METHANOSARCINALES BIOGEOCHEMISTRY,
IMPLICATIONS FOR METHANE CYCLING

A Thesis in
Geosciences
by
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Abstract

The anaerobic oxidation of methane (AOM) is a poorly understood process. It consumes large amounts of methane and is a significant component of the global carbon and methane cycles. I employed an interdisciplinary approach to help enlighten AOM by looking at both the activity of related processes and incubations of AOM-containing sediments.

Trace methane oxidation (TMO) is a process, potentially related to AOM, by which archaeal methane producers (methanogens) oxidize small amounts of methane. I used a stable isotope tracer to examine this process in multiple archaeal sulfate reducer and methanogen cultures (with emphasis on *Methanosarcina acetivorans* cultures). I provide new evidence that co-enzyme M reductase, a key enzyme of methanogenesis, is likely involved in AOM. I also observed that a suite of oxidizing agents included in the incubations did not enhance TMO rates. Finally, I demonstrated that, under specific culture conditions, the rate of TMO to methyl sulfide products in *M. acetivorans* is nearly seven times greater than to any product ever previously reported for TMO. I used incubations of active AOM sediment to demonstrate that hydrogen does not play a metabolic role in AOM consortia. These experiments, combined with thermodynamic and geochemical considerations, suggest that methyl sulfides may be the long-sought intermediate metabolite and crucial to the AOM metabolism.

The potential role of methyl sulfides in AOM increases the need for understanding their formation in related pathways. I demonstrate the first instance of energy conservation through methyl sulfide production by a methanogen, *M. acetivorans*, and the first documented instance of methyl sulfide production from a carbon monoxide substrate.

I also developed a new approach for isolating taxon-specific RNA sequences in preparation for stable isotope analysis. I used a DNAzyme containing a catalytic motif with variable recognition domains. I used *M. acetivorans* RNA to develop and test the approach in preparation for potential application in AOM investigations.
Finally, in testing the potential influence of oxidizing agents on TMO I discovered that *M. acetivorans* was capable of growth under a microaerophilic atmosphere. I tested and compared the ability of other strict anaerobes to withstand similar oxidative stress.
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“The movement of a canoe is like a reed in the wind. Silence is part of it, and the sounds of lapping water, bird songs, and wind in the trees. It is part of the medium through which it floats, the sky, the water, the shores…There is magic in the feel of a paddle and the movement of a canoe, a magic compounded of distance, adventure, solitude, and peace. The way of a canoe is the way of the wilderness, and of a freedom almost forgotten. It is an antidote to insecurity, the open door to waterways of ages past and a way of life with profound and abiding satisfactions. When a man is part of his canoe, he is part of all that canoes have ever known.”


As we paddle through intellect’s oceans may we all find connections to what came before and what may come to follow.
Chapter 1

Introduction

1.1 Background information
Methane is a strong greenhouse gas and its influence on global climate spans billions of years. High methane concentrations during the Archean and Proteozoic helped offset low solar luminosity resulting in an environment warm enough for liquid water, and thus more suitable for expansion of life (Pavlov et al., 2003). Methane remains a strong factor on the modern Earth where it is most abundant form of reduced carbon in the present atmosphere (Cicerone et al., 1988). Current atmospheric methane abundance is 2.5 times that of pre-industrial levels (IPCC, 2001), making methane a major contributor to global climate warming. The total annual methane flux to the atmosphere is approximately 600 Tg, of which roughly 60 % is attributable to anthropogenic sources (IPCC, 2001).

Heightened concern over anthropogenic climate perturbation highlights the need for better understanding sources and sinks in the global methane cycle.

There are two primary methane sources to the atmosphere, thermogenic and biogenic. Approximately 18 % of the atmospheric methane flux is thermogenic methane derived primarily from fossil fuels (Quay et al., 1999), with the remaining flux from biologic sources. Methanogens are a group of microorganisms clustered in the Euryarchaeota branch of the archaeal domain that almost exclusively produce the entire biogenic microbial methane flux. Methanogens inhabit anoxic areas rich in organic matter including wetland and marine sediments, rice paddies, and ruminant digestive systems (Madigan et al., 2000). Methanogen substrates vary but fit into three classes: 1) CO₂ type substrates (including carbon dioxide, carbon monoxide, and formate), 2) simple methylated substrates, and 3) acetotrophic substrates that exclude complex organic compounds (Madigan et al., 2000). The net conversion of large organic compounds into methane first requires fermentor or acetogenic activity to break the compounds down to simple substrates usable by methanogens (White, 2000, Zinder, 1993). Methane is
released from anoxic environments to the atmosphere, forming a critical link between anoxic environments and the global carbon cycle.

Proteomic and genomic investigations of methanogens are revealing the enzymatic steps governing methanogenesis. Interestingly, similar studies are demonstrating the metabolic relatedness of anaerobic oxidation of methane (AOM) to methanogenesis (Hallam et al., 2003, Krüger et al., 2003, and Meyerdierks et al., 2005). AOM occurs primarily in marine sediments and links bacterial sulfate reduction with archaeal methane oxidation to oxidize up to 90% (Hinrichs et al., 2000) of methane transported through anoxic sediment columns in marine systems. Anaerobic degradation of organic material and decomposing methane hydrates are the primary sources of methane feeding AOM. Worldwide, methane hydrates store an estimated 10,000 Gt of methane in an ice matrix (Kvenvolden, 2002). Despite their size, however, atmospheric methane fluxes from hydrates contribute only ~2% of the global flux (Reeburgh, 1996) because AOM oxidizes an estimated 300 Tg of clathrate-originating methane per year (Hinrichs and Boetius, 2001).

Despite their significance to global methane cycling, we are only beginning to understand the AOM process. Genetic investigations identify at least three types of archaeal anaerobic methane oxidizers (ANME-1, ANME-2, ANME-3)(Knittel et al., 2005). Investigations also implicate bacterial sulfate reducers in the process although they do not appear to be required in all cases (Orphan et al., 2002). Tight physical interaction between these two groups (Boetius et al., 2000) suggest a mutualistic metabolism where a by-product of methane oxidation is consumed by the sulfate reducer to maintain favorable energy yields in the process (Hinrichs et al., 1994). The mechanism behind this exchange and the substrate transferred remains unclear, in large part because the organisms participating in AOM are not isolated in pure culture.

*Methanosarcina acetivorans* is a methanogen first isolated from marine sediments dominated by kelp debris (Sowers et al., 1984). This organism shows a high degree of
metabolic diversity, using acetate, methanol, methylamine, dimethylamine, trimethylamine, dimethylsulfide, and carbon monoxide as growth substrates (Sowers et al., 1984, Ni et al., 1994, Rother and Metcalf, 2004). Genomic investigations revealed one of the largest archaeal genomes ever sequenced, complete with proposed genes for yet undiscovered metabolisms and processes (Galagan et al., 2002). Further, this readily culturable organism is one of the most phylogenetically related to ANME-2 and ANME-3 AOM organisms (Hinrichs et al., 1999, Knittel et al, 2005).

1.2 Thesis organization

Work presented in this dissertation exploits *M. acetivorans* as a model organism, capitalizing on its high metabolic diversity and its close link to methane oxidizing organisms, to help reveal new insights to the biogeochemistry of the global methane cycle. I sought to use *M. acetivorans* as a tool for understanding AOM and related processes. First, presented in chapters 2, and 3, *M. acetivorans*, along with several other Euryarchaeota, are used to investigate archaeal methane oxidation, when it occurs at trace levels during microbial metabolism. Second, chapter 4 presents my discovery of a new metabolism for *M. acetivorans*. Third, in a series of incubations using marine methane seep sediment, I build upon insights from chapters 3 and 4 and investigated archaeal methanotrophy (with net methane oxidation) (chapter 5). Further, I used cultures of *M. acetivorans* to develop a stable isotope tool for the *in situ* investigation of metabolism by members of the Methanosarcinales (chapter 6). Finally, I used *M. acetivorans* and other anaerobic microorganisms to explore the limits of habitability under anoxic and microaerophilic conditions (chapter 7).
1.3 Trace methane oxidation studied in several Euryarchaeota under diverse conditions


This chapter uses trace methane oxidation (TMO) as a proxy for exploring AOM. TMO is the small amount of methane oxidized during routine methanogenic growth (Zhender and Brock, 1979). I tested the hypothesis that archaeal sulfate reducers may participate in methane oxidation and assessed methane oxidation by multiple methanogen species. I tested a suite of potential electron acceptors to determine whether any stimulated an increase in the proportion of methane oxidized (versus that produced by the culture). (Co-authors of this manuscript are Christopher H. House, doctoral co-advisor, Katherine H. Freeman, doctoral co-advisor, and James G. Ferry, collaborator).

1.4 Products of trace methane oxidation during non-methylotrophic growth by *Methanosarcina* and implications for anaerobic oxidation of methane

(in press at *JGR Biogeosciences*)

Focusing on *M. acetivorans*, I explored two metabolisms not previously analyzed for TMO. I tracked TMO products into different metabolites using a stable isotope tracer. I observed a novel metabolite (methyl sulfide) that was also the most abundant end product of methane oxidation ever observed by this, or any other isolated archaeal species. (Co-authors of this manuscript are Christopher H. House, doctoral co-advisor, B. Thomas, collaborator, Jennifer Vrentas, collaborator, and Katherine H. Freeman, doctoral co-advisor).
1.5 Methyl sulfide production by a novel carbon monoxide metabolism in *Methanosarcina acetivorans*  

(in review at *Geology*)

Dimethyl sulfide is the largest component of the biogenic sulfur flux to the atmosphere. When cultured on carbon monoxide as its sole carbon and energy substrate, *M. acetivorans* methylates sulfide to form both methane thiol and dimethyl sulfide. These methyl sulfides display a rare link between global sulfur and methane cycles. Additionally, this is the first demonstration of methyl sulfide production by a methanogen species. My work documents energy production by the metabolism and considers the importance of this metabolism to biogeochemical cycles of the present and ancient Earth. (Co-authors of this manuscript are Christopher H. House, doctoral co-advisor, Jennifer Vrentas, collaborator, and Katherine H. Freeman, doctoral co-advisor).

1.6 Methylated sulfides: a proposed metabolic intermediate in the anaerobic oxidation of methane  

(for submission to *Environmental Microbiology*)

The mechanics of methanotrophic and sulfate reducer interaction in AOM consortia are largely unknown. I used a series of natural sample incubations to explore both hydrogen and carbon monoxide as potential substrates passed between consortia members. The results of my incubations suggest that neither of these gases act as a AOM metabolic intermediate. Here, I propose a new model describing AOM, which includes methyl sulfides as the intermediate. The new model is support by past work with TMO (including chapter 2 and 3) and new thermodynamic considerations, as well as published biochemical, proteomic, and genomic reports. (Co-authors for this manuscript are

1.7 Optimizing DNAzyme activity for phylogenetic 16S RNA selection

Biomarkers provide insights to the presence and metabolic activity of microorganisms in a wide variety of investigations. Unfortunately, every organism does not produce a unique biomarker. I am developing a method that utilizes 16S RNA as a phylogenetically specific stable isotope biomarker. This approach enables use of RNA as a taxon-specific biomarker with selectivity ranging from the domain to species level. Here, I use *M. acetivorans* to initially develop this tool with the potential for eventual application to the closely related archaeal methanotrophs. I used a DNAzyme containing catalytic DNA motif with variably recognition domains to selectively cleave targeted RNA. I used gel electrophoresis to isolate only the desired RNA from a mixed RNA sample and a stable isotope trace to assess overall stringency of the approach. I developed and assessed activity of a suite of DNAzymes under varying reaction conditions to identify parameters affecting cleavage efficiency. (Co-authors for this manuscript are Katherine H. Freeman, doctoral co-advisor, Zhidan Zhang, collaborator, and Christopher H. House, doctoral co-advisor).

1.8 Oxygen tolerance in strictly anaerobic prokaryotes

(for submission to Geobiology)

Methanogens are traditionally classified as strict anaerobes and their severe oxygen sensitivity limits potential habitable environments. However, during research presented in chapter 2, I found that *M. acetivorans* can grow under microaerophic conditions. Based on this surprising finding, I tested the oxygen threshold for growth of multiple methanogen and anaerobic prokaryotes. The genome sequence for *M. acetivorans* discloses a hydrogenase of unknown function (Galagan et al., 2002). I also tested
whether this hydrogenase could enhance oxygen resistance. More thoroughly understanding anaerobes’ responses to oxygen can help define the habitability boundary between anoxic and oxic environments. (Co-authors for this manuscript are Zhidan Zhang, collaborator, Katherine H. Freeman, doctoral co-advisor, Victoria J. Orphan, collaborator, and Christopher H. House, doctoral co-advisor).

1.9 References


Chapter 2

Trace methane oxidation studied in several Euryarchaeota under diverse conditions

2.1 Abstract
Using $^{13}$C labeled methane, we document the level of trace methane oxidation by *Archeoglobus fulgidus*, *Archeoglobus lithotrophicus*, *Archeoglobus profundus*, *Methanobacterium thermoautotrophicum*, *Methanosarcina barkeri*, and *Methanosarcina acetivorans*. The results indicate trace methane oxidation during growth varied among different species and among methanogen cultures grown on different substrates. The extent of trace methane oxidation for *M. thermoautotrophicum* (0.05 ± 0.04 %, ± 2 standard deviations ($\sigma$) of the methane produced during growth) is less than *M. barkeri* (0.15 ± 0.04 %) grown under similar conditions using H$_2$ and CO$_2$. *M. acetivorans* oxidized more methane during growth on trimethylamine (0.36 ± 0.05 %) than during growth on methanol (0.07 ± 0.03 %). This may indicate that, in *M. acetivorans*, either a methyltransferase related to growth on trimethylamine plays a role in the oxidation of methane or that methanol is an intermediate of methane oxidation. Additions of various possible electron acceptors (O$_2$, NO$_3^-$, SO$_4^{2-}$, SO$_3^{2-}$) or the addition of H$_2$ to the headspace did not substantially enhance or diminish methane oxidation in *M. acetivorans* cultures. Separate growth experiments with FAD and NAD$^+$ showed that inclusion of these electron carriers also did not enhance methane oxidation. These results suggest trace methane oxidized during methanogenesis cannot be coupled to the reduction of these electron acceptors in pure cultures, and that the mechanism by which methane is oxidized in methanogens is independent of H$_2$ concentration. In contrast to the methanogens, species of the sulfate-reducing genus *Archeoglobus* did not significantly oxidize methane during growth (oxidizing 0.003 ± 0.01, of the methane provided for *A. fulgidus*, 0.002 ± 0.009 % for *A. lithotrophicus*, and 0.003 ± 0.02% for *A. profundus*). Lack of observable methane oxidation in the three *Archeoglobus* species examined may indicate that methyl coenzyme M reductase, which is not present in this genus, is required for the
anaerobic oxidation of methane, consistent with the “reverse methanogenesis” hypothesis.

2.2 Introduction

The anaerobic oxidation of methane (AOM) is a significant methane sink in marine sediments, oxidizing up to 90% of the biologic methane produced in these environments (Hinrichs et al., 2000). Organisms responsible for AOM (ANME-1 and ANME-2) have not been isolated but genetic and lipid analyses indicate the methane-consuming organisms are archaean and closely related to cultured methanogenic genera, specifically Methanosarcina and Methanococcoides (Hinrichs et al., 1999, Boetius et al., 2000, Hinrichs et al., 2000, Orphan et al., 2001a). Lipid analyses and fluorescence in-situ hybridization (FISH) suggest that certain Bacteria form tight consortia with the archaean methanotroph (the ANME-2), coupling methane oxidation to sulfate-reduction and rendering the overall reaction thermodynamically favorable (Boetius et al., 2000, Orphan et al., 2001b). Nauhaus et al. (2002), investigating methane seep sediments, showed a one-to-one reduction of sulfate to sulfide with the oxidation of methane to CO₂ when methane served as the only available electron donor. The bacterial component of these natural consortia is most closely related to isolated sulfate-reducers from the Desulfosarcinales (Orphan et al., 2001a) and stable isotope evidence suggests their biomass is, at least in part, composed of carbon from oxidized methane (Pancost et al., 2000, Hinrichs et al., 2000, Orphan et al., 2001b, Michaelis et al., 2002). Some archaean methane-oxidizing organisms, the ANME-1, may perform AOM and associated sulfate reduction unaided by Bacteria (Michaelis et al., 2002, Orphan et al., 2002).

The enzymatic mechanisms mediating AOM in marine sediments remain unknown. One possible mechanism, proposed by Hoehler et al. (1994), is reverse methanogenesis. In this paper, we refer to “reverse methanogenesis” as the reversal of a methanogen’s typical metabolism such that methane production is replaced by net methane oxidation. Hallam et al. (2004) recently found nearly all genes associated with methanogenesis in environmental ANME-1 sequences. It is hypothesized that reverse methanogenesis
begins with methane activation by either methyl-coenzyme M reductase (MCR) or methyl transferase (MT) as described by Hoehler and Alperin (1996). MCR catalyzes the final reductive step in all known biogenic methane formation and the operon (mcrA) encoding this enzyme is observed in all known methanogen species. Consistent with the reverse methanogenesis hypothesis is the recent discovery of mcrA or a close variant in both ANME-1 and ANME-2 environmental samples (Hallam et al., 2003, Krüger et al., 2003, Hallam et al., 2004). In the event that ANME-2 methanotrophy is initiated by reverse MCR activity, analysis of closely related, culturable species with active MCR may provide a suitable model system from which to study archaeal methane oxidation until the indigenous archaeal methanotrophs can be cultured.

We use the term “trace methane oxidation” (TMO) to refer to conversion of labeled methane into CO₂ in methanogen cultures during typical methanogenic growth (Zehnder and Brock 1979, Harder, 1997). The extent to which reverse methanogenesis and TMO are related is undetermined. Yet, if the two processes are mechanistically similar, providing an organism capable of TMO with favorable environmental conditions could potentially initiate reverse methanogenesis.

While never experimentally demonstrated, Hohler and Alperin (1996) use a thermodynamic approach to argue that under the proper conditions, principally low H₂ pressures, reverse methanogenesis becomes favorable. Also, exposure to typical methanogenesis inhibitors (such as bromoethane or methyl fluoride) halts AOM activity within marine consortia (Krüger et al., 2003), suggesting a possible link to methanogenesis pathways. The reverse methanogenesis hypothesis invokes consortium associations to help maintain thermodynamic favorability by consuming the methanotroph’s metabolic end product, keeping its concentration below a critical thermodynamic threshold. One possible end product is H₂, which could be produced by an archaeal methanotroph and consumed by sulfate-reducing bacteria. Valentine et al. (2000) monitored H₂ production rates in stressed methanogen cultures subjected to high methane concentrations and low methanogenic substrate levels. No link between methane
abundance and H\textsubscript{2} production was observed, suggesting reverse methanogenesis is either not linked to H\textsubscript{2} production or it is not a metabolism adoptable by the methanogens tested. Acetate has been proposed as an alternative AOM intermediate (Valentine and Reeburgh 2000). Yet, Nauhuas et al. (2002) tested multiple potential intermediates (including acetate, formate, hydrogen, and methanol) and demonstrated these, including acetate, did not greatly enhance sulfate reduction rates in methane seep sediment samples, suggesting it is not an intermediate for in situ AOM.

In order to further the understanding of TMO and begin evaluating the reverse methanogenesis hypothesis, we examined TMO in pure cultures of three Archaeoglobus species and three methanogen species. Our experiments were aimed at determining (1) if archaeal species more closely related to AOM methanotrophs exhibited a stronger propensity for methane oxidation expressed through enhanced TMO, (2) whether the inclusion of different potential electron acceptors make conditions more amenable to methane oxidation, increasing the level of TMO, and (3) if archaeal sulfate-reducers, which share genetic similarity to the ANME group but lack the potential enzymatic methane activators (namely MCR) required for reverse methanogenesis, exhibit TMO.

For each experiment, a set of cultures was grown under headspaces containing differing proportions of \textsuperscript{13}C-labeled methane. Following growth, isotopic analysis was used to quantify the amount of \textsuperscript{13}C label converted from methane to CO\textsubscript{2}, indicating the extent of TMO in the culture.

\textbf{2.3 Methods}

Microorganisms

\textit{Archaeoglobus fulgidus} VC16, \textit{Archaeoglobus profundus} AV18, and \textit{Methanobacterium thermoautotrophicum} ΔH were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). \textit{Methanosarcina barkeri} MS was obtained from the Oregon Collection of Methanogens. \textit{Archaeoglobus lithotrophicus} TF2 was a kind gift from Karl Stetter, emeritus Universität Regensburg. \textit{Methanosarcina}

acetivorans 2CA was provided by Dr. J. G. Ferry (Penn State University, University Park PA 16802, USA).

Media

Strict anaerobic technique was employed for cell culturing. Media for *A. fulgidus* and *A. profundus* was prepared as recommended by DSMZ (medias 399 and 519 respectively). *M. thermoautotrophicum* was cultured on media containing (per l media): 3.0 g Na$_2$SO$_4$, 0.2 g KH$_2$PO$_4$, 0.3 g NH$_4$Cl, 0.3 g KCl, 0.2 g CaCl•2H$_2$O, 0.3 g MgCl•6H$_2$O, 1.0 ml 10x Wolfe solution (Wolin et al., 1963), 0.5 mg resazurin, 3.0 ml NaOH solution (10%), and 0.5 g Na$_2$S•9H$_2$O. All reagents except Na$_2$S were mixed in solution and degassed by bubbling with a stream of N$_2$ for 20 minutes followed by Na$_2$S addition. The media (20 ml per bottle) was dispensed into 120 ml culture bottles in an anerobic chamber followed by three flushes with a mixture of H$_2$ and CO$_2$ (80:20) and subsequent filling of the bottles to 3 bars pressure with the gas mixture and sterilization in an autoclave. *A. lithotrophicus* was cultured in the same media but with the addition of 20 g per liter NaCl. *Methanosarcina barkeri* was cultured in media containing, per liter: 0.29 g K$_2$HPO$_4$•3H$_2$O, 0.23 g KH$_2$PO$_4$, 0.23 g (NH$_4$)$_2$SO$_4$, 0.45 g NaCl, 0.06 g CaCl•2H$_2$O, 0.09 g MgSO$_4$•7H$_2$O, 1.0 ml (NH$_4$)$_2$Ni(SO$_4$)$_2$ solution (0.2%), 1.0 ml FeSO$_4$•7H$_2$O solution (0.2%), 0.5 mg resazurin, 1.0 ml 10x Wolfe mineral solution (Wolin et al. 1963), 6.0 ml vitamin solution, 4.0 ml NaOH solution (10%) and prepared as above with the addition of 0.5 g/l Na$_2$S•9H$_2$O. Media for *M. acetivorans* contains (per liter final solution): Mix A: 23.4 g NaCl, 3.8 g NaHCO$_3$, 1.0 g KCl, 1.0 ml 10x Wolfe mineral solution (Wolin et al. 1963), 2.0 ml vitamin solution, 0.5 mg resazurin; Mix B: 11.0 g MgCl$_2$•6H$_2$O, 0.30 g CaCl$_2$•2H$_2$O, 0.68 g KH$_2$PO$_4$; Mix C: 1.0 g NH$_4$Cl, 0.50 g cysteine•HCl, appropriate substrate; Mix D: 0.50 g Na$_2$S•9H$_2$O. Mixes A and B in 500 ml water each were degassed under N$_2$ then combined in an anaerobic chamber followed by addition of mix C and finally the addition of mix D. Substrates used were trimethylamine (9.56 g per l) or methanol (5.0 ml per l). Once dispensed (30 ml per culture bottle), individual bottles were flushed three times then filled to two bars pressure with a mixture of N$_2$ and CO$_2$ (80:20) and autoclaved. For the experiment involving *M.
acetivorans and H₂, the culture bottles were pressurized to 2 bars with a mixture of H₂ and CO₂ (80:20). For experiments involving M. acetivorans and oxygen, neither Na₂S nor resazurin were included in the growth media and sterile oxygen was injected into the bottles subsequent to autoclaving.

Cultivation
Two sets of four culture bottles were used for each species investigated. 40 ml (at 1 atm ≈1.8 mmoles) of total methane was added to each culture bottle with increasing proportions of labeled methane (99% ¹³CH₄, 1% ¹²CH₄) added to each bottle in the series (Cambridge Isotope Labs, Cambridge MA, Lot 00-04). For each tested species, one set of bottles was inoculated for growth while the second was used as an uninoculated negative control. Incubation for all species except M. barkeri lasted one week to ensure maximum growth and substrate consumption. Due to its slower growth, M. barkeri was incubated for two months. The amount of methane produced in each culture was calculated from established stoichiometric relationship for each substrate and final methane production. The cultures were grown to completion, ensuring complete substrate usage. Measurement of methane concentration in parallel experiments, using gas chromatography with a thermal conductivity detector, confirmed the accuracy of calculated methane productivity. Cell counts following growth were performed by phase-contrast microscopy using a counting chamber for all species but M. barkeri where cell clumping prevented accurate counting.

Sample preparation
Following culture growth, two distillations were used to collect and purify CO₂ from each sample for isotope analysis. The first distillation was done using an apparatus (Fig. 2.1) we developed for collection of CO₂ from high-pressure cultures with up to four atmospheres of mixed gases. After growth, a sample bottle was connected to the evacuated distilling apparatus through a gas tight needle. A liquid nitrogen trap froze CO₂ from the culture bottles into a separate collection tube, and gaseous methane was pumped from the sample. A secondary liquid nitrogen trap ensured quantitative CO₂ collection.
During growth, the *Archaeoglobus* spp. produced H$_2$S that interfered with preliminary CO$_2$ isotope analysis. For these samples, approximately 100 mg silver foil was included in the sample collection vial and removed H$_2$S at room temperature over 24 – 48 hours prior to analysis on the mass spectrometer. The foil removed all H$_2$S artifacts from the isotopic analysis. A second distillation cryogenically trapped and removed water (2-propanol and dry ice) and condensed CO$_2$ (liquid nitrogen) to assure residual non-condensable gasses were removed. Isotope analysis to determine $^{13}$C/$^{12}$C ratio for the CO$_2$ in each culture bottle used a Finnigan MAT 252 dual inlet mass spectrometer.

Data analysis
Isotope data were plotted as the $^{13}$C fractional abundance of the initial methane ($F_{CH4} = ^{13}$C / ($^{13}$C + $^{12}$C)) in each culture bottle versus the measured $F_{CO2}$ for CO$_2$ in each bottle after incubation (Figure 2.2). Least-squares calculations provide the slope and intercept for the resulting relationships and a positive slope indicates methane oxidation. We next consider mass balance for CH$_4$ and CO$_2$ produced by *Methanosarcina acetivorans* cultures during growth on either trimethylamine or methanol. The CH$_4$ in a culture bottle after growth represents that contributed during growth and methane lost by oxidation:

$$F_Tn_T = F_in_i + F_bn_b - F_on_ox$$

(1)

$F_T$, $F_i$, $F_b$, and $F_ox$ represent the fractional abundance of carbon isotopes in total, initial, biogenic, and oxidized CH$_4$ respectively. Likewise, $n_T$, $n_i$, $n_b$, and $n_ox$ represent the moles of total, initial, biogenic, and oxidized CH$_4$ respectively. We assume that any fractionation in the methane oxidation process is insignificant in our strongly labeled system, such that $F_ox = F_T$; we can therefore simplify equation 1 and solve for $F_T$:

$$F_T = \frac{F_in_i + F_bn_b}{n_i + n_b}$$

(2)

We assume oxidized CH$_4$ is converted to CO$_2$, such that:

$$F_{TCO2}n_{TCO2} = F_{iCO2}n_{iCO2} + F_{bCO2}n_{bCO2} + F_{TCH4}n_{ox}$$

(3)

$F_{TCO2}$, $F_{iCO2}$, $F_{bCO2}$ represent fractional abundance of carbon isotopes in total, initial, and biogenic CO$_2$ respectively. Similarly, $n_{TCO2}$, $n_{iCO2}$, and $n_{bCO2}$ represent moles of total,
initial, and biogenic CO\textsubscript{2} respectively. In a culture bottle containing unlabeled CH\textsubscript{4}, we can ignore the CH\textsubscript{4} oxidation input into the CO\textsubscript{2} mass balance because the small n\textsubscript{ox} does not significantly affect F\textsubscript{TCO2} or n\textsubscript{TCO2}. In cases with unlabeled CH\textsubscript{4} we have:

\[
F_{\text{TCO2}i}n_{\text{TCO2}i} = F_{\text{TCO2}i}n_{\text{TCO2}i}^* + F_{\text{bCO2}i}n_{\text{bCO2}i}^* \tag{4}
\]

Where terms are defined as in equation three, and \(n^*\) is used to refer to bottles cultured with unlabeled CH\textsubscript{4}. We prepared incubation bottles such that \(F_{i\text{CO2}} = F_{i\text{CO2}}^*\) and \(n_{i\text{CO2}} = n_{i\text{CO2}}^*\). Therefore, unlabeled incubations are used to account for CO\textsubscript{2} produced during cell growth:

\[
F_{\text{TCO2}}n_{\text{TCO2}} = F_{\text{TCO2}}n_{\text{TCO2}}^* + F_{\text{ox}} \tag{5}
\]

Combining 2 with 5 and solving for \(n_{\text{ox}}\) shows:

\[
n_{\text{ox}} = \left( F_{\text{TCO2}i}n_{\text{TCO2}i} - F_{\text{TCO2}i}n_{\text{TCO2}i}^* \right) \times \frac{F_{i\text{CO2}} + F_{\text{bCO2}}}{n_i + n_b} \tag{6}
\]

\(F_{\text{TCO2}}\) and \(F_{\text{TCO2}^*}\) were measured in multiple sample cultures with varied \(F_i\) (figure 2.2). \(F_{\text{TCO2}}\) is interpolated from the linear regression of the data at the median \(F_i\), while \(F_{\text{TCO2}^*}\) was interpolated from the same line at the point with no labeled methane. We controlled the amount of labeled CH\textsubscript{4} added to each bottle, with \(n_i\) was fixed at \(\approx 1.8\) mmoles. We estimated the biogenic methane composition to be \(\delta = -50\ \%\), and therefore \(F_b = 0.01056258\). Because of the strong label, small variations in \(F_b\) become inconsequential to the results. \(n_b\) is estimated based on the stochiometry of methanogenesis for a given substrate and confirmed in parallel experiments using gas chromatography with a thermal conductivity detector. \(n_{\text{TCO2}^*}\) is estimated by summing \(n_{i\text{CO2}^*}\) and \(n_{b\text{CO2}^*}\); CO\textsubscript{2} input from CH\textsubscript{4} oxidation is small in comparison to other CO\textsubscript{2} sources.

We solved for \(n_{\text{ox}}\) using an iterative method. First, \(n_{\text{TCO2}}\) was estimated as equal to \(n_{\text{TCO2}^*}\). We then solved for \(n_{\text{ox}}\) by adjusting \(n_{\text{TCO2}}\) to account for CO\textsubscript{2} derived from CH\textsubscript{4} oxidation. \(n_{\text{ox}}\) values converged within two to three iterations. \(n_{\text{ox}}\) was calculated for both the growth experiments and control bottles (media with labeled CH\textsubscript{4} but no cells) and the amount of CH\textsubscript{4} oxidized in each experiment is reported as the difference between these two values. Error estimates are based upon standard error of the slope used to calculated
Final reported error is estimated by repeating the above calculations using an experimental slope plus twice the slope standard error (i.e., 2σ) and a control slope minus twice the slope standard error. The same system of calculations was used for determining CH₄ oxidation in the other methanogen and sulfate reducer cultures, adjusted to account for stoichiometric input (or uptake) of biogenic methane and CO₂ (n_b, n_bCO₂). Methane oxidation is reported as both the percent of total post-growth methane oxidized in each bottle and, for methanogens, as the percent of methane produced during growth that was oxidized to CO₂.

2.4 Results / Discussion

*Archaeoglobus* is an archael sulfate-reducing genus with multiple culturable representatives (Stetter et al., 1987, Stetter et al., 1993). There are several reasons we chose to look at *Archaeoglobus* for studying TMO. First, recent environmental genomic sequencing of uncultured AOM methanotrophs revealed a general core similarity between the ANME-1 and the Archaeoglobales (Hallam et al., 2004). Second, sulfate-reduction by *Archaeoglobus* could provide an electron sink for methane oxidation. Third, *Archaeoglobus* possess much of the enzymatic machinery associated with methanogenesis (Klenk et al., 1998). *A. fulgidus*, for example, contains enzymes involved with reduction of CO₂ to a methyl group in methanogenesis but operates these enzymes in the reverse direction, oxidizing methyl groups to CO₂. Yet, *Archaeoglobus* species lack MCR (Stetter et al., 1987, Klenk et al., 1998), used in all known methanogenic pathways and hypothesized to potentially be required for reverse methanogenesis. While the role of MCR in methanotrophy is undetermined, its absence from *Archaeoglobus* species would prohibit the initiation steps required for reverse methanogenesis unless an alternative enzymatic pathway for methane activation is present. We examined methane oxidation in three *Archaeoglobus* species, each utilizing different growth substrates for sulfate reduction; *A. fulgidus* utilizes lactate, *A. profundus* utilizes acetate and H₂, and *A. thermolithotrophicus* grows chemolithotrophically on H₂ and CO₂. In each case, the amount of methane oxidized was not detected within the experimental error (Figure 2.3, Table 2.1). Lack of observable TMO in these species
suggests MCR is required for TMO, which is consistent with the reverse methanogenesis hypothesis for AOM.

The methanogens, *Methanobacterium thermoautotrophicum*, *Methanosarcina barkeri*, and *Methanosarcina acetivorans*, each displayed small amounts of methane oxidation (Figure 2.3, table 2.1) consistent with previous reports based on similar experimental work (Zehnder and Brock, 1979, Zehnder and Brock, 1980, Harder, 1997). We chose to explore further the reaction by which *M. acetivorans*, which has a close rRNA phylogenetic relation with the ANME-2 methanotroph lineage (Orphan et al., 2001a), and also has a large and complex genome (~5.8 Mb) with high metabolic diversity (Galagan et al., 2002). Such close relation to indigenous AOM species coupled with its large genome makes *M. acetivorans* a reasonable candidate for harboring genes required for reverse methanogenesis and may make it a valuable model organism for studying archaeal methane oxidation. The TMO we observed suggests a pathway for methane oxidation is present in *M. acetivorans*. Providing the proper chemical environment might promote greater methane oxidation.

We tested whether O\textsubscript{2}, NO\textsubscript{3}\textsuperscript{-}, SO\textsubscript{4}\textsuperscript{2-}, and SO\textsubscript{3}\textsuperscript{2-} could be used as an electron acceptor in methane oxidation by *M. acetivorans*. Enriching the culture media in these electrons acceptors could stimulate methane oxidation if electrons can be transferred to them effectively. Sulfite is a metabolic intermediate in bacterial sulfate reduction and, therefore, we hypothesize it is a possible metabolite that could be transferred between the archaeal and bacterial members of in vivo AOM consortia. In our hypothesized model, sulfite transfer within the AOM consortia from the sulfate-reducer to the archaeal ANME-2 would provide oxidizing potential for the methanotrophic reactions. However, none of the electron acceptors tested with *M. acetivorans* substantially enhanced the amount of methane oxidized in the cultures (Figure 2.3, Table 2.1), indicating that these electron acceptors, including sulfite, are not utilized by this methanogen to oxidize methane, even at trace levels.
Interestingly, most methanogen species are extremely oxygen sensitive, being killed by even trace amounts of oxygen. However, two superoxide dismutases, a superoxide reductase, and a catalase were uncovered in the \textit{M. acetivorans} genome, suggesting the potential for oxygen resistance (Galagan et al., 2002). The presence of a cytochrome \textit{d} oxidase may even potentially provide a link between oxygen and energy conservation by the organism (Galagan et al. 2002). We cultured \textit{M. acetivorans} under a microaerophilic headspace (0.7 \% O\textsubscript{2}). Diffusional constraints likely lowered the percent oxygen in the actual liquid growth media, but \textit{M. acetivorans} still demonstrated oxygen tolerance under microaerophilic conditions. The presence of oxygen had little effect on the cell yields, methane production, or trace methane oxidation in these cultures (Figure 2.3, Table 2.1), indicating that the oxygen reduction likely occurring cannot be coupled to methane oxidation. Ongoing research is now underway to more closely examine the responses of \textit{M. acetivorans} to oxygen.

We also tested the effect of including H\textsubscript{2} in the culture headspace. \textit{M. acetivorans} is capable of growth and methanogenesis using a large array of substrates but does not utilize H\textsubscript{2}. Unlike the other methanogens tested, \textit{M. acetivorans} lacks two essential hydrogenases required for methanogenic growth using hydrogen but does have other hydrogenases frequently associated with methanogenesis (Galagan et al., 2002). Hydrogen transfer between AOM consortia members may be essential for maintaining the low hydrogen concentrations required by thermodynamic constraints (Hoehler et al. 1994). In this experiment, however, the presence of hydrogen did not have a deleterious effect on the amount of methane oxidized (Figure 2.3, Table 2.1).

Nauhaus et al. (2002) suggested that AOM is dependent upon the transfer of electron carriers between consortia members. In this model, ANME-2 would donate electrons from methane to an oxidized electron carrier that is then passed to the bacterial sulfate-reducer. We utilized a simplified protocol to determine whether adding either NAD\textsuperscript{+} or FAD, the oxidized forms of these carriers, to \textit{M. acetivorans} cultures increased methane oxidation during growth. We incubated cultures with trimethylamine (TMA) as a growth
substrate and also provided labeled methane (initial $F_{\text{CH}_4}$ estimated at 0.117) in media containing 0.1 mM NAD$^+$ or FAD. If these common electron carriers could be utilized by *M. acetivorans* during TMO we would observe enhanced methane oxidation. Yet, when compared to the results shown in Table 1, neither NAD$^+$ nor FAD elevated methane oxidation in *M. acetivorans* above typical background levels, suggesting the two electron carriers cannot be coupled to methane oxidation.

We also examined *M. acetivorans* for trace methane oxidation when grown on two different substrates, methanol and TMA. More methane was oxidized when TMA, versus methanol, was utilized as the substrate for methanogenesis. The reason for enhanced methane oxidation in cultures grown on TMA is presently unknown. One potential explanation involves enzymatic differences in the TMA and methanol pathways. Methylamine-based methanogenesis requires three distinct methyltransferase I isozymes specific for mono- di- and tri-methylamines, and only one of two isozymes of methyltransferase II. Methanogenesis from methanol utilizes only one methyltransferase I and one methyltransferase II, both specific for methanol (Ding et al., 2002). Enhanced TMO in cultures grown with TMA may result from enhanced expression of a methyltransferase involved in TMA metabolism or enhanced expression of a closely related homolog of such a methyltransferase. Alternatively, it is possible that methanol may be an end product of TMO. Nauhaus et al. (2002) explored sulfate reduction in marine AOM samples. They found that when methane was removed and replaced by methanol, sulfate reduction continued at approximately half the original rate. Microbial responses to methanol can be very sensitive to concentration (for example, methanol becomes toxic at higher concentrations) and adjusting the methanol concentration used by Nauhuas et al. (2002) may optimize the reactions they observed, increasing sulfate reduction. The ability of consortia sulfate-reducing bacteria to use methanol as a substrate at all might suggest methanol is an intermediate of AOM. The sulfate-reducing bacteria would be crucial for removing excess methanol, preventing its build up from making AOM energetically unfavorable. If a similar mechanism of methane oxidation is used in
our experiments, TMO might be hindered in cultures grown with methanol since high methanol concentrations could limit methane oxidation.
2.5 Figures and Table

Figure 2.1. Distilling apparatus used to cryogenically isolate CO$_2$ from pressurized culture bottles after growth.

The sample is introduced to the system through a needle. Liquid nitrogen collects CO$_2$ in a separate sample tube, allowing for subsequent removal of non-condensable gasses by a vacuum. The auxiliary freezing coil is also cooled by liquid nitrogen and serves as a secondary trap for assuring no CO$_2$ loss. Heating the auxiliary freezing coil after removing non-condensable gasses allows any trapped CO$_2$ to migrate to the sample tube.
Figure 2.2. The $^{13}$C fractional abundance ($^{13}$F) of a culture bottle’s CO$_2$ after incubation versus $^{13}$F of final CH$_4$ tracks methane oxidation to CO$_2$.

A. $^{13}$F CO$_2$ in culture bottle headspace following incubation versus $^{13}$F of the final methane in each bottle for Methanosarcina acetivorans cultures (■) grown with trimethylamine and for identical, but not inoculated, control bottles (□). The difference in slopes between the cultured ($8.0 \times 10^{-3}$) and control samples ($-8.6 \times 10^{-5}$) is used to determine the amount of methane oxidation in the experiment.

B. Results from Archaeoglobus profundus (●) and control samples (○) showing no direct relationship between the extent of final methane enrichment and $^{13}$F of final CO$_2$, indicating no methane oxidation by the cultures.
Figure 2.3. Methane oxidation in each culture.

The amount of methane oxidation (% ± 2 standard deviation) standardized to either the total amount of methane in the culture after growth (shaded area) or the methane produced by the culture during growth (non-shaded area) observed in experiments with varying species and substrates. Added concentrations: lactate, 13.4 mM; H₂ (for all but M. acetivorans experiment), 2.4 bar; H₂ (M. acetivorans experiment), 1.6 bar; Ac (acetate), 12.2 mM; SO₄²⁻ (for A. fulgidus), 14.0 mM; SO₄²⁻ (for A. profundus), 33.0 mM; SO₄²⁻ (for A. thermolithotrophicum), 21.1 mM; MeOH (methanol), 125 mM; O₂, 7.0 mbar; TMA (trimethylamine), 100 mM; NO₃⁻, 6.59 mM; SO₄²⁻ (for M. acetivorans experiments), 21.1 mM; SO₄²⁻, 2 mM.
Table 2.1: Growth conditions and methane oxidation per culture experiment.

<table>
<thead>
<tr>
<th>Species</th>
<th>Growth substrate</th>
<th>Media addition</th>
<th>Incubation temp. (°C)</th>
<th>Cells per mL</th>
<th>Experimental Slope</th>
<th>Control Slope</th>
<th>Methane oxidized (mole)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. fulgidus</em></td>
<td>Lactate</td>
<td>-</td>
<td>85</td>
<td>2E+08</td>
<td>3.1E-05</td>
<td>1.7E-05</td>
<td>4.7E-8 (± 1.7E-7)</td>
</tr>
<tr>
<td><em>A. lithotrophicus</em></td>
<td>H₂, CO₂</td>
<td>-</td>
<td>85</td>
<td>7E+07</td>
<td>9.7E-05</td>
<td>8.1E-05</td>
<td>3.8E-7 (± 1.6E-7)</td>
</tr>
<tr>
<td><em>A. profundus</em></td>
<td>Acetate, H₂</td>
<td>-</td>
<td>85</td>
<td>1E+08</td>
<td>7.6E-06</td>
<td>-1.3E-05</td>
<td>5.2E-8 (± 2.3E-7)</td>
</tr>
<tr>
<td><em>M. thermoautotrophicus</em></td>
<td>H₂, CO₂</td>
<td>-</td>
<td>65</td>
<td>5E+07</td>
<td>6.1E-04</td>
<td>2.8E-05</td>
<td>6.3E-7 (± 5.9E-7)</td>
</tr>
<tr>
<td><em>M. barkeri</em></td>
<td>Methanol</td>
<td>-</td>
<td>33.5</td>
<td>n/a</td>
<td>1.8E-03</td>
<td>6.0E-06</td>
<td>2.0E-6 (± 5.7E-7)</td>
</tr>
<tr>
<td><em>M. azovitale</em></td>
<td>Methanol</td>
<td>-</td>
<td>33.5</td>
<td>5E+08</td>
<td>1.0E-03</td>
<td>4.7E-05</td>
<td>2.0E-6 (± 8.5E-7)</td>
</tr>
<tr>
<td><em>M. aceticum</em></td>
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<td>33.5</td>
<td>5E+08</td>
<td>7.9E-04</td>
<td>1.2E-05</td>
<td>1.6E-6 (± 3.2E-7)</td>
</tr>
<tr>
<td><em>M. acetivorans</em></td>
<td>Methanol</td>
<td>-</td>
<td>33.5</td>
<td>4E+08</td>
<td>1.1E-03</td>
<td>4.9E-06</td>
<td>2.3E-6 (± 2.6E-7)</td>
</tr>
<tr>
<td><em>M. termophilum</em></td>
<td>TMA</td>
<td>-</td>
<td>33.5</td>
<td>1E+09</td>
<td>8.0E-03</td>
<td>-8.6E-05</td>
<td>2.0E-5 (± 3.0E-6)</td>
</tr>
<tr>
<td><em>M. acetivorans</em></td>
<td>TMA</td>
<td>-</td>
<td>33.5</td>
<td>9E+08</td>
<td>7.2E-03</td>
<td>9.2E-05</td>
<td>1.7E-5 (± 1.4E-6)</td>
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<tr>
<td><em>M. acetivorans</em></td>
<td>TMA</td>
<td>-</td>
<td>33.5</td>
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<td>2.0E-5 (± 1.9E-6)</td>
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<tr>
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<td>TMA</td>
<td>-</td>
<td>33.5</td>
<td>5E+08</td>
<td>8.5E-03</td>
<td>3.4E-05</td>
<td>2.0E-5 (± 7.6E-7)</td>
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<tr>
<td><em>M. acetivorans</em></td>
<td>TMA</td>
<td>-</td>
<td>33.5</td>
<td>1E+09</td>
<td>9.4E-03</td>
<td>-2.5E-05</td>
<td>2.3E-5 (± 2.7E-6)</td>
</tr>
</tbody>
</table>

Note: TMA = trimethylamine
Note: Each culture was initially provided 1.79E-3 moles methane. In addition, based on methanogenesis stoichiometry and measurements performed on parallel experiments, the cultures produced 1.3E-3, 2.8E-3, and 5.5E-3 moles methane for the cultures grown on H₂/CO₂, cultures grown on methanol, and cultures grown on TMA respectively.

* Clumping of *M. barkeri* during growth prevents accurate cell counting for these cultures.
2.6 References


Chapter 3

Products of trace methane oxidation during non-methytrophic growth by *Methanosarcina*

3.1 Abstract

Our understanding of the metabolic pathways involved in the anaerobic oxidation of methane (AOM) is challenged by not having an isolated anaerobic methanotroph. Fortunately, trace methane oxidation (TMO) is carried out by isolated organisms closely related to anaerobic methanotrophs. As TMO likely shares metabolic similarities with the AOM metabolism, studies of TMO in culture experiments can serve to enhance our understanding of AOM. Here, we explored TMO in cultures of *Methanosarcina acetivorans* grown separately on acetate and carbon monoxide. We observed no methane oxidation to carbon dioxide in the cultures grown on acetate, but did observe methane oxidation to acetate ($1.1E-6 \pm 3.5E-8$ moles CH$_4$ oxidized, 0.10 % of the CH$_4$ produced). Methane was exclusively converted to the methyl position. Because AOM lipids exhibit strong $^{13}$C depletion in nature, our results imply acetate does not play a major role as an AOM intermediate. The carbon monoxide cultures showed little or no methane conversion to carbon dioxide ($1.9E-7 \pm 2.0E-7$ moles CH$_4$ oxidized), moderate methane oxidation to acetate ($1.8E-7 \pm 7.3E-8$ moles CH$_4$ oxidized, 0.14 % of biogenic CH$_4$ production), and high methane conversion to the methyl groups of product methyl sulfides ($4.0E-6 \pm 7.7E-7$ moles CH$_4$ oxidized, 3.1 % of biogenic CH$_4$ production). Taken together, our results identify methyl sulfides as more likely intermediates in natural AOM than acetate.

3.2 Introduction

The anaerobic oxidation of methane (AOM) couples methane oxidation to sulfate reduction in marine environments and is a significant component of global cycles of both methane and carbon (Hinrichs et al., 2000). Methane is both a potent greenhouse gas and the most abundant organic compound in the Earth’s atmosphere (Cicerone and Oremland, 1988). On the order of 10,000 gigatons (roughly twice the known reserves of oil, coal, and natural gas) of methane are frozen in a water matrix as clathrates (Kvenvolden,
In areas of modern clathrate decomposition, AOM captures and oxidizes over 90% of the released methane before atmospheric escape (Hinrichs et al., 2000). Warming of marine waters caused by global climate change could, however, destabilize methane clathrates and accelerate methane release. Better understanding the mechanisms of methane oxidation and sulfate reduction in AOM will enhance estimates of how AOM would respond to a changing climate with heightened methane availability.

Specific organisms responsible for AOM are not isolated in pure culture but genetic, lipid, and isotopic evidence suggests organisms with close phylogenetic linkage to Methanosarcina and Methanococcoides initiate methane oxidation (Hinrichs et al., 1999, 2000, Boetius et al., 2000, Orphan et al., 2001a). Similarities between enzymes documented in AOM organisms and those found in typical methanogenic metabolisms suggest analogous metabolic pathways may be active in both systems. Hoehler and Alperin (1996) used a thermodynamic approach to suggest AOM could proceed by reversal of typical methanogenic metabolism where the same or similar enzymes are used in the reverse direction. Only under specific environmental conditions would the “reverse methanogenesis” pathway be energetically plausible. Hallam et al. (2003) and Krüger et al. (2003) report both the occurrence of methyl coenzyme M reductase genes (a required gene for methanogenesis) and their high expression within active AOM groups. Hallam et al. (2004) used genetic evidence to support the potential presence of nearly all enzymes required for methanogenesis in AOM organisms.

A number of researchers have explored methane oxidation in culturable methanogenic species (Zehnder and Brock 1979, Harder 1997, Moran et al., 2005). Methane oxidation in each of these studies was at trace levels, far below the quantity of methane produced. Here we use the term “trace methane oxidation” (TMO) in reference to the small amount of methane that is oxidized during dominantly methanogenic growth and in contrast to AOM where net methane oxidation is the primary metabolic process.
Zehnder and Brock (1979) and Moran et al. (2005) explored TMO in multiple methanogen species including *Methanosarcina acetivorans* and observed varying amounts of methane oxidation with different species and by the same species when grown on different substrates. *M. acetivorans* shows close phylogenetic relationship to the AMNE-2 lineage of AOM (Orphan et al., 2001a) making study of TMO in this species particularly relevant. We developed a method in which $^{13}$C labeled methane is provided to methanogen cultures, and isotopic enrichment of carbon dioxide observed after growth permits quantifying low levels of methane oxidation to carbon dioxide (Moran et al., 2005). Based on this approach, Moran et al. (2005) reported greater methane oxidation to carbon dioxide when *M. acetivorans* was cultured on trimethylamine versus methanol. Higher methane oxidation is indicated by increased isotopic enrichment, and thus a steeper slope, when $^{13}$C contents of carbon dioxide are plotted against that of methane.

Valentine and Reeburgh (2000) argued that acetate could be an effective metabolic intermediate for transferring electrons from methanotroph to sulfate reducer during the AOM. In addition to being closely related to archaeal methanotrophs, *M. acetivorans* has multiple metabolic pathways that consume or produce acetate. *M. acetivorans* expresses an acetoclastic methanogenesis pathway by which acetate is converted to both methane and carbon dioxide (Sowers et al., 1984). Secondly, Rother and Metcalf (2004) demonstrated concurrent growth and acetate production in *M. acetivorans* cultures where carbon monoxide was the sole substrate. Moran et al. (in preparation) additionally observed methyl sulfide production in similar cultures. We examined these metabolic pathways for any signs of reversal consistent with trace conversion of methane to acetate, carbon dioxide, or methyl sulfide products (figure 3.1).

Here we report TMO to the methyl group of acetate during acetoclastic methanogenesis and to both methyl sulfides and acetate during growth of *M. acetivorans* on carbon monoxide. Together, these results expand the known suite of methanogenic pathways associated with TMO and help identify potential AOM intermediates.
3.3 Methods

Microorganisms and Media

*Methanosarcina acetivorans* 2CA was kindly provided by James G. Ferry (Department of Biochemistry and Molecular Biology, The Pennsylvania State University). We employed strict anaerobic technique for all cell culturing. Culture medium contained (per liter final solution): Mix A: 23.4 g NaCl, 3.8 g NaHCO₃, 1.0 g KCl, 1.0 mL 10x Wolfe mineral solution, 2.0 mL vitamin solution, and 0.5 mg resazurin; Mix B: 11.0 g MgCl₂•6H₂O, 0.30 g CaCl₂•2H₂O and 0.68 g KH₂PO₄; Mix C: 1.0 g NH₄Cl, 0.50 g cysteine hydrochloride; and Mix D: 0.50 g Na₂S•9H₂O. Mixes A and B in 500 mL water each were degassed under N₂ then combined in an anaerobic chamber followed by the addition of Mix C and finally Mix D. Sodium acetate (3.28 g per L) was added to solution with Mix C for the acetate culture experiments. Once dispensed (30 mL per culture bottle), individual bottles for the acetate experiment were flushed three times then filled to 0.2 MPa pressure with a mixture of N₂ and CO₂ (80:20 (v/v)) and autoclaved. Bottles for the carbon monoxide growth experiment were flushed three times and then filled to 0.3 MPa pressure with CO.

Culturing

A different set of control and experimental culture bottles were used for both the acetate and carbon monoxide experiments with either four or five bottles in each set. A total of 40 mL (at 0.1 MPa ≈ 1.8 mmol) of methane was added to each culture bottle with increasing proportions of labeled methane (99 % ¹³CH₄, lots 00-02 and 02-01, Cambridge Isotope Labs, Cambridge, MA) added to each bottle in the series. One set of bottles was inoculated for growth and the other not inoculated as a negative control. All bottles were incubated at 33.5 °C and cultures were grown to completion; 21 and 17 days respectively for the acetate and carbon monoxide cultures.
Sample preparation and measurement

CO₂ analyses
Carbon dioxide isotope measurement followed that of Moran et al. (2005). Aliquots of headspace gases were collected from each sample and cryogenically distilled to remove water vapor and non-condensable gases (including methane and nitrogen). Distillation was done in line with a Finnigan MAT 252 dual inlet mass spectrometer (Thermo Finnigan, Bremen, Germany) and purified carbon dioxide samples were immediately introduced to the mass spectrometer for $^{13}$C/$^{12}$C measurement (with precision better than 0.5 ‰). Prior to distillation, headspace samples from the carbon monoxide cultures were heated (100 °C for 20 minutes) with 0.45 g silver foil to remove sulfur compounds that otherwise would produce deleterious effects on isotope-ratio determination. Additionally, 40 mL carbon dioxide was added to each control bottle in the carbon monoxide experiment to ensure ample material for isotope measurement since there was no carbon dioxide from microbial growth. The Energy Institute (University Park, PA) performed carbon dioxide and methane quantification in the carbon monoxide growth cultures with precision better than 1 %.

Acetate analyses
Small (~ 1mL) aliquots of mature culture were centrifuged (20 minutes at 8,000 rpm) then decanted to remove cell material. The resulting supernatant was stored at −20 °C until analyzed. Acetate quantification was performed using a Dionex LC30 ion chromatograph with an Ion Pac AS18 analytical column; precision was better than 10 %. Methane and carbon dioxide production in the acetate grown cultures were estimated based on accepted methanogenic stoichiometries and the measured acetate consumption.

Samples for acetate isotope analysis were collected, centrifuged, and stored as above. Immediately prior to isotope analyses, 0.3 M oxalic acid (a volume matching that of the sample) was used to acidify each sample. Compound-specific, continuous-flow stable-isotope analysis targeting acetate in water solution was performed using gas chromatograph separation and $^{13}$C / $^{12}$C ratio determination on a MAT 252.
Chromatography was performed using a Nukol column (30m, 0.32 mm inner diameter, Supelco, Bellefonte, USA) and with an initial oven temperature of 85 °C held for one minute then increased at 20 °C per minute to 137 °C and held isothermal for 34.5 minutes before a final temperature increase of 20 °C per minute to 220 °C and held for 8 minutes to decompose any remaining oxalic acid within the injector and guard column. A combustion furnace operating at 1000 °C oxidized acetate to CO$_2$ permitting isotope determination (Dias and Freeman, 1997) with precision better than 1 ‰. In a separate analysis, the pyrolysis conditions described by Dias et al. (2002) were employed to selectively oxidize acetate’s carboxyl group, allowing intramolecular isotopic measurement (with precision better than 1 ‰). In place of a typical combustion furnace, a non-oxidizing palladium reactor at 600 °C was used. Pyrolysis was slightly non-selective, oxidizing a small amount of methyl in addition to carboxyl carbon. Standard curves derived from serial dilutions of position-labeled acetate reagent allowed us to estimate the fraction of methyl conversion to acetate. In our analyses approximately 10 % or less of the CO$_2$ measured from pyrolysis was methyl carbon.

Methyl sulfide analyses
Both dimethyl sulfide and methyl mercaptan were observed in the cultures grown on carbon monoxide (Moran et al., in prep.). Methyl sulfide quantification used a gas chromatograph with a flame ionization detector and a GS-Q column (30m, 0.32mm ID) (J & W Scientific, Palo Alto, California). Temperatures maintained at 100 °C, 105 °C, and 325 °C were used for the column, injector, and detector respectively. A suite of standard bottles was prepared identically to culture bottles but with addition of known amounts of DMS. Headspace injections from these bottles were used to calibrate the detector response from culture bottle headspace injections with a precision better than 10 %.

We used an FFAP (Alltech, Deerfield, Illinois) column and combustion furnace as described above to oxidize methylated sulfides and to determine $^{13}$C fractional abundance ($^{13}$F = $^{13}$C / ($^{12}$C + $^{13}$C)) for the combined methyl mercaptan and dimethyl sulfide peaks.
in each sample. The GC oven was held isothermal at 45 °C for 12 minutes then heated to 220 °C at 30 °C per minute and maintained at 220 °C for 10 minutes. The injector temperature was maintained at 130 °C and the column head pressure was 29 psi. Carbon dioxide produced from methyl sulfide combustion was analyzed as above on a continuous flow MAT 252.

Data Analysis
Our approach to data analysis followed that of Moran et al. (2005) where a combined mass and isotope balance is used to track incorporation of labeled methane into metabolic products. We used the following master equation to determine methane oxidation into each examined product:

\[
F_{\text{product}} n_{\text{product}} = \sum_{i=1}^{y} F_{\text{source}_i} n_{\text{source}_i}
\]

(1)

where in each case F represents the $^{13}$C fractional isotope abundance of the chemical species and n is moles of that species. Each TMO product of interest had between two and three possible sources. The final source in each summation was $F_{CH4ox}$ were $n_{CH4ox}$ is the moles of methane oxidized to the analyte of interest and equation 1 was solved for $n_{CH4ox}$ for each analyte. We measured $F_{\text{product}}$ in a series of bottles with increasing initial methane isotopic enrichment ($F_{\text{CH4}}$) and plotting the results shows increasing enrichment in $F_{\text{product}}$ when TMO is a source for that product. The midpoint of the best-fit least squares regression was solved to determine $F_{\text{product}}$ and the point on this relationship corresponding to no labeled methane addition was used as $F_{\text{source}}$ for all but the methane source term. While $F_{\text{product}}$ and $F_{\text{source}}$ are single points it is important to remember that they incorporate the relationship between multiple data points corresponding to the multiple cultures in each experiment. $F_{\text{CH4}}$ was estimated as a mass balance between methane initially added to each culture (F varied in relation to the amount of labeled methane added and n = 1.8 mmol) and biogenic methane produced during growth (F assumed to be 0.0106 and n varied). In each case we also assumed that any isotopic fractionation during methane oxidation becomes insignificant in such a
strongly labeled system, permitting us to assume that $F_{CH_4ox}$ for any product of methane oxidation equals $F_{CH_4}$.

Methane oxidation to carbon dioxide
Sources for total amount of carbon dioxide ($n_{CO_2}$) in the acetate grown cultures include that initially present in the medium ($n_{CO_2_{initial}}$), that produced during growth ($n_{CO_2_{biogenic}}$), and that resulting from methane oxidation ($n_{CH_4ox}$). Solving equation 1 for $n_{CH_4ox}$ in this case shows:

$$n_{CH_4ox} = \frac{F_{CO_2}n_{CO_2} - F_{CO_2_{initial}}(n_{CO_2_{initial}} + n_{CO_2_{biogenic}})}{F_{CH_4ox}}$$ (2)

where $F_{CO_2_{initial}}$ is interpolated from the linear best-fit curve at the point corresponding to addition of unlabeled methane. We assume that this point includes contributions from initial and biogenic CO$_2$ but not CO$_2$ from methane oxidation. Likewise, $F_{CO_2}$ is calculated from the best-fit relationship at the median $^{13}F_{CH_4}$. Note the requirement of knowing $n_{CO_2}$ in order to determine $n_{CH_4ox}$. To overcome this limitation we approach the solution iteratively, first assuming $n_{CO_2}$ without an $n_{CO_2_{ox}}$ term and then accounting for that in subsequent iterations. The calculation converges in seven steps. Estimates of methane oxidation in control bottles were determined in the same manner as above but with an $n_{CO_2_{biogenic}} = 0$, since no growth occurred in the control bottles.

To determine methane oxidation to carbon dioxide in the carbon monoxide-grown cultures, we used the following:

$$n_{CH_4ox} = \frac{n_{CO_2}(F_{CO_2} - F_{CO_2_{biogenic}})}{F_{CH_4ox} - F_{CO_2_{biogenic}}}$$ (3)

where the terms are defined as above. $n_{CO_2}$ initial is zero in this case and was excluded from equation 3. Since $n_{CO_2}$ is a measured quantity, the solution of (3) did not require iteration.

Methane oxidation to acetate and methyl sulfides
Total acetate ($n_{\text{ace}}$) in the acetate-grown cultures is derived from two sources: residual acetate provided but not consumed in the medium ($n_{\text{aceinitial}}$) and acetate from methane oxidation ($n_{\text{CH4ox}}$). We calculate $n_{\text{CH4ox}}$ as follows:

$$n_{\text{CH4ox}} = n_{\text{ace}} \frac{(F_{\text{ace}} - F_{\text{aceinitial}})}{F_{\text{CH4ox}} - F_{\text{aceinitial}}}$$

(4)

where F represents the $^{13}$C fractional abundance for each of the n terms respectively. $F_{\text{ace}}$ is calculated at the median $^{13}$F$_{\text{CH4}}$ value on the best-fit relationship of $F_{\text{ace}}$ versus $F_{\text{CH4}}$ values and $F_{\text{aceinitial}}$ is calculated from the midpoint of the same best-fit relationship in control bottles with no acetate consumption.

The carbon monoxide-grown cultures were not provided with acetate although acetate is produced during growth. In this case, determination of $n_{\text{aceox}}$ uses equation 4 with the substitution of acetate produced during growth ($n_{\text{acebiogenic}}$) for initial acetate ($n_{\text{aceinitial}}$). Additionally, $F_{\text{acebiogenic}}$ is interpolated from the best-fit relationship describing $F_{\text{ace}}$ and $F_{\text{CH4}}$ at the point with addition of no labeled methane since control bottles do not contain acetate. Determining methane oxidation to methyl sulfides used equation 4 with substitution of the equivalent methyl sulfide value for each term above.

Error assessment

Determination of methane oxidation to each analyte requires interpolation of $F_{\text{product}}$ from the best-fit relationship describing $F_{\text{product}}$ and $F_{\text{CH4}}$. Methane oxidation error was estimated by carrying one standard error in the calculated $F_{\text{analyte}}$ through each of the above equations. Variation produced by all measurement imprecision as well as slight differences in culture growth can be approximated using the error on $F_{\text{product}}$ since this value is dependant upon the other measured quantities.

3.4 Results and discussion

We examined TMO by *M. acetivorans* during acetoclastic growth and growth on carbon monoxide and looked at multiple potential TMO products.
Cultures grown on acetate

*M. acetivorans* cultured on acetate produced no appreciable isotopic enrichment in post-growth carbon dioxide ($2.2E-8 \pm 1.5E-4$ moles; figure 3.2A). This contrasts to previous work (Moran et al., 2005) where the same organism oxidized between $2.0E-6$ (on methanol) and $2.0E-5$ (on trimethylamine) moles methane into carbon dioxide when cultured under otherwise similar conditions. *M. acetivorans* more effectively oxidized methane into acetate, producing robust $^{13}$C enrichment (figure 3.2B), which represents oxidation of $1.1E-6$ ($\pm 3.5E-8$) moles methane, or 0.10 % of the biogenic methane produced. We measured the amount of methane-derived acetate only at the completion of culture growth and, therefore, have likely underestimated actual methane oxidation because enriched acetate produced early in growth could be reused for methane production.

We used intramolecular isotope analysis (Dias et al., 2002) to separately determine whether oxidation of methane to either acetate’s carboxyl or methyl group was favored. We observed strong preferential enrichment in acetate’s methyl group over the carboxyl group (figure 3.3). We therefore conclude that acetate formation during TMO originates from two distinct carbon pools, an enriched methyl group derived from the labeled methane used in this experiment, and a carboxyl group likely from dissolved inorganic carbon (DIC). Formation of acetate from these two components would be catalyzed by carbon monoxide dehydrogenase (CODH), demonstrating this enzyme’s role in TMO to acetate.

Acetate has also been invoked as a potential intermediate between methanotroph and sulfate reducer components of AOM consortia (Valentine and Reeburgh, 2000, Zehnder and Brock, 1980). Similarity between TMO and AOM suggest CODH would play a role in AOM acetate production and putative CODH sequences have been observed in ANME-1 environmental samples (Hallam et al., 2004, Meyerdierks et al., 2005). CODH is known to produce acetate from carbon dioxide and a methyl group (Madigan et al., 2000). Based on isotopic constraints, however, acetate formation by CODH does not
account for observed extreme $\delta^{13}C$ depletions in AOM associated lipids and biomass (Hinrichs et al., 1999, Orphan et al., 2001b). Acetate is a building block for membrane lipid synthesis and their isotopic depletion strongly indicates that naturally $^{13}C$-depleted methane is the source of both carbon atoms in acetate (Hinrichs et al., 1999, Elvert et al., 2000, Bain et al., 2001). Reported $\delta^{13}C$ values for DIC at AOM systems (Blair and Aller, 1995 and Teske et al., 2002) are not as low as for methane, and CODH incorporation of DIC into acetate would produce intramolecular isotope variations inconsistent with the extreme isotope depletions observed in lipid biomarkers. For instance, Orphan et al. (2002) analyzed AOM sediments from the Eel River Basin and found $\delta^{13}C$=-49.5‰ for methane and $\delta^{13}C$=-21.7‰ for DIC. Acetate formation entirely from methane carbon still requires further fractionation to account for the observed lipid values ($\delta^{13}C$=-107‰), but significantly less than that needed for lipids built from acetate composed of a mixture of methane and DIC carbon ($\delta^{13}C$=-38.3‰). Furthermore, Orphan et al. (2001a) monitored both Archaeal lipid and carbonate $\delta^{13}C$ values down a sediment core containing AOM activity and observed nearly constant, highly depleted $\delta^{13}C$ lipids values even though carbonate $\delta^{13}C$ values decreased from -18.0‰ near the surface to -29.9‰ at 9-12cm depth. If DIC was incorporated into acetate, the $\delta^{13}C$ values of lipids would have decreased with depth to track the carbonate trend. Our observed intramolecular isotope patterns with only methyl carbon showing the methane carbon origin, then, suggest acetate produced by CODH is not an intermediate in AOM.

**Carbon monoxide cultures**

When *M. acetivorans* cultures were grown using carbon monoxide as the only substrate (figure 3.4), oxidation of methane to carbon dioxide was near our detection limit (1.9E-7 ± 2.0E-7 moles) suggesting either no or very little methane oxidation took place. However, methane oxidation to acetate was appreciable in the cultures (1.8E-7 ± 7.3E-8 moles or 0.14 % of the methane produced by the culture). These results, when compared to those of Harder (1997) and Moran et al. (2005) also with *M. acetivorans*, argue for a reverse methanogenesis-type mechanism in which TMO proceeds through exclusively active and expressed methanogenic metabolisms, thereby limiting potential TMO
products. Acetate, then, is a TMO product of *M. acetivorans* growth when an acetate metabolisms is active and either consuming acetate (during acetoclastic methanogenesis) or producing acetate (as during growth on carbon monoxide). Carbon dioxide is a TMO product during *M. acetivorans* methylotrophic growth (Harder 1997, Moran et al. 2005) but the pathway used for carbon dioxide production (Keltjens and Vogels, 1993) is not active during growth on acetate or carbon monoxide. Instead, the sharp partitioning between methyl and carboxyl carbon by CODH prohibits TMO to carbon dioxide in this special case.

We discovered that during growth on carbon monoxide, sulfide methylation by *M. acetivorans* produced both methyl mercaptan and dimethyl sulfide (Moran et al., in preparation). Methyl sulfide isotopic analysis (figure 3.4, B) indicated oxidation of 4.0E-6 (± 7.7E-7) moles methane, or 3.1% of the biogenic methane produced. In absolute terms, conversion of methane to methyl sulfides during growth on carbon monoxide was over 22 times greater than its conversion to acetate by the same culture. Methane is not the main metabolic growth product in this metabolism; when normalizing to percent biogenic methane oxidized we should be mindful that there are fewer moles of methane produced in these cultures. Yet, the ratio of methane oxidation to methane production has historically been used to evaluate TMO amounts between culture species (Zehnder and Brock 1979) and remains a valuable tool for comparing previous work with our current results. As a percent of produced methane, conversion to methyl sulfides during growth on carbon monoxide was over seven times larger than the highest methane oxidation (to CO$_2$) amount reported by Moran et al. (2005) in earlier studies of this organism and nearly ten times greater than that in any pure culture studied by Zehnder and Brock (1979).

In the course of growth on both acetate and carbon monoxide, *M. acetivorans* utilized enzyme pathways interconnecting methane with a potential number of possible AOM methanotroph products; acetate, carbon dioxide, and methyl sulfides. Of these products, methyl sulfides showed the highest amount of methane incorporation. One explanation
involves initiation of methane oxidation by co-enzyme M (CoM) reductase and then transfer as a methyl group to sulfide. Hallam et al. (2003) and Krüger et al. (2003) showed evidence for CoM reductase involvement as a likely initial methane receptor in AOM. Work with a closely related organism (*Methanosarcina barkeri*) suggests methyl group transfer from methyl CoM to methyl mercaptan would need to overcome a modest energy barrier of 0.35 kJ per reaction (Tallant et al., 2001). Further, the carrier protein involved in this transfer is methylated when CoM reductase is inhibited showing that any energy barrier associated with methyl group transfer away from methyl CoM can be overcome (Cao and Krzycki, 1991). Therefore, transfer of a methyl group from the sulfur bond of CoM to a sulfide linkage would encounter few energetic barriers and provide a methane conversion route (figure 3.5). The role of methyl sulfides in AOM has, to the best of our knowledge, never been explored. Such high preference for methyl sulfide production in the TMO of a closely related methanogen combined with low thermodynamic barriers to methyl sulfide formation, however, suggests the occurrence of methyl sulfides in AOM environments should be examined.

### 3.5 Conclusion

TMO was observed during growth of *M. acetivorans* on both acetate and carbon monoxide (figure 3.6). Methane incorporation into acetate was observed in both cases. Intramolecular tracking of a stable isotope label demonstrated selective methane conversion to only acetate’s methyl and not carboxyl carbon in the acetate-grown cultures. Such high intramolecular differences suggest strict discrimination by CODH prevents mixing of reduced and oxidized carbon pools. The heterogeneity of CODH-produced acetate observed during TMO precludes its role as an AOM intermediate where strong isotopic depletions rule out a mixed carbon source.

In sharp contrast to earlier work, we found no methane oxidation to carbon dioxide under either acetate or carbon monoxide growth conditions, and we suggest methane oxidation to carbon dioxide in *M. acetivorans* proceeds only during methylotrophic growth. This suggests, for *M. acetivorans*, that carbon monoxide is oxidized to carbon dioxide through
CODH activity and not via a reversal of the carbon dioxide reduction pathway, which is a known TMO route in *M. acetivorans* and other methanogen species (Zehnder and Brock, 1979; Harder 1997; Moran et al., 2005).

The proportion of biogenic methane conversion to methyl sulfides is higher than for any previously reported TMO product, likely due to low energy barriers for methyl sulfide production. Further, our results demonstrate that acetate produced by CODH is inconsistent with an AOM intermediate and that, unless acetate production occurs by a different pathway, acetate is not an intermediate for AOM. Our results indicate compounds including methyl sulfides (this paper) or methanol (Moran et al., 2005) are more likely AOM intermediates.
3.6 Figures

Figure 3.1 Metabolic pathways examined for TMO in *M. acetivorans* cultures.

Methane production is the hallmark of methanogenic growth and we examined methanogenesis pathways (solid arrows) from both acetate (H$_3$C-COOH) and carbon monoxide (CO) in *M. acetivorans* cultures. Concurrent with methane production in acetate grown cultures is carbon dioxide evolution (CO$_2$), which contributes to the dissolved inorganic carbon pool (DIC). CO$_2$ is also produced in addition to acetate and methyl sulfides in the CO metabolism. We quantified trace reversal of these enzymatic pathways that oxidizes methane to any of the associated metabolic products (dashed arrows). Key metabolic enzymes include those highlighted; carbon monoxide dehydrogenase (CODH) and methyl coenzyme M reductase (CoM Reductase).
Figure 3.2 Isotope analysis of carbon dioxide and acetate in acetate grown cultures.

A) Isotopic CO$_2$ analysis after growth (■) shows minimal methane incorporation in cultures grown on acetate with a nearly identical trend displayed by the control incubations (□). B) In contrast, the residual acetate (●) in post-growth cultures shows clear isotope enrichment distinct from the control (○) incubations suggesting methane conversion to acetate.
Two isotopic measurements of acetate were performed; first, complete combustion permitted analysis of the entire molecule and second, pyrolysis allowed the selective analysis of acetate’s carboxyl group. An isotope mass balance comparison of the total and carboxyl carbon composition permits calculation of the methyl group’s isotopic carbon composition. The stronger slope, and thus higher incorporation of precursor methane, in the methyl position demonstrates the overall preference for methane oxidation to methyl carbon versus carboxyl carbon.
Figure 3.4 Isotopic analysis of headspace products produced during carbon monoxide growth.

A) Isotopic CO$_2$ analysis (■) shows minimal methane incorporation above control incubations (□). B) Post-growth methyl sulfide (▲) isotope enrichment is significantly higher than that of acetate (●) suggesting enhanced methane conversion to the former.
Methyl sulfide production was both observed in cultures grown on carbon monoxide and was the primary receptor for oxidized methane carbon. The solid arrows above propose hypothetical routes for both methane and methyl sulfide production. Metabolic intermediates likely include methyl groups that, when transferred to coenzyme M (CoM), can either be reduced by CoM reductase to methane or transferred to methanethiol or sulfide. Work done by Tallant et al. (2001) on a similar methyltransferase suggests the energetic yield of methyl transfer from methyl sulfides to CoM is low. Taken inversely, the energetic barrier for methyl transfer to a methyl sulfide would be both low and present a potential pathway for methane conversion to methyl sulfides. Dashed arrows represent possible TMO carbon flow from methane to both methyl sulfide and, through metabolic intermediates, acetate.

**Figure 3.5** Potential pathway for TMO to methyl sulfide products through carbon monoxide metabolism in *M. acetivorans* (adapted from Moran et al., in prep.).
Figure 3.6 TMO products in *M. acetivorans* cultured on different substrates.

The above depicts methanogenic routes used by *M. acetivorans* where bold type identifies repositories of methane-derived carbon produced during TMO and underlined type describes TMO products observed in this study. Oxidation of labeled methane (*) to carbon dioxide has been observed only during methylotrophic growth (Harder, 1997 and Moran et al., 2005) while we demonstrate here that methyl sulfides and acetate are potential TMO products of other metabolic routes.
3.7 References


Chapter 4

Methyl sulfide production by a novel carbon monoxide metabolism in *Methanosarcina acetivorans*

4.1 Abstract
Methyl sulfides including dimethyl sulfide and methanethiol are central to the Earth’s sulfur cycle as they link marine and terrestrial sulfur pools. Natural sources of methyl sulfides to the environment include breakdown of dimethylsulfoniumpropionate, hydrogen sulfide methylation, and decomposition of aromatic and amino acid sulfur-containing compounds. Here we report the first known metabolic production of dimethyl sulfide and methanethiol by a methanogen, *Methanosarcina acetivorans*, and also the first reported methyl sulfide production using carbon monoxide (CO) as a sole carbon source. Exposing *M. acetivorans* cultures to increasing initial sulfide concentrations enhanced both growth and methyl sulfide production, suggesting energy conservation through this novel metabolism. Free energy changes during the reaction are not only favorable to growth, but can also be more energetic than other non-methanogenic CO pathways in marine sediments. CO inhibits methanogenesis, and we propose that methyl sulfide production is a mechanism for energy synthesis when cells experience CO exposure. Further, methyl sulfides are known methanogenic substrates and provide an energy source once a CO burden is lifted. Prominence of both methanogenic species and CO during Earth’s early history suggests methyl sulfide production may have been important through the Archean, and it is, therefore, a potentially significant biologic indicator for life on other planets. CO is also a known (but poorly quantified) component of modern anoxic environments, this new metabolism is potentially both a significant sink for CO in marine sediments and an important source for DMS to the modern sulfur cycle.

4.2 Introduction
The global natural biogenic sulfur flux to the atmosphere is estimated at 103 Tg S per year (Andreae and Raemdonck, 1983), and is nearly equal to the anthropogenic flux of approximately 104 Tg S per year (Cullis and Hirschler, 1980). Dimethyl sulfide (DMS)
contributes up to 75% of the biogenic flux (Kelly and Smith, 1991), forming an essential link between the marine and terrestrial sulfur cycles by supplying a volatile sulfur compound to the atmosphere for later deposition onto land surfaces (figure 4.1) (Lovelock et al., 1972). Decomposition products of DMS in the atmosphere increase atmospheric acidity, leading to acid rain (Nriagu et al., 1987) and influence global climate by initiating cloud condensation (Charlson et al., 1987).

Microbial pathways dominate biogenic DMS production and include: breakdown of dimethylsulfoniumpropionate (Wakeham et al., 1987; Lomans et al., 1997; Yoch et al., 2001), sulfide and methanethiol (MeSH) methylation (Zinder and Brock, 1978; Kiene and Hines, 1995; Lomans et al., 1997; Visscher et al., 2003), and decomposition of aromatic sulfur containing compounds or sulfur containing amino acids (Zinder and Brock, 1978; Finster et al., 1990; Lomans et al., 2001). Microbial decomposition pathways largely balance production, degrading up to 90% of DMS produced before its atmospheric release (Lomans et al., 2002). Known DMS decomposition routes include activity by phytoplankton (Wakeham et al., 1987; Fuse et al., 1995) and bacterial sulfate reducers (Kiene, 1988; Tanimoto and Bak, 1994; Visscher et al., 2003). In areas depleted of both oxygen and sulfate, methanogens are the primary DMS consumer in both marine and freshwater environments (Zinder and Brock 1978; Oremland et al., 1989; Lomans et al., 1999a; Lomans et al., 1999b; Lomans et al., 1999c). (For reviews of these processes see Kelly and Smith 1991 and Lomans et al., 2002.)

Rother and Metcalf (2004) recently described a carbon monoxide (CO) metabolism for the methanogen *Methanosarcina acetivorans*. Unlike previously described CO methanogenic pathways (Kluyver and Schnellen, 1947; Daniels et al., 1977; O’Brien et al., 1984), this metabolism produces acetate and formate but not hydrogen. In addition to acetate and formate, here we report the metabolic production of DMS and MeSH by *M. acetivorans* when cultured on CO. We also suggest that methyl sulfide production is a means of energy conservation, chemical detoxification, and substrate storage during exposure to CO and sulfide. CO is associated with metabolic activity in a wide variety of
microbial systems (Conrad et al., 1983; Rich and King, 1999; Bartholomew and Alexander, 1979; Conrad and Seiler 1980; Visscher et al., 2003) but processes regulating CO concentrations in these systems remain largely unquantified. Linking CO reduction with sulfide methylation, as demonstrated here, makes methyl sulfides a potentially significant CO sink throughout anoxic marine sediments and further ties CO to production of DMS, the largest natural sulfur source to the atmosphere.

4.3 Methods

Methyl sulfide monitoring experiment

*Methanosarcina acetivorans* C2A was cultured in media similar to that used by Moran et al. (2005) but with reduced sodium bicarbonate concentration (1. g / L medium), no initial sulfide addition, KH$_2$PO$_4$ addition from a sterile stock solution after media autoclaving, and no organic substrate addition. 150 mL medium was dispensed into 600 mL anaerobic culture bottles that were then pressurized to 3 atmospheres with CO. Sulfide was added to the bottles using an anaerobic 0.78 M sulfide stock solution. Sulfide additions totaled 0.00, 0.16, 0.31, and 0.63 mmoles per culture bottle in four different sets with triplicates in each set. Culture bottles were autoclaved following sulfide addition. Standard bottles for analyte quantification were made in parallel with the experimental bottles above but lacked sulfide addition. Methane was added to the standard bottles prior to autoclaving, and DMS and MeSH were filter sterilized and added following bottle autoclaving.

Cultures were inoculated with 2.0 mL from a CO-grown preculture and incubated at 33.5 °C. Immediately following inoculation and at intervals throughout growth, 1.0 mL liquid samples were collected from each bottle for sulfide analysis, ion chromatograph analysis, and cell counting (performed using phase-contrast microscopy with a counting chamber). Headspace samples of 200 µL were analyzed for CH$_4$, DMS, and MeSH.

Samples were preserved for sulfide analysis by precipitation with ZnCl and refrigerated until analyzed. Sulfide 1 reagent and sulfide 2 reagent (catalog numbers 1816-49 and
1817-49 respectively, HACH Company, Loveland CO) were added to the samples and allowed to develop for 5 minutes. Absorbance ($\lambda = 655$ nm) was measured on a Beckman DU 530 spectrophotometer (Beckman Coulter, Fullerton, CA) and concentrations determined by comparison to standards.

Acetate and formate concentrations were determined using a Dionex LC30 ion chromatograph and an Ion Pac AS18 analytical column with precision better than 10%. Small (~ 0.5 mL) culture aliquots were centrifuged (15 minutes at 5,000 rpm) then decanted to remove particulate and cell material. The resulting supernatant was stored at −20 °C until analyzed.

Gas samples were analyzed using a Hewlett Packard 5890 gas chromatograph with flame ionization detector. A GS-Q column (30m, 0.32mm ID) (J & W Scientific, Palo Alto, California) was used with a column pressure of 21 psi and an isothermal temperature of 60 °C. DMS and MeSH analysis used 200 $\mu$L of headspace from each bottle and quantification (precision better than 10%) was by comparison to standards run daily.

CO toxicity experiment
The media used for testing CO toxicity was the same as that used previously (Moran et al., 2005) with 5.0 mL / L methanol and an initial headspace of 1.5 atmospheres N$_2$: CO$_2$ (80:20). 30 mL medium was dispensed into 120 mL bottles. The bottles were inoculated with 0.3 mL inoculum from a methanol-grown preculture of $M. \ acetivorans$ and incubated at 33.5 °C. We used three sets of bottles with triplicates in each set. The first set received an injection of 0.5 atmospheres CO at the time of inoculation, the second set received 0.5 atmospheres CO after 47 hours, and the third set never received exposure to CO. Growth was monitored throughout incubation by removing 1.0 mL of medium and analyzing its absorbance ($\lambda=550$ nm) on a Beckman (Fullerton, CA) DU 530 UV / Vis spectrophotometer.
4.4 Results / Discussion

Rother and Metcalf (2004) demonstrated growth of *M. acetivorans* with CO as the sole substrate and CO$_2$, acetate, formate, and methane as products. We observed the novel metabolic products of both DMS and MeSH (in addition to acetate, formate, and methane) during growth of *M. acetivorans* in the presence of CO (figure 4.2). Methyl sulfide formation effectively scrubbed out free sulfide in the culture media to less than 4% of the pre-growth value, suggesting sulfide methylation as a pathway for methyl sulfide formation. Higher cell density and increased methane production by *M. acetivorans* cultures exposed to greater sulfide abundance (figure 4.3) suggest cells produce energy during sulfide methylation.

At standard state, energy yields for methyl sulfide production are consistent with energy conservation:

$$CO + \frac{1}{3}H_2S + \frac{1}{3}H_2O \rightarrow \frac{1}{3}CH_3SH + \frac{2}{3}CO_2 \quad [1]$$

$$\Delta G^\circ' = -34.4 \text{ kJ/mol CO consumed}$$

Free energy gains are comparable to those of acetate formation from CO:

$$CO + \frac{1}{2}H_2O \rightarrow \frac{1}{4}CH_3COO^- + \frac{1}{2}CO_2 + \frac{1}{4}H^+ \quad [2]$$

$$\Delta G^\circ' = -39.6 \text{ kJ/mol CO consumed}$$

*M. acetivorans* was isolated from sediments rich in decaying sea-grass and kelp deposits (Sowers et al., 1984) that invariably deviate from standard state conditions. Bladders used to keep kelp upright underwater are filled with up to 12% CO (Langdon, 1917) and their decay is a likely CO source. Microbial sulfate reducers, fermenters, and acetogenic organisms supported by the sediment’s rich organic content would elevate sulfide and acetate activities favoring sulfide methylation (equation 1) over production of acetate (equation 2) by *M. acetivorans*. 
It remains unlikely, however, that methyl sulfide production would be more favorable than methane production:

\[
CO + \frac{1}{2}H_2O \rightarrow \frac{1}{4}CH_4 + \frac{3}{4}CO_2
\]  

\[\Delta G^{\circ} = -46.5 \text{ kJ/mol CO consumed}\]

Slowing of methanogenesis (equation 3) caused by CO inhibition may help explain the diversion of some metabolic carbon flow away from more energetically favorable methane formation to methyl sulfide production. CO is a known methanogenesis inhibitor when *Methanosarcina barkeri* is grown on methanol (O’Brien et al., 1984) and high CO concentrations inhibit methanogenesis in *Methanobacterium thermoautotrophicum* cultures using CO as their sole growth substrate (Daniels et al., 1977). Previous work with *M. acetivorans* demonstrated reduced methanogenesis when cells were exposed to greater CO partial pressure but the same study also reported increased biomass production with higher CO availability (Rother and Metcalf 2004).

We sought to confirm CO inhibition of *M. acetivorans* cultures using methanol as the growth substrate (figure 4.3). CO applied during logarithmic cell growth produced an immediate reduction in growth rate but the cultures recovered and final cell masses were nearly equivalent to those never experiencing CO exposure. Actively growing methanol cultures may be able to neutralize CO toxicity, possibly because actively growing cells have enough energetic reserves to replace affected enzymes or produce detoxifying compounds to overcome the stress.

We hypothesize that CO toxicity targets the methanogenic pathway, helping explain why CO added with culture inoculation inhibits growth (figure 4.4). Unlike actively growing cultures, a freshly inoculated culture lacks the metabolic reserves needed to combat CO inhibition of methanogenic energy production. Interestingly, methanol-grown *M. acetivorans* cultures can eventually overcome this stress and grow using CO, but this metabolic conversion can take weeks (data not shown). Although toxic, CO does not kill
the culture, and once adapted to growth on CO, cells can employ non-methanogenic routes for energy conservation (including methyl sulfide and acetate formation) and culture growth can resume.

Exposure to CO does not inhibit acetogenic or methyl sulfidogenic growth, so we propose that CO inhibits methyl-coenzyme M reductase (MCR) and constricts carbon flow through this enzyme. MCR is the terminal enzyme-mediated step in all methanogenic metabolisms (Ermler et al., 1997), so CO inhibition of MCR is consistent with the observed drop in methane production in the presence of CO. Since the acetogenic and proposed methyl sulfidic pathways of *M. acetivorans* cultured on CO produces energy without MCR activity, it is unaffected by MCR inhibition.

Reduced MCR activity would increase methyl-coenzyme M (methyl-CoM) concentrations by retarding its cycling back to coenzyme M (CoM). CoM is an electron acceptor for energy-producing redox reactions and unchecked methyl CoM buildup would eventually stop energy production by sequestering all CoM. Methyl sulfide formation provides a low-energy method for regenerating CoM without MCR activity. Working with a closely related methanogen (*Methanosarcina barkeri*), Tallant et al. (2001) demonstrated that direct methylation of MeSH to DMS has a modest energy barrier of only 0.35 kJ per reaction. When MCR is inhibited, the transfer protein that normally methylates CoM becomes methylated itself by methyl-CoM (Cao and Krzycki, 1991; Tallant and Krzycki, 1996), and elevated MeSH concentrations promote small-scale methylation of MeSH to DMS (Lomans et al., 1999c); suggesting reversibility in the first step of methanogenic DMS consumption. Thus, in instances of MCR inhibition by CO, methyl sulfide formation is essential for regenerating CoM and maintaining an active metabolism for energy production.

Methyl sulfides are also methanogenic substrates for *M. acetivorans* (Ni et al., 1994), and once a CO stress is removed, the methyl groups from methyl sulfides can be returned to CoM and methanogenesis would resume. Methyl sulfide production, then, serves both as
an energy source when MCR is inhibited and provides a medium for storing methanogenic substrates. *M. acetivorans* was isolated from organic-rich marine sediments containing high amounts of kelp debris (Sowers et al., 1984). Decay of kelp float bladders, which contain up to 12% CO (Langdon, 1917) would provide periodic CO release to sediment microenvironments. Methyl sulfide production would allow *M. acetivorans* to continue growth during periods of CO exposure while producing a methanogenic substrate for periods of low CO concentration.

To the best of our knowledge, the metabolism described here is the first example of a methanogen producing high concentrations of methyl sulfides and of a CO metabolism resulting in sulfide methylation. This novel metabolism is a potentially important source of methyl sulfides in modern anoxic environments. The prevalence of CO throughout anoxic marine sediments remains largely unenumerated, but CO concentrations suggestive of microbial production is observed in other anoxic environments, especially those with high organic content such as paddy soils and eutrophic lake sediments (Robinson, 1930; Conrad et al., 1983; Conrad et al., 1988; Krämer and Conrad, 1993). CO emission has also been observed by methanogen species, likely from its release as a metabolic intermediate (Bott and Thauer, 1987). CO accumulation in sulfide-bearing marine waters or sediments is a potential stimulus for methyl sulfide production by the metabolism described here. The interplay between CO and methyl sulfides in this new path forms a dynamic bridge between global carbon and sulfur cycles by providing volatile organic sulfur compounds for atmospheric release and transport to terrestrial systems (figure 4.5). Understanding the extent of CO utilization for sulfide methylation in anoxic marine sediments and the amount of methyl sulfides later used for methanogenesis will help elucidate the role these sediments play in nutrient cycling and should be further explored.

CO conversion to methyl sulfides may have played a prominent role in ancient sulfur cycling. Methanogens are amongst the earliest evolving species in Earth’s history and were well established by the Late Archean or earlier (Battistuzzi et al., 2004; Ueno et al.,
2006) when biogenic methane was a strong global climate regulator (Kasting and Seifert, 2002). The Earth’s atmosphere may have contained elevated CO concentrations during this period (Kharecha et al., 2005) fostering an environment for methanogen-mediated methyl sulfide production balanced with methane production. Methanogens were frequent components of ancient microbial communities and they remain a recurrent component in modern analogs. Microbial communities dominated the ancient biosphere so even small net methyl sulfide production would create appreciable atmospheric accumulation (Visscher et al., 2003). The quest for life outside our planet depends on searching for chemical signatures of life (Des Marais et al., 2002). If similar, early-evolving organisms are present on other planets, then methyl sulfides provide a valuable target in the search for extraterrestrial microbial life.

4.5 Conclusions
Production of DMS and MeSH by a methanogen culture demonstrates a new metabolism active when *M. acetivorans* is cultured on CO. Correlation between initial sulfide concentration and production of both biomass and methyl sulfides link this pathway to energy conservation. In its native environment, this metabolism could provide *M. acetivorans* with energy-yielding detoxification of CO that simultaneously produces methanogenic substrates (DMS and MeSH) usable when the CO stress is removed. Better constraining the extent of CO production in anoxic marine environments will help establish the significance of this metabolism in global sulfur and carbon cycling. Both methanogens and CO were likely abundant on early Earth making this process potentially important globally and providing an additional biogenic target for the ongoing search for life on other planets.
4.6 Figures

Figure 4.1 Methyl sulfides play a key role in bridging global sulfur pools.

Volatile flux out of the marine sulfur pool is dominated by DMS. This atmospheric source provides sulfur to terrestrial setting through rainout, replenishing sulfur lost by river transport, dust, volcanic, and biogenic gases.
As demonstrated by Rother and Metcalf (2004) CO is converted to acetate, formate, and methane during growth by *M. acetivorans*. We show the additional production of MeSH (■) and DMS (▲) at the expense of sulfide (●) suggesting sulfide methylation as the production mechanism. The points above each represent an average of three parallel cultures having an initial sulfide addition of 0.16 mmoles per bottle.
Figure 4.3 Enhanced methyl sulfide production, methane production, and growth with increasing amounts of sulfide.

We added sulfide in increasing amounts to four different sets of cultures, each set in triplicate. Increased sulfide additions resulted in enhanced MeSH and DMS production with the sum of DMS and MeSH in each bottle shown (○). Methane production (□) and cell density (△) also increased with sulfide addition suggesting both higher rates of methanogenesis and biomass production with increased levels of sulfide.
Figure 4.4 CO inhibition of methanogenic growth by *M. acetivorans*.

These cultures were grown on methanol for which methanogenesis is the only demonstrated energy-yielding metabolism in *M. acetivorans*. Biomass production in three sets of cultures, with triplicates in each set, were monitored using optical absorbance ($\lambda = 550$ nm) with the average of the three cultures shown above. In one set (◊) CO was added with the inoculation and no growth was observed over the monitoring duration. CO was added to the second set (□) after 47 hours of incubation. This set showed decreased growth rate after the CO addition when compared to the third set (▲) which experienced no CO addition. Final biomass production, however, were nearly identical in the second two sets suggesting that, while CO may hinder methanogenesis, an actively growing culture can overcome this stress.
Figure 4.5 Linking carbon and sulfur cycles.

Dimethyl sulfide (DMS) is the major organic sulfur flux to the atmosphere. The metabolism described here links DMS production to the inorganic sulfur pool by consuming sulfide. DMS consumption can link to the carbon cycle by acting as a methane precursor. Carbon dioxide is also produced during both by carbon monoxide consumption and methane production.
4.7 References


Chapter 5

Methyl sulfides: possible metabolic intermediates of anaerobic oxidation of methane

5.1 Abstract
The anaerobic oxidation of methane (AOM) consumes a large portion of the methane released from decomposing marine clathrates, making it a strong regulator in marine methane cycling. Microbial consortia containing archaeal and bacterial constituents can mediate AOM but the interplay between these two groups remains unknown. Previous explorations suggest that H\textsubscript{2} may be transferred between species and if H\textsubscript{2} were the intermediate, low concentrations would be required for energetic favorability. We used a stable isotope methane tracer to test the effects of elevated H\textsubscript{2} pressures on incubations of active AOM sediments from both the Eel River basin and Hydrate Ridge. We observed minimal deleterious effects of the H\textsubscript{2} on methane oxidation, suggesting H\textsubscript{2} does not play an interspecies role in AOM.

In place of H\textsubscript{2}, we propose methyl sulfides as a metabolic intermediate. We use thermodynamic and biochemical evidences to demonstrate the feasibility of methyl sulfides constituting a central role in interspecies carbon transfer in AOM consortia. Incubations of sediment samples with carbon monoxide also demonstrates preference for carbon monoxide over methane as an electron donor and are consistent with demonstrated methyl sulfide production from carbon monoxide by a species (Methanosarcina acetivorans) with close phylogenetic relation to archaeal methanotrophs.

5.2 Introduction
Barnes and Goldberg (1976) and Reeburgh (1976) first observed anaerobic oxidation of methane (AOM) when measuring declining methane and sulfate concentrations through anoxic coastal sediments. Although the microorganisms responsible for AOM are not isolated in pure culture, using sediment incubations, Nauhaus et al. (2002) confirmed methane oxidation is linked to sulfate reduction with the net stochiometery:
\[ \text{CH}_4 + \text{SO}_4^{2-} \rightarrow \text{HCO}_3^- + \text{HS}^- + \text{H}_2\text{O} \quad \text{(equation 1)} \]

Methane clathrates are the dominant methane source for AOM communities and median estimates suggest clathrates store 10,000 Gt of methane carbon worldwide (Kvenvolden, 2002). Clathrates are active geologic formations that can both maintain steady methane escape (Liu and Flemings, 2006) and adjust in size and methane flux by responding to environmental destabilization caused by changes in sediment temperature or pressure (Kvenvolden, 1993). AOM communities oxidize up to 90% of the methane released from clathrates (Hinrichs et al., 2000) making them important regulators of this potent greenhouse gas. AOM oxidizes an estimated 300 Tg of methane a year (Hinrichs and Boetius, 2001), helping minimize methane release from marine environments to 2% of the global flux (Reeburgh, 1996).

The dominant role AOM plays in regulating marine methane makes it a significant component of the global methane and carbon cycles. Its importance in these cycles highlights the need to close gaps in the current understanding of AOM mechanisms. Two microbial groups working in consort mediate AOM. Stable isotope analyses of specific lipid biomarkers trace methane carbon through first an archaeal methanotroph then a bacterial sulfate-reducer (Hinrichs et al., 1999; Hinrichs et al., 2000; Pancost et al., 2000; Michaelis et al., 2002). Fluorescence in situ hybridization (FISH) imaging shows these groups can form close physical relationships in spherical consortia clusters with archaeal cores and bacterial shells (Boetius et al., 2000). Isotopic measurements confirm methane oxidation by the consortia. These analyses also show individual archaeal cells sometimes maintain viable AOM activity (Orphan et al., 2002), although mechanistic similarities between AOM by isolated organisms and by cell consortia remain unknown.

There are at least three distinct groups of Archaea (ANME-1, ANME-2, and ANME-3) associated with AOM (Knittel et al., 2005), and these groups show phylogenetic affinity with cultured methanogen species (Hinrichs et al., 1999; Orphan et al., 2001a; Teske et al., 2002). ANME can be paired with bacterial consortia members which show high
genetic similarity with known sulfate reducers (Orphan et al., 2001a; Teske et al., 2002; Leloup et al., 2006). The exact roles of the archaeal and bacterial components in these consortia remain unclear, although it is likely the ANME group oxidizes methane to a substrate used by the sulfate reducer (Blumeburg et al., 2005). Intermediates transferred between species are unknown, in part because AOM species are not isolated in pure cultures. In this paper, we use seep sediment incubations to explore possible AOM intermediates and substrates.

Hoehler et al. (1994) suggested hydrogen as a potential intermediate for AOM based largely on observations at Cape Lookout Bight. They identified that AOM activity followed seasonal cycles, with increased activity in the winter when hydrogen concentrations were lowest. Nauhaus et al. (2005) tested the ability of various substrates to stimulate sulfate reduction in incubations of sediments with AOM activity. They determined that hydrogen, acetate, formate, methanol, carbon monoxide, and methylamines did not support metabolic activity in AOM-associated sulfate reducers, and concluded these substrates were unlikely intermediates in their AOM incubations.

Our work aims to reconcile the conflicting observations on the role of hydrogen in AOM presented by Hoehler et al. (1994) and Nauhaus et al. (2005). We used $^{13}$C-labelled methane to track the fate of methane in incubations of seep sediments with and without added hydrogen. We used similar incubation experiments to determine whether carbon monoxide inhibits AOM or whether it can substitute for methane as an electron donor. We compare our results to past work by Harder (1997), Moran et al. (2005), and Moran et al. (in press) who explored trace methane oxidation (TMO) in species with close genetic relation to ANME groups. TMO produces very low methane oxidation rates in otherwise methanogenic cultures. The combined contributions of our current environmental incubations and past work with pure cultures suggest methyl sulfides are a metabolic intermediate; a suggestion consistent with documented energetic constraints, enzymatic behavior, and geochemical observations.
5.3 Methods

Sampling

Samples were collected aboard the R/V Western Flier in July and August 2005. Push core samples were collected by the ROV Tiburon. We collected the Eel River Basin samples (site T-867, push core 46 and site T-864, push core 46) along the southern (N 40° 47.2001”; W 124° 35.7078”) and northern (N 40° 48.6682”; W 124° 36.7417”) ridges respectively. Hydrate Ridge samples (site T-872, push cores 62 and 63) were collected at one site (N 44° 39.2845”; W 124° 59.9115”). Samples were flushed with argon then sealed in mylar bags and refrigerated shipboard until shipped directly to Penn State upon docking. We made efforts to limit both oxygen exposure and large deviation from in situ temperature.

Incubations

We used a series of batch incubations to test the effects of CO and H₂ on AOM. We combined homogenized sediment samples with seawater and distributed 18 mL of the resulting slurry into 120 mL culture bottles. We degassed the seawater using a stream of N₂ and CO₂ gas (80:20 N₂:CO₂) and then reduced the anoxic seawater with 0.5 g / L N₂S•9H₂O. We estimate final slurry composition at an approximately 1:5 sediment to seawater ratio. We used ice packs to ensure the sediment and slurry temperature remained cool. We sealed the sample bottles in the anaerobic chamber then removed them for gas addition. We used positive incubation controls designed to ensure our setup and sample handling was conducive to AOM. We flushed control bottles with multiple volumes of CH₄ then filled to two bars with CH₄. On top of the CH₄ we added 10 mL ¹³CH₄ (Cambridge Isotope Laboratories, lots 02-01 and 00-02), 10 mL CD₄ (Cambridge Isotope Laboratories, lot 03-02), 30 mL CO₂, and 50 mL N₂. We prepared experimental bottles identically to the control bottles but with slightly different headspace preparations. We substituted 50 mL H₂ for 50 mL N₂ in bottles used to test the effects of H₂ on AOM. In bottles used to test for AOM activity in the presence of CO, we substituted 50 mL CO for 50 mL N₂. For bottles designed to test the ability of AOM communities to oxidize CO, we first flushed the system with multiple volumes of CO then filled each bottle with
2 bars CO, 10 mL $^{13}$CO (Cambridge Isotope Laboratory, lot KGQ-Y), 30 mL CO$_2$, and 60 mL N$_2$. We estimate initial $\delta^{13}$C for both CH$_4$ and CO (when labeled CO was added) at 4400 ± 200 ‰. We included N$_2$ in the headspace additions to ensure equal initial pressures in each sample. We used triplicates for each set of conditions for both sample locations (Hydrate Ridge and Eel River Basin). We reproduced controls of experiments involving CO in quadruplicate. Once prepared, we incubated the samples at 10 °C.

We monitored H$_2$ during incubation to ensure partial pressures remained between 0.25 and 0.50 bar. We used a gas chromatograph with a pulsed discharge detector and quantified H$_2$ concentration against a series of standards mixed in similar culture bottles to those used for the incubations. We used a Molsiv column (HP-MolSiv (19091P-MS8), 30-m, 0.32-mm inner diameter, 25-µm film thickness, J & W Scientific, Santa Clara, CA, USA), constant oven temperature (50 °C), carrier flow of 2.5 mL / minute of helium, and sample injections were 15 µm. We observed H$_2$ consumption in each sample set and after measuring H$_2$ partial pressures we added H$_2$ to bring it back up to 0.50 bar. We measured H$_2$ approximately every 7 to 10 days during the incubation and pressures never fell below 0.25 bar.

We performed isotope analysis on CO$_2$ in each sample bottle following similar protocols to that used by Moran et al. (in press). We collected aliquots of headspace gasses from each sample and cryogenically distilled them to remove water vapor and non-condensable gases (including CH$_4$ and N$_2$) leaving pure CO$_2$ for analysis. Distillation was in line with a Finnigan MAT 252 dual inlet mass spectrometer (Thermo Finnigan, Bremen, Germany) and we introduced purified CO$_2$ samples directly into the mass spectrometer for $^{13}$C/$^{12}$C measurement (with precision better than 1 ‰). We estimated initial $\delta^{13}$C$_{CO_2}$ at -37.8 ‰ based on measurements of the CO$_2$ added to each bottle.
5.4 Results

Results from the incubations under an elevated H$_2$ atmosphere (figure 5.1) show steadily increasing $\delta^{13}$C$_{CO2}$ in both control and experimental incubations from each sampling location (Hydrate Ridge and Eel River Basin). Isotopic CO$_2$ enrichment demonstrates conversion of labeled CH$_4$ to CO$_2$ and is an indicator of active AOM both with and without H$_2$ addition. Increasing $\delta^{13}$C$_{CO2}$ in the incubations with $^{13}$CO (figure 5.2) demonstrate oxidation of CO to CO$_2$ throughout the experiment. In sharp contrast to controls, $\delta^{13}$C$_{CO2}$ in incubations containing both CO and $^{13}$CH$_4$ (figure 5.2) showed negligible enrichment over time demonstrating cessation of CH$_4$ oxidation in the presence of CO (figure 5.2). Table 1 lists individual isotope data for each sample incubation.

5.5 Discussion

We incubated AOM sediments both with and without H$_2$ (0.5 bar) and observed similar CO$_2$ isotopic enrichment, clearly demonstrating the presence of H$_2$ did not hinder methane oxidation (figure 5.1). If H$_2$ is a reaction intermediate, thermodynamic constraints suggest concentrations below 0.29 nM are required to make the reaction energetically favorable (Hoehler et al. 1994). H$_2$ concentrations in our incubations were estimated using Henry’s law to be 0.43 mM, which is over six orders of magnitude above the critical threshold identified by Hoehler et al. (1994). From these results, we conclude that H$_2$ is not a metabolic intermediate in the AOM we observed. This conclusion fully agrees with results by Nauhaus et al. (2005) who demonstrated that H$_2$ addition did not support sulfate reduction by sulfate reducers associated with AOM in the Desulfosarcina and Desulfococcus groups.

Without the benefit of culturable AOM species it is difficult to probe further the specific mechanisms that drive AOM. Thus, we examined previous published studies of the related process of trace methane oxidation (TMO) for insights. Zehnder and Brock (1979) first identified TMO as the trace amount of methane oxidized in growing methanogenic cultures. These cultures oxidize only a small fraction ($\leq$ 0.3 %) of the
methane they produce. Nevertheless, the enzymatic process by which methane is oxidized in these cultures can help reveal potential oxidation pathways and products for the natural AOM process.

Moran et al. (in press) investigated TMO in *Methanosarcina acetivorans*, a methanogen with close phylogenetic relation to the ANME-2 group (Hinrichs et al., 1999; Orphan et al., 2001a). When cultured on CO, methyl sulfides were by far the dominant TMO product. The cultures converted 3.1% of the biogenic methane produced, which is over seven times more methane oxidized (per methane produced) than the maximum previously observed methane oxidation to CO$_2$ by this organism (0.42%, Moran et al., 2005) or nearly ten times higher than the maximum amounts recorded for any methanogen (0.32%) by Zehnder and Brock (1979). These results indicate the presence of an enzymatic pathway supporting conversion of methane to methyl sulfide and that methyl sulfides should be assessed for their potential role in the natural AOM process.

There are several reasons to believe that methyl sulfides could be involved in AOM. First, methyl sulfides are a known methanogenic substrate for *M. acetivorans* (Ni et al., 1994) meaning this organism maintains an enzymatic pathway linking methyl sulfides with methane. Second, several studies suggest that methyl coenzyme M reductase (MCR) is the first step of AOM (Hallam et al. 2003; Krüger et al., 2003; Moran et al., 2005), and there is a demonstrated low energy barrier for methyl transfer between methyl coenzyme M and sulfide (Tallant et al., 2001). MCR activity in AOM would convert methane to a methyl group bound to coenzyme M (CoM). High concentrations of methyl-CoM cause methylation of transfer proteins in *M. barkeri* cultures (Cao and Krzycki, 1991; Tallant and Krzycki, 1996) potentially leading to sulfide methylation and thus methyl sulfide formation.

Based on the findings in TMO experiments (Moran et al., in press), we propose that AOM methanotrophs consume CH$_4$ by producing methyl sulfides including methanethiol as shown here (normalized to consumption of 1 mole methane):
CH₄ + 1/3 HCO₃⁻ + 5/3 H⁺ + 4/3 HS⁻ \rightarrow 4/3 H₂CSH + H₂O \text{ (equation 2)}

Electrons released when CH₄ binds to CoM provide the electron source for this metabolism and help establish a proton gradient for subsequent ATP synthesis. The net electron acceptor for this electron transport is bicarbonate, which is readily converted to CO₂ by carbonic anhydrase prior to its reduction. CO₂ reduction by a pathway similar to that used in CO₂ reductive methanogenesis would produce methyl groups bound to CoM (figure 5.4). Transferring the bound methyl group to sulfide would regenerate the CoM, needed for both CH₄ activation and CO₂ reduction. The CO₂ reductive pathway typically requires H₂ but Lessner et al. (2006) demonstrated electrons derived from CO oxidation provided reducing power used by \textit{M. acetivorans}. Here (equation 2), methane oxidation is the electron source and CO₂ is the initial electron acceptor.

Low methanthiol concentrations are required for positive energy yields from equation 1. Sulfate reducing bacteria could consume methanethiol to maintain a low concentration. Tanimoto and Bak (1994) demonstrated methyl sulfide oxidation by sulfate reduction. They isolated three strains of sulfate reducing bacteria that consume dimethyl sulfide, methanethiol, or both, and oxidize this carbon to bicarbonate with one example shown here:

\[ 4/3 H₂CSH + SO₄^{2-} \rightarrow 4/3 HCO₃⁻ + 7/3 HS⁻ + 5/3 H⁺ \] \text{ (equation 3)}

Normalizing and combining equations 2 and 3 emphasizes potential interplay between both members of AOM consortia (figure 5.3) by yielding accepted AOM stochiometry (equation 1).

Methanethiol transfers methane-derived carbon from the initial methanotroph to the associated sulfate reducer. The interplays proposed here are bilateral. Initial methanotrophy profits from both sulfate reduction products (HCO₃⁻ and HS⁻) and
produces the substrate (H$_3$CSH) used for sulfate reduction in the mutually beneficial relationship. Intense recycling of substrates and products underscores the overall low energy yields of AOM and the necessity for achieving optimal efficiency in every electron transfer.

Transferring substrates, usable by only specialized groups of sulfate reducers, such as methyl sulfides, between consortia members helps explain why only select sulfate-reducing groups (including Desulfocarcina and Desulfococcus) (Orphan et al., 2001a) participate in AOM clusters. We would expect a wider diversity of sulfate-reducing bacteria associated with AOM if consortia members exchanged a widely used substrate such as acetate or hydrogen. As an example, Nauhaus et al. (2005) observed enrichment of non-AOM associated sulfate-reducing populations in incubations with abundant H$_2$, a competitive substrate.

If methyl sulfides are an intermediate in AOM, their production must provide sufficient energy for growth to the methanotroph. Equation 2 is endergonic under standard conditions at pH 8 ($\Delta G^\circ = +55$ kJ/reaction) but is better evaluated at in-situ conditions:

$$\Delta G' = \Delta G^\circ + RT \ln \frac{[H_3CSH]^{\frac{\beta}{2}}}{[CH_4][HCO_3^-][HS^-]^{\frac{\beta}{2}}}$$

(equation 4)

Where $\Delta G'$ is the Gibbs free energy of the reaction under environmental conditions which we approximate using pH 8. We estimate $[CH_4] = 4$ mM, $[HCO_3^-] = 10$ mM, $[HS^-] = 3$ mM, $[SO_4^{2-}] = 15$ mM, and temperature $= 10$ °C. At methanethiol concentrations below 0.36 nM, equation 2 becomes energetically favorable. This low concentration for an AOM intermediate is comparable to the threshold proposed by Hoehler et al. (1994) for H$_2$. Turner et al. (1988) and Leck et al. (1990) observed sharp variation in seawater DMS concentrations, with concentrations nearly two orders of magnitude higher (and above the energetic AOM threshold) in the summer than in the winter (which were below the threshold) around mainland Britain and in the Baltic Sea respectively. If similar
DMS variations occur at Cape Lookout Bight, they would explain the seasonal AOM activity observed by Hoehler et al (1994).

Lower pH values reduce $\Delta G^\circ$ in equation 2:

$$\Delta G^\circ = \Delta G^\circ + m \Delta G_f'(H^+)$$  \hspace{1cm} (equation 5)

where $m$ is the number of protons consumed per reaction. As pH decreases, equation 2 becomes increasingly favorable and positive energy gains are possible at methanethiol concentrations above 0.36 nM (the energetic threshold at pH 8). Protons released by equation 3 and bicarbonate consumption by equation 2 would both contribute to a pH gradient between archaeal and sulfate-reducer consortial partners. Strong pH gradients through the consortia may help explain their spherical shape, which conserves a low surface area to volume ratio and could help maintain lower pH in consortia cores where methanotroph reactions dominate (Boetius et al., 2000; Orphan et al., 2001b). Bacterial sulfate reducers typically aggregate on consortia crusts where close contact with seawater would buffer the environment near pH 8. A spherical morphology would slow removal of the methanethiol intermediate, but in equation 2 we observe that the energetic stimulation of increased $[H^+]$ (through reduced pH) outweighs energetic restraint of methanethiol increase following their stochiometric ratios; 5/3 for $[H^+]$ and 4/3 for $[H_3CSH]$.

Carbon monoxide incubations
Incubations of AOM sediments showed robust CO oxidation to CO$_2$ (figure 5.2). Oxidation of CO to CO$_2$ is carried out by carbon monoxide dehydrogenase (CODH), and Meyerdierks et al. (2005) observed CODH genes in ANME groups. Further, in *M. acetivorans*, Lessner et al. (2006) observed reduction of CO$_2$ with electrons generated from CO oxidation. Moran et al. (submitted) report DMS production by *M. acetivorans*, likely also using electrons generated from CO oxidation. The presence of a similar pathway in our AOM incubations would help explain cessation of CH$_4$ oxidation in the
presence of CO (figure 5.2) since CO is a more favorable electron source than CH₄, and its oxidation would energetically out-compete CH₄ oxidation. In the presence of CO, therefore, instead of equation 2 we propose:

\[
\text{CO} + \frac{1}{3} \text{HS}^- + \frac{1}{3} \text{H}_2\text{O} + \frac{1}{3} \text{H}^+ \rightarrow \frac{1}{3} \text{H}_3\text{CSH} + \frac{2}{3} \text{CO}_2 \quad \text{(equation 6)}
\]

\(\Delta G^\circ\) (at pH 8, temperature = 10 °C) is more favorable using CO (equation 6) in place of CH₄ (equation 2) as an electron donor by 88 kJ/reaction. Higher energy conservation permits higher methanethiol production by archaeal methanotrophs, providing increased substrate availability for sulfate reducers. This is constant with the higher sulfate reducer abundance observed in fluorescence in situ hybridization preparations (following Orphan et al., 2001a) of incubations with CO versus incubations with CH₄.

5.6 Conclusions

Our work with AOM incubations from Hydrate Ridge and the Eel River Basin suggest methyl sulfides, instead of H₂, are the main AOM reaction products, but are otherwise consistent with the previously proposed reverse methanogenesis mechanism for AOM (Hoehler et al., 1994). Coupling archaeal-produced methyl sulfides with bacterial sulfate reduction accounts for observed AOM stochiometries with energetics sufficient to support microbial growth. Future exploration of methyl sulfide effects on AOM incubations and methyl sulfide profiles through AOM environments may ultimately reveal their prominence in the natural AOM metabolism.
5.7 Figures and Table

![Graph A](image1.png)

![Graph B](image2.png)

**Figure 5.1** Isotopic enrichment of CO₂ during incubation with $^{13}$CH₄.

Increased isotopic enrichment ($^{13}$C) of headspace CO₂ throughout incubation demonstrates oxidation of labeled CH₄ (estimated $\delta^{13}$C ≈ 4400 ‰) to CO₂ in both control incubations void of H₂ addition (■) and in experimental incubations with H₂ partial pressures maintained above 0.25 bars (○). We observed positive AOM concurrent with H₂ exposure in incubations from both the Eel River Basin (A) and Hydrate Ridge (B).
Figure 5.2 Comparing isotopic enrichment in incubations with $^{13}$CH$_4$ to those with either $^{13}$CO or $^{13}$CH$_4$ and CO.

Control incubation with labeled CH$_4$ (estimated $\delta^{13}$C $\approx$ 4400 ‰) showed isotopic enrichment in CO$_2$ indicating active AOM to CO$_2$ end-product in both the Eel River Basin (A) and Hydrate Ridge (B) samples. Similar incubations provided a labeled CO (estimated $\delta^{13}$C $\approx$ 4400 ‰) headspace showed CO$_2$ enrichment demonstrating CO oxidation to CO$_2$. Incubations with a headspace containing labeled CH$_4$ (estimated $\delta^{13}$C $\approx$ 4400 ‰) and 0.5 bar initial CO showed negligible isotopic enrichment in CO$_2$, demonstrating cessation of AOM in each set of samples when CO is present.
Figure 5.3 Proposed consortia interactions in AOM.

CH$_4$ and SO$_4^{2-}$ are consumed by the methanotroph and sulfate reducer with HCO$_3^-$ and HS$^-$ as net process products. H$_2$CSH production by methanotrophy is transient, being consumed by sulfate reduction that in turn produces HS$^-$, HCO$_3^-$, and acidity, which facilitates further methanotrophy.
ANME can use methane (CH$_4$) as an electron source by transferring CH$_4$ (as a methyl group) to coenzyme M (CoM) (outlined shading). ANME uses available electrons to reduce carbon dioxide (CO$_2$) to a methyl group and transfers the methyl group to CoM (black shading). Methanethiol production regenerates CoM, permitting both the electron generating and consuming reactions. Carbon monoxide dehydrogenase generates electrons to fuel the metabolism from carbon monoxide (CO) when this substrate is present (gray shading). Our results showed cessation of CH$_4$ oxidation when both CH$_4$ and CO are available suggesting CO is a preferred electron source.
Figure 5.5 Methanotrophic and sulfate reducer energy yields.

Thermodynamic energy yields for methanotrophy and sulfate reduction show opposite dependence upon methanthiol concentration such that both processes are concurrently favorable (negative $\Delta G'$) over a limited methanthiol range. This range expands when we consider a potential pH gradient through AOM consortia that would lower the pH in methanotroph microenvironments. Shown above (light shading) is the calculated methanethiol concentration consistent with energy conservation by both consortia members at pH 8. When pH in the consortia core drops to 7 the viable methanthiol concentration range expands (darker shading).
### Table 5.1 Headspace CO\(_2\) isotope measurements

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5.8 References


Chapter 6

Optimizing DNAzyme activity for phylogenetic 16 S RNA selection

6.1 Abstract
Small subunit ribosomal RNA contains regions of sequence specificity unique to related phylogenetic groups. Here, we use the catalytic motif in the ‘10-23’ DNAzyme with modified recognition domains to design a series of DNAzymes to selectively cleave target microbial 16 S RNA from the domain (Archaeal) to genus (*Methanosarcina*) level. Cleaved RNA can then be separated from non-target RNA by size. Ability to harvest specific RNA polymers from natural samples enables RNA-focused stable isotope investigations of microbe mediated nutrient (specifically carbon and nitrogen) cycles.

We test yields and selectivity of a series of DNAzymes incubated (at both 37 °C and 4 °C) with RNAs extracted from multiple laboratory-cultured microorganisms. We also use the DNAzymes to target specific sequences from mixed RNA samples. Using a stable isotope (\(^{15}\text{N}\)) tracer, we demonstrate the high purity of products resulting from this approach, suggesting that DNAzymes can be used for the capture of RNA for taxon-specific stable isotope investigations.

6.2 Introduction
Microbial communities play crucial roles in Earth’s nutrient cycles by enabling element cycling between the physical and biological worlds. Immense physiological and phylogenetic diversity within microbial communities permits their prominence in nutrient cycling but also poses analytical challenges to scientific investigations linking physiological processes with specific organisms in natural settings. Systematically assessing the role particular microbes play in such complex systems demands a method for isolating signals from individual microbial groups against a complex microbial matrix. Combined biomarker and stable isotope analysis provide one of the most direct routes to interpreting microbial activity. Stable isotope analysis of selected biomarkers
can tie compounds to particular substrates, helping link physiological pathways and nutrient transfer from one organism to another (see Boschker and Middelburg 2002 for review). An array of compounds, including both structural molecules, nucleoside polymers, and enzymatic structures can all be used as biomarkers, but each type has limitations in the context of complex modern ecosystems.

Membrane-bound lipids are widely used as biomarkers, particularly on long timescales. These preserve well but, unfortunately, many specific lipids are found over wide phylogenetic and functional groups (Jahnke et al., 1999), which limits their taxon or metabolic specificity. Secondly, different physical or chemical growth conditions can affect lipid structures produced by a particular microbe and the quantities of specific lipids within a microorganism’s membrane (Jahnke et al., 1999; DeRosa et al., 1986; Schouten et al., 2002) as well as isotope fractionation (Abraham et al., 1998; Jahnke et al., 1999). Further, lipids sometimes display large or variable isotopic fractionation, such that their isotope composition differs from that of microbial bulk biomass (Hayes 2001), complicating detection of small isotopic shifts associated with changes in substrate utilization.

The use of bulk DNA is a potential highly specific biomarker for microorganisms (Coffin et al., 1997; Créach et al., 1999). However, DNA analysis is limited by the need for bulk DNA extraction, which prohibits species-specific exploration and allows only broadly based conclusions from isotope analysis. In some cases bias in DNA extraction results in preferential yields of DNA from a specific phylogeny (such as gram positive versus gram negative) (Créach et al., 1999) but these biases can be difficult to control and have only limited utility.

16 S ribosomal RNA is another biomarker with potential for a high level of specificity within microbial systems. 16 S RNA analysis has several benefits compared to other biomarker systems. Firstly, variations in 16 S RNA sequences follow phylogenetic divisions and are frequently used as a basis for phylogenetic classifications (Hugenholtz
et al., 1998). A biomarker approach exploiting differences in rRNA sequences permits phylogenetic specificity ranging from the domain to species level (Amann, 1995). Secondly, RNA catalyzes the only known route for protein synthesis in all living organisms (Noller, 1993); meaning it is abundant in every known organism. There can be $10^3$ to $10^5$ copies of 16 S RNA per cell in active cultures (Amann, 1995). Such high copy number provides ample material for stable isotope analysis while the phylogenetic specificity assures analysis is targeted at the specific organism of interest. Finally, RNA biosynthetic pathways are very similar in most organisms, suggesting a more universal fractionation pattern than observed with other biomarkers (MacGregor et al., 2002). Further, one exploration of plant DNA (sharing very similar synthetic routes with RNA) suggests a small, fixed offset between DNA and bulk isotopic compositions (Jahren, et al., 2004). Whether nucleic acids are consistently exposed to less extreme isotope fractionation during synthesis in microbial communities remains to be seen.

Despite the possible benefits of using rRNA for exploring microbial systems, few researchers have sought to utilize this tool. Initial studies involving RNA stable isotope analysis have focused on modified stable isotope probing (SIP) protocols, originally developed for use with DNA (Radajewski et al., 2000). The method exposes a living microbial system to a stable-isotope labeled substrate and allows for incorporation of the substrate into microbial biomass. Organisms actively using the heavily labeled substrate incorporate the label into their DNA, increasing the DNA’s density. Ultracentrifugation separates the denser, targeted DNA from DNA produced by organisms not consuming the label. A similar protocol targets RNA to take advantage of RNA’s high copy number and quicker turnover times (Manefield et al., 2002; Lueders et al., 2004).

While SIP may be useful in exploring some microbial systems it also suffers limitations. First, SIP requires large proportions of label incorporation by the active organisms. Without sufficient growth and label incorporation there is insufficient density differences to separate the desired nucleic materials. Secondly, use of this approach for nitrogen isotope investigations is untested. This method is also completely dependent on labeled
substrate utilization. Exposing a microbial system to large amounts of labeled substrate may alter the interactions present in the system but SIP is not sensitive enough to focus at natural isotope abundance levels.

Expanding RNA stable isotope analysis in the direction of modestly or non-labeled, natural systems requires a method for RNA selection that is not dependant upon labeled substrate consumption. Macgregor et al. (2002) and Pearson et al. (2004) both used magnetic bead capture approaches for selecting specific RNA from a mixed sample. While this approach is not fully optimized, typical yields range only up to ~ 25 % (Pearson et al., 2004), limiting detection sensitivity. Inclusion of unlabeled helper probes, however, have improved some yields to sufficient levels for isotope analysis in labeled experiments of natural systems (MacGregor et al., 2006). Non-specific interactions between RNAs are still a concern, and can result in incomplete contaminant removal from targeted isolates, making this approach an effective preliminary purification method, but on its own does not always produce desired purity in isolated products (MacGregor and Amann, 2003).

Uyeno et al. (2004) used RNase H for selecting targeted RNA in preparation for quantifying populations. Oligodeoxynucleotides were hybridized to complimentary RNA sequences and then cleaved by RNase H, which has directed activity at DNA and RNA hybrid sequences (Donis-Keller, 1979). Macgregor and Amann (2003) however, observed non-selective cleavage at sites of multiple base mis-pairings, which could complicate isotopic investigations.

DNAzymes can also be used to cleave RNA, and exhibit higher sequence specificity than RNase H methods (Joyce, 2001). The 10-23 DNAzyme is a short oligonucleotide containing a catalytic loop flanked by two Watson – Crick binding domains that produces Mg$^{2+}$- dependant, sight-specific RNA cleavage (Santoro and Joyce, 1997; Santoro and Joyce, 1998). Enzyme activity is directed towards RNA sequences exactly matching the
sequence in the DNAzyme binding domains. Suenaga et al. (2005) used a similar DNAzyme approach for quantifying a bacterial species in bioreactor samples.

We tested the use of DNAzymes to cleave specific RNA sequences resulting in smaller sequences that can be separated from a complex bulk RNA extract in preparation for stable isotope analysis. We used RNA extracted from cultures of *Methanosarcina acetivorans* to develop and test 10-23-based DNAzymes ranging in specificity from the domain to genus level. We quantified cleavage yields under different conditions and with DNAzymes targeting different sites across the *M. acetivorans* 16 S RNA. We applied select DNAzymes to mixtures of RNA from different organisms to test enzymatic stringency. Further, we used $^{15}$N-labeled RNA to test the stringency of RNA isolation following cleavage by a DNAzyme. *M. acetivorans* is a marine methanogen with close phylogenetic relationship to marine methane oxidizing Archaea. Understanding the applicability of DNAzymes for isolating RNA from *M. acetivorans* will enable future studies focused on multiple groups of microorganisms.

### 6.3 Methods

**Microorganisms**

*Methanobacterium thermoautotrophicum* ΔH, *Archaeoglobus fulgidus* VC16, *Methanococcus jannaschii* JAL-1, and *Methanococcus thermolithotrophicus* SN-1 were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). *Methanococcus maripaludis* JJ was obtained from the Oregon Collection of Methanogens. *Methanosarcina acetivorans* 2CA and *Methanosarcina thermophila* MCM-B705 were kind gifts from Dr. J. G. Ferry (Pennsylvania State University, University Park, PA 16802, USA). *Escherichia coli* (DSM 10235, ATCC 53868) was a kind gift from Dr. J. E. Brenchely (Pennsylvania State University, University Park, PA 16802, USA). *Clostridium acetobutylicum* DSM 792 was a kind gift from Dr. J. Regan (Pennsylvania State University, University Park, PA 16802, USA).
Culturing

Strict anaerobic technique was employed for all cell culturing except when culturing \textit{E. coli}. Medium for \textit{M. thermoautotrophicum} was prepared as described in Moran et al., 2005. Media for \textit{M. jannaschii}, \textit{M. thermolithotrophicus}, and \textit{M. maripaludis} was prepared as for \textit{M. thermoautotrophicum} but with the addition of 20 g per liter NaCl. \textit{M. acetivorans} and \textit{M. thermophila} were cultured using trimethylamine as substrate following the method used by Moran et al., 2005. \textit{C. acetobutylicum} was cultured using ATCC medium 1500 with 10 g per L glucose in place of corn starch. \textit{E. coli} was cultured aerobically on a minimal media containing (per L medium): 12.8 g Na$_2$HPO$_4$•12H$_2$O, 3.0 g KH$_2$PO$_4$, 0.5 g NaCl, 1.0 g NH$_4$Cl, 2.0 g glucose, 0.494 g MgSO$_4$•7H$_2$O, 0.015 g CaCl$_2$•2H$_2$O, 0.010 g FeSO$_4$•7H$_2$O. In experiments using a nitrogen isotope tracer, the nitrogen source for the media was (per L medium) 0.98 g NH$_4$Cl and 0.05 g (\textsuperscript{15}NH$_4$)$_2$SO$_4$ (98+ atom % \textsuperscript{15}N, Sigma-Aldrich, product number 299286) and had an estimated $\delta^{15}$N = +5400 ‰. All cell cultures were grown to late log phase then harvested by centrifugation.

RNA extraction

Fresh cell pellets were extracted using the RNeasy mini extraction kit (Qiagen, catalog number 74104, Valencia, CA, USA). Species with highly durable cell membranes required additional treatments to ensure cell lysis, and we treated \textit{M. maripaludis} and \textit{M. jannaschii} with Proteinase K to enhance RNA extraction. Extract purity was confirmed by gel electrophoresis in a 1 \% agarose gel and samples were stored at –80 °C.

DNAzyme selection

We constructed a PERL script for identifying all potential targets for the 10-23 DNAzyme; those sections of RNA sequence containing a purine followed by pyrimidine residue (in the 5’ to 3’ direction). This script is available in the appendix. Input for the script is the 16 S gene sequence. We assessed hylogenetic specificity for potential target sites by using a probe match search performed on ARB (Ludwig et al., 2004). Once the script identified all potential sites, we manually selected experimental targets based on phylogenetic sequence specificity and position within the 16 S sequence. We gave
preference to sequences more central in the 16 S that would produce fragments of similar size and therefore more easily resolved from uncut 16 S. We also allotted further preference to target sequences overlapping known FISH probe sites, which likely have less inhibition to DNAzyme binding by secondary RNA structure. We obtained all DNAzymes from Sigma Genosys (The Woodlands, Texas, USA).

We used the script to predict the Gibbs free energy change for binding of a specific DNAzyme to its target at both 37 °C and 4 °C (ΔG°37 and ΔG°4) for each DNAzyme following the method of Sugimoto et al. (1995). This approach looked at the thermodynamics of RNA – DNA duplex formation with each recognition domain of a DNAzyme and then averaged ΔG°37 calculated for each recognition domain. A helix initiation penalty (+3.1 kcal / mol) was assessed each duplex.

DNAzyme cleavage
We performed RNA cleavage reactions in either an EPPS-based or TA-based buffer. The EPPS buffer contained 5 mM EPPS, 15 mM sodium chloride, and 10 mM magnesium chloride and pH 8.0. The TA buffer contained 33 mM tris-acetate, 66 mM potassium acetate, 10 mM magnesium acetate, and 0.5 mM dithiotreitol at pH 7.8 and was obtained from Epicentre (Madison, Wisconsin, USA). We performed RNA cleavage by combining the DNAzyme with RNA and 0.04 % sodium dodecyl sulfate in each of the above buffers. Incubation was either 1 hour at 37 °C or 5 hours at 4 °C. All cutters were originally tested with M. acetivorans RNA in both the EPPS and TA buffers at 37 °C. We then tested cutters that produced observable RNA cleavage in both buffers at 4 °C.

We tested cutters with desired phylogenetic specificity and high cutting yields in both buffers with a variety of different RNA’s and mixtures of RNA from multiple species. All RNA mixtures contained equal proportions of each RNA type (as measured using a spectrophotometer at λ = 260 nm). We separated cleaved products using gel electrophoresis in a 1 % agarose gel and visualized using SYBR gold (Invitrogen, catalog number S-11494, Carlsbad, CA, USA). We assessed yields for each DNAzyme tested.
using a profile analysis of the resulting electropherograms. We defined cutting yields based upon the resulting profile areas:

\[
Yield = \frac{(area_1) + (area_2)}{(area_1) + (area_2) + (area_{16S})} \times 100\%
\]

where area 1 and area 2 refer to the band size of cleaved products and area 16 S refers to the band size of uncleaved, 16 S RNA.

Cutter stringency
We combined equal portions of RNA isolated from *M. acetivorans* and *E. coli*. We provided $^{15}$N-labeled ammonium ($\delta \approx +5400 \text{‰}$) as the only nitrogen source for the *E.coli* culture, ensuring high $^{15}$N enrichment in isolated RNA. After mixing, we used the DNAzyme targeting base 813 in the sequence to selectively cleave only the targeted *M. acetivorans* RNA. We used TA buffer (TA?) for the reaction and incubated for 1 hour at 37 °C. We visualized cleavage products using electrophoresis in a 1 % agarose gel. We manually excised agarose slices containing cleavage products from the reaction. Using a spin column (Ambion, catalog number 10065, Austin, TX, USA), we extracted RNA from the agarose slices. We hydrolyzed the extracted RNA using the method of Crain (1990). Briefly, we heat-denatured the RNA, quickly cooled, then used incubations with nuclease P1, venom phosphodiesterase, and alkaline phosphatase in appropriate buffers to hydrolyze the RNA to its respective nucleosides. We used HPLC followed by fraction collection to isolated the adenosine fraction. The separation used a Symmetry C$_{18}$ column (4.6 x 75 mm, 3.5 µm, Waters, catalog number WAT066224, Milford, MA, USA) with isocratic elution in potassium phosphate buffer (0.02 M K$_2$HPO$_4$, pH 6.30). Resulting fractions were lyophilized to dryness then redissolved in 20 µL H$_2$O, transferred to silver capsules, dried in a desiccator, then used for isotope analysis. We performed isotope analysis using an adapted elemental analyzer (EA) and mass spectrometer system. EA modifications were as outlined in Polissar et al. (2005).
brief, EA sample combustion produced a nitrogen (N₂) pulse, which was cryogenically trapped and focused before introduction to a mass spectrometer.

### 6.4 Results and Discussion

Controls on product yield

We developed 85 DNAzymes based around the 10-23 catalytic domain described by Santoro and Joyce (1997). Each DNAzyme targeted regions in the 16 S sequence of *M. acetivorans*. At 37 °C, 31 of these cutters produced appreciable yields in the EPPS buffer while 14 produced appreciable yields in the TA buffer. Yields ranged up to 71 % and 54 % for activity in the EPPS and TA buffers respectively (figure 6.1). We selected 14 DNAzymes that showed activity in both buffers at 37 °C for testing at 4 °C. We included incubations at 4 °C because background RNase activity is reduced at lower temperatures so we would expect less undesirable RNA cutting at this temperature, reducing collection of non-targeted RNA and potentially improving overall approach stringency. Of the DNAzymes tested at 4 °C, 12 showed activity in the EPPS buffer and 13 showed activity in the TA buffer with yields ranging up to 60 % and 58 % respectively (figure 6.2).

Cutting at both 37 °C and 4 °C revealed an unequal response to DNAzyme activity at different locations in the target sequence. Secondary structure within the 16 S RNA likely inhibits DNAzyme access in some locations (Joyce, 2001). Mapping yields over the length of RNA substrate (figure 6.3) shows higher yields in different regions with successful targets frequently clustered (note for instance the cluster of 6 successful target sites centered around base 493). These clusters of higher cutting yields likely signal greater accessibility for the DNAzyme to bind with the RNA in these regions. Thus, it may be possible to enhance cutting yields by relaxing the RNA secondary structure more fully. Caution should be used with denaturing, as effective binding is required for DNAzyme activity and use of denaturants (including heat or chemicals such as formamide) may limit DNAzyme binding to target RNA and reduce cleavage yields. Helper probes (Fuchs et al., 2000), however, proved useful in opening RNA structure in
work performed by Macgregor et al. (2006) and could have useful application with DNAzymes since they would not adversely effect enzyme binding to RNA.

The thermodynamics of DNAzyme binding to target RNA play a distinct role in cutting yields. Overall reaction rates are governed by rates of DNAzyme binding to its target site, cleavage catalysis, and product release (Joyce, 2001). $\Delta G^\circ$ for duplex formation between the DNAzyme and its target must be balanced to promote stable binding but also permit product release following the cleavage reaction. For instance, a strongly negative $\Delta G^\circ$ would promote DNAzyme and RNA complex formation, but would impede complex separation after the cleavage and thus limit reaction efficiency. We calculated $\Delta G^\circ$ (following Sugimoto et al., 1995) for each DNAzyme tested (data not shown) but no direct correlation was observed between cleavage yields and calculated $\Delta G^\circ$. This likely signals the dominating role of site accessibility and RNA secondary structure in cleavage yields.

Focusing on sequence areas with high DNAzyme activity, which we hypothesize are regions of high RNA accessibility, a pattern between $\Delta G^\circ_{37}$ and product yields is apparent. Plotting calculated $\Delta G^\circ$ against observed cutting yields (figure 6.4) reveals a strong relationship and a predicted maximum yield at roughly $\Delta G^\circ_{37} = -7.5$ kcal/mol. The $\Delta G^\circ$ for maximum yields within a target cluster becomes more positive when comparing data between 37 °C and 4 °C incubations. Enhanced binding strength at lower temperatures may select for more positive $\Delta G^\circ$ to maintain adequate product release following catalysis at the reduced temperature.

Predicted $\Delta G^\circ$ for maximum yields varies at different activity clusters along the 16 S sequence, suggesting factors affecting the local environment may affect DNAzyme activity. The 10-23 DNAzyme catalytic mechanism likely involves positioning of a divalent metal cation to assist in mechanistic activation (Joyce, 2001). Any localized affects on cation availability would affect the cleavage rate and thus the overall yields. For instance, lower cation availability in one area would reduce the rate of catalysis,
favoring a higher $\Delta G^\circ$ that would increase the duration of duplex formation and help overcome a slower catalysis rate.

Observed trends between $\Delta G^\circ$ and cutting yields are contained within relatively short regions of the RNA sequence, suggesting relaxed RNA secondary structure is a very local effect in the RNA sequence. Further, thermodynamic control of DNAzyme yields is submissive to the commanding influence of secondary structure site accessibility. Continued exploration of factors governing DNAzyme binding and activity will facilitate more efficient cleavage design to permit higher cutting yields and proportionally better identification of effective DNAzyme targets. $\Delta G^\circ$ for each DNAzyme and substrate pairing can be manipulated by adjusting the length of the DNAzyme binding domains. Therefore, once an optimal $\Delta G^\circ$ is defined for each target region, it is possible to develop DNAzymes better suited to higher yields in that specific region.

Phylogenetic Stringency
An effective method for specific RNA capture demands high stringency. We focused initial stringency assessment on multiple DNAzymes directed towards order-level selectivity (based on Arb probe matching). Figure 6.5 shows an electropherogram of one stringency test. Visible are cut products of RNA extracted from *Methanosarcina acetivorans* and *Methanosarcina thermophila*. Four other related Archaeal species with mismatches to the target sequence showed no cleavage activity with DNAzyme incubation. When *M. acetivorans* RNA was mixed with each equal parts of other types of RNA and then separated, cut fragments from *M. acetivorans* are visible below the uncut, non-targeted RNA (figure 6.5). These results indicate that DNAzyme activity can be directed towards specific RNA sequences in a complex mixture, enabling size separation of target RNA from non-targeted sequences.

Coupled with high stringency, phylogenetic flexibility is also beneficial to an effective RNA capture method where desired targets may range from broad to narrow phylogenetic groups. We explored a DNAzyme targeting site 863 in the 16 S sequence, a conserved
archaeal region (figure 6.6). The cutter effectively produced cleavage products in every archaeal species tested, including the five species shown in figure 6.6. The DNAzyme did not cut RNA extracted from *E. coli* or *C. acetobutylicum*. Consistent activity in all tested archaeal species suggests the target sequence may be in a region of RNA with accessible structure conserved across the domain. The target sequence also overlaps the often-used archaeal-specific Arch 915 FISH probe which has demonstrated binding access across the domain.

Overall Process Selectivity
We tested our ability to isolate selected RNA by mixing desired RNA (from *M. acetivorans*) with “contaminant” RNA (from *E. coli*) containing a highly enriched isotope (^15N) tracer. Equal portions of the two RNA’s were mixed, then cleaved using the DNAzyme targeting base 813 and enzymatically hydrolyzed. We used HPLC purification to isolate the adenosine fraction from the RNA hydrolysis products and used the adenosine fraction for stable isotope (^15N) analysis. The *M. acetivorans* RNA collected from the cut fragments showed no observable isotope enrichment when compared to a control sample. We used a very strong isotope tracer in the stringency test (δ ≈ +5400 ‰) such that if the sample contained 1 % *E. coli* RNA signature we would expect a > +50 ‰ shift in the measured isotopic value. Lack of any enrichment indicates RNA collection was highly stringent and displayed little artifacts from non-specific RNA cleavage or sheering.

6.5 Conclusion
The ability to target a specific biomarker from any one organism or phylogenetic group for isotopic analysis would enable detailed studies of nutrient cycling through microbial systems. The necessity of rRNA for life makes it a crucial component of every living organism and the RNA structure itself can be made amenable for carbon and nitrogen isotopic studies. Successfully exploiting phylogenetic sequence specificity in 16 S RNA would enable RNA to serve as a phylogenetically specific stable isotope biomarker where investigations could target any organism with known 16 S sequence, whether it is
cultured or observable only in environmental samples. Success of any method using rRNA as a selective biomarker hinges upon stringent selection of RNA target from a complex RNA matrix (figure 6.7). Once selected, enzymatic hydrolysis and HPLC purification permit RNA isotope analysis. The phylogenetic specificity and target flexibility (ranging from domain to genus level specificity) of the DNAzymes we tested demonstrate DNAzyme cleavage of targeted RNA followed by size separation and fraction collection provides an effective method for specific RNA capture. Our test with a stable isotope tracer demonstrates the process introduces minimal contaminant RNA that should not affect isotope analysis and interpretation. Better understanding factors governing DNAzyme yields (including RNA secondary structure and thermodynamic interactions) will increase process efficiency from DNAzyme development and target selection to optimizing conditions for maximum yields.
6.6 Figures

**Figure 6.1** DNAzyme cutting yields with 37 °C, 1 hour incubation

RNA extracted from *M. acetivorans* was cleaved with a series of DNAzymes targeting different base positions in the 16 S sequence. Cutting yields are the relative amounts of the cleaved 16 S RNA versus non-cleaved RNA over the 1 hour incubation (at 37 °C). We performed cleavage reactions in both EPPS buffer (shaded regions) and TA buffer (unshaded regions).
RNA extracted from *M. acetivorans* was cleaved with a series of DNAzymes targeting different base positions in the 16S sequence. All DNAzymes tested showed prior cleavage activity towards target RNA in 37 °C incubations. We performed the reactions in 5 hour incubations at 4 °C and in both EPPS buffer (shaded regions) and TA buffer (unshaded regions).
The cutting yields for reactions performed in EPPS buffer (1 hour incubations at 37 °C) show clusters of heightened activity over the 16 S sequence of the *M. acetivorans* target RNA. Such areas of higher activity likely correlate to regions of relaxed secondary structure in the RNA and suggest this structure has a dominating control on DNAzyme effectiveness.

**Figure 6.3** Cutting yields versus target position in the 16 S sequence
Within regions of relaxed RNA secondary structure, the free energy of DNAzyme to substrate binding strongly affects cutting yields. The predicted ΔG°37 for substrate DNAzyme binding of target sites between bases 472 and 502 correlate well to cutting yields with a third order polynomial fit for reactions in both EPPS buffer (●) and TA buffer (□). The predicted optimal cutting yields correlate to ΔG°37 ≈ -7.5 kcal / mol under both buffering conditions. ΔG°37 can be adjusted for each DNAzyme based on binding domain length, so once an optimal ΔG°37 is defined for an accessible RNA region, optimal DNAzymes can be constructed for the target.

Figure 6.4 Free energy effects on cutting yields

Calculated ΔG°37 for DNAzyme Binding (kcal / mol)
Figure 6.5 Phylogenetic specificity of a genus-specific DNAzyme

We incubated a series of RNAs from different organisms with a DNAzyme developed for genus level specificity towards *Methanosarcina* spp. We observe two fragment bands correlating to RNA cleavage at base pair 813 in both the *M. acetivorans* and *M. thermophila* incubations (lanes 11 and 12). No cut fragments are observed in incubations of RNA from archaeal species outside the *Methanosarcina* genus (lanes 7 through 10). When RNA from *M. acetivorans* is mixed with other archaeal RNA the cut products correlate to the desired, *Methanosarcina* spp. RNA (lanes 2 through 6). Gel extraction from the cut fragments then isolates the desired RNA from the mixed RNA sample. Lanes 1 and 13 contain a RNA ladder, which was not exposed to DNAzyme activity.
We incubated a series of RNAs from different archaeal organisms with a DNAzyme targeting base pair 863 in the 16 S sequence. We observed cut products in each of the species tested (lanes 2 through 7). We developed the DNAzyme tested here to target the RNA sequence used by the Arch 915 FISH probe to attempt designing a general domain cutter. Lane 1 contains a negative control containing only RNA and no DNAzyme.

**Figure 6.6** Phylogenetic specificity breadth of domain-specific DNAzyme
RNA isotope analysis requires general RNA extraction from an environmental sample followed by RNA selection and purification prior to isotope analysis. DNAzymes based on the 10-23 motif provide a mechanism for cleavage of recognized RNA sequences, enabling phylogenetic-specific RNA harvesting.
6.7 Supplemental materials
Numerous investigations are helping develop methods for using small subunit (16 S) ribosomal RNA as a phylogenetic-specific stable isotope biomarker (MacGregor et al., 2002, Pearson et al., 2004, Uyeno et al., 2004, MacGregor et al., 2006, and this chapter). The RNA structure can be used for investigating both $\delta^{13}C$ and $\delta^{15}N$ and to help reveal microbial roles in both carbon and nitrogen cycling. RNA is a polymer composed of four nucleosides: adenosine, guanosine, cytidine, and uridine (Lodish et al., 2000). These monomers can be considered in two groups, the purines (adenosine and guanosine) and pyrimidines (cytidine and uridine). The purines contain two fused rings while the pyrimidines have only one. Formation of the purine and pyrimidine ring systems follows different synthetic pathways. The purine rings are synthesized from a variety of amino acids, tetrahydrofolate, and carbon dioxide while the pyrimidine ring is composed of carbamoyl phosphate and the single amino acid aspartate (Stryer, 1999). Further, the two groups of nucleosides are subject to different reactions. For instance, purines are actively recycled by the purine salvage pathway, which is a less energetic process than de novo synthesis (Stryer, 1999).

The heterogeneity of the RNA polymer may be utilized to trace the sources and fates of different building blocks (amino acids and other catabolic precursors) within a cell. For example, I hypothesize that nitrogen limitation may induce a systematic shift in purine versus pyrimidine $\delta^{15}N$ based upon active recycling of purine nucleosides. Integral to any isotopic exploration of intrapolymer RNA isotopes, however, is methodology for individual nucleoside isotope determination. This methodology was developed for adenosine isotope analysis and presented earlier in this chapter 6. Slight adaptation of the HPLC method permits fraction collection of the pyrimidine (combined cytidine and uridine), adenosine, and guanosine fractions from a bulk RNA sample. Here I present preliminary isotopic data for these individual fractions collected from pure Methanosarcian acetivorans cultures (with data collection as outlined in chapter 6). These measurements were performed in triplicate with data summary shown in table 1.
The data presented as purine isotopes constitute a summation of the adenosine and guanosine data.

Of initial interest in the $\delta^{15}\text{N}$ data is the large offset in the purine and pyrimidine isotope values, having an isotope separation: $^{15}\text{N-}\Delta_{\text{pur-pyr}} = 25.0 \%$. The purines also display large isotope separation between their two respective nucleosides: $^{15}\text{N-}\Delta_{\text{ade-guan}} = 20.0 \%$. Future work with cultures in different growth phases and having different nitrogen availability will help accurately assess both sets of data. However, it is important to note that the nitrogen inputs to the two purines are largely identical except that adenosine receives one additional amine group from the amino acid aspartate and guanosine received one addition amine group from glutamine. The differences observed between adenosine and guanosine likely either stem from isotopic differences in the amines provided by these two amino acids or from fractionation associated with consumption of these nucleosides. A 100 \% difference in the final amine added to each base is required to account for the large $^{15}\text{N}$ shift observed between adenosine and guanosine. More likely, tri-phosphorylated adenosine (ATP) is the energy currency for all known cells so this nucleoside has a very high turnover within the culture. Any fractionation between the adenosine used for RNA synthesis versus that used in energy transport could help establish the observed isotope separation.

Observed $^{13}\text{C}$ isotopic shifts are more subtle than shifts in $^{15}\text{N}$. We observed $^{13}\text{C-}\Delta_{\text{pur-pyr}} = -3.7$ and $^{13}\text{C-}\Delta_{\text{ade-guan}} = +4.3 \%$. The array of carbon sources used to construct nucleic acids is expected to produce overall isotope concentrations near that of bulk biomass in microbial systems (Hayes, 2001). Combining this with similar data from cultures grown on different substrates and using different metabolic pathways will help assess the extent to which nucleic acids can identify microbial carbon sources.
Table 6.1: Isotope measurements of nucleoside fractions

<table>
<thead>
<tr>
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<th>δ^{15}N (%)</th>
<th>Standard Deviation (%)</th>
<th>δ^{13}C (%)</th>
<th>Standard Deviation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyrimidine</td>
<td>25.4</td>
<td>5.5</td>
<td>-35.8</td>
<td>1.6</td>
</tr>
<tr>
<td>Purine</td>
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<td>2.3</td>
<td>-32.1</td>
<td>2.1</td>
</tr>
<tr>
<td>Adenosine</td>
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<td>0.6</td>
<td>-30.0</td>
<td>0.8</td>
</tr>
<tr>
<td>Guanosine</td>
<td>-10.4</td>
<td>2.3</td>
<td>-34.3</td>
<td>1.9</td>
</tr>
</tbody>
</table>
6.9 References


Chapter 7

Oxygen tolerance in strictly anaerobic prokaryotes

7.1 Abstract
Oxygen exposure is traditionally considered lethal to anaerobic microorganisms at even low levels. Yet various reports suggest some previously considered strict anaerobes can survive periods of oxygen stress and, in limited cases, even use oxygen for a microaerophilic respiration. We examined six anaerobic organisms to compare their abilities to grow under an atmosphere containing low levels of oxygen. We observed highly variable levels of oxygen tolerance in three methanogen species: *M. acetovorans*, *M. thermoautotrophicum*, and *M. maripaludis*, consistent with the extent of oxygen exposure expected in their native environments. While *M. acetivorans* contains multiple hydrogenases of unknown function, we concluded that these hydrogenases are not linked to hydrogen consumption that might reduce oxidative stress. In addition, our work with the sulfate reducing bacterium *D. gigas* cultured on lactate is supportive of previously observed oxygen detoxification. We observed high oxygen tolerance in *A. woodii* cultures during acetogenic growth on hydrogen and carbon dioxide. Lastly, we observed oxygen tolerance within previously observed ranges when examining *M. minerva*, a methanogen enrichment from a hypersaline mat environment containing sulfate reducing bacteria with high oxygen tolerance. In all, *M. acetovorans*, *M. Minerva*, *M. thermoautotrophicum*, *D. gigas*, and *A. woodii* were found to be remarkably capable of good growth under a microaerophillic headspace.

7.2 Introduction
Anaerobic microorganisms are present in a wide array of environments including many sediments, wetland soils, and animal digestive systems (Madigan et al., 2000) where they play a key role in both carbon (White, 2000) and nitrogen (Maier et al., 2000) cycling. Anaerobe sensitivity to oxygen exposure varies greatly from little negative effects in facultative anaerobes (which can generally use available oxygen for respiration) (Maier et al., 2000) to presumed lethal consequences in methanogens, the strictest of known
anaerobes (Jarell, 1985). Recent evidence, however, demonstrates oxygen survivability and resistance in a suite of presumed strict anaerobic microorganisms including acetogenic (Boga and Brune, 2003), fermentor (Jenney, et al., 1999), sulfate reducing (Canfield and DesMarais, 1991, Neil et al., 1996, Johnson et al., 1997, Abreu et al., 2000, and Sigalevich et al., 2000), dissimilatory metal reducing (Lin et al., 2004), and even disease-causing (Baughn and Malamy et al., 2004) organisms.

Intertwined with unexpected oxygen resistance in anaerobes is an emerging understanding of specific enzymology to deal with oxidative stress. For instance, superoxide reductase catalyzes the reduction of toxic superoxide to water using complementary activity from rubredoxin, oxidoreductase, and peroxidase in Pyrococcus furiosus (Jenney et al., 1999). The sulfate reducer Desulfovibrio gigas contains neelaredoxin (Silva et al., 1999), rubredoxine-oxygen oxidoreductase (Gomes et al., 1997), catalase, and a heat shock protein (Fareleira et al., 2003) that appear related to oxygen tolerance. The archeal sulfate-reducer Archaeoglobus fulgidus also maintains an oxygen detoxification pathway containing neelaredoxin, catalase, and a NAD(P)H-oxygen oxidoreductase (Abreu et al., 2000).

Most surprising is energy conservation linked to oxygen reduction in some presumed strict anaerobes. For instance, the sulfate reducer Desulfovibrio salexigens can use oxygen to respire polyglucose (van Niel et al., 1996). The sulfate reducer Desulfovibrio vulgaris positions itself along an anoxic / oxic boundary at an optimal oxygen concentration of 0.02 – 0.04 % for oxygen dependent growth (Johnson et al., 1997).

Methoanogens are considered the strictest of anaerobes, requiring a redox potential of less than -330 mV to sustain energy production (Jarell, 1985). Yet, Kiener and Leisinger (1983) and Fetzer et al. (1993) both observed variable viability in methanogen cells following oxygen exposure (with exposure to air for up to 30 hours in same cases). Further, Wagner et al. (1999) described methanogenic activity in aerated soils, although
the extent to which this resulted from the influence of both soil microenvironments and methanogen oxygen tolerance in the observations is unclear.

To assess the potential for methanogenic growth under microaerophilic atmospheres, we tested the oxygen tolerance of three pure methanogen cultures in addition to both sulfate reducer and acetogenic cultures. We used a series of incubations to explore the upper oxygen limits for growth of these cultures. We also investigated oxygen tolerance in a methanogen enrichment from a microbial mat containing sulfate reducers with high oxygen tolerance. Our efforts to test oxygen sensitivity of multiple strictly anaerobic species helps discern the habitable limits of each species and their capability to withstand an environment with variable oxygen abundance.

7.3 Methods

Microorganisms

*Methanococcus maripaludis* JJ, *Methanobacterium thermoautotrophicum* ΔH, *Desulfovibrio gigas* SRL 6146, and *Acetobacterium woodii* WB1 were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). *Methanosarcina acetivorans* 2CA was a kind gift from Dr. James G. Ferry (The Pennsylvania State University, University Park, PA, USA). Victoria J. Orphan (California Institute of Technology, Pasadena, California, USA) provided a *Methanococcoides minerva* enrichment originating from a microbial mat in Guerrero Negro, Baja California Sur, Mexico.

Culturing

*M. acetivorans* was cultured as in Moran et al. (2005) but with only methanol substrate (5.0 mL per L media), only 1.0 g NaHCO₃, excluding sulfide addition, and including 0.5 g (per L) cysteine (hydrochloride, anhydrous). *M. Minerva* was cultured on the same medium but with trimethylamine (9.566 g per L) in place of methanol and with 50 g per L of NaCl. Initial headspace was 1.5 bar 80 % N₂, 20 % CO₂. We cultured *M. maripaludis* and *M. thermoautotrophicum* using the medium described in Moran et al.
(2005) except we excluded sulfide, added 0.5 g per L cysteine (hydrochloride, anhydrous), and excluded sodium chloride from the *M. maripalidus* media. Initial headspace was adjusted to 3 bar 80 % H₂, 20 % CO₂. *D. gigas* medium (adapted from DSMZ medium 149) was prepared as the media above and contained (per L media): 1.0 g KH₂PO₄, 0.5 g NH₄Cl, 0.4 g MgSO₄•7H₂O, 0.1 g CaCl•2H₂O, 1.0 mL 10x Wolfe solution (Wolin et al., 1963), 1.0 mL 2 M H₂SO₄, 2.0 g sodium lactate, 2.0 g NaHCO₃, 5.0 mL vitamin solution, 0.5 g cysteine (hydrochloride, anhydrous), and initial headspace was 1.5 bar N₂. *A. woodii* media (based on DSMZ medium 135) contained (per L): 1.0 g NH₄Cl, 0.33 g KH₂PO₄, 0.45 g K₂HPO₄, 0.10 g MgSO₄•7H₂O, 1.0 mL 10x Wolfe solution (Wolin et al., 1963), 2.0 mL vitamin solution, 10.0 g NaHCO₃, 0.50 g cysteine (hydrochloride, anhydrous). Initial headspace was adjusted to 3 bar of 80 % H₂, 20 % CO₂.

We dispensed media into 540 mL culture bottles in groups containing 10 mL, 20 mL, 30 mL, 50 mL, or 100 mL aliquots. All bottles were sealed with gas tight butyl stoppers. After autoclaving, we added variable amounts of oxygen to each culture from sterile stocks. Likewise, for the *M. acetivorans* experiments with hydrogen, we added hydrogen from a sterile stock and brought the initial concentrations to 5 %.

We used 0.5 mL inoculations for each culture. Each set of experiments was incubated at 33.5 °C with 30 rpm agitation except for *M. thermoautotrophicum* that was incubated at 60 °C with 30 rpm agitation. The cultures were allowed to grow to completion and results were recorded either as positive for growth or negative for no growth (assessed by medium turbidity after incubation).

### 7.4 Results and Discussion

Results from testing oxygen tolerance in *M. acetivorans* are shown in figure 7.1. Our results show both concentrations where growth occurred (filled symbol) and concentrations at which *M. acetivorans* did not grow (open symbols). The true threshold between oxygen tolerance and toxicity is equal to or greater than the highest
concentration having growth and less than the lowest oxygen concentration not producing
growth. Figure 7.2 best displays this threshold, which has its lower boundary along the
points joined by the solid line and its upper limit below the points joined by the dashed
lines. Also on this plot are calculated dissolved oxygen concentrations based on Henry’s
Law, incubation temperature, and the initial headspace oxygen concentrations. Similar
results for *M. minerva* are shown in figure 7.3.

Notable in all cultures is increased oxygen tolerance at higher media volumes. Despite
constant culture agitation, the positive correlation between media volume and oxygen
resistance likely indicates an oxygen gradient through the media. Enhanced media
volumes, then, would enable microenvironments with reduced oxygen concentration to
develop. When present, any such gradient may signal an oxygen sink in the culture, most
likely through a microbial process. This could either be active microbial
decontamination of oxygen or an oxidative process that consumes oxygen by reacting
with microbial enzymes, effectively poisoning the microbe.

The genome sequence for *M. acetivorans* revealed a number of genes potentially
involved in oxygen resistance, including superoxide dismutases, a superoxide reductase,
and a catalase (Galagan et al., 2002). Additionally, Duwat et al. (2001) identified a
cytochrome d oxidase in a *Lactococcus lactis* that enables respiration with oxygen in this
normally anaerobic bacterial fermentor. Identification of a similar gene in *M. acetivorans*
raised the possibility of an aerobic metabolism in this organism. However, the results
here are inconsistent with this possibility. *M. acetivorans* shows oxygen tolerance at
levels consistent with other tested organisms, and oxygen’s toxicity above these levels
suggests it does not enhance growth.

The genome sequence for *M. acetivorans* also revealed numerous hydrogenases of
unknown function (Galagan et al., 2002). Unlike many other methanogens, this
methanogen can not utilize hydrogen for methane formation (Sowers et al., 1984),
making the intended function of the hydrogenase genes uncertain. Boga and Brune
(2003) demonstrated that several acetogen cultures could use hydrogen to reduce oxygen, thus displayed enhanced oxygen tolerance when hydrogen was available. We tested the effects of added hydrogen on oxygen resistance in *M. acetivorans* to assess whether the hydrogenases may function in oxygen detoxification. The similar toxicity threshold for *M. acetivorans* cultures both containing and devoid of added hydrogen, however, suggests that the identified hydrogenases do not function in oxygen detoxification.

In studies using *M. maripaludis*, we observed lower oxygen tolerance in this methanogen than in either *M. acetivorans* or *M. thermautotrophicum*. Different methanogens displayed largely different death curves when exposed to oxygen in a resting state by Kiener and Leisinger (1983) so it is not unexpected to observe different oxygen tolerances during methanogenic growth. Fetzer et al. (1993) suggest methanogen oxygen resistance may, in part, reflect adaptation to their native environments. They discover oxygen tolerance in rice paddy methanogens, which likely experience seasonal oxidative and dessication stress correlating to reduced water levels in the paddies. Conversely, Jones et al. (1983) isolated *M. maripaludis* from a high organic matter salt marsh sediment (Jones et al., 1983) where constant water saturation may limit oxygen exposure. Further, high organic matter content in the sediments would promote heterotrophic respiration to effectively remove oxygen when it was present. It is possible that without recurring oxygen exposure, *M. maripaludis* does not require active oxygen detoxification and thus does not maintain this function. It is interesting, however, that McAllister and Kral (2006) observed little reduction in *M. maripaludis* methane production after washing a cell pellet with aerobic medium. These cells may be able to resist short-term oxygen exposure but not grow under even low oxygen concentration over prolonged periods.

When growing at high oxygen concentrations, *M. thermoautotrophicum* cells clustered together. Sigalevich et al. (2000) observed similar morphology in both *Desulfovibrio oxyclinae* cultures and bacterial sulfate reducers present in a microbial mat. Aggregate formation would shelter internal cells from oxygen stress and may represent a morphological adaptation to oxygen exposure. Of further note for *M.*
thermoautotrophicum is its culture at higher temperature (60 °C) versus the other methanogens. Oxygen is less soluble in the medium at higher temperatures, as reflected by the plotted dissolved oxygen concentration.

The two bacterial species examined, *D. gigas* and *A. woodii*, also showed significant differences in oxygen sensitivity. The media used to culture *A. woddii* contained ample hydrogen, which, as described by Boga and Brune (2003), can be used to reduce oxygen and remove the oxygen stress. Lemos et al. (2001) demonstrated that *D. gigas* can reduce oxygen to water when using fumarate and sulfate as growth substrates. However, Fareleira et al. (2003) suggest oxygen may inhibit energy production in lactate-cultured cells. While the lactate-grown cells in our experiments displayed some resistance to oxygen stress, they did not show signs of metabolic oxygen consumption, supporting the observations of Fareleira et al. (2003) that oxygen respiration in *D. gigas* is substrate-dependant.

*M. minerva* was enriched from a hypersaline microbial mat. These same mats produce high sulfate reduction rates contemporaneous with rapid photosynthesis and oxygen evolution, suggesting dissimilatory sulfate reduction in the presence of oxygen (Canfield and DesMarais, 1991). We tested *M. minerva* to determine whether it too would show high oxygen tolerance. Our results demonstrate that oxygen tolerance in *M. Minerva*, however, was comparable to that in *M. acetivorans* and *M. thermoautotrophicum*.

Methanogen species likely evolved when earth’s biosphere was almost entirely reducing and devoid of free oxygen. The ability of three of the methanogens we tested (*M. minerva, M. acetivorans* and *M. thermoautotrophicum*) to resist oxygen toxicity suggests adaptation to a changing biosphere as free oxygen became abundance. Withstanding oxidative stress would permit methanogenesis to continue as free oxygen became more abundant on earth, and may have helped buffer a sharp decline in methane production that would have occurred without adaptation.
7.5 Conclusions

Anaerobic microorganisms catalyze a number of reactions critical to global carbon and nitrogen cycling. While these organisms can be rapidly poisoned by oxygen, many of them contain enzymatic tools for combating oxygen stress. We observed notable differences in oxygen tolerance amongst the six anaerobic species we examined. The ability of anaerobes to grow under a microaerophilic headspace presumably helps define their habitable extremes. For instance, slight oxygen resistance in methanogens likely enhances methane production along oxic and anoxic boundaries, such as that observed in detrital marine water column particles (Marty, 1993, van der Maarel et al., 1999).
7.6 Figures

Figure 7.1 Growth of *M. acetivorans* under varying degrees of oxygen stress

We cultured *M. acetivorans* in multiple culture bottles having both different media volumes and different headspace oxygen concentrations. The shaded points above represent bottles in which growth occurred (as measured by culture turbidity) while the shaded points describe bottles devoid of growth. The data set can be simplified to include just the region of likely oxygen tolerance. The true oxygen tolerance could range from the highest concentration producing growth to just below the lowest oxygen concentration that did not produce growth.
Figure 7.2 Oxygen tolerance in growing cultures

We tested oxygen tolerance in multiple cultures. The solid lines connect the cultures showing growth in the highest oxygen concentration for each species and the dashed lines connect the lowest, lethal oxygen concentration for each media volume. True oxygen tolerance is between these values but having a range such as this permits comparison of oxygen sensitivity between species.
**Figure 7.3 Oxygen tolerance in *M. Minerva***

*M. minerva* is a methanogen enrichment from a microbial mat showing high sulfate reduction rates under oxic conditions. We tested the enrichment for similar signs of oxygen resistance in the methanogen component. The results, however, suggest oxygen tolerance in *M. minerva* is similar to that in other methanogens tested.
References


Chapter 8

Summary and Future Directions

8.1 Summary

*Methanothermicita acetivorans* is a marine methanogen (Sowers et al., 1984) with a large genome and high metabolic diversity (Galagan et al., 2002). This methanogen shows amongst the highest phylogenetic relationship of all culturable species to anaerobic methane oxidizers, which to date are unculturable (Orphan et al., 2001). Work described in this thesis focuses on both revealing the metabolic pathways and reactions present in *M. acetivorans* and applying these discoveries to better understand the process of anaerobic oxidation of methane (AOM).

Chapters 2 and 3 both focus on trace methane oxidation (TMO), a process with potential metabolic similarity to AOM. We used a stable-isotope tracer to monitor methane oxidation by multiple archaeal species. In contrast to *M. acetivorans* cultures, archaeal sulfate reducers (*Archaeoglobus* spp.) do not catalyze methane oxidation within the limits of detection. These organisms lack methyl coenzyme M reductase (Klenk, et al., 1998), a key enzyme in methane formation (Ermler et al., 1997). The apparent requirement for methyl coenzyme M reductase in TMO agrees with the conclusions of Hallem et al. (2003) and Krüger et al. (2003).

In addition to testing *Archaeoglobus* spp., we compared TMO activity by *M. acetivorans* cultures with that by other methanogen cultures and by *M. acetivorans* cultures containing various substrates and potential electron acceptors. Supplemented electron acceptors induced negligible enhancement in *M. acetivorans* TMO, suggesting they are not linked to the enzymatic TMO pathway. However, these experiments did demonstrate the ability of *M. actievorans* to maintain an active metabolism under a microaerophilic atmosphere (chapter 7). Further, different amounts of TMO were observed in *M.*
acetivorans cultures having different growth substrates. Most notably, we found preferential oxidation of methane by TMO to specific metabolites when grown on different substrates. For instance, methyl sulfide was the preferred TMO end product when M. acetivorans was cultured on carbon monoxide. Not only was the proportion (3.1 %) of methane converted to methyl sulfides in these cultures higher than observed in our previous experiments (0.42 %), but it was also above the highest levels previously reported levels for methanogen-mediated TMO (0.32 %, Zehnder and Brock, 1979).

Methyl sulfide production by TMO in M. acetivorans is groundbreaking on two accounts. First, further investigation (chapter 4) revealed that methyl sulfide production was an energy conserving metabolism in M. acetivorans when cultured on carbon monoxide. To the best of our knowledge, this is the only documentation of both a methanogen metabolically producing methyl sulfides and a carbon monoxide metabolism resulting in methyl sulfide production. Globally, methyl sulfides link the marine and atmospheric sulfur cycles with microalgae considered the dominant source of methyl sulfides in the marine realm. Methyl sulfide production by the metabolism we describe demands further study to decipher the role of this anaerobic source within the modern sulfur cycle. Further, carbon monoxide was potentially abundance in early earth environments (Kharecha et al., 2005), contemporaneous with presumed high methanogen activity (Kasting and Seifert, 2002). Understanding the role this archaeal-mediated metabolism has on the current sulfur cycle may better enlighten both its role in past environments as well as potential roles in searching for life on other planets (Des Marais et al., 2002) with environments similar to that on early earth.

The observation of methyl sulfide production by M. acetivorans is also notable for its potential implications in AOM. We used (chapter 5) incubations of a series of AOM-active sediments to demonstrate that the hypothesized (Hoehler et al., 1994) intermediate in this process (H₂) was not an active AOM component in our samples. We used thermodynamic and biochemical evidence to demonstrate the feasibility of methyl sulfides constituting a central role in the AOM process by serving as a carbon substrate
transferred between the archaeal methanotroph and bacterial sulfate reducer. The proposed mechanism is also consistent with AOM sediment incubations with carbon monoxide, which demonstrated that carbon monoxide can also serve as an electron donor for methyl sulfide production. To the best of our knowledge, we are the first to propose methyl sulfides as the means for interspecies transfer of carbon and electrons crucial to consortial AOM activity.

In chapter 6 we describe efforts to isolate phylogenetically specific RNA sequences from a mixed sample. DNAzymes are sequences of DNA that contain enzymatic properties. We adapted a previously developed DNAzyme (Santoro and Joyce, 1997) to identify and cleave only targeted RNA sequences. When combined with stringent harvesting of cleaved fragments, this approach provides a high fidelity method for stable isotope analysis of specific phylogenetic groups. We determined cutting yields and overall method stringency using *M. acetivorans* RNA as a model organism. Continued development of this approach will permit studies of microbial regulation of nutrient cycles at unprecedented levels of detail. Further, we focused DNAzyme development on *M. acetivorans* RNA due to its high similarity to the sequences of archaeal AOM organisms and to facilitate application of this method in future studies of AOM organisms.

Finally, chapter 7 describes the ability of *M. acetivorans* and other anaerobes to tolerate and metabolize under a microaerophilic atmosphere. Understanding the extent to which *M. acetivorans* and other anaerobes can resist oxygen toxicity will help better define the appropriate niches they can inhabit. We observed methanogenic growth under atmospheres containing up to nearly 2.5% oxygen with sulfate reducing and acetogenic metabolisms showing even higher oxygen tolerance.

The broad metabolic diversity and phylogenetic relation of *M. acetivorans* to AOM methanotrophs provided a rich array of questions and helped direct the work described in
this thesis. *M. acetivorans* has proven to be a highly useful model organism for our investigations.

8.2 Future Directions

Any enlightenment I received through work described in this thesis merely illuminates the boundaries to my own knowledge and enhances my desire to push forth the limits of my understanding. There are many aspects of the current work that demand further exploration, which, in turn, will bring yet more curiosity for the next round of investigation.

Firstly, revealing the potential role methyl sulfides play in AOM demands actual direct testing in AOM incubations of the hypothesis that methyl sulfides are transferred between consortial members. Once this is established, the extent to which a similar process governs AOM by non-consortia forming anaerobic methanotrophs must be assessed. Secondly, a miniature sulfur cycle between methanotroph and sulfate reducing organisms is required if methyl sulfides are transferred in AOM. Sulfide released by sulfate reduction would be methylated by the methanotroph to produce methyl sulfides. The reaction stochiometry demonstrates net sulfide production in the overall metabolism. The high concentration and potential cycling of metabolically active sulfide may instill a sulfur stable isotope signature to AOM communities. In combination with carbon isotopes, any sulfur stable isotope signature produced by AOM could offer a window to past microbial communities preserved in the rock record. Preliminary work with AOM sediment incubations can be used to assess the potential for sulfur isotope fractionation by AOM.

Secondly, work testing the stringency of DNAzyme-based RNA harvesting facilitated method development for assessing both nitrogen and carbon isotopes of individual nucleosides from an RNA sample. Preliminary evidence (Supplementary materials, chapter 6) suggests high intra-polymer nitrogen isotope fractionation may exist both between individual nucleosides and between nucleoside groups (purines and
pyrimidines). Stable nitrogen isotope ($\delta^{15}N$) investigations provide useful guides for deciphering ecological and feeding interactions in plant and animal ecological investigations (please see Peterson and Fry, 1987 for a concise review and case studies). I seek to determine whether similar fractionation is observable in microbial systems. Further, I seek to determine whether recycling of purines by the purine salvage pathway (Stryer, 1999) produces nitrogen fractionation between purines and pyrimidines in microbial systems and whether this fractionation is exacerbated in periods of nitrogen limitation.

Finally, the work in chapter 7 demonstrates the ability of various anaerobic microorganisms to metabolize under an oxygen stress. I seek to use timeseries analysis of batch *M. acetivorans* cultures having oxygen exposure to determine whether this organism removes oxygen prior to growth, during growth, or not at all.

### 8.3 References


Appendix

DNAzyme target identification

A.1 Introduction
The DNAzymes developed for the work outlined in chapter 6 were selected using the script below, run using Perl. The script accepts any gene sequence and returns all potential DNAzymes that can be developed to target regions in the sequence. This selection permits only sites containing a purine followed by pyrimidine in the target sequence (5’ to 3’), in agreement with the activity observed by Santoro and Joyce (1997). The script further estimates and returns the ΔG° based on the methods of Sugimoto et al. (2005). The script allows the user to identify the lengths of the recognition domains and does not require that they be symmetrical. Note that input files must be in simple text format and include any file extension on the infile name. The output file from the script should be named to include any desired file extension.

A.2 Script

#!/usr/bin/perl

print "What is the name of the organism?";
$organism = <STDIN>
print "What is the name of the input file?";
$filename = <STDIN>
print "What shall I call the output1 file?"
$new1 = <STDIN>
print "5' ARM length?";
$arm5 = <STDIN>
print "3' ARM length?"
$arm3 = <STDIN>
open (INFILE, "$filename") || die "cannot find file: $!"
open (OUTFILE1, ">$new1")
while (<INFILE>) {
    @RNA = split //, $_
    $check = 0;
    $counter = 0;
    $lengthRNA = $#R_RNA + 1;
print OUTFILE1 "$organism"
print OUTFILE1 "Input file is: $filename"
print OUTFILE1 "5' Arm length equals: $arm5"
print OUTFILE1 "3' Arm length equals: $arm3"
print OUTFILE1 "G or A purine position t5'DNA arm t5'deltaG tDNA loop t 3'DNA arm t 3'deltaG t target RNA (5' to 3')"

foreach $a (@R_RNA) {
    $counter = $counter + 1;
    if ($a eq "T" or $a eq "C") {
        if ($R_RNA[$counter] eq "A" or $R_RNA[$counter] eq "G") {
            $spotlength = $lengthRNA - $counter;
            print OUTFILE1 "$R_RNA[$counter] t$spotlength t";
            $delG = 3.1;
            $tempC = $counter - $arm5;
            $tempA = $counter - 1;
            foreach $b ($tempC..$tempA) {
                if ($R_RNA[$b] eq "A") {
                    print OUTFILE1 "T"
                }
                if ($R_RNA[$b] eq "G") {
                    print OUTFILE1 "C"
                }
            }
            @newtempARM = reverse @tempARM;
            undef @tempARM;
            foreach $Y (0..$#newtempARM) {
                if ($newtempARM[$Y] eq "A" and $newtempARM[$Y+1] eq "A") {
                    $delG = $delG - 1.0;
                } if ($newtempARM[$Y] eq "A" and $newtempARM[$Y+1] eq "C") {
                    $delG = $delG - 2.1;
                } if ($newtempARM[$Y] eq "A" and $newtempARM[$Y+1] eq "G") {
                    $delG = $delG - 1.8;
                }
} if ($newtempARM[$Y] eq "A" and $newtempARM[$Y+1] eq "T") {
    $delG = $delG -0.9;
} if ($newtempARM[$Y] eq "C" and $newtempARM[$Y+1] eq "A") {
    $delG = $delG -0.9;
} if ($newtempARM[$Y] eq "C" and $newtempARM[$Y+1] eq "C") {
    $delG = $delG -2.1;
} if ($newtempARM[$Y] eq "C" and $newtempARM[$Y+1] eq "G") {
    $delG = $delG -1.7;
} if ($newtempARM[$Y] eq "C" and $newtempARM[$Y+1] eq "T") {
    $delG = $delG -0.9;
} if ($newtempARM[$Y] eq "G" and $newtempARM[$Y+1] eq "A") {
    $delG = $delG -1.3;
} if ($newtempARM[$Y] eq "G" and $newtempARM[$Y+1] eq "C") {
    $delG = $delG -2.7;
} if ($newtempARM[$Y] eq "G" and $newtempARM[$Y+1] eq "G") {
    $delG = $delG -2.9;
} if ($newtempARM[$Y] eq "G" and $newtempARM[$Y+1] eq "T") {
    $delG = $delG -1.1;
} if ($newtempARM[$Y] eq "T" and $newtempARM[$Y+1] eq "A") {
    $delG = $delG -0.6;
} if ($newtempARM[$Y] eq "T" and $newtempARM[$Y+1] eq "C") {
    $delG = $delG -1.5;
} if ($newtempARM[$Y] eq "T" and $newtempARM[$Y+1] eq "G") {
    $delG = $delG -1.6;
} if ($newtempARM[$Y] eq "T" and $newtempARM[$Y+1] eq "T") {
    $delG = $delG -0.2;
    
    
    
    print OUTFILE1 "t$delG"
    print OUTFILE1 "tGGCTAGCTACAACGA\t"
    undef @tempARM;
    $tempD = $counter + $arm3 + 1;
}
$tempE = $tempD - $arm3;
$tempF = $tempD - 1;
foreach $c ($tempE..$tempF) {
    if ($R_RNA[$c] eq "A") {
        print OUTFILE1 "T"
    }
    if ($R_RNA[$c] eq "G") {
        print OUTFILE1 "C"
    }
    if ($R_RNA[$c] eq "T") {
        print OUTFILE1 "A"
    }
    if ($R_RNA[$c] eq "C") {
        print OUTFILE1 "G"
    }
    if ($R_RNA[$c] eq "A" or "G" or "T" or "C") {
        push @tempARM, $R_RNA[$c]
    }
}
@newtempARM = reverse @tempARM;
undef @tempARM;
$delG = 3.1;
foreach $Y (0..$#newtempARM) {
    if ($newtempARM[$Y] eq "A" and $newtempARM[$Y+1] eq "A") {
        $delG = $delG - 1.0
    } if ($newtempARM[$Y] eq "A" and $newtempARM[$Y+1] eq "C") {
        $delG = $delG - 2.1
    } if ($newtempARM[$Y] eq "A" and $newtempARM[$Y+1] eq "G") {
        $delG = $delG - 1.8
    } if ($newtempARM[$Y] eq "A" and $newtempARM[$Y+1] eq "T") {
        $delG = $delG - 0.9
    } if ($newtempARM[$Y] eq "C" and $newtempARM[$Y+1] eq "A") {
        $delG = $delG - 0.9
    } if ($newtempARM[$Y] eq "C" and $newtempARM[$Y+1] eq "C") {
        $delG = $delG - 2.1
    } if ($newtempARM[$Y] eq "C" and $newtempARM[$Y+1] eq "G") {
        $delG = $delG - 1.7
    }


```perl
if ($newtempARM[$Y] eq "C" and $newtempARM[$Y+1] eq "T") {
    $delG = $delG - 0.9;
}
if ($newtempARM[$Y] eq "G" and $newtempARM[$Y+1] eq "A") {
    $delG = $delG - 1.3;
}
if ($newtempARM[$Y] eq "G" and $newtempARM[$Y+1] eq "C") {
    $delG = $delG - 2.7;
}
if ($newtempARM[$Y] eq "G" and $newtempARM[$Y+1] eq "G") {
    $delG = $delG - 2.9;
}
if ($newtempARM[$Y] eq "G" and $newtempARM[$Y+1] eq "T") {
    $delG = $delG - 1.1;
}
if ($newtempARM[$Y] eq "T" and $newtempARM[$Y+1] eq "A") {
    $delG = $delG - 0.6;
}
if ($newtempARM[$Y] eq "T" and $newtempARM[$Y+1] eq "C") {
    $delG = $delG - 1.5;
}
if ($newtempARM[$Y] eq "T" and $newtempARM[$Y+1] eq "G") {
    $delG = $delG - 1.6;
}
if ($newtempARM[$Y] eq "T" and $newtempARM[$Y+1] eq "T") {
    $delG = $delG - 0.2;
}

print OUTFILE1 "\t$delG"
print OUTFILE1 "\n"
$tempsl = $spotlength - 1;
$tempG = $tempsl - $arm3;
$tempH = $tempsl + $arm5;
foreach $d ($tempG..$tempH) {
    print OUTFILE1 "$RNA[$d]";
}
print OUTFILE1 "\n"
```
A.3 References


Vita

James J. Moran

Education

Aug. 2000 – present
Ph.D. candidate, Department of Geosciences with specialization in isotope geochemistry and
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The Pennsylvania State University, Advisors: Katherine H. Freeman and Christopher H. House

B.A. (magna cum laude) in Chemistry and Geology, minor in Biology
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Publications

Lessner, Daniel J., Lingyun Li, Qingbo Li, Tomas Rejtar, Victor P. Andreev, Matthew Reichlen, Kevin
Hill, James J. Moran, Barry L. Karger, James G. Ferry. An unconventional pathway for reduction

Moran, James J., Christopher H. House, Katherine H. Freeman, James G. Ferry (2005) Trace methane
oxidation studied in several Euryarchaeota under diverse conditions. *Archaea*, **1**, 303-309.

Teaching Experience

Spring 2005 Co-lead a one-month module (Geohazards of Climate Change) in Geosc 402 (Natural
Hazards in Geosciences)
Fall 2004 Co-instructed Geosc 597C (Biogeochemical Analysis)
Spring 2002 Teaching assistant for Geosc 21 (Earth and Life)
Fall 2001 Teaching assistant for Geosc 202 (Chemical Processes in Geology)
Spring 1999 and Spring 2000, Laboratory assistant for Organic Chemistry Lab (Lawrence University)
Spring 1998 Laboratory assistant for introductory chemistry course (Lawrence University)

Honors, Fellowships, and Awards

2006 Second place paper award, Penn State Geosciences Student Colloquium
2005 Research fellowship, Penn State Astrobiology Center
2002 – 2005 Research fellowship, Biogeochemical Research Initiative in Education
2005 Tait Scholarship in Microbial Biogeochemistry
2005 American Society of Microbiology Graduate Student Travel Grant
2004 / 2005 Arnulf I. Muan Graduate Fellowship
2003 Tait Scholarship in Microbial Biogeochemistry
2001 Krynine Award
2000 / 2001 Graduate Fellow, Penn State University
2000 Induction to Phi Beta Kappa (Gamma-Delta of Wisconsin)

University and Community Service

2002 – present Leader in a local youth group
2005, 2006 (summer) Volunteer at a YMCA camp
2003 and 2004 Exhibitor at Space Day, Penn State University
2001 Organizing Member for the 4th Annual Environmental Chemistry
Symposium, Penn State University
1999 / 2000 Senior class officer
1998 – 2000 University Honor Council chair