Ether- and ester-bound *iso*-diabolic acid and other lipids in members of *Acidobacteria* subdivision 4

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As published in: *Appl. Environm. Microbiol.* **80**, 5207-5218 (2014).

Abstract

Recently, *iso*-diabolic acid (13,16-dimethyl octacosanedioic acid) has been identified as a major membrane-spanning lipid of subdivisions 1 and 3 of the *Acidobacteria*, a highly diverse phylum within the *Bacteria*. This finding pointed to the *Acidobacteria* as a potential source for the bacterial glycerol dialkyl glycerol tetraethers that occur ubiquitously in peat, soil, lakes and hot springs. Here we examined the lipid composition of seven phylogenetically divergent strains of subdivision 4 of the *Acidobacteria*, a bacterial group that is commonly encountered in soil. Acid hydrolysis of total cell material released *iso*-diabolic acid derivatives in substantial quantities (11-48% of all fatty acids). In contrast to subdivisions 1 and 3 of the *Acidobacteria*, 6 out of the 7 species of subdivision 4 (excepting '*Candidatus* Chloracidobacterium thermophilum') contained *iso*-diabolic acid ether-bound to a glycerol in larger fractional abundance than *iso*-diabolic acid itself. This is in agreement with the analysis of intact polar lipids (IPLs) by HPLC/MS, which showed the dominance of 'mixed' ether-ester glycerides. *Iso*-diabolic acid-containing IPLs were not identified because these IPLs are not released with a Bligh-Dyer extraction, as observed before when studying lipid compositions of subdivisions 1 and 3 of the *Acidobacteria*. The presence of ether bonds in the membrane lipids does not seem to be an adaptation to temperature, because the five mesophilic isolates contained a higher amount of ether lipids than the thermophile '*Ca*. C. thermophilum'. Furthermore, experiments with *Pyrinomonas methylaliphatogenes* did not reveal a major influence of growth temperature over the 50-69°C range.

Introduction

Isoprenoidal ether lipids ubiquitously occur in the membrane lipids of *Archaea* (1), but occasionally ether lipids are also detected in the bacterial domain albeit with non-isoprenoidal chains (2,3). Unusual glycerol dialkyl glycerol tetraethers (GDGTs) with *n*-alkyl chains containing 2-3 methyl groups instead of isoprenoidal chains (so-called branched GDGTs; brGDGTs; e.g. structures **1** and **2** in Fig. 1) were identified for the first time in peat more than a decade ago (4) and subsequently turned out to occur ubiquitously in soil, peat, lake water and sediments, river water and sediments, and coastal marine sediments (5). BrGDGTs have also been observed in thermophilic environments such as terrestrial hot springs(6), where they are believed to be produced in situ by themophilic bacteria (7,8). Despite their widespread occurrence and potential applications in geochemistry and paleoclimatology (5), their microbial source is still unclear. The assessment of the stereochemistry of the glycerol units in brGDGTs revealed that it is opposite of that of archaeal isoprenoidal GDGTs, suggesting that they must derive from *Bacteria* (9). A heterotrophic lifestyle of the source organism(s) of brGDGTs was suggested based on their natural stable carbon isotopic composition in peat (10) and soil (11) and "natural labelling experiments" (11,12). The environmental abundance of *Acidobacteria* has led to the suggestion that these bacteria may be the biological source of the brGDGTs (13). This hypothesis was recently supported by membrane lipid analysis of 13 species of subdivisions (SDs) 1 and 3 of the *Acidobacteria,* which showed that the uncommon membrane-spanning lipid, 13,16-dimethyl octacosanedioic acid (*iso*-diabolic acid; **3**) is a major lipid in all species studied (14). This lipid can be considered as a building block of the brGDGTs but occurs predominantly ester- and not ether-bound in the SD 1 and 3 *Acidobacteria*. In three of the 13 analyzed strains, small amounts of ether-bound *iso*-diabolic acid, including brGDGT **1,** were detected after hydrolysis of the cells. However, the brGDGT distribution in soils is much more complex, and the presence of additional (acido)bacteria might explain the presence of the full complement of brGDGTs in the environment.

Acidobacteria are a highly abundant and diverse phylum of the domain *Bacteria* (15-20). For example, a recent study of bacterial abundance of peat layers of a Siberian wetland using pyrosequencing of 16S rRNA genes revealed that 35-40% of the reads were from *Acidobacteria* (21). Using similar methods, the abundance of *Acidobacteria* in organic matter-rich, low-pH soils was reported to be over 60% (22). Because known whole genomes of *Acidobacteria* only contain one copy of the 16S rRNA gene, in contrast to many other bacteria, their abundance may even be underestimated by these methods (23). The *Acidobacteria* have been divided in 26 subdivisions (SDs), mainly based on environmental sequences (24), but only six of these contain taxonomically characterized representatives. For SD 1, eight genera have been defined: *Acidobacterium* (25), *Acidicapsa* (26), 'Acidipila' (27), *Bryocella* (28), *Edaphobacter* (29), *Granulicella* (30,31), *Telmatobacter* (32), and *Terriglobus* (33,34), while only 1-3 genera have been characterized for SDs 3 [*Bryobacter* (35)], 8 [*Holophaga* (36), *Geothrix (37), Acanthopleuribacter* (38)], 10 [*Thermotomaculum* (39)], and 23 [*Thermoanaerobaculum* (40)]. For SD 4, the number of known genera has recently been expanded. Four genera have now been defined. The thermophilic '*Ca.* Chloracidobacterium thermophilum' was enriched from a hot spring and represents the first phototrophic acidobacterium (41). *Blastocatella fastidosa*, an aerobic chemoorganoheterotroph (42), and two *Aridibacter* species (43) were isolated from semiarid savannah soils. The thermophile *Pyrinomonas methylaliphatogenes* was isolated from a geothermally-heated soil and possesses a chemoheterotrophic and obligately aerobic metabolism (44). Molecular ecological studies based on 16S rRNA genes have indicated that, in wetlands, the most abundant *Acidobacteria* fall in SD 1 and 3 (21), whereas in lakes SDs 1,

6, and 7 thrive (45). In soils, SDs 1-4 and 6 are most dominant, with SD 4 contributing on average 20-30% of total *Acidobacteria* depending on the method used (i.e. clone libraries or pyrosequencing) (19). In contrast to most other SDs, the relative abundance of SD 4 increased with increasing soil pH and at pH >7, 16S rRNA sequences derived from members of this SD typically represent more than half of all acidobacterial sequences (19). The lipids produced by *Acidobacteria* of SD 4 may thus form a major source of the unusual ether lipids in soil. Here we describe in detail the lipid composition of five previously classified bacteria and two newly isolated strains, all belonging to the *Acidobacteria* SD 4, and discuss their distributions.

Materials and Methods

Cultures. The acidobacterial strains used in this study are listed in Table 1. *Blastocatella fastidiosa* A2_16^T, *Aridibacter famidurans* A22_HD_4H^T, *Aridibacter kavangonensis* Ac_23_E3^T, and two other acidobacterial strains from semiarid soils from Namibia were grown at the DSMZ at 28°C by moderate shaking for 9-14 days depending on the strain. All strains were grown in liquid SSE/HD 1:10 medium that was based on soil solution equivalent (SSE) (46) with an increased iron content and supplemented with 0.25 g l^{-1} yeast extract (Difco Laboratories Inc., Detroit, MI), 0.5 g l^{-1} of peptone (Difco), 0.1 g l^{-1} glucose (Sigma-Aldrich, Steinheim, Germany), 0.1 ml l^{-1} ten vitamin solution (47), and 1 ml l^{-1} trace element solution SL 10 (48). 10 mM 2-(4-morpholino)ethanesulfonic acid (MES; Sigma) or 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Sigma) was used to buffer the medium at a pH of 5.5 (*B. fastidiosa*, strain Ac_28_D10^T) or 6.5 (*A. famidurans*, *A. kavangonensis,* strain Ac_11_E3^T), respectively. Biomass was harvested by centrifugation (9000 × g, 30 min; Avanti-J26 XPI, Beckman Coulter), frozen (-20°C, overnight), and lyophilized (0.05 mbar, -30°C).

Pyrinomonas methylaliphatogenes K22^T was isolated from a geothermally heated soil (68 °C, pH 6.9) collected from Mt. Ngauruhoe, an active strato-volcano located in the Tongariro volcano complex on the North Island of New Zealand. Cells were grown at 60°C as described previously (44) using the basal liquid FS1V medium with the addition of 0.1 g l^{-1} casamino acids (Difco) and 0.5 g l^{-1} glucose in an oxic headspace (1:1 ratio of headspace to medium) (49). Subsequently, this bacterium was also grown at three different temperatures (50, 60, and 69°C). The cells were then centrifuged at 5,000 r.p.m. for 30 min and the supernatant decanted off. The subsequent pellet was lyophilized overnight.

'*Ca*. C. thermophilum' was isolated from microbial mats in alkaline siliceous hot springs in Yellowstone National Park, WY, USA (41). The enrichment culture was grown at 53°C as described previously (50). However, carbon and nitrogen sources were changed to 50 mg l^{-1} peptone and yeast extract of each, 365 mg l^{-1} 2-oxoglutarate and 625 mg l^{-1} bicarbonate. 125 mg l^{-1} thioglycolate were added as reduced sulfur source. Cells of '*Ca*. C. thermophilum' were separated from the other members of the enrichment (predominantly *Anoxybacillus* sp.; ca. 20%) by Percoll density centrifugation (50).

Tree calculation. Almost full-length 16S rRNA gene fragments of two strains (Ac_11_E3a and Ac_28_D10a) isolated at the DSMZ were amplified by colony-PCR with primers 8f and 1492r (51). Sequences of purified PCR products (ExoSAP-IT®, USB, Cleveland, OH) were determined by Sanger sequencing on an AB 3730 DNA analyzer (Applied Biosystems, Foster City, CA) using the AmpliTaq FS Big Dye terminator cycle sequencing kit (Applied Biosystems). The 16S rRNA gene sequences of strains Ac_11_E3 and Ac_28_D10 are stored in Genbank under accession numbers KF840370 and KF840371, respectively. These sequences together with those published for the other strains were added to the small subunit

ribosomal RNA non-redundant reference database SILVA version 108 [www.arb-silva.de; (52)] in the ARB software environment (53). After automated alignment with the Fast aligner tool, the alignment was manually refined based on secondary structure information. A phylogenetic tree was calculated using the neighbor-joining algorithm (termini filter; 41,484 valid positions between position 60 and 1438 of the *Escherichia coli* 16S rRNA reference gene; 1000 bootstrap re-samplings).

Lipid analysis For all studied strains lyophilized cells were hydrolyzed with 1N HCl in methanol by refluxing for 3 h, following the procedure described previously (14). The extracts obtained were methylated with diazomethane to transform fatty acids into methyl esters, and an aliquot was silylated with N,O-*bis*(trimethylsilyl)-trifluoroacetamide (BSTFA) in pyridine at 60°C for 20 min and analyzed by gas chromatography (GC) and GC-mass spectrometry (GC-MS) using conditions previously described (14). Another aliquot of the methylated extract was separated over an activated Al_2O_3 column using dichloromethane (DCM) and DCM/methanol (1:1, v/v/) to five an apolar and polar fraction, respectively. The apolar fraction was used to determine the double bond positions of the mono-unsaturated fatty acid methyl esters (FAMEs) using the mass spectra of their dimethyl disulfide derivatives as described by Nichols et al. (54). The polar fraction was dissolved in hexane/propanol (99:1, v/v), filtered over a 0.45 μm polytetrafluorethylene filter, and analyzed by high performance liquid chromatography/atmospheric pressure chemical ionization mass spectrometry (HPLC/APCI-MS) for branched GDGTs.

For all strains, intact polar lipids were extracted from the lyophilized cells using a modified Bligh-Dyer technique (55) as described by Pitcher et al. (56). An aliquot of the obtained extract was dissolved in hexane/2-propanol/water (72:27:1), filtered through a 0.45 μm regenerated cellulose filter, and analyzed by HPLC/ESI-MS^n using conditions previously described (14).

Results

Seven strains of bacteria belonging to the *Acidobacteria* SD 4 were analyzed for their lipid composition; five are species that have previously been characterized (*Blastocatella fastidiosa* (42), *Pyrinomonas methylaliphatogenes* (44), '*Ca.* C. thermophilum' (41), *Aridibacter famidurans*, and *Aridibacter kavangonensis* (43)), and two are novel strains isolated from soils in Namibia (Table 1). Fig. 2 depicts their phylogenetic relationship based on the 16S rRNA gene and the position of SD 4 relative to other characterized phylogenetic branches within the phylum *Acidobacteria*. The maximum phylogenetic diversity within the cited SD 4 strains is quite large with up to >20% sequence dissimilarity, which is substantially larger than that observed for SD 1 and 3 *Acidobacteria* (Fig. 2).

Lipids released by acid hydrolysis. Fig. 3 shows two examples of typical gas chromatograms of total lipid fractions obtained after acid hydrolysis of cells (i.e. for *P. methylaliphatogenes* and *A. famidurans*). All strains contained *iso*-C_{15} as a dominant regular fatty acid, with the unsaturated counterpart, *iso*-$C_{15:1\Delta 9c}$, present in the mesophilic but not in the thermophilic strains (Table 2). The fatty acid distribution of *P. methylaliphatogenes* (Fig. 3a), and to a lesser extent of strain Ac_28_D10, deviates from the other investigated strains because it also contains relatively high amounts of longer *iso* fatty acids, i.e. *iso* $C_{17:0}$, *iso* $C_{19:0}$, and the uncommon *iso* $C_{21:0}$ fatty acid. This latter fatty acid was also encountered in small relative abundance (ca. 2%) in three other investigated strains (Table 2). In the mesophilic strains *n*-$C_{16:1\Delta 9}$ was also present as a relatively abundant fatty acid (Fig. 3b; Table 2). In addition to these regular fatty acids the more unusual, later-eluting (Fig. 3a) lipid,

13,16-dimethyloctacosanedioic acid (or *iso* diabolic acid **3**) was detected in varying amounts (1-47% of total lipids; Table 2).

Strikingly, acid hydrolysis of cell material not only released fatty acids and *iso*-diabolic acid **3** but also released substantial amounts of monoalkyl glycerol ethers (MGE), except for '*Ca.* C. thermophilum', in which no ether lipids were detected (Table 2). The ether lipids were MGE derivatives of the abundant saturated fatty acids, with *iso* C_{15} MGE (**7**) and the MGE derivative (**5**) of *iso*-diabolic acid **3** as the most abundant representative (Table 2; Fig. 3). MGE **5** was previously (14) tentatively identified in two species of SD 1 *Acidobacteria* by its mass spectrum (Fig. 4c) that was virtually identical to that of 15,16-dimethyl-28-glyceryloxydodecanoic acid (57), but a deviating retention time. In the two SD 1 species, MGE **5** represented only ca. 3% of the lipids (14), whereas in the SD 4 species investigated here MGE **5** represents 5-26% of the lipids (Table 2). To confirm fully its structural resemblance with *iso*-diabolic acid **3**, a fraction enriched in MGE **5** (as methyl ester) was subjected to reduction with $LiAlH_4$ to convert the methyl ester to an alcohol. This was followed by treatment with HI and H_2/PtO_2, which yielded the hydrocarbon 13,16-dimethyloctacosane as confirmed by mass spectral analysis and relative retention time data (4).

In addition to *iso*-diabolic acid **3** and its MGE derivative, we also detected two related components containing one additional methyl group (i.e. **4** and **6**). This was apparent from their mass spectra (Figs. 4b and 4d), which revealed a shift of several fragment ions in the high *m/z* region by 14 Th. To elucidate the position of the methyl group, a fraction containing MGE **6** was subjected to $LiAlH_4$ followed by HI treatment and hydrogenation (see above). This yielded 5,13,16-trimethyloctacosane as confirmed by mass spectral analysis and relative retention time data (4). This experiment revealed the position of the methyl group to be at C-5 but still did not elucidate the position of the additional methyl in the MGE derivative to be at C-5 or C-ω5. This was determined by direct HI treatment followed by hydrogenation, which generated the C_{31} monocarboxylic acid **9**. Its mass spectrum in comparison to the monocarboxylic acid **8** formed from MGE derivative **5** revealed that the additional methyl group is in the vicinity of the ether bond, resulting in structure **6**. The mass spectral fragmentation pattern of a methylated *iso*-diabolic acid detected in '*Ca.* C. thermophilum' (Table 2) was also consistent with a methyl group at position C-5.

The 5-methyl *iso*-diabolic acid MGE **6** was detected in 4 out of 5 mesophilic species with strain Ac_11_E3 containing the highest relative amount of the methylated derivative. Because methylation at C-5 was detected for *iso*-diabolic acid from '*Ca.* C. thermophilum', *B. fastidiosa* and *P. methylaliphatogenes* were the only two species out of seven investigated strains that did not contain 5-methyl lipids (Table 2).

Distribution of intact polar lipids. To characterize the intact polar lipids (IPLs) of all species of *Acidobacteria* investigated, the Bligh-Dyer solvent extracts were analyzed by HPLC/ESI-MS^n. The IPLs were dominated by 'mixed' ether-ester mono glycerides (Table 3). IPLs with phosphocholine (PC) head groups dominated except for '*Ca.* C. thermophilum,' for which the dominant IPLs were diacylglycerylhydroxy-methyl-(N,N,N)-trimethylalanine (DGTA) lipids. The overall number of carbon atoms in the acyl/alkyl groups of these IPL is consistent with the dominant fatty acids and MGEs detected after acid hydrolysis (Table 2). However, no membrane-spanning IPLs (i.e. IPLs containing ester-bound *iso*-diabolic acids **3** or **4** or MGEs **5** or **6**) were detected in any of these Bligh-Dyer extracts.

Branched GDGTs. The acid-hydrolyzed biomass of some of the acidobacterial cultures was also analyzed for the presence of GDGTs by HPLC/APCI-MS using SIM. However, we were

unable to identify any branched GDGTs **1** and **2** or any other branched GDGT in the species investigated.

DISCUSSION

Chemotaxonomic relationships. The fatty acid distributions of all studied *Acidobacteria* belonging to SD 4 show a quite consistent pattern: they all contain *iso* $C_{15:0}$ as an abundant fatty acid (13-36% of the total lipids; Table 2). Five of them also contain *iso* $C_{15:1\Delta9c}$ as an abundant fatty acid (7-19%), whilst four of them contain $C_{16:1\Delta9}$ in substantial amounts (6-11%) (Table 2). *Iso*-diabolic acid **3** was detected in all examined species of SD 4 *Acidobacteria* in varying amounts (1-47% of total lipids; Table 2). This lipid was identified previously as an abundant lipid in *Acidobacteria* of SD 1 and 3 (14) and in thermophilic *Thermoanaerobacter* species (58-60), in which they fulfill a role as membrane-spanning lipids. In these studies *iso*-diabolic acid was only detected after hydrolysis of the cell material. In agreement with this mode of occurrence, a previous report on the lipids of '*Ca.* C. thermophilum' likewise did not report *iso*-diabolic acid in the Bligh-Dyer extract (50), whereas after acid hydrolysis of cell material, as performed in this study, it comprises the most abundant lipid (Table 2). In contrast to '*Ca.* C. thermophilum' and *Acidobacteria* of SD 1 and 3 (14), the relative abundance of *iso*-diabolic acid is relatively low (1-4%; Table 2) in the other investigated SD 4 species. However, in these other species *iso*-diabolic acid occurs relatively abundantly (5-25% of total lipids; Table 2) in an ether-bound form as the MGE derivative **5**. This component was previously identified as a minor constituent in *Acidobacteria* of SD 1 and 3 (14). In general, this observation seems to be characteristic for SD 4 *Acidobacteria*; all species, except '*Ca.* C. thermophilum', contain substantial amounts (21-40%) of ether lipids (Table 2). This is consistent with the analysis of IPLs in the Bligh-Dyer extract, which shows that the most dominant IPLs are "mixed" ether/ester lipids (Table 3).

These chemotaxonomic relationships are confirmed when cluster analysis is performed on the lipid distributions, including those of previously reported *Acidobacteria* of SD 1 and 3 (14,26) (Fig. 5). The lipid distributions of all SD 4 *Acidobacteria* form a clearly distinct cluster. The only exception is '*Ca.* C. thermophilum'; its lipid distribution is more similar to that of various SD 1 *Acidobacteria.* In the phylogenetic tree based on the 16S rRNA gene (Fig. 2) '*Ca.* C. thermophilum' is also clearly separated from the other SD 4 *Acidobacteria* (Fig. 2), although it is also distinct from SD 1 and 3 species. The distinct taxonomic position of '*Ca.* C. thermophilum' is consistent with its physiological capabilities; it is the only known phototrophic member of the *Acidobacteria* (41), while all other species are heterotrophs.

The IPL compositions of the SD 4 *Acidobacteria* are also in line with the cluster analysis of the lipid distribution; '*Ca.* C. thermophilum' is the only species that contains predominantly diacyl lipids, whereas the other examined species contain mixed ether/ester lipids. Furthermore, '*Ca.* C. thermophilum' contains predominantly diacylglyceryl-hydroxymethyl-N,N,N-trimethyl-beta-alanine (DGTA) lipids, whereas all other species show a dominance of phosphocholine IPLs (Table 3). It should, however, be noted that the reported IPL distribution probably represents a biased view of the membrane lipid composition, because IPLs containing membrane-spanning lipids were not detected, whereas direct acid hydrolysis of cells generated substantial amounts of these lipids (9-48%; Table 2). As discussed previously for SD 1 and 3 *Acidobacteria* (14), this may be caused by relatively large and polar head groups, which may render the IPLs containing membrane-spanning lipids non-extractable using the Bligh-Dyer protocol. Despite this bias, there is generally a

good overlap between the reported acyl/alkyl composition of the IPLs (Table 3) and the lipid composition (Table 2); the IPLs seem to contain mainly C_{15} and, to a lesser extent, C_{17} acyl/alkyl chains as may be tentatively concluded from a total number of acyl/alkyl carbons of C_{30} and C_{32}.

Variation in lipid composition: influence of environmental variables. The membrane lipids of the SD 4 *Acidobacteria* are quite distinct from the diacyl glycerol membrane lipids that characterize most bacteria. Firstly, they contain a substantial amount of membrane spanning-lipids (9-48%; Table 2) and secondly, they contain a high percentage of ether linkages (up to 40%; Table 2). In contrast to the *Archaea*, membrane-spanning lipids are uncommon in the bacterial domain but diabolic or *iso*-diabolic acid, acids connecting two glycerol moieties, do occur in *Butyrivibrio* sp. (61), *Sarcina ventriculi* (62), members of the *Thermotogales* (2,57,63-65), *Thermoanaerobacter* species (58,59,62) and *Acidobacteria* of SD 1, 3 (14), and 4 (this work), respectively. Ether membrane lipids are the hallmark of the *Archaea* (1,5), but an increasing number of bacterial species has been shown to contain diether, tetraether, or mixed ether/ester lipids. These include (but are not restricted to) *Ammonifex degensii* (66), *Aquifex pyrophylus* (67), *Thermatoga* sp. (2,57) several sulfate-reducing bacteria (68-70), *Mycoplasma fermentans* (71), anammox bacteria (72), and *Acidobacteria* SD 1 and 3 (14), and 4 (this work).

Classically, the presence of membrane-spanning and ether-bound lipids is seen as an adaptation to high temperatures or other extreme conditions, as is the case for isoprenoidal tetraether lipids of *Archaea* (73). Consistent with this idea, most bacterial species that contain membrane-spanning lipids are moderate or extreme thermophiles, although *Butyrivibrio sp.* and most cultured *Acidobacteria* are mesophilic. In a study of different species of the order *Thermotogales* (57), it was shown that in *Thermotoga* spp. the core membrane lipids were characterized by the presence of both ester and ether bonds, whereas no ether bonds occurred in the phylogenetically related *Thermosipho* and *Fervidobacterium* spp. Therefore, both the occurrence of membrane-spanning lipids and the presence of ether bonds in bacteria do not seem to be an adaptation to temperature alone.

In this study we examined two thermophilic species of the SD 4 *Acidobacteria*. '*Ca.* C. thermophilum', grown at 53°C, has the highest percentage of membrane-spanning lipids (48%; Table 2) but its membrane lipids do not contain ether bonds. Compared to the mesophilic species, *P. methylaliphatogenes*, grown at 60°C has a moderate percentage of membrane-spanning lipids (20%; Table 2), but a lower total of ether bonds (21%; Table 2). The most distinct difference in the composition of the thermophilic species compared to the mesophiles is that they contain very little unsaturated lipids (Table 2). To examine the influence of growth temperature on the membrane lipid composition further, *P. methylaliphatogenes* was grown at three temperatures in the 50-69°C range. Subtle changes in the membrane lipid composition were detected, but in contrast with classical ideas on membrane adaptation, a decreasing rather than an increasing trend in the percentage of membrane-spanning lipids and ether bonds with increasing temperature was observed (Figs. 6a-b). Only a small increase in the number of *n*-alkyl chains (Fig. 6c) and slight increase in the average chain length (Fig. 6d), determining the thickness of the membrane, were apparent with increasing temperature. Thus, the lipid data of the SD 4 *Acidobacteria* also indicate that the occurrence of membrane-spanning lipids and the presence of ether bonds in bacteria are probably not only an adaptation to temperature but suggest that other (including genetic) factors are probably in play.

***Acidobacteria* as a potential source for branched GDGTs.** Branched GDGTs (e.g., **1** and **2**) occur ubiquitously in soil, peat bogs, and lakes (5). Their distribution is used to reconstruct past pH and temperature based on a set of empirical relationships (74-76), which are thought to reflect the ability of bacteria in soil and lake water to adjust their membrane composition in response to temperature and pH. *Acidobacteria* have been proposed as probable candidates for the production of branched GDGTs (13) and this has been supported by the recent identification of its "building block" *iso*-diabolic acid **3** in *Acidobacteria* of SDs 1 and 3 (14). Although small amounts of branched GDGT **1** were detected in a few species, *iso*-diabolic acid **3** occurred predominantly in an ester-bound form and not in an ether-bound form, indicating that other *Acidobacteria* are probably the origin of the branched GDGTs. This was one of the reasons to perform this study. It showed that neither SD 4 *Acidobacteria* produce branched GDGTs, at least not the seven species that we investigated. However, six of the seven investigated species produce lipids in which *iso*-diabolic acid **3** or its methylated counterpart **4** occur ether-bound to a glycerol moiety (i.e. MGEs **5** and **6**) in relatively large amounts (i.e. 9-30%; Table 2). Such moieties reflect important structural units of the branched GDGT **1** and **2**. Strikingly, the ether-bound *iso*-diabolic acid moiety occurs only at the *sn*1 but not at the *sn*2 position of glycerol. Apparently, whilst most of the SD 4 *Acidobacteria* are able to produce the ether bond at the *sn*1 position enzymatically, they lack the enzyme(s) able to produce ether bonds at the *sn*2 position. Consequently, the diester/diether lipids **10** and **11**, composed of two esterified MGE **5** and **6** units, which are presumed to be important constituents of the membrane lipids of SD 4 *Acidobacteria*, have the closest structural resemblance to branched GDGTs **1** and **2**.

Another apparent mismatch with the GDGTs occurring in SD 1 *Acidobacteria* and branched GDGT occurring in the environment is that only GDGT **1** was detected in the *Acidobacteria* (14), whereas branched GDGTs with additional methyl substituents (such as **2**) occur widely in the environment (77,78). This additional methylation occurs at one (i.e. **2**) or at both alkyl chains at C-5, although recently branched GDGTs with the methylation at C-6 have also been reported (79). The detection of the 5-methyl *iso*-diabolic acid (i.e. **4**) and MGE **6** in five out of seven species of SD 4 *Acidobacteria* now for the first time reveals that an additionally methylated *iso*-diabolic acid or its ether derivative is biosynthesized by *Acidobacteria*. Interestingly, the two thermophilic species produce no (i.e. *P. methylaliphatogenes*) or only small amounts (i.e. '*Ca.* Chloracidobacterium thermophilum') of additionally methylated *iso*-diabolic acid or its derivative (i.e. **4** and **6**) (Table 2). Four of the five mesophilic SD 4 *Acidobacteria* produce these components, with strain Ac_11_E3 containing them in highest relative abundance (Table 2). This is in agreement with the distributions of brGDGTs in the environment, which generally reveals an increase in the degree of additional branching with decreasing temperature (75,76,78). The mesophilic species *B. fastidiosa* is, however, an exception in this respect since it does not contain **4** or **6** (Table 2). This suggests that although apparently there is a strong environmental control on branched GDGT composition (75,76,78), there may also be a genetic factor involved. In the species investigated we did not identify any additionally methylated *iso* C_{15} fatty acid or *iso* C_{15} MGE. This suggests that, in the biosynthesis of the membrane lipids, the methylation of C-5 occurs after the head-to-head condensation of two *iso* C_{15} fatty acids to *iso*-diabolic acid **3**, i.e. after the membrane-spanning lipid has been synthesized.

Our finding of ether-bound *iso*-diabolic acid and its 5-methyl derivative as important membrane lipids of SD 4 *Acidobacteria* further closes the gap between the presumed origin of brGDGTs in the environment and the occurrence of related lipids in bacteria. Presently, we still lack known *Acidobacteria* that are able to produce glycerol membrane lipids that are ether-linked at the *sn*2 position (although some SD 1 species are able to produce small

amounts of GDGT **1**) and *Acidobacteria* that produce membrane-spanning lipids containing cyclopentane moieties formed by internal cyclization (9). Further studies of the lipids of newly cultivated *Acidobacteria* may lead to identification of the bacterial sources of the ubiquitous branched GDGTs in the environment. This will allow a more fundamental study of the environmental and genetic controls on the distribution of these lipids that are currently widely applied in palaeoenvironmental and palaeoclimate studies (80).

Acknowledgements. Soil samples from Namibia were taken under collection permits 1358/2009 and 1569/2011, and exported under permits ES 24478 (of April 6, 2009) and ES 25691 (of April 12, 2011). Characterization of strains A2_16, A22_HD_4H, Ac_11_E3, Ac_23_E3, and Ac_28_D10 was performed under the Material Transfer Agreements of the NBRI (National Botanical Research Institute, Namibia) of April 05, 2012. The authors thank Alicia Geppert (DSMZ) for help with cultivating *Acidobacteria* for this study. We greatly appreciate the assistance of National Park Service personnel. The research leading to these results has received funding from the European Research Council (ERC) under the European Union's Seventh Framework Program (FP7/2007-2013) ERC grant agreement [226600]. Jörg Overmann received support by the BMBF programs BIOLOG/BIOTA (01LC0621C) and TFO (01LL0912M). Studies of "*Ca.* C. thermophilum" were supported by grant (DE-FG02-94ER20137) from the U. S. Dept. of Energy to D.A.B. and were conducted under Yellowstone National Park research permits YELL-0129 and 5494 (to David M. Ward, Montana State University). GNS staff acknowledge the support of the DCF Geothermal Resources of New Zealand (GRN) work programme.

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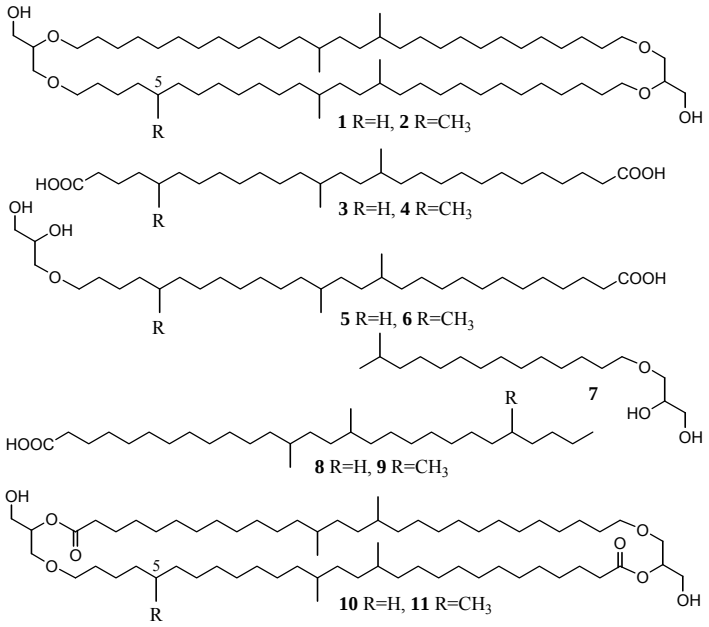

Figure 1. Structures of lipids mentioned in the text. **1-2** are GDGTs ubiquitously occurring in the environment. **3-4** are *iso* diabolic acids. **5-6** are *iso* diabolic acids ether-bound to a glycerol moiety at the *sn*-1 position. **7** is a C_{15} *iso* fatty acid ether-bound to a glycerol moiety at the *sn*-1 position. **8-9** are derivatives of *iso* diabolic acids **3-4** where one of the carboxylic groups is reduced. **10-11** represent hypothetical structures showing the core of the membrane-spanning lipids of the SD4 *Acidobacteria* based on the results reported in this paper.

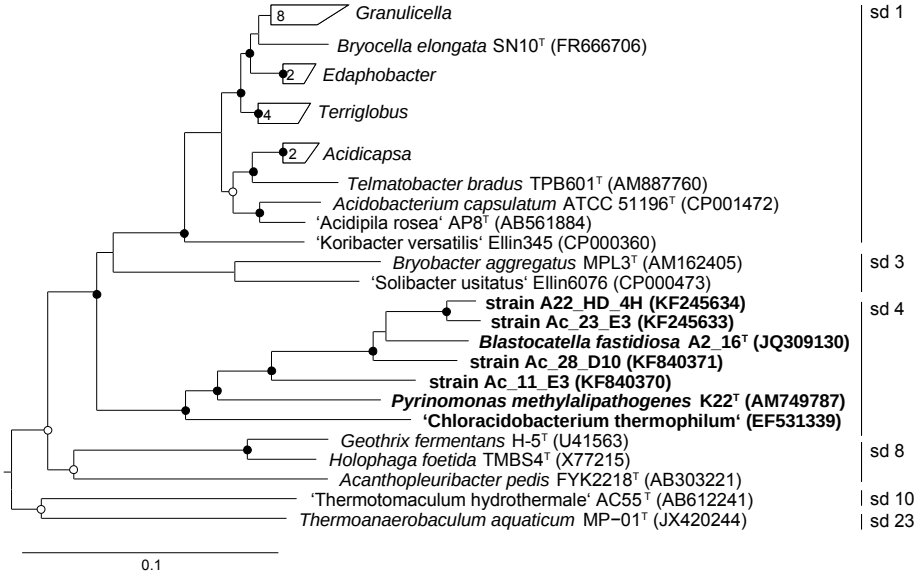

Figure 2. Rooted neighbor-joining phylogenetic tree (Felsenstein correction) based on almost full-length 16S rRNA gene sequences showing the investigated strains of *Acidobacteria* SD 4 (bold) in relation to other described acidobacterial taxa. Open and closed circles indicate bootstrap values (expressed as a percentages of 1000 replicates) of >70% and >90%, respectively. The following sequences were used as outgroup: *Planctomyces brasiliensis* DSM5305^T (AJ231190), *Planctomyces maris* DSM8797^T (AJ231184), and *Planctomyces limnophilus* DSM 3776^T (CP001744). Bar indicates 10% nucleotide divergence.

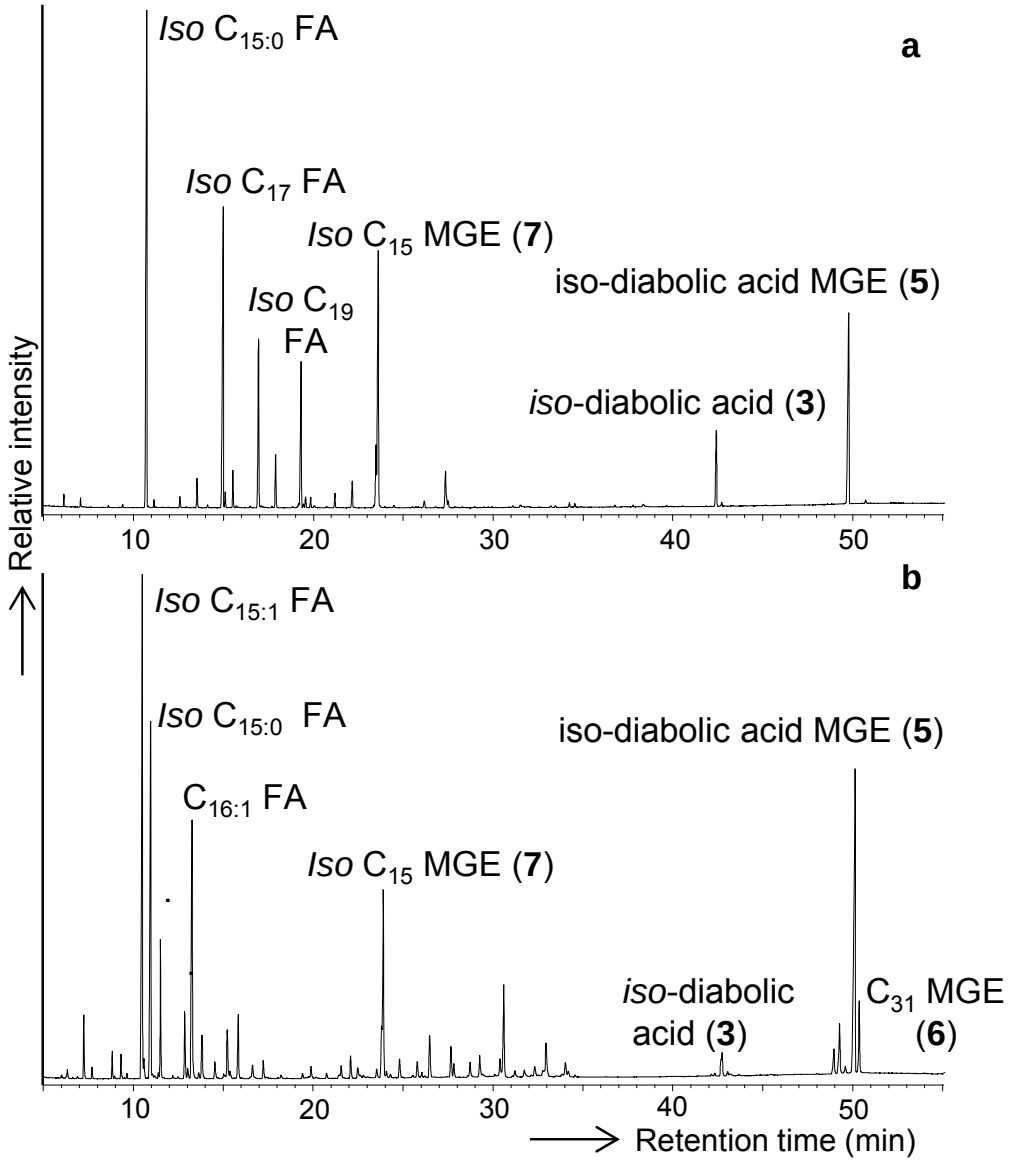

Figure 3. Gas chromatograms of lipids released after acid hydrolysis of whole cell material of (a) the *P. methylaliphatogenes* K22^T and (b) *A. famidurans* A22_HD_4H^T. Carboxylic groups were derivatized to the corresponding methyl esters and alcohol moieties were derivatized to trimethyl silyl ethers prior to gas chromatographic analysis. Numbers refer to structures shown in Fig. 1.

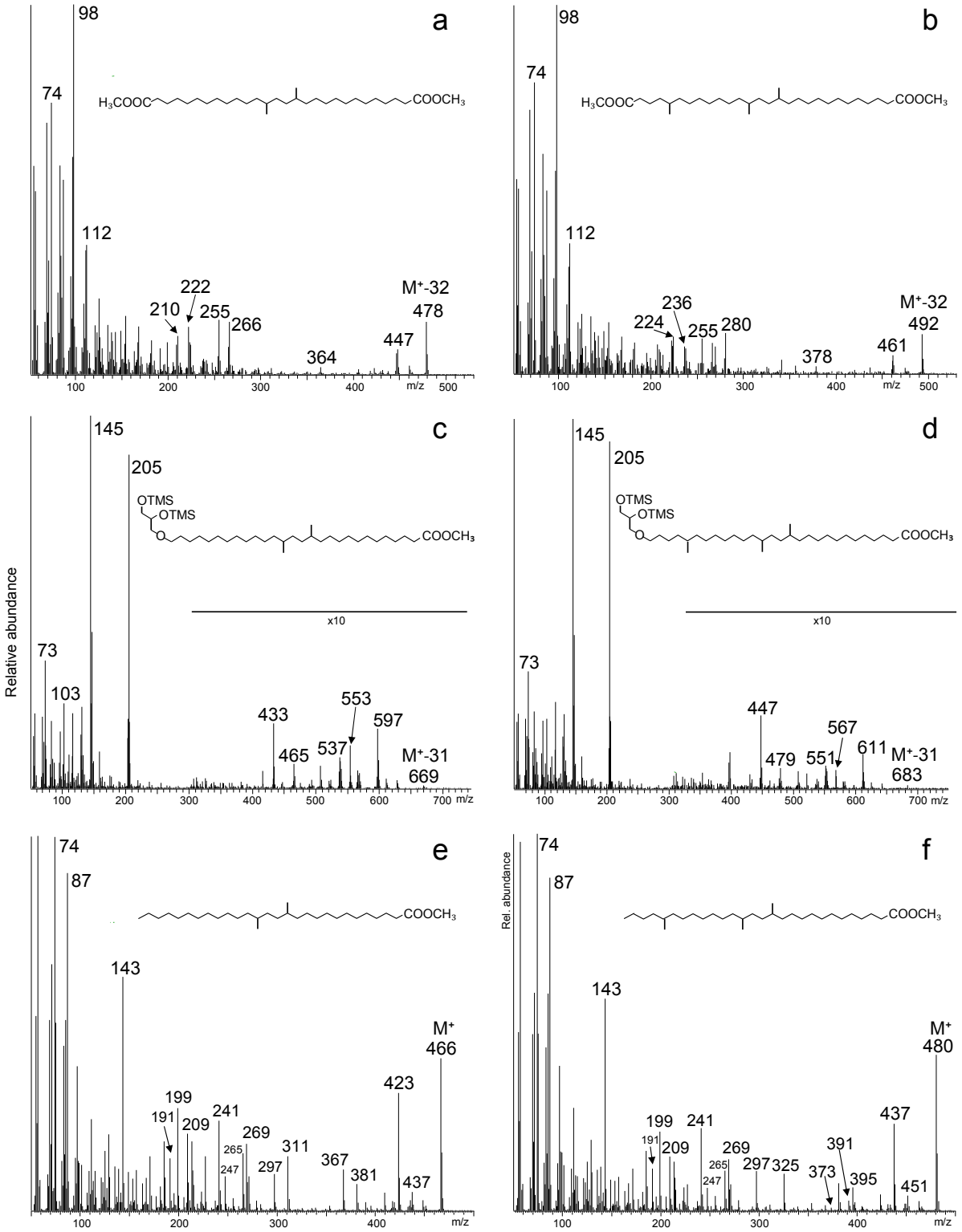

Figure 4. Mass spectra (corrected for background) of the methyl ester and TMS derivatives (where appropriate) of (**a**) *iso*-diabolic acid (**3**), (**b**) 5-methyl *iso*-diabolic acid (**4**), (**c**) *iso*-diabolic acid MGE (**5**), (**d**) 5-methyl *iso*-diabolic acid MGE (**6**), (**e**) 13,16-dimethyl octacosanoic acid, and (**f**) 13,16, 24-trimethyl octacosanoic acid. The latter two components were formed by HI/$LiAlH_4$ treatment of *iso*-diabolic acid MGE (**5**) and 5-methyl *iso*-diabolic acid MGE (**6**).

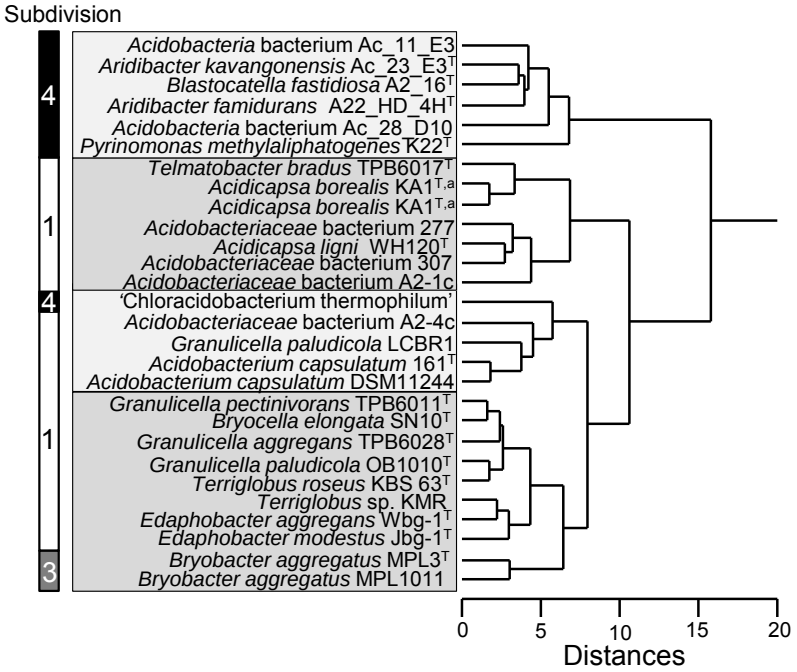

Figure 5. Cluster analysis of the distribution of the lipids released by acid hydrolysis of cell material of the *Acidobacteria* of SD 4 in comparison with results of *Acidobacteria* SD 1 and 3 reported previously (14,26) using an identical method of lipid analysis. The input of the cluster analysis was the Bray–Curtis similarity matrix of lipid profiles (% of total lipids; e.g. Table 2). A hierarchical clustering was performed in SYSTAT 13© using Euclidian distance and the average linking method. [a] Two different batches of cultures were studied.

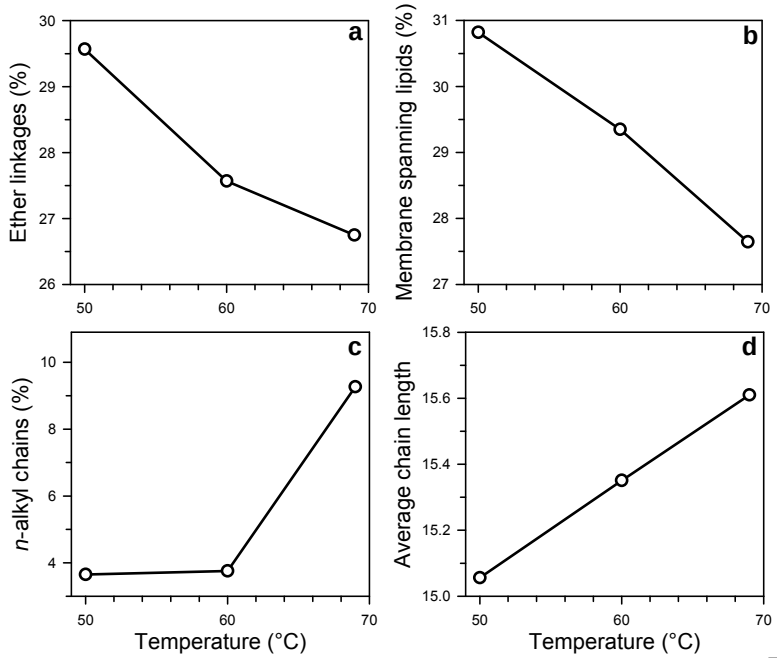

Figure 6. Membrane lipid characteristics of *P. methylaliphatogenes* K22^T as a function of growth temperature. (**a**) fraction of ether linkages, (**b**) fraction of membrane-spanning lipids, (**c**) fraction of *n*-alkyl chains, and (**d**) average chain length. The average chain length was calculated by dividing the chain length of the membrane lipids by a factor two and by ignoring methyl substituents.

1

TABLE 1: *Acidobacteria* of SD4 used in this study

Species	Origin	Substrates used	T range (°C)	T opt. (°C)	pH range	pH opt.	Ref.
Blastocatella fastidiosa A2_16^T (=DSM 25172^T)	Pastureland soil, Erichsfelde, central Namibia	complex protein substrates, protocatechuate[b]	14-40	29-35	4.0-10.0	5.0-7.5	(42)
Aridibacter famidurans A22_HD_4H^T (=DSM 26555^T)	Pastureland soil, Erichsfelde, central Namibia	complex protein substrates, protocatechuate, N-acetylgalactosamine, rhamnose, xylose[b]	15-44	24-36	4.0-9.0	5.5-9.0	(43)
Aridibacter kavangonensis Ac_23_E3^T (=DSM 26558^T)	Fallow soil, Mashare, northern Namibia	complex protein substrates, protocatechuate, N-acetylgalactosamine, maltose, rhamnose, fumarate, isovalerate, laminarin[b]	12-44	36-44	3.5-10.0	5.5-8.0	(43)
Acidobacteria bacterium Ac_11_E3[a]	Bushveld soil, Mashare, northern Namibia	casamino acids, casein hydrolysate, yeast, peptone	11-53	35-45	4.7-8.1	5.4-7.0	-
Acidobacteria bacterium Ac_28_D10[a]	Agricultural soil, Mashare, northern Namibia	casamino acids, yeast, proline, protocatechuate	17-40	29-35	4.3-9.4	5.5-7.9	-
Pyrinomonas methylaliphatogenes K22^T (=DSM 25857^T)	Geothermal soil, New Zealand	Simple mono- and oligosaccharides, and a limited number of complex protein substrates			4.1-7.8	6.5	(44)
'*Ca.* Chloracidobacterium thermophilum'	Hot spring, Yellowstone, USA.	peptone, yeast extract, 2-oxoglutarate, bicarbonate, thioglycolate			n.d.	8.5	-

TABLE 2: Relative abundance of fatty acids and ether lipids after acid hydrolysis of cell material and general characteristics of the membrane lipids in the studied SD 4 *Acidobacteria*

Component	% of total lipids[a] in strain[b]:						
	1	**2**	**3**	**4**	**5**	**6**	**7**
Fatty acids							
Iso C13			1.7	1.6			
C14:1Δ9			0.8				
C14:0							2.9
Iso C15:1Δ9c	9.6	6.6	19.0	8.7	16.8		
Iso C15:1Δ9tr		0.4	0.7	0.3	0.4		
Iso C15:0	13.1	18.9	12.5	23.4	22.8	30.6	35.6
Anteiso C15							1.2
Iso C16	1.6	0.2		1.9			4.6
C16:1Δ9	10.1	3.1	10.5	10.5	5.8		0.9
C16:0	1.0	1.2	1.8	1.3	4.7	1.1	4.1
Iso C17:1Δ9	3.4	2.1	0.7	1.8	4.3		
Iso C17:0	2.4	0.6		1.1	5.4	16.1	2.5
Anteiso C17:0				1.4		1.1	0.6
C18:1Δ9		4.1					
C18:0		0.8				2.1	
Iso C19:1Δ9		1.1					
Iso C19:0						6.8	
C20:1Δ9		1.2					
C20:0		0.9	0.5			1.1	
Iso C21:1Δ9		0.8					
Iso C21:0		2.1	1.8	1.7	4.4	2.6	
Iso-diabolic acid (**3**)[c]	1.8	1.8	1.6	1.8	1.0	3.8	46.5
5-methyl *iso*-diabolic acid (**4**)							1.2
Ethers							
Iso C15-MGE (**7**)	21.6	20.7	15.9	15.7	19.5	14.9	
Iso C16-MGE	4.3		1.2	2.6	1.2		
C16-MGE	2	3.3	4.6	3.6	2.1		
Iso C17-MGE	7.3	0.2	2.2	3.3	2.8	1.9	
Anteiso C17-MGE	2.9		0.9	2.1		0.7	
Iso-diabolic acid–MGE (**5**)	18.9	25.3	20.2	15.4	5.0	17.2	
5-methyl *iso*-diabolic acid-MGE (**6**)		4.6	3.4	1.8	3.8		
Mono-unsaturation (%)[d]	27	21	36	24	31	0	1
Membrane-spanning (%)[d]	21	31	24	18	9	20	48
Ether moieties (%)[d]	40	34	30	29	23	21	0

[a] Normalized on the sum of the components listed. Values for major components (i.e. >5%) are underlined
[b] Strains: (1) *Blastocatella fastidiosa* A2_16^T (=DSM 25172^T); (2) *Acidobacteria* bacterium Ac_11_E3; (3) *Aridibacter famidurans* A22_HD_4H^T; (4) *Aridibacter kavangonensis* Ac_23_E3^T; (5) *Acidobacteria* bacterium Ac_28_D10; (6) *Pyrinomonas methylaliphatogenes* K22^T (=DSM 25857^T); (7) '*Ca*. C. thermophilum'
[c] bold numbers refer to structures in Fig. 1
[d] calculated on a molar basis, where membrane spanning-lipids are counted as two molecules

TABLE 3: Relative abundances[a] and acyl/alkyl composition of IPLs in the seven species[b] of SD 4 *Acidobacteria*

IPL[c]	Species						
	1	**2**	**3**	**4**	**5**	**6**	**7**
DGTA			+ (30:0)	++ (30:0)			+++ (30:0, 32:0)[d]
PE	+ (32:0, 30:0, 33:1, 31:1, 30:1)	+ (30:0, 30:1, 34:1)	+ (30:1, 32:1, 30:0)	+ (30:0, 32:0, 33:1)	+ (30:0, 30:1)	+ (30:0, 34:0)	
MMPE							+ (30:0)[d]
DMPE	+ (32:1, 30:0, 31:1, 30:1,32:0)	+ (30:0, 30:1)	+ (30:1, 32:1, 31:1, 30:0)	+ (30:0, 32:0)	+ (30:0, 30:1)		
PC	+++ (30:1, 32:0, 32:1, 31:1, 30:0)	+++ (30:1, 30:0)	+++ (30:1, 31:1, 32:1)	+++ (30:0, 32:0)	+++ (30:1, 30:0, 32:0)	+++ (30:0, 32:0, 34:0) + (34:0, 32:0)[d]	
unknown			+[e]			++[f]	

[a]Abundance relative to major peak in the LC/MS base peak chromatogram (+++ = base peak; ++ = 50-100% of base peak; + = 10-50 % of base peak). Note that the mass spectral response factors for different IPL groups can be quite different. The predominant fatty acid composition in order of relative abundance is reported as the total number of carbon atoms of the acyl/alkyl moieties and the number of double bond equivalents.

[b] Strains: (1) *Blastocatella fastidiosa* A2_16T (=DSM 25172^T); (2) *Acidobacteria* bacterium Ac_11_E3; (3) *Aridibacter famidurans* A22_HD_4H^T; (4) *Aridibacter kavangonensis* Ac_23_E3^T; (5) *Acidobacteria* bacterium Ac_28_D10; (6) *Pyrinomonas methylaliphatogenes* K22^T (=DSM 25857^T); (7) '*Ca*. C. thermophilum'

[c] IPLs as *sn*1-alkyl-*sn*2-acyl-glycerols, unless mentioned differently. IPLs are listed in order of elution; DGTA= dialylglycerylhydroxy-methyl-(N,N,N)-trimethylalanine, PE= phosphoethanolamine, MMPE= monomethylated PE, DMPE= dimethylated PE, PC = phosphocholine.

[d] diacyl IPL

[e] characterized by *m/z* 1283

[f] characterized by *m/z* 1366