The ISME Journal (2011) 5, 1946–1956
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www.nature.com/ismej

ORIGINAL ARTICLE

Thermophilic anaerobic oxidation of methane by marine microbial consortia

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The anaerobic oxidation of methane (AOM) with sulfate controls the emission of the greenhouse gas methane from the ocean floor. AOM is performed by microbial consortia of archaea (ANME) associated with partners related to sulfate-reducing bacteria. *In vitro* enrichments of AOM were so far only successful at temperatures $\leqslant$25 °C; however, energy gain for growth by AOM with sulfate is in principle also possible at higher temperatures. Sequences of 16S rRNA genes and core lipids characteristic for ANME as well as hints of *in situ* AOM activity were indeed reported for geothermally heated marine environments, yet no direct evidence for thermophilic growth of marine ANME consortia was obtained to date. To study possible thermophilic AOM, we investigated hydrothermally influenced sediment from the Guaymas Basin. *In vitro* incubations showed activity of sulfate-dependent methane oxidation between 5 and 70 °C with an apparent optimum between 45 and 60 °C. AOM was absent at temperatures $\geqslant$75 °C. Long-term enrichment of AOM was fastest at 50 °C, yielding a 13-fold increase of methane-dependent sulfate reduction within 250 days, equivalent to an apparent doubling time of 68 days. The enrichments were dominated by novel ANME-1 consortia, mostly associated with bacterial partners of the deltaproteobacterial HotSeep-1 cluster, a deeply branching phylogenetic group previously found in a butane-amended 60 °C-enrichment culture of Guaymas sediments. The closest relatives (*Desulfurella* spp.; *Hippea maritima*) are moderately thermophilic sulfur reducers. Results indicate that AOM and ANME archaea could be of biogeochemical relevance not only in cold to moderate but also in hot marine habitats.
The ISME Journal (2011) **5,** 1946–1956; doi:10.1038/ismej.2011.77; published online 23 June 2011
Subject Category: microbial ecology and functional diversity of natural habitats
Keywords: anaerobic methanotrophy; ANME; syntrophy; thermophilic microorganisms

Introduction

Methane, the simplest hydrocarbon, is an atmospheric trace gas of climatic relevance. Vast amounts of methane are formed by anaerobic microbial degradation of organic matter as well as by geothermal processes (Schoell, 1980) and may eventually reach the atmosphere. In the ocean, however, most methane migrating upwards from deep sediment strata is already consumed within the anoxic seafloor at distinct horizons characterized by simultaneous methane and sulfate depletion (Reeburgh, 2007). These horizons are usually populated by archaea (ANME groups) and bacteria of distinct phylogenetic lineages clustering with methanogens and Deltaproteobacteria, respectively (Boetius *et al.*, 2000; Knittel and Boetius, 2009). The ANME archaea are thought to oxidize the methane in principle via a reversal of the reactions of methanogenesis (Zehnder and Brock, 1979; Krüger *et al.*, 2003; Hallam *et al.*, 2004; Scheller *et al.*, 2010). Biochemical studies so far focused on the ANME-associated protein that is closely related to methyl-coenzyme M reductase (Mcr), the nickel enzyme catalyzing the terminal step in methanogenesis (Thauer, 1998; Hallam *et al.*, 2003; Krüger *et al.*, 2003; Scheller *et al.*, 2010). The associated Deltaproteobacteria most likely perform the sulfate reduction, using methane-derived reducing equivalents (Hoehler *et al.*, 1994). Yet, the mechanism of the intercellular transfer of reducing equivalents has not been elucidated, and axenic binary cultures have not been reported to date.

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Received 21 December 2010; revised 18 April 2011; accepted 18 April 2011; published online 23 June 2011

ANME-2 is a prominent phylogenetic archaeal group at most marine cold gas seeps (Orphan *et al.*, 2001; Mills *et al.*, 2003; Wegener *et al.*, 2008b; Knittel and Boetius, 2009) and some sulfate–methane transition zones where the temperature is 4–14 °C (Knittel and Boetius, 2009). Another group, ANME-3, occurs for instance at Haakon Mosby Mud Volcano (Niemann *et al.*, 2006; *in situ* temperature −1.5 °C) and the Eastern Mediterranean seepages (Omoregie *et al.*, 2008; 14 °C). Cells of both groups form dense consortia with specific bacterial phylotypes clustering with sulfate-reducing Deltaproteobacteria (most often within *Desulfosarcinales*, Seep-SRB-1a; Schreiber *et al.*, 2010) or relatives of *Desulfobulbus* (Knittel *et al.*, 2003; Lösekann *et al.*, 2007; Pernthaler *et al.*, 2008; Schreiber *et al.*, 2010), respectively. A third phylogenetic group, ANME-1, is dominant in the microbial mats covering chimney structures at methane seeps in the Black Sea (Michaelis *et al.*, 2002; *in situ* temperature of ~10 °C), and in several diffusive methane interfaces (Thomsen *et al.*, 2001; Lanoil *et al.*, 2005; Harrison *et al.*, 2009; Aquilina *et al.*, 2010). The anaerobic oxidation of methane (AOM) habitats with these ANME types exhibit temperatures between −1.5 and 20 °C (Boetius *et al.*, 2009; Supplementary Table 1). First hints of anaerobic methanotrophs thriving at higher temperatures were obtained by identification of 16S rRNA genes of ANME-1 (Teske *et al.*, 2002; Schrenk *et al.*, 2004; Roussel *et al.*, 2008) and ANME-specific core lipids (Schouten *et al.*, 2003) in hydrothermally influenced marine sediments. Also, radiotracer incubation of such sediment indicated thermophilic AOM (Kallmeyer and Boetius, 2004).

Indeed, AOM according to $CH_4 + SO_4^{2-} \rightarrow HCO_3^- + HS^- + H_2O$ should in principle be possible also under thermophilic conditions (ΔG with 100 kPa CH_4, 0.028 M SO_4^{2-}, 0.03 M HCO_3^- and 0.001 M HS^- between 25 and 80 °C ranges from −30 to −34 kJ mol^{-1}; for calculations see Materials and methods). Also from an enzymatic point of view, a restriction of AOM only to low temperatures is not expected. Methanogenesis as a metabolically related process (see above) and sulfate reduction with electron donors other than methane have been reported to occur at temperatures up to 100 °C (Jørgensen *et al.*, 1992; Stetter, 1996).

Here, we investigated possible thermophilic AOM and the responsible microorganisms in hydrothermally influenced sediments from the Guaymas Basin, Gulf of California. We combined rate measurements, enrichment cultivation with methane as sole organic substrate, and analyses of molecular markers. Hydrothermal Guaymas Basin sediments are characterized by rapid thermochemical transformation of labile fractions of settled detritus to volatile fatty acids, methane and higher hydrocarbons (Simoneit *et al.*, 1988; Martens, 1990). These compounds act as substrates for diverse microorganisms such as sulfate reducers. The high microbial sulfide production in these sediments fosters patchy mats of sulfide-oxidizing bacteria such as *Beggiatoa* (Jannasch *et al.*, 1989). From such areas, a variety of sulfate-reducing bacteria (Rueter *et al.*, 1994; Jeanthon *et al.*, 2002), sulfate-reducing archaea (Burggraf *et al.*, 1990; Khelifi *et al.*, 2010) and methanogenic archaea (Kurr *et al.*, 1991) have been isolated.

Materials and methods

Sediment characterization and sampling
Samples from a hydrothermal vent site in the Guaymas Basin were obtained during the RV *Atlantis* cruise AT15-56 in November/December 2009 with the submersible *Alvin* (Dive 4570; 27°00.437 N, 111°24.548 W) from bacterial mats (Supplementary Figure 1). Temperature profiles were measured *in situ* and the retrieved sediment was sectioned into the following horizons: (A) 2–13 cm, 4–30 °C; (B) 14–25 cm, 30–60 °C and (C) 26–45 cm, 60–85 °C (Supplementary Figure 1c). The cored material consisted of unconsolidated, methane-rich clays. The sections were transferred to glass bottles, diluted 1:1 with artificial anoxic seawater medium (Widdel and Bak, 1992) and stored anoxically with a methane headspace until further processing.

Determination of sulfate reduction rates
Sulfate reduction rates were determined by injection of 25 µl $^{35}SO_4^{2-}$ tracer (Hartmann Analytics, Braunschweig, Germany) so as to achieve ~75 kBq per horizon (~30 cm^3) into push cores at intervals of 1 cm and incubation at *in situ* temperature for 12 to 48 h. Cores were sliced into 1 cm sections and transferred to zinc acetate solution (0.9 M) to stop the reaction and fix sulfide. The total reduced inorganic sulfur was separated from the radiolabeled reactant ($^{35}SO_4^{2-}$) by reduction with Cr^{2+} and cold distillation (Kallmeyer *et al.*, 2004). The radioactivity of both pools was quantified by scintillation counting (scintillation cocktail, Lumasafe Plus (Perkin Elmer, Waltham, MA, USA); scintillation counter, 2900TR LSA (Hewlett Packard, Palo Alto, CA, USA)), and rates were calculated as described (Jørgensen and Fenchel, 1974).

Determination of methane oxidation rates
Methane oxidation to inorganic carbon was determined via radiolabeling of homogeneous sediment slurries prepared from defined proportions of seawater medium and sediment. Samples (2 ml slurry; dry weight 195 mg) were distributed to culture tubes (5 ml) and sealed with butyl rubber stoppers inside an anoxic chamber. The headspace was completely filled with methane-saturated (250 kPa) synthetic seawater, while gas was allowed to escape via an inserted hypodermic needle. Samples were pre-incubated for 5 days at the designated temperatures.

Control samples were inactivated with formaldehyde solution (final concentration, 20 g l^{-1}). Upon pre-incubation, carrier-free $^{14}CH_4$ (15 kBq) dissolved in 50 μl of anoxic water was added per tube. Tubes were further incubated for 48 h. Activity was stopped by alkalization with NaOH. The total methane concentration was determined by gas chromatography using a 5890A instrument (Hewlett Packard) equipped with a Porapak-Q column (6 ft, 0.125 in, 80/100 mesh; Agilent, Santa Clara, CA, USA) and a flame ionization detector, and operated at 40 °C with helium as carrier gas. Radioactivity in methane and inorganic carbon was determined after their separation and conversion to gaseous CO_2 as described (Treude *et al.*, 2003). Label in the trapped CO_2 fractions was determined by scintillation counting (cocktail Ultima Gold XR, Perkin-Elmer; instrument 2900TR LSA, Hewlett Packard). Rates were calculated as described previously (Treude *et al.*, 2003).

Enrichment of anaerobic methanotrophic microorganisms

Microorganisms were enriched in culture vials (156 ml) at 37 °C (samples from horizon A), 50 °C (horizon B) and 60 °C (horizon B) with synthetic seawater medium (Widdel and Bak, 1992) under a headspace (56 ml) of 250 kPa CH_4 and 40 kPa CO_2. Controls for each temperature were incubated with N_2 and CO_2. Vials were incubated at the indicated temperatures on rotary shakers at low speed (50 rpm). Activity was followed by quantification of sulfide using a rapid colorimetric test (Cord-Ruwisch, 1985). The sulfidic supernatant was replaced by fresh medium every 40–70 days.

Determination of the methane oxidation to sulfide production ratio

To measure methane consumption coupled to sulfate reduction (and resulting sulfide production), sediment slurries (50 ml) were added to culture vials (250 ml) inside an anoxic chamber. Bottles were completely filled with CH_4-saturated (250 kPa) anoxic seawater medium (see above), incubated at 50 °C, and intensely shaken by hand once per day. Per time point, 1 ml supernatant was withdrawn and replaced with the same volume of sterile medium. The sample was injected into rubber-sealed vials (6 ml) containing 0.5 ml $ZnCl_2$ (50 g l^{-1}) to bind sulfide. Methane was quantified in the headspace by gas chromatography (see above). Sulfide was liberated from ZnS (by acidification) and quantified colorimetrically using the methylene blue-forming reaction in a miniaturized assay (Aeckersberg *et al.*, 1991).

DNA extraction, PCR and clone library construction

DNA was extracted as described by Zhou *et al.* (1996) from a 9-ml subsample of enrichment cultures incubated for 3 and 9 months. After 9 months, only the supernatant after 1 h settling time was used for the extraction. The protocol encompassed three cycles of freezing and thawing, chemical lysis in a high-salt extraction buffer (1.5 M NaCl) by heating of the suspension in the presence of sodium dodecyl sulfate and hexadecyltrimethylammonium bromide, and treatment with proteinase K. The following domain-specific primers were used to amplify almost full-length 16S rRNA genes from the extracted chromosomal DNAs: Bacteria, primers GM3F (Muyzer *et al.*, 1995) and EUB1492R (Kane *et al.*, 1993); Archaea, primers 20F (Massana *et al.*, 1997) and Arc1492R (Teske *et al.*, 2002). In addition, fragments of the gene coding for the α subunit of methyl-coenzyme M reductase (*mcrA*) were amplified using the primers ME1 and ME2 (Hales *et al.*, 1996).

PCR reactions were performed in a Mastercycler Gradient (Eppendorf, Germany) in a 20-μl reaction volume. Each PCR reaction contained: 0.5 μM of each primer, 200 μM of each deoxyribonucleoside triphosphate, 6 μg bovine serum albumin, 1 × PCR buffer (5Prime, Hamburg, Germany), 1 × PCR Enhancer (5Prime), 0.25 U *Taq* DNA Polymerase (5Prime) and 5–10 ng of template DNA. The following cycling conditions were applied: one initial step at 95 °C for 5 min; 26 cycles, each at 95 °C for 1 min, 42 °C (58 °C for Arch20F/Arc1492R; 48 °C for ME1/ME2) for 1.5 min, and 72 °C for 3 min; and final step at 60 °C for 60 min. After PCR, the DNA of 10 reactions was pooled, gel extracted and purified by using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. The DNA was ligated to the pGEM-T-Easy vector (Promega, Madison, WI, USA) and transformed into *Escherichia coli* One Shot Top10 cells (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. Taq cycle sequencing was performed using ABI BigDye Terminator chemistry and an ABI377 sequencer (Applied Biosystems, Foster City, CA, USA).

Phylogenetic analysis

The phylogenetic affiliation was inferred with the ARB software package (Ludwig *et al.*, 2004) based on Release 102 of the ARB SILVA database (Pruesse *et al.*, 2007). Comparative sequence analyses were performed with representative sequences from the Guaymas Basin enrichments together with sequences of related archaea (Figure 4a), Deltaproteobacteria (Figure 4b) and methyl-coenzyme M reductases (Figure 4c) found in public databases. In total, 877 (deltaproteobacterial) and 1090 (archaeal) nearly full-length 16S rRNA gene sequences (>1350 bp) were used for tree construction. For *mcrA* tree construction, 483 sequences (>230 amino acids) were considered. Phylogenetic trees were calculated by maximum likelihood analysis (PhyML, RAxML) and the neighbor-joining algorithm. A 50% base frequency filter was used for 16S rRNA gene tree calculation to exclude highly variable positions. The phylogenetic McrA tree was

generated from *mcrA*-deduced amino-acid sequences using PhyML and RAxML with a 30% amino-acid frequency filter. The resulting trees were compared manually and, if necessary, a consensus tree was constructed. Relevant partial sequences were subsequently added to the tree according to maximum parsimony criteria, without allowing changes in the overall tree topology.

Nucleotide sequences have been deposited at EMBL, GenBank and DDBJ under accession numbers FR682488 to FR682496 (archaeal 16S rRNA genes), FR682813 to FR682818 (*mcrA*) and FR682644 to FR682663 (bacterial 16S rRNA genes).

Design and application of oligonucleotide probes

Oligonucleotide probes HotSeep-1-590, ANME-1-GI812 and ANME-1-GII186 (Supplementary Table 2) were designed using the ARB probe design tool. Specificity of the probes was evaluated by Clone-FISH (fluorescence *in situ* hybridization) (Schramm *et al*., 2002) for probe HotSeep-1-590, or directly on enrichment samples for probes ANME-1-GI812 and ANME-1-GII186. To generate melting curves, the probes were hybridized to clones at formamide concentrations of 0%, 10%, 20%, 30%, 40%, 50%, 60% and 70%. To increase the accessibility of the HotSeep-1-590 probe target site on the 16S rRNA, helper oligonucleotides were designed. In addition, the probes were tested for sensitivity (target group hits) and specificity (outgroup hits) *in silico* with the ARB probe match tool. Probe HotSeep-1-590 has at least three mismatches to non-target sequences. Probes ANME-1-GI812 and ANME-1-GII186 were designed to discriminate between the two ANME-1 Guaymas subclusters: probe ANME-1-GI812 has two mismatches to cluster II and ANME-1-GII186 has five mismatches to cluster I. Oligonucleotide probes were synthesized by Biomers (Ulm, Germany).

Samples for cell hybridization were fixed with formaldehyde ($30\,g\,l^{-1}$, final concentration) for 2 h at room temperature, washed with 1× PBS (phosphate-buffered saline), pH 7.2 and stored in PBS:ethanol (1:1) at $-20\,^{\circ}C$ until further processing. Fixed samples were treated by mild sonication for 20 s with a MS73 probe (Sonoplus HD70, Bandelin, Berlin, Germany) at $\leqslant 10$ W.

CARD-FISH (catalyzed reporter deposition fluorescence *in situ* hybridization) of cells was performed as described previously (Pernthaler *et al*., 2004) with the following modifications: aliquots of the enrichment samples were filtered onto 0.2 μm pore-size polycarbonate filters (GTTP, Millipore, Billerica, MA, USA). For cell wall permeabilization, filters were sequentially incubated in sodium dodecyl sulfate solution (0.5%) for 10 min and proteinase K solution ($15\,\mu g\,ml^{-1}$, 0.1 M Tris–HCl, 0.05 M EDTA, pH 8, 0.5 M NaCl) for 2 min at room temperature. Endogenous peroxidases were inactivated by incubating the filters in 0.01 M HCl for 5 min at room temperature. Permeabilization with higher HCl concentrations or lysozyme caused damage of cells and disturbance of their arrangement within the ANME-1/HotSeep-1 sheaths. The oligonucleotide probes were applied with formamide concentrations according to literature data or Clone-FISH results, respectively (Supplementary Table 2). For dual-CARD-FISH, peroxidases of initial hybridizations were inactivated by 30 min incubation in 0.3% H_2O_2 in methanol at room temperature. Catalyzed reporter deposition was performed using the fluorochromes Alexa Fluor 488 and Alexa Fluor 594. Finally, samples were stained with DAPI (4′,6′-diamidino-2-phenylindole). Micrographs were obtained by confocal laser scanning microscopy (LSM510; Zeiss, Oberkochen, Germany).

Lipid extraction and analyses

Total lipid extracts were retrieved by modified Bligh and Dyer extraction (Sturt *et al*., 2004). Fractions of core glycerol dialkyl glycerol tetraether (GDGT) were purified by preparative liquid chromatography (ThermoFinnigan Surveyor equipped with preparative LiChrosphere Si60 column, 250 mm × 10 mm × 5 μm, Alltech, Unterhaching, Germany) connected to a Gilson FC204 fraction collector (Gilson, Inc., Middleton, WI, USA). Analysis of core GDGTs followed the described procedure (Huguet *et al*., 2006) with C_{46}-GDGT as injection standard. Analysis was performed by combined liquid chromatography–quadrupole mass spectrometry (1200 series, Agilent), using a Prevail Cyano column ($3 \times 150\,mm^2$; Grace, Waukegan, IL, USA). GDGTs were eluted isocratically. Compounds were detected by atmospheric pressure positive ion chemical ionization mass spectrometry.

For isotopic analysis, GDGTs were subjected to ether cleavage using BBr_3 (Sigma-Aldrich, St Louis, MO, USA) in dichloromethane (Bradley *et al*., 2009). Carbon isotopic composition of produced biphytanes was determined using gas chromatography combustion isotope ratio mass spectrometry (gas chromatography: Hewlett Packard 5890 series II equipped with a 30-m TRX-5MS fused silica column; combustion: ThermoFinnigan Combustion Interface-II: mass spectrometry: Finnigan MAT252). Carbon isotopic values are expressed in per mil (‰) deviations from the Pee Dee Belemnite standard. The analytical error was <1‰.

Thermodynamic calculations

The standard free energy change of AOM was calculated from free energies of formation (Stumm and Morgan, 1996). The free energy change for non-standard conditions was calculated using the van t'Hoff equation, concentrations in the medium at the beginning of incubation and activity coefficients from the IUPAC project on ionic strength corrections for stability constants (http://www.iupac.org/web/ins/2000-003-1-500).

Results and discussions

Sediment cores were taken from underneath a bacterial mat (identified as *Beggiatoa* by microscopy, and in prior analyses by Jannasch *et al.* (1989); Supplementary Figures 1a and b). The abundance of *Beggiatoa* was indicative of high rates of sulfate reduction. Sulfate reduction rates determined in replicate cores peaked between 3 and 6 cm with 1500 nmol per day per ml (average throughout the upper 15 cm: 530 nmol per day per ml, corresponding to roughly 0.25 μmol per day per g_{dw}; Supplementary Figure 1d).

Homogenized material from the three horizons was further diluted, equilibrated with methane (250 kPa) and incubated without headspace in a temperature gradient block (0 to 90 °C; Kallmeyer *et al.*, 2003). After 5 days of pre-incubation, methane oxidation rates were determined using ^{14}C-methane. We measured substantial AOM rates between 4 and 70 °C (Figure 1). Highest rates occurred between 42 and 65 °C, with ~1.2 μmol per day per g_{dw}. AOM was not observed at ⩾75 °C.

To confirm the stoichiometry of methane oxidation and sulfate reduction in our enrichments, methane consumption and sulfide production were chemically quantified in headspace-free subsamples (2 months after methane had been added to start AOM activity) at 50 °C. Within 2 weeks methane consumption occurred simultaneously with sulfide production (Figure 2). As in previous low-temperature enrichments (Nauhaus *et al.*, 2002; Holler *et al.*, 2009), the ratio between methane consumed and sulfide formed (corrected against the background) was in accordance with the expected stoichiometry of 1 mol CH_4 oxidized per mol SO_4^{2-} reduced. The proportion of methane-derived reducing equivalents channeled into biosynthesis and therefore not available for sulfate reduction was previously shown to be extremely low (around 1%; Nauhaus *et al.*, 2007; Wegener *et al.*, 2008a) and can be neglected in evaluating the AOM stoichiometry. In controls without methane, no production of methane (methanogenesis) was detected and sulfide production was only ~10% of rates in the presence of methane. Hence, these measurements gave clear evidence for thermophilic AOM in the natural enrichment.

To allow further increase of the biomass and activity of the organisms responsible for thermophilic AOM, horizons A (37 °C) and B (50 and 60 °C) were incubated for 10 months in anoxic synthetic seawater medium with methane as sole organic substrate. To avoid the inhibition of growth by accumulated sulfide (Boetius *et al.*, 2009), the supernatant was replaced by fresh medium at sulfide concentrations of 10 mM, which was every 40–70 days (Figure 3a). After each addition of new medium (dry weight-related) sulfide production with methane was faster than in the previous period (Figures 3a and b). Over the total incubation time of 250 days, methane-dependent sulfate reduction rates of the 50 °C culture increased exponentially from 3 to 34 μmol per day per g_{dw} (Figure 3b). This indicated significant increase of active methanotrophic biomass. The apparent doubling time was 68 days. However, within each individual incubation periods, the increase in sulfide appeared constant (‘linear’ growth). This indicated that the inhibitory effect of accumulating sulfide and increase in the catalytic microbial biomass counteracted such that the actual activity was constant. The doubling time at 50 °C was shorter than in incubations at 37 °C (77 days) and at 60 °C (112 days) (see Supplementary Figure 2). Thus, we assume an apparent optimum for the growth of thermophilic methanotrophs around 50 °C. In all control experiments without methane, sulfate reduction declined and was below detection limit after 5 months of incubation (data not shown).

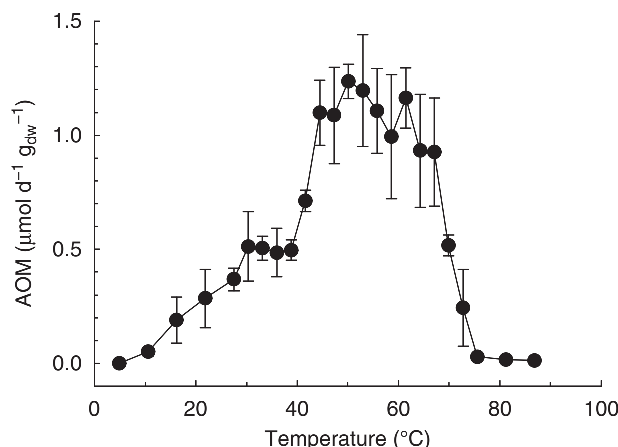


Figure 1 Rates of AOM in Guaymas Basin sediment at different temperatures measured as $^{14}CH_4$ conversion to $^{14}CO_2$. Homogenous samples (2–45 cm sediment depth, *in situ* temperature, 4–85 °C) were pre-incubated for 5 days at designated temperatures followed by incubation with labeled methane for 48 h. Error bars indicate s.d. from triplicates.

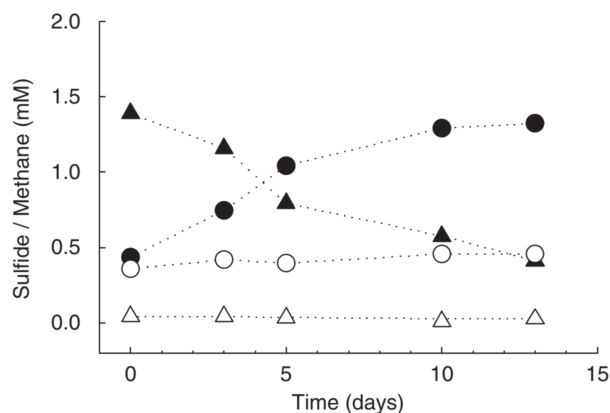


Figure 2 Time course experiment of AOM enrichment incubated without headspace at 50 °C. Sulfide formation (black circles) and methane consumption (black triangles) in the enrichment. A control without methane addition (open triangles, background methane) showed only minor sulfide formation (open circles).

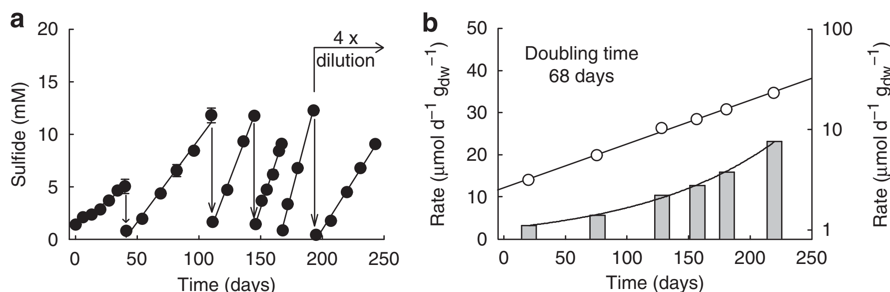


Figure 3 Long-term enrichment of anaerobic methanotrophs at 50 °C. (**a**) Sulfide formation (black circles) in the initial sediment suspension and upon five exchanges of the sulfide-rich supernatant. Medium exchanges are indicated with arrows. (**b**) Linear (vertical bars) and semi-logarithmic (open circles) illustration of methane-dependent sulfide production of the different incubation periods over time; normalized by dry weights.

Table 1 Numbers of retrieved clones for genes encoding 16S rRNA and McrA (subunit A of methyl-coenzyme M reductase) from a methane-oxidizing anaerobic enrichment culture analyzed after 3 months (enrichment subsample) and 9 months (supernatant subsample) of incubation at 50 °C

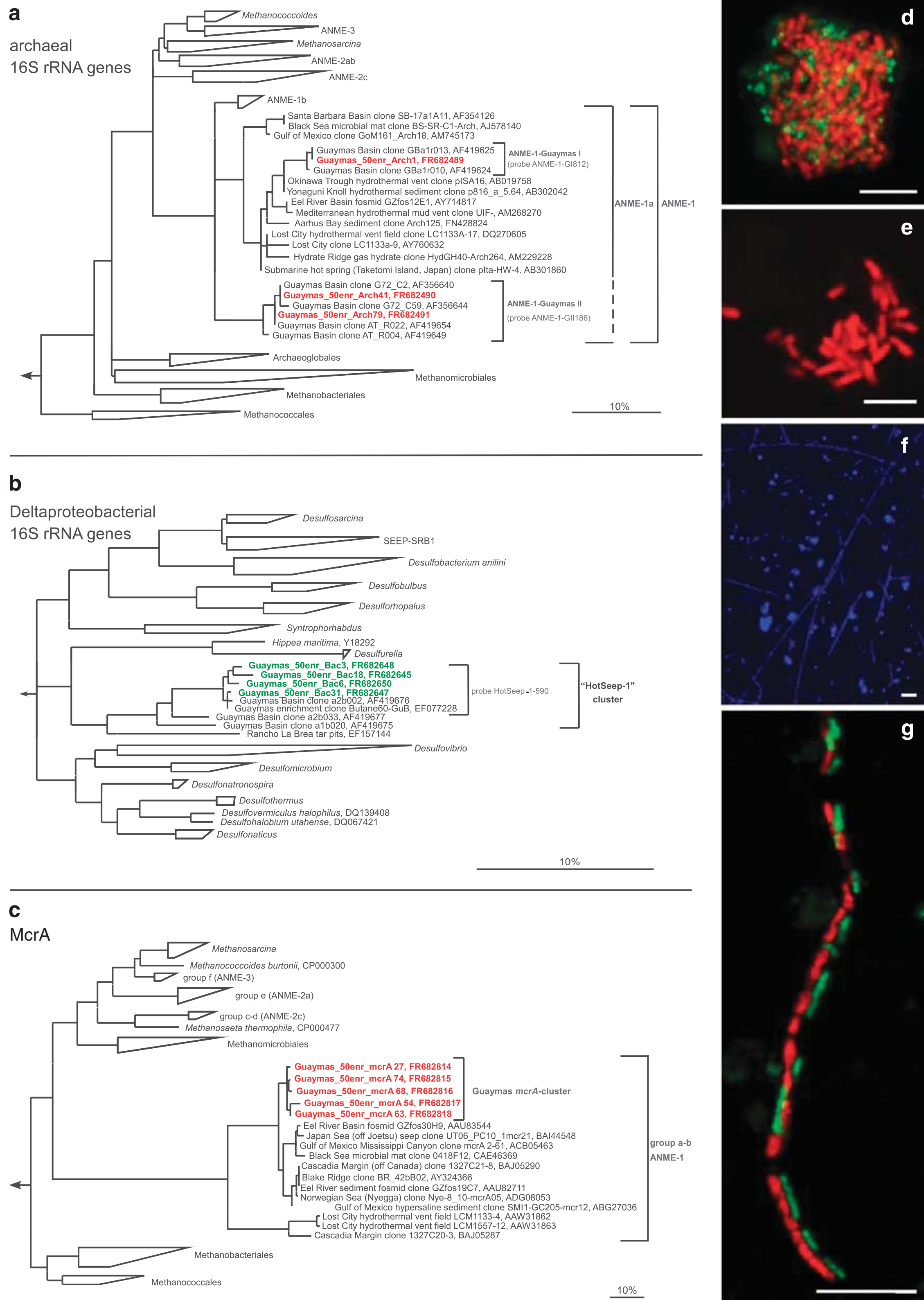
Phylogenetic affiliation	*16S rRNA (3 months)*	*16S rRNA (9 months)*	*mcrA*
Archaea			
Euryarchaeota			
ANME-1			64
ANME-1-Guaymas I	19		
ANME-1-Guaymas II	27		
Methanosarcinales			
ANME-2c			3
Others			1
Thermoplasmatales	6		
Halobacteriales	4		
Total archaeal clones analyzed	**56**	**n.a.**	**68**
Bacteria			
Proteobacteria			
Betaproteobacteria		3	
Gammaproteobacteria	1	10	
Deltaproteobacteria			
HotSeep-1-cluster	53	71	
DSS group (Desulfosarcinales)		1	
Desulfuromonadales	1		
Syntrophobacterales	1		
Others	2	12	
Acidobacteria		1	
Actinobacteria		3	
Bacteriodetes		1	
Candidate division OD1	5	1	
Candidate division OP3	3	2	
Candidate division OP8	8	13	
Chloroflexi	3	5	
Deferribacteres	1		
Planctomycetes		2	
Unaffiliated	2	2	
Total bacterial clones analyzed	**80**	**127**	

Abbreviations: n.a., not analzyed.

To identify phylotypes responsible for AOM with sulfate at 50 °C, the enrichment was examined by cloning 16S rRNA genes and genes encoding methyl-coenzyme M reductase (Mcr) subunit A (*mcrA*). The most frequently obtained archaeal 16S rRNA genes and transcribed *mcrA* genes were affiliated with *mcrA* of ANME-1 (Figures 4a and c; Table 1), matching sequences previously isolated from Guaymas Basin sediment (Teske *et al.*, 2002). The retrieved ANME-1 16S rRNA gene sequences cluster together with other sequences previously retrieved from Guaymas Basin (Teske *et al.*, 2002; clone G72_C2 and G72_C59, Longnecker and Reysenbach, database release) and form two Guaymas-specific subclusters named ANME-1-Guaymas I and II (Figure 4a; Table 1). Sequence similarity within subcluster ANME-1-Guaymas I ranged between 97.9% and 99.9% and within subcluster Guaymas II between 97.9% and 99.8%. The retrieved *mcrA*-derived sequences form a separate cluster with at least 6% amino-acid divergence to other sequences within the *mcrA* groups a–b (Figure 4c). In comparison to *mcrA* from methanogenic archaea, the present sequence contains a modification (motif $VX_2CCX_4CX_5C$), which has been previously observed in the abundant nickel protein of ANME-1 consortia in a cold habitat (Krüger *et al.*, 2003). The most frequently retrieved bacterial 16S rRNA clone sequences represent a lineage distantly related to *Desulfurella* spp., sulfur-reducing thermophilic Deltaproteobacteria (Figure 4b; Table 1). 16S rRNA gene sequences representing this lineage were previously obtained from surficial Guaymas hydrothermal sediments rich in ANME-1, but without detectable ANME-2 (Teske *et al.*, 2002). The same group was found in a heterogeneous sulfate-reducing enrichment culture from Guaymas Basin sediment amended with butane and grown at 60 °C (Kniemeyer *et al.*, 2007). However, the presently obtained AOM enrichment characterized by a high proportion of related 16S rRNA genes did not show detectable sulfate reduction activity with butane instead of methane within an incubation time of 7 weeks. Corresponding to the naming of bacterial seep clusters identified at cold seeps (‘Seep-SRB-1 to -4’ and subgroups; Knittel *et al.*, 2003; Schreiber *et al.*, 2010), we named this new group of thermophilic, ANME-associated bacteria ‘HotSeep-1’.

Fluorescence *in situ* hybridization with specific 16S rRNA targeting oligonucleotide probes (Supplementary Table 2) revealed spherical ANME-1 cell aggregates associated with Deltaproteobacteria of the HotSeep-1 group after 2 months of incubation (Figure 4d). The consortia varied in size and harbored between a few and several hundred

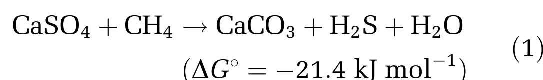
cells. ANME-1 cells in these aggregates were identified by the new probe ANME-1-GII186 as members of subgroup ANME-1-Guaymas II. In addition, hybridization showed ANME-1 aggregates without partner bacteria (Figure 4e). ANME-1 cells in these aggregates typically have a length of 1.2 μm



and a width of 0.3–0.4 μm. After 5 months, unique chain-forming aggregates with >100 ANME-1 cells appeared (Figures 4f and g) associated in a 1:1 ratio with thinner (0.4 μm in width) rod-shaped bacteria (Figure 4g). The ANME-1 cells in these chain-forming aggregates are larger than those in spherical aggregates with a length of 2–2.5 μm and a width of 0.7 μm. Hybridization with the new probe, ANME-1-GI812, identified chain-forming members of subgroup ANME-1-Guaymas I (Figure 4g). The partner bacteria were attached to the ANME-1 cells, and both cell types were enclosed in a common sheath. We observed further growth of these ANME-1 chains. The longest chains observed after 11 months of incubation had a length of >250 μm. Aggregations of coiled ANME-1 chains have also been detected. Hybridization with probe HotSeep-1-590 showed that these partner bacteria belong as well to the HotSeep-1 cluster. Based on the observation of significant increase of activity, and ANME-1 HotSeep-1 chains in the 50 °C enrichments, we propose that members of the ANME-I Guaymas clades and their partner bacteria of the HotSeep-1 cluster are thermophiles and oxidize methane with sulfate at an apparent temperature optimum of 45–60 °C.

As an independent complementary method for the chemotaxonomic identification of ANME, we analyzed the archaeal GDGT lipids in the enrichment. The intact lipids contained mostly diglycosides, which are abundant in lipids in ANME-1 dominated habitats (Rossel *et al.*, 2011). The GDGT cores were dominated by chains without or with four or five cyclopentane rings. GDGTs with one to three rings, which were previously assigned to thermophilic ANME-1 (Schouten *et al.*, 2003), were significantly depleted in ^{13}C as compared with typical planktonic signals (Table 2). The isoprenoids prepared from the GDGTs, especially mono- and bicyclic biphytane, were depleted in ^{13}C ($\delta^{13}C$, −49‰). This indicates incorporation of methane-derived carbon as shown for lipids of anaerobic methanotrophs (Wegener *et al.*, 2008a). These results are consistent with earlier analyses of hydrothermally influenced Guaymas Basin sediment (Schouten *et al.*, 2003).

Apart from the hot Guaymas Basin sediments and some calcified gas vents in cold to moderately thermophilic habitats such as the Black Sea (Michaelis *et al.*, 2002) and the Lost City Hydrothermal Field (Schrenk *et al.*, 2004), most shallow gas-rich habitats investigated so far were dominated by the methanotrophic clades ANME-2 and ANME-3 (Knittel and Boetius, 2009). Also, all prior cultivation attempts using environmental samples from marine cold seeps selected for these groups (Boetius *et al.*, 2009). Here, propagation of methane oxidation (Figures 1–3) as well as comparative sequence analysis, hybridization and membrane lipid information demonstrate that thermophilic AOM up to at least 60 °C is mediated by members of the ANME-1 group. Hence, considering previous findings of ANME sequences and lipids in hot environments (Schouten *et al.*, 2003; Schrenk *et al.*, 2004; Roussel *et al.*, 2008) together with our results, we suggest that AOM could be widespread in hot marine habitats including, for example, hydrothermal crusts, brine lakes and subsurface gas reservoirs. Furthermore, it would be interesting to investigate whether thermophilic ANME groups and their associated bacterial partners may be involved in the widely observed transformation of anhydrite to calcite according to



$$CaSO_4 + CH_4 \rightarrow CaCO_3 + H_2S + H_2O \quad (\Delta G^\circ = -21.4\ \mathrm{kJ\ mol^{-1}}) \qquad (1)$$

in evaporitic cap rocks of moderately heated gas reservoirs (Werner *et al.*, 1988), which would extend the importance of ANME driven methanotrophy in the global carbon cycle and in deep subsurface diagenesis.

Table 2 Concentrations of archaeal glycerol dialkyl glycerol tetraether (GDGT) in the enrichment culture (top) and stable carbon isotopic compositions of GDGT-derived biphytane derivatives obtained by ether cleavage (bottom)

Tetraethers	*Concentration* $\mu g\, g_{dw}^{-1}$	*Rel. abundance (%)*
GDGT-0	1.90	25
GDGT-1	0.49	6
GDGT-2	0.70	9
GDGT-3	0.47	6
GDGT-4	1.78	23
GDGT-cr.	2.24	29
GDGT-cr. (iso)	0.07	1
Isoprenoids	*Isotopic composition ($\delta^{13}C$ vs. PDB)*	*Rel. abundance (%)*
Acyclic biphytane	−25.2‰	40
Monocyclic biphytane	−49.1‰	13
Bicyclic biphytane	−41.6‰	29
Tricyclic biphytane	−20.0‰	18

Structures of the GDGTs are shown in Schouten *et al.* (2003).

Figure 4 Phylotypes and cell aggregates in the methane-oxidizing anaerobic-enrichment culture grown at 50 °C. Phylogenetic trees showing the affiliations of 16S rRNA gene sequences retrieved from Guaymas methane-oxidizing enrichments with selected reference sequences of (**a**) Euryarchaeota and (**b**) Deltaproteobacteria. Sequences from this study are printed in bold red (archaea) and bold green (bacteria). Probe specificity is indicated with brackets. Bar = 10% estimated sequence divergence. (**c**) Phylogenetic tree of amino acid sequences of the α subunit of the methyl-coenzyme M reductase (*mcrA*), (**d–g**) cell aggregates of ANME-1 visualized by CARD-FISH. Scale bars = 10 μm. (**d**, **e**, **g**) Confocal laser scanning micrographs. (**f**) Regular epifluorescence micrograph. (**d**) Spherical ANME-1/HotSeep-1 aggregates stained with probe ANME-1-350 (red) and probe HotSeep-1-590 (green). (**e**) Monophyletic ANME-1 aggregate stained with probe ANME-1-350. (**f**) DAPI staining showing long chain-forming ANME-1 aggregates. (**g**) Chain-forming ANME-1/HotSeep-1 aggregates. ANME-1 cells were identified as members of subcluster ANME-1-Guaymas I (probe ANME-1-GI812, red) and bacterial partners as members of the HotSeep-1 cluster (probe HotSeep-1-590, green).

Acknowledgements

We are indebted to the crew and pilots of Research Vessel *Atlantis* and Research Submersible *Alvin*. We thank Nicole Rödiger, Viola Krukenberg, Philipp Hach, Viola Beier, Gabriele Schüssler, Dennis Enning and Florin Musat for technical support. This study was supported by Grant NSF OCE-0647633 to AT, by the Leibniz program of the Deutsche Forschungsgemeinschaft to AB, the DFG Research Center and Cluster of Excellence MARUM, and the Max Planck Society.

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