Genomic and enzymatic evidence of acetogenesis by anaerobic methanotrophic archaea

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Anaerobic oxidation of methane (AOM) mediated by anaerobic methanotrophic archaea (ANME) is the primary process that provides energy to cold seep ecosystems by converting methane into inorganic carbon. Notably, cold seep ecosystems are dominated by highly divergent heterotrophic microorganisms. The role of the AOM process in supporting heterotrophic population remains unknown. We investigate the acetogenic capacity of ANME-2a in a simulated cold seep ecosystem using high-pressure biotechnology, where both AOM activity and acetate production are detected. The production of acetate from methane is confirmed by isotope-labeling experiments. A complete archaeal acetogenesis pathway is identified in the ANME-2a genome, and apparent acetogenic activity of the key enzymes ADP-forming acetate-CoA ligase and acetyl-CoA synthetase is demonstrated. Here, we propose a modified model of carbon cycling in cold seeps: during AOM process, methane can be converted into organic carbon, such as acetate, which further fuels the heterotrophic community in the ecosystem.
Cold seeps are areas, where hydrocarbon-rich fluid seeps up from below the ocean floor at fluid-flow velocities of centimeters to meters per year, often as hydrogen sulfide and methane. They are common along continental margins worldwide and can be thought of as hot spots of a certain habitat type, providing niches that are strongly different from the surrounding seafloor. The chemosynthetic microorganisms inhabiting cold seeps convert the methane into organic matter and carbon dioxide to generate energy. Anaerobic oxidation of methane (AOM) coupled to sulfate reduction (SR) is the primary energetic process in cold seeps and is catalyzed by a consortium of anaerobic methanotrophic archaea (ANME) and sulfate-reducing bacteria (SRB) of the Deltaproteobacteria. The overall AOM-SR reaction $\text{CH}_4 + \text{SO}_4^{2-} \rightarrow \text{HS}^- + \text{HCO}_3^- + \text{H}_2\text{O}$ generates a Gibbs free energy of only $-20$ to $-40$ kJ/mol of methane oxidized, which is shared between ANME and SRB. Therefore, AOM is considered one of the least exergonic processes supporting life. Meanwhile, the methane-fueled anoxic sediments above gas vents and gas hydrates are one of the most populated marine microbial ecosystems, reaching $10^5$–$10^{10}$ cells/cm$^3$. In addition, a large number of metazoans (such as tubeworms, bivalves, etc.) depend on the energy type, providing niches that are strongly different from the surrounding seafloor or at the fluid seeps up from below. Therefore, we propose a conceptual model of carbon cycling in cold seeps, where acetate release from AOM process is supported by genomic and enzymatic evidence. This AOM-enriched community was subsampled and incubated with $85$ mM dissolved $\text{^{13}C-CH}_4$, which is the calculated methane saturation concentration under $8$ MPa methane partial pressure, and $30$ mM dissolved $\text{^{12}C-HCO}_3^-$ as the carbon sources. Under $8$ or $30$ MPa incubation pressure, the sulfate concentration increased from $9.15$ $\mu$M to $92.01$ or $15.75$ $\mu$M respectively, the $\text{^{13}C-HCO}_3^-$ abundance among total $\text{HCO}_3^-$ in the liquid phase increased from natural abundance of $1.08\%$ to $1.31\%$, the final concentration of $\text{^{13}C-acetate}$ (with molecular weight of $61$) reached to $0.45$ or $0.32$ $\mu$M, respectively (Table 1). $\text{^{13}C-acetate}$ with molecular weight of $62$ was not detected. Therefore, it is likely that $\text{^{13}C-acetate}$ was mainly converted from $\text{^{13}C-CH}_4$ directly, rather than from $\text{^{13}C-CO}_2$ through acetogenesis.

The microbial communities of each incubation stage were analyzed based on the $16$S rRNA gene sequence data. In archaeal community, ANME-2a and Marine_Benthic_Group_D (MBG-D) were the main groups, which contribute $36.3$–$52.3\%$ and $27.7$–$49.3\%$, respectively (Fig. 1b). On the other hand, the bacterial community was relatively more diverse than archaeal community; SRB accounted for less than $11\%$ with Desulfovarcina (3.9–7.3\%), Desulfofabaclla (0.1–0.2\%), SEEP-SRB1 (0.1–0.2\%), and uncultured Desulfiobacteraceae (2.2–4.9\%), while the dominant groups were Clostridiales (55.0–59.8\%), Pseudomonadales (6.8–8.8\%), Oceanospirillales (3.0–7.0\%), Desulfuromonadales (3.7–5.6\%), and Hydrogenophilales (2.3–5.0\%) (Fig. 1b). The ANME and SRB cells ranged from $4.2 \times 10^3$ to $3.1 \times 10^4$ cells/mL and $1.3 \times 10^4$ to $1.4 \times 10^5$ cells/mL, respectively (Fig. 1b).

Genomic evidence of methane to acetate conversion by ANME-2a. To verify whether ANME-2a has the metabolic potential to convert methane into acetate and to identify the key genes involved, a metagenomic approach was applied. DNA was extracted from the biomass after each incubation stage. A total of $126,043,665$ reads passed the quality control criteria. De novo assembly of the metagenomic reads and binning by tetranucleotide signatures revealed a total of five metagenome-assembled genomes (MAGs) belonging to ANME with high quality (Supplementary Table 1). The taxonomic identity of each MAG was verified by the construction of a phylogenetic tree based on whole-genome information (Supplementary Fig. 2). The details of the data processing are described in the "Methods" section. A

**Results**

A microbial community fueled solely by the AOM process. An AOM-enriched community, which originated from a mud volcano in Gulf of Cadiz, was incubated in a simulated cold seep ecosystem where methane and sulfate were supplied as the only energy source within the ecosystem for 8 years prior to this research. The incubation was performed in a continuous high-pressure reactor with independent control of the methane partial pressure and incubation pressure. To test the stability of this AOM-enriched community, the incubation pressure was changed every two months: 8, 15, 30, and 8 MPa (II). The detailed incubation conditions are described in the "Methods" section. The chemical composition in the incubation system was measured every two days to track AOM activity (Supplementary Fig. 1). Approximately $0.99$–$2.30$ $\mu$mol sulfide production per day was observed in all the tested conditions except the one at $30$ MPa, when the community was likely disturbed by such high pressure and a negative average sulfide production was observed ($-12.26$ $\mu$mol sulfide production per day) (Fig. 1a). Moreover, throughout the incubation period, approximately $1.39$–$2.56$ $\mu$M acetate was detected in the slurry, and the highest acetate accumulation was observed under $8$ MPa methane and $8$ MPa incubation pressure (Fig. 1a). The microbial conversion of methane into acetate was further confirmed by isotope-labeling experiments. This AOM-enriched community was subsampled and incubated with $85$ mM dissolved $\text{^{13}C-CH}_4$, which is the calculated methane saturation concentration under $8$ MPa methane partial pressure, and $30$ mM dissolved $\text{^{12}C-HCO}_3^-$ as the carbon sources. Under $8$ or $30$ MPa incubation pressure, the sulfide concentration increased from $9.15$ $\mu$M to $92.01$ or $15.75$ $\mu$M respectively, the $\text{^{13}C-HCO}_3^-$ abundance among total $\text{HCO}_3^-$ in the liquid phase increased from natural abundance of $1.08\%$ to $1.31\%$, the final concentration of $\text{^{13}C-acetate}$ (with molecular weight of $61$) reached to $0.45$ or $0.32$ $\mu$M, respectively (Table 1). $\text{^{13}C-acetate}$ with molecular weight of $62$ was not detected. Therefore, it is likely that $\text{^{13}C-acetate}$ was mainly converted from $\text{^{13}C-CH}_4$ directly, rather than from $\text{^{13}C-CO}_2$ through acetogenesis.

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complete metabolic pathway converting methane to acetate was identified in the ANME-2a MAGs (Fig. 2) (Supplementary Data 1). The oxidation of methane yielding CH$_3$-S-COM is catalyzed by the enzyme Mcr, and then, the methyl group is transferred to H$_2$SPT (tetrahydrosarcinapterin) by membrane-bound methyltransferase (Mtr) to form CH$_3$-H$_2$MPT. Under the function of the reversible CO dehydrogenase/acetyl-CoA synthetase (Cdh) complex, acetyl-CoA is produced from CH$_3$-H$_2$MPT. Moreover, the genes $acs$ and $acd$, which encode AMP-forming acetyl-CoA synthetase (Acs) and ADP-forming acetate-CoA ligase (Acd), respectively, were identified in the ANME-2a genome. $acs$ is widely distributed in all three domains of life and is considered as the predominant enzyme in the acetate to acetyl-CoA reaction.$^{20}$ Acd is a novel enzyme in acetate-forming archaea that catalyzes the conversion of acetyl-CoA to acetate and couples this reaction with the synthesis of ATP (acetyl-CoA + ADP + $P_i$ $\rightarrow$ acetate + $ATP$ + CoA). Acd is considered specific to Archaea, although it is also found in a few genomes of bacteria, such as the propionic acid-producing bacteria $Propionibacterium acidipropionici^{21,22}$. In addition, the ANME-2a genomes identified in the present study also possess a complete reversal of the CO$_2$-dependent methanogenesis pathway, Embden-Meyerhof-Parnas gluconeogenesis pathway and Wood-Ljungdahl pathway. The acetyl-CoA generated in all of the above diverse metabolic reactions can be converted to acetate via Acd, which would allow ANME-2a to produce energy via substrate-level phosphorylation.$^{23}$ Furthermore, 28 $acd$ genes were identified in metagenomes mainly belonging to ANME-2a, MBG-D, and methanogens. $\text{However}$, only ANME-2a presents as a high abundance (55.2–80.0%) and has been known as methane oxidizer, thus to be expected as the major player to convert methane into acetate in this simulated cold seep ecosystem (Supplementary Data 2).

The relationship between ANME and bacteria are further interpreted from the metagenomic data. Unlike in ANME-2d or ANME-1, $ech$ or $mvh$ gene, the key genes to convert energy from hydrogen, have not been identified in ANME-2a MAGs even though the completenesses are over 99%. Therefore it is unlikely that hydrogen is served as electron shuttle between ANME-2a and SRB. Meanwhile, $fr$ gene encoding coenzyme F420-dependent dissimilatory sulfite reductase has been identified, suggesting the possibility of ANME-2a oxidizing methane using sulfite as electron acceptor without a partner. Moreover, MAGs belonging to Desulfobacterales, Desulfotibacteria, and Burkholderales possess genes involved in acetate utilizing pathway and sulfate/thiosulfate reduction pathway in their genomes, which makes them potential candidate partners of ANME-2a (Supplementary Data 3).

Enzyme activities of Acd and Acs. To further verify the metabolic capacity of ANME-2a to produce acetate, we performed heterologous gene expression and activity assays on the two key enzymes Acd and Acs at the final step of acetate formation. Gene sequences were extracted from Bin-8 MPa, and their annotations were verified by constructing phylogenetic trees using IQ-TREE (Fig. 3). The Acd sequence identified in this ANME-2a genome was clustered with those of $Pyrococcus$ sp., which have been verified by heterologous gene expression in previous research.$^{24-26}$ For Acd, the genes coding for the alpha and beta subunits of Acd were synthesized and subsequently cloned into the pET-28a protein expression vector between the sites

diagram

Table 1 Sulfide, $^{13}$C-acetate concentrations and $^{13}$C-$\text{CO}_2$ abundance after incubation in the fed-batch experiment.

| Dissolved $^{13}$C-$\text{CH}_4$ concentration (mM) | Incubation pressure (MPa) | Sulfide concentration after incubation (µM)$^2$ | $^{13}$C-$\text{CO}_2$ abundance among total $\text{CO}_2$ after incubation$^3$ | $^{13}$C-acetate concentration after incubation (µM)$^3$ |
|-----------------------------------------------|--------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|
| 85                                           | 8                        | 92.01 ± 5.82                                  | 1.31% ± 0.00%                                | 0.45 ± 0.27                                  |
| 85                                           | 30                       | 15.75 ± 3.72                                  | 1.31% ± 0.01%                                | 0.32 ± 0.03                                  |

*Data are mean values with standard deviations from triplicate incubations.
The activity of Acd was introduced upstream of the beta subunit. SDS-PAGE revealed an alpha subunit and beta subunit with apparent molecular masses of 55 kDa and 25 kDa, respectively (Fig. 4a). Details about the protein expression and enzyme activity detection are described in the Methods section. The purified enzyme showed catalytic activity for acetate formation. The affinity to acetyl-CoA was $K_m = 31.28 \mu M$ (Fig. 4b), similar to the $K_m = 37 \mu M$ from *Methanocaldococcus jannaschii* Acd$^{23}$. *M. jannaschii* is a thermophilic methanogenic archaeon using CO$_2$ and H$_2$ as primary energy sources and can de novo synthesize acetate and pyruvate from CO$_2$. We also cloned and expressed the ANME-2a *acs* gene in *Escherichia coli*. The gene coding for Acs was synthesized and subsequently cloned into the vector pET-28a between the NdeI and BamHI sites. The enzyme was overexpressed in Rosetta (DE3) and obtained by protein purification with a molecular mass of 70 kDa (Fig. 4c). Normally, Acs is considered as the key enzyme for acetyl-CoA formation from acetate, CoA and ATP (acetate + ATP + CoA $\rightarrow$ acetyl-CoA + AMP + PP$_i$), and the $K_m$ values for acetyl-CoA are 2-fold higher than those of Acd$^{28,29}$. However, the Acs purified in this study revealed a higher affinity for acetyl-CoA ($K_m = 8.5 \mu M$) than for acetate ($K_m = 0.49 \mu M$) (Fig. 4d, e), which indicates a preference for acetate production in this case. The enzyme assays of Acd and Acs provide strong evidence to conclude that the conversion from acetyl-CoA to acetate is favorable for ANME-2a.

Wide distribution of the *acd* gene in cold seep archaea. To explore the distribution of archaeal acetylgenesis in cold seep environments, a phylogenetic tree of *acd* genes from a total of 77 cold seep environments was constructed (Supplementary Fig. 3). A collapsed version of this phylogenetic tree was built to highlight the main taxa (Fig. 5). Among all the ANME and methanogens, only ANME-2, Methanosarcinales, and Ca. Verstraetaarchaeota contained *acd* genes. In particular, the ANME-2 from methane seepages and subsea floor sediment formed a separate cluster. No ANME-1 or ANME-3 were found to possess the *acd* gene in these metagenomes. Meanwhile, *acd* genes were identified in Archaeoglobi, Ca.Bathyarchaeota, Crenarchaeota, Thermococci, and Thermoplasmata (including MBG-D), indicating a wide distribution of acetate metabolism among cold seep archaea.

**Discussion**

In this research, a simplified and stable ecosystem supported solely by the AOM process was successfully maintained using a high-pressure continuous bioreactor, allowing us to precisely monitor the ecological dynamics therein. Because of the low solubility of methane at ambient pressure and the extremely low affinity of AOM process for methane ($K_m$ of 37 mM), high-pressure bioreactors have been applied in AOM studies and obtained higher AOM activity$^{19}$. The high-pressure continuous bioreactor applied in this experiment can control the methane partial pressure and incubation pressure to simulate cold seep eruption at different depths. Furthermore, the continuous flow can remove accumulated metabolites such as CO$_2$ and sulfide in a timely manner, alleviating their inhibition of AOM activity and avoiding the toxic effect of sulfide on sulfate-reducing bacteria$^{30}$. The stable and relatively high AOM activities observed during a one-year incubation demonstrated a healthy AOM-supported ecosystem with a microbial community structure similar to those found in nature. For example, the communities retrieved from 23...
globally distributed methane seeps were dominated by mainly methane-oxidizing archaea (e.g., ANME-2a/b: ~0–50%) and heterotrophic bacteria (e.g., Gammaproteobacteria: ~0–30%; Alphaproteobacteria, ~0–5%; Clostridia: ~0–50%)31, to which high percentages of species in our enriched community were attributed (Fig. 1b). Meanwhile, all seeps harbor extremely diverse bacterial communities including dozens of groups, and approximately 70% of the bacterial community is heterotrophic31. The quick consumption of acetate by the heterotrophic population may keep the acetate concentration low, thus the Gibbs free energy to form acetate from methane and bicarbonate is negative, e.g., under our cultivation condition is down to −4.6 kJ/mol. This makes the acetogenesis process energetically beneficial to ANME-2a cells, although it is insufficient to sustain microbial life. AOM process remains as the major energy source to ANME-2a.

The genomic and enzymatic evidence provided by this study of acetate production by ANME-2a could explain the existence of the large heterotrophic communities in cold seep ecosystems, as acetate is a popular carbon source for marine ecosystems. In surface water, acetate has been identified not only as an energy source but also as an important carbon source incorporated into biomass (accounting for 58% of total uptake) by heterotrophic bacteria32. In anoxic sediments, acetate was found to be utilized as an energy and carbon source, and approximately 10–76% was assimilated into biomass for cell growth10,33. The archaeal acetate-producing enzyme Acd represents the major energy-conserving reaction during the fermentation of sugar, peptides, and pyruvate to acetate34. Because ANME-2a is phylogenetically related to the acetotrophic methanogen group Methanosarcinales and performs the AOM process via a reverse methanogenesis pathway, and acd gene has been identified in ANME-2a and ANME-2d MAGs17,35,36, it is reasonable to expect the intracellular conversion of methane into acetate by ANME-2. In addition to acetate, Wegener et al. proposed that during the AOM process, some methylated compounds (i.e., methanol, methylamines, and methyl sulfides) may leak from ANME and thus support the growth of methanogens37. The production of organic compounds by ANME may be the result of unbalanced enzymatic activities in the reverse methanogenesis pathway. For example, under the scenario that the production of CH₃-SCoM and CH₃-H₄MPT is faster than their consumption, the generation of acetate is easily achieved. As recently reported, ANME-2d produces acetate indirectly from methane, but via intracellular storage compound, when nitrate or nitrite was supplied as an electron acceptor under a rate-limiting condition38. In the natural seeping environments in deep sea, the dissolved methane concentration is much higher than the sulfate reduction rates indirectly from methane, but via intracellular storage compound, when nitrate or nitrite was supplied as an electron acceptor under a rate-limiting condition38. In the natural seeping environments in deep sea, the dissolved methane concentration is much higher than the sulfate reduction rates.
considering the possibility of producing organic carbon during the AOM process, methane consumption through the AOM process may be underestimated. In our high-pressure continuous incubation experiment, when optimal AOM activity was observed, approximately $1.7 \times 10^7$ cellular growth (~0.85 μg carbon) per day were observed (Fig. 1b). Assuming 70% of the cells are heterotrophs and 10–76% of consumed acetate is channeled into biosynthesis according to the previous reports, they would need 0.78–5.95 μg organic carbon per day. Assuming all the organic carbon originated from methane, and considering approximately 24 μg methane-carbon consumption per day (calculated from the AOM activity in Fig. 1a), 3–25% of the total consumed methane is converted to acetate or other organic compounds that have been previously neglected. Based on the previous calculation, the AOM process consumes up to 300 Tg methane/year, equivalent to ~88% of the methane released from subsurface reservoirs. Considering the missing step of methane conversion to acetate, methane consumption via AOM could be even greater. Because methane is one of the most powerful greenhouse gases, revisiting the methane budget of cold seeps, where acetate release from AOM process is considered significant in terms of predicting global climate change.

In conclusion, cold seep environments host abundant and diverse microbial communities, and nearly 70% of the bacteria are heterotrophs. Metabolic reconstruction of ANME-2a and heterologous expression and activity assays of the ADP-forming acetate-CoA ligase gene \( acd \) from ANME-2a demonstrate the capability of ANME-2a to produce acetate during methane oxidation. Based on the diversity and metagenomic analysis, the acetate can be supporting a large number of heterotrophic bacteria besides sulfate reducers, such as Firmicutes, Chloroflexi, Actinobacteria, Atribacteria, and Bacteroidetes. Based on these results, we propose a conceptual model of carbon cycling in cold seeps, where acetate release from AOM process is considered significant. Our findings expand the metabolic repertoire of ANME-2a and increase understanding of the carbon cycle in cold seep ecosystems.

**Methods**

**Continuous high-pressure incubation and activity analysis.** The inoculum was originally from Captain Aryustin Mud Volcano (35° 39.700'N; 07° 20.012'W) at a water depth of 1200 m and had been incubated in a continuous high-pressure bioreactor for years before ANME-2a enrichment was obtained. In this study, the methane partial pressure and the incubation pressure were independently controlled in a flow-through high-pressure system. Suggested by the calculated methane affinity for the same ANME-2/SRB community, 8 MPa of methane was provided in the system to achieve the maximum AOM rate. The incubation pressures were further set as 8, 15, and 30 MPa accordingly to mimic the cold seep environments at different water depths and to create dynamics to the microbial community inside the reactor without changing the substrate concentrations. Every three hours, methane was supplied to the reactor at a flow rate of 0.1 mL/min. Incubation was performed at 15 °C, and the methane pressure and the incubation pressure were independently controlled in a flow-through high-pressure system. In this study, we propose a conceptual model of carbon cycling in cold seeps, where acetate release from AOM process is considered significant in terms of predicting global climate change.

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**Fig. 5** Distribution of acd genes among archaea from cold seeps. A total of 113 acd genes are identified from 77 metagenomic datasets of cold seep samples. An uncollapsed tree is shown in Supplementary Fig. 3. Acd gene from ANME-2a is marked as red asterisk.

**Fig. 6** Proposed conceptual diagram of carbon cycling in cold seeps. A proposed conceptual model of carbon cycling in cold seeps: during the classic anaerobic oxidation of methane process, methane can be converted into organic carbon, such as acetate, which further fuels the heterotrophic community in the ecosystem.
DNA extraction and 16S rRNA gene analysis. DNA was extracted and purified according to the modified SDS-based method described by Natarajan et al.57. Purified DNA was dissolved in 60 μL of ddH2O and stored at −80°C until use. The V4 region of bacterial 16S rRNA genes was amplified by polymerase chain reaction (PCR) with the primer pair 53F (5′-GCTGACGGTCAACAACTCCTAG-3′) and 558R (5′-GGACTACCAGGGTATCTAATCCTGTT-3′)48. PCR products of each sample were quality- and length-checked using an Illumina HiSeq X Ten platform using a 2 × 150-bp PE strategy at BGI Co., Ltd. (Shenzhen, China). The metagenomic reads from raw shotgun sequencing were trimmed with sickle (v1.33, https://github.com/najoshi/sickle) using the “pe” option and default settings. The DNA reads were assembled using a modified IDBA- UD (v1.1.1)55 with the following parameters: -p 4, -m 2, -min_contig 500, --step 4, --min_contig 500, --pre_correction, --seed_kmer 55, --mask 124, --mink 24. Then, the sequencing reads were mapped to the assembled scaffolds using Bowtie (v2.2.8)52. After that, the coverage of the assembled scaffolds was calculated by SAMTools (v1.3.1)52 and the assembly from MetaBAT (v2.12.1)52. Rummaging of the assembled metagenomic sequences was performed by both MetaBAT (v2.12.1)52 and MyCC59 with the meta option. The minimum scaffold length was 2.5 kb for MetaBAT and 1 kb for MyCC59. The quality and rough taxonomy were evaluated by CheckM (v1.0.9)60. Manual correction was performed with mmmgene55 according to coverage and GC content. The last step was repeated until the completeness was higher than 95% and contamination was lower than 5%. Protein-coding DNA sequences were determined using Prodigal (v2.6.3)61 with the “–meta” setting. Functional information for each predicted protein-coding DNA sequence was collected by a sequence of similarity searches against the KEGG62 and COG64 databases using DIAMOND63 with an e-value <1e−5.

Protein expression and purification. The genes coding for the alpha and beta subunits of Acd were synthesized by Shanghai RealGene Biotech using the E. coli BL21 (DE3) codon usage. The T7 expression was introduced upstream of the beta subunit of Acd. The two genes were subsequently cloned into the pET-28a protein expression vector between the sites NdeI and BamHI of the E. coli BL21 (DE3) -grovL vector, which was generally provided by Dr. Jun Luo, Xiamen University. The expression strain was induced with 0.2 mM isopropyl-β-D-thiogalactoside in a total volume of 1.0 L at 20°C for 10 h when the OD600 of the bacterial culture reached 0.8. The gene coding for Acs was synthesized by Shanghai RealGene Biotech and subsequently cloned into the vector pET-28a between the sites NdeI and BamHI of the E. coli codon usage. Rosetta (DE3) was used as the expression strain. The plasmid was obtained from TransGen Biotech. The E. coli strain Rosetta (DE3) was induced with 0.2 mM isopropyl-β-D-thiogalactoside in a total volume of 500 mL when the OD600 of the bacterial culture reached 0.5. The cells were harvested by centrifugation at 5000 rpm for 5 min and then lysed by sonication in lysis buffer (20 mM Tris-HCl, pH 8.0, 300 mM NaCl, 10% glycerol). The cell lysate was centrifuged at 10,000 rpm for 30 min at 4°C to separate the supernatant containing soluble protein, which was collected and applied to a Ni-NTA column. After washing with a buffer (20 mM Tris-HCl, pH 8.0, 300 mM NaCl, 40 mM imidazole, 10% glycerol), the fusion protein was eluted with another buffer (20 mM Tris-HCl, pH 8.0, 300 mM NaCl, 300 mM imidazole and 10% glycerol) and then dialyzed into storage buffer (20 mM Tris-HCl, pH 8.0, 300 mM NaCl, 50% glycerol). An empty pET-28a vector was used as a control. All chemicals were reagent grade and were purchased from Sigma–Aldrich (St. Louis, MO, USA).

Enzyme assay. Acs activity was measured in the direction of acetate formation by following PPI-dependent- and AMP-dependent HSCOa release from acetyl-CoA with the thiol reagent 5-thiobis(2-nitrobenzoic acid) (DTNB) and measuring the formation of thiophenolate anions at 412 nm (ε412 = 13.6 mM−1 cm−1)13. The reaction mixture was contained 200 mM Tris-HCl, pH 8.0, 1.25 M KCl, 1 mM MgCl2, 0.1 mM DTNB, 0.05 mM acetyl-CoA, 2 mM AMP, and 2 mM PPI by a previously modified procedure28. For the reverse reaction in the acetate decomposition direction, the activity was determined by coupling the reaction with malate dehydrogenase (Mdh) and citrate synthase (Cs) and monitoring the formation of NADH. Acd activity was also determined in the direction of acetate formation by following the ADP-dependent release of coenzyme A (HSCoa) from acetyl-CoA using the thiol reagent DTNB25. The assay mixture contained 20 mM Tris-HCl, pH 8.0, 40 mM KPi, 1 mM MgCl2, 0.1 mM DTNB, 0.05 mM acetyl-CoA, 2 mM AMP, and 2 mM PPI by a previously modified procedure28. For the reverse reaction in the direction of the enzyme reaction, and for both the forward and reverse reactions, the thiophenolate anion was measured at 412 nm (ε412 = 13.6 mM−1 cm−1)5.

Phylogenetic tree construction. To verify the acd gene sequence from ANME-2 MAG, acd gene expressed in E. coli from the NCBI database and the sequence in MAG 8 MPa were aligned with MAFFT-Lins (v7.313)59 and trimmed with trimAl (v1.4)59 using “automated1”. The final alignments has 513 trimmed columns. A phylogenetic tree was constructed by IQ-TREE (v1.6.6)50 with “LG + G4” model and 1,000 ultrafast bootstraps. To examine the distribution of acd genes among archaea from cold seeps, 77 cold seep metagenomic datasets were collected. If the dataset did not have available annotations in the IMG database, then its raw reads were trimmed with BBMap (v36.27)62. A total of 113 acd genes were identified by eggNOG-mapper27 against the eggNOG database from 77 cold seep metagenomic datasets. To compare the protein expression levels of Aad gene from Alabam from the NCBI database and the sequence in Suppl. info., both the gene expression was analyzed by MegaPhast (v1.0.6-hotfix1), and ORFs were called by Prodigal. And the taxonomy of each gene is determined by nr database with DIAMOND66. Next,
the alignment with 177 columns was used to construct a phylogenetic tree with the same method as above except with LG + I + G4 models and 1,000 ultrafast bootstraps (Supplementary Fig. 3). The tree of ANME-2 MAGs was constructed with a 700 amino acids-long concatenation of 122 archaeal marker genes (Supplementary Fig. 2). Maft-linsi was used for alignment for each gene set, and we have removed the positions with more gaps than 50% of the actual amino acid sequences. The tree was inferred by IQ-TREE (v1.6.6)70 with LG bootstraps (Supplementary Fig. 3). The tree of ANME-2 MAGs was constructed with the GTDB database79 and SILVA SSU132 database52, which are used as a classiﬁcation of anaerobic methanotrophs of the ANME-2 clade. Environ. Microbiol. 56, 2327–2340 (2010).

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