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Ether- and Ester-Bound *iso*-Diabolic Acid and Other Lipids in Members of *Acidobacteria* Subdivision 4

Jaap S. Sinninghe Damsté,[a] W. Irene C. Rijpstra,[a] Ellen C. Hopmans,[a] Bärbel U. Foesel,[b] Pia K. Wüst,[b] Jörg Overmann,[b] Marcus Tank,[c] Donald A. Bryant,[c] Peter F. Dunfield,[d] Karen Houghton,[e] Matthew B. Stott[e]

NIOZ—Royal Netherlands Institute for Sea Research, Department of Marine Organic Biogeochemistry, Den Burg, the Netherlands[a]; Department of Microbial Ecology and Diversity Research, Leibniz Institute DSMZ—German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany[b]; Department of Biochemistry and Molecular Biology, The Pennsylvania State University, University Park, Pennsylvania, USA[c]; Department of Biological Sciences, University of Calgary, Calgary, Alberta, Canada[d]; GNS Science, Extremophile Research Group, Taupo, New Zealand[e]

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 to 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 high-performance liquid chromatography-mass spectrometry (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 larger amount of ether lipids than the thermophile "*Ca.* Chloracidobacterium thermophilum." Furthermore, experiments with *Pyrinomonas methylaliphatogenes* did not reveal a major influence of growth temperature over the 50 to 69°C range.

Isoprenoidal ether lipids ubiquitously occur in the membrane lipids of *Archaea* (1), but occasionally ether lipids also are detected in the bacterial domain, albeit with nonisoprenoidal 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 also have been observed in thermophilic environments, such as terrestrial hot springs (6), where they are believed to be produced *in situ* by thermophilic bacteria (7, 8). Despite their widespread occurrence and potential applications in geochemistry and paleoclimatology (5), their microbial source still is unclear. The assessment of the stereochemistry of the glycerol units in brGDGTs revealed that it is the 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 labeling experiments (11, 12). The environmental abundance of *Acidobacteria* has led to the suggestion that these bacteria are the biological source of the brGDGTs (13). This hypothesis was recently supported by membrane lipid analysis of 13 species of subdivisions (SD) 1 and 3 of the *Acidobacteria*, which showed that the uncommon membrane-spanning lipid, 13,16-dimethyl octacosanedioic acid (*iso*-diabolic acid), is a major lipid in all species studied (14). This lipid can be considered a building block of the brGDGTs but occurs in predominantly ester- and not ether-bound form in SD 1 and 3 *Acidobacteria*. In 3 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 to 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* contain only 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 into 26 SD, based mainly on environmental sequences (24), but only six of these contain taxonomically characterized representatives. For SD 1, eight genera have been defined, *Acidobacterium* (25), *Acidi-*

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Editor: K. E. Wommack
Address correspondence to Jaap S. Sinninghe Damsté, jaap.damste@nioz.nl.

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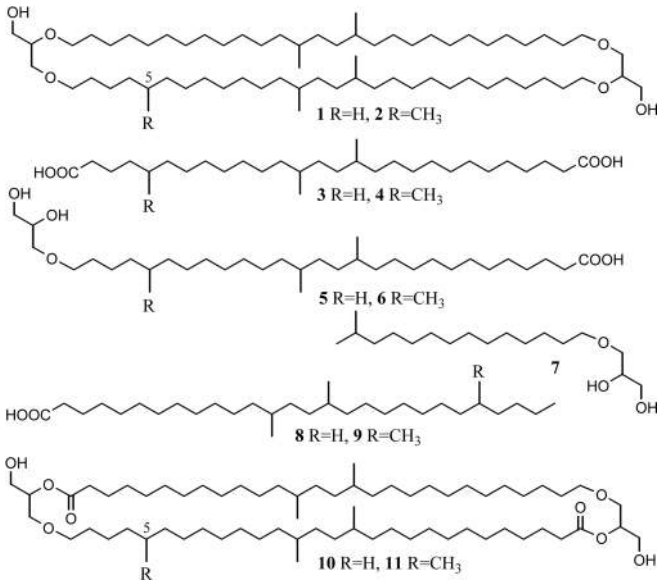

FIG 1 Structures of lipids mentioned in the text. Structures 1 and 2 are brGDGTs ubiquitously occurring in the environment. Structures 3 and 4 are *iso*-diabolic acids. Structures 5 and 6 are *iso*-diabolic acids ether bound to a glycerol moiety at the *sn*1 position. Structure 7 is a C_{15} *iso* fatty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

capsa (26), "*Acidipila*" (27), *Bryocella* (28), *Edaphobacter* (29), *Granulicella* (30, 31), *Telmatobacter* (32), and *Terriglobus* (33, 34), while only 1 to 3 genera have been characterized for SD 3 (*Bryobacter* [35]), 8 (*Holophaga* [36], *Geothrix* [37] and *Acanthopleuribacter* [38]), 10 (*Thermotomaculum* [39]), and 23 (*Thermoanaerobaculum* [40]). For SD 4, the number of known genera recently has been expanded. Four genera now have 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* members fall in SD 1 and 3 (21), whereas in lakes SD 1, 6, and 7 thrive (45). In soils, SD 1 to 4 and 6 are the most dominant, with SD 4 contributing, on average, 20 to 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 pHs above 7, 16S rRNA sequences derived from members of this SD typically represent more than half of all acidobacterial sequences (19). Thus, the lipids produced by *Acidobacteria* of SD 4 may 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 to 14 days, depending on the strain. All strains were grown in liquid SSE-HD (1:10) medium that was based on a soil solution equivalent (SSE) (46) with an increased iron content and supplemented with 0.25 g liter^{-1} yeast extract (Difco Laboratories Inc., Detroit, MI), 0.5 g liter^{-1} of peptone (Difco), 0.1 g liter^{-1} glucose (Sigma-Aldrich, Steinheim, Germany), 0.1 ml liter^{-1} 10 vitamin solution (47), and 1 ml liter^{-1} trace element solution SL 10 (48). Ten mM 2-(4-morpholino)ethanesulfonic acid (MES; Sigma) or 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Sigma) was used to buffer the medium at pH 5.5 (*B. fastidiosa* strain Ac_28_D10^T) or 6.5 (*Aridibacter famidurans* and *A. kavangonensis* strain Ac_23_E3^T), respectively. Biomass was harvested by centrifugation (9,000 × *g*, 30 min; Avanti-J26 XPI; Beckman Coulter), frozen (−20°C overnight), and lyophilized (0.05 mbar at −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 basal liquid FS1V medium with the addition of 0.1 g liter^{-1} Casamino Acids (Difco) and 0.5 g liter^{-1} glucose in an oxic headspace (1:1 ratio of headspace to medium) (49). Subsequently, this bacterium also was grown at three different temperatures (50, 60, and 69°C). The cells then were centrifuged at 5,000 rpm for 30 min and the supernatant decanted off. The subsequent pellet was lyophilized overnight.

"*Ca*. Chloracidobacterium 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 liter^{-1} peptone and yeast extract of each 365 mg liter^{-1} 2-oxoglutarate and 625 mg liter^{-1} bicarbonate. Thioglycolate (125 mg liter^{-1}) was added as a reduced sulfur source. Cells of "*Ca*. Chloracidobacterium 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 BigDye Terminator cycle sequencing kit (Applied Biosystems). The 16S rRNA gene sequences of strains Ac_11_E3 and Ac_28_D10, together with those published for the other strains, were added to the small-subunit rRNA nonredundant 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 positions 60 and 1438 of the *Escherichia coli* 16S rRNA reference gene; 1,000 bootstrap resamplings).

Lipid analysis. For all studied strains, lyophilized cells were hydrolyzed with 1 N HCl in methanol by refluxing for 3 h by 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, vol/vol/) to give an apolar and polar fraction,

TABLE 1 *Acidobacteria* of SD 4 used in this study

Species	Origin	Substrates used	Temp (°C)		pH		Reference
			Range	Optimal	Range	Optimal	
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
Unclassified *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	
Unclassified *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	50–69	65	4.1–7.8	6.5	44
"*Ca.* Chloracidobacterium thermophilum"	Hot spring, Yellowstone, WY	Peptone, yeast extract, 2-oxoglutarate, bicarbonate, thioglycolate	45–60	50–55	ND	8.5	

respectively. The apolar fraction was used to determine the double-bond positions of the monounsaturated 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, vol/vol), filtered over a 0.45-μm-pore-size polytetrafluoroethylene filter, and analyzed by high-performance liquid chromatography/atmospheric pressure chemical ionization mass spectrometry (HPLC–APCI-MS) for brGDGTs.

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-pore-size regenerated cellulose filter, and analyzed by HPLC–electrospray ionization-MS^n using conditions previously described (14).

Nucleotide sequence accession numbers. The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of the acidobacterial strains Ac_11_E3 and Ac_28_D10 are KF840370 and KF840371, respectively.

RESULTS

Seven strains of bacteria belonging to *Acidobacteria* SD 4 were analyzed for their lipid compositions; five are species that have previously been characterized (*Blastocatella fastidiosa* [42], *Pyrinomonas methylaliphatogenes* [44], "*Ca.* Chloracidobacterium thermophilum" [41], *Aridibacter famidurans*, and *Aridibacter kavangonensis* [43]), and two are novel strains isolated from soils in Namibia (Table 1). Figure 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. Figure 3 shows two examples of typical gas chromatograms of total lipid fractions obtained after acid hydrolysis of cells (i.e., for *P. methylaliphatogenes* and *Aridibacter 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 large 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. The latter fatty acid also was encountered in low relative abundance (ca. 2%) in three other investigated strains (Table 2). In the mesophilic strain, *n*-$C_{16:1\Delta 9}$ also was present as a relatively abundant fatty acid (Fig. 3b and 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 various amounts (1 to 47% of total lipids) (Table 2).

Strikingly, acid hydrolysis of cell material released not only fatty acids and *iso*-diabolic acid 3 but also substantial amounts of monoalkyl glycerol ethers (MGE), except for "*Ca.* Chloracidobacterium 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 representatives (Table 2 and Fig. 3). MGE 5 was previously (14) tentatively identified in two species of SD 1 *Acidobacteria* by its mass spectrum (Fig. 4c), which was virtually identical to that of 15,16-dimethyl-

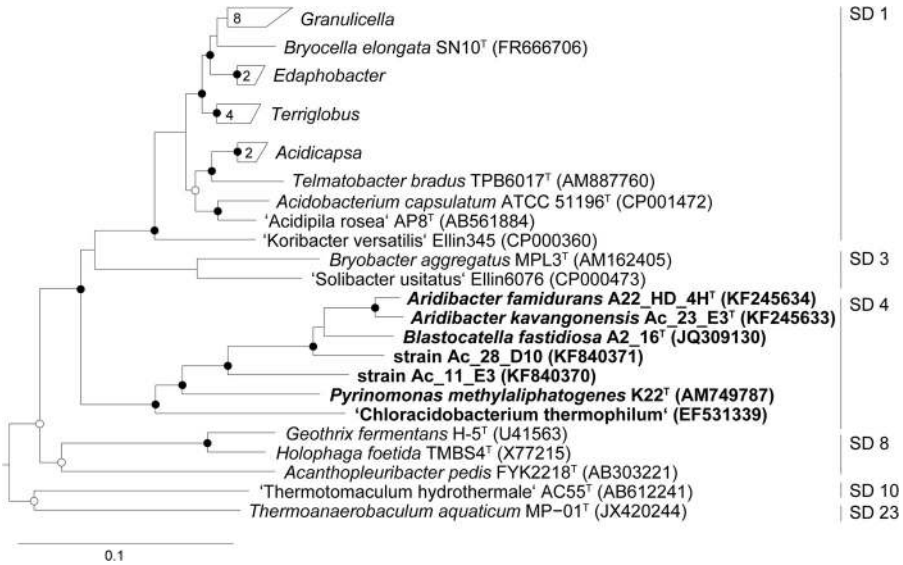

FIG 2 Rooted neighbor-joining phylogenetic tree (Felsenstein correction) based on almost-full-length 16S rRNA gene sequences showing the investigated strains of *Acidobacteria* SD 4 (boldface) in relation to other described acidobacterial taxa. Open and closed circles indicate bootstrap values (expressed as percentages of 1,000 replicates) of >70% and >90%, respectively. The following sequences were used as the outgroup: *Planctomyces brasiliensis* DSM5305^T (AJ231190), *Planctomyces maris* DSM8797^T (AJ231184), and *Planctomyces limnophilus* DSM 3776^T (CP001744). The bar indicates 10% nucleotide divergence.

28-glyceryloxydodecanoic acid (57) but had 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 to 26% of the lipids (Table 2). To confirm fully its structural resemblance with *iso*-diabolic acid 3, a fraction enriched in MGE 5 (as the 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).

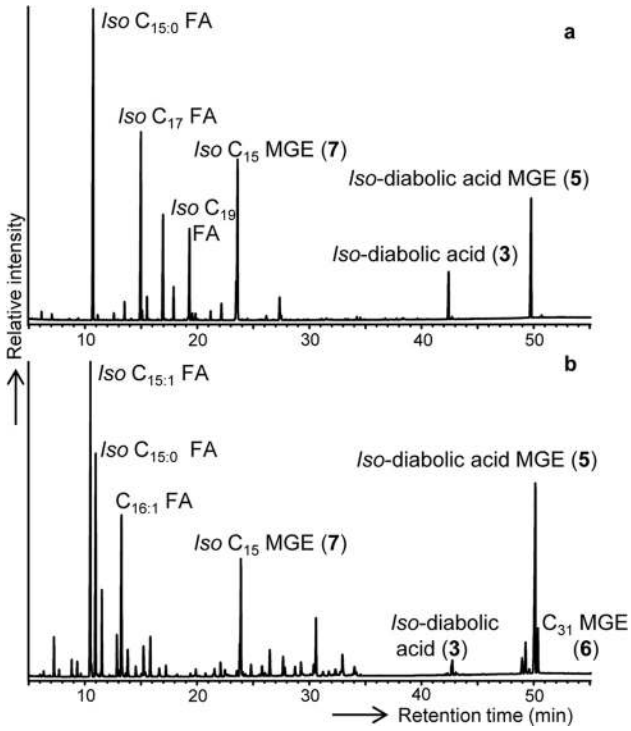

FIG 3 Gas chromatograms of lipids released after acid hydrolysis of whole-cell material of *P. methylaliphatogenes* K22^T (a) and *Aridibacter famidurans* A22_HD_4H^T (b). Carboxylic groups were derivatized to the corresponding methyl esters, and alcohol moieties were derivatized to trimethyl silyl ethers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

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 (Fig. 4b and d), 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 (described 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, compared to that of 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.* Chloracidobacterium thermophilum" (Table 2) also was 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.* Chloracidobacterium thermophilum," *B. fastidiosa* and *P. methylaliphatogenes* were the only two species out of the seven investigated strains that did not contain 5-methyl lipids (Table 2).

Distribution of IPLs. To characterize the intact polar lipids (IPLs) of all species of *Acidobacteria* investigated, the Bligh-Dyer

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-C_{13}			1.7	1.6			
$C_{14:1\Delta9}$			0.8				
$C_{14:0}$							2.9
iso-$C_{15:1\Delta9c}$	9.6	6.6	19.0	8.7	16.8		
iso-$C_{15:1\Delta9tr}$		0.4	0.7	0.3	0.4		
iso-$C_{15:0}$	13.1	18.9	12.5	23.4	22.8	30.6	35.6
anteiso-C_{15}							1.2
iso-C_{16}	1.6	0.2		1.9			4.6
$C_{16:1\Delta9}$	10.1	3.1	10.5	10.5	5.8		0.9
$C_{16:0}$	1.0	1.2	1.8	1.3	4.7	1.1	4.1
iso-$C_{17:1\Delta9}$	3.4	2.1	0.7	1.8	4.3		
iso-$C_{17:0}$	2.4	0.6		1.1	5.4	16.1	2.5
anteiso-$C_{17:0}$				1.4		1.1	0.6
$C_{18:1\Delta9}$		4.1					
$C_{18:0}$		0.8				2.1	
iso-$C_{19:1\Delta9}$		1.1					
iso-$C_{19:0}$						6.8	
$C_{20:1\Delta9}$		1.2					
$C_{20:0}$		0.9	0.5			1.1	
iso-$C_{21:1\Delta9}$		0.8					
iso-$C_{21:0}$		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-C_{15}-MGE (7)	21.6	20.7	15.9	15.7	19.5	14.9	
iso-C_{16}-MGE	4.3		1.2	2.6	1.2		
C_{16}-MGE	2	3.3	4.6	3.6	2.1		
iso-C_{17}-MGE	7.3	0.2	2.2	3.3	2.8	1.9	
anteiso-C_{17}-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		
Monounsaturation[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 to 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, unclassified *Acidobacteria* bacterium Ac_11_E3; 3, *Aridibacter famidurans* A22_HD_4H^T; 4, *Aridibacter kavangonensis* Ac_23_E3^T; 5, unclassified *Acidobacteria* bacterium Ac_28_D10; 6, *Pyrinomonas methylaliphatogenes* K22^T (DSM 25857^T); 7, "*Ca*. Chloracidobacterium thermophilum."
[c] Numbers in parentheses refer to structures shown in Fig. 1.
[d] Calculated on a molar basis, where membrane-spanning lipids are counted as two molecules.

solvent extracts were analyzed by HPLC/ESI-MS^n. The IPLs were dominated by mixed ether-ester monoglycerides (Table 3). IPLs with phosphocholine (PC) head groups dominated, except for "*Ca*. Chloracidobacterium 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 IPLs 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 acid 3 or 4 or MGE 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 selected ion monitoring. However, we were unable to identify any of the brGDGTs 1 and 2 or any other brGDGT 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 to 36% of the total lipids) (Table 2). Five of them also contain *iso*-$C_{15:1\Delta9c}$ as an abundant fatty acid (7 to 19%), while four of them contain $C_{16:1\Delta9}$ in substantial amounts (6 to 11%) (Table 2). *iso*-Diabolic acid 3 was detected in all examined species of SD 4 *Acidobacteria* in various amounts (1 to 47% of total lipids) (Table 2). This lipid was identified previously as an abundant lipid in *Acidobacteria* 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 detected only after hydrolysis of the cell material. In agreement with this mode of occurrence, a previous report on the lipids of "*Ca*. Chloracidobac-

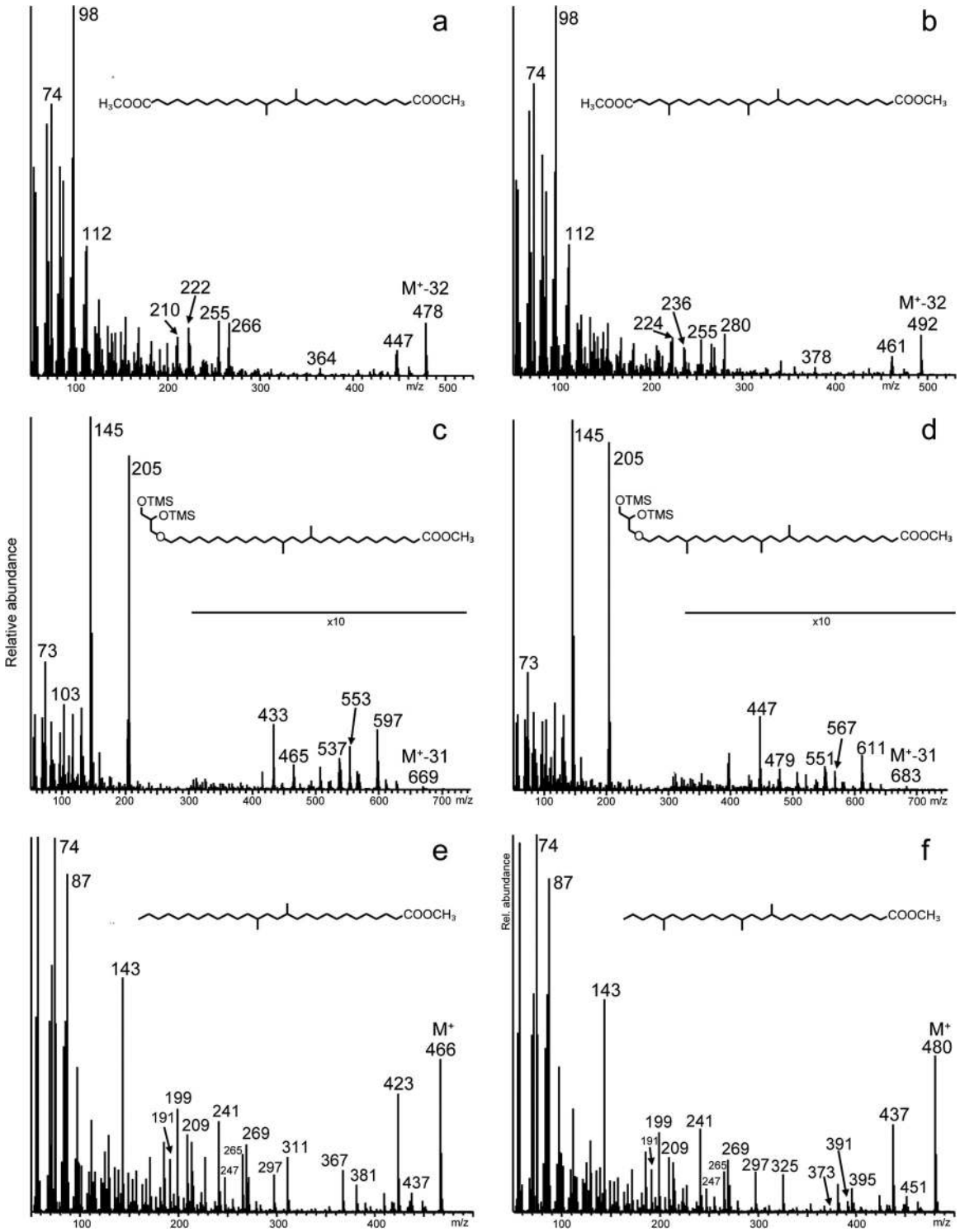

FIG 4 Mass spectra (corrected for background) of the methyl ester and TMS derivatives (where appropriate) of *iso*-diabolic acid (3) (a), 5-methyl *iso*-diabolic acid (4) (b), *iso*-diabolic acid MGE (5) (c), 5-methyl *iso*-diabolic acid MGE (6) (d), 13,16-dimethyl octacosanoic acid (e), and 13,16,24-trimethyl octacosanoic acid (f). 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).

terium 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*. Chloracidobacterium thermophilum" and *Acidobacteria* SD 1 and 3 (14), the relative abundance of *iso*-diabolic acid is relatively low (1 to 4%) (Table 2) in the other investigated SD 4 species. However, in these other species *iso*-diabolic acid occurs relatively abundantly (5 to 25% of

FIG 5 Cluster analysis of the distribution of the lipids released by acid hydrolysis of cell material of the *Acidobacteria* of SD 4 compared to 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 (percentage of total lipids, as in Table 2). A hierarchical clustering was performed in SYSTAT 13 using Euclidian distance and the average linking method. A superscript letter "a" indicates that two different batches of cultures were studied.

total lipids) (Table 2) in an ether-bound form as MGE derivative 5. This component was previously identified as a minor constituent in *Acidobacteria* SD 1 and 3 (14). In general, this observation seems to be characteristic for SD 4 *Acidobacteria*; all species, except "*Ca*. Chloracidobacterium thermophilum," contain substantial amounts (21 to 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 SD 1 and 3 *Acidobacteria* (14, 26) (Fig. 5). The lipid distributions of all SD 4 *Acidobacteria* form a clearly distinct cluster. The only exception is "*Ca*. Chloracidobacterium thermophilum"; its lipid distribution is more similar to that of various members of *Acidobacteria* SD 1. In the phylogenetic tree based on the 16S rRNA gene (Fig. 2), "*Ca*. Chloracidobacterium thermophilum" is also clearly separated from the other SD 4 *Acidobacteria* members (Fig. 2), although it is also distinct from SD 1 and 3 species. The distinct taxonomic position of "*Ca*. Chloracidobacterium 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*. Chloracidobacterium thermophilum" is the only species that contains predominantly diacyl lipids, whereas the other examined species contain mixed ether/ester lipids. Furthermore, "*Ca*. Chloracidobacterium thermophilum" contains predominantly diacylglycerylhydroxymethyl-*N,N,N*-trimethyl-β-alanine (DGTA) lipids, whereas all other species show a dominance of phosphocholine IPLs (Table 3). However, it should be noted that the reported IPL distribution probably represents a biased view of the membrane lipid composition, because

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

IPL[c]	Species[b]						
	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 the major peak in the LC-MS base peak chromatogram (+++, base peak; ++, 50 to 100% of base peak; +, 10 to 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 in parentheses 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*. Chloracidobacterium thermophilum."
[c] IPLs were *sn*1-alkyl-*sn*2-acyl-glycerols, unless mentioned otherwise. IPLs are listed in order of elution. DGTA, diacylglycerylhydroxy-methyl-(N,N,N)-trimethylalanine; PE, phosphoethanolamine; MMPE, monomethylated PE; DMPE, dimethylated PE; PC, phosphocholine.
[d] Diacyl IPL.
[e] Characterized by *m/z* 1,283.
[f] Characterized by *m/z* 1,366.

IPLs containing membrane-spanning lipids were not detected, whereas direct acid hydrolysis of cells generated substantial amounts of these lipids (9 to 48%) (Table 2). As discussed previously for SD 1 and 3 *Acidobacteria* species (14), this may be caused by relatively large and polar head groups, which may render the IPLs containing membrane-spanning lipids nonextractable 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 can be tentatively concluded from the total number of acyl/alkyl carbons of C_{30} and C_{32}.

Variation in lipid composition: influence of environmental variables. The membrane lipids of SD 4 *Acidobacteria* are quite distinct from the diacyl glycerol membrane lipids that characterize most bacteria. First, they contain a substantial amount of membrane-spanning lipids (9 to 48%) (Table 2). Second, 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* species (61), *Sarcina ventriculi* (62), members of the *Thermotogales* (2, 57, 63–65), *Thermoanaerobacter* species (58, 59, 62), *Acidobacteria* SD 1 and 3 (14), and *Acidobacteria* SD 4 (this work). 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 pyrophilus* (67), *Thermotoga* species (2, 57), several sulfate-reducing bacteria (68–70), *Mycoplasma fermentans* (71), anammox bacteria (3), *Acidobacteria* SD 1 and 3 (14), and *Acidobacteria* SD 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* (72). Consistent with this idea, most bacterial species that contain membrane-spanning lipids are moderate or extreme thermophiles, although *Butyrivibrio* species 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*. Chloracidobacterium 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 number 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 few 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 to 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 (Fig. 6a and b). Only a small increase in the number of *n*-alkyl chains (Fig. 6c) and a 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* not only indicate that the occurrence of membrane-spanning lipids and the presence of ether bonds in bacteria are adaptations to temperature but suggest that other (including genetic) factors probably also play a role.

***Acidobacteria* as a potential source for branched GDGTs.** brGDGTs (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 (73–75), 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 candidates for the production of brGDGTs (13), and this has been supported by the recent identification of its "building block" *iso*-diabolic acid 3 in SD 1 and 3 *Acidobacteria* (14). Although small amounts of brGDGT 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* members are probably the origin of the brGDGTs. This was one of the reasons to perform this study. It showed that SD 4 *Acidobacteria* do not produce brGDGTs, 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 to 30%) (Table 2). Such moieties reflect important structural units of the brGDGTs 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, while 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 brGDGTs 1 and 2.

Another apparent mismatch with the GDGTs occurring in SD 1 *Acidobacteria* and brGDGTs occurring in the environment is that only GDGT 1 was detected in the *Acidobacteria* (14), whereas brGDGTs with additional methyl substituents (such as 2) occur widely in the environment (73, 76). This additional methylation occurs at one (i.e., 2) or both alkyl chains at C-5, although recently brGDGTs with the methylation at C-6 also have been reported (77). 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 at the 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

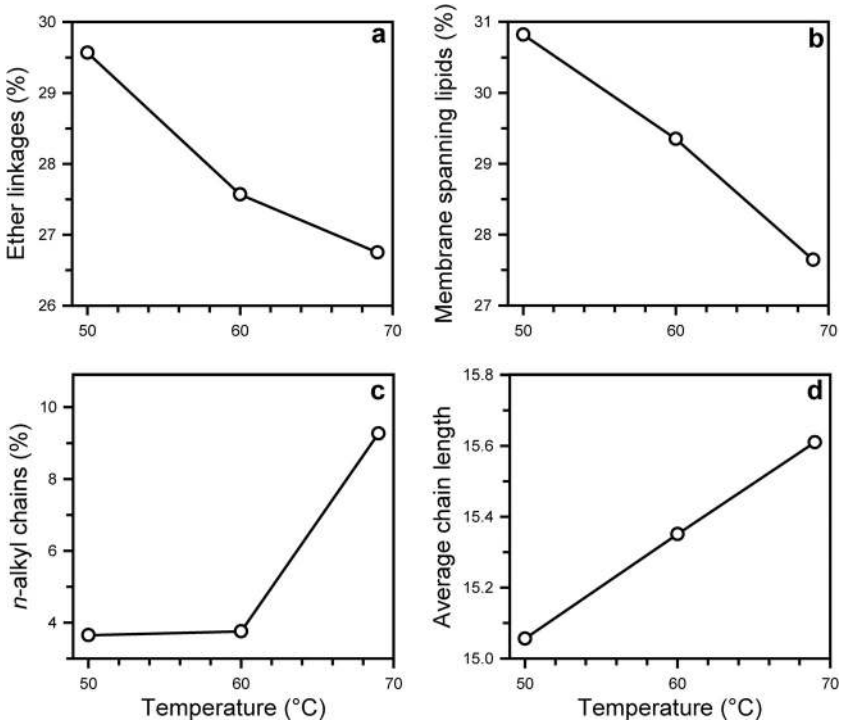

FIG 6 Membrane lipid characteristics of *P. methylaliphatogenes* K22^T as a function of growth temperature. (a) Fraction of ether linkages; (b) fraction of membrane-spanning lipids; (c) fraction of *n*-alkyl chains; (d) average chain length. The average chain length (number of carbon atoms) was calculated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

(73–75). The mesophilic species *B. fastidiosa* is, however, an exception in this respect, since it does not contain structure 4 or 6 (Table 2). This suggests that although there apparently is strong environmental control of brGDGT composition (73–75), there also are genetic factors 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* members 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 brGDGTs 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 paleoenvironment and paleoclimate studies (5).

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