Geochemical Perspectives Letters

Oxygen limitation can trigger the production of branched GDGTs in culture

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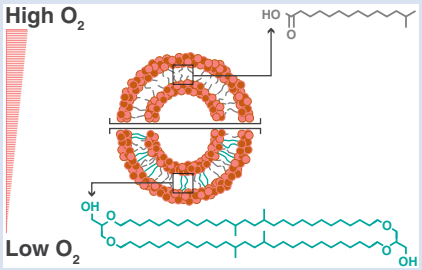
Abstract



Branched glycerol dialkyl glycerol tetraethers (brGDGTs) are ubiquitous and well preserved sedimentary biomarkers. These compounds serve as important palaeoenvironmental indicators due to strong empirical correlations between brGDGT distributions and temperature and pH in modern environments. However, the mechanistic link between temperature, pH, and brGDGT production has been impossible to ascertain thus far due to the absence of a clear biological source for brGDGTs. Here, we report that oxygen limitation triggers brGDGT production in at least one cultured species of Acidobacteria and confirm for the first time the biosynthesis of three structural varieties of brGDGTs, including an uncharacterised isomer of brGDGT Ic. This discovery helps explain why brGDGT producers have been so difficult to identify and provides a pathway towards uncovering the genetic basis and biological function of brGDGTs, which will lead to a more comprehensive understanding of their palaeoenvironmental significance. If the oxygen effects observed here apply more broadly, the empirical calibrations for brGDGT-based temperature and pH reconstructions may currently be missing the effects of oxygen as a relevant and possibly dominant control in the environmental distributions of brGDGTs.

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Introduction

Methodological advances in sample preparation and analysis over the past decade have highlighted the vast global distribution of brGDGTs across terrestrial, aquatic, hydrothermal, and sedimentary systems (Lincoln *et al.*, 2013; De Jonge *et al.*, 2014; Weber *et al.*, 2018; Wang *et al.*, 2019). The relative abundances of structurally unique brGDGTs have been explored in many of these settings in efforts to calibrate brGDGTs as a palaeoenvironmental proxy by establishing empirical correlations with temperature and pH, such as the **M**ethylation index of **B**ranched **T**etraethers (MBT) and the **C**yclization index of **B**ranched **T**etraethers (CBT), respectively (Weijers *et al.*, 2007; Peterse *et al.*, 2012; Naafs *et al.*, 2017). Prior work has demonstrated that many Acidobacteria, a diverse and widespread phylum of soil bacteria, synthesise the potential brGDGT precursor iso-diabolic acid (13,16-dimethyl octacosanedioic acid) in large quantities (Sinninghe Damsté *et al.*, 2018), and that some members of subdivision 1 (SD 1) Acidobacteria, including *Edaphobacter aggregans,* produce trace amounts of at least one brGDGT (Sinninghe Damsté *et al.*, 2011). However, the lack of cultured organisms that consistently produce branched tetraethers raises the question of how these compounds are so structurally diverse and abundant in nature yet so elusive in the laboratory.

Here we investigated the effects of molecular oxygen availability (O_2) on brGDGT production to test the hypothesis that brGDGT production may require a specific environmental constraint. Many Acidobacteria including *E. aggregans* harbour high affinity terminal oxidases in their genomes (Eichorst *et al.*, 2018). These types of oxidases often have half-saturation constants at low nM concentrations of O_2, likely enabling survival and growth in micro-aerobic habitats (Pitcher and Watmough, 2004). Such low O_2 availability is common in many soil, peat, and sedimentary environments and prior work on brGDGT distribution and production across oxygen gradients suggests that some environmental source organisms may preferentially grow at oxic/anoxic transitions (Liu *et al.*, 2014; Weber *et al.*, 2018; Martínez-Sosa and Tierney, 2019). To study the effects of O_2 limitation, we examined the tetraether and fatty acid membrane composition of *E. aggregans* grown in a simplified yeast extract medium under O_2 conditions ranging from fully aerated (21 % O_2) to severely O_2 limited (1 % O_2). An excess additional carbon source (sucrose) was either added or omitted to control for the potential effects of growth rate.

Results and Discussion

The effects of O_2 limitation. *E. aggregans* produced similar fatty acids under all growth conditions with the sum of just four fatty acids (iso-diabolic acid, iso-C15:0, C16:0, and C16:1) constituting over 90 % of the fatty acid fraction (Fig. 1, Table S-1), consistent with previous observations in a different growth medium (91 %; Sinninghe Damsté *et al.*, 2011). Although the relative abundance of individual fatty acids differed significantly between culture conditions, the hypothesised brGDGT precursor

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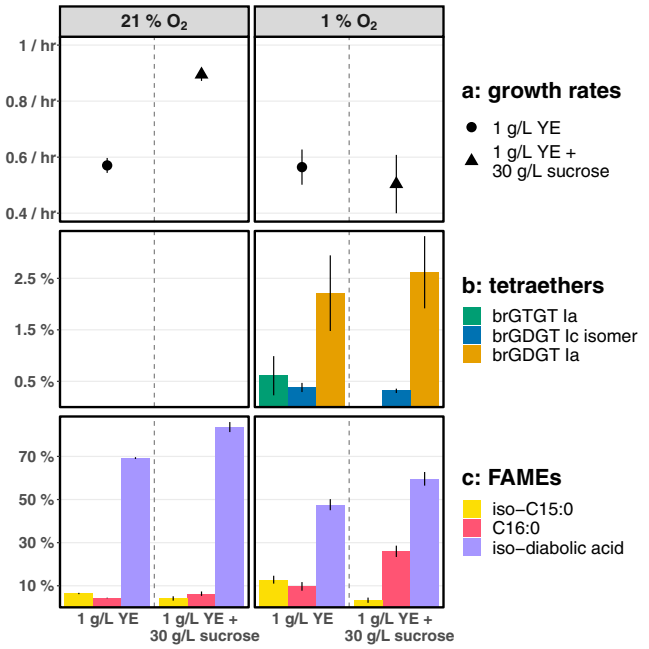

Figure 1. Growth rate and lipid membrane profiles of *E. aggregans* grown in liquid culture at 21 % O_2 (= air, left) and 1 % O_2 (right) with and without carbon excess. Data points represent averages and standard deviations of biological triplicates. **(a)** Growth rates with medium containing 1 g/L Yeast Extract (YE, circle) and 1 g/L YE + 30 g/L sucrose (triangle). **(b)** Tetraethers [% of all detected core lipids]: brGTGT Ia (green), brGDGT Ic isomer (blue), brGDGT Ia (orange). No tetraethers were detected at 21 % O_2. **(c)** Most abundant FAMEs (Fatty Acid Methyl Esters, % of all detected core lipids): iso-C15:0 (light yellow), C16:0 (red), iso-diabolic acid (purple).

iso-diabolic acid was always the dominant membrane core lipid ranging in relative abundance from 40 ± 3 % to 84 ± 2 % with a systematic decrease both at lower O_2 and without sucrose. In liquid culture, the organism produced no detectable tetraethers at 21 % and 5 % O_2, however, at 1 % O_2 *E. aggregans* produced significant quantities (up to 2.9 ± 0.7 %) of up to three different tetraethers (brGDGT Ia, brGTGT Ia, and an uncharacterised isomer of brGDGT Ic; see Fig. 2 for structures).

Growth rates slowed significantly at lower O_2 when grown with excess sucrose (from 0.89 ± 0.02/day at 21 % O_2 to 0.50 ± 0.10/day at 1 % O_2) and remained unchanged when grown without sucrose (0.57 ± 0.03/day at 21 % O_2 to 0.56 ± 0.06/day at 1 % O_2) but tetraether production in liquid culture was limited to 1 % O_2 conditions independent of growth rate (Fig. 1, Table S-2). Temperature and pH were identical for all culturing conditions, suggesting that O_2 limitation is the primary environmental trigger for brGDGT production in *E. aggregans*, with a threshold for biosynthesis in liquid culture between 5 % and 1 % O_2. However, *E. aggregans*, named for its tendency to aggregate, displays significant cellular clumping in liquid culture. Cell aggregates form natural O_2 gradients (Wessel *et al.*, 2014) and may trigger brGDGT biosynthesis by reducing O_2 availability to individual cells deeper within the aggregates. Consequently, the actual biochemical O_2 threshold for brGDGT production may be significantly lower.

Although the physiological role of brGDGTs is not yet understood, we speculate that brGDGT production in *E. aggregans* could be part of an energy stress response imposed by the lack of sufficient O_2 for energy production. Such terminal electron acceptor limitation could place increased importance on the role of lipids in restricting membrane permeability. In Archaea, which produce isoprenoidal GDGTs (isoGDGTs), tetraether lipids are often inferred to play a similar role in combatting energy stress by decreasing the cellular membrane's proton permeability and thus decreasing maintenance energy costs (Qin *et al.*, 2015). Recent work also suggests that the synthesis of ether bonds under oxygen limiting conditions is an energetic adaptation to reducing environments (Boyer *et al.*, 2020). The structural attributes of the tetraether lipids that *E. aggregans* produces that may play a role in decreased membrane permeability include their membrane spanning nature and ether bonds. Specifically, membrane spanning lipids increase membrane rigidity and stability and ether-bound lipids have been found to decrease membrane permeability relative to ester-bound lipids (Van de Vossenberg *et al.*, 1998).

Tetraether structures. The tetraethers of *E. aggregans* were identified based on their retention times and fragmentation patterns as brGDGT Ia, brGTGT Ia (Glycerol *Trialkyl* Glycerol Tetraether), and a structural isomer of brGDGT Ic (Fig. 2). Retention time offsets between the newly described tetraethers (brGDGT Ic isomer and brGTGT Ia) and previously described structurally similar tetraethers (brGDGT Ic and C46 GTGT) are shown in Figure 2b. These data confirm and quantify for the first time the previously suspected production of brGDGT Ia in *E. aggregans* (Sinninghe Damsté *et al.*, 2011), and reveal the capability of this species to produce other forms of tetraethers including a potentially cyclised structure. However, the brGDGT Ic isomer we observe is asymmetric with two unsaturation equivalents on the same alkyl chain. BrGDGT Ic commonly found in environmental samples contains only one unsaturation (a pentacyclic ring) on each alkyl chain instead. While the unsaturation equivalents could stem from cyclisation pointing to an isomer of brGDGT Ic with a bicyclic alkyl chain, other structural variations such as double bonds and cyclohexyl rings could produce the observed mass spectrum (see Fig. 2 for two potential structures). While hydrogenation of the tetraethers did not reduce the brGDGT Ic isomer to brGDGT Ia, suggesting cyclisation instead of double bonds, tetraethers with cyclopentyl rings usually elute later under normal phase chromatography (Liu *et al.*, 2016), not earlier as observed here. Future analyses by NMR or ether cleavage and GC-MS with a purified concentrated fraction of this trace membrane component will help determine the exact structure of this new brGDGT Ic isomer. If *E. aggregans* indeed produces a cyclised brGDGT it must contain a cyclisation pathway different from the archaea, as the previously described archaeal grsAB tetraether cyclisation genes (Zeng *et al.*, 2019) do not have homologues in the *E. aggregans* genome. The third tetraether found in *E. aggregans*, brGTGT Ia, is likewise rarely identified in environmental samples but other tri-alkyl tetraethers have been previously observed. Although the exact biosynthetic pathway for branched tetraethers remains unresolved, we note the correlation between the occurrence of brGTGT Ia and elevated quantities of iso-C15.

Environmental implications. The clumping phenotype of *E. aggregans* highlights the potential importance of micro-scale spatial O_2 gradients in brGDGT production and is supported by *E. aggregans* growth on solid medium; unlike in liquid cultures, we observed measurable quantities of both brGDGT Ia and the brGDGT Ic isomer in fully oxygenated (21 % O_2) plate growth experiments (Table S-1). Aerobic plate growth and colony formation often produce micro-aerophilic environments within colonies, thus a significant portion of the plate culture likely experienced severe O_2 limitation thereby triggering brGDGT production. This mode of growth on a solid substrate, rather than in liquid culture, is much more representative of the lifestyle of soil microorganisms in their natural environment (Kolter and

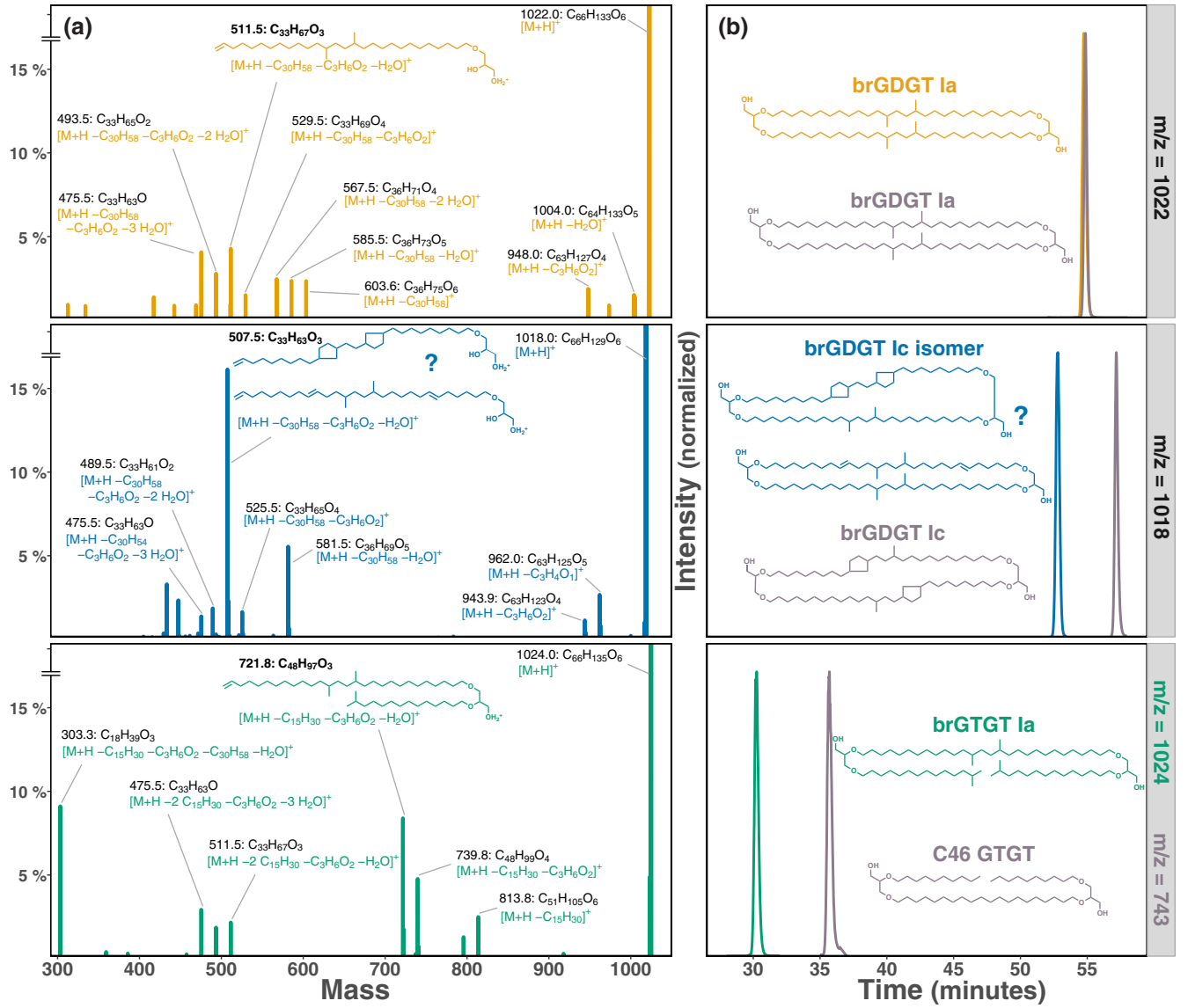

Figure 2. MS2 fragmentation and peak retention time patterns of tetraether lipids identified in *E. aggregans* using normal phase liquid chromatography. **(a)** MS2 fragmentation of brGTGT Ia (green), brGDGT Ic isomer (blue), and brGDGT Ia (orange). Fragment masses and formulae shown in black. Losses from the molecular ion (M+H–*x*.) shown in corresponding compound colour. Structures of three key identifying fragments included. **(b)** Peak retention times of the tetraethers identified in *E. aggregans* in comparison to structurally similar tetraethers from a soil standard and the C46 GTGT analytical standard (grey). Peak intensities are normalised.

Greenberg, 2006) and speaks to the potential importance of O_2 limitation in heterogenous natural settings.

To test if all Acidobacteria exhibit similar suboxia-induced brGDGT production, we conducted follow up experiments with the well studied model organism *Acidobacterium capsulatus* at 1 % O_2. While *A. capsulatus* also produced significant quantities of iso-diabolic acid (23 ± 2 %), we found no evidence of tetraether production at low O_2 (Table S-1). This suggests that brGDGT production at low O_2 is either not a universal trait among Acidobacteria, or that biosynthetic activation thresholds differ between organisms. Future genetic work with *E. aggregans* and additional culturing work with other Acidobacteria will help establish how widespread the genes for brGDGT production are in this phylum and under what conditions brGDGT biosynthesis occurs.

Palaeoclimate proxies. Although *E. aggregans* does not make a sufficiently large number of different tetraethers to test the mechanistic links between physiology and brGDGT-based climate proxies such as MBT and CBT, the results presented here show clearly that O_2 limitation can be a trigger for brGDGT production. Because modern environmental calibrations rely on the relative distribution of brGDGTs in environmental samples, they are susceptible to abundance changes in any one structure. Although calibration data have demonstrated strong relationships between brGDGT distributions and environmental conditions like temperature and pH, dissolved O_2 measurements are limited in existing calibration data (Raberg *et al.*, 2021). It is therefore unknown whether this variable is implicitly captured or mostly unaccounted for in modern calibrations. Recent environmental observations indicate that the abundance of other brGDGTs not detected in this study (*e.g.*, brGDGT IIa and IIIa) may also respond to redox conditions (Weber *et al.*, 2018; Yao *et al.*, 2020). Holocene palaeoclimate records from the Arctic show a decoupling between known temperature trends and brGDGT-inferred temperatures (*e.g.*, Kusch *et al.*, 2019) suggesting an alternative overriding environmental control that is

unaccounted for. Our results suggest that for at least one source organism dissolved oxygen is the primary gradient to which brGDGT biosynthesis responds.

Conclusions

Our results indicate that O_2 availability controls biosynthesis of branched tetraethers by *E. aggregans,* with low O_2 required for production. This is the first confirmed organism to consistently produce significant quantities of multiple brGDGTs, thus opening the door to rigorous laboratory examination to elucidate the biosynthetic pathways and biological function of these enigmatic lipids. The identification of the enzymes involved in the synthesis of brGDGTs in *E. aggregans* will aid in the identification of other bacterial species that produce brGDGTs and help uncover the effect that O_2 limitation may have on brGDGT biosynthesis and palaeoclimate proxies.

Author Contributions

TAH, ADY and SHK designed the research. TAH, JMM, ADY, JD and ND performed the research. TAH and SHK analysed the data. TAH, JMM and SHK wrote the paper.

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Additional Information

Supplementary Information accompanies this letter at https://www.geochemicalperspectivesletters.org/article2132.

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Geochemical Perspectives Letters

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Supplementary Information

The Supplementary Information includes:

- Extended Methods
- Tables S-1 and S-2
- Figures S-1 and S-2
- Supplementary Information References

All data and code are available at https://github.com/KopfLab/2021_halamka_et_al and archived with DOI 10.5281/zenodo.4651686.

Extended Methods

Culturing

Edaphobacter aggregans strain Wbg-1 (DSM 19364; Koch *et al.*, 2008) and *Acidobacterium capsulatum* strain 161 (DSM 11244) were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). *E. aggregans* was grown on yeast extract (YE) at 20 °C and pH 5.0 in phosphate-buffered *Acidobacterium* subdivision 1 medium with (PSYL5) or without (PYL5) added sucrose (1.8 g/L KH_2PO_4, 0.2 g/L $MgSO_4 \cdot 7\ H_2O$, 1 g/L YE, +/- 30 g/L sucrose) (Campanharo *et al.*, 2016). *A. capsulatum* was grown at 20 °C at pH 4.3 in DSMZ 269 rich medium with glucose. Both strains were grown routinely in aerobic culture tubes (20.8 % O_2). For suboxic growth, the strains were grown in 100 mL bottles continuously stirred and flushed at 100-133 SmL/min with a sterile hydrated blend of N_2 and Air using digital mass flow controllers (Alicat Scientific) at appropriate ratios to obtain 1.04 % and 5.2 % O_2 (v/v) in the headspace (throughout the manuscript we refer to 1.04 % O_2 as 1 %, 5.2 % as 5 %, and 20.8 % as 21 %). Attempts to study *E. aggregans* at O_2 mixing ratios < 1 % were unsuccessful because of cell clumping and insufficient growth yield for lipid analysis. Attempts to grow *E. aggregans* anaerobically by fermentation were likewise unsuccessful, matching previous physiological observation for many Acidobacteria (Eichorst *et al.*, 2018). No attempts were made to grow *E. aggregans* with alternative electron acceptors because the organism does not have the genetic potential for anaerobic respiration. *E. aggregans* was grown aerobically on plates containing PSYL5 with 1.5 g/L agar. Culture experiments were conducted in biological triplicates. For liquid cultures, optical density (OD) was measured at 600 nm (Thermofisher, Genesys 30 Visible Spectrophotometer) and growth rates were calculated by non-linear least squares fitting of OD measurements to a logistic function.

Sample Preparation

Cells from liquid culture were harvested in stationary phase by centrifugation (5000 RPM for 3 minutes). Cells from plate culture were harvested by scraping with a spatula once no more visible growth was observed. Harvested cells were lyophilized overnight and then physically disrupted in 2 mL microcentrifuge tubes by vortexing with methanol and 250 µL of 100 µm muffled glass beads for 10 minutes at 3000 rpm using a Disruptor Genie (Scientific Industries, SI-DD38). This additional cell disruption step increased fatty acid extraction yields for *E. aggregans* by an estimated >1000 % for iso-C15:0 and iso-diabolic acid. Excess MeOH was evaporated and 25 µg 23:0 PC (1,2-ditricosanoyl-sn-glycero-3-phosphocholine), 25 µg 24:0 FA (tetracosanoic acid), and 25 ng C46 GTGT (Huguet *et al.*, 2006) were added to all samples as internal quantification standards. Lipids were extracted for 90 minutes at 65 °C with 500 µL 3N HCl in MeOH (33 % final water content) to cleave tetraether headgroups and transesterify fatty acid esters to fatty acid methyl esters (FAMEs). Samples were cooled for 10-minutes before the addition of 500 µL methyl tert butyl ether. The upper organic phase was extracted 3 times with 500 µL n-hexane and total lipid extracts (TLEs) were evaporated under N_2. TLEs were resuspended in n-hexane for solid phase extraction and separated into 3 lipid classes using 500 mg aminopropyl columns (Sigma Aldrich Discovery DSC-NH2): FAMEs and ketones were eluted using 6 mL of n-hexane followed by 7 mL of 4:1 Hexane:DCM; alcohols including tetraether core lipids were eluted using 7 mL of 9:1 DCM:Acetone; free acids were eluted using 8 mL of 2.5 % formate in DCM. The fatty acid fraction was only used to verify quantitative derivatization to FAMEs during the initial extraction step.

Lipid Analysis

Tetraethers were analysed in the Organic Geochemistry Laboratory at the University of Colorado Boulder on a Thermo Scientific Ultimate 3000 HPLC coupled to a Q Exactive Focus Orbitrap-Quadrupole MS with APCI source using previously published methods (Hopmans *et al.*, 2016) with the following modification: the initial eluent gradient was 14 % 90:1 Hexane:IPA instead of 18 % 90:1 Hexane:IPA in order to achieve better separation between isomers. The compounds were confirmed together with retention time and MS2 spectra generated by data dependent acquisition mode (ddMS2). FAMEs were analysed on a DB-5 capillary column (Agilent Technologies, 30 m length, 0.25 mm I.D., 0.25 µm film thickness) with a Thermo Trace 1310 Gas Chromatograph (2 min at 80 °C, ramped to 140 °C at 20 °C/min, ramped to 325 °C at 5 °C/min, held for 10 min at 325 °C) equipped with PTV injector and coupled to a TSQ 8000 Evo triple quadrupole MS on full scan mode. Cellular tetraether abundances were calculated relative to FAMEs using the C24 and C46 internal standards.

Hydrogenation

To determine if any of the tetraether structures identified in this study contained unsaturations, a hydrogenation experiment was conducted on a portion of the alcohol fraction of *E. aggregans*. The sample was suspended in 1.5 mL of ethyl acetate in a 2 mL glass vial, with an excess of platinum catalyst added to the vial. The sample was then connected to a pressured H_2 gas manifold for 90 minutes. The resulting sample was run on the HPLC-MS using the same method described below for all other samples and compared to a non-hydrogenated fraction of the same sample.

Supplementary Tables

Table S-1 Lipid data.

Table S-1 is available for download (Excel) from the online version of the article at http://www.geochemicalperspectivesletters.org/article2132.

Table S-2 Growth rate data.

Organism	% O_2	C source	Replicate	Growth rate [d^{-1}]	*K* [OD600]
e.agg	21	1 g/L YE	rep1	0.54	0.60
e.agg	21	1 g/L YE	rep2	0.59	0.54
e.agg	21	1 g/L YE	rep3	0.58	0.57
e.agg	21	1 g/L YE + 30 g/L sucrose	rep1	0.87	1.56
e.agg	21	1 g/L YE + 30 g/L sucrose	rep2	0.89	1.54
e.agg	21	1 g/L YE + 30 g/L sucrose	rep3	0.92	1.51
e.agg	5	1 g/L YE + 30 g/L sucrose	rep1	0.88	1.20
e.agg	5	1 g/L YE + 30 g/L sucrose	rep2	0.91	1.23
e.agg	5	1 g/L YE + 30 g/L sucrose	rep3	0.88	1.16
e.agg	1	1 g/L YE	rep1	0.53	0.37
e.agg	1	1 g/L YE	rep2	0.52	0.29
e.agg	1	1 g/L YE	rep3	0.64	0.30
e.agg	1	1 g/L YE + 30 g/L sucrose	rep1	0.38	0.44
e.agg	1	1 g/L YE + 30 g/L sucrose	rep2	0.55	0.36
e.agg	1	1 g/L YE + 30 g/L sucrose	rep3	0.58	0.35
a.cap	1	DSMZ 269	rep1	1.10	0.20
a.cap	1	DSMZ 269	rep2	1.31	0.19
a.cap	1	DSMZ 269	rep3	0.94	0.25

Supplementary Figures

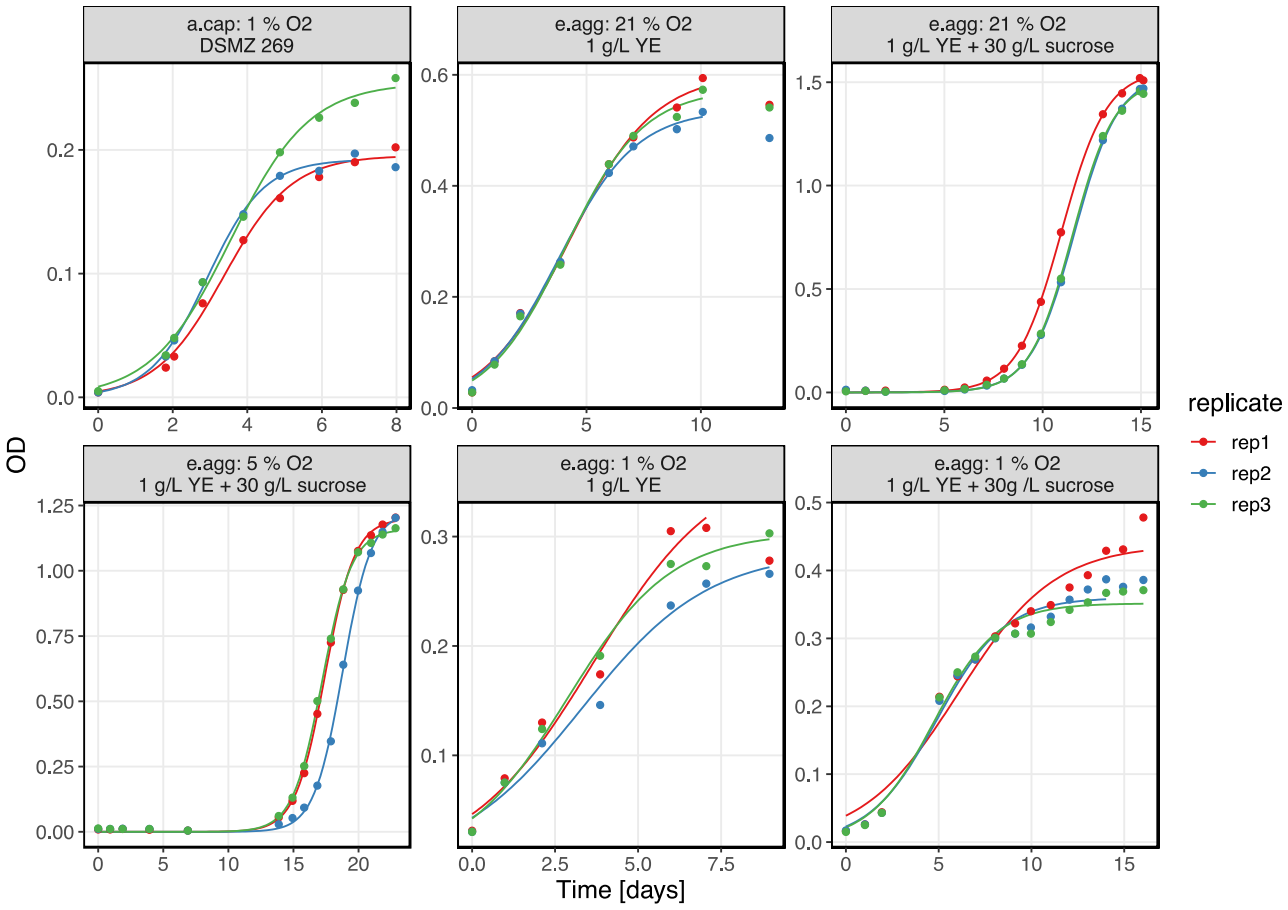

Figure S-1 Growth curves from *E. aggregans* and *A. capsulatum* liquid culture experiments. Panels are labelled with organism name, oxygen content, and carbon source. Colours represent biological replicates. Data points (circles) are optical density measurements at 600 nm *vs*. time (in days). Lines are fitted growth curves estimated by fitting optical density measurements to the following logistic equation, where t is time, OD is the optical density, and fit parameters μ and K represent the growth rate and carrying capacity (max OD), respectively:

$$OD_t = \frac{K}{1 + (K/OD_{t0} - 1) \cdot e^{-\mu t}}$$

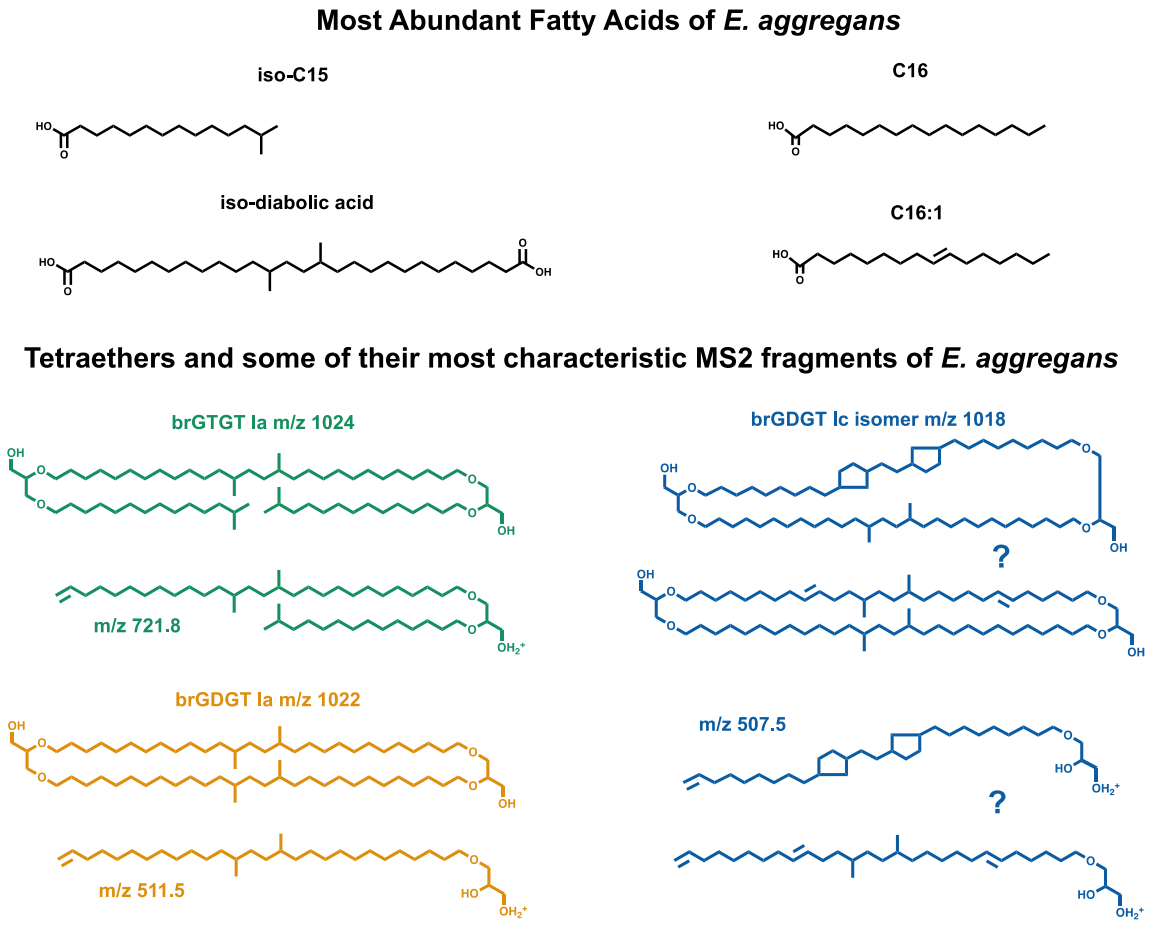

Figure S-2 Structures of the most abundant fatty acids and tetraethers (with characteristic fragments) of *E. aggregans*.

Supplementary Information References

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