Microbial diversity of Loki's Castle black smokers at the Arctic Mid-Ocean Ridge

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ABSTRACT

Hydrothermal vent systems harbor rich microbial communities ranging from aerobic mesophiles to anaerobic hyperthermophiles. Among these, members of the archaeal domain are prevalent in microbial communities in the most extreme environments, partly because of their temperature-resistant and robust membrane lipids. In this study, we use geochemical and molecular microbiological methods to investigate the microbial diversity in black smoker chimneys from the newly discovered Loki's Castle hydrothermal vent field on the Arctic Mid-Ocean Ridge (AMOR) with vent fluid temperatures of 310–320 °C and pH of 5.5. Archaeal glycerol dialkyl glycerol tetraether lipids (GDGTs) and H-shaped GDGTs with 0–4 cyclopentane moieties were dominant in all sulfide samples and are most likely derived from both (hyper)thermophilic *Euryarchaeota* and *Crenarchaeota*. Crenarchaeol has been detected in low abundances in samples derived from the chimney exterior indicating the presence of *Thaumarchaeota* at lower ambient temperatures. *Aquificales* and members of the *Epsilonproteobacteria* were the dominant bacterial groups detected. Our observations based on the analysis of 16S rRNA genes and biomarker lipid analysis provide insight into microbial communities thriving within the porous sulfide structures of active and inactive deep-sea hydrothermal vents. Microbial cycling of sulfur, hydrogen, and methane by archaea in the chimney interior and bacteria in the chimney exterior may be the prevailing biogeochemical processes in this system.

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INTRODUCTION

Deep-sea hydrothermal vents are unique environments that support highly productive ecosystems driven by geochemical energy and have been proposed as a possible site for the origin and early evolution of life (Baross & Hoffman, 1985; Martin *et al.*, 2008). The distinctive black smoker chimneys at submarine hydrothermal environments are formed when sulfates and sulfides precipitate due to mixing of hot, acidic, and anoxic vent fluids with cold oxic seawater, thus creating steep thermal and chemical gradients along and inside the chimney (Tivey, 1995). Migrating fluids contain reduced inorganic and organic components providing metabolic energy for diverse thermophilic microbial communities that inhabit either specific microniches in different parts of the porous chimney structure, appear as free-living micro-organisms in vent fluids and plumes, or occur as symbionts of vent macrofauna (Karl, 1995). These micro-organisms are adapted to a habitat characterized by extreme environmental conditions such as high temperature and pressure, low pH as well as elevated concentrations of dissolved gases (H_2S, H_2, CO_2, CH_4) and metal sulfides (Miroshnichenko, 2004).

Since the discovery of deep-sea hydrothermal vent systems in the late 1970s (Corliss *et al.*, 1979), enrichment and isolation studies as well as culture-independent approaches, which mainly involved molecular studies of the 16S rRNA gene, were performed. These studies revealed a remarkable microbial diversity with numerous so far uncultivated organisms thriving in these extreme and unstable habitats (Takai *et al.*, 2001; Schrenk *et al.*, 2003; Alain *et al.*, 2004; Kormas *et al.*, 2006; Sogin *et al.*, 2006).

Hyperthermophilic archaea tend to be dominant in the high temperature zones of the chimney interiors, whereas both archaea and bacteria are present in the cooler exterior parts where mixing with seawater is more prevalent (Schrenk et al., 2003). Along with the apparent temperature zonation, where distinctive micro-organisms can thrive, different metabolisms are thermodynamically available. For example, oxidation of methane, ammonia, and sulfur are favored at low temperatures toward the chimney exterior, while methanogenesis and reduction of sulfate or sulfur is favored at higher temperatures toward the chimney interior (McCollom & Shock, 1997; Takai et al., 2001; Schrenk et al., 2003; Kormas et al., 2006; Takai & Nakamura, 2011).

In addition to molecular techniques, organic geochemical approaches can be employed to reveal microbial community structures by analyzing lipid biomarkers. Archaea synthesize distinctive membrane lipids predominantly composed of diether lipids (i.e., archaeol, hydroxyarchaeol) and isoprenoid glycerol dialkyl glycerol tetraethers (GDGTs). GDGTs with an additional covalent bond, so-called H-shaped GDGTs, have so far only been reported for cultivated isolates of (hyper-) thermophilic archaea (Morii et al., 1998; Sugai et al., 2004; Koga & Morii, 2005; Schouten et al., 2008). GDGTs are excellent biomarkers to use in hydrothermal vents as they are community-specific and resistant to extreme environmental conditions (Derosa & Gambacorta, 1988; van de Vossenberg et al., 1998; Macalady et al., 2004). There are, however, only few studies carried out on biomarker lipids in deep-sea hydrothermal vents indicating possible microbial communities with their potential metabolisms (Blumenberg et al., 2007; Blumenberg et al., 2012; Bradley et al., 2009).

Loki's Castle is a deep-sea hydrothermal vent field located at the ultra-slow spreading Arctic Mid-Ocean Ridge (AMOR) in the Norwegian-Greenland Sea at 74°N. Discovered in 2008, it is the northernmost black smoker field known to date and hosts a unique ecosystem (Pedersen et al., 2010). It is a sediment-associated system, and therefore, hydrothermal fluids contain an unusual enrichment of carbon dioxide, methane, and ammonia, providing potential for various energy metabolisms and diverse microbial populations. Here, we report first insights into the microbial assemblages inhabiting active and inactive sulfide chimneys of the Loki's Castle hydrothermal vent field based on biomarker lipid analysis and 16S rRNA gene-based taxonomy.

STUDY AREA AND SAMPLING

The Knipovich Ridge is one of the AMOR spreading centers in the Norwegian-Greenland Sea, and the southernmost part of this ridge is one of the slowest spreading ridge segments on Earth. The Loki's Castle vent field is located at 2400 m water depth on an axial volcanic ridge (AVR) where the magma-starved end of the Mohns Ridge migrates into the Knipovich Ridge through a sharp northward bend in the direction of the spreading axis at 73°30′N and 8°E (Fig. 1) (Pedersen et al., 2010). At the seafloor, black smoker fluids are discharging from four, up to 13 m tall, chimneys. The chimneys are situated on two hydrothermal mounds that are approximately 150 m apart and are estimated to be 20–30 m high and about 150–200 m across. The mounds of Loki's Castle are comparable in size with the TAG-mound (Trans-Atlantic Geotraverse) on the Mid-Atlantic Ridge (Pedersen et al., 2010). The four active chimneys were named João, Menorah, Camel, and Sleepy. João is the tallest of the four chimneys, situated on the eastern sulfide mound.

The hydrothermal fluids from Loki's Castle reach temperatures of 310–320 °C and have a pH of 5.5. The vent fluid compositions are characterized by high concentrations of CH_4, H_2, and NH_4, as well as elevated concentrations of higher hydrocarbons, and are indicative of a sediment-impacted hydrothermal vent system (Pedersen et al., 2010; Baumberger, 2011). Significant sediment accumulation is not present at the volcanic ridge hosting the field. However, the rift valley of the southern Knipovich Ridge in the vicinity of Loki's Castle is partly buried by a thick sediment cover. These sediments, derived from the nearby Bear Island fan, likely underlie the AVR and influence hydrothermal fluid compositions (Pedersen et al., 2010; Baumberger, 2011).
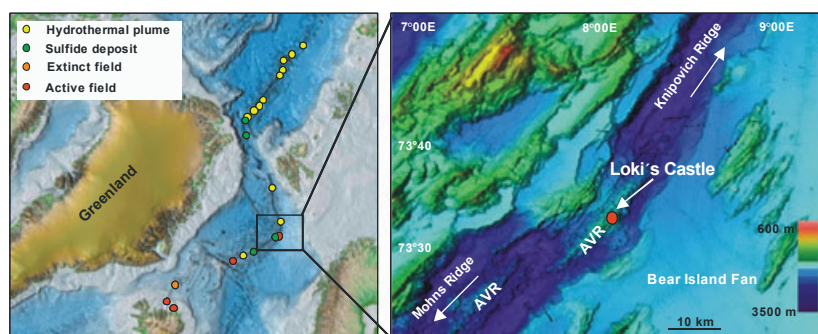

Fig. 1 Location of the Loki's Castle vent field at the Arctic Mid-Ocean Ridge (AMOR). AVR, Axial Volcanic Ridge.

Table 1 Bulk data of chimney samples and microbial filaments from Loki's Castle black smokers

Dive	Chimney	Sample material	Sample part	TOC (%)	$\delta^{13}C$ (‰)	$\delta^{34}S$ (‰)	Major mineral composition, color, texture
GS08-ROV10(1)	*João*	Active	Interior	0.09	−19.0	4.3	Amorphous silica rich; gray, relatively hard material
GS08-ROV10(2)	*João*	Active	Interior	0.10	−24.9	N.a.	Anhydrite; gray, soft material
GS09-ROV9	*João*	Filaments	Exterior	12.11	−27.1	n.a.	Thin, white filaments
GS10-ROV9(1)	*João*	Active	Middle	0.86	−36.0	1.0	Marcasite, sphalerite; black soft material
GS10-ROV9(2)	*João*	Active	Middle	0.17	−39.3	1.7	Marcasite, sphalerite, pyrite; black soft material
GS08-ROV11(1)	*Menorah*	Active	Interior	3.12	−14.2	2.1	Sphalerite, pyrrhotite, pyrite; black soft material
GS08-ROV11(2)	*Menorah*	Active	Interior	0.11	−24.3	2.3	Pyrite; black soft material
GS08-ROV11(3)	*Menorah*	Active	Interior	0.09	−22.7	2.5	Sphalerite, pyrrhotite; black soft material
GS08-ROV11(4)	*Menorah*	Active	Interior	0.03	−15.3	n.a.	Sphalerite, pyrrhotite; black soft material
GS09-ROV6(1)	*No name*	Inactive	Exterior	0.06	−6.8	18.5	Anhydrite, gypsum, talc; gray, relatively soft material
GS09-ROV6(2)	*No name*	Inactive	Exterior	0.11	−6.0	21.0	Anhydrite, gypsum; gray, relatively soft material

n.a. not analyzed. Numbers in brackets indicate subsamples taken from same chimney wall.

Table 2 Taxonomic affiliation, abundances and 16S rRNA gene numbers of microbial populations of *Menorah* bulk chimney material (GS08-ROV11)

Taxonomic level Phylum	Taxonomic level Class	% of prokaryotic SSU 454 sequence reads
Proteobacteria	*Gammaproteobacteria*	0.2
Proteobacteria	*Betaproteobacteria*	0.1
Proteobacteria	**Epsilonproteobacteria**	**36.1**
Proteobacteria	*Deltaproteobacteria*	0.1
Firmicutes	*Clostridia*	0.1
Deferribacteres	*Deferribacterales*	0.1
Thermotogae	*Thermotogae*	0.6
Aquificae	**Aquificae**	**26.1**
Thermodesulfobacteria	Thermodesulfobacteria	0.8
Candidate division SR1	–	0.8
Chloroflexi	*Dehalococcoides*	0.1
Crenarchaeota	*Thermoprotei*	0.9
Euryarchaeota	*Thermoplasmata*	0.3
Euryarchaeota	**Methanococci**	**2.8**
Euryarchaeota	**Archaeoglobi**	**1.8**
Euryarchaeota	**Thermococci**	**28.4**
Thaumarchaeota	*Marine Group I*	0.1

Bold font indicates taxonomic groups represented by more than 1% of the total 16S rRNA gene pool.

The chimney samples analyzed in this study were collected during R/V *G.O. Sars* cruises in 2008, 2009, and 2010 using a Bathysaurus XL remotely operated vehicle (ROV) equipped with a hydraulically operated box sampler. In total, eleven samples recovered from two active and inactive sulfide structures (*Menorah*, *João*, and one unnamed chimney) at Loki's Castle hydrothermal vent field were subsampled and analyzed for mineral composition and lipid biomarkers (Table 1). From *Menorah*, a bulk sample from the chimney wall was sampled for 16S rRNA gene analysis (sample GS08-ROV11; Table 2). In addition, microbial filaments at the surface of the *João* structure were sampled by a suction sampler and analyzed for lipid biomarkers. All samples were stored at -20°C until used for analysis.

MATERIALS AND METHODS

Rock analyses

Freeze-dried, crushed, and powdered chimney samples were analyzed by X-ray diffraction (Bruker, AXS D8 Advance) to determine the mineralogy. Total organic carbon (TOC), carbon isotope ratios ($\delta^{13}C$), and sulfur isotope ratios ($\delta^{34}S$) of the bulk rock samples were measured after decarbonation on a ThermoFisher Flash-EA 1112 elemental analyzer coupled via a Conflo IV interface to a ThermoFisher Delta V isotope ratio mass spectrometer. The system was calibrated with the reference materials NBS22 ($\delta^{13}C = -30.03$) and IAEA CH-6 ($\delta^{13}C = -10.46$) for carbon and IAEA –S-1 ($\delta^{34}S = -0.3$), IAEA –S-2 ($\delta^{34}S = +22.67$), IAEA –S-3 ($\delta^{34}S = -32.55$) for sulfides and NBS 127 ($\delta^{34}S = +21.1$), IAEA-SO-5 ($\delta^{34}S = +0.49$) and IAEA-SO-6 ($\delta^{34}S = -34.05$) for sulfates. All analytical results are reported in the conventional δ notation, in per mil relative to the Vienna Pee Dee belemnite (VPDB) standard for carbon and the Vienna Canon Diablo Troilite (VCDT) standard for sulfur. Reproducibility of the measurements was better than 0.2‰.

Molecular techniques and taxonomic analysis

DNA extraction and PCR amplification

Ten-gram of bulk chimney wall material from the active *Menorah* structure (GS08-ROV11) was pulverized in a sterilized steel mortar, and DNA was extracted from this homogenized material (approximately 0.5 g) using a FastDNA® spin kit for soil in conjunction with the FastPrep instrument (MP Biomedicals, Santa Ana, CA) following manufactures protocol and applying the modifications described by Hugenholtz *et al*. (1998). The extracted DNA was PCR amplified in triplicates using the prokaryotic primer set 787F (5′ATTAGATACCCNGG TAG3′) (Roesch *et al*., 2007) and Uni1391R (5′ACGGG CGGTGWGTRC3′) modified from Lane *et al*. (1985), as

described by Lanzén *et al.* (2011). The resulting amplicons were purified and sequenced using multiplex GS FLX pyrosequencing (without Titanium chemistry) at the Norwegian High-Throughput Sequencing Centre (NSC) in Oslo, Norway.

Filtering, removal of noise and taxonomic assignment of 16S rRNA gene amplicon sequence data

The dataset (5485 reads) was filtered and cleaned from noise by using the software AmpliconNoise (Quince *et al.*, 2011). The protocol has been described previously (Lanzén *et al.*, 2011). In short, bad-quality reads are removed (flow intensity 0.5–0.7), so are sequences not matching the applied primer sequence as well as chimeric reads. The resulting 3646 high-quality reads distributed on 122 unique reads with an average read length of 231 bp were used for taxonomic evaluation. To assign each sequence read to a taxon, we compared our reads with the SILVA SSUref database release 100 (Pruesse *et al.*, 2007), using *blastn*. A manual revision of this database was conducted, and the taxonomy updated with respect to *Epsilonproteobacteria*, *Acidobacteria*, *Chloroflexi*, and the Archaea as well as a more restrictive quality filter (pintail score >75, alignment quality >75 and length >1200 bp; database available at http://services.cbu.uib.no/supplementary/community-profiling/). Taxomomical assignments were then evaluated using the software MEGAN version 3.7 (Huson *et al.*, 2007) by applying a last common ancestor algorithm (for details see Lanzén *et al.*, 2011). Taxonomic affiliation of sequence reads and the relative abundances within the bulk chimney wall of the *Menorah* structure are given in Table 2. Pyrosequencing flowgrams (SSF files) have been deposited in the NCBI Sequence Read Archive under the accession number SRA052614.

Lipid extraction, derivatization, and fractionation

About 6–12 g of each chimney sample was freeze-dried, crushed to a fine powder, and ultrasonically extracted using methanol (MeOH), dichloromethane (DCM)/MeOH (1:1 v/v), and DCM (three times). The extracts were combined and the bulk of the solvent subsequently removed by rotary evaporation under vacuum. Elemental sulfur was removed from the total lipid extract (TLE) by flushing with n-hexane over a small pipette filled with HCl-activated copper. The TLE was further transmethylated with MeOH/HCl (10% w/v) at 70 °C for 2 h to convert free and ester-bound fatty acids into their corresponding methyl esters (FAMEs), and silylated with bis (trimethyl) trifluoroacetamide (BSTFA) in pyridine at 60 °C for 20 min to convert alcohols in trimethylsilyl (TMS) ether derivatives. An aliquot of the TLE was chromatographically separated into apolar and polar fractions using a column with activated silica as stationary phase. Apolar compounds were obtained

using n-hexane/DCM (9:1 v/v) as eluant. Polar fractions containing the GDGTs (i.e., structures I-XII, Fig. 3) were eluted with DCM/MeOH (1:1 v/v, 3 column volumes). After solvent evaporation, the polar fractions were redissolved in 200 µL of HPLC-grade n-hexane/isopropanol (99:1 v/v) and were filtered through a 0.45-µm PTFE filter prior to HPLC/APCI/MS analysis. Microbial filaments were extracted using a modified Bligh-Dyer procedure (Bligh & Dyer, 1959). A solvent mixture of phosphate-buffer (0.05 M, pH 7.4)/methanol (MeOH)/dichloromethane (DCM) 0.8/2/1 (v/v) was added to the frozen cell material. The mixture was sonicated for 10 min after which further DCM and phosphate-buffer were added to a volume ratio of 0.9/1/1. After centrifuging (5 min at 1120 *g*), the DCM layer was collected. The residue was re-extracted twice following the same procedure. The extracts were combined and the bulk of the solvent subsequently removed by rotary evaporation. An aliquot of the extract was further hydrolyzed in 2 M HCl/MeOH (1/1, v/v) for 3 h at 75 °C. The pH of the hydrolyzed extract was adjusted to pH 3 using 1 M KOH (MeOH 96%). The extract was derivatized as described previously. The position of the double bonds in the fatty acids was determined by analysis as their dimethyl disulfide (DMDS) adducts according to the method of Nichols *et al.* (1986). Briefly, an aliquot of the sample dissolved in 50 µL of n-hexane was treated with 100 µL of DMDS and 20 µL of iodine solution (6% w/v in diethyl ether). The reaction was carried out in 2-mL screw-cap glass vials at 50 °C for 48 h. The mixture was cooled and diluted with 500 µL of n-hexane. The excess of iodine was reduced by addition of 500 µL of sodium thiosulfate (5% w/v in MilliQ water). The organic phase was removed, and the aqueous phase extracted twice with 500 µL of n-hexane. Combined organic phases were evaporated under a stream of nitrogen and diluted with 100 µL of n-hexane prior to GC-MS analysis.

Analysis and identification of biomarkers

High-performance liquid chromatography-mass spectrometry (HPLC-MS)

Glycerol dialkyl glycerol tetraethers analysis was performed at the Geological Institute of the ETH Zurich using high-performance liquid chromatography/atmospheric pressure chemical ionization–mass spectrometry (HPLC/APCI–MS) with a Thermo Surveyor LC system coupled to an LCQ Fleet ion trap mass spectrometer equipped with a PAL LC autosampler and Xcalibur software, as described by Hopmans *et al.* (2000). Normal phase separation was achieved with an Alltech Prevail Cyano column (150 mm × 2.1 mm; 3 µm) maintained at 30°C. Flow rate of the n-hexane/isopropanol (IPA) (99:1) mobile phase was 0.3 mL min^{-1}, isocratically for the first 5 min, thereafter

with a linear gradient to 2% IPA in 30 min, and a column cleaning step with 10% IPA in n-hexane. Injection volume was 20–50 μL. Scanning was performed over the m/z ranges 740–746, 1016–1054, and 1280–1318. Relative abundances of GDGTs were calculated using peak areas of the $[M+H]^+$ ions vs. those of the C20-diol internal standard (m/z 743). GDGTs were identified and distinguished via their MS^2 spectra. MS^2 experiments were performed with conditions according to Knappy et al. (2009). Briefly, eluting species were monitored using the positive ionization mode of the APCI source. Conditions for APCI-MS were as follows: vaporizer temperature 300 °C, sheath gas (N_2) flow rate 40 (arb. units), auxiliary gas (N_2) flow rate 5 (arb. units), capillary temperature 200 °C, capillary voltage 23 V, and corona discharge current 5 μA. Positive ion MS spectra were obtained by scanning a narrow mass range from m/z 1220 to 1350. MS^2 spectra were recorded using the data dependent ion scan feature, in which the base peak of an MS scan is selected for collision induced dissociation (CID) in MS^2 (collision energy was set at 30%).

Gas chromatography-mass spectrometry (GC-MS)

Compound identification was done by combined GC-MS. GC-MS was conducted using a Hewlett Packard 6890 gas chromatograph equipped with an on-column injector. A fused silica capillary column (HP-5, 30 m length, 0.25 mm inner diameter, 0.25 μm film thickness) with helium as a carrier gas was used. The gas chromatograph was interfaced to a HP 5973 mass selective detector (MSD) with a mass range of m/z 50–800. The samples were injected at 60 °C. The GC oven temperature was subsequently raised to 120 °C at a rate of 10°C min^{-1} and then to 320 °C at 4°C min^{-1}. The temperature was then held constant for 20 min. The structural characterization of lipids was evaluated by comparing their mass spectral fragmentation pattern with published spectra.

## RESULTS AND DISCUSSION

Mineralogy and bulk isotope data

The mineralogy of the samples recovered from active (João and Menorah) and inactive sulfide chimneys (Table 1) indicated that they were mostly derived from the interior zones of the chimney wall. The sulfide-poor samples GS09-ROV6(1 + 2) were dominated by abundant anhydrite, and less gypsum and talc. In general, during chimney growth, anhydrite precipitates around a black smoker vent at the leading edge of chimney growth, where hot hydrothermal fluids first encounter cold seawater (Haymon, 1983), talc forms in hot chimneys from seawater magnesium and hydrothermal silica (Haymon & Kastner, 1981). Thus, we can assume that our samples containing anhydrite were derived from the exterior zones of the chimney wall. The

porous sulfide samples mainly consisted of pyrite, pyrrhotite, sphalerite, and marcasite (Table 1), reflecting intermediate temperatures of formation (<240 °C for marcasite) (Haymon, 1983). A number of studies have shown that once the anhydrite walls of a chimney are in place, and hydrothermal fluid is protected from extensive mixing with seawater, which subsequently leads to the precipitation of Zn-Cu-Fe sulfides toward the chimney interior. During this stage, anhydrite is partially dissolved again and replaced by sulfides (Haymon, 1983).

$\delta^{34}S$ values generally ranged from 1‰ to 2‰ in the sulfide samples (Table 1) indicating a mid-ocean ridge basalt (MORB) source (Shanks & Seyfried, 1987). The sulfate samples (i.e., anhydrite) showed $\delta^{34}S$ values of 19‰ and 21‰, reflecting seawater sulfate values. Total organic carbon (TOC) was generally low in the chimney samples ranging from 0.03% to 0.9%; however, GS08-ROV11(1) of active Menorah revealed an extraordinarily high TOC content of 3% (Table 1). These strong variations in the organic content of samples from the same chimney indicate that hydrothermal vents provide small, patchy, and unstable habitats for microbes. The $\delta^{13}C$ of organic carbon varied between −6 and −7‰ for the inactive chimney samples which is in the range of the isotopic composition observed for mantle derived CO_2. $\delta^{13}C$ values of organic carbon in the active chimney samples varied from −14 to −39‰, yielding a range of 25‰ (Table 1). The low $\delta^{13}C$ values in the marcasite-bearing samples GS10-ROV9 (1 + 2) of João may indicate the presence of chemolithoautotrophs preferentially using ^{12}C in a distinct layer within the middle/outer chimney wall where metastable pyrrhotite is being rapidly replaced by either pyrite or marcasite, depending on the pH of the fluid (for marcasite pH < 5) (Murowchick & Barnes, 1986). Thus, besides variations in temperature and availability of reduced chemical species, the mineralogy and habitat type could be important factors affecting the composition of microbial communities (Kato et al., 2010).

Microbial diversity

The sulfide chimney matrix of the active Menorah structure (GS08-ROV11) harbored a diverse range of thermophilic and hyperthermophilic archaea and bacteria, as shown both by our 16S rRNA gene-based taxonomy (Table 2, Fig. 2) and lipid analysis (Fig. 3). However, lipids such as GDGTs are common lipids of many archaea, and therefore, it is difficult to link them with a specific archaeal group without additional microbial- or compound-specific isotope data. In the following sections, we use the information obtained from the 16S rRNA gene amplicon library to discuss the link between possible source organisms and different compound classes detected in samples originating from the same chimney wall.
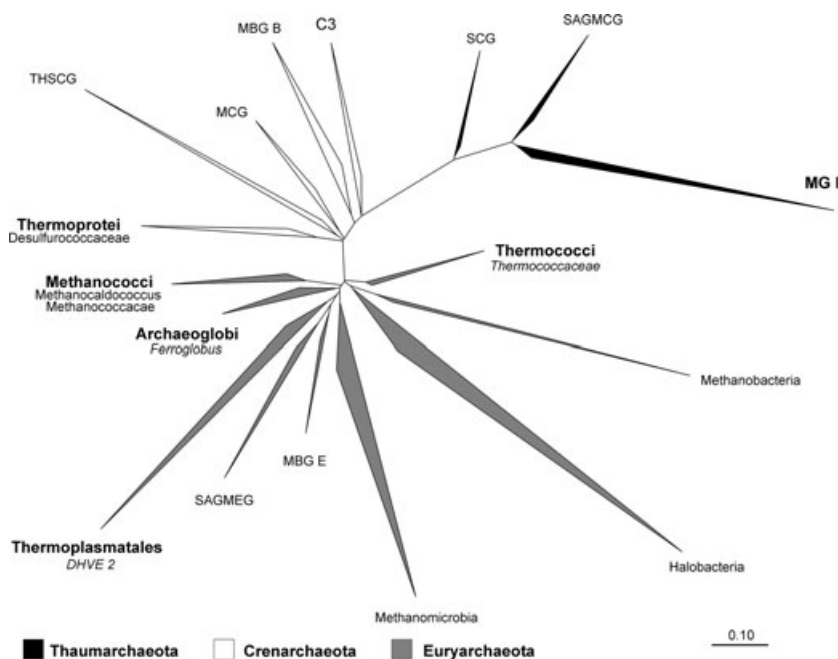
Fig. 2 Phylogenetic Neighbor-joining tree based on archaeal 16S rRNA gene sequence information. The taxonomic affiliation of the sequences obtained from the *Menorah* active sulfide chimney is highlighted in bold. Further the group to which these sequences could be assigned at a lower taxonomic level is given below the phylum name in italic. The relative abundance of each group can be found in Table 2. THSCG, Terrestrial hotspring crenarchaeotic group; MCG, Miscellaneous crenarchaeotic group; MBG, Marine Benthic Group; SCG, Soil crenarchaeotic group; SAGMCG, South African gold mine crenarchaeotic group; MG I, marine group I; SAGMEG, South African gold mine Euryarchaeotic group.

Archaea

Distribution and origin of archaeal GDGTs (I–VII)

All samples from the chimney structures contained a range of isoprenoid GDGTs (Fig. 3), indicating that archaea are present throughout the chimney walls. GDGT I, a trialkyl-type GDGT (Fig. 3), was detected as a minor constituent (<1%) of the total GDGT pool from *Menorah* and *João* active sulfide chimneys (Fig. 4). GDGT I has been reported in a number of cultivated thermophilic and hyper-thermophilic Crenarchaeota (Gulik *et al.*, 1988; de la Torre *et al.*, 2008) and has been proposed as an intermediate in the biosynthesis of GDGT II from archaeol (Koga & Morii, 2007). GDGT II (Fig. 3) was detected in all samples analyzed and was also the most abundant compound with relative abundances between 29 and 61% of all GDGTs, followed by GDGTs containing 1–4 cyclopentane rings (Fig. 3 structures III-VI) with relative abundances between 1 and 19% (Fig. 4). Methanogenic archaea and members of the family *Archaeoglobaceae* have been found to produce predominantly GDGT II (Koga & Morii, 2005). Sequences related to both groups were found in our taxonomic data, namely members of the family *Methanococcales* and of the genus *Ferroglobus* belonging to the family of *Archaeoglobales*. *Methanococcales* (2.8% of the total community) is a group that constitutes strictly anaerobic autotrophs that gain energy by the reduction of CO_2 with H_2, generating CH_4. *Ferroglobus* made up 1.8% of the total community (Fig. 2) and is a hyperthermophilic (growth between 65 and 95 °C) member of the *Archaeoglobales* that oxidizes ferrous iron (Fe^{2+}) but also molecular hydrogen, and sulfide under strictly anaerobic conditions (Hafenbradl *et al.*, 1996). Nitrate and thiosulfate ($S_2O_3^{2-}$) are used as electron acceptors that are known from a variety of hyperthermophiles (Stetter *et al.*, 1987; Stetter, 2002). Although it seems likely that the origin of the detected GDGT II stems from the above-mentioned groups, we cannot exclude that there are other, still uncultivated archaeal groups that also produce this as a major lipid.

Detection of 16S rRNA genes related to hyperthermophilic members of the family *Thermoproteaceae* (Crenarchaeota) and *Thermoplasmataceae* (Euryarchaeota) (Table 2, Fig. 2) suggest that, at least partly, they may be the source organisms for GDGT III–VI containing 1–4 cyclopentane rings (Fig. 3). As reported by Koga & Morii (2005), these groups have been found to predominantly synthesize GDGT II-VI in culture. Indeed, members of the *Desulfurococcus*, a group composed of hyperthermophilic heterotrophs, growing at temperatures up to 95°C (no growth is reported for temperatures of ≤ 65 °C) and gaining energy by oxidation of hydrogen using elemental sulfur (Huber & Stetter, 2006; Stetter, 2006), were detected as minor components representing 0.9% of the microbial community (Fig. 2). It is interesting to know that we found GDGTs with a maximum of only four rings in such high temperature environment. In general, the number of cyclopentane rings incorporated in tetraether lipids increases with increasing growth temperature (i.e., Uda *et al.*, 2001; Schouten *et al.*, 2002; Boyd *et al.*, 2011). GDGTs with up to eight rings were reported from Yellowstone hot springs (Schouten *et al.*, 2007), where
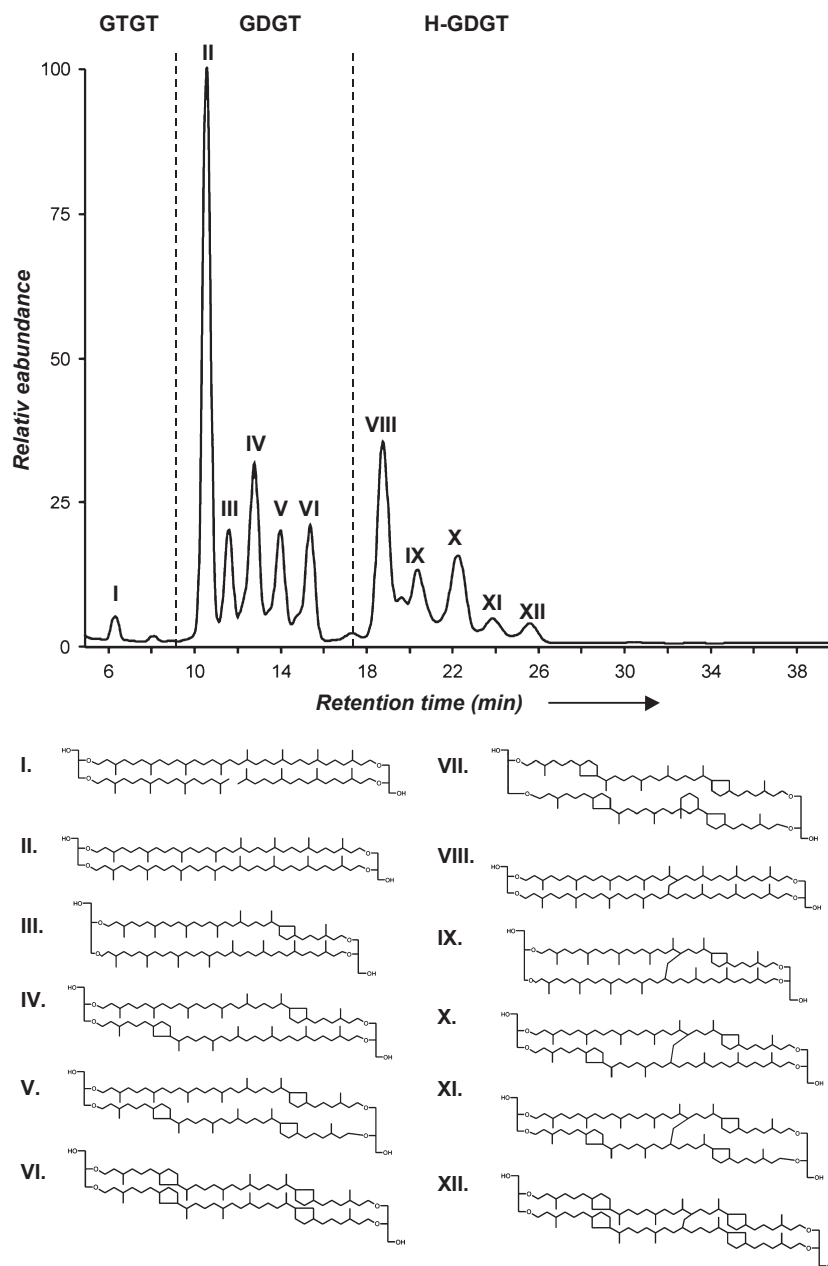
Fig. 3 High-performance liquid chromatography (HPLC)/APCI/MS base peak chromatogram showing the distribution of GDGTs in *Menorah* active black smoker material GS08-ROV11(1), and structures of GDGTs present in the black smokers of Loki's Castle. The position of the covalent bond between the isoprenoid hydrocarbon chains in GDGTs VII–XI is tentative (Morii *et al.*, 1998). Cren, Crenarchaeol; GTGT, glycerol dialkyl glycerol tetraether; GDGT, glycerol dialkyl glycerol tetraether; H-GDGT, H-shaped GDGT (see text).

temperatures were generally lower than in black smokers. Thus, there are possibly other parameters controlling the GDGT lipid composition in archaea, for example, pH, pressure, heavy metal content as well as biological factors. For the thermoacidophilic archaea *Sulfolobus acidocaldarius*, it has been shown that the incorporation of cyclopentane rings leads to a more tightly packed membrane than one without rings, thus regulating membrane behavior, that is, fluidity or proton permeability (Gabriel & Chong, 2000).

Crenarchaeol (GDGT VII, Fig. 3) was found in the middle and exterior zones of the chimneys, and only traces could be detected in the interior parts of the chim-

ney wall at *Menorah* GS08-ROV11(1) (Fig. 5A). In the *João* sample GS10-ROV9(2), crenarchaeol and the regioisomer accounted for 5% and <0.1%, respectively, of the total GDGTs (Fig. 4) (concentration was 1 ng/g chimney material). The mineralogy of this sample suggests that it is derived from the chimney middle to exterior where marcasite precipitated during chimney growth (Haymon, 1983). As for GDGT II–VI, very low concentrations of about 20 pg/g chimney material were detected in an anhydrite-dominated chimney sample GS09-ROV6(2), clearly showing a seawater sulfate signal (Table 1). Here, crenarchaeol accounted for 25% of all GDGTs (Fig. 4). Crenarchaeol was originally thought to be a specific
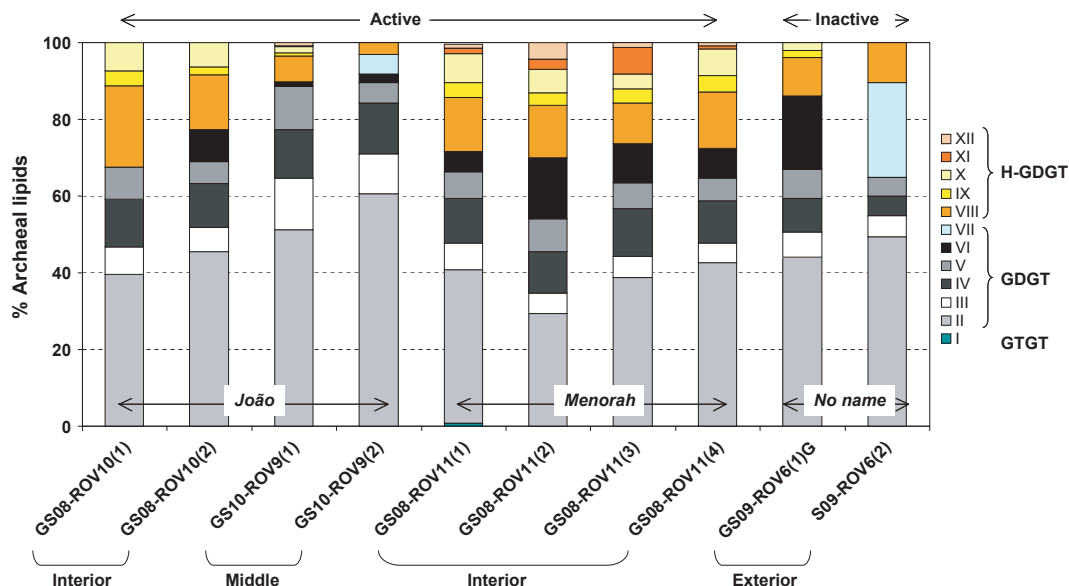
Fig. 4 Relative abundances of archaeal GDGTs present in samples derived from different parts of active and inactive chimneys. Roman numerals refer to structures in Fig. 3. Numbers in brackets indicate subsamples taken from same chimneys.

biomarker for mesophilic *Crenarchaeota* (recently, the phylum has been split up into two phyla; *Crenarchaeota* and *Thaumarchaeota*: Brochier-Armanet *et al.*, 2008; Spang *et al.*, 2010). This group is composed of putative aerobic ammonia oxidizers (AOA) (Könneke *et al.*, 2005; Wuchter *et al.*, 2006) and is ubiquitously found in marine systems between 2 and 30 °C (Sinninghe Damsté *et al.*, 2002). However, thermophilic members of the ammonia-oxidizing archaea have also been found to synthesize cren-archaeol at temperatures up to 87 °C (Pearson *et al.*, 2004; Zhang *et al.*, 2006; de la Torre *et al.*, 2008). Loki's Castle is a sediment-impacted hydrothermal system with high ammonium concentrations (6.1 mmol kg^{-1}; Pedersen *et al.*, 2010), suggesting that AOA may be active at this site as well. Moreover, Zhang *et al.* (2006) suggested that crenarchaeol could be an original and ancient biochemical property of the thermophilic *Crenarchaeota*, which occupy a deeply branching point in the phylogenetic tree of life (Forterre *et al.*, 2002). Our lipid and 16S rRNA gene-based data, however, suggest that hyperthermophilic *Crenarchaeota* only account for a small fraction of the total microbial community in the chimney (see discussion previously; Table 2, Fig. 2). Low relative abundances of 16S rRNA genes related to marine group I *Thaumarchaeota* (Table 2, Fig. 2) indicate the presence of AOA. However, chimney walls are sufficiently permeable to allow the influx of small amounts seawater; therefore, our data could also be interpreted as an introduction of *Thaumarchaeal* cells from the ingression of ambient seawater where they have been found to be ubiquitous (Takai *et al.*, 2004).

Distribution and origin of archaeal H-GDGTs (VIII–XII)

In addition to GDGTs I-VII, another group of later-eluting compounds was present in the HPLC/MS chromatogram (VIII–XII, Fig. 3). These compounds showed mass spectra characteristic of GDGTs with base peak ions of 1300, 1298, 1296, 1294, and 1292, respectively, which are the [M+H]$^+$ ions (Fig. 5A). The distribution of these compounds is similar to that of GDGTs II-VII, and they were further identified and distinguished via their MS^2 spectra. MS^2 experiments revealed that GDGTs VIII-XII have a different fractionation pattern than GDGT II-VII, exhibiting a far less pronounced degree of dissociation (Fig. 5B,C). This is due to the covalent bond between the two hydrocarbon chains in these compounds, which stays intact during the dissociation, generating a product ion that maintains the C$_{80}$ hydrocarbon core, and two small fragments resulting from the loss of an OH and a glycerol group. The MS^2 spectra are identical to those previously published by Knappy *et al.* (2009) who analyzed cells from a pure culture of *Methanobacter thermoautotrophicus* grown at 70 °C. The specific MS^2 spectrum indicates that compounds VIII-XII are GDGTs with an additional covalent bond between the isoprenoid chains, so-called H-shaped GDGTs (H-GDGTs) (Morii *et al.*, 1998). Abundances of H-GDGTs VIII-XII are generally lower than those of regular GDGTs (Fig. 3), and contribute with 3–21% to the total lipid pool (Fig. 4). H-shaped isoprenoid GDGTs have so far been identified in several cultivated archaea, for example, in the above-mentioned hyperthermophilic *Methanobacter thermoautotrophicus*, in the hyperthermophilic methanogen *Methanothermus fervidus* (Morii *et al.*, 1998),
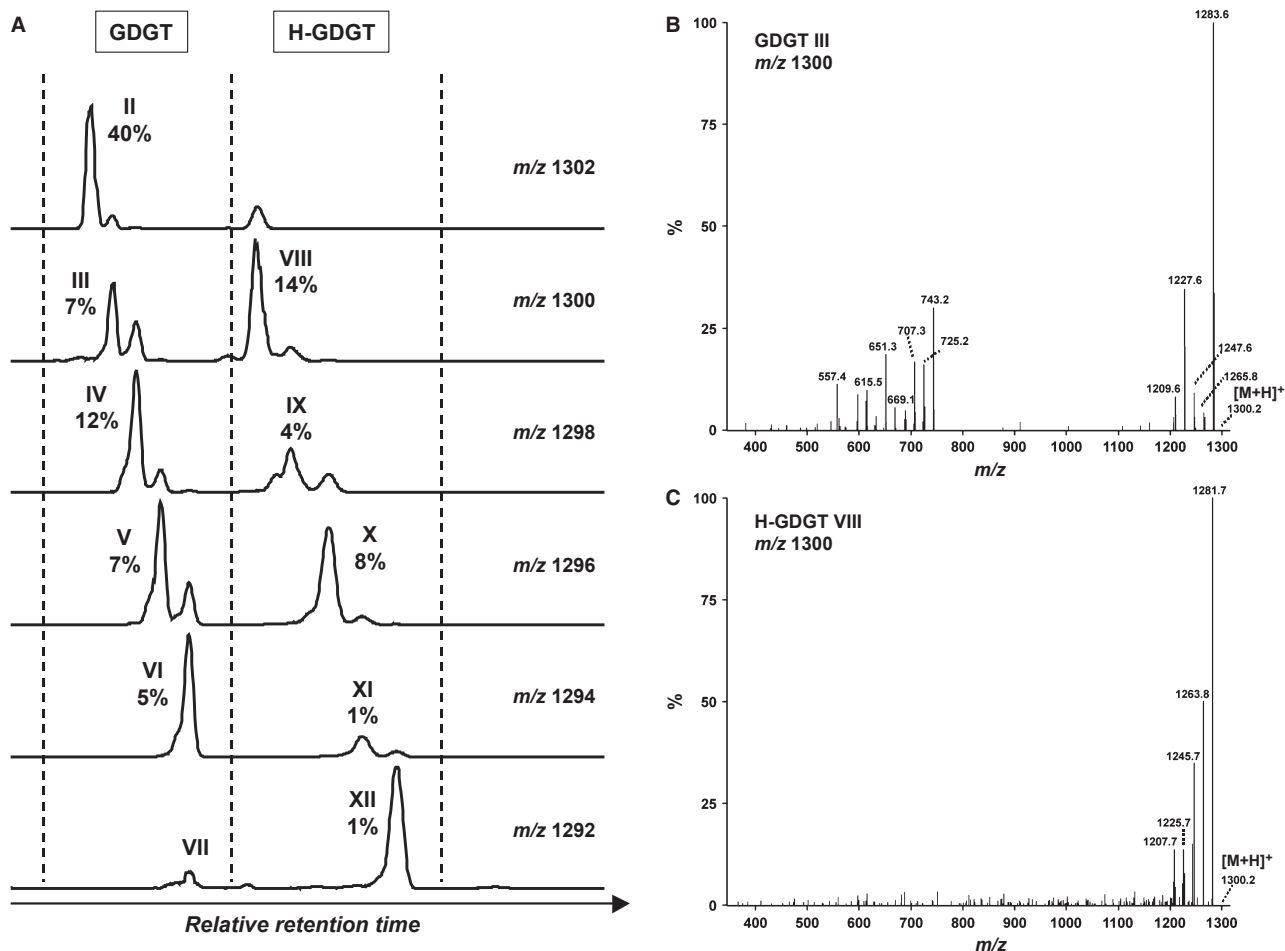
Fig. 5 (A) Individual ion chromatograms for glycerol dialkyl glycerol tetraethers (GDGTs) II-X detected in *Menorah* active chimney material GS08-ROV11(1) in the positive ion MS base peak chromatogram. The percentage of the total extract contributed by each lipid is also shown. MS^2 spectra for (B) GDGT II and (C) H-GDGT VIII with [M+H]$^+$ at *m/z* 1300.2.

as well as in different species of the order *Thermococcales* growing at neutral pH with optimal growth temperatures >80 °C (Godfroy *et al.*, 1997; Sugai *et al.*, 2004). In congruence with this, we found a high abundance of *Thermococcales*-related 16S rRNA gene sequences (28.4% of total community) (Table 2, Fig. 2), suggesting that this group is the main source of H-GDGTs. *Thermococcus* is an obligate anaerobic, sulfur-reducing heterotroph belonging to the *Euryarchaeota*, which is often detected as a member of vent communities (Takai *et al.*, 2001; Schrenk *et al.*, 2003; Kormas *et al.*, 2006; Takai & Nakamura, 2011). Sulfur reduction has been suggested to be the thermodynamically favored reaction at higher temperatures (>38 °C), while sulfur oxidation is more viable at lower temperatures (McCollom & Shock, 1997). Elemental sulfur is either stimulatory or is required for growth of *Thermococcus*. Elevated hydrogen concentrations measured in the vent fluids (Pedersen *et al.*, 2010) can serve as an electron donor for the reduction of elemental sulfur to H_2S.

Another candidate group for the high abundance of H-GDGTs was identified by Schouten *et al.* (2008) who detected H-shaped GDGTs with up to four cyclopentane rings in *Aciduliprofundum boonei*, a cultivated thermoacidophilic sulfur- and iron-reducing *Euryarchaeota* from a deep-sea hydrothermal vent. This organism belongs to the DHVE2 cluster and is capable of growing from pH 3.3 to 5.8 and between 55 and 75 °C (Reysenbach *et al.*, 2006). However, at Loki's Castle, 16S rRNA genes related to the DHVE2 cluster contributed only 0.3% to the total microbial community (Table 2, Fig. 2), indicating that only a minor part of the H-GDGTs originates from those archaea.

Our finding of high abundances H-shaped GDGTs is not surprising as the introduction of an additional covalent cross-link between the isoprenoid chains is thought to help maintain membrane structure at high temperatures (Morii *et al.*, 1998; Schouten *et al.*, 2008). Our results indicating the prevalence of archaeal communities near the warm interior of black smoker chimneys is also in agreement with

earlier findings by Schrenk *et al.* (2003). Higher rigidity and stability of archaeal tetraether lipids that form monolayer membranes are better suited to extreme environments than the ester type of bilayer lipids of bacteria or eukarya (van de Vossenberg *et al.*, 1998). Moreover, relatively high abundances of both GDGTs and H-GDGTs with 1–4 cyclopentane rings detected in our samples is compatible with culture studies showing that (hyper)thermophilic *Crenarchaeota* and *Euryarchaeota* produce GDGTs containing more cyclopentane moieties with increasing growth temperature (Uda *et al.*, 2001, 2004). As discussed previously, the distribution of archaeal GDGTs and H-GDGTs could also be controlled by other parameters such as pH, pressure, heavy metal content or it could be related to a specific metabolism (Uda *et al.*, 2004; Boyd *et al.*, 2011). GDGT abundances in samples derived from the exterior wall of the inactive chimney GS09-ROV6 (1 + 2) were about three orders of magnitude lower than those derived from active chimneys, suggesting that once a chimney stops venting, conditions are no longer favorable for archaeal communities. In our sample of the exterior wall of the inactive chimney, high abundances of unsaturated (and branched) fatty acids were detected and may indicate the presence of sulfur-oxidizing

Table 3 Concentration of distinctive compounds extracted from active and inactive chimney material and chimney-associated filaments

Compound	Active GS08-ROV11(1) Menorah (ng g^{-1})	Filaments GS09-ROV9(2) João (µg g^{-1})	Inactive GS09-ROV6(1) unknown (ng g^{-1})	Inactive GS09-ROV6(2) unknown (ng g^{-1})
Fatty acids				
i-C15:0	nd	778	nd	nd
ai-C15:0	nd	549	nd	nd
ai-C16:0	nd	236	nd	12.5
C16:1ω7c	nd	17577	192.9	1074.2
C16:1ω7t	nd	1985	26.3	114.2
C16:1ω5c	nd	2485	26.2	77.7
C16:0	nd	5350	37.1	314.0
10-Me-C16:0	nd	nd	nd	10.8
C18:1ω9c	nd	nd	15.6	24.9
C18:1ω7c	nd	6361	31.6	100.2
C18:0	nd	266	nd	nd
C22:1ω9c	nd	nd	732.2	282.1
Alcohols				
Archaeol	102.2	3.1*	nd	nd
sn-2-hydroxyarchaeol	68.0	nd	nd	nd
Hydrocarbons				
Crocetane	3.1	nd	nd	nd
PME	6.8	nd	nd	nd
PME:4	25.9	nd	nd	nd
Hopanoids				
diploptene	29.2	nd	nd	nd
diplopterol	15.2	nd	nd	nd

nd, not detected.*sample contained small amounts of rock debris. Also glycerol dialkyl glycerol tetraethers (GDGTs) and H-GDGTs were detected in low abundances (<1 µg g^{-1}).

(and sulfate-reducing) members of the ε subclass of the Proteobacteria (the ε-Proteobacteria) (Table 3; see section on bacteria below; H. Dahle, unpublished data).

Isoprenoid diether lipids and hydrocarbons

Isoprenoid compounds of archaeal origin, such as archaeol, were also found in the *Menorah* sample GS08-ROV11(1). Archaeol is produced by a variety of different archaeal groups, such as halophiles, thermophiles, and acidophiles (Koga & Morii, 2005), and was present with a concentration of 102 ng/g (Table 3). Another isoprenoid compound, *sn*-2-hydroxyarchaeol, was detected as both mono- and di-trimethylsilyl (TMS) derivatives with a concentration of 68 ng/g. *sn*-2-hydroxyarchaeol is a diagnostic biomarker for the thermoacidophilic archaeal order *Thermoplasmatales* and the methanogenic order *Methanococcales* (Koga *et al.*, 1998), which is consistent with the findings of abundant GDGTs with cyclopentyl moieties as well as 16S rRNA gene-based taxonomy data (see section above; Table 2). Concentrations of crocetane, irregular isoprenoids pentamethyleicosane (PME) and PME 4 were 3, 7, and 26 ng/g chimney material, respectively. These compounds have been attributed to anaerobic, methanotrophic archaea (ANME) (Elvert *et al.*, 1999; Bian *et al.*, 2001), although PME was originally believed to be synthesized by methanogenic archaea (Holzer *et al.*, 1979; Risatti *et al.*, 1984). Whereas the presence of methanogenic archaea is supported by our 16S rRNA gene data (*methanococci*), no sequences related to any of the ANME groups were detected.

Bacteria

The lipid composition of large filamentous bacteria collected from the *João* active sulfide chimney (GS09-ROV9) revealed the dominance of C$_{16:1ω7c}$ (47%), C$_{18:1ω7c}$ (17%), and C$_{16:0}$ (14%) fatty acids. Concentrations of these compounds are about three orders of magnitude higher in this sample as in the chimney material (Table 3). The lipid pattern is consistent with lipid profiles of known sulfur-oxidizing bacteria from sediments and hydrothermal vents (Jacq *et al.*, 1989; Guezennec *et al.*, 1998; Zhang *et al.*, 2005). The filaments attached to the outer chimney wall have been found to be related to sulfur-oxidizing bacteria *Sulfurovum*, a group belonging to the ε-Proteobacteria that thrive at lower ambient temperatures (H. Dahle, unpublished data). Minor abundances of C$_{16:1ω5c}$ and C$_{18:1ω9c}$ detected in the filaments as well as in the inactive chimney samples GS09-ROV6(1 + 2) may be derived from sulfate-reducing bacteria (Dowling *et al.*, 1986; Elvert *et al.*, 2003; Londry *et al.*, 2004).

16S rRNA gene analysis with subsequent taxonomic analysis of Menorah active chimney GS08-ROV11 revealed that members of the *Aquificales* and ε-*Proteobacteria* were the dominant bacteria accounting for 26 and 36% of the total

prokaryotic community (Table 2). The *Aquificales* is thought to be the earliest branching lineage within Bacteria and have often been detected in hot springs (Spear *et al.*, 2005; Purcell *et al.*, 2007) and deep-sea vent ecosystems (Kormas *et al.*, 2006; Blumenberg *et al.*, 2012). Among the bacteria, *Aquificales* exhibit one of the highest growth temperatures (95 °C). They are anaerobic lithoautotrophs, gaining metabolic energy from the oxidation of molecular hydrogen (H_2) or sulfur compounds. Lithotrophic sulfur reduction by oxidation of H_2 is believed to be one of the most ancient types of catabolism (Fischer *et al.*, 1983), and the preferred source of energy at temperatures >38 °C (McCollom & Shock, 1997). The apparent lack of dialkyl glycerol diethers (DAGE) specific for the *Aquificales* (Jahnke *et al.*, 2001) at *Menorah* may be because the distinct zones where the *Aquificales* thrive within the chimney structure were not present in our subsamples for lipid analysis. Another possibility of the observed discrepancy could be a primer bias in the 16S rRNA gene approach, leading to a skewed relative abundance estimate. The same may be the case for the deep-branching ε-Proteobacterial group *Nautiliales* that were detected as one of the main organisms within the chimney wall of *Menorah* (Table 2). *Nautiliales* is believed to be constituted of thermophilic sulfur-reducing bacteria that are found to be key players in sulfidic habitats (Alain *et al.*, 2004; Campbell *et al.*, 2006). Other members of the ε-Proteobacteria, for example, the *Campylobacteriales*, made up only a very small fraction of the total population (data not shown). The dominant lipids of *Nautilia profundicola* have been shown to be $C_{18:1\omega7c}$ and $C_{16:1\omega7c}$ fatty acids (Smith *et al.*, 2008). High abundances of branched and monounsaturated fatty acids detected in samples derived from the outer chimney wall GS09-ROV6 (1 + 2) (Table 3) may be produced by sulfur-oxidizing and sulfate-reducing bacteria. In general, lower growth temperatures of 45–53 °C indicate that bacteria mainly thrive within the exterior chimney walls, which has also been reported for a white smoker by Kormas *et al.* (2006). The hopanoids diploptene and diplopterol with low concentrations (Table 3) have predominantly been found in aerobic bacteria, that is, methanotrophs, heterotrophs, and cyanobacteria. Hopanoids also occur in anaerobic bacteria, for example, members of the *Planctomycetes* capable of anaerobic ammonium oxidation (Sinninghe Damsté *et al.*, 2004), in *Geobacter* species (Fischer *et al.*, 2005; Härtner *et al.*, 2005), and in sulfate-reducing bacteria of the genus *Desulfovibrio* (Blumenberg *et al.*, 2006). Concentrations of these lipids, however, are about 1–2 orders of magnitude lower than measured for GDGTs.

CONCLUSIONS

In the present study, we gave first insights into the diversity of microbial communities present in sulfide structures of active and inactive vents from the newly discovered Loki's Castle black smoker field at the Arctic Mid-Ocean Ridge. Evidence for both archaea and bacteria was provided by a combination of lipid biomarker and 16S rRNA gene-based techniques. The specific lipid distribution observed in samples derived from different parts of the active and inactive chimneys indicate the presence of diverse consortia of (hyper)thermophilic *Euryarchaeota* and *Crenarchaeota* within the warmer interior zones, while bacterial lipids were only a minor constituent. Our study particularly showed that H-GDGTs, which have to date not been reported from archaea under environmental conditions, were abundant in all samples. With additional 16S rRNA gene data available, these compounds could also be linked with their potential source organisms which may be (hyper)thermophilic members of the *Thermococcaceae*. Based on biomarker lipid and 16S rRNA gene analyses, we conclude that sulfur reduction by (hyper)thermophilic archaea and bacteria as well as archaeal methanogenesis are the most likely metabolic activities within the interior zones of the black smoker chimney walls at Loki's Castle. Bacterial sulfur oxidation and sulfate reduction as well as ammonia oxidation are favorable metabolisms in the exterior zones of the chimney walls.

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