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13,16-Dimethyl Octacosanedioic Acid (*iso*-Diabolic Acid), a Common Membrane-Spanning Lipid of *Acidobacteria* Subdivisions 1 and 3▽†

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The distribution of membrane lipids of 17 different strains representing 13 species of subdivisions 1 and 3 of the phylum *Acidobacteria*, a highly diverse phylum of the *Bacteria*, were examined by hydrolysis and gas chromatography-mass spectrometry (MS) and by high-performance liquid chromatography-MS of intact polar lipids. Upon both acid and base hydrolyses of total cell material, the uncommon membrane-spanning lipid 13,16-dimethyl octacosanedioic acid (*iso*-diabolic acid) was released in substantial amounts (22 to 43% of the total fatty acids) from all of the acidobacteria studied. This lipid has previously been encountered only in thermophilic *Thermoanaerobacter* species but bears a structural resemblance to the alkyl chains of bacterial glycerol dialkyl glycerol tetraethers (GDGTs) that occur ubiquitously in peat and soil and are suspected to be produced by acidobacteria. As reported previously, most species also contained *iso*-C_{15} and $C_{16:1\omega7C}$ as major fatty acids but the presence of *iso*-diabolic acid was unnoticed in previous studies, most probably because the complex lipid that contained this moiety was not extractable from the cells; it could only be released by hydrolysis. Direct analysis of intact polar lipids in the Bligh-Dyer extract of three acidobacterial strains, indeed, did not reveal any membrane-spanning lipids containing *iso*-diabolic acid. In 3 of the 17 strains, ether-bound *iso*-diabolic acid was detected after hydrolysis of the cells, including one branched GDGT containing *iso*-diabolic acid-derived alkyl chains. Since the GDGT distribution in soils is much more complex, branched GDGTs in soil likely also originate from other (acido)bacteria capable of biosynthesizing these components.

Acidobacteria form one of the most diverse phyla of the domain *Bacteria*. Twenty-six subdivisions have been reported, mainly based on environmental sequences (4), but only a few of these include taxonomically characterized representatives. Subdivision 1 is best characterized by *Acidobacterium capsulatum* (25), *Terriglobus roseus* (12), *Edaphobacter aggregans*, *Edaphobacter modestus* (26), and four *Granulicella* species (40). *Bryobacter aggregatus* (30) is the only species described for subdivision 3, and subdivision 8 contains three described species: *Holophaga foetida* (33), *Geothrix fermentans* (7), and *Acanthopleuribacter pedis* (16). A whole-genome study has been performed for *A. capsulatum* and two isolates, "*Candidatus* Koribacter versatilis" and "*Candidatus* Solibacter usitatus," from subdivisions 1 and 3, respectively (48). These studies indicate that acidobacteria are generally versatile heterotrophs that are common in peats and acidic soils. Molecular ecology studies have indicated that acidobacteria form a substantial part of the microbial community of wetlands (10, 29, 34, 39) and soils (19, 21). In soils, the relative contribution of these bacteria to the overall bacterial community is negatively correlated with pH (21); especially species from subdivision 1 are more abundant at lower pH (12).

The abundance of acidobacteria in soils and peat has led to the suggestion that they may be the biological source of a group of structurally uncommon tetraether membrane lipids (49). These components (e.g., structure 1 in Fig. 1) were first identified in peat (45) and subsequently turned out to occur ubiquitously in soils (51). Determination of the stereochemistry of the glycerol moieties in these molecules revealed that they must derive from *Bacteria* (50), possibly *Acidobacteria* (49). The natural ^{13}C abundance of these bacterial tetraethers in soil is consistent with a heterotrophic lifestyle of its source organism(s) (53). Examination of a suite of bacterial cultures, including a few acidobacterial strains, for the bacterial tetraethers did not reveal their origin (49). However, in recent years many more members of the phylum *Acidobacteria* have been characterized.

In the present paper, we describe in detail the lipid compositions of 17 different strains representing 13 species of subdivisions 1 and 3 of the phylum *Acidobacteria* and discuss their distributions.

MATERIALS AND METHODS

Cultures. The acidobacteria used in this study are listed in Table 1. Four members of the genus *Granulicella*, i.e., *Granulicella paludicola* OB1010^T, *G.*

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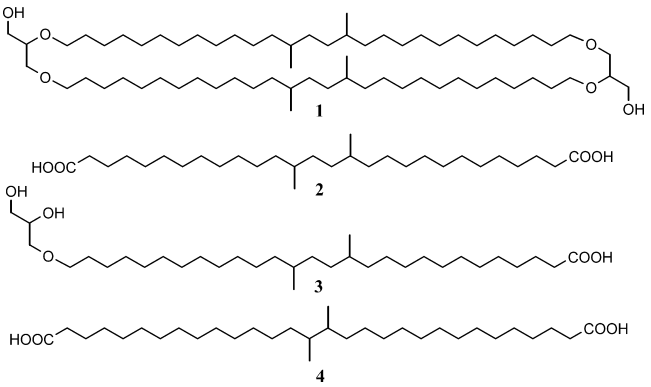

FIG. 1. Structures of lipids discussed in the text. Shown are the branched GDGT with four methyl substituents (structure 1), *iso*-diabolic acid (structure 2), the glycerol ether derivative of *iso*-diabolic acid (structure 3), and diabolic acid (structure 4).

paludicola LCBR1, *G. aggregans* TPB6028^T, and *G. pectinivorans* TPB6011^T, were grown at the Winogradsky Institute in liquid medium MM1 with 0.5 g liter^{-1} fructose as described by Pankratov and Dedysh (40). Two strains of *B. aggregatus*, *Bryocella elongata* SN10, *Terriglobus* sp. KMR, *A. capsulatum* 161^T, and *Acidobacteriaceae* bacterium KA1 were grown at the Winogradsky Institute in liquid medium MM with 0.5 g liter^{-1} glucose (30). *B. aggregatus* MPL3^T and *Acidobacteriaceae* bacterium KA1 were grown at both pHs 4.2 and 7.0 in medium MM buffered with 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) or HEPES, respectively. For most strains, the cell biomass used for analysis was collected after 1 week of incubation at 24°C. The biomass of the slow-growing species *B. aggregatus* was collected after 2 weeks of incubation.

Cells of all other acidobacterial strains and *A. capsulatum* 161^T were grown at the DSMZ at room temperature with vigorous shaking for 1 to 5 weeks, depending on the strain. Strains 277, 307, A2-1c, and A2-4c were grown at pH 5.5 in a medium consisting of soil solution equivalent (1) with the iron content changed from 5 to 50 μM and buffered with 10 mM MES as the basal medium and supplemented with 10-fold-diluted HD medium (0.5 g liter^{-1} of peptone, 0.25 g liter^{-1} of yeast extract, and 0.1 g liter^{-1} of glucose), Balch's vitamin mixture (2) 10-fold diluted, and trace element solution SL-10 (47). The two *Edaphobacter* strains (Jbg-1^T and Wbg-1^T) were grown at pH 5.5 in a 5-fold-diluted HD medium containing 1.0 g liter^{-1} of peptone, 0.5 g liter^{-1} of yeast extract, 0.2 g liter^{-1} of glucose, and 10 mM MES. *T. roseus* KBS 63^T was grown at pH 7.2 in DSMZ medium 830 (see http://www.dsmz.de/microorganisms/media_list.php). *A. capsulatum* 161^T was grown at pH 3.5 in DSMZ medium 269 with 0.1 g liter^{-1} instead of 0.3 g liter^{-1} yeast extract. Biomass was harvested by ultracentrifugation (9,000 × *g*, 30 min; Avanti-J26 XPI, Beckman Coulter), frozen (−20°C, overnight), and lyophilized (5 Pa at −30°C).

TABLE 1. Acidobacteria used in this study

Species	Subdivision	Origin	Substrates used	pH range	pH optimum	Reference(s)
Acidobacteriaceae strain 277	1	Sandy soil from river bank, Okavango, Namibia	Most sugars, some sugar alcohols, starch, some amino acids, acetate, succinate, some heteropolysaccharides	3.5–7.5	5.0–6.5	DSMZ[a]
Acidobacteriaceae strain 307	1	Sandy soil from river bank, Okavango, Namibia	Most sugars, some sugar alcohols, starch, some amino acids, fumarate, succinate, some heteropolysaccharides	3.5–8.5	5.0–6.5	DSMZ[a]
Acidobacteriaceae strain A2-1c	1	Savanna soil, Erichsfelde, Namibia	Most sugars, some sugar alcohols, starch, some amino acids, gluconate, pyruvate, succinate, some heteropolysaccharides	ND[b]	ND	DSMZ[a]
Acidobacteriaceae strain KA1 (= DSM 23886)	1	*Sphagnum* peat, Tver region, European north Russia	Most sugars, glucuronate, galacturonate, some heteropolysaccharides	3.5–7.3	5.0–5.5	WIM[c]
Acidobacteriaceae strain A2-4c	1	Savanna soil, Erichsfelde, Namibia	ND	ND	ND	DSMZ[a]
A. capsulatum 161^T (= DSM 11244^T)	1	Acidic mine drainage, Japan	Most sugars, glucuronate, starch, some heteropolysaccharides	3.0–6.0	ND	12, 25
B. elongata SN10^T (= DSM 22489^T)	1	*Sphagnum* peat, Archangelsk region, European north Russia	Most sugars, lactate, ethanol, some heteropolysaccharides	3.2–6.6	4.7–5.2	9
G. pectinivorans TPB6011^T (= DSM 21001^T)	1	*Sphagnum* peat, Tomsk region, western Siberia	Several sugars, acetate, lactate, galacturonate, gluconate, starch, some heteropolysaccharides and polyalcohols	3.0–7.5	3.8–4.5	40
G. aggregans TPB6028^T (= LMG 25274^T)	1	*Sphagnum* peat, Tomsk region, western Siberia	Most sugars, malate, pyruvate, galacturonate, gluconate, starch, some heteropolysaccharides and sugar alcohols	3.0–7.5	3.8–4.5	40
G. paludicola OB1010^T (= DSM 22464^T)	1	*Sphagnum* peat, Yaroslavl region, European north Russia	Most sugars, starch, some heteropolysaccharides, galacturonate	3.0–7.5	3.8–4.5	40
G. paludicola LCBR1	1	*Cladonia* sp. collected from peat bog, Tomsk region, western Siberia	Most sugars, starch, some heteropolysaccharides, galacturonate, gluconate, glucuronate	3.0–7.5	3.8–4.5	40
T. roseus KBS 63^T (= DSM 18391^T)	1	Agricultural soil, Michigan	Several sugars, succinate, glucuronate, gluconate	5.0–7.0	6.0	12
Terriglobus sp. KMR (= ATCC BAA-1395)	1	Sphagnum peat, Yaroslavl region, European north Russia	Most sugars, starch, some heteropolysaccharides	4.5–7.2	5.5–6.5	41
E. modestus Jbg-1^T (= DSM 18101^T)	1	Soil deciduous forest, Würzburg, Germany	Most sugars, some sugar alcohols, glutamate, glutamine	4.0–7.0	5.5	26
E. aggregans Wbg-1^T (= DSM 19364^T)	1	Alpine soil, Kochel, Germany	Several sugars, glutamate, glutamine, aspartate, ornithine, glucuronate	4.5–7.0	5.5	26
B. aggregatus MPL3^T (= DSM 18758^T)	3	*Sphagnum* peat, Tomsk region, western Siberia	Most sugars, pyruvate, starch, some heteropolysaccharides, galacturonate, glucuronate	4.5–7.2	5.5–6.5	30
B. aggregatus MPL1011	3	*Sphagnum* peat, Tomsk region, western Siberia	Most sugars, pyruvate, lactate, starch, some heteropolysaccharides, galacturonate, glucuronate	4.5–7.2	5.5–6.5	30

[a] Isolated at DSMZ.
[b] ND, not determined.
[c] WIM, isolated at the Winogradsky Institute of Microbiology.

Lipid analysis. For all of the strains studied, lyophilized cells were hydrolyzed with 1 N 5% HCl in methanol by reflux for 3 h. The hydrolysate was adjusted to pH 4 with 2 N KOH-methanol (MeOH) (1:1, vol/vol) and, after addition of water to a final 1:1 ratio of H_2O-MeOH, extracted three times with dichloromethane (DCM). The DCM fractions were collected and dried over sodium sulfate. The extract obtained was methylated with diazomethane and separated over an activated Al_2O_3 column into an apolar and a polar fraction using DCM and DCM-MeOH (1:1, vol/vol) as the eluent, respectively. The apolar fraction (containing the fatty acid methyl esters [FAMEs]) was analyzed by gas chromatography (GC) and GC-mass spectrometry (MS). Double-bound positions of the monounsaturated FAMEs were determined on the basis of the mass spectra of their dimethyl disulfide derivatives as described by Nichols et al. (35). The polar fraction was dissolved in hexane–2-propanol (99:1, vol/vol), filtered over a 0.45-μm polytetrafluoroethylene filter, and analyzed by high-performance liquid chromatography (HPLC)–atmospheric pressure chemical ionization (APCI) mass spectrometry (MS) for branched GDGTs.

For selected strains, intact polar lipids were extracted from the lyophilized cells using a modified Bligh-Dyer technique (5) as described by Pitcher et al. (42). An aliquot of the extract obtained was dissolved in hexane–2-propanol–water (72:27:1), filtered through a 0.45-μm regenerated cellulose filter, and analyzed by HPLC-electrospray ionization (ESI) MS.

For *G. aggregans* TPB6028^T, a more extensive lipid characterization was performed. The lyophilized cells were also hydrolyzed with 1 N KOH-MeOH (96%) by reflux for 1 h. The hydrolysate was neutralized with 2 N HCl-MeOH (1:1, vol/vol) to pH 4 and, after addition of water, extracted with DCM three times. The extract was methylated with diazomethane, silylated with *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide in pyridine at 60°C for 20 min and analyzed by GC and GC-MS. Lyophilized cells were also directly extracted using a modified Bligh-Dyer technique (see above) without prior acid or base hydrolysis, and the extract was methylated and silylated as described above and analyzed by GC and GC-MS. Aliquots of the Bligh-Dyer extract were also hydrolyzed with HCl-MeOH and with KOH-MeOH, derivatized, and analyzed by GC and GC-MS as described above. The cell residue after Bligh-Dyer extraction was hydrolyzed with HCl-MeOH and KOH-MeOH, derivatized, and analyzed by GC and GC-MS as described above.

GC and GC-MS. GC was performed using a Hewlett-Packard (HP6890) instrument equipped with an on-column injector and a flame ionization detector. A fused silica capillary column (25 m by 0.32 mm) coated with CP Sil-5 CB (film thickness, 0.12 μm) was used with helium as the 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 Finnigan Trace ultra gas chromatograph interfaced with a Finnigan Trace DSQ mass spectrometer operated at 70 eV with a mass range of *m/z* 40 to 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. GDGT lipids were analyzed by HPLC-APCI-MS according to Hopmans et al. (17), 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 Cyano column (150 mm by 2.1 mm; 3 μm). The flow rate of the hexane–2-propanol (99:1, vol/vol) eluent was 0.2 ml min^{-1}, isocratically for the first 5 min and thereafter with a linear gradient to 1.8% propanol in 45 min. The column was subsequently rinsed by eluting 10% propanol in hexane for 10 min and back to 1% propanol for the last 10 min. The injection volume of the samples was usually 10 μl. Branched GDGTs were analyzed mostly by APCI-MS in selected ion monitoring mode (SIM) (44).

IPL analysis by HPLC-ESI-MS^2. Intact polar lipids (IPLs; i.e., glycerol ester membrane lipids with attached polar head groups) were analyzed according to a previously published method (46), with some modifications. The Agilent 1200 series liquid chromatograph used was equipped with a thermostat-controlled autoinjector and a column oven and coupled to a Thermo LTQ XL linear ion trap with an Ion Max source with an ESI probe (Thermo Scientific, Waltham, MA). Separation was achieved on a Lichrosphere diol column (250 mm by 2.1 mm, 5-μm particles; Alltech Associates Inc.) maintained at 30°C using the mobile-phase system described by Sturt et al. (46) but with a modified elution program of 100% A for 1 min, followed by a linear gradient to 66% A and 34% B in 17 min, maintenance for 12 min, followed by a linear gradient to 35% A and 65% B in 15 min, where A is 79:20:0.12:0.04 hexane–2-propanol–formic acid–14.8 M aqueous NH_3 and B is 88:10:0.12:0.04 2-propanol—water–formic acid–14.8 M aqueous NH_3. The flow rate was 0.2 ml min^{-1}, and the total run time was 60 min, with a reequilibration period of 20 min. Positive-ion ESI settings were as follows: capillary temperature, 275°C; sheath gas (N_2) pressure, 25 arbitrary units (AU); auxiliary gas (N_2) pressure, 15 AU; sweep gas (N_2) pressure, 20 AU; spray voltage, 4.5 kV. The lipid extract was analyzed by positive-ion scanning (*m/z* 400 to 2,000), followed by data-dependent, dual-stage tandem MS (MS^2), where the four most abundant masses in the mass spectrum were fragmented successively (normalized collision energy, 25; isolation width, 5.0; activation Q, 0.175). Each MS^2 was followed by data-dependent, triple-stage tandem MS (MS^3), where the base peak of the MS^2 spectrum was fragmented under identical fragmentation conditions.

Preparation of an authentic standard. 13,16-Dimethyloctacosanedioic acid (ca. 3 mg; 96% pure by GC) was isolated by thin-layer chromatography from a large-size culture of *Thermoanaerobacter thermohydrosulfuricus* strain TLO^T (3). Its structure was confirmed by two-dimensional nuclear magnetic resonance techniques, which gave spectra identical to those reported previously (24).

RESULTS

Seventeen strains belonging to the phylum *Acidobacteria* were analyzed for their lipid composition; 15 of them are species in subdivision 1 (*A. capsulatum*, two *Terriglobus* strains, two *Edaphobacter* species, four *Granulicella* species, *B. elongata* SN10, and five additional representatives of the family *Acidobacteriaceae*), and two are in subdivision 3 (two *B. aggregatus* strains) (Table 1; see Fig. S1 in the supplemental material).

Composition of total lipid fractions. Fig. 2a shows a typical gas chromatogram of a total lipid fraction obtained after acid hydrolysis of cells of *G. aggregans* TPB6028^T. The dominant regular fatty acids are *iso*-C_{15}, *n*-$C_{16:1\omega7c}$, and *n*-C_{16}, a distribution commonly observed in the acidobacterial strains studied, although other dominant regular fatty acids are *iso*-$C_{17:1\omega8c}$, *iso*-C_{17}, and *n*-$C_{18:1\omega9}$, depending on the phylogenetic positions of the strains studied (Table 2). In addition to these regular fatty acids, a more unusual, much later-eluting (Fig. 2a) lipid was detected in relatively large amounts (22 to 43% of the total fatty acids; Table 2). This lipid was identified as a long-chain dicarboxylic acid, 13,16-dimethyloctacosanedioic acid (or *iso*-diabolic acid; structure 2 in Fig. 1), based on mass spectral data reported previously (24) and coelution experiments with an authentic standard isolated from cell material of *T. thermohydrosulfuricus* (3).

iso-Diabolic acid could be identified only after hydrolysis of the cell material. We used *G. aggregans* TPB6028^T to examine the mode of occurrence of *iso*-diabolic acid in the phylum *Acidobacteria*. The lipids released after alkaline hydrolysis of the cell material were similar to those obtained after acid hydrolysis (Fig. 2b). However, *iso*-diabolic acid was absent from the Bligh-Dyer extract of *G. aggregans* TPB6028^T (Fig. 2c) and also did not appear after base or acid hydrolysis of the Bligh-Dyer extract (Fig. 2d and e). Acid and base hydrolyses of the residue of the cell material after Bligh-Dyer extraction did release *iso*-diabolic acid; the distribution of the lipids released revealed an enrichment of *iso*-diabolic acid relative to the regular fatty acids (e.g., Fig. 2f). Bligh-Dyer extraction of *G. aggregans* TPB6028^T at an elevated temperature (i.e., 40°C) did not result in release of *iso*-diabolic acid in any form in the extract, as revealed by acid hydrolysis of the obtained extract and subsequent GC-MS analysis.

Acid hydrolysis of cell material of *E. aggregans* Wbg-1^T and *Acidobacteriaceae* bacterium A2-4c released not only *iso*-diabolic acid but also its glycerol ether derivative (i.e., structure 3 in Fig. 1). This component was tentatively identified by its mass

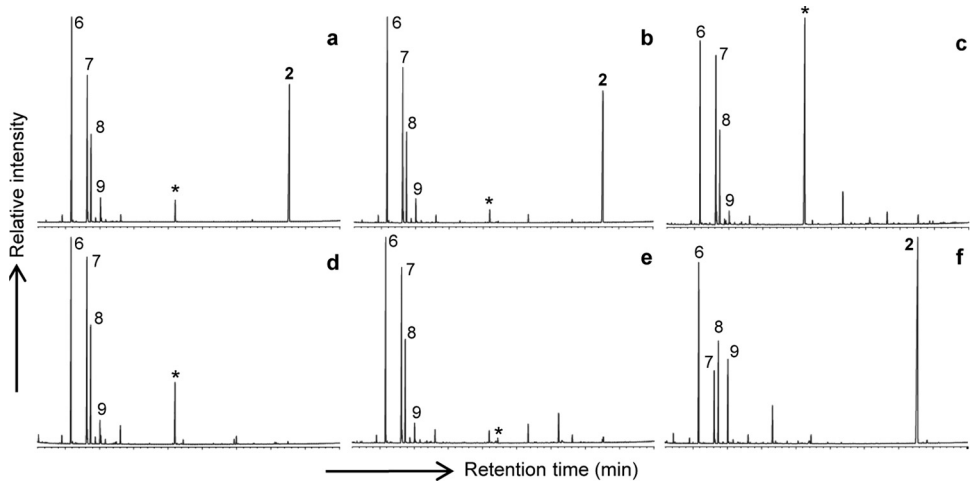

FIG. 2. Gas chromatograms of various lipid extracts of *G. aggregans* TPB6028^T. Shown are the fatty acids released after acid (a) and alkaline (b) hydrolyses of whole-cell material and the fatty acids present in the Bligh-Dyer extract (c), the acid-hydrolyzed Bligh-Dyer extract (d), the alkali-hydrolyzed Bligh-Dyer extract (e), and the alkali-hydrolyzed residue after Bligh-Dyer extraction (f). Fatty acids were derivatized to the corresponding methyl esters prior to GC analysis. Key: 2, *iso*-diabolic acid; 6, *iso*-C_{15} fatty acid; 7, $C_{16:1\omega7c}$ fatty acid; 8, $C_{16:0}$ fatty acid; 9, *iso*-C_{17} fatty acid. An asterisk denotes a plasticizer contaminant (phthalate). Note the abundance of *iso*-diabolic acid in the hydrolysates from the cell material and residue after extraction and its absence from the solvent extract.

spectrum, which was virtually identical to that of 13,14-dimethyl-28-glyceryloxydodecanoic acid (8), which was identified on the basis of the full identification of its homologue (11). Although the mass spectrum was almost identical, there was a difference in the retention time, consistent with the different methyl substitution pattern of the glycerol ether derivative of *iso*-diabolic acid.

Distribution of intact polar lipids in *Acidobacteria*. To characterize the IPLs of the species belonging to the phylum *Acidobacteria*, the Bligh-Dyer solvent extract of *G. aggregans* TPB6028^T, *T. roseus* KBS 63^T, and *E. aggregans* Wbg-1^T were analyzed by HPLC-ESI-MS^2. The IPLs were dominated by phosphatidylethanolamine (PE) lipids with *iso*-C_{15}, *n*-$C_{16:1}$, and *n*-$C_{16:0}$ diglyceride moieties as determined by the molecular weight and characteristic fragmentation. However, no membrane-spanning IPLs were detected in any of these Bligh-Dyer extracts.

Presence of branched GDGTs in *Acidobacteria*. The acid-hydrolyzed biomass of the acidobacterial cultures was also analyzed for the presence of GDGTs by HPLC-APCI-MS using SIM. In the cases of *E. aggregans* Wbg-1^T and *Acidobacteriaceae* bacteria A2-4c and 307, positive identification of a branched GDGT (i.e., structure 1 in Fig. 1) was obtained, while the other cultures were negative (Table 2). The concentration in strain 307 was low, but in the cases of *E. aggregans* Wbg-1^T and strain A2-4c, its identification by SIM was confirmed by full-scan HPLC-APCI-MS; the APCI mass spectrum was characterized by peaks at *m/z* 1,022 $[M+H]^+$, *m/z* 1,004 $[M+H]^+$-18 (loss of water), and *m/z* 948 $[M+H]^+$-74 (loss of glycerol), the characteristic fragmentation pattern for isoprenoid (17) and nonisoprenoidal GDGTs (45). In both cases, no other branched GDGTs with additional methyl groups, which normally co-occur with branched GDGT in peats and soils (50, 52), were detected.

DISCUSSION

Fatty acid distribution. All of the fatty acid distributions of the acidobacteria studied show a quite consistent pattern; they all contain *iso*-$C_{15:0}$ as an abundant fatty acid (12 to 39%; Table 2) in combination with $C_{16:1\omega7C}$ and $C_{16:0}$, $C_{18:1\omega9}$, or *iso*-C_{17} fatty acids (Table 2). This is generally consistent with data reported in the literature for these species (9, 12, 26, 30, 40), except that most of these studies report "summed feature 1" (i.e., 16:1ω7c and/or 15:0 *iso*-2-OH). Our GC-MS data indicate that in all of these species, $C_{16:1\omega7C}$ is a dominant fatty acid and that 2-hydroxy *iso*-C_{15} is not. Another apparent mismatch with the literature data is observed for *E. aggregans* Wbg-1^T: Koch et al. (26) reported $C_{17:1\omega8C}$ (49%), *iso*-$C_{16:0}$ (19%), and $C_{17:0}$ (8%) to be the dominant fatty acids, in strong contrast to the fatty composition reported here, i.e., dominated by *iso*-$C_{15:0}$ (26%), $C_{16:1\omega7C}$ (24%), and $C_{16:0}$ (6%) (Table 2). The fatty acid distribution reported here matches those of phylogenetically related species (e.g., *E. modestus* Jbg-1^T; Table 2) quite well, and we therefore cannot explain the results reported by Koch et al. (26).

Multivariate statistical analysis of the distribution of the lipids released by acid hydrolysis of cell material revealed four distinct clusters (Fig. 3). The first cluster is composed of *Acidobacteriaceae* bacterial strains 277, 307, A2-1c, and KA1; they are all characterized by relatively large amounts of (un)saturated *iso*-C_{17} fatty acids (23 to 30%; Table 2). The second cluster is formed by *A. capsulatum* 161^T, containing $C_{18:1\omega9}$ as an important constituent (ca. 13%), which is uncommon in the other acidobacteria studied (Table 2). The third cluster, composed of many of the other species, is characterized by the presence of $C_{16:0}$ and $C_{16:1}$ fatty acids (Table 2). The *Bryobacter* species, characterized by a relatively high abundance of the $C_{16:0}$ fatty acid, form a fourth distinct cluster. This classification is generally in line with the 16S rRNA gene phylogeny (see

TABLE 2. Fatty acid composition after acid hydrolysis of cell material and presence of branched GDGTs in the acidobacterial strains studied

Component	% of total fatty acids[a] in strain[b]:																	
	1[c]	2[c]	3[c]	4[d]	5[c]	6[c]	6[d]	7[d]	8[d]	9[d]	10[d]	11[d]	12[c]	13[d]	14[c]	15[c]	16[d]	17[d]
iso-C_{13}					0.2				0.2		5.4	5.2	2.3					
iso-C_{14}				2.0							1.6							
$C_{14:1\omega5}$								0.1	1.7	0.1	0.4[e]	0.2	0.4	0.1	0.5	0.6	0.1	0.3
$C_{14:0}$						0.5	0.5	0.5	1.8	0.8	0.9	0.5	1.5	1.9	1.1	1.0	1.2	1.7
iso-$C_{15:1\omega6}$				0.4	3.8[f]		0.2	0.1	0.2					0.1				
iso-C_{15}	38.7	34.2	29.2	46.0	32.1	36.1	38.8	35.0	37.0	30.0	33.0	32.5	35.1	27.0	22.4	26.0	16.0	12.0
anteiso-C_{15}			7.3					0.2	0.1		1.1	0.8		0.5			0.8	
$C_{15:1\omega6}$								0.1	0.8	0.3	4.4[g]	0.4		0.1			0.3	0.5
$C_{15:0}$									0.2		1.4	0.2		0.2			0.6	0.4
iso-C_{16}		0.8	2.6	0.6														
$C_{16:1\omega7c}$		0.2		0.3	9.9	2.0	3.2	26.0	21.0	22.0	8.4	18.5	20.3	18.0	21.5	23.7	23.0	32.0
$C_{16:1\omega7t}$								0.4	0.3	1.2	0.4	0.2		0.3	2.1		0.8	0.4
$C_{16:1\omega6+\omega5}$								0.3						1.6				0.1
$C_{16:0}$		3.0		0.6	4.8	5.8	3.2	7.0	5.0	11.0	9.8	6.0	6.8	7.3	7.6	5.6	15.0	18.0
iso-$C_{17:1\omega8c}$	19.5	14.6	17.4	18.4	1.0	0.5	0.8	0.8	0.2	0.6	0.1	0.2		0.4	0.3	1.1	0.3	
iso-$C_{17:1\omega8t}$	0.9	1.3	0.5	1.5														
iso-C_{17}	5.8	6.1	8.5	6.8	1.0	1.7	1.5	1.7	1.6	3.0	2.2	1.7	0.5	0.7	0.7	2.0	2.7	1.0
anteiso-C_{17}	0.4	0.9	4.1	1.8				0.2	0.6	0.6	1.0	1.0		0.7			2.4	0.5
$C_{17:1\omega8}$					0.3	1.4	0.9							0.2[h]			0.1[h]	0.1
$C_{17:0}$		0.8			0.2	1.3	0.3	0.1	0.5	0.4	0.6	0.7		0.3			0.5	0.4
$C_{18:1\omega9}$					3.4	11.1	15.4	0.4										2.0
$C_{18:1\omega7}$		2.9						0.7	0.1	0.1				3.8	0.2		0.9	0.4
$C_{18:0}$		2.6			0.6	4.0	1.8	0.4	0.4	1.0	0.5	0.5		1.4	0.8		1.5	1.2
iso-C_{19}		2.7																
iso-Diabolic acid[i]	34.7	29.9	30.3	21.5	39.4	35.6	33.4	26.0	28.0	29.0	29.0	31.5	33.1	36.0	42.8	36.1	33.0	29.0
iso-C_{15}-monoglycerol ether					0.2											0.5		
C30 glycerol ether[j]					3.2											3.5		
GDGT[k]	−	±	−	−	√	−	−	−	−	−	−	−	−	−	−	√	−	−

[a] Values for major components (i.e., >3% of the total fatty acids) are underlined.
[b] Strains: 1, *Acidobacteriaceae* strain 277; 2, *Acidobacteriaceae* strain 307; 3, *Acidobacteriaceae* strain A2-1c; 4, *Acidobacteriaceae* strain KA1; 5, *Acidobacteriaceae* strain A2-4c; 6, *A. capsulatum* 161^T; 7, *B. elongata* SN10^T; 8, *G. pectinivorans* TPB6011^T; 9, *G. aggregans* TPB6028^T; 10, *G. paludicola* LCBR1; 11, *G. paludicola* OB1010^T; 12, *T. roseus* KBS63^T; 13, *Terriglobus* sp. strain KMR; 14, *E. modestus* Jbg-1^T; 15, *E. aggregans* Wbg-1^T; 16, *B. aggregatus* MPL3^T; 17, *B. aggregatus* MPL1011^T.
[c] Grown at DSMZ.
[d] Grown at the Winogradsky Institute of Microbiology.
[e] ω7 instead of ω5.
[f] ω8 instead of ω6.
[g] ω7 instead of ω6.
[h] ω6 instead of ω8.
[i] Structure 2 in Fig. 1.
[j] Structure 3 in Fig. 1.
[k] Structure 1 in Fig. 1. −, not detected; ±, detected by single-ion monitoring MS but not confirmed by full mass spectrum; √, present, confirmed by full mass spectrum.

Fig. S1 in the supplemental material), which also shows *Acidobacteriaceae* bacterial strains 277, 307, A2-1c, and KA1 and *Bryobacter* species as distinct clusters. In the 16S rRNA gene-based tree, *A. capsulatum* is most closely related to *Acidobacteriaceae* strain A2-4c, whereas this is not apparent from its lipid profile, which most closely resembles that of the *Granulicella-Terriglobus-Edaphobacter* group (Fig. 3). The apparent ordering of the different *Granulicella*, *Terriglobus*, and *Edaphobacter* species in the 16S rRNA gene tree is not so strictly followed in the clustering on the basis of the lipid profiles, which is likely caused by the rather similar lipid distributions of these species (Table 2).

***iso*-Diabolic acid in acidobacteria.** In contrast to all previous studies, *iso*-diabolic acid was detected in all examined species of subdivisions 1 and 3 of the phylum *Acidobacteria* in relatively large amounts (22 to 43% of all fatty acids). This is most likely due to the fact that it could be released only by hydrolysis of total cell material, a procedure that is not typically applied in most microbiological studies. *iso*-Diabolic acid has previously been encountered only in thermophilic *Thermoanaerobacter* species (3, 24, 32), where it fulfills a role as a membrane-spanning lipid. In our study of *T. thermohydrosulfuricus* (3), *iso*-diabolic acid was also detected only after hydrolysis of total cell material. However, Lee et al. (32) reported IPLs containing *iso*-diabolic acid in an esterified form in an extract obtained by extracting *T. ethanolicus* at an elevated temperature (i.e., 40°C). When *G. aggregans* TPB6028^T cells were subjected to hot extraction, no material could be released that, upon hydrolysis, generated *iso*-diabolic acid. This indicates that these acidobacteria probably contain complex lipids that are hard to extract and contain *iso*-diabolic acid in a bound form in substantial amounts. Since *iso*-diabolic acid is released by both acid and base hydrolyses, it is likely that it is predominantly bound via ester linkages and not by glycosidic or amide bonds, which are not hydrolyzed by treatment with a base.

Membrane-spanning lipids are far less common in the bacterial than in the archaeal domain but do occur there. Clarke et al. (6) provided evidence that diabolic acids (e.g., structure

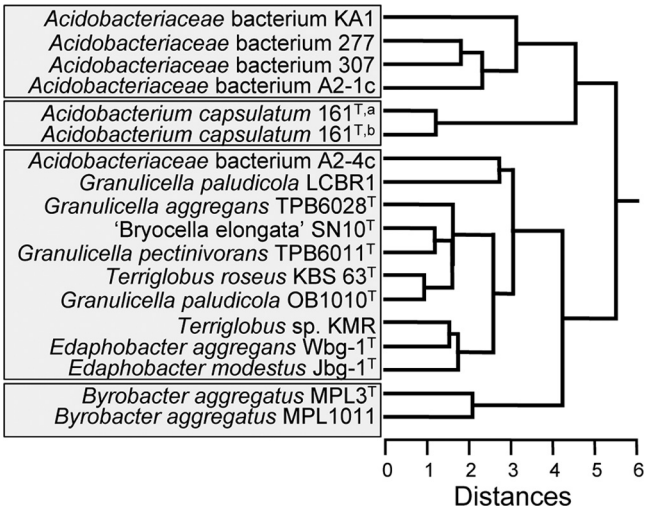

FIG. 3. Multivariate statistical analysis of the distribution of the lipids released by acid hydrolysis of cell material of the acidobacteria studied. The input of the cluster analysis was the Bray-Curtis similarity matrix of lipid profiles (% of total lipids; Table 2). A hierarchical clustering was performed in SYSTAT 13 using Euclidian distance and the average-linking method. *A. capsulatum* 161 was grown at both the DSMZ (a) and the Winogradsky Institute (b).

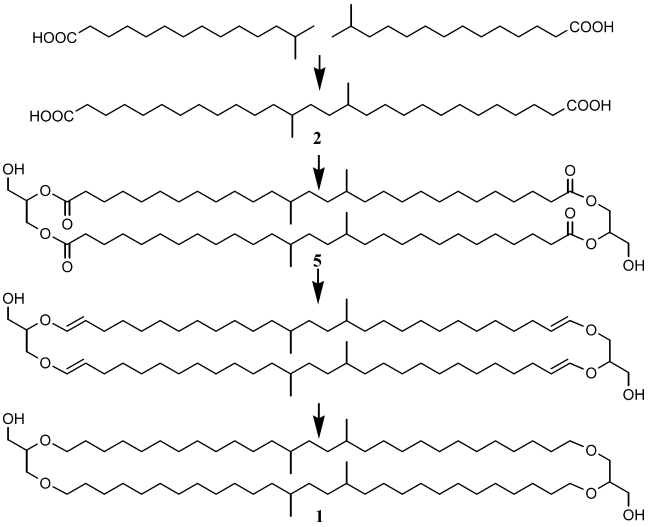

FIG. 4. Hypothetical scheme outlining a potential biosynthesis route from the C_{15} iso fatty acid to branched GDGT **1**. Intermediates are *iso*-diabolic acid formed by the coupling of two C_{15} iso fatty acids as previously proposed by Jung et al. (23), a glycerol dialkyl glycerol tetraester in which *iso*-diabolic acid forms the backbone, and an intermediate in its conversion into branched GDGT. The latter step is based on a biosynthetic route of branched dialkyl glycerol diethers in *M. xanthus* proposed by Ring et al. (43).

4 in Fig. 1) may act as linkers between two glycerol moieties in polar membrane lipids of *Butyrivibrio* spp. Diabolic acids and their ether derivatives have also been identified in members of the order *Thermotogales* (11, 18, 20, 54) and in *Sarcina ventriculi* (22). In all of these cases, the membrane-spanning lipids could be extracted from the cells, in contrast to what is reported here for the acidobacteria. Sinninghe Damsté et al. (8) examined nine different species of the order *Thermotogales* by HPLC-MS and demonstrated the presence of membrane-spanning diglycerol lipids comprised of diabolic acid-derived moieties. In *Thermotoga* spp., the core membrane lipids were characterized by the presence of both ester and ether bonds, whereas in the phylogenetically more distinct *Thermosipho* and *Fervidobacterium* spp., only ester bonds occurred.

It is interesting that in the acidobacteria *E. aggregans* Wbg-1^T and *Acidobacteriaceae* strain A2-4c, we detected the glycerol ether derivative of *iso*-diabolic acid and a branched GDGT (Table 2; structures 1 and 3 in Fig. 1). Both components bear a strong structural resemblance to *iso*-diabolic acid since the carbon skeleton of the diether-bound alkyl moieties is the same as that of *iso*-diabolic acid (Fig. 1). In *E. aggregans* Wbg-1^T and strain A2-4c, we detected only a branched GDGT with four methyl groups and not any further methylated branched GDGTs, which normally co-occur with branched GDGT in peats and soils (50, 52). We also detected only one monoether (i.e., structure 3 in Fig. 1). At the same time, *iso*-diabolic acid was the only dicarboxylic acid encountered in this species (and all other species). This suggests a biosynthetic relationship between the branched GDGT, the glycerol ether derivative of *iso*-diabolic acid, and *iso*-diabolic acid. Ring et al. (43) provided evidence that ether linkages in IPLs in the aerobic bacterium *Myxococcus xanthus* were formed by the reduction of ester linkages via vinyl ether intermediates. This pathway could perhaps also explain the presence of both ester and ether bonds in the membrane-spanning lipids of *Thermotoga* species and the presence of only tetraesters in *Thermosipho* and *Fervidobacterium* spp. (8), of which the latter two would thus apparently lack the enzymes to perform this reaction. Similarly, this may explain the presence of branched GDGT and the glycerol ether derivative of *iso*-diabolic acid in *E. aggregans* Wbg-1^T and their absence in the other acidobacteria biosynthesizing *iso*-diabolic acid. This would indicate that a glycerol dialkyl glycerol tetraester in which *iso*-diabolic acid forms the backbone would be an intermediate (structure 5 in Fig. 4) which in the other acidobacteria (which would lack the ability to transform an ester into an ether linkage) could form the core of the difficult-to-extract IPLs. The presence of mixed esters/ethers with *iso*-diabolic acid as the core lipid in *E. aggregans* Wbg-1^T and strain A2-4c would also explain the presence of the glycerol ether derivative of *iso*-diabolic acid after hydrolysis and its absence in the other acidobacteria studied.

It has been demonstrated that diabolic acids are produced from condensation reactions of fatty acids at the ω-1 positions (15). This proposed biosynthetic pathway is in good agreement with the carbon number distributions of lipids in various species of *Thermotogales* (8). Jung et al. (23) suggested that the *iso*-diabolic acids would be biosynthesized by ω,ω′ coupling of two iso fatty acids. In *Archaea*, isoprenoid GDGTs are also thought to be produced from (partial) condensation of two glycerol diethers by ω,ω′ coupling of isoprenoid alkyl chains (27, 28, 36); note that the tail (where the condensation takes place) of the isoprenoid chain is identical to that of iso fatty acids. The proposed ω,ω′ coupling of two iso fatty acids to obtain *iso*-diabolic acids is in full agreement with the results obtained here: *iso*-C_{15} fatty acid is the dominant iso fatty acid present, and the C_{30} *iso*-diabolic acid is the only dicarboxylic acid present. This would suggest the hypothetical overall bio-

TABLE 3. Major fatty acids (>5% of the total fatty acids) present in the acid hydrolysates of two acidobacteria grown at different pHs

Fatty acid	% of total fatty acids			
	Acidobacteriaceae strain KA1		*B. aggregatus* MPL3^T	
	pH 4.2	pH 7.0	pH 4.2	pH 7.0
iso-C_{15}	44.5	49.1	14.4	16.9
$C_{16:1\omega7c}$			28.1	34.1
$C_{16:0}$			8.7	8.8
iso-$C_{17:1\omega8c}$	15.1	9.5		
iso-$C_{17:0}$	8.4	9.5		
iso-Diabolic acid[a]	21.3	22.1	39.8	33.7

[a] Structure 2 in Fig. 1.

synthetic pathway indicated in Fig. 4. Incorporation of isovaleryl coenzyme A in the pathway of fatty acid biosynthesis results in the formation of *iso*-C_{15} fatty acid. Subsequent ω,ω′ coupling of two *iso*-C_{15} fatty acids results in the formation of *iso*-diabolic acid, which can be incorporated into tetraester (structure 5) and tetraether (structure 1) membrane-spanning lipids that subsequently are attached to polar head groups to produce membrane-spanning IPLs. Some of these IPLs have previously been identified in *T. ethanolicus* (32). It should be noted that the membrane-spanning IPLs do not necessarily contain only *iso*-diabolic acid but can also contain, in addition, other fatty acid moieties, as observed for the extractable PE IPLs. However, the results of the hydrolysis of the residue after Bligh-Dyer extraction indicate that *iso*-diabolic acid represents the majority (Fig. 2f). The identity of the head groups in these membrane-spanning IPLs remains unknown, as it was not possible to extract them, even at elevated temperature.

Most bacterial species that contain membrane-spanning lipids are moderate or extreme thermophiles (various *Thermotoga*, *Thermosipho*, *Fervidobacterium*, and *Thermoanaerobacter* species and *S. ventriculi*), although *Butyrivibrio* sp. is a mesophile. All of the analyzed acidobacteria studied here are also mesophilic. Therefore, the occurrence of membrane-spanning IPLs in bacteria seems not only to be an adaptation to temperature. In their study of the presence of diabolic acids in *S. ventriculi*, Jung et al. (23) showed that this bacterium produced these lipids only when grown at pH 3 and not when grown at pH 7, suggesting a response to pH. When, at neutral pH, the growth temperature was increased from 37 to 45 to 55°C, diabolic acids were also produced. Some of the bacteria studied that produce membrane-spanning IPLs are (slightly) acidophilic, and the acidobacteria studied here are also acidophilic. However, when *Acidobacteriaceae* strain KA1 and *B. aggregatus* MPL3^T, which are representatives of subdivisions 1 and 3, respectively, were grown at pH 7.0 instead of pH 4.2, *iso*-diabolic acid was still produced in similar relative quantities (Table 3). Therefore, it remains to be seen why some bacteria produce membrane-spanning IPLs.

Geobiological implications. In sedimentary records, soil-derived branched GDGTs are widely used to reconstruct past pHs and temperatures. This is based on empirical relationships developed on the basis of differences in branched GDGT distributions observed in soils from a wide variety of geographical locations and their relationship with environmental variables such as temperature and pH (52). This was explained by the presence of ubiquitously occurring bacteria in soil that adjust their membrane composition in response to temperature and pH. Acidobacteria were proposed as likely candidates (49) because they occur in substantial cell numbers in soil (13, 14, 19) and peat (10, 34) and because branched GDGT concentrations in soil are higher at lower pH (51) and acidobacteria, especially those in subdivision 1, are often relatively more abundant in soil at lower pH (21, 31).

The identification of a branched GDGT in *E. aggregans* Wbg-1^T and *Acidobacteriaceae* strain A2-4c is the first evidence that the bacterial branched GDGTs may indeed be produced by acidobacteria, as suggested earlier (49). The presence of *iso*-diabolic acid in all examined species of *Acidobacteria* in subdivisions 1 and 3 is a further confirmation of this, since the carbon skeleton of *iso*-diabolic acid is the most common alkyl moiety in the branched GDGTs in soil (51). A potential acidobacterial origin of branched GDGTs is consistent with their heterotrophic lifestyle (i.e., they use a wide variety of organic components; Table 1); the natural ^{13}C abundance of branched GDGTs in soil also suggests that they must derive from heterotrophic microbes (37, 38, 53). Nevertheless, there is still an important mismatch with the GDGTs occurring in *E. aggregans* Wbg-1^T and *Acidobacteriaceae* strain A2-4c and those occurring in soil: in *E. aggregans* Wbg-1^T and *Acidobacteriaceae* strain A2-4c, only a branched GDGT with four methyl groups was detected, whereas soils contain branched GDGTs with additional methyl substituents (51, 52). Another issue is that the acidobacteria analyzed here are all aerobes, while in peatlands the highest concentrations of branched GDGTs are found below the water table, in the zone where oxygen quickly becomes limiting (49, 50). Therefore, branched GDGTs in soil likely also originate from other (acido)bacteria capable of biosynthesizing these components, and until we have discovered what they are, biological experiments using cultures to validate the relationships between the degree of methylation and cyclization of branched GDGTs and environmental parameters such as temperature and pH (52) have to wait.

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