Characterization of hydrogenotrophic methanogenic Archaea enriched from Trunk River

Brittni L. Bertolet

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INTRODUCTION

Inland lakes, rivers, and reservoirs are increasingly recognized for their contribution to atmospheric methane (CH\(_4\)) concentrations. Globally, inland waters are estimated to produced 25% of the continental land sink in CH\(_4\) every year (Bastviken et al. 2011). However, in a changing world, these estimates are difficult to project and this is largely due to a lack of process-based models that consider the biological complexity of these biogeochemical processes. In inland freshwater and brackish ecosystems, CH\(_4\) is primarily produced biologically by methanogenic Archaea (hereafter referred to as “methanogens”), which regulate the rate of sediment diagenesis and carbon fate (Borrel et al. 2011). Despite this, few ecosystem models of CH\(_4\) production explicitly consider the microbial community and little is known about how differences between methanogens may contribute to variation in ecosystem CH\(_4\) production.

Biological methanogenesis occurs via three major metabolic pathways, characterized by their electron donors and terminal acceptors: acetoclastic (using acetate), hydrogenotrophic, (using H\(_2\)/CO\(_2\)), and methylotrophic (using methyl-compounds as substrates) (Liu & Whitman 2008). In freshwater and brackish sediments, methane is assumed to result primarily from the acetoclastic pathway (Conrad 1999), with many suggesting that the genus *Methanoseta* to dominate lake sediment communities (Zepp Falz et al. 1999; Chan et al. 2002). However, community biomarker surveys (16S rRNA and *mcrA* genes) increasingly document higher-than-expected relative abundances of known or putative hydrogenotrophic methanogens in lakes and soils environments (Nusslein et al. 2001; Conrad et al. 2010). Although sequence-based analyses do not directly consider the active component of a microbial community, the pervasiveness of these groups across regions and ecosystems suggests they may be functionally important constituents of sediment methanogen communities. Unfortunately, few methanogens have ever been isolated from sediments and much is still to be understood about the distribution of physiological traits, such as substrate use, growth rate, and CH\(_4\) yield.

In the present study, I sought to isolate and characterize hydrogenotrophic methanogens from Trunk River sediments using both physiological assays and genomics. Although isolation was unsuccessful, I determined how the methanogenic consortium responded to the availability of alternative electron donors using experimental incubations with formate additions. Additionally, using shotgun metagenomics, five metagenome assembled genomes (MAGs) were constructed and used to determine both phylogeny and metabolic potential of the methanogenic consortium.
METHODS

Sample collection and enrichment:

Sediment was collected from the sediment-water interface of Trunk River (TR), a brackish river in Woods Hole, MA (Lat: 41.534850, Long: -70.641458). Sediment was transported back to the laboratory and placed in an anaerobic chamber until inoculation. Prior to inoculation, selective media to enrich for hydrogenotrophic methanogens was prepared anaerobically. To prepare 1 liter of media, 5 mL of 1M ammonium chloride solution, 0.1 mL of 100mM potassium phosphate (pH 7.2) solution, 5 mL of 1M MES buffer (pH 5.5), 1 mL of trace elements, 0.1 mL of 1% Resazurin, and 10 mL of 100X fresh water base (consisting of 100 g NaCl, 40 g MgCl2.6H2O, 10 g CaCl2.H2O, 50 g KCl per liter) were added per liter of deionized water. The media was then boiled for 10 minutes under a stream of N2/CO2 (80%:20%) gas to boil out oxygen. The media was also cooled under N2/CO2. Once cooled, 1 mL of multivitamin solution, 25 mL of 1M sodium bicarbonate, and 1 mL of 1M sodium sulfide were added. Media was then brought inside the anaerobic chamber, and once the media became clear, 25 mL of media was dispensed into 100 mL serum bottles. Serum bottles were then crimped shut with air-tight rubber septa and autoclaved. After cooling from the autoclave, 0.5 mL of antibacterial solution (1 g Rifampcin SV per 100 mL water) was added to each bottle.

To begin enrichments, serum bottles containing sterile anaerobic methanogen media were brought into the anaerobic chamber and uncrimped. Approximately 1 g of fresh sediment was then inoculated into each bottle and serum bottles were crimped shut with air-tight rubber septa. At the gassing station, headspace was replaced with a H2:CO2 gas mixture (20%:80%), using three rounds of repeated vacuum and gas replacement. After headspace replacement, all enrichments were stored in the dark at 30 degrees Celsius, where they remained for the duration of the study.

Liquid enrichments were passaged every 7-9 days in the same liquid media using an anaerobic syringe. A total of three passages were conducted before isolation strategies were employed. At each step of passage, production of CH4 was determined through gas chromatography with a flame ionization detector (FID) and enrichments were visualized for auto-florescence at 420 nm using a florescent microscope and the Alexa488 filter. Enrichments without CH4 production were discarded.

Isolation approaches:

To isolate hydrogenotrophic methanogens, a number of different strategies were employed. First, enrichments were serially diluted with the intent to dilute-to-extinction in liquid media. This occurred in the same media described prior in the same 100 mL serum bottles with a H2/CO2 headspace. To obtain colonies, agar shakes, flat-bottom bottles, and plates were prepared (Fig. 1). Agarose was added to the methanogen media described above to a final concentration of 1% agar for each method.

Shakes were constructed in 25 mL Balch tubes, in which 9 mL of sterile anoxic 1% agar media were added to each and mixed with 1 mL of inoculum before solidification. Media and inoculation was prepared under under a stream of N2/CO2 at the gassing station. For the flat-bottom bottles, media was prepared as described above and inoculum was spread on the surface of the agar after solidification and occurred in the anaerobic chamber. For plates, two
different strategies were employed: top agar and no top agar. For all three methods, inoculum was serially diluted to determine best inoculation concentration. Additionally, for all three methods, headspace was replaced with the same H₂:CO₂ gas mixture and replenished every day to continuously supply the necessary substrates. All cultures were stored in the dark at 30 degrees Celsius for the length of the study.

**Figure 1.** Agar shakes (A), flat-bottom bottles (B), and petri plates (C) used to isolate hydrogenotrophic methanogen colonies.

**Formate growth experiment:**
To determine how the availability of formate affected CH₄ production rates of the enrichment methanogenic consortium, experimental incubations were constructed in 25 mL anaerobic Balch tubes (Fig. 2). Each Balch tube contained a final volume of 12 mL (10 mL of selective methanogen media, 1 mL of inoculation from a single source enrichment, and 1 mL of additional media based on treatment). The consortium was subjected to three different treatments in which the availability of electron donors was manipulated. Treatments included H₂-only, formate-only, and H₂-formate. For treatments receiving formate, formic acid was added to a final concentration of 2mM formate. Additionally, the formate-only treatment contained a N₂/CO₂ headspace instead of H₂/CO₂. Finally, 1 mL of sterile ultra-pure water was added to the H₂-only treatments to keep total volume constant across treatments.

**Figure 2.** Experimental incubations for the formate growth experiment.
CH₄ production was monitored every day until the concentration of CH₄ was unchanging and stationary phase was obtained. H₂/CO₂ was never replenished. Incubations were visualized using microscopy to determine any cursory changes in relative abundances of unique cell types.

To calculate CH₄ production rates, peak area was first converted to parts per million (ppm) of CH₄ using a standard curve produced on July 7th, 2018. The concentration of CH₄ (ppm) was then converted to micromolar (umol) using the ideal gas law and normalized by the volume of the media (mL). To determine the rate of CH₄ production, only data from the growth stage of the experiment (8/13/2018-8/15/2018) was used in a linear regression against time. Thus, the CH₄ production rate is reported as umol CH₄ mL⁻¹ hr⁻¹. To determine differences in CH₄ production rates between samples, a one-way ANOVA was performed in R.

**Metagenomic analyses:**

DNA was extracted using a PowerFecal extraction kit from the initial enrichments after 21 days of growth. DNA was sequenced on an Illumina Hi Seq. Raw reads were trimmed using Trimmomatic, co-assembled using MegaHit, mapped using Bowtie, and binned using concoct. Once binned, MAGs were checked for completion using CheckM, which considers the presence of single copy genes. To determine the phylogeny of constructed MAGS, 16 ribosomal proteins used in Hug et al. 2016 were extracted using a phylogenetic workflow described in Graham et al. 2018. This workflow uses a curated reference database of the same ribosomal proteins extracted from available methanogen genomes from NCBI. Several phylogenetic trees were constructed with the entire database, as well as only subsets of the database. Finally, MAGs were annotated using the Rapid Annotation using Subsystem Technology (RAST) platform and metabolic models were built using ModelSeed in Kbase.

**RESULTS AND DISCUSSION**

**Microscopy characterization and isolation:**

Three unique methanogenic cell types were confirmed in the TR enrichments through microscopy (cocci, rod-like, and spiral shaped) (Fig. 3). Additionally, 2 non-florescent cell types were also observed, with one highly motile non-florescent cell type. All cell types were observed in every passage, with no noticeable difference between passages after a few days of growth had occurred. However, further quantification, either with flow cytometry or specific FISH probes, would have greatly benefitted this.

Dilution-to-extinction in liquid media was unsuccessful in producing pure cultures. Cultures either produced CH₄ and continued to have a mixed consortium, or no CH₄ was produced and few cells were ever detected. Shakes and flat-bottom bottles were also unsuccessful in producing colonies, however all cultures produced CH₄. The agar plates were the most successful. Although I never obtained colonies, on the last day of incubation, small specks were noticeable on the surface of the agar that may have produced colonies if longer growth was possible. This potential for colony formation is likely due to the increased surface area of the plates, as well as the large amount of headspace available in the plate canisters (Fig. 1C). It is also worth noting that these specks were observed on plates without top agar and inoculated with undiluted inoculum, so dilutions are likely unnecessary in future attempts.
Methanogens are visualized based on auto-fluorescence at 420 nm, which is due to the presence of redox cofactor F420. Unique cell types are denoted with arrows.

**Effects of formate availability on CH₄ production rates of enrichments:**

Formate availability had a significant effect on consortium CH₄ production rates (Fig. 3B). All treatments were significantly different from each other, and incubations with both formate and H₂ had the highest rate of CH₄ production. Interestingly, incubations receiving only formate as the sole electron donor had negligible CH₄ production. While a trace amount of CH₄ was detected on the first day of sampling, this is most likely due to residual H₂ that was introduced in the inoculation as CH₄ concentrations did not increase otherwise throughout the length of the incubation. However, this is unconfirmed.

It is worth noting that the significant increase in CH₄ production when both electron donors are present is greater than the sum of the H₂-only and formate-only treatments, suggesting some interaction that is promoting CH₄ production when both electron acceptors are available. Previous researchers have seen evidence of formate-dependent reduction of CO₂ with H₂ in other members of Methanobacteriales (Yang et al. 2016), which may be contributing to higher CH₄ production rates. However, because community assembly was not determined at the end of the experiment, it is difficult to determine the mechanism behind this effect. Analyses of metabolic models from the constructed MAGs may provide further insight.
Figure 3. Average CH₄ concentration in the headspace of experimental incubations for each day and treatment (A). CH₄ production rate of each treatment during the growth phase (8/13/18-8/15/18) (B). Error bars represent standard error.

**Metagenomic analysis of enrichments:**

Shotgun metagenomic sequencing of the consortium enrichments retrieved four methanogen bins (M1-M4) and one bacterial bin (B1) (Fig. 4). MAGs M1-M3 were near complete (90.1-98.9%) with less than 1% contamination. MAG M4 was moderately complete (65.8%) with less than 1% of contamination, and MAG B1 was semi-complete (82.7%) with 1.3% contamination. These MAGs constituted only 25.4% of the total base-pairs sequenced, and further refinement of other bins could provide additional complete or semi-complete MAGs.

Figure 4. Visualization of complete metagenome assembled genomes (MAGs) constructed from shotgun metagenomic sequencing of TR sediment enrichments. Visualization was generated using Anvi’o.
Phylogenetic analysis of 16 ribosomal proteins extracted from the constructed MAGs placed all methanogen MAGs within known hydrogenotrophic methanogen genera with high confidence (Fig. 5). Four different trees were generated, each with a different number of reference genomes, and all trees displayed the same placement of MAGs. Thus, the phylogeny is visualized in Figure 5 with only a subset of the reference database.

The phylogenetic analysis of MAG B1 was not as conclusive. Ribosomal proteins placed B1 within the Tenericutes phylum, within the Mollicutes class. Although no 16S or 23S rRNA genes were assembled, one 5S rRNA gene could be extracted from the MAG, and this was 92% identical to the gene in a Tenericutes MAG assembled from enrichments from a methane seep (Skennerton et al. 2016). Members from this group are hypothesized to be anaerobic fermenters, that may supply methanogens with substrates such as acetate and ethanol. It is inconclusive, however, whether this is a true syntrophic relationship, and further research is still needed to understand the role of the free-living Tenericutes within methanogenic communities. Members of Mollicutes have also been shown to possess resistance to Rifampicin antibiotics, which may explain the presence of this group in our enrichments.

**Figure 5.** Phylogeny of methanogen MAGs constructed from TR sediment enrichments. *Desulfurococcus amylovorans* was used as an out-group and bootstrap values are reported.
CONCLUSIONS AND FURTHER DIRECTIONS

Hydrogenotrophic methanogenic consortium from TR sediments were enriched and characterized. At least 5 distinct microorganisms were present and CH₄ production rates were sensitive to the availability of electron donors. Additions of formate significantly increased CH₄ production as compared with incubations receiving only H₂. This has implications for understanding methanogenesis in nature, as rarely are communities isolated with only one available electron donor, and may point to ecologically important metabolisms. Further, analysis of constructed MAGs also supported the use of formate as an important intermediate in the reduction of CO₂ to methane in this hydrogenotrophic community. In both MAGs M2 and M3, formate dehydrogenases were present, which are necessary for formate donation of electrons to heterodisulfide in the last step of methanogenesis. However, further research is needed to confirm the mechanism for how formate is being utilized by this consortium.

In this study, the coupled use of functional assays and genomics analyses was a powerful tool for understanding the metabolic capacity of microbial communities, but further work would greatly benefit these conclusions. Particularly, understanding dynamic changes in the microbial consortium after experimentation would have helped support hypotheses for how formate additions affected CH₄ production. This could have been accomplished through either 16S sequencing, flow cytometry, or FISH visualization or any other way to quantify microbial communities, and subsequent experiments should consider employing such monitoring.

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REFERENCES


