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Tracing the production and fate of individual archaeal intact polar lipids using stable isotope probing

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ABSTRACT

Analysis of cellular membrane lipids has been widely applied to describe the microbial community composition in natural systems. When combined with stable isotope probing (SIP) experiments, deuterium label (D_2O) uptake into lipids enables assessment of microbial biomass production. We performed SIP on methane-rich, hydrothermally-heated sediments to examine the de novo production of individual archaeal intact polar lipids (IPLs) by mesophilic anaerobic methane oxidizing group-1 archaea (ANME-1). The greatest extent of label uptake was by phosphatidyl glycerol archaeol (PG-AR), reaching 50% of the medium's label concentration in only 10 days. This indicates PG-AR as an important cell membrane lipid during the active growth phase of mesophilic ANME-1. Much less label uptake was into intact polar glycerol dibiphytanyl glycerol tetraethers (GDGTs), especially for those bound to diglycosidic head groups. The low production of these GDGTs contrasts with their predominance in ANME-1-dominated natural samples. We attribute the differential label uptake among individual IPLs to the ANME-1 tetraether biosynthetic pathway. This mechanism likely involves head-to-head condensation of two molecules of PG-AR and progressive substitution of PG by glycosidic head groups. The observation that ANME-1 invest in the synthesis of diethers during optimum growth suggests intact ARs and/or phosphate-bearing tetraethers as important biomarkers for actively growing populations in natural environments, while the diglycosidic GDGTs appear to signal stationary ANME-1 communities.

1. Introduction

Intact polar lipids (IPLs), the building blocks of every cellular membrane, have been proposed as biomarkers for both Archaea and Bacteria in sedimentary environments (e.g. Zink et al., 2003; Sturt et al., 2004). While abundant archaeal glycosidic ether lipids may reflect accumulation in deeply buried sub-seafloor sediments (Lipp and Hinrichs, 2009; Schouten et al., 2010; Logemann et al., 2011; Xie et al., 2013), the presence of diverse diether and tetraether lipids containing both phosphate-based and glycosidic polar head groups in methane seep sediments may reflect metabolically active communities (Rossel et al., 2008, 2011; Schubotz et al., 2011). However, to fully assess the dynamics of microbial lipid biosynthesis, the production and turnover of individual lipid species (e.g. diether and tetraether lipids with different head group combinations) need to be better understood.

Lipid biosynthesis in natural samples has been examined using radiolabeled [e.g. ^{14}C-labeled compounds (Nemoto et al., 2003), $^{33}PO_4^-$ (van Mooy et al., 2006)] and stable isotope probing (SIP; e.g. Boschker et al., 1998). Lipid-based SIP has been applied to verify the microbial activity in sediments and microbial mats hosting anaerobic methane oxidizing communities (e.g. Blumenberg et al., 2005; Wegener et al., 2008; Kellermann et al., 2012) and in marine sub-surface sediments (Lin et al., 2012; Wegener et al., 2012). These studies have shown a more rapid response of bacteria to label addition, especially in shallow sediments where turnover times for bacterial lipids were estimated to be several orders of magnitude shorter than those for archaeal lipids (Wegener et al., 2012). However, through inclusion of a large pool of archaeal fossil core lipids (ca. 90%; Lipp and Hinrichs, 2009; Liu et al., 2011), as well as a substantial fraction of glycosidic archaeal ether lipids accumulated extracellularly (Xie et al., 2013), these calculations may have underestimated the archaeal activity.

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In a previous study, we examined lipid production and C fixation in enrichments of mesophilic methane oxidizing archaea (ANME) from Guaymas Basin sediments using a dual SIP approach (deuterated water and ^{13}C-labeled inorganic C; Kellermann et al., 2012). To our knowledge, meso- and thermophilic AOM enrichments from this hydrothermal vent are the only laboratory cultures with proliferating ANME-1 communities (Wegener et al., 2016). In that study, which assessed isotope label incorporation into bulk lipid pools (analysis of both fossil and intact lipids), we observed an imbalance between the high concentration of archaeal tetraether lipids and their strikingly low label uptake. On the other hand, we measured a relatively high production rate for archaeal diethers, but their concentration was unexpectedly low in the samples investigated.

To comprehensively understand the dynamics of biosynthesis of archaeal membrane lipids, we focued here on the deuterium uptake of individual archaeal IPLs (hereafter IPL-SIP). This required a sophisticated analytical procedure involving preparative liquid chromatography (e.g. Kellermann et al., 2011) of the bulk lipids and subsequent isotopic analysis (D) of the polar ether lipid-derived isoprenoid hydrocarbons (cf. phytane and biphytane) in each fraction (Kellermann et al., 2012; Supplementary material, Fig. S1). We demonstrate that the differential label uptake into individual IPLs reflects the dynamics of ANME-1 archaea lipid biosynthesis, which likely involves head-to-head condensation of diethers to afford tetraether lipids.

2. Material and methods

2.1. Study design and sample handling

A detailed description of the design of the SIP experiment has been provided by Kellermann et al. (2012), where we demonstrated that these ANME enrichments from the Guaymas Basin oxidized methane but, instead of methane-derived C, assimilated inorganic C. That study focused only on bulk or total lipid extracts (TLEs) from incubations grown under a CH_4 headspace. In brief, all anaerobic incubations were performed in 250 ml incubation containers at 37 °C with CH_4 atmosphere at 200 kPa + 50 kPa CO_2. Each sediment slurry [each ca. 4 g dry mass (g_{dm})] had been prepared with artificial anaerobic seawater medium, which had been spiked with both D_2O (ca. 5% labeled) and ^{13}C-labeled dissolved inorganic C ($^{13}C_{DIC}$, ca.15% labeled). Note that this IPL-SIP experiment focused entirely on D uptake, while IC assimilation (through $^{13}C_{DIC}$; Kellermann et al., 2012) is not further discussed. In total, four bottle incubations with identical conditions were harvested at four different time points (t_0, t_{10}, t_{17} and t_{24} days). At the end of each incubation, the sediment slurry was extracted using a modified Bligh and Dyer (1959) method in order to obtain the total lipid extract (TLE; for details see Sturt et al., 2004).

2.2. Methods for microbial community analysis

DNA was extracted as described by Zhou et al. (1996) from a 15 ml sub-sample of the enrichment culture. The archaeal 16S rRNA genes were amplified from the extracted chromosomal DNA using primer set 20F (Massana et al., 1997)/Arc1492R (Teske et al., 2002). PCR reactions were performed with a Mastercycler Gradient (Eppendorf) in a 20 µl reaction volume. The PCR reactions were carried out at 0.5 µM concentration of each primer, 200 µM of each deoxyribonucleoside triphosphate, 6 µg bovine serum albumin, 1× PCR buffer (5Prime), 0.25 unit Taq DNA polymerase (5Prime) and 5–10 ng template DNA.

The PCR started with an initial heating step (95 °C for 5 min), followed by 30 cycles, each at 95 °C for 1 min, 58 °C for 1.5 min, 72 °C for 3 min, and a final step at 72 °C for 10 min. The DNA from 10 reactions was pooled, gel extracted and purified using the QIAquick PCR Purification Kit (Qiagen). The DNA was ligated to the pGEM-TEasy vector (Promega) and transformed into *Escherichia coli* One Shot Top10 cells (Invitrogen). Taq cycle sequencing was performed using ABI BigDye Terminator chemistry and an ABI377 sequencer (Applied Biosystems). Archaeal nucleotide sequences (16S rRNA genes) have been deposited at EMBL, GenBank and DDBJ under accession numbers: FR682479–FR682487 and HE817766–HE817767. The phylogenetic affiliation was inferred with the ARB software package (Ludwig et al., 2004) based on Release 115 of the ARB SILVA database (Quast et al., 2013).

For CARD-FISH, aliquots of the enrichment were fixed for 2 h with CH_2O (30 g/l final concentration at room temperature), washed with 1× PO_4^{3-} buffered saline (PBS), pH 7.2, and stored in PBS:EtOH (1:1) at −20 °C until further processing. Fixed samples were treated via mild sonication for 20 s with an MS73 probe (Sonopuls HD70; Bandelin) at 10 W. Aliquots were filtered onto polycarbonate filters (GTTP, 0.2 µm pore size; Millipore). CARD-FISH was performed as described by Pernthaler et al. (2004) with the following modifications: cell walls were permeabilized with lysozyme (10 g/l in 0.1 M Tris–HCl, 0.05 M EDTA, pH 8.0) for 20 min at 37 °C and proteinase K (15 µg/ml in 0.1 M Tris–HCl, 0.05 M EDTA, 0.5 M NaCl, pH 8.0) for 2 min at room temperature. Endogenous peroxidases were inactivated with 0.3% (vol/vol) H_2O_2 in MeOH for 30 min at room temperature. The oligonucleotide probes, anaerobic methane oxidizing archaea ANME-1-350 (AGTTTTCGCGCCTGATGC; Boetius et al., 2000) for ANME-1 archaea, and ANME-2-538 (GGCTACCACTCGGGCCGC; Treude et al., 2005) for ANME-2 archaea were applied at a CH_2O concentration of 40% and 50% (vol/vol), respectively. Subsequently samples were stained with 4′,6′-diamidino-2-phenylindole (DAPI). Samples were analyzed via epifluorescence microscopy (Axioplan; Zeiss) and confocal laser scanning microscopy (LSM510; Zeiss).

2.3. Lipid detection and separation into individual fractions

An aliquot (5%) of each extract was analyzed using high performance liquid chromatography–mass spectrometry (HPLC–MS). The system consisted of a ThermoFinnigan Surveyor HPLC instrument, equipped with a LiChrosphere Diol-100 column (150 × 2.1 mm, 5 µm particle size; Alltech, Germany), coupled to a ThermoFinnigan LCQ Deca XP Plus ion trap MS instrument with an electrospray ionization (ESI) interface; the settings were as described previously (Sturt et al., 2004). Archaeal IPLs were identified according to their fragmentation patterns during MS^2 experiments (Yoshinaga et al., 2011).

For purification of individual IPLs, a combination of normal and reversed phase preparative HPLC was applied. Normal phase separation was performed with a LiChrosphere Diol-100 column (250 × 10 mm, 5 µm particle size; Alltech, Germany), connected to a guard column of the same packing material, both operated at room temperature (cf. Kellermann et al., 2011). The flow rate was 1.5 ml/min and the elution gradient was: 100% A to 65% B in 120 min, held at 65% B for 30 min, then 30 min column re-equilibration with 100% A; where A was *n*-hexane/2-propanol (8:2, v:v) and B 2-propanol/MilliQ water (9:1, v:v). The fraction collector (Gilson FC204) provided a total of 12 fractions over 120 min (Fig. S1). The co-elution of 2G-GDGT and 2G-AR in normal phase fraction 4 (F_N-4) was resolved using reversed phase preparative HPLC (cf. Lin et al., 2012). This step was performed with an Eclipse XDB-C_{18} column (250 × 9.4 mm, 5 µm particle size; Agilent, Böblingen, Germany) operated at 40 °C and at a constant 2 ml/min using the gradient: 100% A to 100% B in 50 min, then held for 20 min at 100% B, and finally 30 min column re-equilibration with 100% A, where eluent A and B were 100% MeOH and 100%