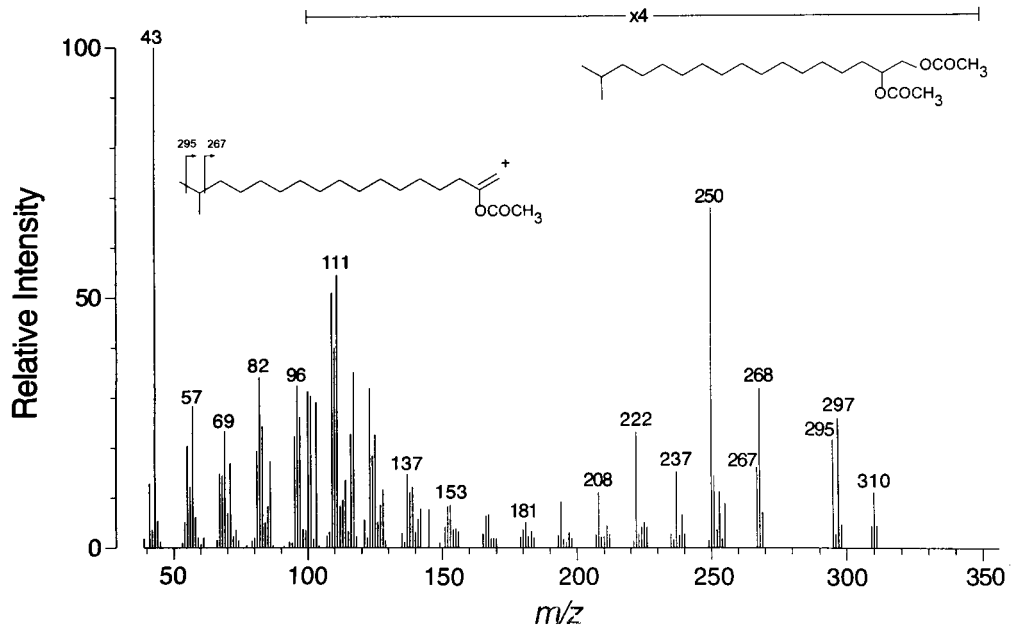

FIG. 1. EI mass spectrum of the acetate derivative of 16-methyl-1,2-heptadecanediol from GL-1 of *T. scotoductus* X-1(t2). The inset structure indicates the fragmentation of the M-60 ion, from which the iso and anteiso 1,2-diols can be distinguished. The absence of the fragment at *m/z* 281 indicates an iso-branched alkyl chain.

bium roseum, together with smaller amounts of three others (Table 3). Their elution order, relative proportions, and EI and chemical ionization mass spectra were consistent with those of the compounds reported by Pond et al. (23) and confirmed the identification of the compounds from *Thermus* strains as long-chain 1,2-diols. None of the compounds from *Thermomicrobium roseum*, however, matched the retention times of the diols from *Thermus* strains. Moreover, the mass spectra of the TMS ethers provided little information on the structure of the alkyl chains and in particular did not enable determination of the positions of methyl branches. It was therefore impossible to verify from these spectra if the octadecanediols from *Thermus* spp. were branched. Since the EI mass spectra of the acetates of 1,2-diols are more sensitive to variations in the structure of the alkyl chain (10), the acetate derivatives of the diols from *Thermus* were also analyzed by GC-MS (Fig. 1 and Fig. 2). No molecular ions were observed, but elimination of an acetate group accounts for the ion at *m/z* 310 (M-60). A very weak fragment at *m/z* 328 (M-42) due to loss of ketene (C_2H_2O) was also sometimes present. Combined losses of both acetate and ketene accounted for the ions at *m/z* 268 [(M-60)-42], while the abundant fragment at *m/z* 250 (M-2×60) was formed by loss of both acetate groups. The ion at *m/z* 297 (M-73) was attributable to cleavage between C-1 and C-2, expelling acetic acid forming a secondary fragment at *m/z* 237. These acetic acid and ketene elimination fragments were present at similar relative abundances in both compounds and afforded no information about the structure of the alkyl chain.

The structure of the alkyl chain was defined by an important group of ions at odd mass number (*m/z* 295, 281, 267, and 253) which were formed by cleavage, without rearrangement, of the terminal region of the alkyl backbone. The spectrum of the more abundant, earlier-eluting diol suggested that the alkyl chain was iso branched, because of the absence of the fragment at *m/z* 281 which can be formed only by cleavage of two bonds (Fig. 1). In the spectra of the unbranched diols from *Thermomicrobium roseum*, weak ions analogous to *m/z* 281 [(M-60)-29] were always present. In the spectrum of the later-eluting of the two diols from *Thermus*, the fragment at *m/z* 267 was absent (Fig. 2), suggesting an anteiso-branched structure. In support of this interpretation, the abundance of the fragment at *m/z* 281 was enhanced compared to straight-chain compounds, presumably because cleavage between C-15 and C-16 would result in a stabilized secondary carbon cation of this mass.

To confirm this interpretation, the diols were deuteroacetylated and their EI mass spectra were recorded. A deuteroacetyl group is 3 Da greater in mass than an acetyl group, and so ions in the mass spectra will be shifted by 3 *m/z* units (compared to the corresponding acetyl derivative) per incorporated deuteroacetyl group. Thus, the ions at *m/z* 310, 295, 281, 267, and 253 in the acetate spectra were shifted to *m/z* 313, 298, 284, 270, and 256, respectively, in the spectra of the deuteroacetate derivatives, showing that each contained a single acetate group. The fragment at *m/z* 284 was absent from the spectrum of the iso-branched diol, whereas that at *m/z* 270 was not present in the spectrum of the anteiso compound, which was consistent with the behavior of the acetate derivatives. Fragment ions which did not contain acetate groups, such as those at *m/z* 268, 250, and 222, were unaffected by deuteroacetylation. The ion attributable to combined loss of acetate and ketene (observed at *m/z* 268 in the acetate spectra) shifted to *m/z* 269 in the deuteroacetates, showing that, as expected, a single deuterium is retained in the charge-bearing fragment. The spectra of the acetates and deuteroacetates of the minor C_{17} and C_{19} diols indicated that these were also iso-branched species.

This structure-specific fragmentation of the alkyl chains of the acetate derivatives thus enables routine determination of methyl branch positions without the complex reduction procedure of Pond et al. (23). Taken together, these data show that the glycolipids from *T. scotoductus* X-1 and *T. filiformis* Tok4 A2 contain, as a major component, 16-methyl-1,2-heptade-

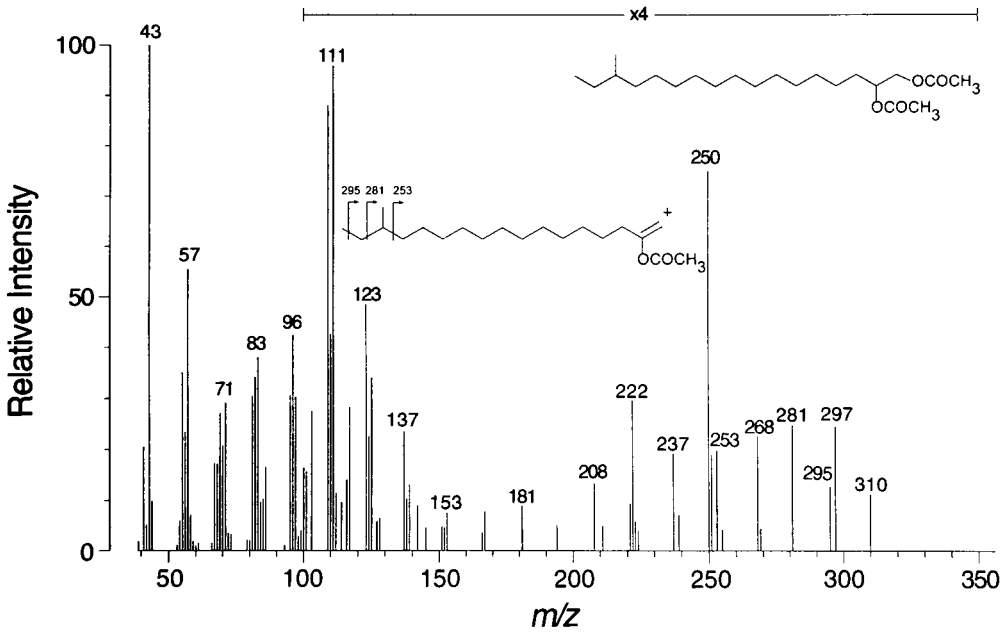

FIG. 2. EI mass spectrum of the acetate derivative of 15-methyl-1,2-heptadecanediol from GL-1 of *T. scotoductus* X-1(t2). The absence of a fragment at *m/z* 267 and the enhanced abundance at *m/z* 281 are consistent with an anteiso-branched alkyl chain.

canediol, lower levels of its anteiso-branched isomer, 15-methyl-1,2-heptadecanediol, and trace amounts of 15-methyl-1,2-hexadecanediol and 17-methyl-1,2-octadecanediol.

FAB MS of GL-1 and GL-2. Intact GL-1 from *T. filiformis* Tok4 A2, and GL-1 and GL-2 from *T. scotoductus* X-1(t1) and X-1(t2), were analyzed by FAB MS. Minor signals observed at *m/z* 1,439, 1,453, and 1,467 (nominal mass) in the negative-ion spectra (Table 4) were consistent with the presence of diglycosyl-(*N*-acyl)glycosylaminyl-glucosyl-diacylglycerols substituted with C_{15}, C_{16}, and C_{17} fatty acids (shown to be primarily iso branched by GC), as previously detected in the spectra of GL-1 from strains SPS-11 and CG-2 (2). However, the abundant signal at *m/z* 1,409 and those at *m/z* 1,395 and 1,381 would not be expected from glycosylated diacylglycerols with these sugar and acyl group compositions and were absent from the spectra of GL-1 from strains SPS-11 and CG-2. The negative-ion mass spectrum of GL-1 from *T. scotoductus* X-1(t2) was similar to the spectrum of *T. filiformis* Tok4 A2 except that the relative abundances of the glycosylglycerol species with deprotonated molecules at *m/z* 1,439.0, 1,453.0, and 1,466.9 were somewhat higher in relation to *m/z* 1,409, 1,395, and 1,381 (Table 4).

The molecular ions observed in the negative-ion spectrum of underivatized GL-2 from *T. scotoductus* X-1(t1) were 162 lower in mass than the corresponding ions in the spectrum of GL-1 (Table 4), suggesting that GL-2 was a truncated analog of GL-1, in which one hexose residue was missing. Since the FAB spectra of the underivatized samples did not unambiguously define their monosaccharide sequence, the glycolipids were reanalyzed after peracetylation. Signals at 1,937.1, 1,923.1, and 1,909.1 in the spectrum of GL-1 from *T. filiformis* Tok4 A2 (Fig. 3) were consistent with sodium cationization and addition of 12 acetyl groups to the species observed at *m/z* 1,409, 1,395, and 1,381 in the negative-ion spectrum. This was confirmed by the spectra of the perdeuteroacetyl derivatives, in which the corresponding sodiated molecules were observed at *m/z* 1,973, 1,959, and 1,945. The sequence of monosaccharide residues was indicated by glycosidic cleavage fragments, predominantly B-type carbenium ions (5). The terminal residue was hexose (from the B_1 glycosidic fragment at *m/z* 331), which was linked to hexose (from the abundant B_2 ion at *m/z* 619), which in turn was linked to *N*-acylated hexosamine (from the B_3 ions at *m/z* 1,088, 1,102, and 1,116). The inner residue was also hexose (from the weak B_4 ion at *m/z* 1,404). The B_3 ions at *m/z* 1,088, 1,102, and 1,116 were present in an abundance ratio of 0.5:0.5:1, showing that the hexosamine was *N*-acylated with about 50% C_{17} fatty acids, the balance being made up of C_{15} and C_{16} fatty acids in approximately equal amounts. In a previous study of *Thermus* glycolipids, it was shown that the relative proportions of *N*-linked fatty acids deduced from the FAB spectra agreed well with the results independently obtained from GC analysis of the hydrolyzed glycolipids (2).

GL-2 was identified as a triglycosyl analog of GL-1 with the sequence Hex-Hex*N*Acyl-Hex because of the presence of B-type carbenium ions at *m/z* 331 (B_1), 828 (B_2), and 1,116 (B_3) in the spectrum of the peracetylated material (Fig. 4). This interpretation was consistent with the spectra of the deuteroacetyl derivatives in which the B_1, B_2, and B_3 fragments were shifted to *m/z* 343, 848, and 1,143. The relative intensities of the B_2 ions at *m/z* 828, 814, and 800 indicated that the hexosamine was predominately *N*-acylated by C_{17} fatty acids (64%), the remainder comprising about 21% C_{15} and 15% C_{16} fatty acids.

The terminal monosaccharides of the glycolipids were identified by collisional activation of their B_1 carbenium ions (at *m/z* 331 and 343 in the acetyl and deuteroacetyl derivatives, respectively [2]). The resulting daughter ion spectra of the B_1 ions of acetylated and deuteroacetylated GL-1 from *T. scotoductus* were identical to those from authentic standards of glucopyranose, whereas those from GL-2 were in good agreement with galactopyranose.

These results, in conjunction with the sugar analysis and the FAB MS fragmentation data, established the glycan sequence of GL-2 of *T. scotoductus* X-1(t1) as Gal*p*-Glc*N*Acyl-Glc and GL-1 of *T. scotoductus* X-1(t2) as Glc*p*-Gal*p*-Glc*N*Acyl-Glc.

TABLE 4. Measured and calculated masses of molecular ions in the FAB spectra of glycolipids in strains of *T. filiformis* and *T. scotoductus*

Composition	$[M - H]^-$ (% RI) (observed)[a]	$[M - H]^-$ (calculated)[b]	$[M + Na]^+$ (% RI) (observed)[c]	$[M + Na]^+$ (calculated)[d]
GL-1 of *T. filiformis* Tok4 A2				
$(Hex)_2$-Hex*N*Acyl-Hex-LCD[e] $(15:0)_2$	1,380.9 (88)	1,380.93	1,909.1 (70)	1,909.06
$(Hex)_2$-Hex*N*Acyl-Hex-LCD (15:0) (16:0)	1,395.0 (39)	1,394.95	1,923.1 (48)	1,923.07
$(Hex)_2$-Hex*N*Acyl-Hex-LCD (15:0) (17:0)	1,408.9 (100)	1,408.97	1,937.1 (100)	1,937.09
$(Hex)_2$-Hex*N*Acyl-Hex-LCD (17:0) (16:0)	1,422.9 (32)	1,422.98	1,951.2 (35)	1,951.11
$(Hex)_2$-Hex*N*Acyl-Hex-Gro[f] $(15:0)_2$ (17:0)	1,438.9 (34)	1,438.94	1,967.1 (40)	1,967.06
$(Hex)_2$-Hex*N*Acyl-Hex-Gro (15:0) (16:0) (17:0)	1,452.8 (32)	1,452.95	1,981.1 (28)	1,981.08
$(Hex)_2$-Hex*N*Acyl-Hex-Gro $(17:0)_2$ (15:0)	1,466.8 (28)	1,466.97	1,995.1 (30)	1,995.09
$(Hex)_2$-Hex*N*Acyl-Hex-Gro $(17:0)_2$ (16:0)	ND[g]	1,480.10	2,009.0 (14)	2,009.11
GL-1 of *T. scotoductus* X-1(t2)				
$(Hex)_2$-Hex*N*Acyl-Hex LCD $(15:0)_2$	1,380.7 (55)	1,380.93	1,908.9 (48)	1,909.06
$(Hex)_2$-Hex*N*Acyl-Hex LCD (15:0) (16:0)	1,394.8 (73)	1,394.95	1,923.0 (60)	1,923.07
$(Hex)_2$-Hex*N*Acyl-Hex LCD (15:0) (17:0)	1,408.9 (100)	1,408.97	1,937.0 (100)	1,937.09
$(Hex)_2$-Hex*N*Acyl-Hex LCD (17:0) (16:0)	1,422.9 (65)	1,422.98	1,951.0 (38)	1,951.11
$(Hex)_2$-Hex*N*Acyl-Hex Gro $(15:0)_2$ (17:0)	1,439.0 (83)	1,438.94	1,967.1 (50)	1,967.06
$(Hex)_2$-Hex*N*Acyl-Hex Gro (15:0) (16:0) (17:0)	1,453.0 (65)	1,452.95	1,981.1 (33)	1,981.08
$(Hex)_2$-Hex*N*Acyl-Hex-Gro $(17:0)_2$ (15:0)	1,466.9 (72)	1,466.97	1,995.1 (52)	1,995.09
$(Hex)_2$-Hex*N*Acyl-Hex-Gro $(17:0)_2$ (16:0)	1,481.2 (40)	1,480.10	2,009.1 (20)	2,009.11
GL-2 of *T. scotoductus* X-1(t1)				
Hex-Hex*N*Acyl-Hex-LCD $(15:0)_2$	1,218.8 (40)	1,218.88	1,621.2 (37)	1,620.97
Hex-Hex*N*Acyl-Hex-LCD (15:0) (16:0)	1,232.7 (38)	1,232.90	1,635.2 (43)	1,634.99
Hex-Hex*N*Acyl-Hex-LCD (15:0) (17:0)	1,246.9 (100)	1,246.91	1,649.2 (100)	1,649.01
Hex-Hex*N*Acyl-Hex-LCD (17:0) (16:0)	1,260.9 (37)	1,260.93	1,663.3 (34)	1,663.02
Hex-Hex*N*Acyl-Hex-Gro $(15:0)_2$ (16:0)	1,262.8 (38)	1,262.87	1,665.3 (36)	1,664.96
Hex-Hex*N*Acyl-Hex-Gro $(15:0)_2$ (17:0)	1,276.8 (76)	1,276.89	1,679.3 (52)	1,678.98
Hex-Hex*N*Acyl-Hex-Gro (15:0) (16:0) (17:0)	1,290.9 (47)	1,290.90	1,693.3 (35)	1,693.00
Hex-Hex*N*Acyl-Hex-Gro $(17:0)_2$ (15:0)	1,304.9 (74)	1,304.92	1,707.3 (55)	1,707.01

[a] Measured masses of the deprotonated molecules in the negative-ion spectra of the underivatized glycolipids. RI, relative intensity.
[b] Calculated monoisotopic masses of the deprotonated molecules of the underivatized glycolipids.
[c] Measured masses of the sodium-cationized molecules in the positive-ion spectra of the peracetylated glycolipids.
[d] Calculated monoisotopic masses of the sodium-cationized molecules of the peracetylated glycolipids.
[e] LCD, long-chain diol.
[f] Gro, glycerol.
[g] ND, not detected above background.

Similar collisional activation experiments showed that the terminal residue of GL-1 of *T. filiformis* Tok4 A2 was glucopyranose, which, together with the compositional data in Table 1, indicated that the glycan head group was identical in sequence to that of GL-1 from *T. scotoductus* X-1(t2). The FAB MS results also showed that the polar head group of GL-2 of *T. oshimai* SPS-11 was a truncated version of GL-1 from the same strain (2) which lacked the terminal glucose residue, establishing its glycan sequence as Glc*p*-Glc*N*Acyl-Glc (results not shown).

The spectra of the acetate derivatives indicated that the monosaccharide sequence and fatty acid acylation patterns of the glycan head group of GL-1 from *T. scotoductus* X1 and *T. filiformis* Tok4 A2 were identical to those of GL-1 from *Thermus* strain CG-2 (2). However, as the *m/z* values of the major molecular ions of the glycolipids from the former two species differed from those of strain CG-2, it followed that the difference must reside in the lipid anchor. Since hydrolysis of the purified glycolipids liberated several previously uncharacterized long-chain diols, and less than the expected amount of glycerol was detected in the hydrolysates, a proportion of the glycan head groups of GL-1 and GL-2 in *T. filiformis* and *T. scotoductus* could be linked to acylated long-chain diols rather than to glycerol. A diglycosyl-(*N*-acyl)glycosaminyl-glucosyl-acyloctadecanediol, O-acetylated with pentadecanoic acid and N-acylated with heptadecanoic acid, would yield a deprotonated molecule at *m/z* 1,408.9 on negative-ion FAB MS and, after peracetylation, an $[M + Na]^+$ at *m/z* 1,937.0, in excellent agreement with the observed values. Calculated *m/z* values for other patterns of N- and O-acylation are listed in Table 4. These structures are supported by the presence of an abundant fragment ion at *m/z* 493 in the spectra of the peracetylated glycolipids, which is attributable to loss of the glycan head group from the lipid moiety and is consistent with a C_{18} diol O-acylated with a C_{15} fatty acid. The ions at *m/z* 507 and 521 are formed analogously from the corresponding C_{16} and C_{17} acylated species. In the case of GL-1 from *T. filiformis* Tok4 A2, the ratio of the relative abundances of *m/z* 493, 507, and 521 is 1.0:0.18:0.24, indicating that C_{15} fatty acids are the predominant *O*-acyl substituents, whereas the B_3 fragments derived from the glycan head group show that glucosamine is predominantly acylated with C_{17} fatty acids.

A higher proportion of glycerol-linked glycolipids are present in the samples from *T. scotoductus* X-1, and therefore the signals at *m/z* 523 and 551, which are acylglycerol fragments originating by loss of the polar head group, are more abundant. Structures for the major glycosylated acyl diols from *T. filiformis* Tok4 A2 and *T. scotoductus* X-1(t1) are proposed in Figs. 3 and 4.

DISCUSSION

Bacterial long-chain 1,2-diols were first identified by Pond et al. (23) in methanolysates of polar lipid fractions from *Thermomicrobium roseum*, in which it appears that they completely replace glycerolipids. These polar lipids were not characterized, but it was shown by mild alkaline saponification and

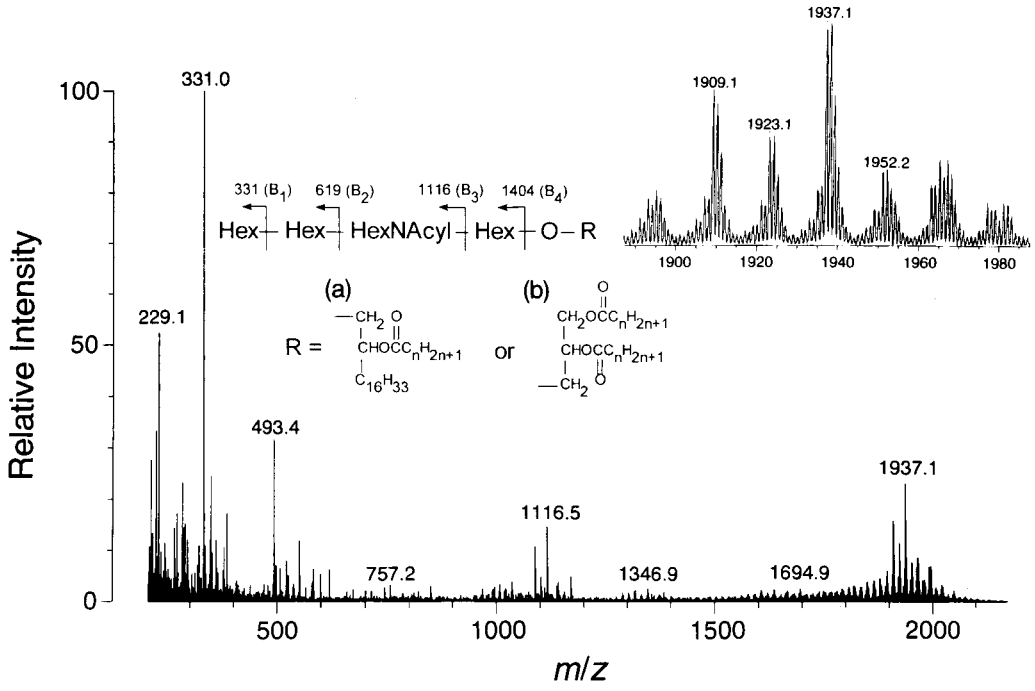

FIG. 3. Positive-ion FAB mass spectrum of peracetylated GL-1 from *T. filiformis* Tok4 A2. The inset shows an expansion of the $[M + Na]^+$ region. The structure diagram indicates that the glycan head group may be linked either to a long-chain diol (R = a) or to acylglycerol (R = b). The major species with $[M + Na]^+$ at *m/z* 1,937.1 is attributable to a diol-linked glycolipid in which the *N*-acyl substituent is 17:0 and the *O*-acyl group is 15:0.

permethylation that the polar head groups were linked to the C-1 hydroxyl group and the fatty acyl substituents were esterified to the C-2 hydroxyl group (22, 23).

The present study appears to be the first to determine the partial structures of intact diol-containing lipids in bacteria, and it seems likely that these glycosylated acyldiols are functionally equivalent to glycero-glycolipids, with the hydrophobic tail of the diol replacing one of the fatty acyl groups of the latter (22, 23). We also show that unlike *Thermomicrobium roseum*, some strains of the genus *Thermus* contain a mixture of diol- and glycerol-based glycolipids. The diol contents of the glycolipid of *T. scotoductus* X-1 and of GL-1 of *T. filiformis* Tok4 A2 appear to be about 40%, while glycerol-linked glycolipids constitute about 60% of the total. By contrast, GL-1 and GL-2 from strains SPS-11 and CG-2 have only minor amounts of diol-based lipids. We also show that the long-chain 1,2-diols from the *Thermus* strains examined are iso and anteiso branched, as are their fatty acids, while *Thermomicrobium roseum* has straight-chain and internally branched long-chain diols. We have not yet explicitly determined the relative positions of *O*-acyl substituents and the polar head groups, but we have provisionally assumed that the C-1 hydroxyl is glycosylated, by analogy with the partially characterized glycolipids from *Thermomicrobium roseum*.

The lipid compositions of several *Thermus* species have been extensively investigated, but the presence of long-chain diols has not been previously reported, possibly because they are not efficiently recovered by the saponification and transmethylation procedure used for the preparation of FAMEs and also because the diol concentration is very low in several of the strains examined (2, 24).

We have also determined the structures of GL-2 from two strains of the genus *Thermus* and show that in both cases, the terminal glucose found in GL-1 from the corresponding strain is absent. The terminal residue of GL-2 from *T. scotoductus* X-1(t1) was shown, by collisional activation MS, to be galactopyranose. Since the terminal galactose in previously characterized *Thermus* glycolipids invariably has the furanose configuration, whereas internal galactose residues have the pyranose form, it seems likely that the terminal galactose of GL-2 is equivalent to the subterminal residue of GL-1. We therefore hypothesize that *T. scotoductus* X-1(t1) is unable to attach glucose to the subterminal galactopyranose residue of GL-1, perhaps because of mutation in the gene coding for the appropriate glucosyltransferase, resulting in the accumulation of GL-2, its biosynthetic precursor. It seems unlikely that GL-2 is a degradation product of GL-1 because it almost completely replaces the latter glycolipid in *T. scotoductus* X-1(t1). Moreover, GL-2 of *T. oshimai* SPS-11 is also a truncated analog of the major glycolipid lacking the terminal hexose, reinforcing the view that GL-2 is a precursor of the normally predominant GL-1. Further investigation is necessary to ascertain the effect of the absence of GL-1 on the growth and physiology of this variant, which, other than forming smaller colonies on solid media, seems indistinguishable from *T. scotoductus* X-1(t2).

Long-chain diols have not thus far been identified in any bacterial species other than *Thermomicrobium roseum*, in which the polar lipids are apparently exclusively 1,2-diol linked (22, 23), and possibly in its relative, the moderate thermophile *Chloroflexus* sp. (31), although this latter report requires confirmation. The relationship of these highly unusual compounds to the ability of *Thermus* to grow at high temperatures is unclear. Many unusual lipids have been detected in the membranes of thermophilic bacteria, such as the alkylglycerol diethers (1,2-di-*O*-alkylglycerol), found in *Aquifex pyrophilus* and *Thermodesulfotobacterium commune* (11, 14) and the long-chain α,ω-dicarboxylic fatty acids of the order *Thermotogales*,

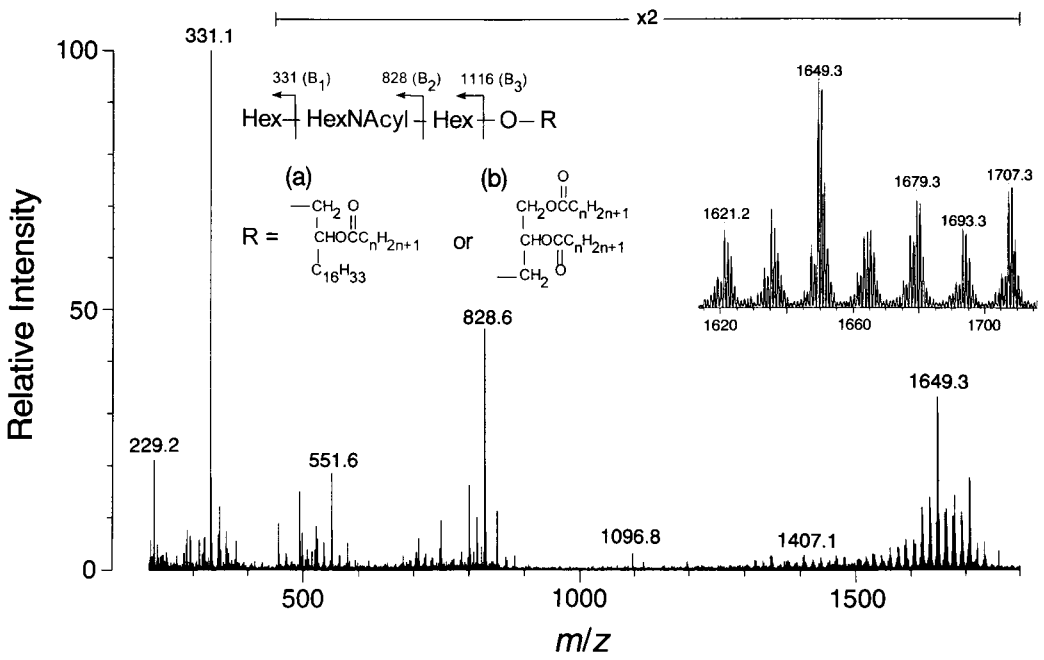

FIG. 4. Positive-ion FAB mass spectrum of peracetylated GL-2 from *T. scotoductus* X-1(t1). The inset shows an expansion of the $[M + Na]^+$ region. The structure diagram indicates that the glycan head group may be linked either to a long-chain diol (R = a) or to acylglycerol (R = b). The major species with $[M + Na]^+$ at *m/z* 1,649.2 is attributable to a diol-linked glycolipid in which the *N*-acyl substituent is 17:0 and the *O*-acyl group is 15:0.

although similar compounds can often also be found in mesophilic species (4, 13).

It seems unlikely that long-chain 1,2-diols represent a specific adaptation to growth at elevated temperatures, since *Thermus* strains in which diols are absent, or are present at low abundance, have the same optimum growth temperature as the strains in which diols are present. The occurrence of long-chain 1,2-diols in both strains of the genus *Thermus* and in *Thermomicrobium roseum* is, therefore, more likely to be the result of a phylogenetic affinity between them.

Several rRNA-based phylogenetic investigations indicate a distant relationship between the *Deinococcus/Thermus* and the GNS lines of descent (7, 9, 28, 31). However, Weisburg et al. (29) suggested that the clustering observed in the rRNA-derived phylogenies was an artifact caused by thermophilic convergence due to high G+C ratios of the rRNA. A more recent phylogenetic analysis of heat shock protein 70 sequences also questioned the relationship between *Thermus* and the GNS and placed *Thermomicrobium roseum* as an outgroup of a clade comprising chlamydia and proteobacteria, with no significant relatedness to the *Deinococcus/Thermus* phylum (8). Nevertheless, phylogenetic studies of the domain *Bacteria* based on the sequence of 16S rRNA place the GNS and the *Deinococcus/Thermus* phylum as separate but distantly related lines of descent or as sister lines of descent with a common origin (28, 31).

Caution is clearly required given the paucity of information about the distribution of long-chain diols among bacteria, but the presence of these highly unusual lipids in both *Thermus* and *Thermomicrobium* is certainly more consistent with those phylogenies in which the *Deinococcus/Thermus* and the GNS lines of descent form sister clades and may indicate a relationship between them. The identification of glycolipids that are simultaneously diol and glycerol linked also serves as a starting point for the investigation of the factors that lead to the preferential synthesis of one or the other type of glycolipid.

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