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.

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 Methylation index of Branched Tetraethers (MBT) and the Cyclization index of Branched Tetraethers (CBI), 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 (O2) 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 O2, likely enabling survival and growth in micro-aerobic habitats (Pitcher and Watmough, 2004). Such low O2 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 O2 limitation, we examined the tetraether and fatty acid membrane composition of E. aggregans grown in a simplified yeast extract medium under O2 conditions ranging from fully aerated (21 % O2) to severely O2 limited (1 % O2). 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 O2 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...
brGDGTs may trigger brGDGT biosynthesis by reducing O2 availability to individual cells deeper within the aggregates. Consequently, the actual biochemical O2 threshold for growth may be significantly lower.

Environmental implications. The clumping phenotype of E. aggregans highlights the potential importance of micro-scale spatial O2 gradients in brGDGT production and is supported by E. aggregans growth on solid medium. Unlike in liquid culture, we observed measurable quantities of both brGDGT Ia and the brGDGT Ic isomer in fully oxygenated (21% O2) 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 O2 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...
While the mechanistic links between physiology and brGDGT-based production at low O2 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 O2 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 O2 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 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 O2 availability controls biosynthesis of branched tetraethers by *E. aggregans*, with low O2 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 O2 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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**References**


Yao, Y., Zhang, J., Vachula, R., Xi, J., Wu, J., Song, X., Huang, Y. (2020) Correlation between the ratio of 5-methyl hexamethylated to pentamethyalted branched GDGTs (H5PS) and water depth reflects redox variations in stratified lakes. Organic Geochemistry 147, 104076.


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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


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 KH₂PO₄, 0.2 g/L MgSO₄ ⋅ 7 H₂O, 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 % O₂). 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 N₂ and Air using digital mass flow controllers (Alicat Scientific) at appropriate ratios to obtain 1.04 % and 5.2 % O₂ (v/v) in the headspace (throughout the manuscript we refer to 1.04 % O₂ as 1 %, 5.2 % as 5 %, and 20.8 % as 21 %). Attempts to study *E. aggregans* at O₂ 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 N2. 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 H2 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](http://www.geochemicalperspectivesletters.org/article2132).

**Table S-2** Growth rate data.

<table>
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<th>Organism</th>
<th>% O₂</th>
<th>C source</th>
<th>Replicate</th>
<th>Growth rate [d⁻¹]</th>
<th>K [OD600]</th>
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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


