Title

Community Structure and Microbial Associations in Sediment-Free Methanotrophic Enrichment Cultures from a Marine Methane Seep

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Abstract

Consortia of anaerobic methanotrophic archaea (ANME) and sulfate-reducing bacteria (SRB) consume large amounts of methane and are the primary producers in marine methane seeps. Despite their importance in the carbon cycle, research on the physiology of ANME-SRB consortia has been hampered by the slow growth and complex physicochemical environment which the consortia inhabit. Here, we report successful sediment-free enrichment of ANME-SRB consortia from a deep-sea methane seep in the Santa Monica Basin, California. Anoxic density gradients and size selective filtration were used to separate ANME-SRB consortia from sediment particles and single cells to accelerate the cultivation process. Over a three year period, a subset of the sediment-associated ANME and SRB lineages, predominantly comprised of ANME-2a/2b and SEEP-SRB1/2, adapted and grew under defined laboratory conditions. Metagenome-assembled genomes from several enrichments revealed that ANME-2a, SEEP-SRB1 and *Methanococcoides* in different enrichments from the same inoculum represented distinct species, whereas other co-enriched microorganisms were closely related at the species level. This suggests that ANME, SRB and *Methanococcoides* are more genetically diverse than other members in methane seeps. Flow cytometry and sequencing of cell aggregates revealed that *Methanococcoides*, *Anaerolineales* and SEEP-SRB1 were overrepresented in the ANME-2a cell aggregates relative to the bulk metagenomes, suggesting their physical association and interaction. Overall, our study represents a successful case of pre-selecting microorganisms based on their physical characteristics to accelerate the cultivation of anaerobic slow-growing microorganisms from sediments, and enables detailed analysis, such as flow cytometry and

genomic sequencing, of ANME-SRB consortia and their associated community members in vitro.

Importance

Biological anaerobic oxidation of methane (AOM) coupled to sulfate reduction represents a large methane sink in global ocean sediments. Methane consumption is carried out by syntrophic archaeal-bacterial consortia and fuels an unique ecosystem, yet these interactions in the slow growing syntrophic consortia and between the associated community members remain poorly understood. The significance of our research is the establishment of in vitro enrichments performing AOM with sulfate by selective cultivation of microorganisms based on size, density and metabolism. By reconstructing microbial genomes and analyzing community composition of the enrichment cultures and single aggregates, we shed light on the microbial assemblage that thrive on methane and could be physically associated together. These in vitro enrichments will serve as the model system to study microbial interactions in marine methane seeps.

Introduction

At marine methane seeps, large amounts of the greenhouse gas methane rise up through the seafloor. These ecosystems harbor unique microbial assemblages that consume the majority of methane before it can be released to the atmosphere. With methane as the main energy source, anaerobic methanotrophic archaea (ANME) and sulfate-reducing bacteria (SRB) partners serve as the primary producers of these ecosystems. Together, ANME and SRB catalyze anaerobic oxidation of methane (AOM) with sulfate and consume 85-300 Tg methane annually (1). The

resulting organic carbon and metabolic products such as sulfide provide electron donors to fuel ecosystems in the shallow and deep ocean. Because ANME-SRB consortia are at the foundation of methane seep functioning, enhanced understanding of their physiology and interactions is key to understanding these ecosystems.

Cultivation of microorganisms remains an important step in understanding microbial physiology and interactions, the molecular basis for biogeochemical processes, and selecting for rare members from environmental samples. For microorganisms that live in sediments such as ANME and SRB, cultivation has the added benefit of separating microbes of interest from their complex physical, chemical and biological surroundings. Since the discovery of ANME-SRB or AOM consortia two decades ago, researchers have continued to apply different approaches to cultivate these slow growing consortia in vitro. Repeated dilution and consecutive sub-culturing have led to in vitro sediment-free enrichments of thermophilic ANME-1b, mesophilic and psychrophilic ANME-2a and ANME-2c archaeal lineages with HotSeep-1 and SEEP-SRB1 bacterial partners (2, 3), as well as HotSeep-1 bacteria (*Candidatus* Desulfofervidus auxilii) without ANME-1b (4). However, eliminating sediment requires long incubation times and numerous dilutions, especially for psychrophilic AOM consortia from marine methane seeps that double every 3-7 months (5–8). A previous study obtained a sediment-free enrichment of psychrophilic ANME-2a/2c and SEEP-SRB1 after 84 months using the repeated dilution and consecutive sub-culturing approach (3). To accelerate the cultivation process, bioreactors have been designed to simulate in situ methane seep conditions such as high pressure and continuous nutrient supply (7, 9–18). These bioreactors have led to microbial communities enriched in ANME-1 and ANME-2a/2c lineages (7, 13, 14, 18, 19), but their operation could be technically

challenging especially over the long-term. More recently, archaea belonging to the mesophilic ANME-2c partnered with *Desulfobulbaceae*-affiliated SEEP-SRB2 were cultivated from a shallow coastal methane seep in the Mediterranean after separating the AOM consortia from coarser sand grains by shaking and settling (20). This approach was successful because of the loose association between the microbial consortia and their sandy habitat (20, 21). This physical-separation method is much simpler than bioreactor-based approaches and accelerated the cultivation process, but simple shaking is incompatible with separating AOM consortia in deep-sea clay-rich methane seep sediments due to their tight physical association with the fine-grain sediment matrix.

To generate a sediment-free enrichment of AOM consortia from deep-sea methane seeps, we used a density-based approach with mild sonication followed by size selective filtration under anoxic conditions to separate AOM consortia from their sediment matrix. A similar approach has been commonly used to dissociate paraformaldehyde or ethanol-fixed AOM consortia from sediment particles for microscopy analysis (22, 23) and adapted for flow cytometry sorting and 16S rRNA gene analysis without chemical fixation (24). Cultivation of viable AOM consortia in batch cultures supplied with methane and sulfate over a 3 year period followed by consecutive dilution over 2 years resulted in 4 sediment-free enrichments showing active sulfate-coupled AOM activity. We analyzed the community composition of these sediment-free enrichments using 16S rRNA gene amplicon analysis, fluorescence microscopy, metagenomics, and flow cytometry sorting and sequencing of individual AOM consortia.

Materials and Methods

Sediment collection

Methane seep sediment was collected from Santa Monica Basin (lat. 33 47.3388, long. 118 40.0775) at 860 m water depth (in situ temperature 4 °C) during a research cruise organized by the Monterey Bay Aquarium Research Institute (MBARI) on board the R/V *Western Flyer* using the ROV *Doc Ricketts* on May 7, 2013. The intact sediment core used for the experiments described here (push core 76) was collected under a white textured microbial mat and 15 cm in length. Sediments were extruded from the push core liner within a few hours of collection, and transferred into whirlpak bags in 3 cm horizons under a stream of argon, heat-sealed in a mylar bag flushed with argon for 5 minutes and stored at 4 °C for transport back to the laboratory. In the laboratory on June 18, 2013, the sediment horizons were then transferred into 0.5 L pyrex bottles sealed with butyl rubber stoppers and incubated at 4 ºC with a methane headspace (0.2 MPa) and N_2-sparged 0.2 µm filter sterilized benthic seawater collected near the seep site (vol/vol ratio of 2:1 for seawater:settled wet sediment). This incubation ran for 30 days with one replacement of N_2-sparged 0.2 µm filter sterilized benthic seawater and 0.2 MPa methane headspace when sulfide levels exceeded 15 mM, as measured by methylene blue assay described previously (25). The 3-6 cm and 6-9 cm horizons (sediment #7047-7048) from this push core were determined to be most active based on sulfide production rates. A 50 ml subsample of sediment-seawater slurry was removed from #7047 and #7048 incubation bottles, and combined in a new 0.5 L pyrex bottle under methane headspace (0.2 MPa) on July 18, 2013. This sediment slurry served as the starting material for AOM consortia enrichment on the following day.

Aggregate selection and cultivation

Aggregate-forming AOM consortia were separated from sediment particles and single cells based on their density and size (Supplementary Figure 1). The selection procedure was modified from a previous microscopy protocol and performed on ice in anaerobic chamber with N_2:H_2 (95:5) atmosphere; all reagents and media were made anoxic by N_2 sparging for >10 min and equilibrated in anaerobic chamber overnight. For initial cultivation, the seawater media was described in (13).

In the percoll density separation procedure, we tested the addition of pyrophosphate, an ionic detergent to assist with dissociating cells from sediments (26), and sonicating the sediment slurry to separate cell aggregates from sediment particles while keeping the aggregates intact and viable. A set of 4 treatments were performed in separate Axygen 2-ml screw cap tubes (Corning Life Sciences SCT-200-C-S, Union City, CA, USA) in an anaerobic chamber with N_2:H_2 atmosphere (ca. 2-4% H_2, <1 ppm O_2). In treatment 1, no pyrophosphate addition or sonication was performed on the 5 ml sediment slurry in 1 ml aliquots. In treatment 2, no sonication was performed, and 0.5 ml pyrophosphate (0.2 μm filtered 100 mM) was mixed with 5 ml sediment slurry by repeated pipetting and incubated >10 min at room temperature prior to splitting into 1 ml aliquots. In treatment 3, 1 ml aliquots of 5 ml sediment slurry (without pyrophosphate) were sonicated for 3 times using a sonicating wand (Branson sonifier 150, BransonUltrasonics/Emerson Electric, Danbury, CT, USA) at setting 1.3 (2 W) for 10 s with >30 s rest in between on ice. In treatment 4, 0.5 ml pyrophosphate was mixed with 5 ml sediment slurry then sonicated in 1 ml aliquots (a combination of treatment 2 and 3). Following these treatments, 1 ml of N_2-sparged 0.2 μm filtered percoll solution (Sigma-Aldrich P4937, St Louis, MO, USA) was carefully injected into the bottom of the 2 ml tubes containing the sediment

slurry aliquots using a 1-ml syringe and 18G needle. The tubes were capped tightly and taken out of the anaerobic chamber for centrifugation at 16100 × g for 30 min at 4 ºC. The tubes were then immediately transferred back into the anaerobic chamber without mixing.

For size selective filtration, an autoclaved glass filtering tower with a 25 mm 3 µm Isopore polycarbonate membrane filter (EMD Millipore, Darmstadt, Germany) on top of a 25 mm 5 µm Durapore PVDF membrane as backing filter (Merk Millipore) was used. Prior to filtering the percolled samples, ~10 ml of N_2-sparged 0.2 µm filter sterilized seawater medium (13) was filtered through to wash the filtration setup. The top and middle layers of the percoll density gradient containing cells and cell aggregates (~1 ml) were then transferred to the filter tower and slowly filtered through with gentle vacuuming. Without letting the filter run dry, another 50 ml of the N_2-sparged 0.2 µm filter sterilized seawater medium was added to the filter tower to wash away the percoll and anything smaller than 3 µm. Filtration was stopped with ~5 ml of seawater and >3 µm sample fraction remaining in the filter tower. The sample seawater mixture was then repeatedly pipetted to dislodge any biomass stuck on the filter. The mixture was transferred into a 30-ml serum vial and capped with a black butyl rubber stopper. To retain any biomass that might still remain stuck on the filter, the polycarbonate filter was also transferred into a separate 30 ml serum vial with 1 ml of seawater media, and capped with a black butyl rubber stopper (Supplementary Figure 1). Prior to capping the butyl rubber stoppers, a subsample of 50 µl was taken from the serum vials to check for presence of AOM aggregates and successful separation from sediment particles via microscopy. Treatment 3 and 4 contained numerous intact AOM consortia, whereas treatment 1 and 2 without sonication contained few AOM consortia and therefore were discarded. An additional 20 ml N_2-sparged 0.2 µm filter sterilized seawater

medium was added to treatments 3 and 4 serum vials (4 total, Supplementary Figure 1) and the serum vial headspaces were sparged and pressurized with CH_4:CO_2 (95:5, 0.3 MPa). The serum vials were then incubated at 10 ºC in the dark.

For continuous dilutions and transfers of the enrichment cultures, we used a seawater medium recipe described in (27). The media salinity is similar to that of the initial cultivation, but contains 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) as the buffer instead of bicarbonate. Sulfide production in the cultures was monitored using the methylene blue assay described previously (25). The media was partially exchanged in the cultures when sulfide levels exceeded 10 mM. Specifically, this included removing approximately half of the volume of the overlying seawater in the serum vials was removed using a 4 cm 18 G long needle under positive N_2 pressure without disturbing the settled biomass, and fresh N_2-flushed 0.2 µm filter sterilized seawater medium was added. Transfers of the cultures were made in 30-ml or 120-ml serum vials by taking half of the volume of the original culture into a new N_2-flushed serum vial capped with a black butyl rubber stopper, and adding fresh N_2-flushed 0.2 µm filter sterilized seawater medium. The serum vials were flushed and pressurized with 0.3 MPa CH_4 and incubated at 10 ºC in the dark.

Microscopy

Subsamples of the enrichment cultures (0.1 ml) were collected anaerobically and immediately fixed in paraformaldehyde (2.67% final concentration in 1× PBS) overnight at 4 °C. The next day, the fixed samples were washed twice with 1× PBS after centrifuging 16100× g for 5 min, and stored in 0.1 ml 1× PBS:ethanol (1:1 v/v) at -20 °C until microscopy analysis. For DNA

staining, 50 µl of the fixed samples were dried onto separate wells of a 10-well poly-l-lysine pretreated slide (Tekdon, Myakka City, FL, USA). The slide was then washed sequentially in 50%, 80% and 96% ethanol for 2 min each. After air drying the slide at room temperature, 1 mg/ml of 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) in Citifluor AF-1 anti-fading solution (Electron Microscopy Sciences, Hatfield, PA, USA) (28) was added to the wells and incubated for 30 min in the dark prior to imaging. Fluorescence in situ hybridization (FISH) of the samples was also conducted on 10-well poly-l-lysine pretreated slides as described above. Three dual-labelled (29) oligonucleotide probes (Integrated DNA Technologies, San Diego, CA, USA) were used: ANME-2a-828 probe with Alexa488 fluorophore (30), ANME-2c-760 probe with Cy3 fluorophore (31), DSS658 probe with Cy5 fluorophore (32). FISH was performed at 40% formamide concentration following procedures previously described (33) followed by applying DAPI-Citifluor solution as described above. Light and epifluorescence imaging was performed on a BX51 epifluorescence microscope with 20× and 40× dry objectives (Olympus, Center Valley, PA, USA). DAPI images were adjusted using Adobe Photoshop Lightroom Classic 10 (Adobe, San Jose, CA, USA) to improve the image clarity for large cell aggregates using +50 setting in the texture, clarity and dehaze sliders.

Community analysis via 16S rRNA gene amplicon sequencing

Biomass was harvested from 3-5 ml of the 4 sediment-free enrichments and a culture transferred from one of the enrichments (Supplementary Figure 1) for genomic DNA extraction. The culture fluid was sampled anaerobically into a 2-ml Eppendorf tube and centrifuged at 8000× g for 5 min. After carefully removing the supernatant by pipetting, the biomass pellet was immediately extracted using DNeasy Blood and Tissue kit (Qiagen, Valencia, CA, USA) following the

manufacturer's instructions for Gram-positive bacteria. In total, enrichments were sampled at 3 different times over the course of the 5-year enrichment (2016/04/22, 2017/05/03, and 2017/11/01). DNA extracts were purified using Protocol A of the CleanAll DNA/RNA Clean-up and Concentration Micro Kit (Norgen Biotek, Thorold, Ontario, Canada) following the manufacturer's instructions.

For 16S rRNA gene amplicon sequencing, the V4-V5 region of the 16S rRNA gene was amplified from the DNA extracts using archaeal/bacterial primers with Illumina (San Diego, CA, USA) adapters on 5' end (515F 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-GTGYCAGCMGCCGCGGTAA-3'and 926R 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-CCGYCAATTYMTTTRAGTTT-3'). PCR reaction mix was set up in duplicate for each sample with Q5 Hot Start High-Fidelity 2× Master Mix (New England Biolabs, Ipswich, MA, USA) in a 15 µL reaction volume according to manufacturer's directions with annealing conditions of 54 °C for 30 cycles. Duplicate PCR samples were then pooled and barcoded with Illumina Nextera XT index 2 primers that include unique 8-bp barcodes (P5 5'-AATGATACGGCGACCACCGAGATCTACAC-XXXXXXXX-TCGTCGGCAGCGTC-3' and P7 5'-CAAGCAGAAGACGGCATACGAGAT-XXXXXXXX-GTCTCGTGGGCTCGG-3'). Amplification with barcoded primers used Q5 Hot Start PCR mixture but used 2.5 µL of product in 25 µL of total reaction volume, annealed at 66 °C, and cycled only 10 times. Products were purified using MultiScreen Plate MSNU03010 (Millipore-Sigma, St. Louis, MO, USA) with vacuum manifold and quantified using QuantIT PicoGreen dsDNA Assay Kit (ThermoFisher Scientific, Waltham, MA, USA) on the CFX96 Touch Real-Time PCR Detection System (BioRad, Hercules, CA, USA). Barcoded samples were combined

in equimolar amounts into single tube and purified with PCR Purification Kit (Qiagen) before 250 bp paired end sequencing on Illumina's MiSeq platform with the addition of 15-20% PhiX (Laragen, Culver City, CA).

The resulting sequence data was processed in QIIME v1.8.0 (34) following a recently published protocol (35). Raw sequence pairs were joined, requiring a 50 bp overlap, with a maximum of 4 mismatches in overlapping sequence. Joined sequences were then quality-trimmed, with minimum Phred quality score of 30 and any sequences with unknown base call ("N") were removed, and clustered de novo into operational taxonomic units (OTUs) with 99% similarity using UCLUST, and the most abundant sequence was chosen as representative for each de novo OTU (36). For the purposes of general tracking of major microbial species in the enrichments over time, the use of OTUs rather than amplicon sequence variants (ASVs) was deemed sufficient. Taxonomic identification for each representative sequence was assigned using UCLUST with a custom SILVA database modified from SILVA database release 138 (37), requiring minimum similarity of 90% to assign a taxonomy; 9 of the top 10 hits were required to share a taxonomic assignment to assign that taxonomy to a query. Our SILVA database had been appended with 1,197 in-house high-quality, methane seep-derived bacterial and archaeal clones. Any sequences with chimera-check pintail values of <50 or alignment scores of <75 were removed. The modified SILVA database is available upon request from the corresponding authors. Singleton OTUs were removed. Alpha diversity indexes (Chao1 and Shannon) were computed using QIIME alpha_diversity.py script (34) after rarefaction to the minimum number of sequences in the samples and compared using two tailed t-test with equal variance.

Enrichment culture metagenomics and binning

Purified DNA extracts (time point 2017/11/01) of the 5 enrichment cultures, which were used for 16S rRNA gene amplicon sequencing, were also used for shotgun metagenomic sequencing. Sequencing libraries were constructed from 2-50 ng of genomic DNA as described previously (38). Libraries were sequenced on HiSeq2500 (Illumina) in paired end mode with the read length of 250 nt following manufacturer's instructions.

The metagenomic sequencing reads were trimmed using Trimmomatic v0.36 with default settings and adaptor clipping profile Truseq3-PE, and assembled using Spades v3.11.1 with default settings for standard dataset. Metagenome contigs were annotated using the Integrated Microbial Genomes (IMG) (39) Annotation Pipeline. Metagenome-assembled genomes (MAGs) were binned from separate assemblies of each dataset using Anvi´o v6.2 (40). Contigs were binned based on sequence composition and read coverage across all datasets. The MAGs resulting from the binning of the five datasets were de-replicated using dRep v2.6.2 (41) with a 99% average nucleotide identity threshold. MAG analysis showed that ANME and SRB MAGs were missing. Manual inspection of the contigs of the assemblies using crossplots of coverage and GC content revealed clusters of unbinned contigs with high coverage. These clusters were binned using these crossplots as previously described (42), and resulted in fragmented but highly complete MAGs of ANME and SRB from all enrichments, except for an SRB MAG from enrichment I.

Single aggregate sorting and community composition analysis

AOM consortia from sediment-free enrichments were stained with a DNA stain (SYBR Green I Nucleic Acid Gel Stain; Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) and sorted based on size and fluorescence intensity at the Department of Energy Joint Genome Institute (JGI). Briefly, an Influx cell sorter (Becton Dickinson, Franklin Lakes, NJ, USA) was sterilized, and sheath fluid was prepared using 1× PBS, as previously described (24). After passing through a 70 µm nylon mesh filter, samples were sorted using a 200 µm nozzle at 0.21 bar into 384-well plates. The sort mode was "1.0 drop enriched". DNA stain (SYBR Green I) was excited using a 488 nm laser, and fluorescence was captured with a 530 nm filter. Gates were defined using a combination of SSC and FSC characteristics vs. 530 emission. Cell aggregates were differentiated from single cells based on a combination of scatter characteristics and SYBR brightness (Supplementary Figure 2). Test sorts of aggregates were examined via microscopy to verify successful sorting of AOM consortia. Two samples, one from enrichment II and one mixed sample from enrichments I and II (Supplementary Figure 1), were sorted, resulting in 80 aggregate-containing wells and 40 cell-containing wells from each sample.

The sorted cell-containing and aggregate-containing wells, in addition to 24 no template control wells, were genome amplified using the RepliG single cell kit (Qiagen) as previously described (43), but with the addition of Epicentre Ready-Lyse lysozyme at 50U/ul to the lysis step. A 300 bp insert standard shotgun library was constructed and sequenced using the NextSeq platform (Illumina). BBTools software tools (https://sourceforge.net/projects/bbmap/) were used to remove Illumina artifacts, PhiX, reads with more than one "N" or with quality scores (before trimming) averaging less than 8 or reads shorter than 51 bp (after trimming), and reads with >95% identity mapped to masked versions of human, cat and dog references. Then, sequencing

adapters were removed using trim-galore v0.6.6 (https://github.com/FelixKrueger/TrimGalore/) with cutadapt v3.1 (44).

Community composition analysis was performed using reads from our sorted cells and aggregates, as well as no template controls after whole genome amplification, in addition to using reads from bulk metagenomes from the enrichment cultures. Each set of reads were mapped to 77 de-replicated MAGs reconstructed from the bulk metagenomes using CoverM v0.6.1 (https://github.com/wwood/CoverM) with settings set at 80% read length and 95% identity.

Data Availability

The 16S rRNA gene amplicon sequences, shotgun metagenome sequences and metagenome-assembled genomes from the sediment-free enrichment cultures have been deposited in National Center for Biotechnology Information (NCBI) under BioProject PRJNA758896 (reviewer link: https://dataview.ncbi.nlm.nih.gov/object/PRJNA758896?reviewer=r73he4r75o5vhmm1u7bcssjrd9). The raw sequences for flow sorting controls, sorted single cells and aggregates from the sediment-free enrichments have been deposited in the Joint Genome Institute (JGI) Genomes Online Database under Study ID Gs0133461.

Results

We separated viable sediment-hosted AOM consortia from non-physically attached cells and sediment particles based on density and size. The initial starting material was sourced from a deep-sea methane seep sediment core collected in the Santa Monica Basin (California, USA)

where specific depth horizons (6-12cm) exhibiting high sulfate-coupled AOM activity were selected for enrichment cultivation. We performed mild sonication, percoll density separation and size selective filtration from aliquots of this sediment slurry under anoxic conditions to separate AOM consortia from single cells and sediment particles, and tested to see if pretreatment with pyrophosphate, an ionic detergent (26), prior to sonication resulted in greater recovery of consortia (Supplementary Figure 1). Light microscopy after treatments indicated that sonication was effective for separating intact AOM consortia from sediment particles without prior chemical fixation, while no difference was observed with pyrophosphate addition. We used 4 sonicated, density separated and size selected samples with or without pyrophosphate addition as inocula for sediment-free enrichments (Supplementary Figure 1). After 3 years of incubation with methane and sulfate, high levels of sulfide were produced in all 4 enrichments suggesting active AOM coupled to sulfate reduction. DNA staining and FISH microscopy revealed cell aggregates ranging from 5 to 300 μm in diameter composed of individual or multiple AOM consortia embedded in an exopolymeric matrix (Figure 1). The consortia were observed in larger aggregations, forming visible black flecks that settled on the bottom of the serum bottles. Similar observations were reported for sediment-free enrichments of ANME-1 and ANME-2c consortia (20). The extracellular substance, presumably produced by members of the AOM consortia, appeared to physically aggregate multiple AOM consortia and cells. Results from these microscopy and sulfide measurements indicated that the AOM consortia were alive and viable after sonication and percoll treatments. Sulfide production continued in subsequent dilutions and transfers into a defined seawater media and was dependent on methane addition (data not shown). Due to repeated subsampling for experiments and slow growth of psychrophilic ANME-2, we cannot estimate doubling times for the ANME-2a and ANME-2b consortia in our

enrichment cultures based on sulfide production rates. Based on the slow accumulation of biomass we observed, the earlier reported range of doubling times between 3-7 months is a reasonable estimate (5–8). Taken together, AOM consortia from a deep-sea methane seep were alive and grew after our pre-selection procedure.

Community analysis based on the partial 16S rRNA gene revealed significant decreases in species richness (Chao1 index, p=0.028) and diversity (Shannon index, p=0.00025) in the sediment-free enrichments compared to the original sediment. Specifically for AOM consortia, the original sediment contained 7 ANME taxa (taxonomy based on SILVA SSU rRNA database v138, including ANME-1a/1b/other and ANME-2a/2b/2c) and 2 SRB taxa (SEEP-SRB1/2) at >0.5% relative abundance (Supplementary Table 1). After 3 years, the enrichments only contained 2 abundant ANME lineages (ANME-2a/2b) and 2 abundant SRB lineages (SEEP-SRB1/2) at >0.5% relative abundance (Figure 2 and Supplementary Table 1). ANME-2a and SEEP-SRB1 were the most abundant ANME and SRB lineages in all 4 enrichments, with one enrichment (Enrichment I) also containing abundant ANME-2b and SEEP-SRB2 at 12% and 3%, respectively, of the total taxa recovered (Figure 2). Other microorganisms also grew in the enrichment cultures and varied in abundance between enrichments and time (Figure 2 and Supplementary Table 1). Multiple taxa present in the enrichments have no cultured representatives, including those in the phyla *Cloacimonadota*, *Eremiobacterota*, *FCPU426* and *Nanoarchaeota* (order *Woesearchaeales*). Our enrichment process not only reduced the sample complexity and cultivated AOM consortia, but also cultivated other taxa that likely depend on the AOM consortia and methane.

We used DNA from the 4 sediment-free enrichments and a culture transferred from one of the enrichments (Supplementary Figure 1) for shotgun metagenomic sequencing. We reconstructed genome bins from the enrichment metagenomes individually and de-replicated at 99% average nucleotide identity (ANI) to obtain 77 metagenome-assembled genomes (MAGs) (Supplementary Table 2). Methane metabolism, as indicated by the marker *mcrA* gene, is restricted to MAGs belonging to ANME lineages and two lineages in the *Methanosarcinaceae* family including *Methanococcoides*, a genus of methylotrophic methanogens that has been found in other AOM enrichments as well (20). Several taxa were represented by MAGs recovered from 2 or more of our sediment-free enrichments. Comparing the MAGs belonging to the same taxon from separate enrichment bottles revealed differences in the average nucleotide identity (ANI) (Figure 3). MAGs of ANME-2a, SEEP-SRB1 and *Methanococcoides* were more dissimilar, at the level of different species, using 95% ANI for species delineation (minimum ANI values of 89.6%, 94.4% and 92.8%, respectively) (45). MAGs of other taxa from different enrichment bottles in comparison were closely related at the species level (ANI values >98.7%). While the source sediment and cultivation conditions were the same for all the enrichment cultures, separate cultures still led to cultivation of distinct species of the same taxon as shown by our genomic comparisons.

Given that these enrichment cultures still contained at least 40 taxa (Supplementary Table 1), we applied flow cytometry to focus our analysis on the AOM consortia and develop a broader understanding of the range of microbial taxa forming physical associations in our cultures. Putative AOM consortia could be distinguished from single cells based on their larger size and higher fluorescence from DNA stain, and we leveraged these properties to sort and analyze the

single cell and cell aggregate fractions independently (Supplementary Figure 2). Two samples were used for flow sorting: sample II from enrichment II and sample I+II from a mixture of enrichments I and II. Eighty individual cell aggregates were sorted, lysed, genome amplified, and sequenced from each sample. As controls, we also sorted single cells and included no template empty wells as contamination checks using the same procedure. The background contamination in our flow cytometry procedure was low, with only 1 out of 24 no template control wells amplified (Supplementary Table 3). Likewise, the amplification success rate with single cells was low in our procedure, with only 1 out of the 80 wells with sorted single cells amplified (Supplementary Table 3). In comparison, the sorted aggregates yielded a much higher amplification success rate, with 53 or 33 of the 80 wells successfully amplified from sample I or I+II, respectively. Taken together, our flow cytometry procedure is suitable to examine individual cell aggregates while avoiding background and single cell contamination.

We then confirmed that the sorted cell aggregates were mainly composed of AOM consortia as observed in fluorescence microscopy. The community composition analysis was based on the occurrence of 77 taxa using de-replicated MAGs from the enrichment culture metagenomes and enabled by read mapping. Given that whole genome amplification introduces biases in the coverage of genomic regions, we only analyzed for presence and absence of taxon with greater than 1% mapped reads as the cutoff without delving into their relative abundances. Of the sorted aggregates that successfully amplified, 90% (87% from II and 94% from I+II) contained ANME-2a and 83% (79% from II and 88% from I+II) contained SEEP-SRB1. Based on our fluorescence microscopy data, we did not observe any of AOM consortia that contained ANME but not SRB, and therefore the sorted aggregates showing only ANME-2a present but not any SRB was likely

due to partial lysis or preferential whole genome amplification of ANME-2a over their SRB partner. ANME-2b was found in 5% of the sorted aggregates that also contained ANME-2a. These data show that the vast majority of the sorted aggregates were ANME-2a in partnership with SEEP-SRB1.

We further explored potential microbial associations with ANME-2a among the sorted aggregates. By comparing the number of sorted ANME-2a aggregates where non-ANME organisms were detected to the relative abundance of these same organisms in the bulk enrichment culture metagenomes, we identified 3 species that occurred more frequently in ANME-2a aggregates than the bulk samples (Figure 4A). As expected, the dominant sulfate-reducing bacterial partner of ANME-2a, SEEP-SRB1 was significantly more abundant in the ANME-2a aggregates compared to their relative abundance in the bulk samples (Figure 4B). Additionally, *Methanococcoides* archaea and *Anaerolineales* bacteria also had higher than expected occurrences in ANME-2a aggregates in both of our flow cytometry samples (Figure 4B, Supplementary Tables 4 and 5). Moreover, *Methanococcoides* and *Anaerolineales* tend to co-occur, with 68% or 75% *Methanococcoides*-containing aggregates also contained *Anaerolineales* in both flow cytometry samples. Given these co-occurrences in sorted aggregates, *Methanococcoides* and *Anaerolineales* might be physically attached and interact with each other and with the ANME-2a and SEEP-SRB1 consortia in our enrichment cultures.

Discussion

Microorganisms do not exist in isolation but in interconnected communities in the environment (46). While ANME-SRB consortia are responsible for methane consumption in marine methane seeps, their interactions with a broader array of microbial community members are also likely to influence the methanotrophic capacity and geochemical cycling of carbon and nutrients within these ecosystems. From deep-sea methane seep sediment, we selected for microbial methanotrophic consortia and cultivated them in defined media with methane and sulfate, establishing sediment-free enrichments performing AOM with sulfate in vitro. By delving into community composition at the bulk and single aggregate levels, our results revealed the identities of the microorganisms not only co-enriched in the incubation bottles but also those that might be physically associated and potentially interacting with ANME-SRB consortia. Such insights help broaden our understanding on how microorganisms are interconnected and influence the functioning of methane seep ecosystems.

Our sediment-free enrichment community compositions resemble previous enrichment cultures from marine methane seeps. *Methanococcoides* and others belonging to phyla *Caldatribacteriota* (*JS1* class, formerly *Atribacteria*), *Bacteroidota*, *Firmicutes* and *Cloacimonadota* (formerly *Cloacimonetes*) that were abundant in our enrichments (Figure 2) have also been identified in previous AOM enrichments (13, 14, 20, 47–49). Amongst these taxa, *Methanococcoides* (as part of the *Methanomicrobia*) and *Caldatribacteriota* were identified as part of the core methane seep microbiome (50). *Fermentibacterota* (formerly *Hyd24-12*) frequently occurs in methane seep diversity surveys and is also part of the core methane seep microbiome (50, 51), but until now has not been reported to be an abundant member of seep enrichment cultures. These organisms are likely anaerobic heterotrophs based on genomic predictions and known or inferred

metabolisms of related taxa (47, 52–55). The main metabolic product of AOM consortia - sulfide - could be used by microorganisms in the *Campilobacterota* phylum (formerly *Epsilonproteobacteria*) such as *Sulfurimonas* or *Arcobacter* in our enrichments (Figure 2) (56, 57). Accordingly, we observed decreases in *Sulfurimonas* abundance in all enrichments with more frequent media exchanges that kept sulfide levels low (Figure 2). Some taxa in phyla such as the *Patescibacteria/Candidate Phyla Radiation*, *Cloacimonadota* and *Nanoarchaeota* may be in symbiotic association (53, 54, 58–60), dependent on, or parasitizing, other microorganisms in the enrichments. Among these potential symbionts, *Woesearchaeales* (*Nanoarchaeota*) has not previously been reported to be abundant in AOM enrichments except ours, but were recently reported in high abundance in Arctic methane sediments (61). Their reduced genomes suggest a fermentative and symbiotic lifestyle (62, 63). However, the abundance of *Woesearchaeales* decreased with successive transfers (Figure 2), suggesting that it is not growing in proportion to and likely not directly interacting with AOM consortia.

Although all 4 of our sediment-free enrichments originated from the same sediment slurry, different enrichments led to multiple distinct species of ANME-2a, SEEP-SRB1 and *Methanococcoides* (Figure 3), suggesting that the in situ populations of these taxa are more genetically diverse than other taxa from the same methane seep habitat. This observation is supported by a previous 16S rRNA gene survey of methane seeps worldwide, which reported that ANME and SRB lineages distributed globally but experienced selective pressure to diversify locally (50). Our observation of coexistence of multiple ANME and SRB species was also consistent with the high fragmentation (>100 contigs) of ANME and SRB MAGs in our study (Supplementary Table 2) as well as in previous work (64, 65). This is likely due to the high strain

heterogeneity of ANME and SRB given the coexistence of multiple species or strains, even though their high relative abundance and sequencing coverage in the methane seep shotgun metagenomes should facilitate their genome assembly (66).

Comparing community composition in flow sorted cell aggregates and bulk enrichment cultures revealed potential physical association between microorganisms. Neither *Methanococcoides* nor *Anaerolineales* was previously reported to be physically associated with AOM consortia, when investigated using immunomagnetic capture or fluorescence activated flow sorting (24, 28, 67). This could be due to differences in microbial interactions in the refined enrichment culture communities and the more complex sediment communities, where ANME-SRB consortia were hypothesized to form a broad range of symbiotic relationships (24, 67). Also, ANME-2a was the most abundant ANME lineage in this study whereas ANME-1a or ANME-2c was the most abundant ANME lineage in the previous work (24, 28, 67). The majority of the organisms found to be associated with AOM consortia in previous studies, including *Burkholderiaceae*, *Planctomycetota* (formerly *Planctomycetes*, AKAU3564 sediment group), *Fermentibacterota* (formerly *Hyd24-12*), *Caldatribacteriota* (*JS1* class, formerly *Atribacteria*), *Bacteroidota* (formerly *Bacteroidetes*), *Sphaerochaeta* (formerly *Spirochaeta*), *Pseudomonas*, and *Desulfobacterota* (formerly *Deltaproteobacteria*) (24, 28, 67), were present in our enrichment cultures (>0.1% of the community based on 16S rRNA gene, Figure 2 and Supplementary Table 1) but did not have higher occurrence in ANME-2a aggregates than the bulk enrichment cultures (Supplementary Tables 4 and 5). The interactions between *Methanococcoides* and *Anaerolineales* and potentially with AOM consortia remains to be elucidated, but could affect

methanotrophic capacity of AOM consortia given their potential methanogenic metabolism, close physical association and tendency to co-occur in cell aggregates.

Our enrichment cultures represent a stable system to investigate the microbial interactions. Of the cultured *Methanococcoides* (52, 68–70), the *Methanococcoides* in our enrichments were most similar to *Methanococcoides burtonii* (68); they had nearly identical 16S rRNA genes (98% identities over 252-253 bp available) and belonged to the same genus but different species based on their genomes (91-92% average nucleotide identity) compared to *M. burtonii*. Metabolically, the *Methanococcoides* in our enrichments contained nearly all of the methanol, methylamine, dimethylamine and trimethylamine utilization genes, suggesting they are also methylotrophic methanogens like *M. burtonii* (68). The sustained presence of methylotrophic methanogens in the methanotrophic cultures is intriguing. The methyl compounds may originate from the *Anaerolineales* that *Methanococcoides* associated with. Given that all cultured *Anaerolineales* are obligate anaerobic chemoorganoheterotrophs (71, 72), a symbiotic cross-feeding relationship between *Anaerolineales* and *Methanococcoides* is possible, perhaps fueled by the degradation of organic substrates produced by ANME-SRB or the breakdown of consortia biomass in these multi-year enrichment cultures. There is precedence for this as partnerships between *Anaerolineales* and methanogens have been previously reported, where members of the *Anaerolineales* were successfully co-cultured with hydrogenotrophic methanogens resulting in enhanced growth (71, 72). In other heterotrophic enrichments, *Anaerolineales* and hydrogenotrophic or acetoclastic methanogens were observed to co-occur and hypothesized to form a syntrophic relationship (73–75). However, members of the *Anaerolineales* have not been reported to co-occur with methylotrophic methanogens like *Methanococcoides*. The known

degradation products of *Anaerolineales* include acetate and hydrogen but not methyl compounds (72), yet all known *Methanococcoides* exclusively metabolize methyl compounds but not acetate or hydrogen as methanogenic substrates (52, 68–70). Discerning the metabolic interactions between the *Anaerolineales* and *Methanococcoides* requires additional investigation, but this association is clearly distinct from previously described methanogenic associations. Direct or mediated interspecies electron transfer could be a possibility (76, 77). Future microscopy and functional studies at single aggregate level may reveal if the cellular activities of different taxa in cell aggregates also co-occurred and provide insight on the functions and interactions of *Methanococcoides* and *Anaerolineales* in a methanotrophic system.

Selecting microorganisms based on their physical traits has often been used to separate them from their complex biological and physical surroundings. For example, percoll density separation allowed the purification of anaerobic ammonium-oxidizing bacteria (78) and selective size filtration enabled the description of *Candidate Phyla Radiation* (or *Patescibacteria*) (79). Here by pre-selecting AOM consortia using both density separation and size filtration, we accelerated the cultivation process of these slow-growing microorganisms from sediment and established sediment-free AOM enrichments in vitro. We demonstrated that the methane seep microorganisms, including AOM consortia and multiple core methane seep taxa, were alive and viable after percoll density separation, sonication and filtration, establishing a protocol for future enrichment efforts from sediments and soils. These sediment-free enrichments can act as simplified model systems, not only for microscopic and genomic interrogation, but also to investigate the physiology and study the interactions of microorganisms in a laboratory setting to shed further light on their biology.

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Competing interests

The authors declare no competing interests.

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Figures

Figure 1. Microscopy of sediment-free methanotrophic enrichment cultures. (A) Light microscopy of a large cell aggregate. (B-E) Fluorescence microscopy of zoomed in regions with DNA stain (4′,6-diamidino-2-phenylindole; DAPI) showing distinct cell clusters that resemble AOM consortia. Note that due to the large size, only the periphery of the cell aggregate was stained. (F-I) Fluorescence microscopy of cell aggregates using taxon specific oligonucleotide probes: ANME-2a (red) and partner SRB (green). F) AOM consortia clustered together in a large cell aggregate. (G-I) Individual AOM consortia. Scale bars = 5 μm.

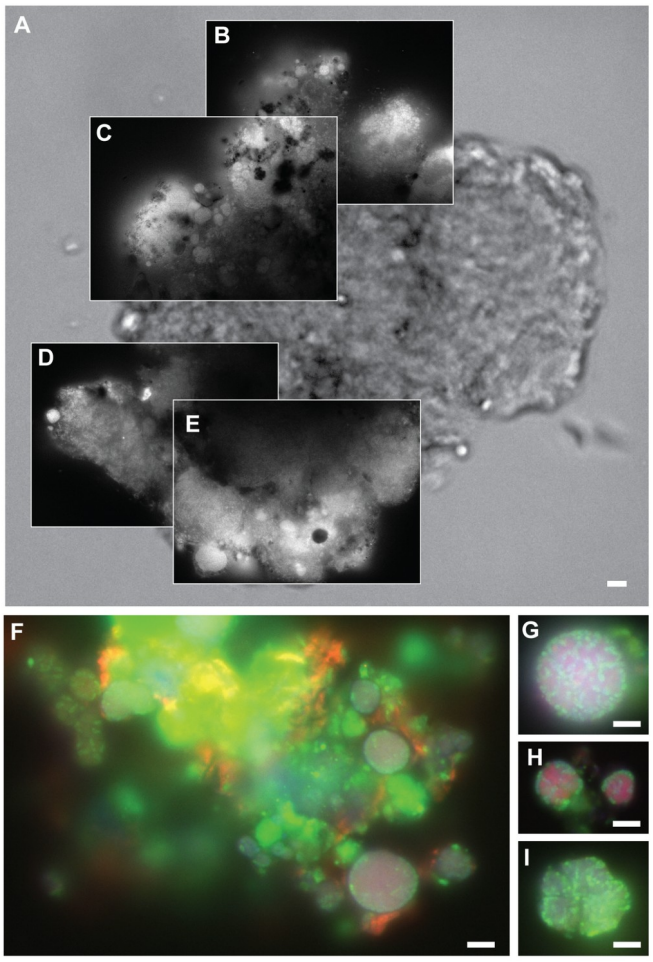

Figure 2. Community analysis of the original sediment inoculum and 4 sediment-free AOM enrichments over time by 16S rRNA gene amplicon sequencing. Taxonomic classification is based on the SILVA SSU rRNA database v138.

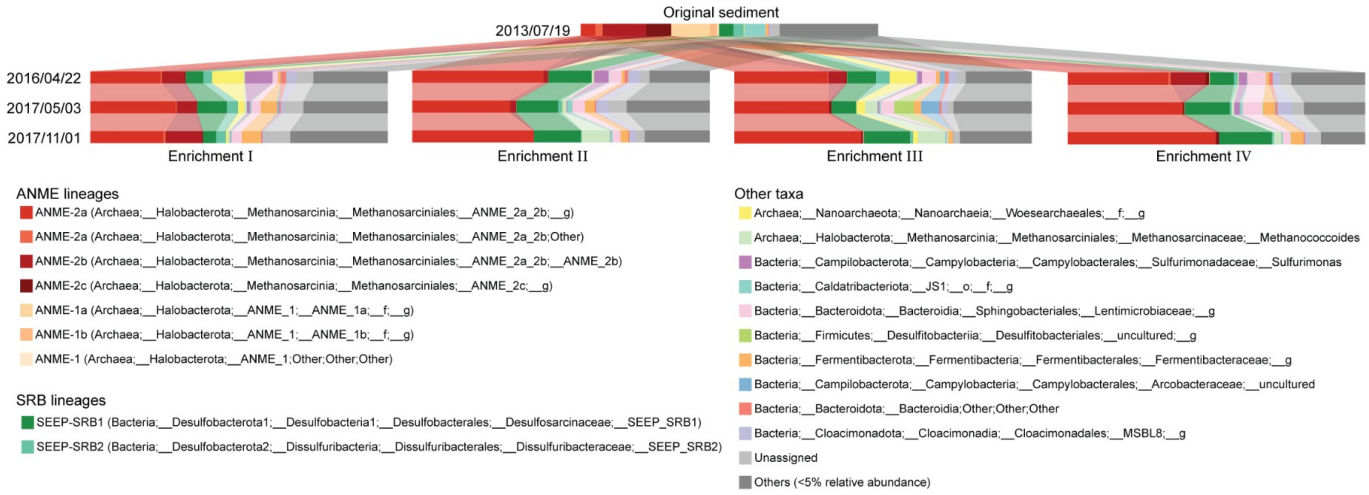

Figure 3. Average nucleotide identity (ANI)-based genome diversity of metagenome-assembled genomes recovered from multiple enrichments. ANME-2a, SEEP-SRB1 and *Methanococcoides* genomes have lower within-group ANI than other community members, and therefore have higher genotypic diversity in the methane seep sediment that they originated from.

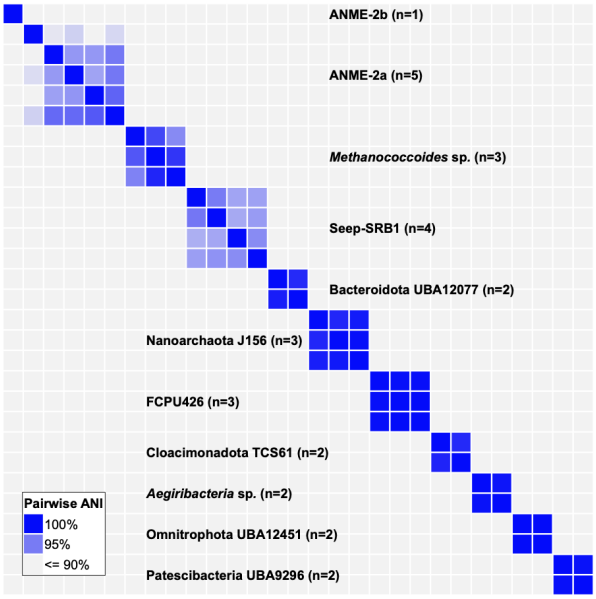

Figure 4. Potential physical association of microorganisms in ANME-2a aggregates. (A) Taxon relative abundance in sediment-free enrichment culture metagenomes (enrichment II or mixed I+II) compared to taxon occurrence based on read mapping to metagenome-assembled genomes in sorted cell aggregates. (B) Residues from the robust linear models indicate that *Methanococcoides* and *Anaerolineales*, in addition to the known syntrophic SEEP-SRB1 partner of ANME-2a, have higher residues in robust linear models and thus higher occurrences in sorted aggregates than the enrichment cultures. Taxonomic classification is based on the Genome Taxonomy Database release 95.