ORIGINAL ARTICLE

Acetoclastic *Methanosaeta* are dominant methanogens in organic-rich Antarctic marine sediments

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Despite accounting for the majority of sedimentary methane, the physiology and relative abundance of subsurface methanogens remain poorly understood. We combined intact polar lipid and metagenome techniques to better constrain the presence and functions of methanogens within the highly reducing, organic-rich sediments of Antarctica's Adélie Basin. The assembly of metagenomic sequence data identified phylogenetic and functional marker genes of methanogens and generated the first *Methanosaeta* sp. genome from a deep subsurface sedimentary environment. Based on structural and isotopic measurements, glycerol dialkyl glycerol tetraethers with diglycosyl phosphatidylglycerol head groups were classified as biomarkers for active methanogens. The stable carbon isotope ($\delta^{13}\text{C}$) values of these biomarkers and the *Methanosaeta* partial genome suggest that these organisms are acetoclastic methanogens and represent a relatively small (0.2%) but active population. Metagenomic and lipid analyses suggest that Thaumarchaeota and heterotrophic bacteria co-exist with *Methanosaeta* and together contribute to increasing concentrations and $\delta^{13}\text{C}$ values of dissolved inorganic carbon with depth. This study presents the first functional insights of deep subsurface *Methanosaeta* organisms and highlights their role in methane production and overall carbon cycling within sedimentary environments.

The ISME Journal (2018) 12, 330–342; doi:10.1038/ismej.2017.150; published online 17 October 2017

Introduction

Marine sediment constitutes Earth's largest reservoir of methane (CH_4) (Valentine, 2011), including continental shelf environments, which produce 0.7–14 Tg CH_4 year⁻¹ (Valentine, 2002; Ferry and Lesser, 2008). Even though CH_4 oxidizers consume most of this greenhouse gas (Reeburgh, 2007), it is important to understand all major subsurface sources. Sedimentary methanogens include representatives of the Methanosarcinales, Methanomicrobales, Methanococcales, Methanopyrales, Methanobacteriales and Methanomassiliicoccales orders (Sowers and Kestead, 1984; Sowers and Ferry, 2003; Ferry and Kestead, 2007; Lang *et al.*, 2015; Katayama *et al.*, 2016). Collectively, these organisms compose a curiously

small proportion of sequenced communities observed in CH_4 -rich, subsurface environments (Valentine, 2011). Less than 4% of archaeal 16S rRNA gene clone libraries generated from the CH_4 -bearing sediment of the Peru and Cascadia margins represented methanogens (Parkes *et al.*, 2005; Inagaki *et al.*, 2006). Functional gene surveys of these margins also identified fewer copies of the methyl coenzyme M reductase (*mcrA*) than predicted based on CH_4 concentrations (Colwell *et al.*, 2008; Biddle *et al.*, 2008). This scarcity of detectable methanogens has raised the following hypotheses: (i) that primer-based sequencing may be biased against methanogens or (ii) that methanogens may be highly active relative to the total microbial population (Parkes *et al.*, 2005; Inagaki *et al.*, 2006). In any case, limited observations of methanogens in subsurface environments restrict our understanding of CH_4 production in these settings.

The Adélie Basin, Antarctica is a long, narrow shelf with high surface water diatom primary productivity (Leventer *et al.*, 2006), which results in high sedimentation rates and anoxic conditions.

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Received 17 January 2017; revised 16 June 2017; accepted 24 June 2017; published online 17 October 2017

The sediments are characterized by negligible sulfate and increasing CH₄ concentrations with depth, indicating active methanogenesis (Expedition Scientists 318 *et al.*, 2011b). Despite geochemical evidence for CH₄ production, a sequencing survey of the 16S rRNA gene identified <0.1% of the sequenced community as methanogens (Carr *et al.*, 2015). To further investigate the scant methanogenic community in this basin, this study circumvented primer-based methods and generated three metagenomes from sample depths 14, 25 and 100 m below seafloor (mbsf). The identification of methanogen genomic markers at depth 14 m prompted further characterization of the communities within the top 20 m of sediment. This study utilized structural and isotope analyses of intact polar lipids (IPLs) and bacterial polar lipid fatty acids as an independent and complimentary method for identifying and quantifying viable microorganisms *in situ* (Zink *et al.*, 2003; Lipp *et al.*, 2008). Using IPLs with glycerol dialkyl glycerol tetraether (GDGT) core lipids as a proxy to estimate viable archaeal populations has been recently challenged because the observed decay of glycosidic ether lipids is one to two orders of magnitude slower than bacterial phospholipids (Xie *et al.*, 2013), suggesting that glycosidic ether lipids may be remnants of fossil planktonic archaea (Schouten *et al.*, 2010). Therefore, this study focused on phosphatidic IPLs as diagnostic markers for viable archaea.

The $\delta^{13}\text{C}$ values of diagnostic lipids can also help elucidate carbon metabolisms (Pancost and Sinninghe Damsté, 2003; Biddle *et al.*, 2006; Schubotz *et al.*, 2011; Mills *et al.*, 2013; Carr *et al.*, 2013). Methanogens are known to convert carbon dioxide (CO₂), acetate and/or small methylated compounds to CH₄. CO₂ reduction is thought to be the principle pathway in marine sediments (Whiticar *et al.*, 1986); however, consumption of acetate can be significant in sulfate-depleted environments, while methanogens using non-competitive methylated substrates can exist wherever those compounds are abundant (Ferdeman *et al.*, 1997; Fitzsimons *et al.*, 1997). The family Methanosarcinaceae is considered to be the most versatile group of methanogens: some species can metabolize all three types of substrates (Sowers and Ferry, 2003; Ferry and Kestead, 2007). A study of *Methanosarcina barkeri* suggested that stable carbon isotope fractionation between lipids and substrate ($\Delta_{\text{substrate-lipid}}$) was greatest when cultures were grown on methylated substrates ($\Delta_{\text{substrate-lipid}} = 33\text{--}46\text{‰}$) (Londry *et al.*, 2008). Less fractionation was observed during growth on CO₂ ($\Delta_{\text{substrate-lipid}} = 11\text{--}20\text{‰}$) and acetate ($\Delta_{\text{substrate-lipid}} = 2\text{--}6\text{‰}$). Notably, variations in growth conditions also affected fractionation, suggesting that $\delta^{13}\text{C}_{\text{lipids}}$ values alone are not sufficient for predicting the metabolic pathway. Here we compare $\delta^{13}\text{C}_{\text{IPLs}}$ values to dissolved inorganic carbon (DIC) and total organic carbon (TOC) to estimate $\Delta^{13}\text{C}_{\text{lipid-substrate}}$ values. $\Delta^{13}\text{C}_{\text{lipid-substrate}}$ values were combined with the

metagenomic evidence to predict the dominant methanogenic pathway occurring within the sediments. Additionally, we measured $\delta^{13}\text{C}$ values of polar lipid fatty acids to better characterize bacterial carbon cycling and identify potential synergistic interactions between bacteria and methanogens (for example, cycling of fermentation products such as small organic substrates or hydrogen, Nobu *et al.*, 2015).

Methods

Site description and sampling strategy

Sediments were collected from IODP Hole U1357C (Expedition 318 Scientists, 2011a), directly off the Wilkes Land Margin (66°24.8'S, 140°25.5'E, water depth 1017 m, sedimentation rate $\sim 2\text{ cm year}^{-1}$). A complete description of our sampling strategy was reported by Expedition 318 Scientists (2011b) and Carr *et al.* (2015). Briefly, alternate 10-cm-length whole round samples were taken for lipid and interstitial water geochemistry analyses within the top 20 m. Additional 5 cm³ syringe samples were collected for DNA analyses reaching a depth of 103.65 mbsf. Ship-board analyses included DIC, CH₄ and sulfate concentrations (Expedition 318 Scientists, 2011a). DIC increases from 40 to 80 mM at 18.27 mbsf (Figure 1). CH₄ concentrations suggest a maximum of 12.8 mM at 21.61 mbsf; however, gas expansion likely caused gas loss during core recovery. Thus it is possible that CH₄ concentrations were saturated throughout the shallow core sections. Samples for $\delta^{13}\text{C}_{\text{CH}_4}$ analysis were not taken; however, ethane concentrations were negligible (<1 ppmv), indicating that the bulk of the CH₄ was biogenic. Sulfate is depleted to below detection within 2 m, higher concentrations of sulfate observed at the top of each 9-m-core section suggest seawater contamination. These potentially contaminated samples were excluded from shore-based analyses. Sulfide gas was not measured, although a sulfidic odor was apparent. Total organic nitrogen and TOC concentrations for the top 18 of core were reported previously (Carr *et al.*, 2016). TOC concentrations decreased with depth from $\sim 2.0\text{ wt\%}$ to $1.4\text{ wt\%}$ at 18.37 mbsf. Total organic nitrogen concentrations were relatively constant and averaged $0.30\text{ wt\%}$. Consequently C/N decreased with depth, averaging 5.6 and suggesting a large marine contribution to the TOC pool.

To confirm the validity of unique methanogen lipid biomarkers, IPLs from the Adélie Basin were compared with those of a CH₄-free control site located on the continental rise—IODP Hole U1359B (64°54.2'S, 143°57.7'E, water depth 3021 m, sedimentation rate $\sim 20\text{ m My}^{-1}$). Ten-cm whole rounds samples were collected for IPL analysis.

Stable carbon isotope analyses of carbon substrates

$\delta^{13}\text{C}$ analyses of DIC were conducted at the Stable Isotope Biogeochemistry Laboratory at Stanford University. CO₂ was evolved from DIC using

Figure 1 Depth profiles for concentrations of dissolved inorganic compounds, carbon isotopic compositions of DIC and TOC (0–20 mbsf, this study) and dissolved CH_4 concentrations (0–171 mbsf) in sediment pore waters at IODP Site U1357 (Expedition 318 Scientists, 2011b, figure modified with permission).

cryogenic vacuum separation techniques (Gruber *et al.*, 1999). The $\delta^{13}\text{C}_{\text{DIC}}$ values were measured on a Finnigan MAT 252 isotope ratio mass spectrometer (IRMS). Sediment for TOC measurements were lyophilized, homogenized, loaded into silver capsules and acidified with a 6% sulfurous acid solution. Isotopic measurements were made with a Carlo Erba NA 1500 elemental analyzer/Conflo II system (CE Elantech, Inc., Lakewood, NJ, USA) coupled to a Finnigan DeltaPlus IRMS (Thermo Fisher Scientific, Waltham, MA, USA). The $\delta^{13}\text{C}$ values of DIC and TOC were reported in the standard delta notation relative to Vienna Pee Dee Belemnite (VPDB) with 0.05‰ precision.

Metagenome analyses

Genomic DNA was extracted from Hole U1357C sample depths 14, 25 and ~100.5 mbsf (97.4 and 103 mbsf pooled) using a phenol chloroform protocol modified from Zhou *et al.* (1996) as described in Carr *et al.* (2015). DNA was concentrated using a PowerClean Pro DNA Clean-Up Kit (Mo Bio Laboratories, Inc., Solana Beach, CA, USA). Metagenomic sequencing was performed at the WM Keck sequencing facility at the Marine Biological Laboratory. Library preparation, sequencing, quality control and assembly are described within the Supplementary Information. Briefly, paired-end sequencing of DNA was performed on an Illumina NextSeq 500 (Illumina, Inc., San Diego, CA, USA). Raw reads are available in NCBI's Short Read Archive, accession numbers SAX2769041–SRX2769043. Adapter removal, quality assessment and filtering was performed using the BBmap software version 35.14 (Bushnell, 2015). Community coverage was estimated using Nonpareil (Rodriguez-R and Konstantinidis, 2014). Sequence reads from all depths were co-assembled using MEGAHIT (Li *et al.*, 2015) and binned using CONCOCT (Alneberg *et al.*, 2014) via the platform anvi'o (Eren *et al.*, 2015). This assembly

is available on the Joint Genome Institute's Integrated Microbial Genome Expert Review platform (IMG/M, ID 3300008470, Markowitz *et al.*, 2014). Binning generated 70 bins, one of which was classified as a methanogen (genus *Methanosaeta*). The original paired-end reads of this *Methanosaeta* bin and any contig that contained coding regions for methanogenic genes were reassembled (SPAdes genome assembler v3.7.1, Bankevich *et al.*, 2012), binned (CONCOCT via anvi'o) and manually inspected twofold. Completeness and contamination of the final *Methanosaeta* genome from a metagenome (GFM) was assessed against an established list of Euryarchaeota single copy genes (UID49) using CheckM (Parks *et al.*, 2015). The *Methanosaeta* GFM was annotated using the IMG automated pipeline (Taxon ID 2687453729, Markowitz *et al.*, 2014). Annotations were verified against those obtained using myrast (Aziz *et al.*, 2008). Genomes average nucleotide identity (ANI) comparisons were calculated using the IMG/ER 'pairwise ANI tool' (Supplementary Information) and orthologous gene clusters (OGCs) were compared using OrthoVenn, using the default settings (Wang *et al.*, 2015).

Putative 16S rRNA genes were assembled from individual sample depths using Meta-RNA (Huang *et al.*, 2009) and EMIRGE (Miller *et al.*, 2011), aligned and classified against the SILVA non-redundant database (v123, Quast *et al.*, 2013) and quantified using kallisto 0.42.4 (Bray *et al.*, 2016; Supplementary Information).

Lipid extraction, analysis and quantification

Total lipids extracts were generated from 10 10-cm whole round samples and bacterial-derived polar lipid fatty acids were converted to fatty acid methyl esters (FAMES) and were identified and quantified according to Carr *et al.* (2016). Total lipids extract aliquots were also analyzed both in the Summons laboratory at MIT and the Hinrichs laboratory at the MARUM. Identification and quantification of IPLs

and core GDGTs was achieved on a Agilent 1200 series HPLC system coupled to an Agilent 6520 accurate-mass quadrupole time-of-flight mass spectrometer (Summons laboratory, Agilent, Santa Clara, CA, USA) and Dionex Ultimate 3000 UHPLC (Thermo Fisher Scientific, Waltham, MA, USA) coupled to a Bruker maXis ultra-high-resolution orthogonal acceleration quadrupole time-of-flight tandem MS2 instrument (Hinrichs laboratory, Bruker Daltonics Inc., Billerica, MA, USA) following previously described protocols (Becker *et al.*, 2013; Wörmer *et al.*, 2013; Zhu *et al.*, 2013; Supplementary Information). Archaeal IPLs were identified by exact masses and diagnostic fragmentation patterns (Yoshinaga *et al.*, 2011) using automated data-dependent fragmentation of base peak ions. Quantification of IPLs occurred using C46 GDGT as an internal standard (Huguet *et al.*, 2006), taking response factors of available archaeal IPLs into account (see Supplementary Information for details of quantification and error estimations) with a detection limit of ca. 100 pg on column. The Methane Index (MI) and Branched and Isoprenoid Tetraether (BIT) Index were calculated after Zhang *et al.* (2011) and Hopmans *et al.* (2004), respectively.

Preparative HPLC-MS was performed on five samples (sample depth: 2.55, 7.17, 14.25, 17.25, and 18.87 mbsf) to separate total lipids extracts into fractions that contain different archaeal IPLs for determining head group-specific stable carbon isotopic compositions. Separation of IPL-GDGTs was achieved by reverse phase chromatography using a Zorbax Eclipse XDB C18 column (5 μ m, 10 $\times$ 250 mm, Agilent Technologies, Santa Clara, CA, USA) on an Agilent 1200 series high-performance liquid chromatography (HPLC) system equipped with an Agilent 1200 series fraction collector (Supplementary Information).

Preparation of lipid derivatives for GC-isotope ratio mass spectrometric analysis

The $\delta^{13}\text{C}$ of the FAMES was determined by GC-combustion-IRMS at the US Geological Survey (Denver, CO, USA). FAMES were separated on an Agilent 6890 GC equipped with an Agilent J&W DB-Petro column (100 m length $\times$ 0.25 mm id $\times$ 0.50 μ m film thickness). FAMES were combusted on-line and a VG Optima IRMS measured the resulting CO_2 . The $\delta^{13}\text{C}$ values were corrected for the added carbon during methylation and values are reported in δ notation, expressed against VPDB.

To obtain $\delta^{13}\text{C}$ values of head group-separated archaeal IPLs, individual IPL-GDGT fractions were subjected to ether cleavage after Jahn *et al.*, (2004) yielding biphytanes (Supplementary Information). The $\delta^{13}\text{C}$ values of biphytanes were determined in the Summons and the Hinrichs laboratory using a ThermoFinnigan GC coupled to a ThermoFinnigan Deltaplus XP isotope ratio MS via a GC-combustion interface (Thermo Fisher Scientific, Waltham, MA,

USA) as described previously (Schubotz *et al.*, 2011, 2013). Samples were subjected to duplicate or triplicate measurements if enough material was present. Values are reported in the δ notation relative to the VPDB standard.

Results and discussion

Genomic support for Methanogens

Metagenomic overview. The metagenomic sequence data set from 14 mbsf (45 363 249 paired-end reads) was twice as large as the other two data sets (23 662 967 and 23 748 505 paired-end reads for 25 and 100 mbsf, respectively). Quality control steps reduced data sets to 45 353 241, 23 657 716 and 23 743 085 paired-end reads, respectively. Community coverage was calculated using Nonpareil, a program that evaluates the redundancy of overlapping unassembled reads. The coverage at each sample depth was estimated to be >97% using the default settings, suggesting a sequencing depth >400 $\times$ (Supplementary Figure S1). The final co-assembly was 1.2 Gbp on 1 368 184 contigs with an N50 of 860 bp.

A total of 362 putative full-length 16S rRNA gene sequences were assembled using Emirge from identified 16S rRNA gene fragments. These sequences resemble those of previous pyrosequenced libraries (Figure 2, Carr *et al.*, 2015), demonstrating congruity between the two different approaches. Candidate phylum Atribacteria was the most abundant phylum in all libraries and increased with abundance with depth. A single-cell amplified genome, classified as Atribacteria, was recently isolated from this site (Carr *et al.*, 2015) and suggested that these Atribacteria produce fermentation products such as acetate, ethanol and CO_2 , which could support a methanogenic community (Carr *et al.*, 2015). Other relatively abundant phyla shared between the data sets included Bacteroidetes, Gammaproteobacteria, Planctomycetes and Alphaproteobacteria. Euryarchaeota represented only 1–2% relative abundance in both the pyrosequenced and metagenomic data sets. Only one known methanogen, classified within the genus *Methanosaeta*, was identified in the 14 mbsf, representing 5 and 0.2% of the archaeal and total 16S rRNA sequences assembled with Emirge, respectively.

General description of *Methanosaeta* GFM. From our metagenomic co-assembly, we reconstructed the first known methanogen GFM from a *Methanosaeta* sp. originating from deep-sea marine sediments. *Methanosaeta* species are speculated to be the predominant CH_4 producers on Earth (Smith and Ingram-Smith, 2007), although relatively few have been isolated from the environment (Patel, 1984; Ma *et al.*, 2006; Barber *et al.*, 2011). To our knowledge, *Methanosaeta pelagica*, sampled from estuarine tidal

Figure 2 Relative abundances of putative full-length 16S rRNA genes identified in metagenomic libraries compared with those identified from previous pyrosequenced libraries (Carr et al., 2015).

Table 1 Genome characteristics of *Methanosaeta* genomes

Name	Environment	Size	GC%	16S rRNA similarity	mcrA similarity	ANI
This study	Deep-sea sediment	1.4 MB	54%	—	—	
<i>M. pelagica</i> ^I	Tidal flats	NA	NA	95%	100%	NA
<i>M. harundinacea</i> ^{II}	Anaerobic digester	2.6 MB	60.6	96%	85%	73.6
<i>M. thermophila</i> PT ^{III}	Anaerobic digester	1.9 MB	53.6	92%	81%	70.4
<i>M. concili</i> ^{IV}	Anaerobic digester	3.0 MB	51.0	92%	78%	70.1

Abbreviations: ANI, average nucleotide identity; GC, guanine–cytosine; NA, not applicable. Accession numbers: (I) AB679167, (II) CP003117, (III) CP000477, (IV) CP002565.

flats of Tokyo Bay is the only marine isolate (Mori et al., 2012). Its genome is not sequenced. This *Methanosaeta* GFM has an assembly size of 1.3 Mbp and is estimated to be 64% complete, with minimal contamination (1.65%, Parks et al., 2015). In all, 85% of the GFM was assembled from the 14 mbsf sample (25 mbsf = 15%, 97 mbsf < 0.1%). One thousand six hundred and fifty-four predicted coding regions were identified and 1098 were annotated. The *Methanosaeta* GFM contained a 16S rRNA gene sequence that was most similar to uncultured clones collected from marine sediments and mud volcanoes (Supplementary Figure S2). In comparison to cultured species, the GFM 16S rRNA gene sequence clustered most closely with *M. pelagica* (95% sequence pairwise similarity, Table 1). The *Methanosaeta* GFM also contained an *mcrA* gene. The *mcrA* gene was identical to that of *M. pelagica* but only 78–85% similar to those from other genomes (Table 1). Genomic similarity was measured according to ANI values and by comparing OGCs for the

available genomes (Table 1). Of the 1177 OGCs identified within the GFM, all but 3 were identified within other *Methanosaeta* species, and 851 (72%) were shared by all genomes, suggesting high functional similarity (Supplementary Figure S3). The three OGC clusters could not be annotated. The GFM was most similar to *Methanosaeta harundinacea*, sharing 1068 OGCs and an ANI of 73.55 (Table 1; Supplementary Table S2; Supplementary Figure S3).

Methanogenic potential of *Methanosaeta* GFM. The *Methanosaeta* GFM contained many of the proteins for acetoclastic methanogenesis via the Wood–Ljungdahl pathway (Figure 3, Supplementary Table S3). Using this pathway, acetate is activated to acetyl-CoA by acetyl-CoA synthetase (*acs*). Acetyl-CoA is then cleaved to produce CO₂ and a methyl group, which is transferred to tetrahydromethanopterin and then to 2-mercaptoethanesulfonate by the carbon monoxide dehydrogenase (*cdh*)/acetyl-CoA decarbonylase complex and tetrahydromethanopterin S-methyltransferase

Figure 3 The enzymatic pathways of autotrophic (white arrows) and acetoclastic (black arrows) methanogenesis. Carbon substrates, intermediates and products appear in gray ovals. Enzymes present within this *Methanosaeta* GFM are in bold; missing enzymes or subunits appear gray. Enzymes that are absent from the *Methanosaeta* GFM are outlined. Enzymes: fwd: formylmethanofuran dehydrogenase; ftr: formylmethanofuran:H4SPT formyltransferase; mch: methenyl-H4SPT cyclohydrolase; mtd: F420-dependent methylene H4SPT dehydrogenase; mer: F420-dependent methylene-H4SPT reductase; mtr: methyl-H4SPT: coenzyme M methyltransferase; mcr: methyl-coenzymeM reductase; ack: acetate kinase; pta: phosphotransacetylase. acs: acetyl-CoA synthetase; cdh: carbon monoxide dehydrogenase. Carbon compounds: CO₂: carbon dioxide; formyl-MF: formyl-methanofuran; formyl-H4SPT: formyl-tetrahydrosarcinapterin; methenyl-H4SPT: methenyl-tetrahydrosarcinapterin; methylene-H4SPT: methylene-tetrahydrosarcinapterin; methyl-H4SPT: methyl-tetrahydrosarcinapterin; methylene-S-CoM: 2-(Methylthio)ethanesulfonate. Acetyl-CoA: acetyl-Coenzyme A; Acetyl-PO₄: acetylphosphate.

(mtr), respectively. The *Methanosaeta* GFM lacks subunits *mtrEGH*; however, considering the incompleteness of the genome bin, it is possible that all subunits are present within the organism. During the final step of methanogenesis, the original methyl group is reduced to CH₄ by the three-subunit *mcr*, all of which were present in the GFM.

Laboratory experiments have demonstrated that *M. harundinacea* is capable of utilizing CO₂ as a substrate for one-third of its CH₄ production (Rota et al., 2014). Indeed, *Methanosaeta concilii*, *M. harundinacea* and *M. pelagica* genomes contain most of the gene-encoded proteins required for CO₂ reduction. However, the lack of an energy-conserving hydrogenase, and thus reducing electrons, was hard to reconcile. The experiments with *M. harundinacea* demonstrated that electrons can be acquired from syntrophic partners via electrically conductive pili (Rota et al., 2014). This GFM also contains genes for CO₂ reduction (Figure 3). However, given that organisms with known electrically conductive pili such as *Geobacter* were not dominant in these sediments (<0.5% of the pyrosequenced libraries, Carr et al., 2015), it is more likely this GFM is living syntrophically with

acetogenic bacteria. To test this hypothesis, the co-assembled metagenome was screened for formyltetrahydrofolate synthetase, a conserved enzyme in heterotrophic and autotrophic acetogens (Lovell et al., 1990). One hundred and thirteen formyltetrahydrofolate synthetase genes were identified (Supplementary Table S4) and classified primarily as Anaerolinea (Chloroflexi) and Atribacteria, which represented 6% and 24% of the 16S rRNA gene sequences assembled from 14 mbsf using Emirge, respectively. Both lineages are believed to be heterotrophic and have been hypothesized to fuel methanogenic communities with their fermentation products (Dodsworth et al., 2013; Gies et al., 2014; Carr et al., 2015; Liang et al., 2015). For example, in North Sea sediments, a significant and positive covariance between Atribacteria and *Methanosaeta* organisms suggested that the groups were metabolically interactive (Nobu et al., 2015). Similarly, Anaerolineaceae and *Methanosaeta* organisms were found to be co-abundant in alkane degradation, CH₄-producing enrichments (Liang et al., 2015). Together these observations suggest that *Methanosaeta* is benefiting from the fermentation products of Anaerolinea and Atribacteria and living acetoclastically in this basin.

Characterization of archaeal communities based on IPL structures

More than 90% of the recovered archaeal lipids contained saccharide (glycosidic) head groups, primarily monohexose (1G) or dihexose (2G) (Supplementary Table S4; Figures 4 and 5). Both 1G- and 2G-GDGTs were also present as their hydroxylated derivatives, 1G-OH-GDGT and 2G-OH-GDGT and minor amounts of unsaturated 2G-GDGTs (2G-unsat-GDGT) were detected at deeper depths. Phospho-GDGTs (2G-PG-GDGT, PH-GDGT and HPH-GDGT) comprised 0.8–5% of total archaeal lipids. 1G- and 2G-Archaeol were present at all depths (2–6%). 1G- and 2G-Archaeols and G-GDGTs, as well as their hydroxylated counterparts, are commonly recovered from deep-sea sediments (Lipp and Hinrichs, 2009; Liu et al., 2012) and have been linked to viable benthic archaea (Lipp et al., 2008). However, 1G-GDGT has also been suggested to represent a planktonic source (Schouten et al., 2010; Xie et al., 2012), because it is abundant in planktonic archaea (Schouten et al., 2008; Elling et al., 2015) and is a potential degradation product of 2G-GDGT and HPH-GDGT (Lengger et al., 2012). Considering the amount of planktonic-derived sedimentation, high sedimentation rate and a relatively low input of terrestrial material (low BIT index, Table 2) at Hole U1357C, we exclude 1G-GDGT as a marker for viable archaea.

Phosphatidic GDGTs are more suitable biomarkers for the indigenous archaeal community as they are proposed to degrade faster than their glycosidic counterparts (Schouten et al., 2010; Lengger et al.,

Figure 4 (a) Representative HPLC-MS chromatogram (HILIC-ESI-MS) from site U1357, 7.15 mbsf, showing the presence of major intact polar lipid isoprenoidal glycerol dialkyl glycerol tetraethers (IPL-GDGTs) and bacterial lipids in the mass range m/z 1200–1800. The HPLC-MS chromatogram is depicted as a density map showing retention time on the x axis, m/z on the y axis and the relative peak intensity by grey shading. (b) Structural variety in the head groups and biphytanyl moieties of the detected IPL-GDGTs. Note that during the ether cleavage reaction the hydroxyl group in the biphytane (bp) side chain becomes dehydrated and forms biphytene (bp0:1). Position of the hydroxyl group and double bond were not determined. 1G, monohexose; 2G, dihexose; PG, phosphatidyl glycerol; HPH, hexose-phosphohexose; cren, crenarchaeol.

2012). The most abundant phosphatidic GDGTs recovered were with hexose-phosphohexose head groups (HPH, Figures 4 and 5). These lipids likely originated from benthic Thaumarchaeota, the most abundant archaeal organisms according to 16S rRNA gene sequences assembled with Emirge (Figure 2). The majority of these sequences were classified as the genus *Nitrosopumilus* (54% of the archaeal sequences at 14 m). Cultured *Nitrosopumilus* species produce substantial amounts of HPH-GDGTs with 0–4 cyclopentyl rings and crenarchaeol and have thus been used as environmental biomarkers for Thaumarchaeota (Schouten *et al.*, 2008; Schubotz *et al.*, 2009; Pitcher *et al.*, 2011; Könneke *et al.*, 2012; Elling *et al.*, 2015).

We consider acyclic diglycosyl phosphatidylglycerol-GDGTs (2G-PG-GDGT) as a diagnostic biomarker for the *in situ* methanogens of this basin. The rationale for this suggestion builds on the following observations: (i) acyclic GDGT is the sole GDGT

found in methanogenic archaeal cultures (Koga and Nakano, 2008), (ii) 2G-PG-GDGT has so far only been detected in CH_4 -rich sediments with comparably depleted $\delta^{13}\text{C}$ values (Schubotz *et al.*, 2011; Yoshinaga *et al.*, 2015), (iii) it is absent from our CH_4 -free control site (Figure 5) and (iv) 2G-PG-GDGT is not present in known Thaumarchaeota cultures (Elling *et al.*, 2017); thus Thaumarchaeota sources for this lipid can be excluded. So far, only two strains of Methanosaeta have been investigated for their IPL composition: *M. concilii* contained mainly archaeol-based lipids, while *Methanosaeta thermophila* contained GDGT-0. Their detected head groups were glycosidic, phosphatidyl ethanolamine and phosphatidyl inositol (Koga and Nakano, 2008). We note that, even though PG was not detected as head group in these two representatives, it cannot be excluded that other species might produce PG in the environment. Absolute concentrations of 2G-PG-GDGTs increase from $\sim 60 \text{ ng g}^{-1}$ at 2 mbsf to 150 ng g^{-1} at

Figure 5 (a) Relative abundances of archaeal intact polar lipid with depth at Hole U1357C. (b) Absolute concentrations of 2G-PG-GDGTs and HPH-GDGTs compared with total intact polar lipids at Hole U1357C. (c) Relative abundances of archaeal intact polar lipid with depth at Hole U1359B. (d) Absolute concentrations of HPH-GDGTs compared with intact polar lipids at Hole U1359B. For abbreviations of intact polar lipid compound classes and source assignments, see Figure 4. Note that 1G-GDGT is excluded in all plots owing to a large assumed fossil component of this lipid, see text.

Table 2 Methane Index (MI) and Branched/Isoprenoid Tetraether (BIT) index at sites U1357 (Adélie Basin site) and U1359 (margin site)

	Site U1357 (mbsf)							Site U1359 (mbsf)		
mbsf	2.55	5.95	7.15	14.25	15.36	17.25	18.87	0.25	5.00	9.50
Index										
MI	0.08	0.08	0.07	0.08	0.06	0.08	0.11	0.13	0.14	0.09
BIT	0.01	0.03	0.02	0.02	0.03	0.02	0.02	0.86	0.43	0.42

Abbreviation: mbsf, meters below seafloor. MI values > 0.4 indicate the presence of microbial communities conducting the anaerobic oxidation of methane (Zhang *et al.*, 2011). The BIT index was determined after Hopmans *et al.* (2004) and the MI after Zhang *et al.* (2011) using the following Equations: $BIT = ([GDGT-I] + [GDGT-II] + [GDGT-III]) / ([Crenarchaeol] + [GDGT-I] + [GDGT-II] + [GDGT-III])$; $MI = ([GDGT-1] + [GDGT-2] + [GDGT-3]) / ([GDGT-1] + [GDGT-2] + [GDGT-3] + [Crenarchaeol] + [Crenarchaeol\ Isomer])$. BIT values > 0.4 indicate a high contribution of terrestrial material to the organic material (Hopmans *et al.*, 2004).

17.25 mbsf (Figure 5, Supplementary Table S5). 2G-PG-GDGTs were below detection limits at 18 mbsf, possibly because sediment quantity was limited at this depth. The relative abundance of 2G-PG-GDGTs compared with total archaeal IPL, and excluding 1G-GDGT, varies between 0.6% and 3.9% and compares well to the relative abundance of *Methanosaeta* 16S rRNA gene sequences, confirming the relatively low abundances of methanogenic biomass.

$\delta^{13}C$ values of IPL derivatives. We investigated head group-specific $\delta^{13}C$ of biphytanes to further

understand IPL sources (Figure 6). The $\delta^{13}C$ of biphytanes derived from core-GDGT, (including biphytane 0:1, which is the ether cleavage product from core OH-GDGT) range from -24‰ to -21‰ (Supplementary Table S6) and represent end member values presumed to be derived from planktonic archaea (Pearson *et al.*, 2016). The $\delta^{13}C$ of biphytanes derived from 1G-GDGT mirror core-GDGTs values, confirming that these IPLs are likely planktonic signatures. More depleted $\delta^{13}C$ values were observed for biphytanes derived from 2G-GDGT and 2G-PG-GDGT (-31‰ to -23‰), indicating indigenous

Figure 6 Stable carbon isotopic composition ($\delta^{13}\text{C}$) of biphytanes derived from IPL-GDGTs with depth. Estimated ($\delta^{13}\text{C}$) values were calculated for autotrophic and acetoclastic metabolisms based on observed fractionation patterns of *Methanosarcina barkeri* (Londry *et al.*, 2008). The $\delta^{13}\text{C}$ range for lipids of autotrophic methanogens was estimated using predicted $\delta^{13}\text{C}$ bicarbonate values, which were calculated from measured $\delta^{13}\text{C}$ values of DIC using the observed relation of Mook *et al.* (1974) and assuming summer bottom water to be -0.5°C . The $\delta^{13}\text{C}$ range for lipids of acetoclastic methanogens was estimated using $\delta^{13}\text{C}$ values of TOC.

benthic archaeal communities. 2G-PG-GDGT (bp0) were most ^{13}C -depleted (-31‰), suggesting that compared with the general archaeal community, the source organism used either a lighter carbon source or a metabolism with a larger isotope fractionation during lipid production. These observations support the use of 2G-PG-GDGT as a diagnostic biomarker for *in situ* methanogens.

Interpretation of carbon cycling. Increasing ammonium and DIC concentrations with depth indicate remineralization of organic matter by heterotrophic archaea and bacteria (Figure 1). The increasing $\delta^{13}\text{C}_{\text{DIC}}$ values with depth are contrary to the expectation for simple OM remineralization (Burdige, 2006) but compare to other CH_4 -rich sediments (Paull, *et al.*, 2000; Heuer *et al.*, 2009; Torres and Kastner, 2009; Wehrmann, *et al.*, 2011). Increasing $\delta^{13}\text{C}_{\text{DIC}}$ values could result from three processes: (i) the oxidation of ^{13}C -depleted CH_4 in shallow sediments by anaerobic oxidation of methane (AOM), (ii) selective removal of $^{12}\text{CO}_2$ by autotrophic microorganisms, including methanogens, and (iii) acetoclastic methanogenesis, where the disproportionation of acetate results in ^{13}C -depleted CH_4 and ^{13}C -enriched CO_2 .

We exclude the possibility of AOM (i) because we did not detect any AOM genomic biomarkers, hydroxyarchaeols or ^{13}C -depleted biphytanes derived from 2G-GDGT, as would be expected if

ANME-2 or ANME-1 archaea were present (Blumenberg *et al.*, 2004; Schubotz *et al.*, 2011; Yoshinaga *et al.*, 2015). Additionally, we used the MI of core GDGTs to assess the potential presence of CH_4 -oxidizing archaea by comparing relative abundances of GDGTs with 1, 2 and 3 pentacyclic rings relative to crenarchaeol (Zhang *et al.*, 2011). MI values were <0.17 at all depths (Table 2), indicating that AOM is not a dominant process in sediments up to 20 m depth.

We can exclude bacterial autotrophy (ii) based on metagenomic and lipid analyses. Most bacterial 16S rRNA gene sequences assembled with Emirge were classified as heterotrophs based on the metabolisms of closely related cultured organisms (Carr *et al.*, 2015). Heterotrophic bacteria are consistent with bacterial $^{13}\text{C}_{\text{FAMES}}$ values, which do not track changing $\delta^{13}\text{C}_{\text{DIC}}$ values (Supplementary Figure S4, Supplementary Table S8) but fall within the range of ^{13}C values expected for bacterial anaerobic heterotrophs, which can be depleted by -12‰ relative to $\delta^{13}\text{C}_{\text{TOC}}$ values (Teece *et al.*, 1999). The presence of autotrophic archaea is less clear. The genus *Nitrosopumilus* represents the most abundant archaeal lineage. Cultured *Nitrosopumilus* species can fix carbon using the 3-hydroxypropionate/4-hydroxybutyrate (HP/HB) cycle and assimilate organic carbon (Walker *et al.*, 2010; Könneke *et al.*, 2012; Qin *et al.*, 2014; Hurley *et al.*, 2016). The

co-assembled metagenome contained 32 gene sequences for 3-hydroxypropionyl-coenzyme A dehydrogenase, a required HP/HB cycle enzyme (Supplementary Table S9). All sequences were classified as Thaumarchaeota using BLASTp, suggesting that *Nitrosopumilus* autotrophy is possible. Considering *Nitrosopumilus* HPH-GDGT and PH-GDGT biomarkers, previous studies have demonstrated that isotopic fractionation during lipid production by autotrophic *Nitrosopumilus* species ($\epsilon_{\text{DIC-biphytanes}}$) is -20% (Könneke *et al.*, 2012). Here $\delta^{13}\text{C}$ values of HPH-GDGTs are approximately -24% resulting in $\epsilon_{\text{DIC-biphytanes}}$ values of approximately -10% at 2.5 mbsf and -20% at 14 mbsf (Supplementary Tables S6 and S8). This suggests that *Nitrosopumilus* CO_2 fixation is possible at 14 mbsf but does not fully explain $\delta^{13}\text{C}$ values at 2.5 mbsf. Alternatively, given that HPH-GDGT $\delta^{13}\text{C}$ values are constant with depth, maybe *Nitrosopumilus* are heterotrophic or mixotrophic. If mixotrophic, Thaumarchaeota may be contributing to DIC enrichment with depth but using an isotopically heavy fraction of the TOC pool as substrates for lipid production or maybe recycling the relic and ^{13}C -enriched isoprenoids that are prevalent in these sediments (Takano *et al.*, 2010).

Based on $\delta^{13}\text{C}_{\text{Lipids}}$ calculations of the methanogenic biomarker 2G-PG-GDGT(0) and the observations made by Londry *et al.*, 2008, we conclude that acetoclastic methanogenesis (iii) is a likely control on ^{13}C enrichment of DIC with depth and that acetoclastic methanogenesis is responsible for the majority of the produced CH_4 . Assuming that pure culture studies with *Methanosarcina* by Londry *et al.* (2008) represents environmental metabolisms, we calculated theoretical fractionation effects for both autotrophic CO_2 reduction and acetoclastic methanogenesis (Figure 6). As $\delta^{13}\text{C}_{\text{DIC}}$ values become continuously heavier with depth, we would expect the $\delta^{13}\text{C}_{\text{Lipids}}$ of CO_2 -reducing archaea to follow this trend; however, this is not the case for the biphytane lipids derived from 2-PG-GDGT (Figure 6). Instead, these lipids fall within the expected $\delta^{13}\text{C}$ range of acetoclastic methanogenesis, being between 2% and 7% lighter than $\delta^{13}\text{C}_{\text{TOC}}$. This is in agreement with previous environmental investigations where it was consistently observed that the carboxyl carbon of acetate is $4\text{--}20\%$ heavier than the methyl carbon (Blair and Carter, 1992; Heuer *et al.*, 2009). Consequently, the disproportionation of acetate in sedimentary environments often contributes to a ^{13}C -enriched DIC pool (Heuer, *et al.*, 2010), despite increasing concentrations of DIC due to overall organic matter remineralization.

Conclusion

Subsurface methanogens are difficult to detect and metabolically characterize in sediment environments. We employed genomic and lipid analyses to identify *Methanosaeta* as the sole methanogen at

14 mbsf. The resulting GFM represents the first partial genome for deep-subsurface *Methanosaeta* and verifies that deep-subsurface *Methanosaeta* contain gene-encoded proteins for acetoclastic and CO_2 -reducing methanogenesis, similar to surface isolates. The lack of an energy-conserving hydrogenase and known electron transferring syntrophs suggest that these *Methanosaeta* use acetate and benefit from the fermentation products of heterotrophs. IPL analysis identified 2G-PG-GDGT as a diagnostic marker for methanogens in these sediments. The $\delta^{13}\text{C}$ of this lipid was around -30% , consistent with acetoclastic methanogenesis. Relative abundances of 2G-PG-GDGT represent $0.6\text{--}3.9\%$ of archaeal IPLs and support the metagenomic results, which suggest that *Methanosaeta* represent 5% and 0.2% of the sequenced archaeal and total community at 14 mbsf, respectively. Although this methanogenic population is relatively small, the phosphatidic biomarkers suggest that these organisms were active when sampled, and an important CH_4 source in the Adélie basin, and may fill a similar ecological role in other anoxic basins.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

We thank the shipboard party of IODP Expedition 318 for supporting this work, especially co-chiefs Escutia and Brinkhuis, Staff Scientist Klaus and geochemistry team: James Bendle, Tina van de Flierdt, and Francisco Jimenez-Espejo. We thank Charles Pepe-Ranney and Benjamin Tully for bioinformatics assistance, David Mucciarone for isotope geochemistry assistance, Kai-Uwe Hinrichs and John Spear for the use of their facilities, Julius Lipp for provision of IPL standards and Beth Orcutt for manuscript comments. The Deep Carbon Observatory's Census of Deep Life project funded by the Alfred P Sloan Foundation provided sequencing services. Carr was funded by the US National Science Foundation (NSF OCE-0939564) and the Center for Dark Energy Biosphere Investigations (funded by NSF OCE-1521614, publication no. 390). FS and RES acknowledge funding from NASA Astrobiology grant (NNA13AA90A) and the Central Research Development Fund, University of Bremen. Any use of trade, firm or product names is for descriptive purposes only and does not imply endorsement by the US Government.

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