ORIGINAL ARTICLE

Methanotrophic archaea possessing diverging methane-oxidizing and electron-transporting pathways

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Anaerobic oxidation of methane (AOM) is a crucial process limiting the flux of methane from marine environments to the atmosphere. The process is thought to be mediated by three groups of uncultivated methane-oxidizing archaea (ANME-1, 2 and 3). Although the responsible microbes have been intensively studied for more than a decade, central mechanistic details remain unresolved. On the basis of an integrated analysis of both environmental metatranscriptome and single-aggregate genome of a highly active AOM enrichment dominated by ANME-2a, we provide evidence for a complete and functioning AOM pathway in ANME-2a. All genes required for performing the seven steps of methanogenesis from CO_2 were found present and actively expressed. Meanwhile, genes for energy conservation and electron transportation including those encoding $F_{420}H_2$ dehydrogenase (Fpo), the cytoplasmic and membrane-associated Coenzyme B–Coenzyme M heterodisulfide (CoB-S-SCoM) reductase (HdrABC, HdrDE), cytochrome *C* and the Rhodobacter nitrogen fixation (Rnf) complex were identified and expressed, whereas genes encoding for hydrogenases were absent. Thus, ANME-2a is likely performing AOM through a complete reversal of methanogenesis from CO_2 reduction without involvement of canonical hydrogenase. ANME-2a is demonstrated to possess versatile electron transfer pathways that would provide the organism with more flexibility in substrate utilization and capacity for rapid adjustment to fluctuating environments. This work lays the foundation for understanding the environmental niche differentiation, physiology and evolution of different ANME subgroups.

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Introduction

Anaerobic oxidization of methane (AOM) coupled to sulfate reduction is a key process that effectively controls the methane emission from anoxic marine waters and sediments to the oxygenated ocean (Reeburgh, 1976, 2007). The microorganisms thought to be mediating AOM have been designated as anaerobic methane-oxidizing archaea (ANME), close phylogenetic relatives of the methanogenic archaea. These microorganisms consist of three established groups (ANME-1, ANME-2 and ANME-3) that can form syntrophic aggregates with sulfate-reducing bacteria (SRB) or exist as single cells (Hinrichs *e a .*, 1999; Boetius *e a .*, 2000; Orphan *e a .*, 2001; Knittel and Boetius, 2009). AOM has been hypothesized to be operated via a reversal of the methanogenesis reaction (Hoehler *e a .*, 1994), and this

However, the evidence remains inconclusive because one of the genes in canonical CO_2-dependent methanogenesis, the N^5,N^{10}-methylene-tetrahydromethanopterin (methylene-H_4MPT) reductase (Mer, which catalyzes the reaction from methylene-H_4MPT to methyl-H_4MPT) gene *e*, has not been found in the metagenomes of ANME-1. Meanwhile, very limited genomic information is available for

aggregates were transferred into sterile 0.2 ml tubes that were pre-filled with 3.5 µl of phosphate-buffered saline. Each tube contained one aggregate. These aggregate samples were immediately stored at − 70 °C for further characterization.

Wh e-ge e a ifica i

Whole-genome multiple displacement amplification (MDA) on single aggregate was conducted by REPLI-g Mini kit reagents (Qiagen, Hilden, Germany) following the manufacturer's protocol. DNA (500–800 ng) was generated after each MDA reaction and the first-round MDA product was used for 16S rRNA gene characterization. Forward primer Arch21F (Delong, 1992) and three reverse primers ANME-2-538 (Treude *e a .*, 2005), Arch915 (Stahl and Amann, 1991) and Arch958 (Delong, 1992) were applied for archaeal 16S rRNA gene amplification. The bacterial 16S rRNA gene fragments were amplified with the primer set of Bac27F and Bac1492R. The PCR was initiated with a denaturing step at 94 °

non-rRNA reads were included in further analysis and assembled using SOAP alignment software (Li *e a .*, 2009). Open reading frames were predicted with MetaGene Annotator (Noguchi *e a .*, 2008) and compared with the NCBI NR database using blastx with an *E*-value cutoff of $<10^{-5}$. Those sequences that had reliable hits from NR database were compared with the KEGG and COG databases (with *E*-value $<10^{-5}$) for functional annotation.

Es i a i f ge e e essi i si g e-agg ega e ge e f e a a sc i ic a sc i s

Metatranscriptomic reads were assigned to single-aggregate genes by blastx with *E*-value cutoff 10^{-10} and >90% identity, and then subject to manual inspection to ensure exact mappings between single-aggregate genes and their assigned metatranscriptomic reads. The expression level of each transcript in the transcriptome was quantified by RPKM (reads per Kb per million reads) to measure the read density, which reflected the molar concentration of a transcript in the starting sample by normalizing for RNA length and for the total read number in the measurement (Mortazavi *e a .*, 2008). This approach facilitated comparisons of transcript levels both within and between samples. The expression levels of single-aggregate genes were roughly estimated by the RPKM values of the mapped gene transcripts in the metatranscriptome.

Results and discussion

Ge ic a d a sc i ic da a a a sis

The AOM enrichment investigated in this study contains ANME-2a as the dominant archaeal group (Zhang *e a .*, 2011), mainly in the form of aggregates (Supplementary Figure S1). A few cell aggregates were captured from the enrichment, with their DNA isolated, amplified and sequenced as described in the Materials and methods. One captured single aggregate named M25 was found to have ANME-2a as the sole archaeal group with no bacterial partners: no bacterial 16S rRNA gene fragments were amplified, and only ANME-2a 16S rRNA gene was retrieved by archaeal 16S rRNA gene amplification (for details see Materials and methods). M25 was then subjected to in-depth genomic sequencing. Initially, 11 111 112 reads with a length of 90 bp were generated for this single-aggregate genome M25, and resulted in 3.64 Mbp of assembly (Supplementary Table S2). No bacterial 16S rRNA gene fragments were detected from the M25 assembly, and only one 16S rRNA gene sequence assigned to ANME-2a was found (with its phylogeny displayed in Supplementary Figure S2). Meanwhile, as demonstrated in the phylogeny of the methyl-coenzyme M reductase subunit A (McrA, a key enzyme for methane production and oxidation), *c A* retrieved from the M25 genome was also assigned to the ANME-2a subgroup (Supplementary Figure S3). The genomic evidences (the annotated 16S rRNA gene and *c A* analysis, the absence of bacterial marker genes for sulfate reduction) together with the direct 16S rRNA gene amplification confirmed that M25 contained only ANME-2a without bacterial partners. The genome size for our ANME-2a is estimated to be 3.96 Mbp, with a recovery of ~90% by the current M25 assembly (Supplementary Figure S4, see conserved archaeal single copy gene analysis in Materials and methods). Based on the results of blastx (see taxonomic assignment in Materials and methods), all the genes discussed in this study (as listed in Table 1 and Supplementary Tables S3 and S4) were assigned to Methanosarcinales, where ANME-2a belongs. At present, our understanding on these yet uncultivated ANME-2 genome is limited to a few studies: 11 ANME-2a originated fosmids, totaling ~367 Kb sequence data (Hallam *e a .*, 2004); short sequence read on ANME-2c of 99 bp each (~380 Kb in total) (Pernthaler *e a .*, 2008); and a recently near completed genome for ANME-2d (with a genome size of 3.2 Mbp) capable of methane oxidization coupled to nitrate reduction (Haroon *e a .*, 2013). The genomic data on ANME-2a reported here will improve our understanding on the molecular mechanisms of AOM substantially.

The metatranscriptome of the enrichment contained 1.50E+07 sequence reads, 2262.9 Mb of sequences, and 43.3% of which were non-rRNA thus used for further analysis. Approximately 45% of all non-rRNA transcripts in the metatranscriptome were of archaeal origin (determined by MEGAN+blastx results), and the majority (57%) of these archaeal sequences showed highest similarities to Methanosarcinales. The above data sets were exploited to a detailed investigation on the methane metabolizing pathway in ANME-2a (Table 1 and Supplementary Tables S3 and S4).

Me ha e- idi i g a h a

As shown in Table 1, all genes that encode enzymes responsible for the seven central steps in the methanogenic pathway have been identified in the single-aggregate M25 genome and have corresponding transcripts in the metatranscriptome. Our previous activity tests on the same enrichment have proven that the sulfate reduction is dependent on methane oxidation and no methane production was observed when methane was eliminated from the medium (Zhang *e a .*, 2010). ANME-2a is demonstrated to have all the required genes for a complete methane-oxidizing pathway from CH_4 to CO_2 following reversed-methanogenesis hypothesis, and all these genes were actively expressed during the cultivation (Figure 1 and Table 1). Although we cannot completely rule out the possibility of methane productions by ANME-2a under certain conditions (such as the *i si* environmental conditions), under the controlled incubation condition where

Table 1 Identification of methanogenesis-associated genes in the ANME-2a enrichment sample

S e	*Ge e a e*	*Abb e ia i*	*M25 scaff d ID*	*T a sc i e*
1	*Me h -c e e M ed c ase s b i A*	*c A*	65	2409
	S b i B	*c B*	65	4618
	S b i C	*c C*	65	4112
	S b i D	*c D*	65	3943
	S b i G	*c G*	65	3112
2	*Te ah d e ha e i S- e h a sfe ase s b i A*	*A*	84, 11	1429, 230
	S b i B	*B*	84, 11	608, NM
	S b i C	*C*	84, 11	5359, NM
	S b i D	*D*	84, 11	5080, 173
	S b i E	*E*	84, 11	6057, 5128
	S b i F	*F*	84, 11	2948, NM
	S b i G	*G*	84, 11	470, 50
	S b i H	*H*	84, 11	1720, 167
3	*C e e F420-de e de N5N10- e h e e e ah d e ha e i ed c ase*	*e 1*	5	2652
		e 2	11	393
4	*Me h e e e ah d e ha e i deh d ge ase*	*d*	272	66
5	*Me he e ah d e ha e i c c h d ase*	*ch*	13	4872
6	*F e ha f a e ah d e ha e i N-f a sfe ase*	*f*	1, 1	1581, NM
7	*F e ha f a deh d ge ase s b i A*	*f dA*	95, 243	6030, NM
	S b i B	*f dB*	95	3030
	S b i C	*f dC*	95, 243	7392, 7392
	S b i D	*f dD*	95, 243	4536, NM
	S b i E	*f dE*	NI	NM
	S b i F	*f dF*	225	346
	S b i G	*f dG*	84	193
	S b i H	*f dH*	NI	NM

Abbreviations: NI, not identified; NM, no mapping (transcripts to this SCA gene).Numbers in M25 represent the scaffold ID of the aggregate genome assemblies, and numbers in transcriptome column represent the RPKM (reads per Kb per million reads) value of each gene.

the enrichment was generated, methane oxidation instead of methane production is solely observed.

From our M25 assembly, two complete *e* genes were identified with 49% sequence identity and designated as *e -1* and *e -2*. Phylogenetic analysis of Mer clearly showed a vertical transfer of *e* gene sequences within different orders of methanogenic archaea (Figure 2). The gene *e -1* clustered closely with that from ANME-2d (Haroon *e a .*, 2013), classified within the Methanosarcinales, to which ANME-2 belongs, whereas *e -2* substantially differed from *e* genes of all known methanogenic archaea. The phylogenetic position of *e -2* is still unclear, it may come from an unknown archaeon through horizontal gene transfer. The *e* -1 gene was adjacent to *f F* (the gene encoding F_{420}-phenazine oxidoreductase subunit F). This type of gene organization is conserved in *Me ha sa ci a* species (Baumer *e a .*, 2000; Kulkarni *e a .*, 2009) (Supplementary Figure S5). The finding of canonic *e* is suggesting that the conversion of methyl-H_4MPT to methylene-H_4MPT in ANME-2 is the reversal of the corresponding reaction operating forward in methanogenesis; the same reversal reaction was not identified in ANME-1 (Hallam *e a .*, 2004; Meyerdierks *e a .*, 2010). The *e* -2 gene was adjacent to one of two copies of (the gene encoding tetrahydromethanopterin *S*-methyltransferase), which was designated as -2 (Supplementary Figure S6). The whole sequence identity between two *se s* is ~40%, with the highest identity (45%) found between *A* and the lowest (27%) between *C*. Considering the low sequence similarity between the two sets of genes, it is unlikely that one set of the genes is an artifact resulting from MDA. The Mtr methyltransferase complex MtrA-H catalyzes methyl transfer from N5-methyltetrahydromethanopterin (or N5-methyltetrahydrosarcinapterin) to coenzyme M in methanogenic archaea (Thauer, 1998). The presence of two sets of *e* and in this ANME-2a is intriguing and, to our knowledge, there is no report of an organism possessing two complex sets. It is still a question of whether these two sets of *e* and come from a single organism, as M25 contained multiple cells that may have undertaken extensive genomic differentiation. Nevertheless, the data presented here demonstrate that ANME-2a archaeal group harbors two sets of *e* and genes. The expression levels of *e* -1 and *-1* were nearly one order of magnitude higher than the expression levels of *e* -2 and -2, respectively, as evaluated by the gene expression RPKM values (Table 1), suggesting a major role of *e -1* and *-1* in methane oxidation. It remains unclear whether *e -2* and *-2* take part in the same enzymatic reactions as *e -1* and *-1* or have other functions and why ANME-2a maintains an additional set of these genes.

H_2-i de e de e e g -c e i g echa is s

No canonical hydrogenase was found in either genomic or transcriptomic data, and thus ANME-2a from our

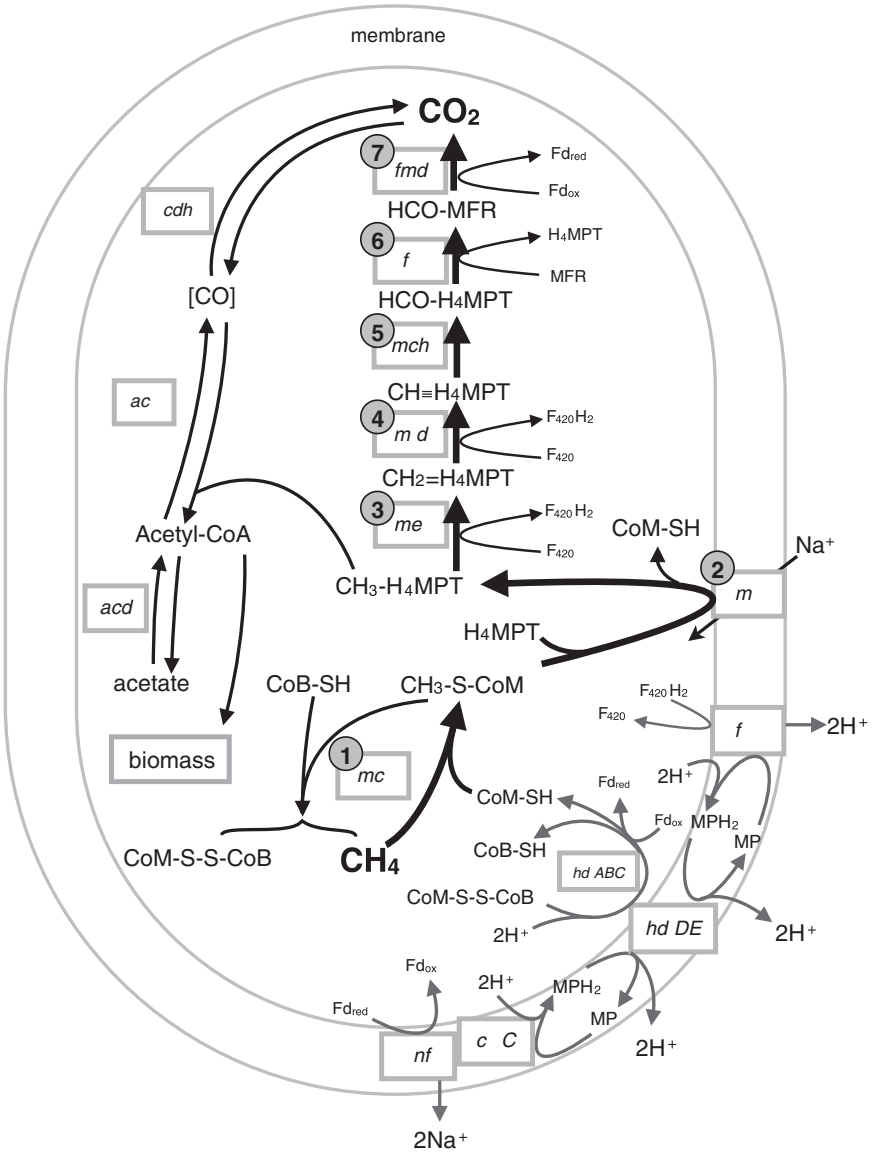

Figure 1 The proposed methane-oxidizing pathway and energy-converting mechanisms in ANME-2a. Only positive gene identifications were displayed (in boxes). The carbon flow was demonstrated with black arrows; the electron flow was indicated with grey arrows. Detailed information of methanogenesis-associated genes is displayed in Table 1, and the names of genes involved in electron transport are displayed in Supplementary Table S3.

enrichment is most probably performing AOM without H_2 as the intracellular electron carrier or intercellular electron shuttle to SRB. Instead, all of the components in the energy-conserving electron transport pathways of non-H_2-utilizing methanogenic archaea, in particular genes for the membrane-associated Coenzyme B–Coenzyme M heterodisulfide reductase HdrDE, genes for CytC (cytochrome *c*) and genes for the Rnf (Rhodobacter nitrogen fixation) complex, which are missing in the H_2-utilizing species, are all identified and expressed in ANME-2a (Supplementary Table S3). Therefore, versatile electron transfer pathways independent of H_2 are postulated within ANME-2a cells. Redox components F_{420}/ $F_{420}H_2$ (the oxidized/reduced forms of a 5′deazaflavin derivative with high concentrations in methanogenic archaea), Fd_{ox}/Fd_{red} (the oxidized/reduced forms of ferredoxin) and MP/MPH_2 (the oxidized/reduced forms of membrane-soluble methanophenazine (MP)) can be recycled to couple methane oxidation with adenosine triphosphate (ATP) synthesis through multiple pathways (Figure 1). For example, the oxidation of $F_{420}H_2$ can be completed via the $F_{420}H_2$:heterodisulfide oxidoreductase system while reducing CoM-S-S-CoB, comprising membrane-bound HdrDE and Fpo, as commonly found in *Me ha sa ci a* (Baumer *e a .*, 2000; Deppenmeier and Mueller, 2008; Kulkarni *e a .*, 2009). Similarly, Fd_{red}/Fd_{ox} is cycled via a Fd_{red}:heterodisulfide oxidoreductase

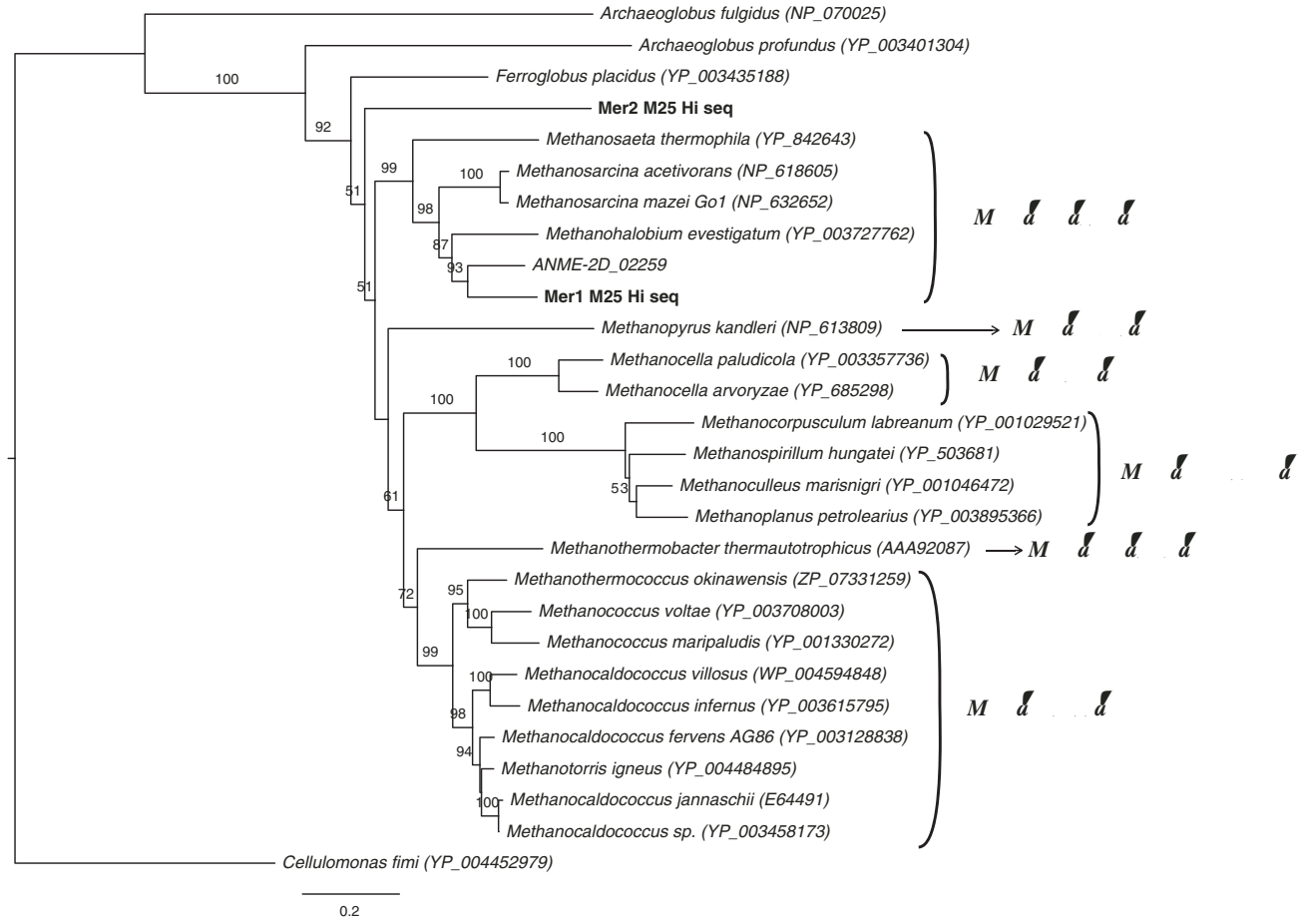

Figure 2 The maximum likelihood tree showing the deduced amino acid sequences of *e* -1 and *e* -2 genes to the selected reference sequences. A total of 25 full-length amino acid sequences of *e* were aligned. Bootstrap values were based on 1000 replicates and shown at the nodes. Mer-1 and Mer-2 from M25 identified in the single-aggregate genome were highlighted, with their detailed information displayed in Table 1.

system including CytC, the Rnf complex and HdrDE that results in a sodium gradient that is exchanged for a proton gradient (Ferry and Lessner, 2008; Wang *e a .*, 2011). This is in agreement with the previous studies that the electron transport pathways in non-H_2-utilizing marine methanogenic species of the *Me ha sa ci a* genus are fundamentally distinct from those in H_2-utilizing freshwater species (Guss *e a .*, 2009). For example, the non-H_2-utilizing, marine methanogen *Me ha sa ci a ace i a s* replaces the Ech hydrogenase with the Rnf complex to generate a transmembrane ion gradient for ATP synthesis, which has been interpreted as an adaptation strategy of this species to the marine environment (Wang *e a .*, 2011). In addition, cytoplasmic HdrABC, another type of heterodisulfide reductase, has been detected with multiple copies (Supplementary Table S3). From the phylogenetic analysis, our ANME-2a-originated *hd A* genes were placed into clades with methyltrophic or/and acetoclastic methanogenic archaea but not H_2-dependent methanogenic archaea (Supplementary Figure S7) (Buan and Metcalf, 2010). We note here that the identified electron transport components HdrDE and the Rnf complex in ANME-2a have not been reported in ANME-1 metagenomes (Hallam *e a .*, 2004; Meyerdierks *e a .*, 2010), indicating that the methane-oxidizing archaea ANME-1 and ANME-2 may have evolved distinct electron transport pathways or strategies, which warrant detailed investigations in the future.

Ace a e- e ab i i g ssibi i

Interestingly, Acd (ADP-forming acetyl-CoA synthetase), a recently identified enzyme in archaea that catalyzes the conversion of acetyl-CoA to acetate coupled with the conversion of ADP to ATP ($\text{acetyl-CoA} + \text{ADP} + \text{P}_\text{i} \rightarrow \text{acetate} + \text{ATP} + \text{CoA}$) (Brasen *e a .*, 2008), was detected in the single-aggregate genome and the metatranscriptome (Supplementary Table S4). This finding supports the possible integration of acetate to acetyl-CoA at the cost of ATP before entering reverse methanogenesis and/or biomass synthesis. As a corollary, if acetate from acetyl-CoA is excreted, it could be utilized by SRB. The potential for ANME-2a to metabolize acetate is further supported by its possession of an

Rnf complex in the energy-converting system that has only been found in non-H_2-utilizing acetoclastic *Me ha sa ci as* species (Ferry and Lessner, 2008; Wang *e a .*, 2011). The utilization of acetate as an intermediate contradicts results obtained from *i i* experiments (Nauhaus *e a .*, 2005), but is consistent with extreme ^{13}C depletion in acetate with $\delta^{13}C$ values approaching $-90‰$ in some methane seep sediments (Heuer *e a .*, 2006). The possibility that the ANME-2a from our enrichment utilizes other methylated C1 compounds such as methylamines or methylsulfides for AOM (Moran *e a .*, 2008) is not supported by this study (Supplementary Table S4).

Ec gica a d e i a i ica i s

Although we cannot entirely rule out the potential presence of hydrogenase because of incompleteness of our current ANME-2a genome, the results from this study suggest that the interspecies hydrogen transfer does not play a major role in forming syntrophic consortia of ANME-2a and bacteria in cold seep ecosystems. It is commonly assumed that interspecies electron transfer is a key process in shaping syntrophic communities, especially in methanogenic, anaerobic methanotrophic, sulfate-reducing and even subsurface ecosystems (Nealson *e a .*, 2005; Stams and Plugge, 2009). The utilization of H^+/H_2 as the electron shuttle is facilitated by the relatively easy transfer of protons and the lacking requirement of enzymes with complex active centers (Vignais and Billoud, 2007). On the other hand, the low midpoint redox potential of this redox couple ($E^\circ = -414$ mV, which is lower than those of NAD^+/NADH, FADH/$FADH_2$, Fd_{ox}/Fd_{red}) causes an energetic problem unless hydrogen is continuously produced and removed by two distinct organisms. A recent publication described a mechanism in which zero-valent sulfur produced via sulfate reduction by ANME-2 served as intermediate for an ANME/SRB syntrophic community and was disproportionated by SRB (Milucka *e a .*, 2012). It needs to be tested in the future whether sulfur/acetate rather than H_2 serves as electron shuttle between ANME-2a and its SRB partner.

This report demonstrates that ANME-2a oxidizes methane to CO_2 with genes from versatile electron transport pathways that do not likely involve H_2, and that ANME-2a possesses the potential ability to produce and utilize acetate. It is shown that ANME-2a in our enrichment clearly shares similarities with members of the Methanosarcinales, in particular with non-H_2-utilizing acetoclastic *Me ha sa ci a* species. Both ANME-2 and *Me ha sa ci a* contain cytochrome and MP and

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