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Brief Report

ANME-1 archaea may drive methane accumulation and removal in estuarine sediments

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Summary

ANME-1 archaea subsist on the very low energy of anaerobic oxidation of methane (AOM). Most marine sediments shift from net AOM in the sulfate methane transition zone (SMTZ) to methanogenesis in the methane zone (MZ) below it. In White Oak River estuarine sediments, ANME-1 comprised 99.5% of 16S rRNA genes from amplicons and 100% of 16S rRNA genes from metagenomes of the Methanomicrobia in the SMTZ and 99.9% and 98.3%, respectively, in the MZ. Each of the 16 ANME-1 OTUs (97% similarity) had peaks in the SMTZ that coincided with peaks of putative sulfate-reducing bacteria *Desulfatiglans sp.* and SEEP-SRB1. In the MZ, ANME-1, but none of the putative sulfate-reducing bacteria or cultured methanogens, increased with depth. Our meta-analysis of public data showed only ANME-1 expressed methanogenic genes during both net AOM and net methanogenesis in an enrichment culture. We conclude that ANME-1 perform AOM in the SMTZ and methanogenesis in the MZ of White Oak River sediments. This metabolic flexibility may expand habitable zones in extraterrestrial environments, since it enables greater energy yields in a fluctuating energetic landscape.

Introduction

Methanogenesis and the anaerobic oxidation of methane (AOM) are key astrobiological targets because (i) they occur in subsurface environments similar to those of Mars, Europa, Enceladus, and Titan; (ii) they have been hypothesized to be key to the origin of life; and (iii) they are some of the lowest energy metabolisms known to support life (Knittel and Boetius, 2009; Mckay, 2016; Russell and Nitschke, 2017; Jones *et al*., 2018; Lipps *et al*., 2020). Many species of methanogens have been cultured and characterized, but they are usually grown under conditions of extremely high substrate concentrations (80% hydrogen) and therefore high-energy yields (Mukhopadhyay *et al*., 2000). Such high-energy conditions almost never occur in the vast majority of marine sediments (Hoehler and Jørgensen, 2013) or extraterrestrial environments (Jones *et al*., 2018). Such environments nevertheless have the ability to produce large amounts of methane; marine sediments are the third largest producers of methane on Earth, after rice production and wetlands (Reeburgh, 2007). It is therefore possible that the methanogens in marine sediments, and potentially extraterrestrial environments, are phylogenetically and functionally divergent from the well-characterized high-energy methanogenic cultures, a notion that is supported by the near-lack of cultured methanogenic groups in some methane-producing coastal sediments (Kendall *et al*., 2007). Uncultured archaea of the Methanomicrobia, called ANaerobic MEthane oxidizers (ANME-1), catalyse AOM in marine sediments in a syntrophy with sulfate reducing microbes (Knittel and Boetius, 2009; Holler *et al*., 2011). Unlike methanogenesis, AOM always occurs at low-energy yield since it requires substrates to be kept at moderate concentrations that satisfy the energetic requirements of both syntrophic partners (Hoehler *et al*., 1998). The direction of exergonicity of low-energy chemical reactions, such as methanogenesis and AOM, can reverse when the relative concentrations of products and reactants shift (Hoehler *et al*., 1998). Acetogenesis, another low-energy metabolism subject to exergonic reversals, has been shown to be reversible in a single species (Zinder, 1994). Cultured methanogens appear to be irreversible (Valentine *et al*., 2000). ANME are therefore good candidates for reversibility. Previous studies have suggested that they are capable of methane production, since they are the most abundant methane-metabolizing organisms in methane-producing sediments (Orcutt *et al*., 2005; House *et al*., 2009; Lloyd *et al*., 2011; Jagersma *et al*., 2012; Bertram *et al*., 2013).

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In marine sediments, the balance between diffusive supply from above of electron acceptors for respiration and their removal via biological respiration drives a down-core shift from sulfate reduction to methanogenesis in the methane zone (MZ), with net removal of methane through AOM in the sulfate methane transition zone (SMTZ) at intermediate depths (Martens and Berner, 1977). Most methanogens in marine sediment are hydrogenotrophic, conserving energy by producing methane from hydrogen plus carbon dioxide (Crill and Martens, 1986; Reeve *et al*., 1997). In the SMTZ, sulfate reducers keep hydrogen concentrations low enough to make hydrogenotrophic methanogenesis exergonic in the reverse direction, resulting in AOM (Hoehler *et al*., 1994). Being able to reverse between methanogenesis and AOM could offer a growth advantage to an organism being buried in a subsurface environment, since they would experience reversals of exergonicity during burial, as the depth of the SMTZ shifts seasonally (Hoehler *et al*., 1994), and microscale alternation between AOM and methanogenesis throughout the SMTZ (Iversen and Jorgensen, 1985; Knab *et al*., 2008; Beulig *et al*., 2019).

Evidence that ANME-1 perform both AOM and methanogenesis in marine sediments is that they are present in both SMTZs and MZs (Harrison *et al*., 2009; Lloyd *et al*., 2011; Underwood *et al*., 2016; Beulig *et al*., 2019), express methyl coenzyme M reductase subunit A (*mcrA*) genes, a key gene in methanogenesis and AOM (Shima *et al*., 2012), in both of these zones (Lloyd *et al*., 2011), and they belong to the Methanomicrobia, a group for which all cultured strains are methanogens (Whitman, 2015). Methane is produced in ANME-1 enrichments (Jagersma *et al*., 2012), and its biomass has variable stable carbon isotope values in nature, suggesting that it can use substrates other than methane to make biomass (House *et al*., 2009). Initial incomplete genomes from ANME-1 contained all of the genes required for methane production except for the one encoding N5, N10-methylene-tetrahydromethanopterin reductase (*mer*) (Hallam *et al*., 2004; Meyerdierks *et al*., 2010). More recently, *mer* has been found in ANME-1 genomes (Beulig *et al*., 2019). Although ANME-1 genomes contain homologues for the hydrogenases of cultured methanogens, they have thus far been found to lack the active site (Meyerdierks *et al*., 2010; Wegener *et al*., 2015; Krukenberg *et al*., 2018). One possible explanation is that the active subunit of a typical methanogenic hydrogenase is present in ANME-1 genomes but has not yet been sequenced because the genomes are incomplete, in a similar situation to the *mer* gene which was discovered when more genomes became available (Beulig *et al*., 2019). Another possibility is that ANME-1 genomes contain a novel hydrogenase active site as an adaptation to low hydrogen concentrations common in nature. Adaptation of ANME-1 to low hydrogen conditions may explain the abundance of enzymes utilizing F_{420} rather than hydrogen (Meyerdierks *et al*., 2010). ANME-1 contains Mtd, which alone is sufficient for metabolizing hydrogen (Hendrickson and Leigh, 2008). However, the lack of the alpha catalytic subunit suggests that ANME-1 may have a variation of Mtd with high hydrogen affinity, similar to the Hmd_{II} variant of Hmd, which has a high hydrogen affinity (Walker *et al*., 2012). Accordingly, Beulig *et al*. 2019 speculate a possible biochemical route for hydrogenotrophic methanogenesis, given enzymes that have been found in ANME-1 genomes (Beulig *et al*., 2019). Well-characterized hydrogenases are phylogenetically diverse (Vignais and Billoud, 2007), suggesting that novel hydrogenases may yet be discovered. All enzymes in the methanogenic pathway are reversible (Scheller *et al*., 2010), allowing for the possibility that a low-energy hydrogenotrophic methanogen could to reverse to AOM and vice versa.

We examined ANME-1 population sizes in 1 cm depth resolution in sediments of the White Oak River estuary through a transition from an SMTZ to an MZ. We also performed a meta-analysis of mRNA expression of 16S rRNA genes and genes of the methanogenesis pathway on a published enrichment culture of ANME-1 that demonstrated net methane production (i.e., methanogenesis) (Wegener *et al*., 2015). Our results are consistent with ANME-1 reversing between AOM and methanogenesis in marine sediments.

Results

We examined the Fraction Read Abundance of 16S rRNA gene sequences times Cell abundance (FRAxC) with 3 cm intervals in the upper 10 cm, 1 cm intervals between 10 and 60 cm, and then 3 cm intervals from 60 to 80 cm below the sediment–water interface throughout a diagenetic sequence in sediments of the marine-influenced White Oak River estuary. The reason for using FRAxC rather than fraction read abundance alone is that it helps account for changes in overall population size (Kevorkian *et al*., 2018). Due to sediment volume restrictions from the small depth intervals, 16S rRNA gene amplicon libraries, cell counts, and hydrogen concentrations were measured for one core (core 6), sulfate concentrations were measured for the other (core 1), and methane concentrations and $\delta^{13}C$ values were measured on both cores.

White Oak River estuary cores

Total cell abundance decreased sharply within the upper 10 cm from 2.0×10^8 to 2.6×10^7 cells g^{-1} and remained

steady at $\sim 1.3 \times 10^7$ cells g^{-1} for the rest of the core (Fig. 1A). Sulfate concentrations in core 1 decreased from a surficial concentration of 11.2 mM to a constant concentration of 0.06 ± 0.02 mM at 65–78 cm (Fig. 1E). This concave decrease in sulfate is consistent with it being consumed by sulfate-reducing bacteria (Martens *et al*., 1998). Aqueous methane concentrations were ~0.004 mM in near-surface sediment and increased to >0.6 mM at 60 cm in core 6 (Fig. 1F) and 75 cm in core 1 (Fig. 1B), with a generally concave shape indicating AOM. Below this, methane increased to 0.73–0.87 mM in the 75–78 cm depth interval of both cores. In core 6, the methane concavity shifted between ~55 cm and 73 cm, consistent with methanogenesis. Between 2.5 cm and 64.5 cm, hydrogen concentrations were low (0.07–2.05 nM) in core 6 (Fig. 1D), consistent with those predicted for sulfate reducers operating at their minimum energy (1.22 ± 0.45 nM in similar sediments, which yields a ΔG of -20 kJ mol^{-1} sulfate, just slightly above the minimum free energy conservation;Hoehler *et al*., 1998). Below this, hydrogen increased above 6 nM, similar to the threshold for energy conservation for hydrogenotrophic methanogenesis at 5.11 nM in similar sediments (Hoehler *et al*., 1998). In core 1, $\delta^{13}C$ of methane decreased from −41‰ ± 1.17 at 41.5 cm to −72‰ ± 0.10 at 73.5 cm (Fig. 1G). In core 6, $\delta^{13}C$ of methane decreased from −46‰ ± 0.20 at 29.5 cm to −74‰ ± 0.02 at 67.5 cm (Fig. 1C). These values are consistent with the biogenic production of methane deeper in the sediments, resulting in enrichment of the lighter carbon isotope, ^{12}C (Whiticar, 1999). Within the SMTZ, methane diffusing up through the sediment became gradually depleted in the lighter carbon isotope, consistent with AOM (Jorgensen and Kasten, 2006). This is because biological AOM has a preference for ^{12}C over ^{13}C, leaving the residual methane ^{13}C-enriched (Alperin *et al*., 1988; Holler *et al*., 2009). The shift in $\delta^{13}C$—CH_4 towards 'heavier' values between ~60 and 30 cm indicates methane oxidation occurs in this depth interval. Methane concentrations in samples shallower than the AOM zone were too low to get accurate $\delta^{13}C$ values, so the values in the upper parts of the cores were not used. Together, these geochemical measurements suggest the location of AOM (30 to ~60 cm) and methanogenesis (>60 cm) in core 6, the core where DNA measurements were made. The depth of the onset of net methanogenesis

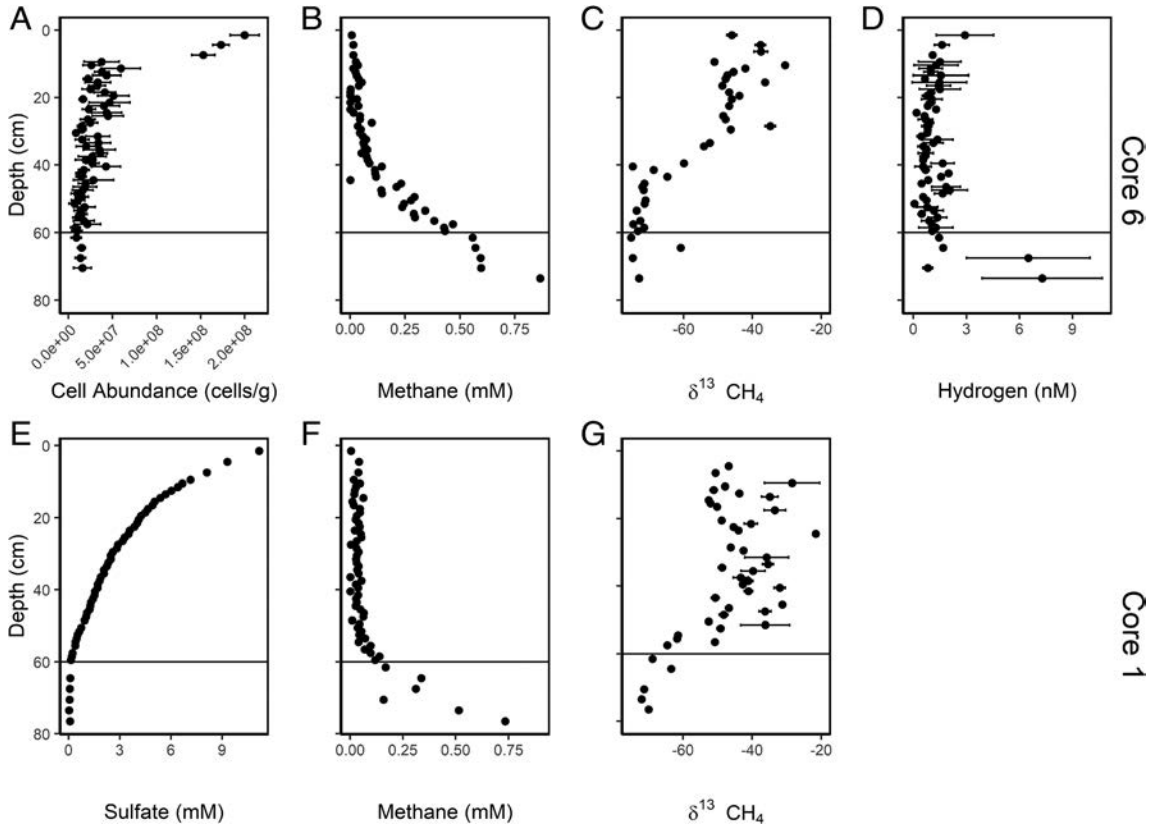

Fig. 1. White Oak River estuary cores show methane-consuming or AOM sediments in the SMTZ and methane-producing or methanogenic sediments below it. Aqueous geochemistry for cores 6 (top row) and 1 (bottom row), with (A) cell abundance, (B and F) methane concentration, (C and G) $\delta^{13}C$of methane, (D) hydrogen concentration, and (E) sulfate concentration. Black line shows approximate transition from AOM to methanogenesis.

was chosen at ~60 cm because it is the depth where (i) sulfate is depleted in core 1, (ii) hydrogen concentrations are consistently elevated in core 6, (ii) methane concentrations are concave-down, and (iv) $\delta^{13}C$ values become consistent with methanogenesis. The upper limit of AOM is defined by the depth at which methane concentrations begin to continuously increase downcore, which is 25 cm in core 6 and 45 cm in core 1. For consistency in nomenclature based on the available substrates, we refer to the depths where methane is present above 60 cm the SMTZ, and the depths below 60 cm the MZ, for methane zone.

ANME-1 dominated the Methanomicrobia in 16S rRNA gene amplicon libraries in both the SMTZ and MZ (99.5% and 99.9%, respectively, Fig. 2). ANME-1 comprised six OTUs of ANME-1a, nine OTUs of ANME-1b, and one OTU that could not be placed into one of those two subgroups. This agrees with previous observations from Aarhus Bay (Beulig *et al*., 2019), White Oak River estuary (Lloyd *et al*., 2011), Gulf of Mexico deep-sea (Underwood *et al*., 2016), and Santa Barbara Basin deep-sea (Harrison *et al*., 2009) that ANME-1 were the dominant or only organisms with the genes for methane-cycling in AOM and methanogenic sediments. Each of these analyses utilized 16S rRNA primers capable of amplifying cultured methanogens, so the absence of cultured methanogens was not likely to be an artefact of primer bias. However, primer bias can greatly skew the relative abundance of different clades (Polz and Cavanaugh, 1998), so we analysed 16S rRNA gene sequences from unamplified metagenomes from the White Oak River estuary (Lazar *et al*., 2016) and found that ANME-1 comprised 100% of the Methanomicrobia in the AOM zone and 92.86% in the MZ, agreeing with amplicon data that the Methanomicrobia were mostly ANME-1 (Fig. 2).

The four most abundant ANME-1 OTUs, comprising 96% of all ANME-1 16S rRNA gene sequences, had FRAxC peaks in the SMTZ that coincided with peaks in putative sulfate-reducing bacteria *Desulfatiglans sp*. and SEEP-SRB1 (Supporting Information Fig. S1). Specifically, the two most abundant ANME-1 OTU (ANME-1b; Supporting Information Fig. S1A and C) had peaks at

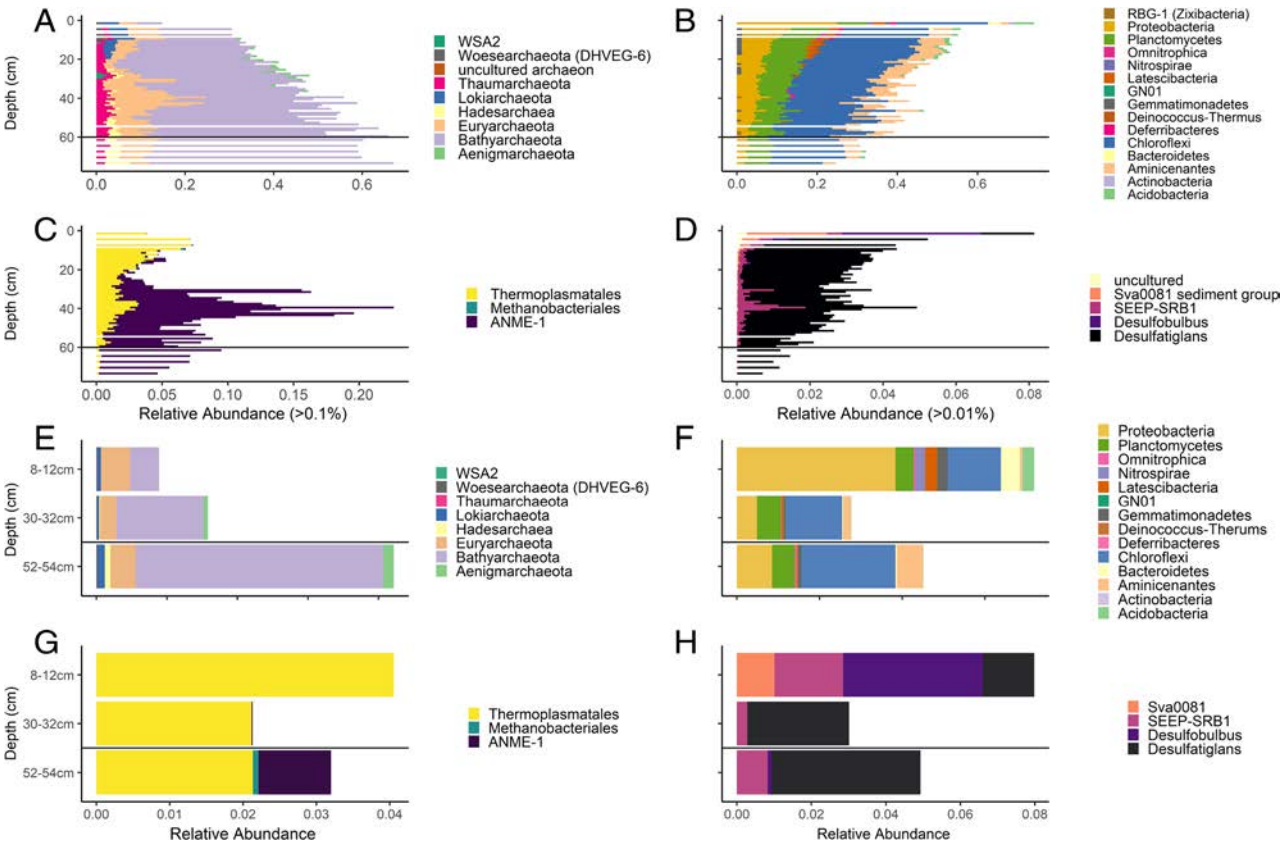

Fig. 2. ANME-1 and *Desulfatiglans* sp. dominate methane- and sulfur-cycling organisms in both methane-consuming and methane-producing sediments. Relative abundance of 16S rRNA gene sequences for all archaea (left panels) and all bacteria (right panels), for amplicon libraries (A–D) and metagenomes (E–H), grouped at the phylum level. C, D, G, and H show putative sulfate-reducing bacteria and Euryarchaeota, grouped at the family level. Only phyla with >1% relative 16S rRNA gene sequence abundance for bacteria and >0.1% for archaea are shown. Black lines show transition from AOM to methanogenesis.

32.5 cm, which coincided with a peak in the most abundant *Desulfatiglans sp*. OTU (Supporting Information Fig. S1B). The third most abundant ANME-1 OTU (ANME-1a; Supporting Information Fig. S1E) had a peak at 37.5 cm, coinciding with the peak in the third most abundant *Desulfatiglans sp*. (Supporting Information Fig. S1F). The first and fourth most abundant ANME-1 OTUs (ANME-1b, Fig. 1A and G) had peaks at 39 cm coinciding with the peak in the fourth most abundant *Desulfatiglans sp*. (Supporting Information Fig. S1H). SEEP-SRB1 have been shown to form syntrophies with ANME-1 (Wegener *et al*., 2015). *Desulfatiglans sp*. have not previously been associated with syntrophies with ANME-1 but were the most abundant sulfate-reducing bacteria in ANME-1-dominated methane seeps in the Gulf of Mexico (Lloyd *et al*., 2006). In White Oak River estuary sediments, *Desulfatiglans sp*. and SEEP-SRB1 comprised 99.9% of 16S rRNA gene sequences of known sulfate-reducing bacteria clades in both the SMTZ and MZ. They were distinct from the dominant clades present in the upper, methane-free sulfate reduction zone: *Desulfobulbus sp*. and the uncultured Sva0081 clade within the Desulfobacteraceae (94% of sulfate-reducing bacteria above 25 cm, Fig. 2). The dominance patterns of these potentially sulfate-reducing clades are supported by metagenomic data as well (Fig. 2). This suggests that *Desulfatiglans sp*. and SEEP-SRB1 may be adapted to syntrophy with ANME-1 in the SMTZ, and this may allow them to out-compete other sulfate-reducing bacteria deeper in the core when ANME-1 are abundant. Many cultured sulfate-reducing bacteria are not dependent on sulfate, and can survive with fermentation in the absence of sulfate (Zhou *et al*., 2011). The second and fourth most abundant *Desulfatiglans sp*. had peaks in the upper 10 cm that were not matched by peaks in ANME-1, suggesting they can be independent of ANME-1.

ANME-1 also had peaks at 41 and 43 cm that were deeper than those of any *Desulfatiglans sp*. peaks (Supporting Information Fig. S1), suggesting that they may also be capable of acting independently of sulfate reducers in these sediments. The most abundant ANME-1b OTU, which accounted for 83% of total ANME-1 reads, did not undergo a population decrease at the base of the SMTZ followed by an increase in the MZ (Supporting Information Fig. S1A). Such a population decrease was suggested in a previous experiment with lower depth resolution (Lloyd *et al*., 2011). This suggests that, for most ANME-1b, if they switch from methane-oxidizing to methane-producing, this either does not require a die-off followed by a separate population growing up, or the population decrease happens in a smaller depth interval than we could observe with 1 cm intervals. A third possibility is that a population decrease does occur between the SMTZ and MZ, but seasonal variability of SMTZ depth (Lloyd *et al*., 2011) dampens the coherence of this signal. One ANME-1a OTU (Supporting Information Fig. S1E), which accounted for 3% of the total ANME-1 reads, did decrease at the base of the SMTZ and increase in the MZ, suggesting that die-off and regrowth may be required for some ANME-1 populations to switch between methane oxidation and production. In total, ANME-1 populations increased relative to those of sulfate-reducing bacteria throughout the transition from SMTZ to MZ (Supporting Information Fig. S2).

The most abundant *Desulfatiglans sp*. OTU maintained its population through the MZ, and all others decreased, suggesting that successively smaller populations were capable of meeting their energetic needs on either cryptic sulfur cycling or fermentation as substrates were depleted with depth (Supporting Information Fig. S1). The coupling of ANME-1 and sulfate-reducing bacteria populations in the SMTZ, and their decoupling in the MZ, is consistent with ANME-1 switching from AOM, which requires a sulfate-reducing partner, to methanogenesis, which does not. The sum of all OTUs of ANME-1a and ANME-1b were three- and five-fold higher in the SMTZ than in the MZ. The amount of methane that is produced over many tens of centimetres of sediment depth in the MZ is consumed over 10–20 cm sediment depth in the AOM zone. Therefore, a reversible methanogen operating at similar cell specific metabolic rates in the SMTZ and MZ would be expected to be in a higher cell density in the SMTZ than in the MZ, as was observed.

The only other type of *Methanomicrobia* detected was one OTU of *Methanobacterales*, which comprised only 0.7% of total *Methanomicrobia* sequences and 0.3% of *Methanomicrobia* in the MZ. They also decreased with depth in the MZ (Fig. 2). The uncultured phylum, *Bathyarchaeota*, has been suggested to perform methane-cycling (Kubo *et al*., 2012; Evans *et al*., 2015) and increased in relative abundance with depth in the MZ (Fig. 2). However, the inference that *Bathyarchaeota* perform methane-cycling is based solely on the presence of an evolutionarily divergent *mcrA* gene found in genomes from a terrestrial coal bed (Evans *et al*., 2015). Orthologues to this *mcrA* have been shown to catalyse butane rather than methane oxidation (Laso-Peréz *et al*., 2016) and none of the *Bathyarchaeota* genomes obtained from the White Oak River estuary sediments have this gene (Lazar *et al*., 2016). Instead *Bathyarchaeota* in marine sediments appear to perform acetogenesis and fermentation of organic substrates such as proteins and lignin (Lloyd *et al*., 2013; He *et al*., 2016). Ten *Hadesarchaeota* OTUs increased in relative abundance in the MZ (Fig. 2). This uncultured archaeal phylum has numerous carbon metabolism

genes in common with *Methanomicrobia* but does not have a methanogenic enzymatic pathway. Instead *Hadesarchaeota* are hypothesized to have a heterotrophic and/or nitrogen cycling lifestyle (Baker *et al*., 2016). A proposed methyl-reducing methanogenic archaeal lineage, WSA2 (Nobu *et al*., 2016), was also detected in our samples. However, the 11 OTUs all declined below 27–30 cm (Fig. 2).

No bacteria or other archaea changed in relative or FRAxC abundance with changes in methane and sulfate concentrations. OTUs from sulfide-oxidizing bacteria such as *Sulfurimonas*, *Thiotrichales*, and *Thiomicrospira* were either low in abundance, not detected, or demonstrated no significant increases in relative abundance with depth. Thirty-one OTUs of the organoheterotrophic genus *Caldithrix* and phylum *Defferibacteres* (Miroshnichenko *et al*., 2010) declined with depth. Obligate iron- and manganese-reducing bacteria either did not meet abundance thresholds or were not detected. Lokiarchaeota and Woesearchaeota both decreased in relative abundance sharply with depth. *Acidobacteria*, *Bacteroidetes*, *Latescibacteria*, *Nitrospirae*, *Deferribacteres* and *Gemmatimonadetes* all decreased with depth and accounted for less than 1% of total reads per sample. *Proteobacteria* declined steadily from ~25% of total reads at the top of the core to ~4% at the bottom (Fig. 2). *Chloroflexi*, *Aminicenantes* and *Planctomycetes* all declined gradually throughout the core profile.

Meta-analysis of enrichment studies

ANME-1 enrichments have been shown to reverse between net AOM and net methanogenesis based on hydrogen and sulfate availability (Holler *et al*., 2009, 2011; Wegener *et al*., 2015). One ANME-1 enrichment was shown to contain ANME-1 as the sole archaea, as well as SEEP-SRB1 sulfate reducers, and other bacteria (Holler *et al*., 2011), and demonstrated that 'methane consumption was reversibly inhibited' by hydrogen additions (Wegener *et al*., 2015). Methane concentrations increased over a period of 3 days when hydrogen concentrations were high (>0.5 mM), even when sulfate was present. We calculated the rate of this methane increase and found it to be equivalent to the rate of methane decrease after hydrogen was consumed (Supporting Information Fig. S3A). As in most AOM enrichment cultures, AOM was assumed to be driven by the dominant archaea present (ANME-1); we tested whether ANME-1 were also the dominant archaea present when the enrichments switched to net methane production.

We performed a Blast search of *mcrA* genes from all cultured methanogens and uncultured groups containing *mcrA* against the transcriptomes from these enrichments. Transcriptomes provide an accounting of all the genes that are being actively transcribed to RNA at the time of sampling, reporting a combination of abundance and activity. We found that only ANME-1 had hits at an e-value cutoff of $<1 \times 10^{-10}$, during both net AOM and net methanogenesis, with the exception of <0.01% hits to ANME-2, another clade of AOM-performing organisms (Orphan *et al*., 2009) in two of the AOM-performing incubations (Supporting Information Fig. S3). Therefore, the only organism with the genes capable of methane metabolism in each of these incubations was ANME-1, and possibly ANME-2. Wegener *et al*. suggested that ANME-1 decreased in metabolic activity under conditions of net methanogenesis, since sulfate reducers prefer electrons from hydrogen than from AOM (Krukenberg *et al*., 2018). In partial agreement with this interpretation, sulfate-reducing bacteria transcripts and those of dissimilatory sulfite reductase subunits A and B (*dsrAB*) increased relative to non-ANME organisms after hydrogen addition, suggesting sulfate-reducing bacteria were stimulated by the hydrogen additions (Supporting Information Fig. S3C). However, total ANME-1 transcripts did not decrease relative to background populations of other organisms after hydrogen addition (Supporting Information Fig. S3C), suggesting that ANME-1 populations were similarly abundant and active under both AOM and methanogenesis. ANME-1 *mcrA* transcripts, however, were greatly elevated under AOM conditions with low H_2 (Supporting Information Fig. S3). This is consistent with observations for cultured methanogens, which have been shown to up-regulate *mcrA* gene expression in response to low H_2 concentration (Reeve, 1997; Kato *et al*., 2008). Collectively, these transcript data support active methane metabolism by ANME-1, and only ANME-1, during net AOM and net methanogenesis in these enrichment experiments.

Discussion

Our results show that ANME-1 is the dominant organism with the genetic capability for AOM and methanogenesis in White Oak River estuary sediments, based on 16S rRNA amplicons and metagenomic DNA sequences. ANME-1 populations have peaks coincident with those of sulfate reducing bacteria in the SMTZ, but their populations increase relative to sulfate reducing bacteria through the MZ. They also do not undergo a major population decrease in the transition from the SMTZ to the MZ. The participation of ANME-1 in both AOM and methanogenesis either occurs through a metabolic reversal (Fig. 3), or through the growth of subclades with identical 16S rRNA sequences that drive only one, but not both of AOM or methanogenesis. However, the lack of a major decrease in the ANME-1 population at the base of the SMTZ is consistent with a continuous population of

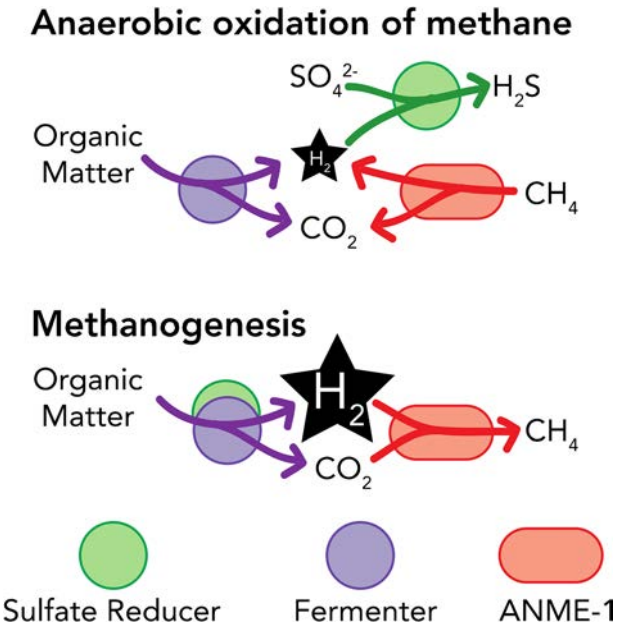

Fig. 3. Model for AOM and methanogenesis in non-seep marine sediments. Fermenters produce hydrogen from organic matter under both conditions. During anaerobic oxidation of methane, sulfate reducers bring hydrogen concentrations so low that methane oxidation is exergonic. During methanogenesis, sulfate reducers have run out of sulfate so hydrogen concentrations build up to a level that makes ANME-1 switch to forward methanogenesis. Sulfate reducers may persist by switching to fermentation. Purple = fermenters, green = sulfate reducers, and red = ANME-1.

ANME-1 reversing between AOM and methanogenesis through the transition from AOM to MZ during burial.

Our meta-analysis of enrichments that toggle between methane production and AOM, based on hydrogen concentrations (Wegener *et al*., 2015), showed that ANME-1 was the only organism expressing methanogenic genes under both of these conditions. Another study with marine sediments from Hydrate Ridge and Amon Mud Volcano show similar H_2-dependent reversibility, demonstrating the principle of reversibility, although the experiments were not examined for the identity of the organisms present (Yoshinaga *et al*., 2014). In this study, washing the enrichments free of sulfate caused methane to increase over a period of 30 days, presumably fueled by hydrogen produced by fermentation of organic matter present in the enrichments. The fact that methane started being produced in less than a day and did not increase in production rate over 30 days suggests that the methane was produced by a low-energy methanogen that were already there, not a growing subpopulation of a different organism, since this would have caused an exponential methane increase and a substantial lag time. Adding H_2 to the headspace increased the rate 100-fold, further supporting hydrogenotrophic methanogenesis. The rate of methanogenesis in these experiments was much lower than that of AOM, likely because some of the cells were likely washed out of the system along with the sulfate.

Our conclusion that ANME-1 participate in methanogenesis as well as AOM in marine sediments is further supported by geochemical models (Hoehler *et al*., 1994, 1998), reversibility of the methanogenic biochemical pathway (Scheller *et al*., 2010; Beulig *et al*., 2019), and dominance of ANME-1 among potential methanogens in non-seep marine sediments in geographically widespread areas (Lloyd *et al*., 2011; Underwood *et al*., 2016; Beulig *et al*., 2019). Although known methanogenic clades have been cultured from marine sediments (Kendall and Boone, 2006; Kendall *et al*., 2007) and identified by gene surveys targeting cultured clades (Lever, 2012), we are not aware of any study that identified cultured methanogens in methanogenic marine sediments when using universal primers, except for salt marsh tidal flats and sandy surface sediments which are periodically exposed to air or have non-competitive methanogenic substrates available (Wilms *et al*., 2006; Ruff *et al*., 2015). This suggests that ANME-1, and not cultured methanogens, are widespread and abundant in MZs of marine sediments. These findings are consistent with the observation that ANME-1 may inhabit more deeply buried marine sediments, due to a possible sensitivity of oxygen (Knittel and Boetius, 2009; Ruff *et al*., 2015). Deep-sea sediments, however, often lack both ANME-1 and cultured methanogens (Vetriani *et al*., 1999; Biddle *et al*., 2006), meaning that other types of low energy methanogens may be present in such locations.

Reversibility may confer an advantage to ANME-1 in marine sediments. ANME-1 may gain energy through AOM in the SMTZ, increasing their cell abundance relative to other methanogens. Then, when hydrogen concentrations increase after sulfate is depleted, ANME-1 have a head start on these other low-energy methanogens and can outcompete them for meagre resources. Reversibility would also be advantageous when the depth of the SMTZ shifts, reversing direction of exergonicity of hydrogenotrophic methanogenesis. The prevalence of metabolically reversible ANME-1 on Earth suggests that this level of extreme metabolic flexibility may be a more widespread feature of organisms specialized to survive in ultra-low energy environments. This could be used as a guide in the search for habitable places on Earth and extraterrestrial environments.

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Data archiving

16S rRNA gene sequences can be found at the NCBI Genbank short read archive with accession number PRJNA565996.

References

Alperin, M.J., Reeburgh, W.S., and Whiticar, M.J. (1988) Carbon and hydrogen isotope fractionation resulting from anaerobic methane oxidation. *Global Biogeochem Cycles* **2**: 279–288.

Baker, B.J., Saw, J.H., Lind, A.E., Lazar, C.S., Hinrichs, K. U., Teske, A.P., and Ettema, T.J.G. (2016) Genomic inference of the metabolism of cosmopolitan subsurface archaeaHadesarchaea. *Nat Microbiol* **1**: 1–7.

Bertram, S., Blumenberg, M., Michaelis, W., Siegert, M., Krüger, M., and Seifert, R. (2013) Methanogenic capabilities of ANME-archaea deduced from 13C-labelling approaches. *Environ Microbiol* **15**: 2384–2393.

Beulig, F., Røy, H., McGlynn, S.E., and Jørgensen, B.B. (2019) Cryptic CH4 cycling in the sulfate–methane transition of marine sediments apparently mediated by ANME-1 archaea. *ISME J* **13**: 250–262.

Biddle, J.F., Lipp, J.S., Lever, M.a., Lloyd, K.G., Sørensen, K.B., Anderson, R., *et al*. (2006) Heterotrophic archaea dominate sedimentary subsurface ecosystems off Peru. *Proc Natl Acad Sci U S A* **103**: 3846–3851.

Crill, P.M., and Martens, C.S. (1986) Methane production from bicarbonate and acetate in an anoxic marine sediment. *Geochim Cosmochim Acta* **50**: 2089–2097.

Evans, P.N., Parks, D.H., Chadwick, G.L., Robbins, S.J., Orphan, V.J., Golding, S.D., and Tyson, G.W. (2015) Methane metabolism in the archaeal phylum Bathyarchaeota revealed by genome-centric metagenomics. *Science (80-)* **350**: 434–438.

Hallam, S.J., Putnam, N., Preston, C.M., Detter, J.C., Rokhsar, D., Richardson, P.M., and DeLong, E.F. (2004) Reverse methanogenesis: testing the hypothesis with environmental genomics. *Science* **305**: 1457–1462.

Harrison, B.K., Zhang, H., Berelson, W., and Orphan, V.J. (2009) Variations in archaeal and bacterial diversity associated with the sulfate-methane transition zone in continental margin sediments (Santa Barbara Basin, California). *Appl Environ Microbiol* **75**: 1487–1499.

He, Y., Li, M., Perumal, V., Feng, X., Fang, J., Xie, J., *et al*. (2016) Genomic and enzymatic evidence for acetogenesis among multiple lineages of the archaeal phylum Bathyarchaeota widespread in marine sediments. *Nat Microbiol* **35**: 1–9.

Hendrickson, E.L., and Leigh, J.A. (2008) Roles of coenzyme F420-reducing hydrogenases and hydrogen- and F420-dependent methylenetetrahydromethanopterin dehydrogenases in reduction of F420 and production of hydrogen during methanogenesis. *J Bacteriol* **190**: 4818–4821.

Hoehler, T.M., Alperin, M.J., Albert, D.B., and Martens, C.S. (1994) Field and laboratory studies of methane oxidation in an anoxic marine sediment: evidence for a methanogen-sulfate reducer consortium. *Global Biogeochem Cycles* **8**: 451–463.

Hoehler, T.M., Alperin, M.J., Albert, D.B., and Martens, C.S. (1998) Thermodynamic control on hydrogen concentrations in anoxic sediments. *Geochim Cosmochim Acta* **621**: 1745–1756.

Hoehler, T.M., and Jørgensen, B.B. (2013) Microbial life under extreme energy limitation. *Nat Rev Microbiol* **11**: 83–94.

Holler, T., Wegener, G., Knittel, K., Boetius, A., Brunner, B., Kuypers, M.M.M., and Widdel, F. (2009) Substantial 13 C/12 C and D/H fractionation during anaerobic oxidation of methane by marine consortia enriched in vitro. *Environ Microbiol Rep* **1**: 370–376.

Holler, T., Widdel, F., Knittel, K., Amann, R., Kellermann, M. Y., Hinrichs, K.-U., *et al*. (2011) Thermophilic anaerobic oxidation of methane by marine microbial consortia. *ISME J* **5**: 1946–1956.

House, C.H., Orphan, V.J., Turk, K.a., Thomas, B., Pernthaler, A., Vrentas, J.M., and Joye, S.B. (2009) Extensive carbon isotopic heterogeneity among methane seep microbiota. *Environ Microbiol* **11**: 2207–2215.

Iversen, N., and Jorgensen, B.B. (1985) Anaerobic methane oxidation rates at the sulfate-methane transition in marine sediments from Kattegat and Skagerrak (Denmark). *Limnol Oceanogr* **1983**: 944–955.

Jagersma, C.G., Meulepas, R.J.W., Timmers, P.H.a., Szperl, A., Lens, P.N.L., and Stams, A.J.M. (2012) Enrichment of ANME-1 from Eckernförde Bay sediment on thiosulfate, methane and short-chain fatty acids. *J Biotechnol* **157**: 482–489.

Jones, R.M., Goordial, J.M., and Orcutt, B.N. (2018) Low energy subsurface environments as extraterrestrial analogs. *Front Microbiol* **9**: 1–18.

Jorgensen, B.B., and Kasten, S. (2006) . In *Sulfur Cycling and Methane Oxidation*. Schulz, H., and Zabel, M. (eds). Berlin/Heidelberg: Springer.

Kato, S., Kosaka, T., and Watanabe, K. (2008) Comparative transcriptome analysis of responses of Methanothermobacter thermautotrophicus to different environmental stimuli. *Environ Microbiol* **10**: 893–905.

Kendall, M.M., and Boone, D.R. (2006) Cultivation of methanogens from shallow marine sediments at Hydrate Ridge, Oregon. *Archaea* **2**: 31–38.

Kendall, M.M., Wardlaw, G.D., Tang, C.F., Bonin, A.S., Liu, Y., and Valentine, D.L. (2007) Diversity of Archaea in marine sediments from Skan Bay, Alaska, including cultivated methanogens, and description of Methanogenium boonei sp. nov. *Appl Environ Microbiol* **73**: 407–414.

Kevorkian, R., Bird, J.T., Shumaker, A., and Lloyd, K.G. (2018) Estimating population turnover rates by relative

quantification methods reveals microbial dynamics in marine sediment. *Appl Environ Microbiol* **84**: 1–16.

Knab, N., Cragg, B., Borowski, C., Parkes, R., Pancost, R., and Jorgensen, B. (2008) Anaerobic oxidation of methane (AOM) in marine sediments from the Skagerrak (Denmark): I. Geochemical and microbiological analyses. *Geochim Cosmochim Acta* **72**: 2868–2879.

Knittel, K., and Boetius, A. (2009) Anaerobic oxidation of methane: progress with an unknown process. *Annu Rev Microbiol* **63**: 311–334.

Krukenberg, V., Riedel, D., Gruber-Vodicka, H.R., Buttigieg, P.L., Tegetmeyer, H.E., Boetius, A., and Wegener, G. (2018) Gene expression and ultrastructure of meso- and thermophilic methanotrophic consortia. *Environ Microbiol* **20**: 1651–1666.

Kubo, K., Lloyd, K.G., Biddle, J., Amann, R., Teske, A., and Knittel, K. (2012) Archaea of the miscellaneous Crenarchaeotal group are abundant, diverse and widespread in marine sediments. *ISME J* **6**: 1949–1965.

Laso-Peréz, R., Wegener, G., Knittel, K., and Widdel, F. (2016) Thermophilic archaea activate butane via alkyl-CoM formation. *Nature* **539**: 396–401.

Lazar, C.S., Baker, B.J., Seitz, K., Hyde, A.S., Dick, G.J., Hinrichs, K.U., and Teske, A.P. (2016) Genomic evidence for distinct carbon substrate preferences and ecological niches of Bathyarchaeota in estuarine sediments. *Environ Microbiol* **18**: 1200–1211.

Lever, M.A. (2012) Functional gene surveys from ocean drilling expeditions - a review and perspective. *FEMS Microbiol Ecol* **84**: 1–23.

Lipps, J.H., Delory, G., Pitman, J.T., and Rieboldt, S. (2020) Astrobiology of Jupiter' s icy moons. *Proc SPIE* **8**: 78–92.

Lloyd, K.G., Alperin, M.J., and Teske, A. (2011) Environmental evidence for net methane production and oxidation in putative ANaerobic MEthanotrophic (ANME) archaea. *Environ Microbiol* **13**: 2548–2564.

Lloyd, K.G., Lapham, L., and Teske, A. (2006) An anaerobic methane-oxidizing community of ANME-1b archaea in hypersaline gulf of Mexico sediments. *Appl Environ Microbiol* **72**: 7218–7230.

Lloyd, K.G., Schreiber, L., Petersen, D.G., Kjeldsen, K.U., Lever, M.A., Steen, A.D., *et al*. (2013) Detrital proteins. *Nature* **496**: 215–219.

Martens, C.S., Albert, D.B., and Alperin, M.J. (1998) Biogeochemical processes controlling methane in gassy coastal sediments - part 1. A model coupling organic matter flux to gas production, oxidation and transport. *Cont Shelf Res* **18**: 1741–1770.

Martens, S., and Berner, R.A. (1977) Interstitial water chemistry of anoxic Lbng Island sound sediments. 1. Dissolved gases. *Limnol Oceanogr* **22**: 10–25.

Mckay, C.P. (2016) Titan as the abode of life. *Life* **6**: 8.

Meyerdierks, A., Kube, M., Kostadinov, I., Teeling, H., Glöckner, F.O., Reinhardt, R., and Amann, R. (2010) Metagenome and mRNA expression analyses of anaerobic methanotrophic archaea of the ANME-1 group. *Environ Microbiol* **12**: 422–439.

Miroshnichenko, M.L., Kolganova, T.V., Spring, S., Chernyh, N., and Bonch-Osmolovskaya, E.A. (2010) Caldithrix palaeochoryensis sp. nov., a thermophilic, anaerobic, chemo-organotrophic bacterium from a geothermally heated sediment, and emended description of the genus Caldithrix. *Int J Syst Evol Microbiol* **60**: 2120–2123.

Mukhopadhyay, B., Johnson, E.F., and Wolfe, R.S. (2000) A novel pH2 control on the expression of flagella in the hyperthermophilic strictly hydrogenotrophic methanarchaeaon Methanococcus jannaschii. *Proc Natl Acad Sci U S A* **97**: 11522–11527.

Nobu, M.K., Narihiro, T., Kuroda, K., Mei, R., and Liu, W.T. (2016) Chasing the elusive Euryarchaeota class WSA2: genomes reveal a uniquely fastidious methyl-reducing methanogen. *ISME J* **10**: 2478–2487.

Orcutt, B., Boetius, A., Elvert, M., Samarkin, V., and Joye, S. B. (2005) Molecular biogeochemistry of sulfate reduction, methanogenesis and the anaerobic oxidation of methane at Gulf of Mexico cold seeps. *Geochim Cosmochim Acta* **17**: 4267–4281.

Orphan, V.J., House, C.H., Hinrichs, K., Mckeegan, K.D., and Delong, E.F. (2009) Methane-consuming by directly coupled isotopic phylogenetic. *Adv Sci* **293**: 484–487.

Polz, M.F., and Cavanaugh, C.M. (1998) Bias in template-to-product ratios in multitemplate PCR. *Appl Environ Microbiol* **64**: 3724–3730.

Reeburgh, W.S. (2007) Oceanic methane biogeochemistry. *Chem Rev*, **107**, 486–513.

Reeve, J.N. (1997) Growth- and substrate-dependent transcription of the formate dehydrogenase (fdhCAB) operon in Methanobacterium thermoformicicum Z-245. *Microbiology* **179**: 899–908.

Reeve, J.N., Nölling, J., Morgan, R.M., and Smith, D.R. (1997) Methanogenesis: genes, genomes, and who's on first? *J Bacteriol* **179**: 5975–5986.

Ruff, S.E., Biddle, J.F., Teske, A.P., Knittel, K., Boetius, A., and Ramette, A. (2015) Global dispersion and local diversification of the methane seep microbiome. *Proc Natl Acad Sci U S A* **112**: 4015–4020.

Russell, M.J., and Nitschke, W. (2017) Methane: fuel or exhaust at the emergence of life ? *Astrobiology* **17**: 1053–1066.

Scheller, S., Goenrich, M., Boecher, R., Thauer, R.K., and Jaun, B. (2010) The key nickel enzyme of methanogenesis catalyses the anaerobic oxidation of methane. *Nature* **465**: 606–608.

Shima, S., Krueger, M., Weinert, T., Demmer, U., Kahnt, J., Thauer, R.K., and Ermler, U. (2012) Structure of a methyl-coenzyme M reductase from Black Sea mats that oxidize methane anaerobically. *Nature* **481**: 98–101.

Underwood, S., Lapham, L., Teske, A., and Lloyd, K.G. (2016) Deep-Sea research II microbial community structure and methane-cycling activity of subsurface sediments at Mississippi canyon 118 before the Deepwater horizon disaster. *Deep Res Part II* **129**: 148–156.

Valentine, D.L., Blanton, D.C., and Reeburgh, W.S. (2000) Hydrogen production by methanogens under low-hydrogen conditions. *Arch Microbiol* **174**: 415–421.

Vetriani, C., Jannasch, H.W., Gregor, B.J.M.A.C., Stahl, D. A., and Reysenbach, A. (1999) Population structure and phylogenetic characterization of marine benthic archaea in Deep-Sea sediments. *Appl Environ Microbiol* **65**: 4375–4384.

Vignais, P.M., and Billoud, B. (2007) Occurrence, classification, and biological function of hydrogenases: an overview. *Chem Rev* **107**: 4206–4272.

Walker, C.B., Redding-johanson, A.M., Baidoo, E.E., Rajeev, L., He, Z., Hendrickson, E.L., *et al*. (2012) Functional responses of methanogenic archaea to syntrophic growth. *ISME J* **6**: 2045–2055.

Wegener, G., Krukenberg, V., Riedel, D., Tegetmeyer, H.E., and Boetius, A. (2015) Intercellular wiring enables electron transfer between methanotrophic archaea and bacteria. *Nature* **526**: 587–590.

Whiticar, M.J. (1999) Carbon and hydrogen isotope systematics of bacterial formation and oxidation of methane. *Chem Geol* **161**: 291–314.

Whitman, W.B. (2015) *Bergey' Manual of Systematics of Archaea and Bacteria*, New York, NY: John Wiley & Sons.

Wilms, R., Sass, H., Ko, B., Cypionka, H., and Engelen, B. (2006) Specific bacterial , archaeal , and eukaryotic communities in tidal-flat sediments along a vertical profile of several meters. *Appl Environ Microbiol* **72**: 2756–2764.

Yoshinaga, M.Y., Holler, T., Goldhammer, T., Wegener, G., Pohlman, J.W., Brunner, B., *et al*. (2014) Carbon isotope equilibration during sulphate-limited anaerobic oxidation of methane. *Nat Geosci* **7**: 190–194.

Zhou, J., He, Q., Hemme, C.L., Mukhopadhyay, A., Hillesland, K., Zhou, A., *et al*. (2011) How sulphate-reducing microorganisms cope with stress: lessons from systems biology. *Nat Rev Microbiol* **9**: 452–466.

Zinder, S.H. (1994) Syntrophic acetate oxidation and reversible acetogenesis. In *Acetogenesis*. Drake, H.L. (ed). Boston, MA: Springer, pp. 386–415.

Supporting Information

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Figure S1 Supporting information
Appendix S1 Supporting information