Structural characterization of diabolic acid-based tetraester, tetraether and mixed ether/ester, membrane-spanning lipids of bacteria from the order Thermotogales

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Abstract The distribution of core lipids in the membranes of nine different species of the order Thermotogales, one of the early and deep branching lineages in the Bacteria, were examined by HPLC/MS and demonstrated to consist of membrane-spanning diglycerol lipids comprised of diabolic acid-derived alkyl moieties. In the Thermotoga species the core membrane lipids are characterized by the presence of both ester and ether bonds, whereas in the phylogenetically more distinct Thermosipho and Fervidobacterium spp. only ester bonds occur. A tentative biosynthetic route for the biosynthesis of these membrane-spanning lipids is proposed. Since species of the order Thermotogales are assumed to have occurred early during the evolution of life on Earth, as suggested by its position in the phylogenetic tree of life, these data suggest that the ability to produce both ether and ester glycerol membrane lipids developed relatively early during microbial evolution.

Keywords Diabolic acid · Ether lipids · Ester lipids · Thermotoga · Thermosipho · Fervidobacterium

Abbreviations
APCI Atmospheric pressure chemical ionization
BSTFA N,O-bis(trimethylsilyl)trifluoroacetamide
DCM Dichloromethane
GDGT Glycerol dialkyl glycerol tetraethers
TLF Total lipid fraction

Introduction

The group of anaerobic bacteria falling in the order Thermotogales represents a very deep phylogenetic branch within the phylogenetic tree of life based upon the ribosomal RNA gene (Woese 1987). Within this order, members of the genus Thermotoga have an upper growth limit of 90°C and represent, together with species in the order Aquificales, cultivated bacteria with the highest known growth temperatures. Bacteria belonging to the Thermotogales occur widespread in the environment: they thrive within continental solfatara springs of low salinity, shallow and deep-sea marine hydrothermal systems and high-temperature marine and continental oil fields (see Huber and Hannig 2005 for a recent review). Their strict organotrophic way of life makes them consumers of microbial organic matter within high temperature ecosystems. Within the order Thermotogales, the genera Thermotoga, Thermosipho, Fervidobacterium, Geotoga, Petrovibrio and Marinitoga have been described, all belonging to the single family Thermotogaceae (Huber and Hannig 2005). The type species T. maritima for the genus Thermotoga, also the first identified member of the Thermotogales, was isolated from a geothermally heated, shallow marine sediment in Italy (Huber et al. 1986). Subsequently, quite a number of phylogenetically closely related (T. neapolitana, T. petrophila, T. naphtophila) and slightly more distant (T. thermarum, T. elfii, T. subterranea,
**Materials and methods**

**Cultures**

Cells were grown in 500-ml batch cultures in 1.2-l-bottles at temperatures of 55–80°C and with either medium A or B (see Table 1). Medium A was composed (per liter demineralized water) of KCl 0.355 g; MgCl₂·6H₂O 4.0 g; MgSO₄·7H₂O 3.45 g; NH₄Cl 0.25 g; K₂HPO₄ 0.14 g; NaCl 10.0 g; glucose 3.8 g; yeast extract 0.5 g; peptone 0.5 g; NaHCO₃·2.0 g; Na₂S·7·9H₂O 0.40 g; trace element solution, 10 ml and vitamin solution, 10 ml, which were based on medium 141 of DSM ([http://www.dsmz.de](http://www.dsmz.de)); CaCl₂·2H₂O, 0.140 g; resazurin, 0.5 mg. The medium was anaerobically dispensed into serum bottles and a gas phase of 180 kPa N₂/CO₂ (80/20 v/v) was applied. NaHCO₃, Na₂S·7·9H₂O, glucose, CaCl₂·2H₂O and vitamin solution were added after sterilization. The pH was 7.0–7.2. Medium B is a modified DSM medium 640 and composed (per liter demineralized water) of NH₄Cl 0.90 g; NaCl 0.90 g; MgCl₂·6H₂O 0.40 g; KH₂PO₄ 0.75 g; K₂HPO₄ 1.50 g; peptone 0.50 g; yeast extract 0.50 g; trace element solution SL-10 (see DSM medium 320), 1.00 ml; FeCl₃·6H₂O 2.50 mg; glucose 3.8 g; cysteine–HCl·H₂O, 0.75 g; resazurin 0.5 mg. The medium was anaerobically dispensed into serum bottles and a gas phase of 180 kPa N₂ (100%) was applied. Glucose and cysteine–HCl·H₂O were added after sterilization. The pH was adjusted to pH 7.2. The cultures were inoculated with cells from the DSM collection of microorganisms (see Table 1 for strain number) and were harvested in their exponential growth phase. Two different batches of *T. lettingae* were grown and analysed separately.

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**Table 1**  Bacteria from the order *Thermotogales* with some background information and the culture conditions used in this study

| Bacterial species         | Source for the isolate                  | Optimal growth temperature (°C) | Reference          | DSM strain number | Culture conditions | Medium*  | T (°C) |
|---------------------------|----------------------------------------|---------------------------------|--------------------|-------------------|--------------------|----------|--------|
| *Thermotoga maritima*     | Anaerobic marine sediment, Italy        | 80                              | Huber et al. (1986) | 3109              | A                  | 80, 55   |        |
| *T. neapolitana*          | Submarine hot spring, Italy             | 85                              | Jannasch et al. (1988) | 4359              | A                  | 80       |        |
| *T. elfi*                 | Oil-producing well, Africa              | 65                              | Ravot et al. (1995) | 9442              | A                  | 65       |        |
| *T. lettingae*            | Sulfate-reducing bioreactor, The Netherlands | 65                          | Balk et al. (2002)   | 14385             | A                  | 65       |        |
| *T. hypogea*              | Oil-producing well, Africa              | 70                              | Fardeau et al. (1997) | 11164             | B                  | 70       |        |
| *Thermosipho sp.*         | Spring, New Zealand                     | 65                              |                   | 6568              | B                  | 65       |        |
| *T. africanus*            | Marine hydrothermal spring, Obock       | 75                              | Huber et al. (1989) | 5309              | B                  | 75       |        |
| *T. melanesiensis*        | Deep sea hydrothermal vent, Pacific Ocean | 70                              | Antoine et al. (1997) | 12029             | B                  | 70       |        |
| *Fervidobacterium pennivorans* | Hot mud of spa, Italy                | 65                              | Koch et al. (1997)  | 9078              | B                  | 65       |        |

*a* See Materials and method sections for a description of the media used
Lipid analysis

Lyophilized cells were repeatedly (×5) ultrasonically extracted with dichloromethane (DCM)/methanol (MeOH) (2:1 v/v). The solvent of the combined extracts was removed by rotary evaporation at reduced pressure. The extract was taken up in DCM and weighed in a small vial by removing the solvent under a gentle stream of nitrogen. An aliquot (ca. 1 mg) of the extract to which a known amount of the internal standard, 2-methylheicosane, was added, was methylated with BF₃/MeOH, filtered over a small pipette filled with silica using ethyl acetate as the eluent, and subsequently silylated with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) in pyridine at 60°C for 15 min. These so-called total lipid fractions (TLF-1) were analysed with gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS). In selected cases, acetylation instead of silylation of the eluate obtained after chromatography over silica was performed following a procedure described elsewhere (Cox et al. 1972).

The cell residue after solvent extraction was, after addition of a known amount of the internal standard, 2-methylheicosane, hydrolysed with 1 N KOH/MeOH (96%) by refluxing for 1 h. The hydrolysate was neutralized with 2 N HCl/MeOH and after addition of water extracted with DCM. This fraction was methylated, chromatographed over silica and silylated as described above and the resulting TLF-2 fraction was analyzed by GC and GC–MS. In specific cases alkaline hydrolysis was also performed with an aliquot of the solvent extract following the same procedure as described above. This resulted in a fraction named TLF-1-OH which was also analyzed by GC and GC–MS.

For analysis of core lipids by high performance liquid chromatography/atmospheric pressure chemical ionization mass spectrometry (HPLC/APCI–MS) aliquots of the solvent extracts (ca. 2 mg) were separated over a small Al₂O₃ column using hexane/DCM (1:1 v/v) and DCM/MeOH as eluent to obtain apolar and polar fractions, respectively. The polar fraction was dissolved in hexane/propanol (99:1 v/v), filtered over an 0.4 μm PTFE filter, and analysed by HPLC/APCI–MS.

GC and GC–MS

GC was performed using a Fisons GC8000 instrument, equipped with an on-column injector and a flame ionization detector. A fused silica capillary column (25 m × 0.32 mm) coated with CP Sil-5 (d₁ 0.12 μm) was used with helium as carrier gas. The samples were injected at 70°C and the oven temperature was programmed to 130°C at 20°C/min and then at 4°C/min to 320°C, at which it was held for 10 min. GC–MS was performed on a HP 5890 gas chromatograph interfaced with a VG Autospec Ultima mass spectrometer operated at 70 eV with a mass range of m/z 40–800 and a cycle time of 1.7 s (resolution 1,000). The gas chromatograph was equipped with a fused silica capillary column as described for GC. The carrier gas was helium. The same temperature program as for GC was used.

HPLC/APCI–MS

Membrane-spanning core lipids were analysed by HPLC/APCI–MS according to Hopmans et al. (2000) with minor modifications. Analyses were performed on an Agilent 1100 series HPLC/MS instrument, equipped with an autoinjector and HP Chemstation software. Separation was achieved on an Alltech Prevail Cyan column (150 mm × 2.1 mm; 3 μm). Flow rate of the hexane/propanol 99:1 (v/v) eluent was 0.2 ml min⁻¹, isocratically for the first 5 min, thereafter with a linear gradient to 1.8% propanol in 45 min, subsequently to 10% propanol in 20 min, and subsequently isocratically for the last 20 min. Injection volume of the samples was usually 10 μl.

Results

Nine species of bacteria belonging to the order Thermotogales were analysed for their lipid composition; five of them are Thermotoga species (T. maritima, T. lettingae, T. elii, T. neapolitana, T. hypogea), three are Thermosiphon species (T. africanus, T. melanesiensis, T. sp.) and one is a Fervidobacterium species (F. pennivorans) (Table 1).

Composition of total lipid fractions

Figure 1a shows a typical gas chromatogram of a total lipid fraction (TLF-1) for T. maritima. In addition to straight-chain fatty acids (predominantly n-C₁₆ and n-C₁₄), a number of more unusual lipids were detected. The first group comprises long-chain dicarboxylic acids; 15,16-dimethyltriacontanedioic acid (or diabolic acid 1; see Fig. 2 for structures) and 13,14-dimethyloctacosanedioic acid (2), identified on basis of mass spectral data reported previously (Carballaire et al. 1997; Klein et al. 1979). The mass spectra of 1 and 2 as methyl ester derivatives are shown in Fig. 3. The relative abundance of the C₃₀ and C₃₂ diabolic acids varied substantially between different species (Table 2). F. pennivorans also contained small amounts of a tentatively identified C₃₄ diabolic acid. In some species, isomers of these diabolic acids, with virtually an identical mass spectrum but with a different retention time, were present in varying quantities. These probably represent stereoisomers, possibly related to the presence of two chiral centres in the middle of the carbon chain of the diabolic
The second group of unusual components comprises glycerol monoethers. 1-O-hexadecyl glycerol (3) and 1-O-tetradecyl glycerol (4) were identified by mass spectral data (m/z 205 base peak and appropriate molecular ion) and a co-elution experiment with an authentic standard. The higher molecular weight monoethers are 15,16-dimethyl-30-glyceryloxytriacontanoic acid (5) and 13,14-dimethyl-28-glyceryloxyoctadecanoic acid (6). Glycerol monoether 5 has been identified previously and the mass spectrum of 5 as acetyl derivative was reported (DeRosa et al. 1988). For identification purposes we used acetylation in addition to silylation to be able to compare the mass spectrum of the derivative of 5 with that reported in the literature (DeRosa et al. 1988). The mass spectrum of 5 as TMS derivative is shown in Fig. 3. Component 6 was tentatively identified by interpretation of its mass spectrum in comparison with that of 5 (Fig. 3). The relative amounts of monoethers 5 and 6 in the TLF-1 vary widely between the examined species of the Thermotogales (Table 2). As observed for the diabolic acids 1 and 2, two additional minor isomers with similar mass spectrometric characteristics but different retention times are observed. The third group of lipids are glycerol dialkyl diesters and glycerol mixed ester/ethers. They were predominantly found in the TLF-1 of T. lettingae (Fig. 4a) and are composed of 1,2- and 1,3-diacyl glycerols (e.g. 7 and 8) and 1-alkyl, 2-acyl (e.g. 9) and 1-alkyl, 3-acyl (e.g. 10) glycerols. They were identified by comparison with mass spectra reported in the literature (Wood et al. 1980). Some Thermotoga species contained small amounts of 1,2-di-O-alkyl glycerols.

The TLF after alkaline hydrolysis of the cell residue after extraction (TLF-2) contained a similar suite of lipids
as the TLF-1 (Table 2), albeit that some other components (e.g. unsaturated C\textsubscript{16:1} and C\textsubscript{18:1} fatty acids) were also present but only in the examined *Thermosipho* species. Most striking was that the characteristic long-chain diacids 1 and 2 and the complex monoethers 5 and 6 were relatively much more abundant in TLF-2 (Figs. 1c,d and 4b; Table 2), suggesting that they are predominantly formed by alkaline hydrolysis from larger lipid structures. A similar distribution was observed when the extract of *T. lettingae* (2nd batch) was subjected to alkaline hydrolysis (TLF-1-OH): the long-chain diacids 1 and 2 and the monoethers 5 and 6, which were not observed in the TLF-1 (Fig. 4a), are one of the most abundant lipids in the TLF-1-OH fraction (Fig. 4b). This indicates that these lipids are building blocks of larger, more complex lipids not amenable to GC.

The absolute amount of the most abundant lipids (in mg g\textsuperscript{-1} dry weight) was for a number of species measured in both the TLF-1 and TLF-2 (Table 3). This showed that the amounts released by alkaline hydrolysis from the residue after extraction are still substantial. It also showed that the highest concentrations occur in the *Thermotoga* species and in *F. pennivorans*, while concentrations in the *Thermosipho* species are substantially lower. Quantification of the lipids in the TLF-1-OH fraction of *T. lettingae* (2nd batch) showed that the concentration increases substantially after alkaline hydrolysis of the extract.

Distribution of membrane-spanning core lipids in *T. maritima*

To characterize the membrane-spanning core lipids of the species belonging to the *Thermotogales*, the polar fraction of the solvent extract was analysed by an HPLC/APCI–MS method (Hopmans et al. 2000) slightly adjusted to enable the elution of more polar components than glycerol dialkyl glycerol tetraethers (GDGTs) from the LC column (see Materials and methods for details). Analysis of the polar lipids of *Thermotoga maritima* revealed a complex composition exemplified by the base peak chromatogram (Fig. 5a). The first eluting, small peak with a retention time of 29.0 min (labelled 11a in Fig. 5b) showed an APCI mass spectrum (Fig. 6a) characterised by peaks at [M + H]\textsuperscript{+}, [M + H]\textsuperscript{+}-18 (loss of water) and [M + H]\textsuperscript{+}-74 (loss of glycerol). This characteristic pattern is well known from archaean GDGTs (Hopmans et al. 2000) and non-isoprenoidal GDGTs (Sinninghe Damsté et al. 2000), suggesting a tetraether structure. Indeed, upon alkaline hydrolysis of this fraction and re-analysis by HPLC/APCI–MS, this minor peak was now the most abundant peak in the base peak chromatogram. These data indicate a GDGT structure for the major peak comprised of two glycerol units connected by two C\textsubscript{32} alkyl chains, resulting in a molecular mass of 1077 (C\textsubscript{70}H\textsubscript{140}O\textsubscript{6}). Since 15,16-dimethyltriacontanedioc
Table 2  Relative distribution (in % of total quantified) of lipids in different species of the order Thermotogales

| Bacterial species                  | Fractiona | Lipidsb |
|------------------------------------|-----------|---------|
|                                    | C14:0 FA  | C16:0 FA | C18:0 FA | 1 | 2 | 3 | 4 | 5 | 6 |
| Thermotoga maritima (80°C)         | TLF-1     | 4.2     | 44.2    | 1.1 | 21.3 | 1.7 | 9.1 | 5.1 | 10.6 | 2.7 |
|                                    | TLF-2     | 1.7     | 25.9    | 0.9 | 38.7 | 4.2 | 2.2 | 1.0 | 17.6 | 7.8 |
| T. maritima (55°C)                 | TLF-1     | 7.5     | 54.7    | 0.5 | 14.3 | 2.3 | 11.1 | 2.5 | 6.6 | 1.2 |
|                                    | TLF-2     | 0.7     | 15.0    | 0.4 | 48.4 | 4.6 | 0.7 | 0.2 | 23.8 | 6.2 |
| T. neapolitana                     | TLF-1     | 4.6     | 61.3    | 2.8 | 17.3 | 1.3 | 3.7 | 0.7 | 7.1 | 1.2 |
|                                    | TLF-2     | 1.9     | 46.5    | 2.8 | 26.4 | 2.1 | 0.6 | 0.7 | 15.8 | 3.9 |
| T. elfii                           | TLF-1     | 3.5     | 61.0    | 4.0 | 28.2 | 2.6 | 0.5 | 0.1 | 0.2 |
|                                    | TLF-2     | 1.2     | 20.0    | 1.2 | 70.7 | 6.4 | 0.2 | 0.3 |
| T. lettingae (1st batch)           | TLF-1     | 3.0     | 37.2    | 5.9 | 24.6 | 5.2 | 6.9 | 3.9 | 8.3 | 5.0 |
|                                    | TLF-2     | 0.8     | 11.6    | 1.0 | 48.3 | 7.8 | 2.2 | 0.9 | 18.7 | 8.7 |
| T. lettingae (2nd batch)           | TLF-1-OH  | 6.3     | 26.4    | 1.1 | 32.6 | 8.8 | 4.8 | 5.8 | 8.5 | 5.6 |
|                                    | TLF-2     | 0.5     | 7.4     | 0.3 | 57.6 | 14.1 | 0.4 | 0.2 | 11.8 | 7.7 |
| T. hypogea                         | TLF-1     | 4.8     | 54.5    | 2.3 | 14.1 | 2.3 | 7.4 | 3.5 | 8.1 | 3.0 |
|                                    | TLF-2     | 0.5     | 4.5     | 1.0 | 47.4 | 1.5 | 0.6 | 0.1 | 33.3 | 11.1 |
| Thermosipho sp.                    | TLF-1     | 7.9     | 51.5    | 28.0 | 12.6 |
|                                    | TLF-2     | 2.6     | 16.0    | 5.0 | 66.8 | 9.6 |
| T. africanus                       | TLF-1     | 4.4     | 57.6    | 32.5 | 2.7 |
|                                    | TLF-2     | 2.5     | 61.8    | 8.1 | 25.4 | 2.2 |
| T. melanesiensis                   | TLF-1     | 5.5     | 62.1    | 30.8 | 1.6 |
|                                    | TLF-2     | 5.6     | 84.7    | 7.1 | 2.6 |
| Fervidobacterium penivorans        | TLF-1     | 1.0     | 54.1    | 9.5 | 35.1 | 0.3 |
|                                    | TLF-2     | 0.2     | 13.5    | 3.0 | 82.3 | 1.0 |

a TLF-1 Total lipid fraction, TLF-2 total lipid fraction obtained after alkaline hydrolysis of the residue after extraction;
TLF-OH Total lipid fraction after alkaline hydrolysis of the extract

b Numbers refer to Fig. 2. Blank entries indicate that the lipid was not detected

Acid (1) and 15,16-dimethyl-30-glyceryloxytriacantoic acid (5) are abundant lipids in the TLF-1 of T. maritima, it seems likely that the alkyl chains in this GDGT are the same, resulting in structure 11a. At the flank of peak 11a, at slightly higher retention time, a similar mass spectrum was observed with all ions shifted by 28 Da to lower m/z values. This compound is likely a GDGT with a C13,14-dimethyl alkyl chains (12a), consistent with the presence of small amounts of 13,14-dimethyloctacosanedioic acid (2) and 13,14-dimethyl-28-glyceryloxyoctadecanoic acid (6) in the TLF-1 (Table 2).

The first large peak in the base peak chromatogram at a retention time of 33.0 min (labelled 11b or 11c in Fig. 5c) shows an APCI mass spectrum (Fig. 6b) in which all ions are shifted by 14 Da to higher m/z values relative to those in the mass spectrum of GDGT 11a (Fig. 6a). In addition, the [M + H]+-18 peak is substantially increased relative to the [M + H]+ peak and an [M + H]+-74 peak is also observed. Since this peak disappears when the fraction is subjected to alkaline hydrolysis, this component is likely to contain an ester moiety. This information, together with its molecular mass, indicates that this component is structurally related to GDGT 11a but with one of the ether bonds replaced by an ester bond: a glycerol dialkyl glycerol triether/monoester with two C32 dimethyl alkyl chains (11b), resulting in a molecular mass of 1091 (C70H138O7). The presence of the ester moiety likely results in the enhanced [M + H]+-18 peak in its APCI mass spectrum. Mass chromatography of m/z 1,074 (Fig. 5c), the abundant [M + H]+-18 peak, reveals that there is an additional, much later eluting isomer with an identical mass spectrum. This could relate to the position of the ester moiety in this type of components: with the parallel configuration of the glycerol groups, as indicated in 11, there are two possible isomers (11b and 11c). However, it is at present unclear why these positional isomers would differ to a large degree in polarity (i.e. the second isomer elutes much later and is, thus, much more polar than the first one). At the flank of the peak of the triether/monoester a component elutes with a mass spectrum similar to that of 11b but with all the major ions shifted by 28 Da to lower m/z values. This component is the triether/monoester with C32 and C30 dimethyl alkyl chains (12b). Mass chromatography of m/z 1,046 (Fig. 5d), the abundant [M + H]+-18 peak of this component, reveals a second, much later eluting isomer for this component as well.

The most abundant peak in the base peak ion chromatogram at a retention time of 37.0 min (Fig. 5a) shows a mass spectrum similar to that of the triether/monoester 11b but in which all major ions are shifted by 14 Da to higher
m/z values (cf. Fig. 6b and c). This component likely represents a glycerol dialkyl glycerol diether/diester with two C_{32} dimethyl alkyl chains (11d), resulting in a molecular mass of m/z 1,105 (C_{70}H_{136}O_{8}) and an [M + H]^+ of m/z 1,088. This component is composed of two esterified 15,16-dimethyl-30-glyceryloxytriacontanoic acid (5) units. Moreover, mass chromatography of m/z 1,060 (Fig. 5f) reveals the presence of a much later eluting isomer with similar mass spectra. This latter cluster of peaks contains co-eluting components with a mass spectrum with peaks at m/z 1,120, 1,106, 1,046 and 1,028, presumably the corresponding glycerol dialkyl glycerol monoether/triester with two C_{32} dimethyl alkyl chains (11e). Mass chromatograms of m/z 1,074 (Fig. 5h) and m/z 1,102 (Fig. 5g) show the presence of a monoether/triester with two C_{32} dimethyl alkyl moieties (11e) and C_{32} and C_{30} building blocks (12e) and much later eluting isomers for both components. These isomers possess identical mass spectra (e.g. Fig. 6d). Finally, small amounts of tetraesters with two C_{32} dimethyl alkyl moieties (11f) and a C_{32} and a C_{30} dimethyl alkyl moiety were detected by mass chromatography of m/z 1,116 (Fig. 5i) and m/z 1,088 (Fig. 5j), respectively. A typical mass spectrum is shown in Fig. 6e. In these traces three distinct isomers were observed.

The distribution of membrane-spanning lipids in T. maritima is not depending on the growth temperature; cells grown at 80 and 55°C showed almost the same distribution.

Distribution of membrane-spanning core lipids in T. lettingae

The distribution of the membrane-spanning lipids in T. lettingae was quite different (Fig. 7) from that observed in T. maritima: it was dominated by the diether/diesters with the components with two C_{32} dimethyl alkyl moieties (12d) and those with a C_{32} and a C_{30} dimethyl alkyl chain (12d; m/z 1,060; Fig. 7c) in approximately equal abundance. Diether/diesters with two C_{30} building blocks are present in relatively small amounts as revealed by the m/z 1,032 mass chromatogram (Fig. 7d). This is consistent with the substantially higher abundance of the C_{30} diabolic acid (2) relative to the C_{32} diabolic acid (1).
and the C₃₃ glycerol monoether 6 relative to the C₃₅ glycerol monoether 5 in the TLF-2 fraction of T. lettingae when compared to T. maritima (Table 2). The relatively high abundance of lipids with a C₃₂ and a C₃₀ dimethyl and, to a lesser extent, with two C₃₀ dimethyl alkyl chains is also observed for the monoether/triesters (Figs. 7e–g) and tetraesters (Figs. 7h–j). Tetraethers (e.g. 11a), which were present in small quantities in T. maritima, were not encountered in T. lettingae.

An additional cluster of early eluting peaks not observed in the HPLC/MS analysis of the extract of T. maritima is present in the base peak chromatogram of the polar fraction of T. lettingae (Fig. 8a). The mass spectra of the peaks comprising this cluster are characterized by one dominant fragment ion which is 2 Da higher than that of the [M + H]+-18 peak of the diether/diesters (cf. Fig. 6c). Mass chromatography of m/z 1,090, 1,062 and 1,034 (Fig. 8b–d) reveals that this cluster of peaks is build up in a similar way as the clusters reflecting the diether/diesters, ether/triesters and tetraesters (cf. Fig. 7). These mass chromatograms also reveal that these compounds are accompanied by a later eluting isomer which possess similar mass spectra. These components likely represent glycerol trialkyl glycerol diether/diesters (13d) comprising one long-chain C₃₂ or a C₃₀ dimethyl alkyl chain and two shorter linear (predominantly C₁₆) alkyl chains. It is well known from archaeal GDGT analyses that so-called “open” or acyclic (in comparison to the macrocyclic components described so far) GDGTs elute substantially earlier under these LC conditions (and are, thus, less polar) and possess APCI mass spectra that do not show an [M + H]+-74 ion resulting from the loss of a glycerol moiety (Hopmans et al. 2000). Instead, they easily loose one of the shorter alkyl chains upon APCI. Indeed, when the polar fraction of T. lettingae was analysed using an extended mass range (m/z 100–1,400), an abundant ion at m/z 792 was observed, which is likely formed by loss of a C₁₆ moiety. Mass chromatography of ions 2 Da higher than used to detect the monoether/triesters and tetraesters reveals the presence of acyclic monoether/triesters and tetraesters (Fig. 8e–g) and tetraesters (Fig. 8h–j).

Fig. 5 HPLC/APCI–MS base peak chromatogram (a) and specific mass chromatograms (b–j) of polar lipids of Thermotoga maritima grown at 55°C. The mass chromatograms, indicative for specific components, are m/z 1.078 (b [M + H]+ C₇₀ tetraether), m/z 1.074 (c [M + H]+-18 C₇₀ triether/monoester), m/z 1.046 (d [M + H]+-18 C₆₈ triether/monoester), m/z 1.088 (e [M + H]+-18 C₇₀ diether/diester), m/z 1.060 (f [M + H]+-18 C₆₈ diether/diester), m/z 1.102 (g [M + H]+-18 C₇₀ monoether/triester), m/z 1.074 (h [M + H]+-18 C₆₈ monoether/triester), m/z 1.116 (i [M + H]+-18 C₇₀ tetraester), m/z 1.088 (j [M + H]+-18 C₆₈ tetraester). Filled and blocked peaks indicate membrane-spanning lipids based on C₃₂/C₃₂ and on C₃₂/C₃₀ branched alkyl chains, respectively. Note that some mass chromatograms are shown twice as they reveal the presence of different components

Distribution of membrane-spanning core lipids in F. pennivorans

Membrane-spanning lipids were also detected in F. pennivorans (data not shown). In this case the distribution was relatively simple: only tetraesters with predominantly two C₃₂ dimethyl alkyl chains (11f) were detected in a distribution similar to those observed in T. maritima (Fig. 5i) and T. lettingae (Fig. 7h), i.e. dominated by three isomers. Smaller amounts of tetraesters with a C₃₂ and a C₃₄ dimethyl alkyl chain were also detected. In addition, the TLF-1 also contained a glycerol esterified with two 15,16-dimethyltriacontanediolic acid moieties or 15,16-dimethyltriacontanediolic acid and 15,16-dimethyldotriacontanediolic acid.
Discussion

Our results indicate that the diabolic acids and their ether derivatives previously identified in members of the *Thermotogales* (DeRosa et al. 1988; Windberger et al. 1989; Jeanthon et al. 1995; Huber et al. 1986) are synthesized to produce membrane-spanning lipids and probably represent biosynthetic intermediates. The relative concentration of the diabolic acids and their ether derivatives is substantially higher in the extract obtained after alkaline hydrolysis of the extracted cell residue (TLF-2) (Table 2). The HPLC/APCI–MS results clearly revealed the presence and, in some cases, the structural complexity of the membrane-spanning core lipids in species of the order *Thermotogales* for the first time. Previous investigators (Clarke et al. 1980) have suggested that diabolic acids may act as linkers between two glycerol moieties in polar membrane lipids of *Butyrivibrio* spp. It is generally thought that the production of membrane-spanning lipids in prokaryotes is an adaptation to high growth temperature, although *Butyrivibrio* is a mesophile. The ubiquitous presence of diabolic acid-based membrane-spanning lipids in members of the *Thermotogales*, which are all (hyper)thermophiles, is in line with this general idea (Table 2). However, the distribution of membrane-spanning lipids in *T. maritima* is not directly affected by the temperature of growth: at both 80 and 55°C diabolic acid-based membrane-spanning lipids were produced.

Despite the overall similarity in the lipid composition, there is a clear dichotomy: all members of the genus *Thermotoga* produce ether lipids (i.e. 3–6, 9–11e, 12a–12e, 13a–13e) in addition to ester lipids, whereas ether lipids are absent in the genera *Thermosipho* and *Fervidobacterium* (Table 2). For example, the HPLC/APCI–MS results showed only the presence of tetraesters in *F. pennivorans*, whereas the *Thermotoga* species investigated showed a wide variety of mixed ester/ether membrane-spanning core lipids (Figs. 5, 7, 8). This suggests that only *Thermotoga* species possess enzymes to produce ether linkages. This classification based on lipid biochemistry is in good agreement with the phylogenetic classification based on the sequence of the 16S rRNA gene (Fig. 9), which reveals that the genera *Thermosipho* and *Fervidobacterium* are quite distinct from the genus *Thermotoga*, although the phylogenetic diversity within the *Thermotoga* cluster is also substantial.

The composition of the low-molecular-weight lipids (i.e. fatty acids, glycerol monoethers 3 and 4), higher molecular-weight lipids (1–2, 5–6) and the membrane-spanning lipids suggest a biosynthetic relationship between these different lipid classes although the relatively high concentrations of
lipids 1–6 also suggest a direct functional role. It has been demonstrated that diabolic acids are produced from condensation reactions of fatty acids at the ω-1 positions (Fitz and Arigoni 1992). These authors showed by the use of [16-2H3]palmitic acid and [14-2H2]palmitic acid that unsaturated fatty acids are involved in this condensation reaction and that it may be mediated by a free radical reaction. In good agreement with the proposed biosynthetic pathway.
for diabolic acids is that in all species investigated in our study the C\textsubscript{16:0} fatty acid is the major fatty acid and 15,16-dimethyltriacontanedioic acid (1), composed of two condensed C\textsubscript{16:0} fatty acid moieties, is the major diabolic acid (Table 2). In cases where the C\textsubscript{34} and C\textsubscript{30} diabolic acids are higher in concentration (e.g. \textit{F. pennivorans}, and \textit{T. lettingae} and \textit{Thermosiph sp.}, respectively) also a concomitant increase of the required monomer, i.e. the C\textsubscript{18:0} and C\textsubscript{14:0} fatty acids, respectively, is observed (Table 2). This is also seen in the presumed biosynthetic end-products, i.e. the membrane-spanning core lipids. For example, \textit{T. lettingae} contains relatively more of these lipids based on C\textsubscript{30} (2) diabolic acids, whereas in \textit{F. pennivorans} the C\textsubscript{34} diabolic acid is a more important building block. This is consistent with the published observation that during growth of \textit{T. maritima} in batch culture the concentration of the C\textsubscript{16:0} fatty acid decreased with a concomitant increase of tentatively identified diabolic acid (Zhang et al. 2002). This was interpreted to reflect the result of synthesis of diabolic acid using the C\textsubscript{16:0} fatty acid. The same observations can be made for the ether lipids only encountered in the \textit{Thermotoga} species. The presence of mono alkyl glycerol ethers in all \textit{Thermotoga} species (Table 2) and mixed glycerol ether/esters in \textit{T. lettingae} (2nd batch) (Fig. 4a) suggests that formation of the ether bond may also occur before the condensation reaction, resulting in the synthesis of membrane-spanning core lipids, takes place. In archaea, GDGTs are also thought to be produced from (partial) condensation of two glycerol diethers (Nishihara et al. 1989; Kon et al. 2002; Koga et al. 1993).

Generally, the distribution of the membrane-spanning lipids is quite complex in the species from the \textit{Thermotogales}. It is noteworthy that several isomers with a quite different polarity were detected in the HPLC traces. The structural composition of the diacyl glycerols and the mixed glycerol ester/ethers in \textit{T. lettingae} perhaps provides a clue to the observed complex composition of the membrane-spanning core lipids. Our analyses revealed that the ester moiety in these lipids can be at both at position 2 and 3 of the glycerol unit when the ether or ester moiety is at position 1 (Fig. 4a). It is quite likely (although this remains to be proven) that this also holds for membrane-spanning core lipids. This introduces a wide variety of structural isomers, which may explain why more than one peak was detected in the various HPLC mass chromatograms (Figs. 5, 6 and 8).

Our data show that some members of the \textit{Thermotogales} (i.e. the \textit{Thermotoga} genus) produce membrane-spanning lipids which are comprised of mixed glycerol ether/ester derivatives, whereas all examined species produce membrane-spanning diabolic acids. Mixed glycerol ether/ester lipids have been increasingly recognized in the Bacterial Kingdom, e.g. in \textit{Planctomycetes} capable of anaerobic ammonium oxidation (Sinninghe Damsté et al. 2002; Sinninghe Damsté et al. 2005), in deep-branching thermophilic bacteria such as \textit{Aquibex} (Huber et al. 1992) and in two mesophilic sulfate-reducing bacteria (Rütters et al. 2001). The tetraethers 11a and 12a, which occur in low amounts in \textit{T. maritima}, are, to the best of our knowledge, the first example of this kind of lipid in bacteria. Non-isoprenoidal GDGTs have been detected before in peats (Sinninghe Damsté et al. 2000) but their biological origin is as yet unknown. Membrane spanning lipids are quite common in the Kingdom Archaea (Koga et al. 1993) but have also been identified in a very limited number of bacteria, e.g. in \textit{Butyribrio} spp. (Clarke et al. 1980), and in \textit{Thermoanaerobacter} species (Jung et al. 1994). It is tempting to associate the peculiar membrane biochemistry of the order \textit{Thermotogales} to its position in the phylogenetic tree of life, i.e. close to the root of the tree (Woese et al. 1990). The unusually numerous archaeal-like genes (almost 25%) found in the \textit{Thermotoga maritima} genome (Nelson et al. 1999) reflect that these are deep-branching bacteria. If species from the order \textit{Thermotogales} occurred early during the evolution of life on Earth, as suggested by its position in the phylogenetic tree of life, our results indicate that the ability to

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**Fig. 9** Phylogenetic tree based on the 16S rRNA gene outlining the relationship between molecular phylogeny and lipid composition for the studied species of the \textit{Thermotogales}. The neighbor-joining tree was reconstructed from distance matrices and \textit{Escherichia coli} served as outgroup. The bar indicates an evolutionary distance of 0.10. Since no 16S rRNA gene sequence is available for \textit{F. pennivorans}, two other \textit{Fervidobacterium} species were used in the construction of the tree. Gene Bank accession numbers are given in parentheses.
produce both ether and ester glycerol membrane lipids developed relatively early during evolution. All Archaea produce ether membrane lipids, whereas Bacteria and Eukaryotes typically produce glycerol esters. Some members of the Thermotogales, thus, represent a hybrid in this way.

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