ANAEROBIC CARBON CYCLING PATHWAYS IN THE SUBSEAFLOOR INVESTIGATED VIA FUNCTIONAL GENES, CHEMICAL GRADIENTS, STABLE CARBON ISOTOPES, AND THERMODYNAMIC CALCULATIONS

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ABSTRACT

MARK A. LEVER: Anaerobic Carbon Cycling Pathways in the Subseafloor Investigated via Functional Genes, Chemical Gradients, Stable Carbon Isotopes, and Thermodynamic Calculations
(Under the direction of Andreas Teske, Marc Alperin, Carol Arnosti, Barbara MacGregor and William Whitman)

Deep subseafloor environments are the largest carbon sink on Earth, and play a vital role in global climate control. The activity of microorganisms inhabiting these environments is a key determinant in the long-term storage of organic carbon, and yet poorly understood. Microbes performing the terminal oxidation step in organic carbon remineralization play a key ecological role, as they facilitate other carbon degradation pathways, such as fermentation, by consuming and hence preventing accumulation of their metabolic waste products to inhibitory concentrations. In this doctoral thesis, I examined patterns in the distributions of terminal carbon-oxidizing microbes that produce methane (methanogens), oxidize methane (methanotrophs), or synthesize acetate (acetogens) in the context of thermal, lithological, and geochemical gradients at three deep sea sites located on the Juan de Fuca Ridge Flank, the Peru Trench, and the Guaymas Basin. Newly-designed PCR primers made the widespread detection of marker genes possible and enabled me to construct the first detailed community profiles of methanogens, methanotrophs, and acetogens in the subseafloor. Known groups occur in addition to unknown groups and both appear zonated along sulfate concentration gradients. Stable carbon isotopic signatures of methane and acetate indicate *in situ* methanogenic, methanotrophic, and acetogenic activity. Possible reactions of methanogenesis, methanotrophy, and acetogenesis are discussed taking into consideration stable carbon isotopic signatures, calculated free energy yields, and substrate use by closest known relatives.

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INTRODUCTION

i. A Traditional View of Methanogenesis, Anaerobic Methane Oxidation, and Acetogenesis in Marine Sediments

In the presence of their respective electron acceptors, denitrifying, metal- and sulfate-reducing microbes are believed to outcompete acetogens and methanogens for common substrates due to higher energy yields (Lovley and Goodwin 1988). High water-column sulfate concentrations (~28 mM) in marine systems result in a high flux of sulfate into sediments that frequently exceeds the supply of readily bioavailable electron donors. In sediments where sulfate concentrations remain high, methanogens are limited to the utilization of non-competitive substrates, whereas acetogens are believed to gain energy from other processes, such as fermentation, or in some cases even sulfate, metal and nitrate reduction (Drake and Daniel 2004, Drake et al. 2006).

Methanogenesis becomes the dominant terminal remineralization pathway in sediments with high rates of organic matter deposition, i.e. coastal, shelf and continental slope sediments, below upwelling areas, at methane seeps, or in deep-sea basins with high rates of organic matter sedimentation, where supplies of bioavailable electron donors exceed those of O_2, nitrate, metal oxides, and sulfate, and result in their depletion. It is generally believed that methanogens over time outcompete acetogens for the same substrates (e.g. H_2/CO_2,

formate, methanol) due to higher energy yields from competitive substrates (Cord-Ruwisch and Ollivier 1986, Hoehler et al. 1999).

Methanogenesis and its main competing process, sulfate reduction, are coupled via the process of anaerobic oxidation of methane (AOM; e.g. Boetius et al. 2000). In AOM, close relatives of methanogens oxidize methane to an unknown product, which is subsequently taken up by sulfate reducers (Orphan et al. 2001, Nauhaus et al. 2002). The energy yield of AOM is typically poor; hence, AOM is only sufficiently exergonic to support life, if it is tightly coupled to sulfate reduction, which prevents build-up of metabolic products and hence its thermodynamic inhibition.

ii. Methanogens, Anaerobic Methanotrophs, and the Marine Environment

Known pathways of methanogenesis fall into three groups: (1) CO_2 or CO fixation using H_2, formate, or certain alcohols (e.g. methanol, ethanol, 2-propanol, 2-butanol) as electron donors, (2) methylotrophic; methyl groups of C_1 compounds (e.g. methanol, mono-, di- and trimethylamine, dimethylsulfide, methylmercaptan) are cleaved and reduced to methane, and (3) aceticlastic; acetate is fermented to methane and CO_2 (Whitman et al. 2006). Due to the inability of methanogens to utilize more complex compounds, organic matter degradation via biotic processes such as fermentation or high-temperature abiotic processes are necessary to provide suitable substrates. H_2 and acetate, often referred to as central intermediates in organic matter breakdown, are typically the most available and quantitatively most important methanogenic substrates (e.g. Valentine 2006). The products of AOM are yet to be established. Methyl sulfides were proposed in a recent study (Moran et al. 2008); yet it is unclear if AOM always proceeds to the same products; similar to methanogenesis, which can

rely on different substrates, the reversal of methanogenesis, AOM, may not always proceed to the same products.

Stable carbon isotopic signatures of cultured methanogens grown on different substrates suggest substrate-dependent differences in $\delta^{13}C\text{-}CH_4$ values. Based on fractionations determined in cultured methanogen strains, $\delta^{13}C\text{-}CH_4$ in marine sediments suggest biogenic methane production predominantlyfrom H_2/CO_2 (Whiticar et al. 1986). The kinetic isotope effect, ε_{CO_2/CH_4}, associated with hydrogenotrophic methanogenesis was found to exceed 95‰, while that associated with methanogenesis from methylated substrates ranged from 40-60‰ (Whiticar 1999). The inference of methanogenic pathway from ε_{CH_4} is disputable, however: (1) at high temperature, abiotic processes, e.g. Fischer-Tropsch-type synthesis, can yield $\delta^{13}C\text{-}CH_4$ signatures in the range otherwise attributed to biological methanogenesis (McCollom and Seewald 2006, 2007) (2) while numerous hydrogenotrophic Methanococcales, Methanomicrobiales and Methanosarcinales have been isolated from the marine environment (Ferry and Lessner 2008), only two marine aceticlasts have been cultivated, *Methanosarcina acetivorans* (Elberson and Sowers 1997) and *Methanosarcina siciliae* (Sowers et al. 1984). In neither were isotopic fractionations associated with aceticlastic growth determined. To date there has been no marine isolate of the obligately aceticlastic genus *Methanosaeta* (Liu and Whitman 2008), even though *Methanosaeta* occur widely in the marine environment (Dhillon et al. 2005, Inagaki et al. 2006, Parkes et al. 2007). Inferences based on comparisons with the related aceticlastic genus *Methanosarcina* are of limited value, since *Methanosaeta* uses a different aceticlastic pathway than *Methanosarcina* (Smith and Ingram-Smith 2007). (3) So far, stable carbon isotopic fractionations have been determined only for few cultured marine methanogens, mostly

thermo- and hyperthermophilic hydrogenotrophs (Whiticar et al. 1986, Valentine et al. 2004), and it remains unclear if the same fractionations occur at lower temperatures. (4) Growing methanogens under *in situ* conditions, especially the high pressures and low substrate concentrations typical of the deep subsurface, can be difficult; yet, both pressure and substrate concentrations affect the free energy yield of methanogenic reactions, which in turn partially control isotopic fractionation (Penning et al. 2005, Takai et al. 2008). (5) Tracer and enrichment studies in the marine environment suggest that acetate is a major substrate of methanogenesis (Crill and Martens 1986, Parkes et al. 2005, Parkes et al. 2007), and that aceticlastic methanogenesis rates can exceed those of hydrogenotrophic methanogenesis (e.g. Orcutt et al. 2005). (6) Only in a few cases have the *in situ* composition and activity of methanogen communities been investigated in parallel, by complementing determinations of natural or tracer C-isotopes with sequencing of phylogenetically and functionally conserved marker genes (e.g. Orcutt et al. 2005, Parkes et al. 2005, Parkes et al. 2007).

There have been few studies focused on methanogen and anaerobic methanotroph distribution in marine sediments. The "traditional" molecular approach, targeting 16S rRNA genes of Archaea using general PCR probes, has led to detection (e.g. Reed et al. 2002, Teske et al. 2002, Mills et al. 2005, Parkes et al. 2005), but typically not in a sufficient number of samples to infer a relationship between methanogen and methanotroph distribution and habitat characteristics. PCR assays of functional genes carry promise. By focusing on a phylogenetically informative gene unique to methanogens and anaerobic methanotrophs, it is possible to sort minority populations of methanogens and methanotrophs from the plethora of non-methanogenic and non-methanotrophic Archaea. The gene for the alpha subunit of methyl coenzyme M reductase (*mcrA*) is a suitable target gene, as it is found

in all known methanogens and anaerobic methanotrophs, has never been found in any other metabolic groups, and is phylogenetically conserved (Reeve et al. 1997, Hallam et al. 2003). To date, *mcrA* gene profiles have been generated for coastal sediments (Parkes et al. 2007; Lloyd et al., unpublished), and for surface environments from the deep sea, such as hydrothermal sediments (Dhillon et al. 2005) and methane seeps (Lloyd et al. 2006, Orcutt et al. 2008). PCR assays of *mcrA* genes have also been conducted with deep-subsurface sediments, but detection succeeded in too few depth horizons to establish a relationship between *mcrA* distribution and habitat (Newberry et al. 2004, Parkes et al. 2005, Inagaki et al. 2006). Phylotypes detected belonged to Methanosarcinales and Methanobacteriales. No anaerobic methanotrophs, and no members of the Methanomicrobiales, Methanococcales, or Methanopyrales groups were detected (Teske and Biddle 2008). And no methanogens or anaerobic methanotrophs have been detected in the deep subseafloor oceanic crust.

iii. Acetogens and the Deep Seafloor

Acetogens are organisms capable of acetate synthesis as an energy-conserving process. They are distinguished from other acetate producers, e.g. fermenters and sulfate reducers, by their ability to synthesize acetate *de novo* via the reduction of CO_2 (Drake 2006). In anaerobic marine sediments, nitrate, metal oxide, and sulfate reducers, as well as methanogens, are believed to drive H_2 concentrations below the thermodynamic threshold for acetogenesis, due to the higher energy yields from competitive substrates.

Autotrophic acetogenesis ($2\ HCO_3^- + 4\ H_2 + H^+ \rightarrow CH_3COO^- + 4\ H_2O$) can be detected in stable carbon isotopes of acetate. There are three key determinants of the $\delta^{13}C$-signature of acetate: (1) $\delta^{13}C$ of the molecule(s) metabolized, (2) fractionation during production, and

(3) fractionation during consumption (Abelson and Hoering 1961, Gelwicks et al. 1989, Blair and Carter 1992, Heuer et al. 2006). The best-studied acetogenic model system is *Acetobacterium woodii*, where autotrophic acetogenesis results in a strongly depleted δ^{13}C-acetate relative to CO_2 (-39.7 to -58.6‰; Gelwicks et al. 1989, Preuss et al. 1989). Additional carbon isotopic studies are required to test whether the isotopic depletion of autotrophic acetogenesis found in *A. woodii* is representative of those found in other cultured strains or natural communities.

Besides autotrophic acetogenesis, there are mixo- and methylotrophic pathways of acetogenesis (Drake et al. 2006 and references therein). All have in common that acetate is directly synthesized via reduction of CO_2, not from cleavage of organic matter via disproportionation reactions or hydrolysis as in other pathways. In mixotrophic acetogenesis, acetate is produced by the combination of methyl groups from organic compounds, e. g. methoxylated aromatic compounds, lactate, methanol, ethanol, or methyl chloride, with carboxyl moieties from CO_2 (e.g. Bache and Pfennig 1981, Eichler and Schink 1984, Traunecker et al. 1991, Liu and Suflita 1993, Drake and Daniel 2004, Peters et al. 1998, Drake et al. 2006). In methylotrophic pathways, methyl groups from organic substrates, e.g. formate, formate + methanol, or lactate, are oxidized to produce CO_2; the CO_2 is subsequently reduced and combined with a methyl group that directly derives form organic matter to produce acetate. The widespread availability of mixo- and methylotrophic substrates (Finke et al. 2007 and references therein, Prahl et al. 1994, Baldock et al. 2004) suggests a vast potential for these reactions in marine sediments. While autotrophic reactions might be oucompeted by metabolic reactions with higher energy yields, acetogenesis might nonetheless take place via mixo- and methylotrophic reactions. δ^{13}C-signatures of acetate

produced mixo- or methylotrophically can be expected to be mixed: lighter than bulk organic matter, due to the fractionation step during CO_2 reduction; but heavier than if produced autotrophically, since the methyl group in acetate preserves the isotopic signature of the organic source material.

Molecular biological identification of acetogens using 16S rRNA gene probes is not possible: known acetogenic species frequently have non-acetogenic sister groups (Drake et al. 2002). Hence, functional genes have been targeted. All known acetogens have the capacity to utilize the acetyl-CoA pathway in carbon metabolism. The *fhs* gene encodes formyl tetrahydrofolate synthetase (FTHFS), which catalyzes the ATP-dependent activation of formate. It is conserved (Lovell et al. 1990, Ragsdale 1991), and has been used in phylogenetic studies (e.g. Lovell and Hui 1991, Leaphart and Lovell 2001, Pester and Brune 2006); due to its occurrence in other metabolic groups, e.g. methanogens (Zhao et al. 1989), sulfate reducers (e.g. Tebo et al. 1998), aerobic heterotrophs (e.g. Greenberg et al. 2006)

Prior to a study by Heuer et al. (2006), there had been no determination of δ^{13}C-acetate in deep-sea sediments. Signatures suggested acetogenic contributions to acetate at some deep-sea sites (Black Sea, Juan de Fuca Ridge Flank). To date there has been no published functional gene survey of acetogens in deep-sea sediments or underlying crust, and the thermodynamic potential for mixo- and heterotrophic acetogenesis has not been shown. The existence and occupation of an ecological niche for acetogens in these environments is yet to be demonstrated.

iv. Goals of this study

The main aim of this dissertation is to examine distribution patterns of methanogens, anaerobic methanotrophs, and acetogens in the context of thermal, lithological, and geochemical (substrate and sulfate concentration) gradients in deep-sea sediments. A basic knowledge of the variables that determine the occurrence of these organisms and structure their communities is necessary to ask ecologically relevant questions. The approach differs from the traditional "top-down" or "black box" approach used by geochemists and ecosystem ecologists, who have traditionally focused on bulk measures, such as net rates of reactions, *in situ* energy yields of reactions, or isotopic compositions of metabolic substrates, metabolic products and biomass. The approach also differs from that of traditional environmental microbiology and molecular biology, where the focus has been on descriptive measures, such as assessment of microbial or genetic diversity, or isolation and characterization of individual strains, which provide valuable information about metabolic potential, but not necessarily *in situ* metabolism. I would like to combine tools from molecular biology (PCR/RT-PCR) with those from geochemistry (stable carbon isotopes, calculated *in situ* energy yields, inference of process rates from geochemical gradients), thereby helping to bridge the gap between the two approaches, and improving our understanding of methanogenesis, anaerobic methanotrophy, and acetogenesis in the marine (deep (subsurface)) realm.

My three study sites are the Juan de Fuca Ridge Flank (47°45.2'N, 127°45.8'W), the Peru Trench (9°6.7'S, 80°35.0'W) and the Guaymas Basin (27°00.8'N, 111°24.6'W), the former two located in the east Pacific, and the latter in the Sea of Cortez. While the former two were drilled to depths of several hundred meters below seafloor to obtain sediment (Peru Trench) or sediment and basalt samples (Juan de Fuca), only surface sediments in the hydrothermally active Guaymas Basin were sampled, since the temperature rises to >100ºC

within 20 cm below seafloor. Energy is probably strongly limiting to diversity and activity at the first two sites, while the Guaymas site is rich in labile organic matter and microbial metabolism is probably more limited by physical disturbance and temperature range than energy shortage. The Juan de Fuca Ridge Flank is in a way an intermediate between the cold, energy-poor Peru Trench sediment and the hydrothermally active Guaymas sediment, in that it combines low energy availability with hydrothermal activity and a consequently large temperature change with depth. Physicochemical characteristics of the three sites relevant to this study are summarized in Table 1.1.

In Chapter II, I will use three independent lines of evidence (stable carbon isotopes, free energy yields, fhs gene composition) to examine the likelihood of acetogenesis at IODP Site 1301 sediments. In Chapters III and IV, I will examine the community profiles of *mcrA* genes at IODP Site 1301 and ODP Site 1230, respectively, in the context of *in situ* temperature, geochemical gradients (sulfate, acetate, formate), lithostratigraphy, and calculated *in situ* energy yields. In Chapter V, I will characterize the *mcrA* community profile in the context of temperature at Everest Mound, and compare potential primer biases and differences in detection limit between a plethora of general and group-specific *mcrA* primers. In Chapter VI, I will examine general trends and differences observed between the three study sites, and postulate explanations to be tested in future studies.

TABLE 1.1: Overview of relevant characteristics of the three study sites.

	Peru Trench[a]	Juan de Fuca Ridge Flank[c]	Guaymas Basin, Everest Mound[e]
Water depth (m)	5,086	3,500	2,000
Coring depth (mbsf)	268	583	0.3
Temperature (°C)	2 - 12	2 - 65	2 – 160[e]
TOC (weight%)	0.21 - 4.45	0.07 - 0.91	>2%
SMTZ Depth (mbsf)	8 – 12	60 - 65	tbd (<0.1)
Methane (mM)	0 - >76[b]	0 - >6	unknown
Acetate (µM)	6.2 - 219.8	0 – 53[d]	tbd (probably 0 - >1,000)[f]
Formate (µM)	2.7 - 14.8	0 – 59[d]	tbd
Dihydrogen (nM)	0.09 - 1.64	unknown	Unknown

a) From ODP Leg 201 Shipboard Scientific Party (2003) , except where indicated.
b) Value calculated in Chapter IV, using 2-phase model by Sun and Duan (2007).
c) From Fisher et al. (2005), except where indicated.
d) From Chapter II, quantified by Verena Heuer, MARUM, Bremen, Germany.
e) From Weber and Jørgensen (2002).
f) Based on values in Martens (1990) and Dhillon et al. (2005).

CHAPTER II

EVIDENCE FOR ACETOGENESIS IN THE DEEP SUBSEAFLOOR

ABSTRACT

Isotopically light acetate in deep subsurface sediments of the Juan de Fuca Ridge Flank indicates an acetogenic component to total acetate production. Thermodynamic calculations indicate acetogenesis from common fermentation products or lignin monomers to be a likely source. Autotrophic acetogenesis is also possible provided that dihydrogen (H_2) concentrations are not drawn down to the thermodynamic thresholds of the competing processes of sulfate reduction and methanogenesis. A high diversity of novel formyl tetrahydrofolate synthetase (*fhs*) genes throughout the upper half of the sediment column indicates the genetic potential for acetogenesis. Our results diverge from the widespread view that under steady-state conditions acetate production in marine sediments is only from fermentation and sulfate reduction.

INTRODUCTION

Next to H_2, acetate is the key intermediate in microbial carbon cycling in anoxic environments (Valentine 2001). Like H_2, it is both a degradation product and substrate in the terminal step of organic matter remineralization (Cappenberg 1974, Sørensen et al. 1981). In addition, certain microbes, e.g. several lithoautotrophic methanogens and sulfate reducers,

assimilate acetate into biomass and require it for growth rather than metabolism (Brysch et al. 1987, Whitman et al. 2001). While concentration profiles and consumption rates have been examined in numerous studies (Sansone and Martens 1982, Crill and Martens 1986, Wellsbury et al. 1997, Wellsbury et al. 2002, ODP Leg 201 Shipboard Scientific Party 2003), little remains known about acetate production in anoxic marine sediments. Fermentation and sulfate reduction reactions are often invoked as sources. In deep subseafloor sediments, acetate concentrations have been shown to increase with temperature at depth, possibly due to enhanced bio- or thermogenic acetate generation from organic matter at high temperatures (Wellsbury et al. 1997, Egeberg and Barth 1998).

Terminal oxidation of organic matter in marine sediments is carried out by the microbial processes of (1) oxygen (O_2), (2) nitrate (NO_3^-), (3) metal (Mn^{4+}, Fe^{3+}), (4) sulfate (SO_4^{2-}), and (5) carbon dioxide (CO_2) reduction, with their vertical distribution influenced by *in situ* energy yields (Froelich et al. 1979, Canfield et al. 1993). Two dissimilatory pathways for CO_2 reduction are known: methanogenesis and acetogenesis. Under steady-state conditions, methane producers (methanogens) are believed to drive H_2 concentrations below the thermodynamic threshold of acetate synthesizers (acetogens) due to the higher energy yield of hydrogenotrophic methanogenesis compared to autotrophic acetogenesis (Lovley and Goodwin 1988, Hoehler et al. 1998).

Acetogens are nonetheless able to thrive due to their ability to use a wide range of substrates and alternate electron-accepting processes, including fermentation, sulfate or nitrate reduction (Drake et al. 2002, Drake et al. 2004, Drake et al. 2006). Moreover, exceptions appear possible if H_2 concentrations are non-limiting, as under non-steady-state conditions resulting from fluctuating depositional regime, temperature, or redox conditions

(Zeikus and Winfrey 1976, Hoehler et al. 1999); when populations are under predatory or viral control; or when high methane concentrations inhibit methanogenesis.

Acetogenesis differs from other pathways of acetate production, such as hydrolysis, fermentation or sulfate reduction, in that acetate is synthesized *de novo* via the reduction of CO_2 (Müller 2003). Synthesis is auto-, mixo- or methylotrophic (Table 2.1). In mixotrophic reactions, methyl groups from organic compounds are combined with carboxyl moieties from CO_2 (Drake et al. 2002, Drake et al. 2004, Bache and Pfennig 1981, Eichler and Schink 1984, Liu and Suflita 1993; Table 2.1). In methylotrophic acetogenesis, organic substrates are used (e.g., methanol, lactate), but at least one catabolic step involves CO_2 reduction and acetate

TABLE 2.1: Overview of auto-, mixo-, and methylotrophic substrates, reactions, and ratios of carbon of organic to carbon of inorganic origin in the product acetate (N/A = not applicable).

	Substrate	**Reaction**	**C_{org}:C_{inorg}**
Autotrophy	H_2/CO_2	$2\ HCO_3^- + 4\ H_2 + H^+ \rightarrow CH_3COO^- + 4\ H_2O$	N/A
	CO	$4\ CO + 4\ H_2O \rightarrow CH_3COO^- + 2\ HCO_3^- + 3\ H^+$	N/A
	CO-H_2	$2\ H_2 + 2\ CO \rightarrow CH_3COO^- + H^+$	N/A
Mixotrophy	ethanol + CO_2	$2\ CH_3CH_2OH + 2\ HCO_3^- \rightarrow 3\ CH_3COO^- + 2\ H_2O + H^+$	2:1
	lactate + H_2/CO_2	$CH_3CHOHCOO^- + 6\ H_2 + 3\ HCO_3^- + H^+ \rightarrow 3\ CH_3COO^- + 6\ H_2O$	1:1
	methanol + CO_2	$4\ CH_3OH + 2\ HCO_3^- \rightarrow 3\ CH_3COO^- + 4\ H_2O + H^+$	2:1
	methanol + H_2/CO_2	$CH_3OH + H_2 + HCO_3^- \rightarrow CH_3COO^- + 2\ H_2O$	1:1
	syringate + CO_2	2 syringate$[-OCH_3]_2 + 2\ HCO_3^- \rightarrow 2$ gallate$[-OH]_2 + 3\ CH_3COO^- + H^+$	2:1
	syringate + H_2/CO_2	syringate$[-OCH_3]_2 + 2\ HCO_3^- + 2\ H_2 \rightarrow$ gallate$[-OH]_2 + 2\ CH_3COO^- + 2\ H_2O$	1:1
	vanillate + CO_2	4 vanillate$[-OCH_3] + 2\ HCO_3^- \rightarrow 4$ protocatechuate$[-OH] + 3\ CH_3COO^- + H^+$	2:1
Methylotrophy	formate	$4\ HCOO^- + H^+ \rightarrow CH_3COO^- + 2\ HCO_3^-$	N/A
	formate/H_2	$2\ HCOO^- + 2\ H_2 + H^+ \rightarrow CH_3COO^- + 2\ H_2O$	N/A
	lactate	$2\ CH_3CHOHCOO^- \rightarrow 3\ CH_3COO^- + H^+$	N/A
	methanol + formate	$CH_3OH + HCOO^- \rightarrow CH_3COO^- + H_2O$	N/A

acetogenic substrates (Prahl et al. 1994, Finke et al. 2007) suggests a vast potential for mixo- and methylotrophic acetogenesis in marine sediments.

Acetogenesis can be inferred from the carbon isotopic composition ($\delta^{13}C$) of acetate. The $\delta^{13}C$ value of a metabolite is driven by the $^{13}C/^{12}C$ ratio of its precursor and the kinetic isotope effects associated with its production and consumption (Abelson and Hoering, Gelwicks et al. 1989, Blair and Carter 1992, Heuer et al. 2006). Strong discrimination against ^{13}C is the norm in autotrophic pathways and, though the database is small, has been observed in methanogenic and acetogenic CO_2 reduction. The best-studied acetogenic model organism is *Acetobacterium woodii*, which, grown on H_2/CO_2, produces a strong ^{13}C depletion relative to CO_2 (-39.7 to -58.6‰; Gelwicks et al. 1989, Preuß et al. 1989).

Molecular biological detection of acetogens using 16S rRNA gene probes is complicated by the fact that acetogens are distributed over 19 bacterial (20) and 2 archaeal (Rother and Metcalf 2004, Henstra et al. 2007) genera, and frequently have close non-acetogenic relatives (Drake et al. 2006). Hence, functional genes of acetogenesis have been targeted. All known acetogens utilize the acetyl-CoA pathway. The *fhs* gene, which encodes formyltetrahydrofolate synthetase (FTHFS), an enzyme that catalyzes the ATP-dependent activation of formate, is fairly well conserved (Lovell et al. 1990, Ragsdale 1991), and has been the subject of environmental surveys (Lovell and Hui 1991, Leaphart and Lovell 2001, Pester and Brune 2006).

In this study, we measured concentrations and carbon isotopic compositions of porewater acetate in a 265-m thick sediment column on the Juan de Fuca ridge flank (Fisher et al. 2005) with a ~60°C increase in *in situ* temperature with depth. We used multiple approaches including (1) a $\delta^{13}C$-acetate profile, (2) porewater geochemical profiles, (3) calculation of

energy yields, (4) consideration of geologic and geothermal characteristics, and (5) an *fhs* gene profile, to examine the potential contribution of acetogenesis to acetate production in deep subsurface marine sediments.

METHODS

Field Site

The sediment and underlying basalt column of IODP Site U1301 were sampled during IODP Expedition 301 in the summer of 2004 (Fisher et al. 2005). IODP Site U1301 is on the eastern flank of the Juan de Fuca Ridge in the northwestern part of Cascadia Basin at latitude 47°45N, longitude 127°45W, and water depth ~2,656 m (Appendix Fig. 1A, Fisher et al. 2005). The sediment column is ~265m thick and covers a basaltic ridge (Second Ridge 1; Appendix Fig. 1B). The lithostratigraphy, temperature gradient, and porewater chemistry of the sediment column are very similar to those 1-2 km north at ODP Site 1026 during Ocean Drilling Program (ODP) Leg 168 (Shipboard Scientific Party 1997, Fisher et al. 2005). The basaltic basement dates back to 3.5 Ma. Overlying sediments were deposited during the Miocene (265-215 mbsf), and Pleistocene to present (215-0 mbsf).

The sediment column is heterogeneous with interbeds of diatomaceous hemipelagic clay, and turbidite deposits of various grain sizes, including clay, silt, sand, gravel and mixtures thereof (Appendix Fig. 1C; Fisher et al. 2005). Turbidites derive from a polymictic assemblage of igneous, metamorphic, metasedimentary, and sedimentary rocks and probably originate from Vancouver Island, the Olympic Peninsula, the northern Cascades, and western British Columbia (Underwood et al. 2005). Transport from the continental shelf to the

abyssal plain occurs via submarine canyons from Vancouver Island, and the larger Barkley, Nitinate, and Juan de Fuca canyons further south (Underwood et al. 2005).

Due to the hydrothermally active basement below, the temperature increases from ~2°C at the seafloor to ~64°C at the sediment-basement interface. Heat transfer through the sediment column is conductive; hence temperature increases linearly with depth (Shipboard Scientific Party 1997).

The porewater geochemical profile has two sulfate reduction zones (~0-70 and 120-265 mbsf), separated by a zone of methanogenesis (~70-120 mbsf; Fig. 2.1; Fisher et al. 2005). In the SMTZs (~60-70 and 120-130 mbsf), virtually all methane produced in the methanogenesis zone is consumed by AOM.

Sampling

Sediment cores were sampled with an Advanced Piston Corer (APC), by which a drill is "advanced" to the desired sediment depth, and a 9.5-m piston core then hydraulically thrust into underlying sediments (Graber et al. 2002). Contamination, monitored by addition of chemical tracer to drilling fluid, was minute ($\leq$0.09 $\mu L\ g^{-1}$) in the core interior (Lever et al. 2006). The one sample with higher contamination was excluded from the analyses.

Porewater samples for geochemical analyses were obtained from 20-40 cm long whole-round intervals using a hydraulic press as described previously (Fisher et al. 2005). For carbon isotope analyses, 5-mL subsamples were frozen in precombusted glass vials.

For molecular biological analyses, 5-cm whole-round intervals of cores were frozen at -80°C. Only sediment from the nearly contamination-free interior was used (Ludwig et al. 2004).

Acetate concentrations and $\delta^{13}C$

Concentrations and carbon isotope compositions of volatile fatty acids (VFAs) were analyzed by isotope ratio monitoring-liquid chromatography/mass spectrometry (irm-LC/MS) using a modification of a previously published protocol (Heuer et al. 2006). We used an optimization of the original method consisting of a VA 300/7.8 Nucleogel Sugar 810H column (300 mm length; 7.8 mm i.d.; Macherey-Nagel) equipped with a guard column (CC30/4 Nucleogel Sugar 810H; 30 mm length; Macherey-Nagel), and 5 mM phosphoric acid as mobile phase with a flow rate of 300 μL min^{-1}. The column was kept at room temperature. Primary standardization on the Delta Plus XP was based on three to six injections of reference CO_2 ($\delta^{13}C$=-28.0±0.1‰ vs. Vienna Pee Dee Belemnite (VPDB), 4.5±0.5 V at m/z 44) before and after each sample. $^{13}C/^{12}C$ ratios and $\delta^{13}C$-values were calculated according to Ricci et al. (1994) and Santrock et al. (1985). Our quantitative analysis is based on the linear correlation between signal area recorded by irm-LC/MS and injected amount of carbon (Heuer et al. 2006). We assessed precision and accuracy as described previously (Heuer et al. 2006); aqueous standard solutions were prepared from sodium salts of VFAs (Aldrich). Analyses of standards were performed regularly during analyses of samples, and $\delta^{13}C$-values obtained by irm-LC/MS compared to corresponding salts determined via irm-elemental analyzer-MS (irm-EA/MS). The precision was 0.6‰ (1 σ, n=4). Mean $\delta^{13}C$-values of dissolved VFAs deviated by <1.4‰ from those determined by irm-EA/MS. The precision of quantitative analyses was 6% (1 σ). The detection limits were 2 μM for concentrations and 10 μM for carbon isotopic analyses.

The carbon isotopic composition of DIC was analyzed using a GasBench coupled to a Finnigan MAT irm-MS. 0.7- to 1.0-mL samples of interstitial water were acidified with 100 μL of phosphoric acid in glass tubes that had been sealed with butyl septa and plastic caps and purged 5× with helium. Samples were allowed to degas CO_2 for 5+ hrs, before gas phases were analyzed for $\delta^{13}C$-CO_2. The analytical precision was 0.1‰ (1 σ).

DNA Extraction

Samples were thawed until soft enough to obtain 6-g aliquots, which were transferred to 50-mL Falcon tubes, homogenized in 10 mL of artificial seawater (3% NaCl, 0.3% MgSO4, 0.2% KCl + 0.312 g $NaH_2PO_4 \bullet 2H_2O$ (20 mM)) and kept at room temperature for 2 hrs. To remove extracellular DNA, tubes were then centrifuged for 20 min at 4,000×g. Sediment pellets, presumably containing intact cells, were kept.

A modification of the ISOIL Large for Beads kit (Nippon Gene, Tokyo, Japan) was used to extract DNA. Beads, 9.5 mL Lysis Solution BB, and 0.5 mL Lysis Solution 20 S were added to pellets, slurried by vortexing, and shaken to break cells for 30s at room temperature, using a SPEX 6850 freezer/mill (SPEX SamplePrep, Metuchen, New Jersey). PCR-inhibitory proteins were removed via proteinase K addition (500 μg mL^{-1}). Samples were incubated while gently being rotated on a shaker table at 50°C for 2 hrs. Temperature was raised to 65°C and samples incubated for one more hour to denature the proteinase. For the rest of the extraction, we followed the manufacturer's protocol.

DNA was purified and concentrated using the Amicon Ultra-15 10K followed by the Montage PCR Cleanup kit (both Millipore Corporation, Billerica, USA). A final DNA

purification was performed using the Mag Extractor – PCR & Gel Clean Up kit (Toyobo, Tokyo, Japan).

PCR Amplification

We amplified *fhs* genes with the FTHFS (Lovell and Leaphart 2005) and *fhs* 49F/574R (see below) primer pairs. DNA was PCR-amplified with the Takara SpeedStar Polymerase Kit (Takara Bio Inc., Shiga, Japan) with Taq concentrations twice those in the manual, and bovine serum albumin added to 4 $\mu g\ \mu L^{-1}$ of reaction mix. The PCR protocol with the FTHFS primer pair was as outlined previously (Lovell and Leaphart 2005).

The FTHFS primer pair yields a ~1.1 kb fragment and is highly degenerate. Successful *fhs* amplifications were limited to three samples. To improve amplification efficiency, and hence lower the detection limit, we designed a primer pair with fewer degeneracies that targeted *fhs* genes detected with FTHFS primers at Site 1301. This new primer pair (*fhs 49F*: 5'- GATGATCGACAACCACRTCTA, *fhs 574R*: 5'- GGCACTTGATGTCGAAGAA) was designed using a gene database and sequence alignment created with the ARB phylogenetic analysis software (Ludwig et al. 2004). DNA fragments are nested within ones targeted by the FTHFS primer pair. In designing this primer pair, we aimed to maximize diversity covered while keeping number of degeneracies to a minimum. The PCR protocol used was (1) 1 × 2 min denaturation (98°C), (2) 40 × 30s denaturation (95°C), 30s annealing (60°C), 1 min extension (72°C), and (3) 1×5 min extension (72°C). PCR was performed using Veriti model thermal cyclers (Applied Biosystems, California, USA).

Cloning and Sequencing

PCR products were purified with the Montage PCR Cleanup kit (Millipore Corp., Billerica, USA), cloned using the Topo TA Kit (Invitrogen, Carlsbad, USA), and inserted into chemically competent *E. coli* as outlined in the Topo TA Kit Manual (Invitrogen, Carlsbad, USA).

Phylogenetic analysis of *fhs* sequences

We imported *fhs* sequences from GenBank into ARB, and created an alignment. Sequences from published articles (Lovell and Leaphart 2005), and BLAST hits of sequences closely related to our environmental sequences were imported. Sequences from IODP Site U1301 were incorporated. Phylogenetic trees were created with ARB neighbor-joining and Jukes Cantor correction. Nucleotides were used since bootstrap calculations yielded higher confidence in branching patterns with these than with amino acids.

Meta-analysis of acetogen substrates

We compiled all literature we could find by February 2008, on known acetogens, their substrate use, and habitat (Appendix, Table 1).

Thermodynamic calculations

We calculated H_2 concentrations and energy yields of likely mixo- and methylotrophic acetogenesis reactions (formate, lactate, methanol, syringate, vanillate) as well as competing fermentation and methanogenesis reactions where relevant (formate, lactate, methanol). Standard Gibbs free energies (ΔG_r^o), entropies (ΔH_r^o), and molar volumes (ΔV_r^o) of biochemical reactions were calculated from standard free Gibbs energies of formation (ΔG_f^o),

standard entropies (ΔH_f^o), and standard molar volumes (ΔV_f^o) of reactants and products (Thauer et al. 1977; Table 2.2). Corrections for temperature and pressure were made using the Nernst and Van't Hoff equations, respectively (Stumm and Morgan 1981).

TABLE 2.2: Standard values of Gibbs free energy of formation ($\Delta G_f°$), partial molar enthalpy ($\Delta H_f°$), and partial molal volume ($\Delta V_f°$) used in thermodynamic calculations.

Thermodynamic Data at Standard Conditions:	**$\Delta G_f°$ (kJ mol^{-1})**	**$\Delta H_f°$ (kJ mol^{-1})**	**$\Delta V_f°$ (mol^{-1})**	**Reference**
acetate	-369.41	-486.42	40.5	Shock and Helgeson (1990)
bicarbonate	-586.85	-691.99	24.6	Wagman et al. (1968)
ethanol	-181.75	-287.42	55.08	Shock and Helgeson (1990)
formate	-351.04	-425.71	26.16	Shock and Helgeson (1990)
gallate	-706.00			Kaiser and Hanselmann (1982)
H^+	0.00	0.00	0.00	Shock et al. (1997)
hydrogen (H_2)	17.57	-4.16	25.2	Shock and Helgeson (1990)
lactate	-513.01	-686.93	56.25	from Shock (1995)
methane	-34.47	-87.96	37.30	Shock and Helgeson (1990)
methanol	-175.39	-246.48	38.17	Shock and Helgeson (1990)
protocatechuate	-551			Kaiser and Hanselmann (1982)
sulfate	-744.96	-910.21	13.88	Shock et al. (1997)
sulfide (HS^-)	11.97	-16.12	20.65	Shock et al. (1997)
syringate	-564.00			Kaiser and Hanselmann (1982)
vanillate	-480.00			Kaiser and Hanselmann (1982)
water	-237.18	-285.83	18.02	Amend and Shock (2001)

Due to the absence of H_2 concentration data, we calculated H_2 concentrations in both sulfate reduction zones (~0-70 and ~120-265 mbsf) and the methanogenesis zone (~70-120 mbsf; Fig. 2.2) assuming that H_2 concentrations were under thermodynamic control by hydrogenotrophic sulfate reducers and methanogens. We assumed *in situ* energy yields to be constant and at the biological energy quantum (BEQ; Hoehler 2005) and used an estimated BEQ of 10 kJ mol^{-1} (Hoehler 2005) in all calculations.

Concentrations of formate were measured, whereas those of lactate and methanol were estimated due to lack of measurement data. We used a 0.2 μM lactate concentration in all calculations, which is lower than measured literature values for marine sediments (0.8-32 μM; Finke et al. 2007). Methanol concentrations in marine sediments have, to our knowledge, never been measured. We used a concentration of 1 nM in all calculations, a value we consider likely to be an underestimate of *in situ* concentrations. Calculated energy yields for reactions involving lactate and methanol are therefore probably underestimates of *in situ* energy yields.

We compared energy yields of mixo- and methylotrophic acetogenesis reactions to those of competing methanogenesis and fermentation reactions. Sulfate reducers are also likely to compete with acetogens for formate and lactate, but due to lack of concentration data for sulfide, we omitted these calculations; *in situ* energy yields of sulfate reduction reactions from formate and lactate are, however, likely to exceed those of mixo- and methylotrophic acetogenesis.

For lignin monomers (syringate, vanillate), no published ΔH_r^o or ΔV_r^o values were available. We therefore calculated energy yields at standard temperature and pressure, over a range of concentrations (10^{-20}-10^0 M) that we considered likely to include *in situ* concentrations.

We used measured activity coefficients, γ, of 1.24 for CH_4, and 0.532 for HCO_3^- (Millero and Schreiber 1982) and $\{H_2O\} = 1.0$. Activity coefficients of methanol and H_2 were approximated with the one for methane. Similarly, activity coefficient for the anions formate, acetate, lactate, syringate, vanillate, gallate, and protocatechuate were approximated with the measured value for bicarbonate. We performed sensitivity analyses by varying

activity coefficients of compounds with no published values by ± 20% and found calculated energy yields, even under the most extreme scenarios, to change by <2 kJ mol^{-1}.

RESULTS AND DISCUSSION

Geochemical Profile

Concentrations of porewater acetate fluctuate with depth, with peaks near the sediment surface, at 20, 82, 190, and 260 mbsf (Fig. 2.1). These peaks are clear evidence for *in situ* acetate production. There is a steady increase from ~35 mbsf to the highest concentrations measured, at 82 mbsf, followed by a steep decrease to ~110 mbsf.

There is no apparent relationship between acetate concentrations and temperature (Fig. 2.1). Furthermore, there is no evidence for (1) enhanced bio- or thermogenic acetate production with increasing temperature, or (2) an underlying thermogenic source of acetate, as suggested for other sites (Wellsbury et al. 1997, Egeberg and Barth 1998). Increased acetate production should be reflected in increased sulfate reduction rates in the lower sulfate reduction zone; however, the almost linear sulfate concentration profile between the basaltic basement and lower sulfate-methane-transition-zone (SMTZ) virtually no sulfate reduction in most of the lower sulfate reduction zone. The nearly linear gradient indicates diffusive mixing between the two end members, sulfate-rich basalt fluid, and (nearly) sulfate-free sediment porewater in the methanogenesis zone. Deviations from linearity in the sulfate concentration profile from 130-265 mbsf are probably caused by sulfide reoxidation during

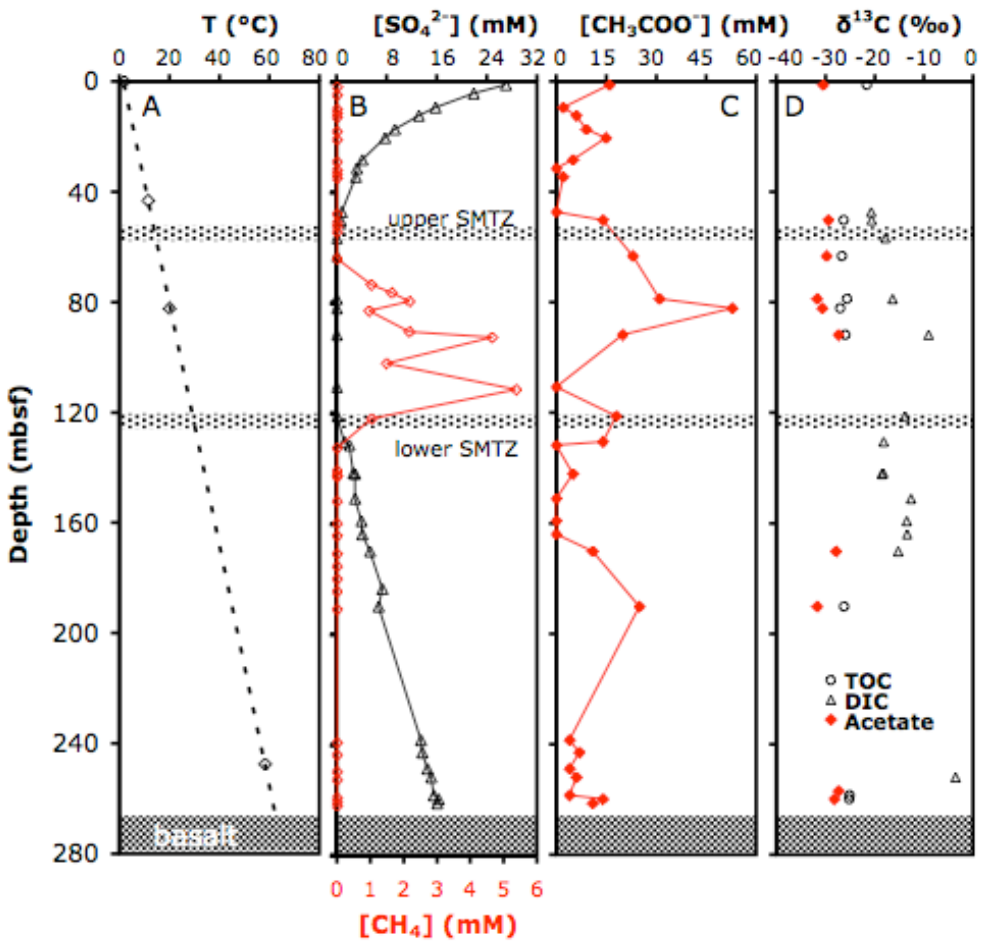


FIGURE 2.1: Depth profiles of (A) temperature, (B) sulfate and methane concentrations, (C) acetate concentrations, (D) $\delta^{13}C$ of acetate, TOC, and DIC. Data shown in (A) and (B) from Fisher et al. (2005).

retrieval, not *in situ* activity of sulfate reducers. By contrast, the lower SMTZ (~120-130 mbsf), where anaerobic oxidation of methane (AOM) takes place, is a deep hotspot of microbial activity, and the main sink of basalt-derived sulfate in the sediment column.

With $\delta^{13}C$-values averaging around -29.5 ± 1.7‰, acetate is depleted in ^{13}C compared to total organic carbon (TOC) and dissolved inorganic carbon (DIC) throughout the entire sediment column. Relative to TOC, acetate is lighter by 9‰ at the surface, by 3-6‰ down to

~82 mbsf, and by 1-5‰ below that. Relative to DIC, the ^{13}C-depletion ranges from 9-24‰. Since δ^{13}C-values of lipids are typically ~2-6‰ lighter than bulk organic matter (Abelson and Hoering 1961), hydrolysis or fermentation of lipids can explain the values in all but the surface horizon, while fermentation of carbohydrates or amino acids is an unlikely source as both tend to be similar or enriched in δ^{13}C relative to TOC (e.g. Abelson and Hoering 1961, Dauwe and Middelburg 1998, Keil and Fogel 2001, Wang and Druffel 2001). While lipid degradation is likely to account for a portion of the acetate produced, lipids are probably not the predominant source of acetate: organic matter is progressively depleted in lipids as it sinks through the water column; consequently, lipids only represent a small portion (0.1-1%) of total organic matter deposited to the seafloor (Wakeham et al. 1997). Instead, most of the acetate likely derives from hydrolysis and fermentation of complex carbohydrates (Brüchert and Arnosti 2003). Without an acetogenic contribution to acetate production, we would therefore expect δ^{13}C values of acetate to be close to or enriched relative to TOC.

Regardless of whether acetogenesis is auto-, mixo- or methylotrophic, it involves a CO_2 reduction step via the acetyl CoA-pathway. Hence, the end product of acetogenesis, acetate, is likely to in all cases be isotopically depleted relative to the bulk δ^{13}C of its carbon sources. Maximal fractionations can be expected for autotrophic acetogenesis, since both carbon atoms of acetate derive from CO_2 reduction. Part of the carbon in mixo- and methylotrophically produced acetate derives from organic matter directly, and is therefore unlikely to undergo significant, if any, fractionation. We thus expect mixo- and methylotrophically produced acetate to have δ^{13}C-signatures that are intermediate, i.e. heavier than autotrophically produced acetate, and lighter than acetate that is the product of heterotrophic microbial processes with no CO_2 reduction steps, such as hydrolysis or

fermentation. Support for our postulate comes from studies of δ^{13}C-fractionations in the Calvin-Benson-Bassham cycle: δ^{13}C-values of mixotrophically produced phytoplankton biomass are intermediate, that is lighter than δ^{13}C of suspended particulate organic matter and heavier than δ^{13}C of autotrophically produced phytoplankton biomass (Bentaleb et al. 1996, Bentaleb et al. 1998).

Potential Acetogen Substrates

If H_2 concentrations at IODP Site U1301 are under thermodynamic control (Fig. 2.2), as in coastal and freshwater sediments (Lovley and Goodwin 1988, Hoehler et al. 1998), sulfate reducers and methanogens will preclude autotrophic acetogenesis (Lovley and Goodwin 1988, Hoehler et al. 1998, Cord-Ruwisch et al. 1988) by driving H_2 concentrations below threshold concentrations required for autotrophic acetogenesis to meet the biological energy quantum (BEQ) of ~10 kJ mol^{-1} (Hoehler 2005). Due to the lack of reliable H_2 profiles, it remains to be demonstrated that deep subseafloor H_2 concentrations are under thermodynamic control, however. A recent study has revealed the cooccurrence of the competing processes of microbial metal reduction, sulfate reduction, and methanogenesis in deep subsurface sediments of the eastern equatorial Pacific (Wang et al. 2008). Hence, we cannot rule out the possibility of autotrophic acetogenesis contributing at least in part to the light δ^{13}C of acetate at IODP Site U1301.

A compilation of literature data on substrates utilized by acetogens demonstrates the ability to perform mixo- and methylotrophic reactions to be essentially as widespread as the ability to perform the classic autotrophic reaction (Table 2.3, Appendix Table 1). Mixo- or methylotrophic acetogenesis therefore provides an alternative explanation for isotopically

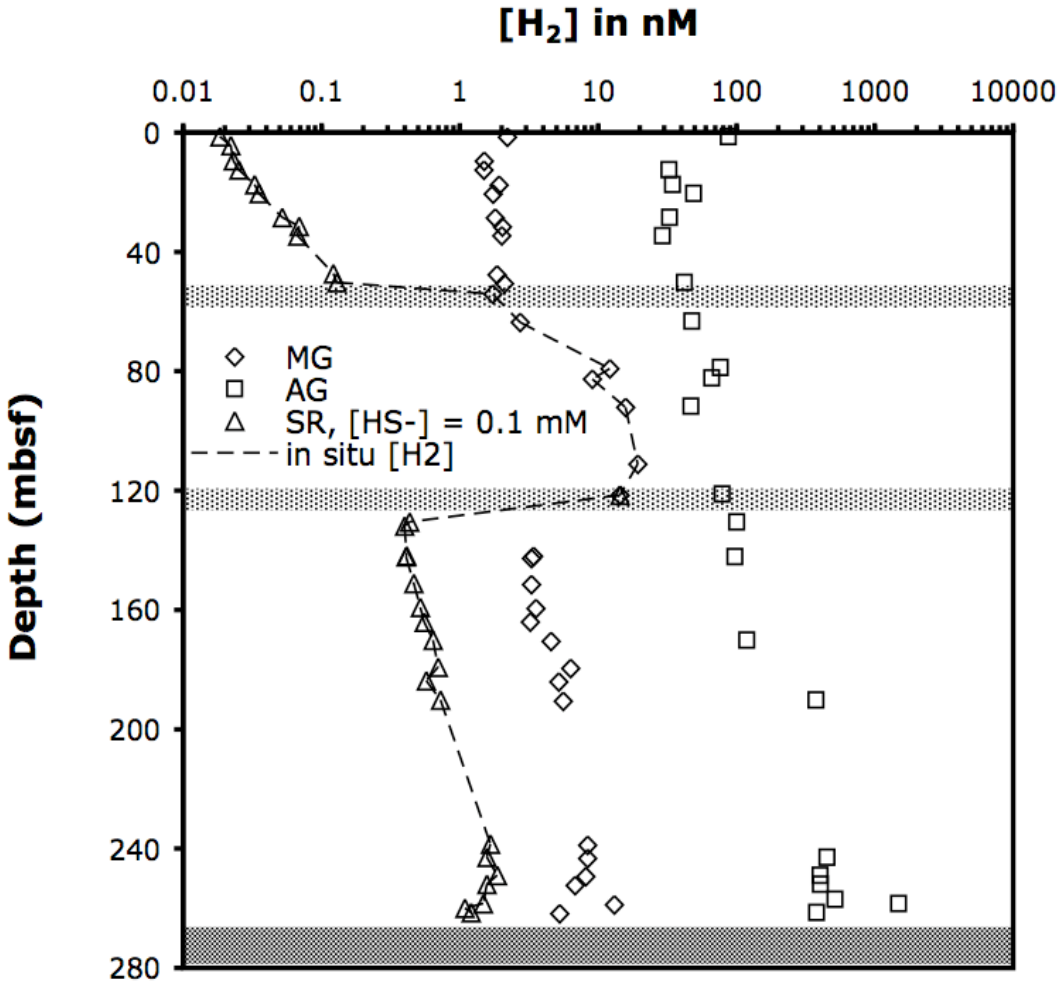


FIGURE 2.2: H_2 concentrations required for ΔG_r = -10 kJ mol^{-1} for sulfate reduction (SR), methanogenesis (MG), and acetogenesis (AG) from H_2-CO_2 under *in situ* conditions. The dashed line indicates the *in situ* H_2-concentration profile if H_2 concentrations are controlled thermodynamically, i.e. by the most energetically favorable metabolic reactions. Note: for calculations of SR, we used $[HS^-]$ = 0.1 mM.

light acetate at IODP Site U1301. We examined the energetic feasibility of some of these mixo- and methylotrophic reactions (involving formate, lactate, methanol, syringate, vanillate) via calculations of energy yields. Where relevant, we compared these energy yields to those of competing methanogenesis and fermentation reactions.

The BEQ for methylotrophic acetogenesis from formate +H_2 is exceeded in the depth intervals from ~30-85 mbsf and near the basement, suggesting the potential for acetogenesis

TABLE 2.3: Summary of published information on substrate use by auto- and mixotrophic acetogens. 'Total # tested' = total number of strains for which use of a substrate was tested; 'Total # positive' = number of strains which tested positive for use of a substrate; '% positive' = percentage of total number of strains that tested positive for use of a substrate. Abbreviations: MeOH = methanol; EtOH = ethanol; Aliphatic = aliphatic compounds other than EtOH (most notably lactate); O-CH_3 = methoxylated aromatic compounds (all references and strains examined are in Appendix Table 1).

	Substrates						
	H_2/CO_2	**CO**	**$HCOO^-$**	**MeOH**	**EtOH**	**Aliphatic**	**O-CH_3**
Total # tested	100	41	79	69	51	59	58
Total # positive	90	27	59	46	27	42	51
% positive	**90**	**66**	**75**	**67**	**53**	**71**	**88**

reactions from formate in certain depth horizons (Fig. 2.3a). By comparison, methylotrophic acetogenesis from formate is not feasible at any depth. Acetogenesis from lactate (Table 2.1) at conservatively estimated concentrations (see Methods) is energetically favorable throughout the sediment column, while mixotrophic reactions from lactate +H_2/CO_2 are only favorable in the methanogenesis zone (Fig. 2.3b). Mixotrophic reactions of methanol as sole substrate, and methylotrophic reactions of methanol +formate are thermodynamically favorable throughout (Fig. 2.3c), whereas reactions of methanol +H_2/CO_2 are only favorable in the upper half of the sediment column.

Thermodynamic calculations suggest that mixo- and methylotrophic acetogenesis reactions from three common fermentation products are likely to be thermodynamically favorable under *in situ* conditions. *In situ* energy yields for lactate and methanol are probably higher than our calculated values, since the lactate and methanol concentration values used in our calculations are likely to be underestimates of *in situ* concentrations.

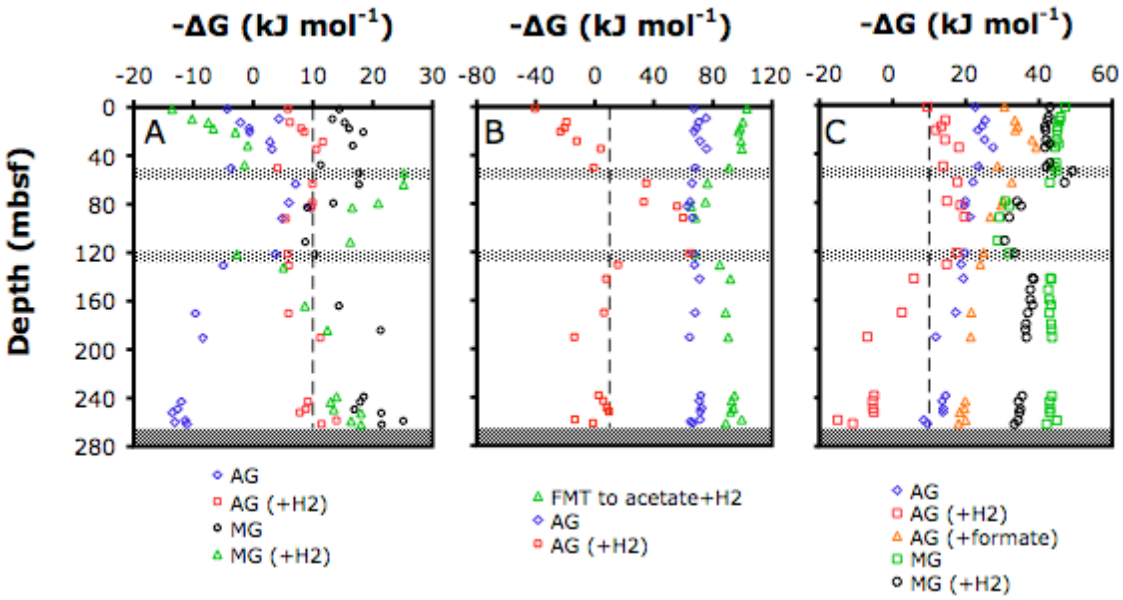


FIGURE 2.3: Calculated *in situ* energy yields of (A) acetogenesis (AG) and methanogenesis (MG) from formate, (B) acetogenesis and fermentation (FMT) from lactate, and (C) acetogenesis and methanogenesis from methanol. We assumed [lactate] = 0.2 μM, [methanol] = 1 nM, and thermodynamically controlled [H_2].

Reactions performed by methanogens and fermenters provide more energy (Fig. 2.3a-c), and one might expect these organisms to outcompete acetogens over time. Acetogens have the advantage of higher metabolic versatility, however. The ability to pool energy from a greater number of substrates and pathways may be a successful alternative survival strategy in environments where energy sources are scarce, such as deep subsurface sediments.

Near the sediment surface, δ^{13}C-TOC suggests predominantly water column-derived organic matter (~ -22‰; Fig. 2.1), while δ^{13}C-TOC in deeper layers is isotopically lighter (-25 to -27‰), in a range typical of terrestrial C_3 plants (Fry and Sherr 1984). The lighter δ^{3}C-TOC below the surface is consistent with the occurrence of coarse sand layers of terrestrial origin (Underwood et al. 2005), transferred to the Cascadia Basin via turbidity currents from the nearby continental shelf (Prahl et al. 1994). A major contributor to the TOC pool in terrestrially-derived marine sediments is lignin, a structural component of cell walls in

vascular plants (Gelwicks et al. 1989). δ^{13}C-signatures of lignin are in the range typical of terrestrial plants (Benner et al. 1987, Goñi and Eglinton 1996). Lignin molecules consist of methoxylated aromatic monomers (Sarkanen and Ludwig 1971). Among anaerobic organic carbon degraders, the ability to O-demethylate lignin monomers, an initial step in the degradation of these highly refractory compounds (DeWeerd et al. 1988), is widespread among acetogens. Of 58 acetogen strains tested, 88% were able to O-demethylate aromatic compounds (Table 2.3). Mixotrophic acetogenesis from lignin monomers (Table 3) provides high energy yields, even at lignin concentrations far below the likely environmentally relevant range (Fig. 2.4). A substantial increase in the proportion of genes associated with aromatic compound metabolism with depth was recently documented for a subsurface sediment column (Biddle et al. 2007). Since lignin monomers are non-competitive substrates for acetogens, acetogens may be essential to the degradation of these monomers in the deep subsurface. The significance of the contribution of mixotrophic acetogenesis from lignin monomers to total acetogenesis, total acetate production, and hence δ^{13}C-acetate remains to be determined, but its investigation has the potential to provide valuable insights into the diagenesis of highly recalcitrant organic matter, as well as the continued persistence of actively metabolizing microorganisms, in ancient sediments.

Fhs Phylogeny

We performed PCR assays with 16 samples from throughout the sediment column (Table 2.2, include these in Fig. 1). We detected a high diversity of mostly novel fhs genes (Fig. 2.5). The distribution of acetogens has been investigated in many anoxic environments,

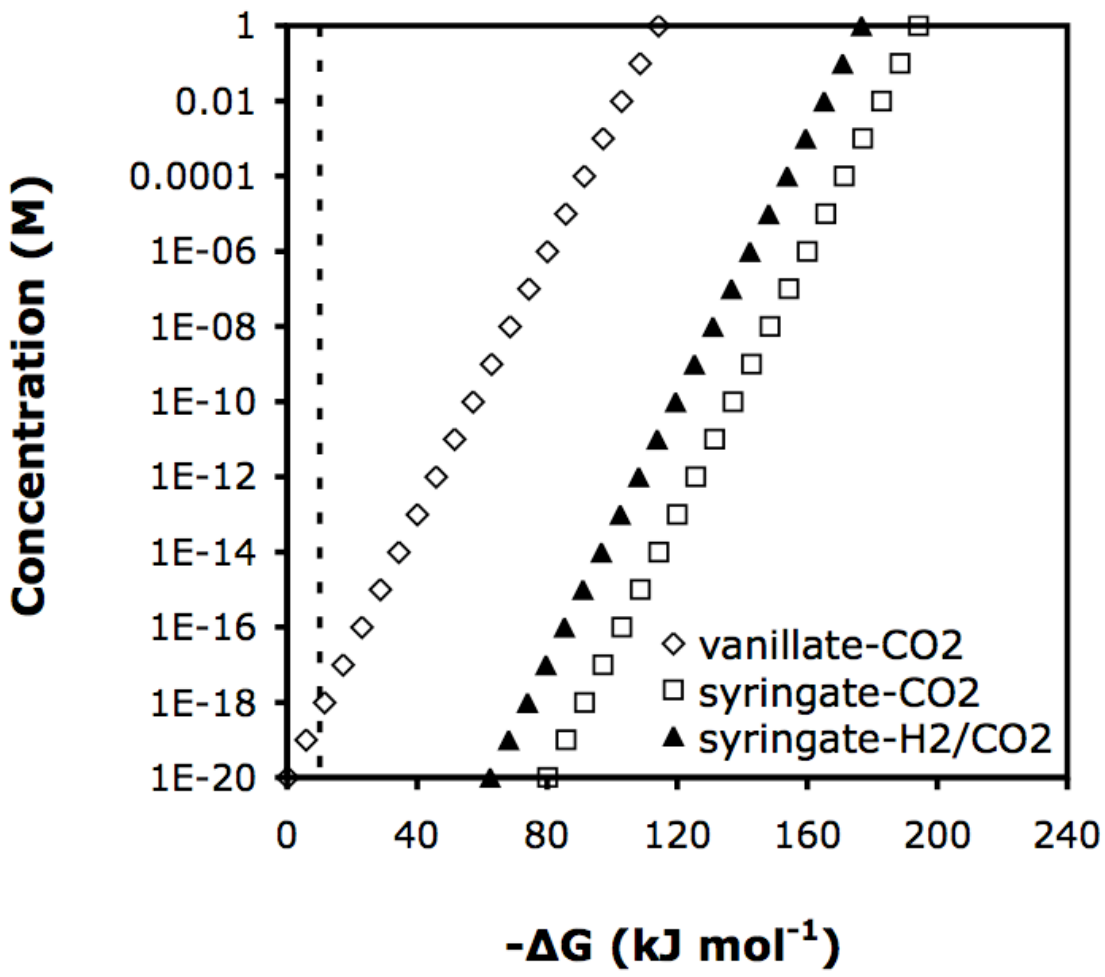


FIGURE 2.4: Calculated energy yields from mixotrophic reactions involving a wide range of lignin monomer concentrations. We used uniform pH = 8, $[HCO_3^-]$ = 5 mM, $[CH_3COO^-]$ = 20 μM, $[H_2]$ = 1 nM, [gallate/protocatechuate] = 1 μM, T = 298.15 K, and P = 1 atm.

including termite guts (Pester and Brune 2006), mammalian intestines (Miller and Wolin 1995), sewage sludge (Frings et al. 1994), rice field soil (Chin and Conrad 1995), tundra soil (Simankova et al. 2000), plant roots (Leaphart and Lovell 2001, Küsel et al. 1999), and freshwater sediment (Wieringa 1940). To our knowledge the community composition in marine sediments has never been studied. Three major sequence groups have good bootstrap support: (1) a proteobacterial group consisting of several clusters, that has the largest and most diversified number of sequences; (2) a *Desulfotomaculum* cluster with close relatives of

known sulfate-reducing Bacteria; and (3) a deeply branching novel cluster that we call JdF Deeply-Branching Cluster.

Using the published FTHFS primer pair (Lovell and Leaphart 2005) we were able to amplify fhs genes from two samples of the upper sulfate reduction zone, and one in the lower AOM zone (Table 2.4). We also obtained a weak PCR band of the correct length from one depth in the methanogenesis zone (11H-1, 91.7 mbsf). Sequences had *fhs* sequences as their closest BLAST hits, but had too many misreads for reliable phylogenetic placement. With the newly designed *fhs 49F/574R* primer pair, we amplified *fhs* genes from 4 additional horizons, all located in deeper horizons of the upper sulfate reduction zone, with one in the AOM zone (Table 2.4). Diversity detected with the new primer pair was higher: we detected 11 clusters in total, 4 with the FTHFS, and 10 with the *fhs 49F/574R* primer pair. This could be due to the greater number of sequences and horizons from which sequences were obtained with the new primer pair. The example of 1H-2, where more sequences were obtained with the FTHFS primer pair, and yet diversity was higher with *fhs 49F/574R* does not support this conclusion, however (Table 2.4). Higher diversity with *fhs 49F/574R* could also be due to the lower number of PCR cycles necessary for successful amplification, as phylogenetic diversity correlates negatively with number of PCR cycles (von Wintzingerode 1997). It remains open whether the new primer pair performs well elsewhere. The importance of high primer specificity in low biomass environments is, nonetheless, apparent. In the methanogenesis zone and below the lower AOM zone, fhs genes were below detection, although our thermodynamic calculations suggest high *in situ* energy yields for several acetogenic reactions, at least in the methanogenesis zone (Fig. 2.3). Possible explanations

range from PCR detection issues to absence of fh*s* genes to insufficient availability of substrates for acetogens to meet maintenance energy requirements.

The *fhs* gene profile is stratified, with the highest diversity in the surface sediment: 7 of the 11 clusters detected were found in this sample (Table 2.4; Fig. 2.5). The *Desulfotomaculum reducens* Cluster was detected only in surface and sulfate-rich sediments, consistent with the metabolism of *Desulfotomaculum reducens*, a known metal and sulfate reducer. The JdF Deeply-Branching Cluster was found only in lower horizons of the upper sulfate reduction and the upper AOM zone. The *Corynebacterium*-affiliated Cluster was found in one horizon in the upper sulfate reduction zone. Clusters within the Proteobacterial Group vary in distribution: phylotypes related to sulfate reducers (*Desulfovibrio desulfuricans* Cluster, *Desulfomicrobium baculatum* Cluster), as well as three other clusters (*Sphingomonas* Cluster, *Granulibacter*-affiliated Cluster, JdF Proteobacterial Cluster) were found only in the upper sulfate reduction zone. The phylogenetically most diverse cluster, named after *Granulibacter bethesdensis*, and the cluster named after *Methylobacter*, both does the Mixed Proteobacterial Cluster. We did not detect any phylotypes that fall into Cluster A, the only *fhs* sequence cluster known to consist almost entirely of known acetogenic bacterial species (Fig. 2.5).

The fact that all three clusters named after sulfate reducers are restricted to the sulfate reduction zone suggests that sequences may belong to sulfate-reducing microbes. A number of sulfate-reducing bacteria utilize the acetyl CoA pathway in reverse during acetate-oxidizing sulfate reduction (Lee and Zinder 1988, Schauder et al. 1989). However, neither aerobic heterotrophs, do not follow the sulfate or methane profile in distribution, and neither *Desulfovibrio desulfuricans* nor *Desulfomicrobium baculatum* are capable of acetogenesis or

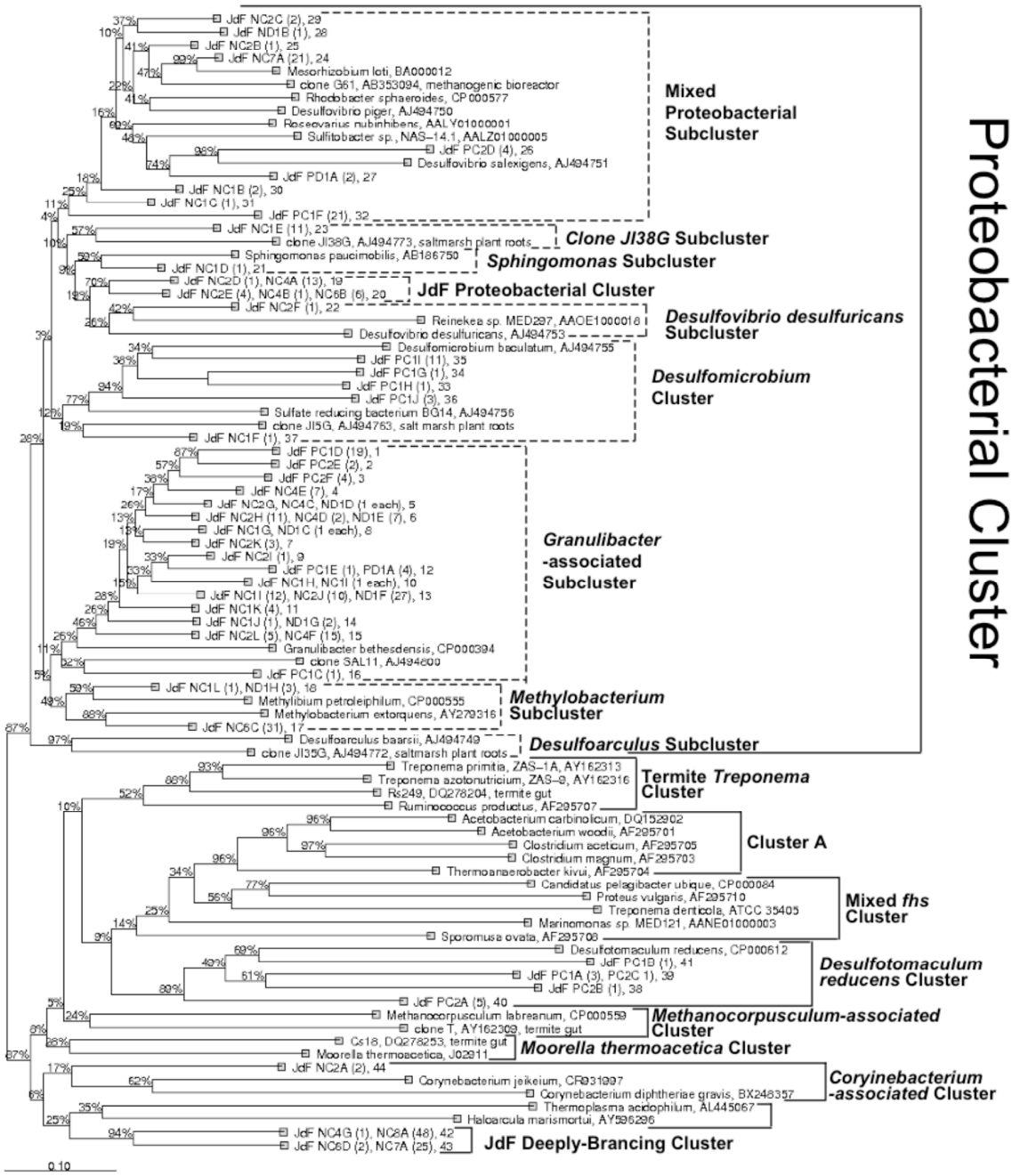


FIGURE 2.5: Phylogenetic tree of *fhs* genes with bootstrap values. Sequences obtained in this study were labeled the following way: 'JdF' for Juan de Fuca, 'N' or 'P' for New or Published primer respectively, 'C' or 'D' for borehole C or D, number for core number in borehole C or D, and A-? for the cluster obtained from that core number and borehole.

acetate oxidation, even though both have a key gene of the acetyl CoA pathway (Postgate and Campbell 1966, Rabus et al. 2006). Similarly, neither *Desulfovibrio salexigens* nor *Desulfovibrio piger*, both in the Mixed Proteobacterial Cluster, are known to oxidize acetate or perform acetogenesis. *Desulfotomaculum reducens* is not acetogenic, but its close relatives *Desulfotomaculum thermobenzoicum* and *D. gibsoniae* are (Tasaki et al. 1992, Kuever et al. 1993). Another member of the genus, *Desulfotomaculum acetoxidans*, is known to oxidize acetate via the acetyl CoA pathway (Spormann and Thauer 1988). Whether *fhs* genes of acetogenic and acetate-oxidizing *Desulfotomaculum* species cluster with *Desulfotomaculum reducens* is uncertain. Other sulfate reducers known to perform acetogenesis are *Desulfoarculus baarsii* (Jansen et al. 1984), *Desulfosporosinus orientis* (Hanselmann et al. 1995), and *Desulfosporomusa polytropa* (Sass et al. 2004; Appendix Table 1), but their fhs genes have not been sequenced . To our knowledge all *fhs* sequences that have so far been obtained from sulfate reducers are included in the phylogenetic tree (Fig. 2.5).

The interpretation of *fhs* phylogeny is constrained by the fact that of the ~100 known acetogen strains, sequences have only been obtained for one fifth, of which most belong to the 'classic' acetogen genera *Acetobacterium*, *Clostridium*, and *Sporomusa*. Sulfate-reducing acetogens and genera with fewer cultured strains are underrepresented. While certain genera, relatedness is not always a good indicator of metabolism, as *Treponema primitia* and *T. azotonutricium* and *T. primitia* demonstrate, where the former is acetogenic and the latter not (Appendix Table 2). The fact that many sequences detected at IODP Site belong to unknown clusters or ones with few cultured representatives highlights the importance of cultivation and continued *fhs* gene sequencing from pure cultures.

TABLE 2.4. Borehole, core identity, depth, likely terminal metabolism, and number of sequences from a given *fhs* cluster from depths analyzed. In parentheses, first the number of sequences obtained with the FTHFS, secondly the number obtained with the *fhs 49F/574R* primer pair. '?' indictates that *fhs* was detected, but there were too many misreads in gene sequences obtained to identify phylogenetic affiliation.

Bore-hole	Core ID	Depth (mbsf)	Likely terminal metabolism	Proteobacterial Cluster								*Desulfotomaculum reducens*	*Coryne-bacterium affiliated*	JdF Deeply-Branching	Clones Total	Clusters Present
				Mixed Proteo-bacterial	Clone JI38G	*Sphingomonas*	JdF Proteobacterial	*Desulfovib. desulfu-ricans*	*Desulfomicrobium baculatum*	*Granulibacter* -affiliated	*Methylo-bacterium*					
C	1H-2	1.5	SR	24 (21, 3)	11 (0, 11)	1 (0, 1)	-	-	17 (16, 1)	42 (21, 21)	1 (0, 1)	4 (4, 0)	-	-	100 (62, 38)	7 (4, 6)
C	2H-3	9.4	SR	7 (4, 3)	-	-	6 (0, 6)	1 (0, 1)	-	37 (6, 31)	-	7 (7, 0)	2 (0, 2)	-	59 (17, 43)	6 (3, 5)
C	4H-5	31.3	SR	-	-	-	14 (0, 14)	-	-	25 (0, 25)	-	-	-	1 (0, 1)	40 (0, 40)	3 (0, 3)
C	6H-6	50.6	SR	-	-	-	6 (0, 6)	-	-	-	31 (0, 31)	-	-	2 (0, 2)	39 (0, 39)	3 (0, 3)
C	7H-2	54.1	SR	21 (0, 21)	-	-	-	-	-	-	-	-	-	25 (0, 25)	46 (0, 46)	2 (0, 2)
C	8H-2	63.6	AOM	-	-	-	-	-	-	-	-	-	-	48 (0, 48)	48 (0, 48)	1 (0, 1)
C	9H-5	78.7	MG	bd	bd	bd	bd	bd	bd	bd	bd	bd	bd	bd	n/a	n/a
C	11H-1	91.7	MG	?	?	?	?	?	?	?	?	?	?	?	n/a	n/a
C	13H-2	111.1	MG	bd	bd	bd	bd	bd	bd	bd	bd	bd	bd	bd	n/a	n/a
D	1H-2	121.5	AOM	3 (0, 3)	-	-	-	-	-	42 (4, 38)	3 (0, 3)	-	-	-	48 (4, 44)	3 (1, 3)
D	3H-2	141.8	SR	bd	bd	bd	bd	bd	bd	bd	bd	bd	bd	bd	n/a	n/a
D	5H-4	162.5	SR	bd	bd	bd	bd	bd	bd	bd	bd	bd	bd	bd	n/a	n/a
C	16H-2	190.2	SR	bd	bd	bd	bd	bd	bd	bd	bd	bd	bd	bd	n/a	n/a
C	18H-3	249.3	SR	bd	bd	bd	bd	bd	bd	bd	bd	bd	bd	bd	n/a	n/a
C	19H-3	258.8	SR	bd	bd	bd	bd	bd	bd	bd	bd	bd	bd	bd	n/a	n/a
C	19H-5	262.9	SR	bd	bd	bd	bd	bd	bd	bd	bd	bd	bd	bd	n/a	n/a
Clones Total:			N/A	55 (25, 30)	11 (0, 11)	1 (0, 1)	26 (0, 26)	1 (0, 1)	17 (16, 1)	146 (31, 115)	35 (0, 35)	11 (11, 0)	2 (0, 2)	76 (0, 76)	379 (83, 296)	11 (4, 10)

Acetogenesis has so far been documented in ~100 bacterial strains, and only one archaeal strain. However, *fhs* has been found in three non-acetogenic Archaea, the methanogen *Methanocorpusculum labreanum* and two facultative organoheterotrophs (*Thermoplasma acidophilum* and *Haloarcula marismortui*; Fig. 2.5; Appendix Table 2). The acetyl CoA pathway is found in all methanogens (4), as well as the archaeal sulfate reducer *Archaeoglobus fulgidus* (Möller-Zinkhan et al. 1989). The archaeal proportion of the active community in the subsurface is often high, sometimes exceeding that of Bacteria (Biddle et al. 2006). Given the lack of information on subsurface acetogens, widespread distribution of the acetyl CoA pathway, and high biomass of Archaea in the subsurface, the existence of more archaeal acetogens appears likely. Two clusters detected at IODP Site U1301 (JdF and *Marinomonas*) form deep branches along with archaeal fhs gene clusters.

CONCLUSIONS

Isotopically light acetate throughout the sediment column at IODP Site U1301 is most easily explained by an acetogenic contribution to acetate production. Under thermodynamically controlled H_2 concentrations, mixo- and methylotrophic acetogenesis reactions are the most plausible sources of light acetate. If H_2 concentrations are not thermodynamically controlled, autotrophic acetogenesis is likely to contribute to light acetate as well.

An overwhelming majority of cultured acetogens can grow mixo- and methylotrophically (Table 2.3), utilizing a wide range of substrates (Liu and Suflita 1993, Prahl et al. 1994, Finke et al. 2007). Calculations suggest that mixo- and methylotrophic acetogenesis reactions from common fermentation products, such as formate, lactate, and methanol, or

from lignin monomers, are energetically favorable in subsurface sediments at IODP Site 1301 (Fig. 2.3, 2.4), even if H_2 concentrations are under thermodynamic control by sulfate reducers or methanogens. With the lignin-derived monomer syringate, *in situ* energy yields may even exceed those of hydrogenotrophic methanogenesis or sulfate reduction, and allow acetogens to successfully compete with methanogens and sulfate reducers for H_2.

Given the probably highly refractory nature of most organic matter in subsurface sediments thousands to millions of years old, extreme energy limitation is likely. Under extreme energy limitation substrate generalists, such as many acetogens, may have an advantage over substrate specialists like methanogens or many sulfate reducers. The ability to pool energy from a wide range of substrates and metabolic pathways may allow acetogens to meet minimum energy requirements, where substrate specialists, like methanogens and many sulfate reducers, are unable despite higher energy yields from certain substrates. Acetogens would not be the first group of organisms to demonstrate the success of the "jack of all trades, master of none" strategy under conditions of extreme resource limitation, as there are similar examples in macroecology (Emlen 1966, Pianka 1994).

We detected mostly novel *fhs* phylotypes that were related to a wide range of aerobic and anaerobic (sulfate-reducing) Proteobacteria, Gram-positive sulfate-reducing Bacteria, and uncultured branches of Bacteria or Archaea. The vertical stratification of *fhs* phylotypes suggests environmental controls on acetogen community composition. The *fhs* sequences from the Juan de Fuca Ridge flank provide molecular evidence for acetogenic organisms and pathways in the subsurface that are more phylogenetically diverse than cultured model strains. In general, the ability to perform acetogenesis may be much more widespread than previously thought: the recently discovered capacity of *Methanosarcina acetivorans* and

Archaeoglobus fulgidus to perform acetogenesis (Rother and Metcalf 2004, Henstra et al. 2007) demonstrates that even among cultured organisms this metabolism has been overlooked.

Our study showcases a new and promising direction in deep subsurface microbiology: functional genes, coding for key reactions of the subsurface carbon cycle, provide a basis to infer microbial community structure and activity in the context of physicochemical habitat characteristics. With a targeted effort to improve existing functional gene assays and to develop new assays, it should become possible to infer microbial processes and community composition in the deep subsurface on a more specific and informative basis than previously possible based on 16S rRNA genes. The combination of functional gene assays, stable isotope signatures, and thermodynamic modeling will significantly advance our knowledge of subsurface microbial ecosystems.

CHAPTER III

DEEP SUBSEAFLOOR METHANE-CYCLING ARCHAEA IN RIDGE FLANK SEDIMENT AND BASALT

ABSTRACT

Ridge flanks cover most of Earth's ocean basins and harbor a microbial subsurface ecosystem of great importance in the global carbon cycle. Yet not much is known about the *in situ* microbial communities involved in carbon cycling and the physicochemical controls structuring these communities. Here, we examine the vertical distribution of methane-cycling microbes at a deep subsurface drilling site located on the eastern flank of the Juan de Fuca Ridge. Methane-producing and anaerobic methane-oxidizing Archaea occur throughout the sediment column and >200 m into the underlying Earth's crust with peak abundances in sulfate-reducing sediments. Sulfate concentrations are a key determinant of community composition, appearing more important than other variables, such as temperature or lithology. The widespread occurrence of methane-metabolizing Archaea suggests an active subseafloor methane cycle that extends throughout sediments and into the Earth's crust.

INTRODUCTION

Though subseafloor sediments and the underlying Earth's Crust are the largest biosphere on Earth, with sediments alone hosting up to one third of living biomass (Whitman et al. 1998), the processes within are poorly understood. Intact cells, 16S rRNA gene sequences

(Roussel et al. 2008), intact membrane lipids (Lipp et al. 2008), and geochemical profiles (ODP Leg 201 Shipboard Scientific Party 2003) of samples obtained by the Ocean Drilling Program (ODP) and Integrated Ocean Drilling Program (IODP) have revealed that microbial life persists even in the deepest sediment columns. Known metabolic groups, e.g. sulfate (SO_4^{2-}) reducing Bacteria or methane (CH_4) producing Archaea (methanogens) occur in addition to many novel groups with unknown physiologies (Biddle et al. 2006, Inagaki et al. 2006). A definite confirmation of life in subseafloor basalt is still missing (Bach and Edwards 2003, Lever et al. 2006), but multiple lines of evidence, such as textural alterations of basalt (Fisk et al. 1998), $\delta^{13}C$ of carbonate, cell counts, and DNA sequences from volcanic glass (Thorseth et al. 2001), and deep boreholes (Cowen et al. 2003, Nakagawa et al. 2006), point towards its widespread occurrence.

Past studies of subseafloor communities have focused mostly on microbial community composition (16S rRNA genes) or microbially driven processes. Links between community composition and microbially driven processes have hardly been established. For instance, even though biological methanogenesis is a widespread process (D'Hondt et al. 2002, Haveman and Pedersen 2002), the organisms involved are largely unknown, as are the factors controlling their community composition. With well-characterized thermal, geochemical, and lithological regimes (ODP Leg 168 Shipboard Scientific Party 1997, Fisher et al. 2005), the eastern flank of the Juan de Fuca Ridge is ideal for the study of controls on the subseafloor CH_4 cycling community. In this study, we obtain an integrated view of the CH_4 cycle at IODP Site U1301 on the Juan de Fuca Ridge Flank by linking functional gene zonation to depth gradients of temperature, lithology, and SO_4^{2-}.

METHODS

Site Description

In summer 2004, Integrated Ocean Drilling Program Expedition 301 drilled the sediment column and upper basement of IODP Site U1301 (Fisher et al. 2005; Appendix Fig. 1). The 265-m thick sediment column is dominated by interbeds of hemipelagic clay and coarser grained sand and silt turbidites, while underlying basalts are primarily composed of pillow and massive lavas that are fractured by veins (Fisher et al. 2005). The temperature increases linearly from the seafloor (2°C) to the basaltic basement (64°C; Fig. 3.1A) and is uniform in the upper basement (Fisher et al. 2005). The occurrence of pyrite at various basement depths indicates the presence of SO_4^{2-} from seawater-derived crustal fluids (~16 mM) in veins throughout (ODP Leg 168 Shipboard Scientific Party 1997). SO_4^{2-} enters the sediment column from overlying seawater and underlying basalt (Fig. 3.1B). Thus, IODP Site U1301 exemplifies the bimodal distribution of seawater-derived oxidants that is a widespread characteristic of the marine deep subsurface[4]; in addition to or combined with geothermal heating, basalt-derived oxidants may foster life in deep sediment layers (Roussel et al. 2008, ODP Leg 201 Shipboard Scientific Party 2003). IODP Site U1301 also illustrates the profound impact of SO_4^{2-} concentrations and SO_4^{2-} reduction on other processes: the zone of net methanogenesis is confined to a SO_4^{2-}-depleted depth interval (~70-115 mbsf; Fig 3.1C) that is demarcated by SO_4^{2-}-CH_4-transition zones, where CH_4 is presumably consumed via anaerobic oxidation of CH_4 (AOM) coupled to SO_4^{2-} reduction (Fig. 3.1C). Outside the net methanogenesis and AOM zones (0-60 and 135-265 mbsf), CH_4 concentrations are 2-3 orders of magnitude lower (immediately measured: 0.8-4.1 μM, after 5-week incubation: 1-30 μM; Fig. 3.1C insert).

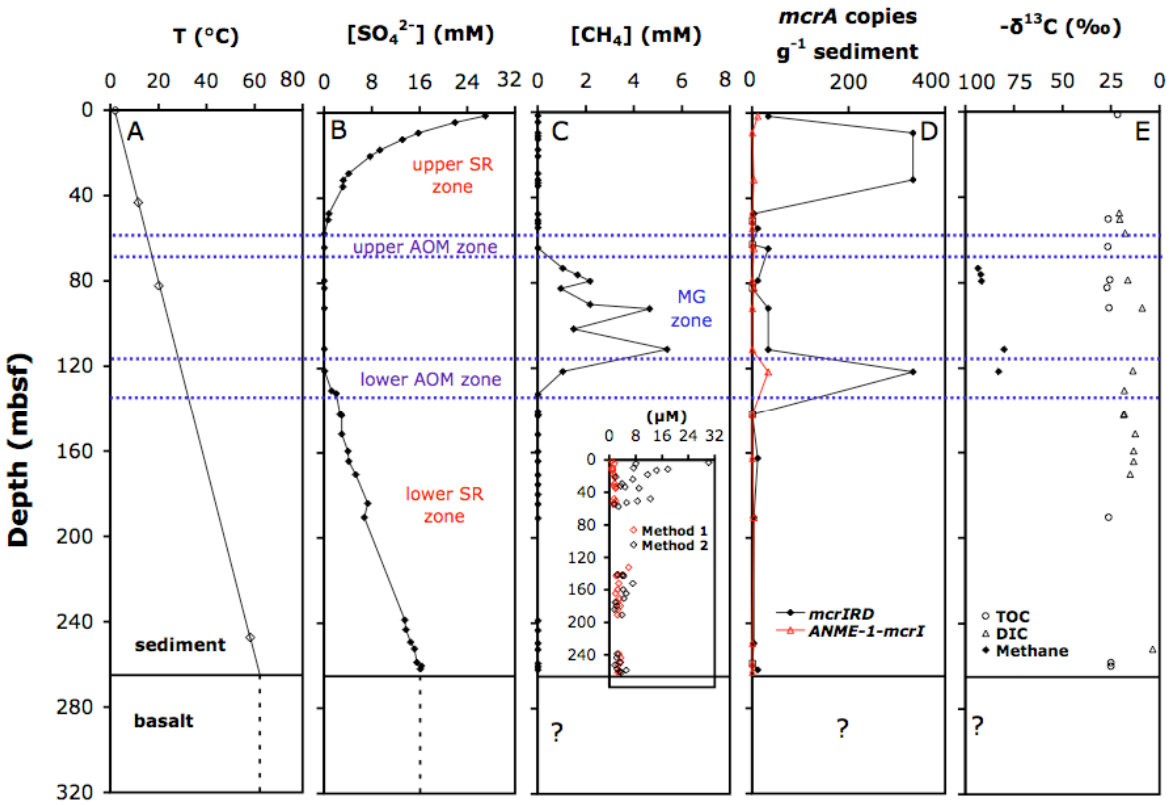


FIGURE 3.1: Depth profiles at IODP Site U1301: (A) temperature (°C), (B) measured SO_4^{2-} concentrations (SR = SO_4^{2-} reduction), (C) measured porewater CH_4 concentrations (MG = methanogenesis); insert: concentrations of CH_4 in SO_4^{2-} reduction zones (Method 1 = measured immediately, Method 2 = measured after 5-week incubation), (D) depth profile of *mcrA* copy numbers based on PCR with dilution series of DNA extracts using the *mcrIRD* and *ANME-1-mcrA* primer pairs, (E) $\delta^{13}C$ of total organic carbon (TOC), dissolved inorganic carbon (DIC), and CH_4.

Sample collection

Sediment cores were obtained by Advanced Piston Corer, whereas basalt cores were sampled using a Rotary Core Barrel (RCB; Fisher et al. 2005). Samples for onboard and shore-based gas analyses were taken on the catwalk immediately after core retrieval. Interstitial water samples for analyses of $\delta^{13}C$-DIC were obtained as described previously (Fisher et al. 2005). Sediment cores used for molecular biological analyses were immediately sectioned and frozen at -80°C. Basalt cores used for molecular biological

analyses were decontaminated (Lever et al. 2006), cracked, and shards from the interior frozen at -80°C.

Quantification of [CH_4] and δ^{13}C-CH_4 in Sediment

On-board analyses of dissolved CH_4 concentrations were performed as described previously (Fisher et al. 2005). For shorebased δ^{13}C-analyses of dissolved CH_4, 3-mL subcores were placed into 20-mL glass serum vials containing 5 mL of 1 N NaOH, sealed with rubber stoppers and crimped immediately, shaken for 20 min to form a homogenous slurry, and frozen for shore-based analyses. After δ^{13}C-CH_4 analyses, headspaces of these vials were flushed completely, and slurries incubated at room temperature for 5 weeks to release and quantify remaining CH_4 (Hinrichs et al. 2006). We do not know if CH_4 released during incubation was dissolved but trapped in fine porespaces or sorbed under *in situ* conditions. Hence, we do know which measured concentration is more relevant to methanogenesis and AOM. See Supplementary Methods for more details on the quantification of [CH_4] and δ^{13}C-CH_4.

Determination of δ^{13}C-DIC and –TOC in Sediment

The δ^{13}C-DIC was analyzed using a gasbench coupled to a Finnigan MAT irm-MS. 0.7- to 1.0-mL samples of interstitial water were acidified with 100 µL of phosphoric acid in glass tubes that had been sealed with butyl septa and plastic caps and purged 5 times with helium. Samples were allowed to degas CO_2 for 5+ hours, before gas phases were analyzed for δ^{13}C-CO_2. The analytical precision was 0.1‰ (1 σ).

For δ^{13}C-TOC analyses, 5-mL aliquots were freeze-dried on board and stored at room temperature. The freeze-dried sediment was ground and homogenized in a mortar. Subsamples (1.5 g) were decalcified with 6N HCl, dried at 60°C overnight, and δ^{13}C-TOC analyses performed on 100-mg subsamples using a dual-inlet mass spectrometer (Finnigan MAT Delta E) with a precision of 0.1‰ (1 σ). Accuracy was checked with an internal lab standard.

DNA Extraction

Aliquots from core centers (6 g) were homogenized in 50-mL Falcon tubes with artificial seawater (10 mL; 3% NaCl, 0.3% MgSO4, 0.2% KCl + 0.312 g NaPh (20 mM)), incubated (room temperature, 2 hrs), and extracellular DNA removed by centrifugation (20 min, 4,000×g). Sediment pellets, containing intact cells, were kept. A modification of the ISOIL Large for Beads kit (Nippon Gene, Tokyo, Japan) was used to extract DNA. Beads, 9.5 mL Lysis Solution BB, and 0.5 mL Lysis Solution 20 S were added to pellets, slurried by vortexing, and shaken to break cells (30s, room temperature), using a SPEX 6850 freezer/mill (SPEX SamplePrep, Metuchen, New Jersey). PCR-inhibitory proteins were removed by proteinase K addition (500 μg mL^{-1}) and gentle rotation on a shaker table (50°C, 2 hrs). Proteinase was denatured (65°C, 1 hr) and the manufacturer's protocol followed afterwards. DNA was purified and concentrated with the (1) Amicon Ultra-15 10K, (2) Montage PCR Cleanup kits (both Millipore Corporation, Billerica, USA), and (3) Mag Extractor – PCR & Gel Clean Up kit (Toyobo, Tokyo, Japan).

PCR Amplification

McrA genes were below detection with published *mcrI* (Springer et al. 1995) and *ME1/ME2* (Hales et al. 1996) primers, but amplified successfully with the *mcrIRD* primer pair (F: 5'-TWYGACCARATMTGGYT; R: 5'-ACRTTCATBGCRTARTT), a revision of the *mcrI* primer pair (Springer et al. 1995) with fewer degeneracies but the same phylogenetic breadth. Since neither the *mcrIRD* nor the *mcrI* primer pairs target ANME-1 *mcrA* sequences well, we designed the group-specific *ANME-1-mcrI* primer pair (F: 5'-GACCAGTTGTGGTTCGGAAC; R: 5'- ATCTCGAATGGCATTCCCTC). Primers were designed using a newly constructed *mcrA* alignment (Lever, unpubl.) in the phylogenetic software program ARB (Ludwig et al. 2004). The PCR protocol consisted of (1) 1 × 2 min denaturation (98°C), (2) 40 × (a) 30s denaturation (95°C), (b) 30s annealing (*mcrIRD*: 55°C; *ANME-1-mcrI*: 63°C), (c) 1 min extension (72°C), and (3) 1×5 min extension (72°C). PCRs were performed using Veriti model thermal cyclers (Applied Biosystems, Foster City, USA).

Cloning and Sequencing

PCR products were purified with the Montage PCR Cleanup kit (Millipore Corp., Billerica, USA), and cloned and inserted into chemically competent *E. coli* using the Topo TA Kit (Invitrogen, Carlsbad, USA).

RESULTS AND DISCUSSION

We extracted DNA and PCR-amplified the gene for the alpha subunit of methyl coenzyme M reductase (*mcrA*), a highly conserved, phylogenetically informative gene unique to methanogens and anaerobic methanotrophs (Friedrich et al. 2005), using samples that had been monitored for drilling fluid (surface seawater) contamination (Lever et al.

2006). Since PCR assays using published *mcrA* primers (Springer et al. 1995, Hales et al. 2006) yielded negative results, we attempted amplification with two newly designed primer pairs with enhanced specificity. The new primer pairs allowed detection of *mcrA* genes in both sediment and basalt. In sediment, PCR with serially diluted DNA extracts demonstrated two peaks in *mcrA* copy number, one near the sediment surface (9.4-31.3 mbsf) and one in the lower AOM zone (121.5 msf; Fig. 3.1D). *McrA* genes occurred in low copy numbers in (1) deeper layers of the upper SO_4^{2-} reduction zone (50.6-54.1 mbsf), (2) the upper AOM zone (61.6-63.6 mbsf), and (3) throughout the lower SO_4^{2-} reduction zone (141.8-261.8 mbsf; Fig. 3.1D). A minor increase in copy numbers occurred in the methanogenesis zone (78.7-111.1 mbsf; Fig. 3.1D). The molecular record of *mcrA* genes at U1301 is by no means limited to sediments, but extends into the underlying basement, where we detected *mcrA* genes in the upper 100-210m (Table 3.1).

Overall diversity is highest among the *Methanosarcinales-Methanomicrobiales*, with 6 out of 8 *mcrA* clusters falling into this order (Fig. 3.2). Out of the 8 clusters detected, 2, the uncultivated Unidentified Rice Field Soil McrA (URFS) group and the anaerobic methanotroph ANME-1, are found throughout the sediment column, independent of temperature, presence of SO_4^{2-}, or lithology (Table 3.1). The URFS and ANME-1 lineages are not only found in sediment, but also at several depths in SO_4^{2-} rich veins in basement basalt. The remaining 6 clusters only occur in the methanogenesis and AOM zones, i.e. in the sediment column in horizons with very low SO_4^{2-} concentrations. Relatives of obligately aceticlastic *Methanosaeta* are found in a single depth horizon in the

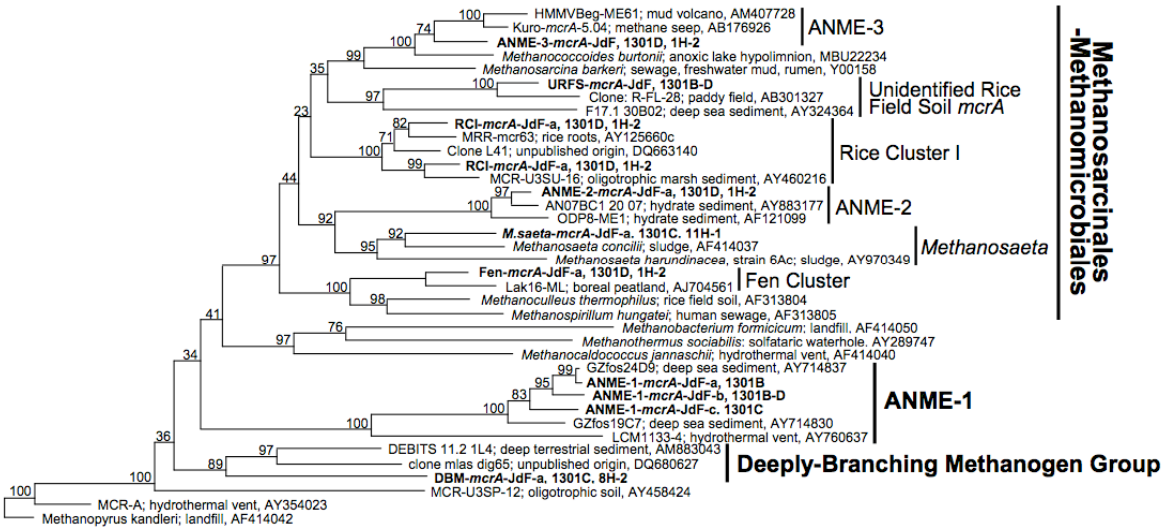


FIGURE 3.2: *McrA* gene phylogeny at IODP Site 1301. Phylotypes from IODP Site U1301 are in bold type face. Constructed in ARB Neighbor Joining using Jukes-Cantor correction[49]. Bootstrap support (in %, 1,000 replications) is indicated at each branching point.

methanogenesis zone (Table 3.1). An uncharacterized monophyletic group, which we call Deeply-Branching Methanogen Group (DBM), is found at only one depth in the upper AOM zone. The lower AOM zone at 121.5 mbsf has the highest overall *mcrA* diversity of all depth horizons sampled, with 6 out of 8 lineages (Table 3.1). Four groups, the obligately hydrogenotrophic Rice Cluster I (RCI), the anaerobic methanotrophs ANME-2 and ANME-3, and Fen Cluster (Fen), a group related to hydrogenotrophic Methanomicrobiaceae (Fig. 3.2, Table 3.1), only occur here. The *mcrA* community profile therefore highlights the importance of SO_4^{2-}-CH_4-transition zones as population density and diversity hot spots in the deep subsurface (Parkes et al. 2005). The presence/absence of certain methanogen groups in the presence of SO_4^{2-} is likely to result from the ability of SO_4^{2-} reducers to outcompete methanogens for the same substrates.

TABLE 3.1: Overview of origins, depths, calculated *in situ* temperatures, lithologies, zones of terminal metabolism, phylogenetic groups , number of clones, and minimum *mcrA* copy numbers per gram sample.

Borehole	Core ID[1]	Depth (mbsf)	T (°C)	Lithology[2]	Terminal metabolism	clone library composition (# of clones)		*mcrA* copy # g^{-1} [5]
						mcrIRD[3]	*ANME-1-mcrI*[4]	
C	1H-2	1.5	2.2	clay	SR	URFS (4)	43	44
C	2H-3	9.4	4.1	clay	SR	URFS (37)	bd	333
C	4H-5	31.3	9.3	fine sand	SR	URFS (40)	45	337
C	6H-3	47.2	13.1	sand	SR	URFS (44)	bd	3
C	6H-6	50.6	13.9	clay	SR	bd	bd	0
C	7H-2	54.1	14.7	clay	SR	URFS (73)	bd	11
C	7H-7	61.6	16.5	fine sand	AOM	bd	bd	0
C	8H-2	63.6	17.0	fine sand	AOM	URFS (44)	45	37
C	9H-5	78.7	20.5	clay	MG	URFS (14)	bd	11
C	10H-1	82.2	21.4	sand	MG	bd	29 (+2 URFS)	0
C	11H-1	91.7	23.6	fine sand	MG	URFS (48); M.saeta (36)	bd	33
C	13H-2	111.1	28.2	clay	MG	URFS (44)	bd	33
D	1H-2	121.5	30.7	clay	AOM	URFS (6); ANME-2 (13); ANME-3 (14); Fen (9); RCI (3)	43	367
D	3H-2	141.8	35.5	silty clay	SR	bd	bd	0
D	5H-4	162.5	40.4	fine sand	SR	bd	bd	11
C	16H-2	190.2	47.0	clay	SR	bd	DBM	7
C	18H-3	249.3	61.0	clay	SR	URFS (38)	bd	3
C	19H-3	258.8	63.3	clay	SR	bd	bd	0
C	19H-5	262.9	64.3	clay	SR	URFS (33)	bd	11
B	1R-1	351.2	~65	breccia, glass	SR	URFS (47)	44	nd
B	2R-2	358.8	~65	pillow basalt	SR	URFS (45)	bd	nd
B	4R-3	369.4	~65	pillow basalt	SR	bd	bd	nd
B	5R-2	378.1	~65	pillow	SR	bd	bd	nd

				basalt				
B	6R-2	386.3	~65	pillow basalt	SR	URFS (44)	bd	nd
B	14R-1	434.3	~65	massive basalt	SR	bd	37	nd
B	17R-1	461.7	~65	pillow basalt	SR	URFS (46)	47	nd
B	20R-1	480.8	~65	pillow basalt	SR	bd	bd	nd
B	23R-1	500.2	~65	pillow basalt	SR	bd	bd	nd
B	26R-1	515.9	~65	pillow basalt	SR	bd	bd	nd

[1] the code is as follows: 1H-2 = core 1, section 2, sampled by Advanced Piston Core (H); 1R-1 = core 1, section 1, sampled by Rotary Core Barrel (R)
[2] sediment data from Lever et al. (2006)[8], basalt data from Fisher et al. (2005)[16]
[3] generated with *mcrIRD* primer pair
[4] generated with *ANME-1-mcrI* primer pair
[5] minimum numbers; could only be calculated for sediments.

URFS dominate the methanogenesis zone, where they occur in all 4 horizons examined (Table 3.1). Isotopically light δ^{13}C-CH_4 (-80 to -93.5‰) suggests that URFS might be producing CH_4 from H_2/HCO_3^- (Fig. 3.1E; Whiticar et al. 1986). On the other hand, the URFS group also occurs in the presence of high SO_4^{2-}-concentrations. The ability to utilize non-competitive substrates, i.e. compounds not used by most SO_4^{2-} reducers, such as methanol, methyl amines or methyl sulfides, is a plausible explanation. Precedents for such metabolic diversity in methanogens exist: methylated substrates and H_2/HCO_3^- are both used as methanogenic carbon sources by members of the genus *Methanosarcina* (Whitman et al. 2006).

ANME-1 is not only present in the lower AOM zone, but also in both SO_4^{2-} reduction zones, including SO_4^{2-}-rich basalt veins, and in the methanogenesis zone (Table 3.1). Though unexpected, their occurrence in both SO_4^{2-} reduction zones is consistent with high

SO_4^{2-} (Fig. 3.1B) and micromolar CH_4 concentrations (Fig. 3.1C insert), conditions that may favor AOM outside SO_4^{2-}-CH_4-transition zones. The finding of methanotrophic ANME-1 at one depth in the methanogenesis zone is consistent with the recent suggestion that ANME-1 can survive as facultative methanogens (Orcutt et al. 2005).

Members of known hydrogenotrophic methanogens (Rice Cluster I) and putatively methanogenic clusters with no cultured representatives (Fen Cluster, DBM) occur in the lower AOM zone. These microbes could be (1) (facultatively) methanotrophic, (2) methanogenic, using a substrate different from the product(s) of AOM, (3) performing a metabolism not involving CH_4 (e.g. anaerobic acetate oxidation), or (4) surviving cells from a time when the methanogenesis zone extended deeper and included sediment horizons within the present AOM zone.

An unexpected outcome of this investigation is the wide temperature range (2-64°C) at which URFS and ANME-1 *mcrA* genes occur. This temperature range surpasses that of any cultured microorganism, the current record holder being a *Methanothermobacter thermoautotrophicus*-like strain with a growth temperature range of 55°C (Wagner and Wiegel 2008). Similarly, ANME-2 and ANME-3 Archaea were recently detected in hot, deep sediments with a temperature range of 50-70°C[2], in contrast to the cold surficial and CH_4 seep sediments where they were previously found (Orphan et al. 2001, Lösekann et al. 2007). A recent study has shown maximum growth temperature of the methanogen *Methanopyrus kandleri* to increase under high pressure, from 110 to 122°C (Takai et al. 2008). Hence, tolerance to the wide temperature range at which URFS and ANME-1 occur at IODP Site U1301 might be facilitated by high pressure.

The habitat of URFS and ANME-1 spans across a wide range of lithologies, from clay and sand to basaltic breccia, pillow and massive lava (Table 3.1), suggesting versatility in surface attachment or unattached growth. The depth distribution raises more general questions about origins and mechanisms of dispersal of subsurface methanogens. Advective transport is possible through basalt, but does not occur vertically through sediment at Site U1301 (Fisher et al. 2005). Cells may have first reached and colonized exposed basement basalt via seawater transport, and expanded their range into sediment, once sediment started accumulating at IODP Site U1301, roughly 1.7 mya (ODP Leg 168 Shipboard Scientific Party 1997). Alternatively, cells, e.g. from anaerobic Metazoan intestines or deposited via turbidites, may have colonized sediment from the surface down. Dispersal into deeply buried sediments could be slowed or hindered by small pore space resulting from sediment compaction, in which case early colonization by URFS and ANME-1, before or soon after sediment started accumulating, would be more likely than a more recent invasion.

CONCLUSIONS

Ridge flank environments may extend over >50% of global ocean basin area (Walker et al. 2008). Biogeochemical cycles in ridge flank sediments and underlying basalt are of global significance and a thorough grasp thereof is required to understand the global C cycle. The vast potential for redox reactions between seawater and basalt to support microbial metabolism (Bach and Edwards 2003) is backed by cell abundances on exposed seafloor basalt that exceed those in overlying seawater by 3-4 orders of magnitude (Santelli et al. 2008). The detection of *mcrA* genes deep into 3.6-million-year-old subseafloor basalt underscores the vast spatial and temporal scale over which these reactions may occur.

Seawater-derived compounds not only stimulate life in the basaltic aquifer, however: electron acceptors from basement fluids control microbial community zonation in deep sediments in a manner similar to surface sediments.

CHAPTER IV

ZONATION OF THE ACTIVE METHANE-CYCLING COMMUNITY IN DEEP SUBSURFACE SEDIMENTS OF THE PERU TRENCH

ABSTRACT

In past surveys of Archaea in subsurface sediments of the Peru Margin, few, if any, methane producers or oxidizers were detected, even in sediment with biologically produced methane hydrate. Here we show that non-detection of 16S rRNA and methyl coenzyme M reductase genes was related to deficiencies in primers used in polymerase chain reactions (PCR). Novel primers with enhanced specificity demonstrate that anaerobic methanotrophs occur throughout the upper 20 m in hydrate-bearing sediment of the Peru Trench and that methanogens occur throughout the underlying methanogenesis zone. The distribution of methanogens and anaerobic methanotrophs reflects the $\delta^{13}C$ profile of dissolved inorganic carbon, but not the concentration profiles of methane and sulfate. Differences of -80‰ between $\delta^{13}C$ of dissolved inorganic carbon and methane suggest methanogenesis predominantly from H_2/CO_2. Calculated free energy yields of methanogenesis from H_2/CO_2, formate, and acetate suggest extreme energy limitation, and illustrate the importance of accurate determination of *in situ* concentrations of substrates and products for a reliable interpretation of microbial ecosystem functioning in the deep subsurface.

INTRODUCTION

The Peru-Chile Margin has been the subject of numerous studies on the interplay of plate tectonics and global oceanic circulation (Shipboard Scientific Party 1988). Subduction of the Nazca Plate under the South American Plate produces an accretionary wedge close to a coastal upwelling region with high rates of primary production and sedimentation. The tectonic history of the edge of the subducted plate is recorded in the sediment column, as are past variations in the upwelling regime resulting from changes in wind patterns, ocean circulation, and sea level (von Huene et al. 1986 and references within). The Peru Continental Margin therefore provides an excellent study area for the examination of plate movements and climate change since the Miocene (e.g. Walsh 1975, Suess et al. 1981, von Huene et al. 1985).

First investigations of the microbiota in sediments of the Peru Continental Margin were performed as part of Ocean Drilling Program (ODP) Leg 112 (Cragg et al. 1990). This study documented active microbial populations in sediment layers to 80 meters below seafloor (mbsf), and was the first demonstration of a marine deep subsurface biosphere. Multiple lines of evidence, including intact cells (Parkes et al. 2008), 16S ribosomal RNA (rRNA) sequences (Inagaki et al. 2006), intact membrane lipids (Lipp et al. 2008), and porewater geochemical profiles (D'Hondt et al. 2004), have since shown that metabolically active microbes persist to at least 1,500 mbsf (Parkes et al. 2008). In addition to groups with cultured representatives, evidence for a large number of uncharacterized groups has been found in the marine deep subsurface (e.g., Li et al. 1999, Reed et al. 2002, Inagaki et al. 2006), many with close relatives in surficial marine sediments (e.g. Vetriani et al. 1999, Takai and Horikoshi 1999).

The first ODP expedition to focus on microbiology, ODP Leg 201, took place in 2002 (D'Hondt et al. 2003). The main destination, with five of the seven sampling sites, was the Peru Continental Margin. A key cruise objective was the study of controls on microbial communities in deeply buried sediments. Since then, the microbial community composition of several sites has been characterized via PCR assays of 16S rRNA genes (Sørensen et al. 2004, Parkes et al. 2005, Inagaki et al. 2006, Webster et al. 2006) and 16S rRNA (Sørensen and Teske 2005, Biddle et al. 2006), fluorescence-*in-situ*-hybridization (Mauclaire et al. 2004, Schippers et al. 2004), and metagenomic signatures of whole-genome amplified DNA (Biddle et al. 2008). In addition, genes diagnostic of microbial metabolisms have been targeted at two sites (Parkes et al. 2005, Webster et al. 2006): the gene for dissimilatory sulfite reductase (*dsrAB*), a key enzyme of dissimilatory sulfate reduction (Wagner et al. 2005), and the gene for the α subunit of methyl coenzyme M reductase (*mcrA*), an enzyme that catalyzes the terminal step of biological methanogenesis and is also present in anaerobic methanotrophs (Friedrich et al. 2005, Hallam et al. 2003). These functional genes reveal metabolic capacity in addition to identity and can provide insights on activities of microbes that lack close cultured relatives. Moreover, minority populations within a given functional group may be more easily detected.

In the following study, we examine the *in situ* community of methanogens and anaerobic methanotrophs in the sediment column of Ocean Drilling Program (ODP) Site 1230 in the Peru Trench via PCR assays of *mcrA*. We investigate the relationship between community zonation and environmental gradients (temperature, sulfate and substrate concentration, lithostratigraphy). To identify members of the methanogen and anaerobic methanotroph community most likely to be living and active, we complement PCR assays of *mcrA* with

reverse transcription-PCR (RT-PCR) of 16S rRNA and *mcrA* mRNA. Using δ^{13}C-CH_4 and calculated *in situ* energy yields of several common methanogenesis and methanotrophy reactions we constrain the likelihood of these reactions and link them to the members of the methanogen and methanotroph community that we detect *in situ*.

METHODS

Field Site

The Peru Trench is part of the larger Atacama Trench that is located between the continental South American Plate and the accretionary wedge of the oceanic Nazca Plate. It stretches parallel to the west coast of South America for 5,900 km. It is unique for a deep sea trench in that it combines slope deposits of mostly diatomaceous mud mixed with sediments from an accretionary wedge, high sedimentation rates, high organic carbon content in the sediment (up to 8%; Suess et al. 1988), and exceptionally high microbial activity in the sediment column (D'Hondt et al. 2004). ODP Site 1230 is located on the lower slope of the Peru Trench at a water depth of 5,086 m (D'Hondt et al. 2004, Appendix Fig. 2). Its sediment column was drilled to ~270 mbsf during ODP Leg 201 in 2002 (D'Hondt et al. 2003). Sediments can be divided into two intervals that are separated by a lithostratigraphic break of 4.5 million years (~200 mbsf): Unit I consisting of clay-rich diatomaceous mud from the Pleistocene to Holocene (0-200 mbsf), and Unit II below the break (~200+ mbsf) consisting of Miocene diatomaceous ooze (Suess et al. 1988, D'Hondt et al. 2003). Sediments are uniformly cold, increasing slightly in temperature from ~2°C at the seafloor to 12°C at 270 mbsf. The geological and geophysical properties of the location were documented in detail at nearby ODP Site 685 during ODP Leg 112 (Suess et al. 1988).

The sediment organic carbon content is high, averaging over 2% even in deep sediment horizons (Meister et al. 2005). The highest alkalinity value ever measured in deep-sea sediments (160 mM), a sulfate-methane-transition-zone (SMTZ) from ~6 to 11 mbsf, and sulfate depletion at ~11 mbsf indicate unusually high biological activity for a deep-sea site (Fig. 2). CH_4 concentrations reach saturation by 28 mbsf (Spivack et al. 2005). Hydrates begin to form at ≤50 mbsf, and are present to at least 200 mbsf (D'Hondt et al. 2003).

Sampling

The upper ~210 m were sampled with an Advanced Piston Corer (APC; D'Hondt et al. 2003), by which a drill is "advanced" to directly above the depth interval of interest, and then a 9.5-m piston core hydraulically thrust into underlying sediments (Graber 2002). Below 210 m, the sediment was too firm for piston coring and was obtained by drilling with an Extended Core Barrel (XCB; Graber et al. 2002). APC sampling is preferable for microbiological analyses due to on average lower contamination of core interiors with drilling fluid; the interior of XCB samples from ODP Site 1230 showed remarkably low levels of contamination, however, and were suitable for microbiological studies (House et al. 2003). Three boreholes were drilled within close proximity to another (~20 m; D'Hondt et al. 2003).

Porewater samples for geochemical analyses were obtained from 20-40 cm long whole-round intervals using a hydraulic press as described previously (Fisher et al. 2005). For carbon isotope analyses, 5-mL subsamples were frozen in precombusted glass vials.

For molecular biological analyses, 5-cm whole-round intervals of cores were frozen at -80°C. Only sediment from the nearly contamination-free interior was used (House et al. 2003, Lever et al. 2006).

Porewater geochemical gradients

Depth profiles of alkalinity, sulfate, CH_4, H_2, formate and acetate (Fig. 4.1A-E) were measured as described previously (D'Hondt et al. 2003). Due to outgassing during core retrieval, CH_4 concentrations measured in porewater from sediments below 15-20 mbsf are underestimates of *in situ* concentrations. Since CH_4 concentrations were near saturation by

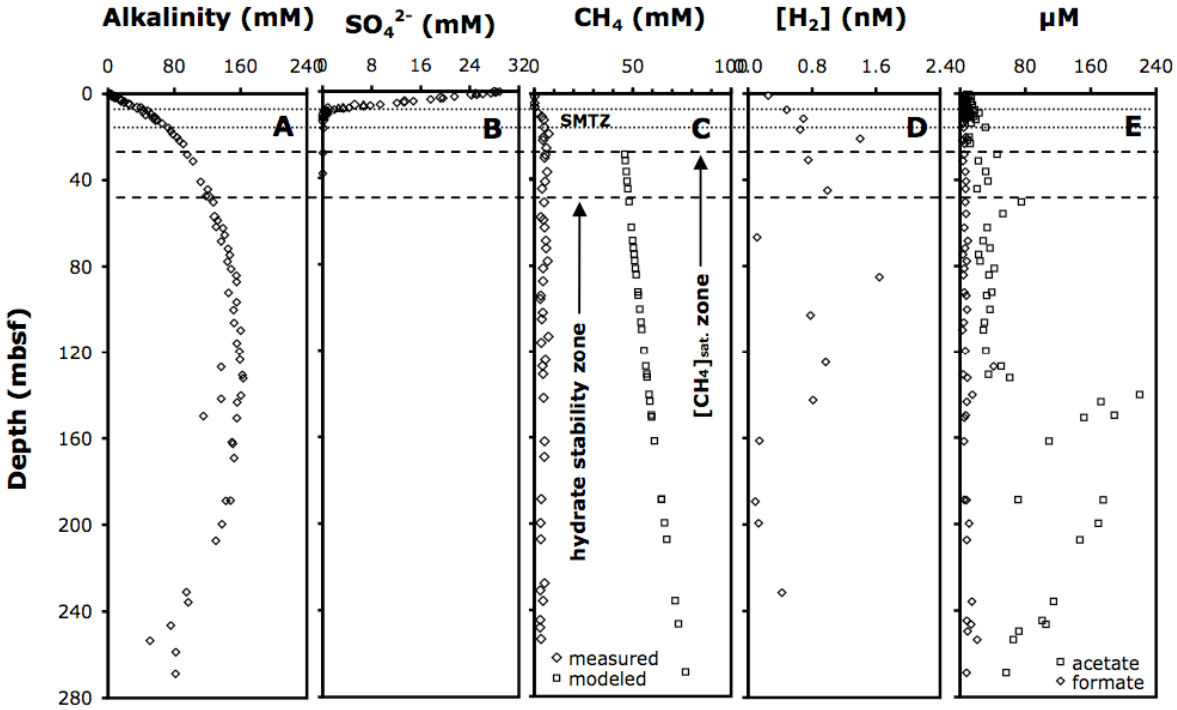


FIGURE 4.1: Relevant porewater concentration profiles at ODP Site 1230: (A) alkalinity, (B) sulfate, (C) measured methane concentrations, modeled methane saturation concentration, and extent of the hydrate stability (D'Hondt et al. 2003) and methane saturation (Spivack et al. 2005) zones, (D) dihydrogen, and (E) formate and acetate. All data except modeled methane concentrations from D'Hondt et al. (2003).

28 mbsf (Spivack et al. 2005), we calculated saturating CH_4 concentrations under *in situ* temperature, pressure, salinity, and pore size using the model for CH_4-seawater-porous media at H-L-equilibrium of Sun and Duan (2007), and used these concentrations in all

thermodynamic calculations. Due to the absence of published data, we used a uniform pore size of 1.0 μM, a value likely to overestimate *in situ* pore size in the highly compacted clay sediments at ODP Site 1230. Since a smaller pore size would require higher CH_4 concentrations for saturation to occur, our model calculations are probably slight overestimates of *in situ* CH_4 concentrations.

$\delta^{13}C$-C_1 and DIC

All ^{13}C-C_1 (~99% ^{13}C-CH_4, ~1% other hydrocarbon gases, mostly ethane and propane) and -DIC were measured as described in D'Hondt et al. (2003).

DNA/RNA Extraction

RNA was extracted as in Biddle et al. (2006), except that the extraction buffer was supplemented with 120 mM sodium phosphate, in addition to 250 mM sodium acetate and 50 mM ethylenediaminetetraacetic acid. DNA was extracted following the same protocol as for RNA, except that the pH of the extraction buffer and phenol was raised to 8.0, the bead beating time reduced to 15s, and the bead beating speed reduced to 4.0 (Qbiogene, Carlsbad, CA). Moreover, the DNase incubation was omitted, and DNA purified with the PowerClean DNA Clean-Up Kit (MOBIO laboratories, Carlsbad, CA) instead of the RNeasy Mini Kit (Qiagen, Valencia, CA).

PCR Amplification

To target all *mcrA* genes we used previously designed mcrIRD and ANME-1-mcrI primer pairs (Lever et al., in prep). Two previously published general *mcrA* primer pairs yielded no

amplification (ME1/ME2; Hales et al. 1996), or amplification at only one depth interval (44.3 mbsf; mcrI; Springer et al. 1995). Several new group-specific *mcrA* and 16S rRNA primers were designed using gene alignments (Lever, unpubl.) constructed in the phylogenetic software program ARB (Ludwig et al. 2003; Table 4.1). PCR assays were performed using the Takara SpeedSTAR HS DNA polymerase kit (TaKaRa Bio USA, Madison, WI) using the following PCR protocol: (1) 1 × 2 min denaturation (98°C), (2) 40 × (a) 10s denaturation (98°C), (b) 30s annealing (Table 8 for temperatures), (c) 1 min extension (72°C), and (3) 1 × 5 min extension (72°C).

To examine the distribution of the active and living methanogen community, we also attempted RT-PCR of *mcrA*-mRNA and 16S rRNA in several depth horizons using new group-specific primers for maximum amplification efficiency and hence sensitivity (mRNA: ODP1230, ANME-1; 16S rRNA: Msaeta 268F/927R, ANME-1 42F/898R; ANMG-1 35F/1038R). RT-PCR assays were carried out using TaKaRa RNA PCR Kits (AMV) Version 3.0 (TaKaRa Bio USA, Madison, WI). The following RT-PCR protocol was used: (1) 1 × 15 min reverse transcription, (2) 5 min denaturation (98°C), (2) 40 × (a) 30s denaturation (98°C), (b) 30s annealing (Table 7 for temperatures), (c) 1 min extension (72°C), and (3) 1×5 min extension (72°C).

Negative controls for mRNA and rRNA transcription, cDNA-PCR, and DNA-PCR were included, in addition to reaction blanks. All negative controls were negative.

Gene	Primer pair	Nucleotide sequences	Ref.	Target organisms	Annealing T (°C)
mcrA	mcrI	F: 5' TAYGAYCARATHTGGYT; R: 5' ACRTTCATNGCRTARTT	Springer et al. (1995)	All mcrA	51.0
mcrA	ME1/ME2	F: 5' GCMATGCARATHGGWATGTC; R: 5' TCATKGCRTAGTTDGGRTAGT	Hales et al. (1996)	All mcrA	58.0
mcrA	*mcrIRD*	F: 5' TWYGACCARATMTGGYT; R: 5' ACRTTCATBGCRTARTT	Lever et al., in prep	All mcrA except ANME-1	55.0
mcrA	*ANME-1-mcrI*	F: 5' GACCAGTTGTGGTTCGGAAC; R: 5' ATCTCGAATGGCATTCCCTC	Lever et al., in prep	ANME-1 and ANMG-1	63.0
mcrA	ODP1230-mcrI	F: 5' GCTACATGTCCGGTGG; R: 5' CGGATAGTTGGGTCCTCT	This study	ODP 1230 M. saeta	59.0
16S	M.saeta 268F/927R	F: 5' CCTACTAGCCTACGACGGGT; R: 5' CCCGCCAATTCCTTTAAGTTT	This study	All *Methanosaeta*	63.0
16S	ANME-1 42F/898R	F: 5' GAGTTCGATTAAGCCATGTTAGT; R: 5' CGACCGTACTCCCCAGAT	This study	ANME-1	61.0
16S	ANMG-1 35F/1038R	F: 5' GCTATCAGCGTCCGACTAAGC; R: 5' TAATCCGGCAGGGTCTTCA	This study	ANMG-1	65.0

TABLE 4.1: Overview of PCR primer pairs used with ODP Site 1230 DNA extracts.

Cloning and Sequencing

PCR products were purified in a 2.5 % low-melting point agarose gel using 1 × Tris acetate - EDTA buffer (TAE). Gel slices containing PCR fragments of the correct length were excised and purified using a S.N.A.P. Mini Kit (Invitrogen, Carlsbad, USA). Purified PCR products were cloned using the Topo TA Kit (Invitrogen, Carlsbad, USA) and transformed into TOP10 electrocompetent cells following the manufacturer's instructions. Plasmid extraction, purification and cycle sequencing were performed at the Josephine Bay Paul Center at MBL (Woods Hole, MA). Sequences were BLAST analyzed using the nucleotide collection in GenBank (www.ncbi.nlm.nih.gov/blast). Phylogenetic trees were created and bootstrap analyses (1,000 replicates) performed in ARB.

Thermodynamic calculations

Energy yields of methanogenesis reactions from H_2/CO_2, formate, and acetate were calculated from standard free Gibbs energies of formation (ΔG_f^o), standard entropies (ΔH_f^o), and standard molar volumes (ΔV_f^o) of reactants and products (Table 2.2). With H_2/CO_2, we also performed calculations in which we assumed fixed free energy yields (-10 kJ mol^{-1}), and calculated the H_2 concentrations necessary for this free energy yield. Corrections for temperature and pressure were made using the Nernst and Van't Hoff equations, respectively.

We used measured activity coefficients for dissolved gasses and anions in seawater, γ, of 1.24 for CH_4, and 0.532 for HCO_3^- (Millero and Schreiber 1982), and $\{H_2O\} = 1.0$ (ref). Due to the absence of published values, the activity coefficient of H_2 was approximated with that of CH_4, and the activity coefficients of formate and acetate approximated with that of HCO_3^-. We performed sensitivity analyses by varying activity coefficients of H_2, formate, and acetate by ± 20% and found that, even under the most extreme scenarios, calculated *in situ* energy yields changed by <2 kJ mol^{-1}.

RESULTS

Carbon isotope geochemistry

δ^{13}C-CH_4 was ~65‰ in the upper 8 mbsf, fluctuated between -50 and -76‰ from 8 to 35 mbsf, stabilized at ~70‰ from 35 to 40 mbsf, and gradually declined to -65‰ at 246 mbsf (Fig. 4.2A). δ^{13}C-DIC decreased from -10‰ at the seafloor to -13‰ by 7 mbsf, increased sharply to 6‰ from 8 to 20 mbsf, continued to increase gradually to reach a maximum of ~20‰ at 123 mbsf, and gradually fell off in deeper layers, reaching 15‰ at 246 mbsf (Fig. 4.2A). The isotopic depletion of ^{13}C-CH_4 relative to ^{13}C-DIC ranges around -53‰ in the upper 6 mbsf, then fluctuates, probably as an artefact of differential outgassing rates during

core retrieval, overall increases to reach its maximum of -86‰ at 40 mbsf, and gradually decreases downward to reach -80‰ at 246 mbsf (Fig. 4.2B). The difference of ~80‰ between ^{13}C-CH_4 and -DIC indicates H_2/CO_2 as the main substrate of methanogenesis (Whiticar et al. 1986). The increasing $\delta^{13}C$-DIC gradient from 19 mbsf upwards indicates an increase in CO_2-reduction rates. The highest CO_2 reduction rates are between 8-9 mbsf, as indicated by the maximum gradient in $\delta^{13}C$-DIC (also see Fig. 4.4).

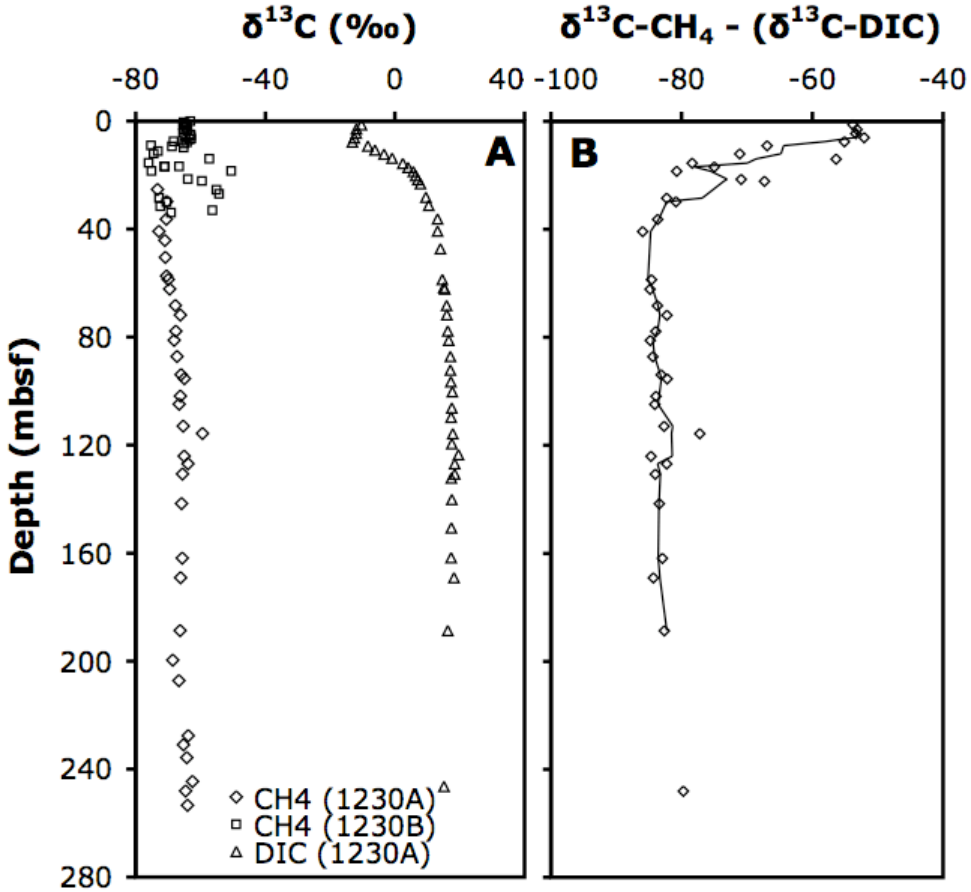


FIGURE 4.2: (A) Depth profile of $\delta^{13}C$-CH_4 and $\delta^{13}C$-DIC. (B) Depth profile of difference between $\delta^{13}C$-CH_4 and $\delta^{13}C$-DIC.

Molecular diversity

We detected *mcrA* sequences from three phylogenetic groups (Fig. 4.3): (1) a sister group of the known aceticlast *Methanosaeta harundinacea* that lacks cultured representatives, (2) close relatives of [suspected] anaerobic methanotrophic ANME-1, and (3) a sister group of ANME-1, which we refer to here as ANMG-1 (Fig. 4.3). There is a clear distribution to the three detected groups. ANME-1-like *mcrA* sequences were found in one horizon within the SMTZ, and five horizons in the upper methanogenesis zone (Table 4.2, Fig. 4.4). Sequences from the ANMG-1 group overlap with ANME-1 sequences in one sample in the upper methanogenesis zone (~20 mbsf) and were detected in three more horizons down to 189 mbsf (Table 4.2; Fig. 4.4). *Methanosaeta*-like *mcrA* sequences show a distribution similar to ANMG-1, but were detected in more depth horizons and to 227 mbsf (Table 4.2, Fig. 4.4).

We detected *mcrA*-mRNA of ANMG-1 in one of three horizons examined, 44 mbsf (Table 4.2). We did not detect *mcrA*-mRNA of ANME-1 or the *Methanosaeta* sister group (Table 4.2). In 16S rRNA analyses, we detected *Methanosaeta* sequences in all three horizons examined (tree not shown), but did not detect 16S rRNA of ANME-1 or ANMG-1.

Thermodynamic calculations

Our thermodynamic calculations are at odds with the inferred pathway of methanogenesis based on isotopes as they suggest that hydrogenotrophic methanogenesis is not exergonic in the methanogenesis zone (Fig. 4.5). The only depth horizons where ΔG_r' are negative are in the sulfate reduction zone, the SMTZ, and a single horizon in the upper methanogenesis zone. Even in the upper sediment horizons, calculated *in situ* energy yields

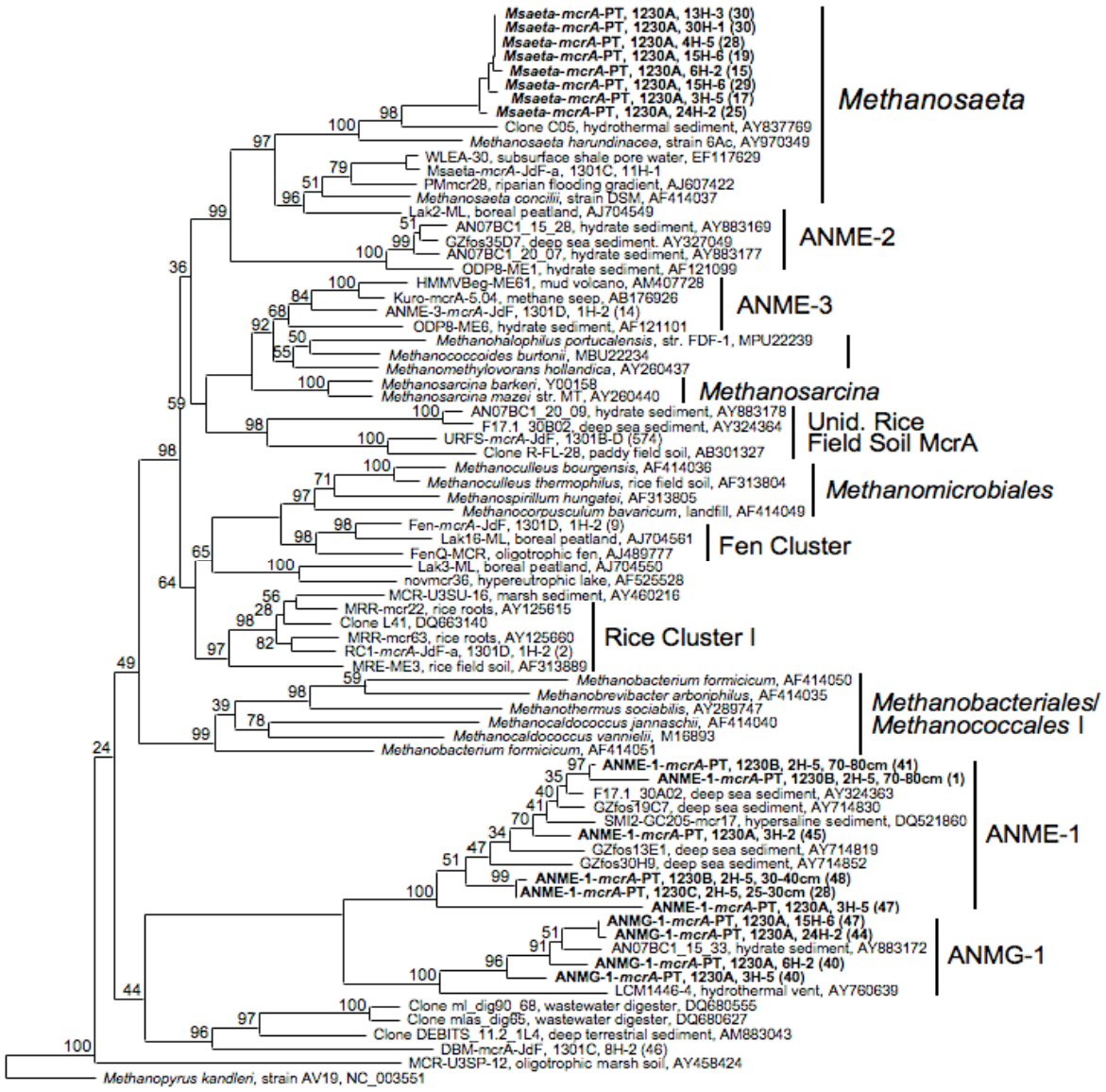


FIGURE 4.3: Bootstrap phylogenetic tree based on *mcrA* nucleotide sequences at ODP Site 1230. Created using Jukes-Cantor correction in ARB neighbor-joining (Ludwig et al. 2004).

remain low and below the critical free energy requirement reported for hydrogenotrophic methanogens in marine sediments (-11.3 to -9.5 kJ mol^{-1}; Hoehler et al. 1994, Hoehler et al. 2001).

TABLE 4.2: Samples analyzed, dominant terminal organic carbon remineralization pathway, depths with positive PCR and RT-PCR amplification, and number of clones obtained per depth (in parentheses); bd = below detection, nd = not determined].

Hole	Core, section, interval (cm)	Depth (mbsf)	terminal C_{org} remineralization pathway	*mcrIRD*-DNA (# of clones)	*ODP1230-mcrA*-DNA (# of clones)	*ANME-1-mcrI*-DNA (# of clones)	*ANME-1-mcrI*-mRNA (# of clones)	*Msaeta-16S*-rRNA-268F/927R
A	1H-1, 25-30	0.25	SZ	bd	nd	bd	nd	nd
A	1H-3, 25-30	3.25	SZ	bd	nd	bd	nd	nd
B	2H-2, 120-125	5.70	SZ/AOM	bd	nd	bd	bd	nd
B	2H-3, 30-40	6.30	SZ/AOM	bd	nd	bd	bd	nd
B	2H-4, 30-40	7.80	AOM	bd	nd	ANME-1	bd	nd
B	2H-5, 70-80	9.70	MG	bd	nd	ANME-1	bd	nd
B	2H-5, 120-125	10.20	MG	bd	nd	ANME-1	bd	nd
C	2H-5, 25-30	10.75	MG	bd	nd	ANME-1	bd	nd
A	3H-2, 25-30	16.05	MG	bd	nd	ANME-1 (45)	nd	nd
A	3H-5, 25-30	20.55	MG	Msaeta (17)	nd	ANME-1 (1), ANMG-1 (40)	nd	nd
A	4H-5, 35-40	30.15	MG	Msaeta (28)	nd	bd	nd	nd
A	6H-2, 25-30	44.55	MG	Msaeta (9)	nd	ANMG-1 (40)	ANMG-1 (1)	Msaeta (7)
A	9H-5, 23-28	65.68	MG	bd	bd	bd	nd	nd
A	13H-3, 20-25	102.00	MG	Msaeta (30)	nd	bd	nd	nd
A	15H-6, 25-30	124.37	MG	bd	Msaeta (19)	ANMG-1 (47)	bd	Msaeta (12)
A	18H-3, 35-40	142.15	MG	bd	Msaeta (20)	bd	nd	nd
A	21H-3, 25-30	160.53	MG	bd	nd	bd	nd	nd

A	24H-2, 24-29	189.04	MG	Msaeta (25)	nd	ANMG-1 (44)	nd	nd
A	30X-1, 108-115	227.38	MG	Msaeta (30)	nd	bd	bd	bd
A	38X-1, 130-135	268.50	MG	bd	bd	nd	nd	nd

Calculated *in situ* energy yields of methanogenesis from acetate and formate suggest that both reactions are exergonic. With the exception of the surface core, where they exceed

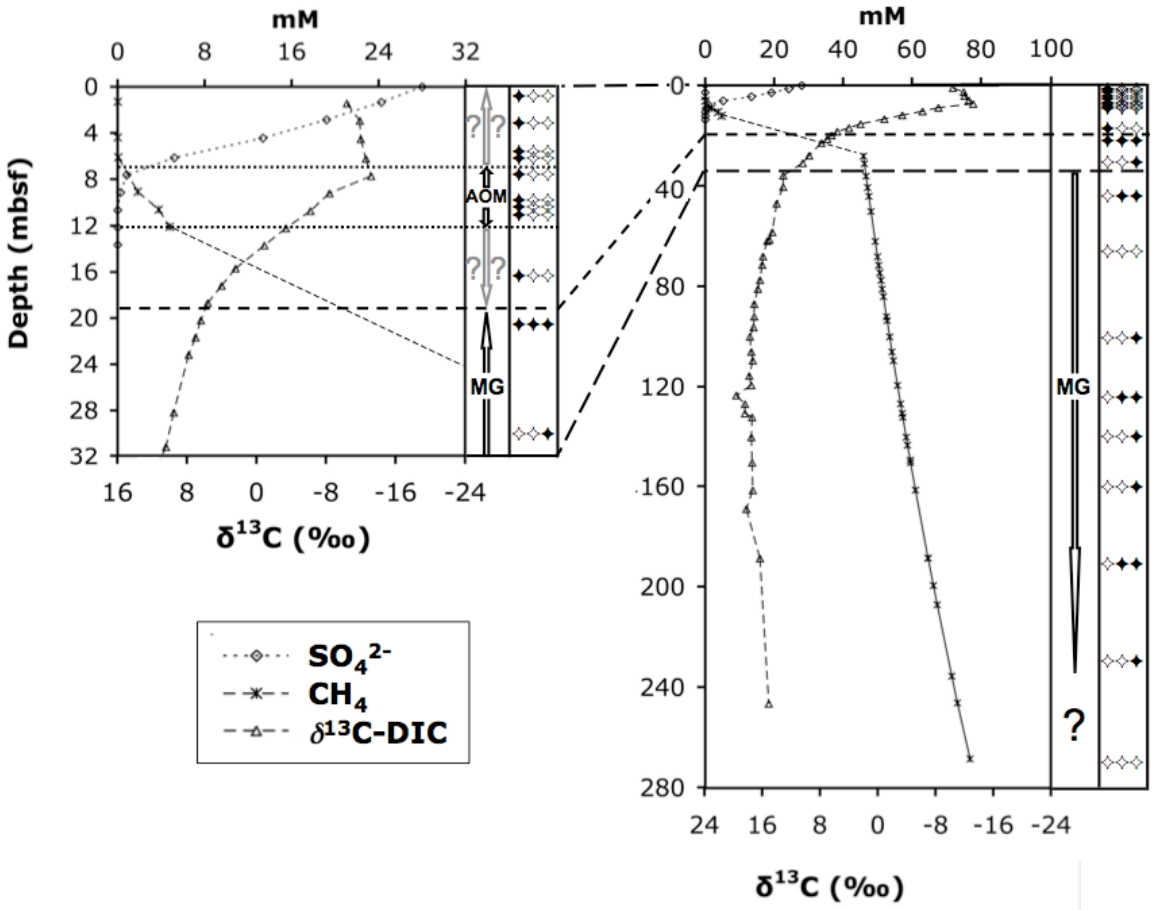


FIGURE 4.4: Distribution of mcrA groups along depth and geochemical gradients of sulfate, methane, and δ^{13}C-DIC. Panel on right side of each graph indicates detection/absence of detection of (1) ANME-1, (2) ANMG-1, and (3) *Methanosaeta* sequences. Solid black symbols indicate detection, empty symbols indicate lack of detection (example: ✦✧✧ indicates presence of ANME-1, and absence of ANMG-1 and *Methanosaeta*).

the previously documented critical free energy requirement of aceticlastic methanogens in marine sediments (-12.8 to -9.1; Hoehler et al. 1994, Hoehler et al. 2001), they range from -0.7 to -5.9 kJ mol^{-1} for formate, and from -0.3 to -8.0 kJ mol^{-1} for acetate (Fig. 4.5).

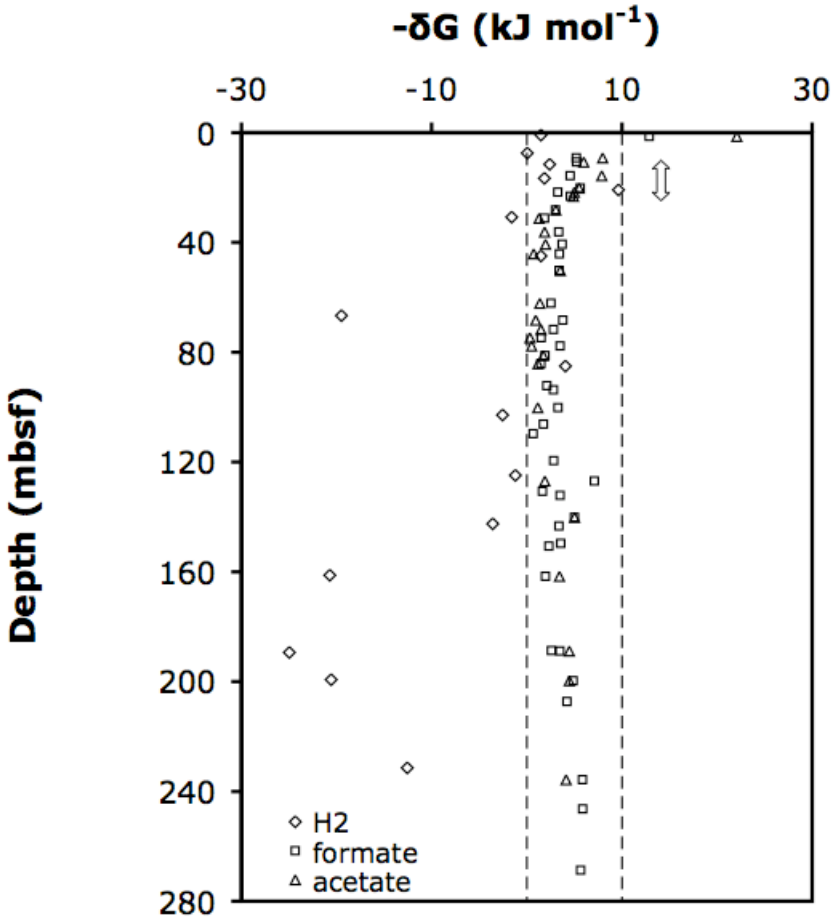


FIGURE 4.5: Calculated *in situ* energy yields of methane production from H_2/CO_2, formate, and acetate. The arrow indicates a depth range in which calculated energy yields are likely to be overestimates of *in situ* energy yields, as measured dissolved methane concentrations were used due to the absence of other data.

DISCUSSION

The apparent paradox of hydrate-rich sediments at ODP Site 1230 being mostly devoid of known, PCR-detectable methanogens in the methanogenesis zone (Inagaki et al. 2006), and completely devoid of anaerobic methanotrophs in the SMTZ (Biddle et al. 2006), led to the hypotheses that (1) methanogens and methanotrophs represent a small, patchily distributed minority of the total archaeal population, or (2) CH_4 production and oxidation are carried out by organisms different from known methanogens and methanotrophs. We provide evidence that the lack of PCR detection in previous studies was to a large extent PCR primer-related: newly designed *mcrA* primers allow us to detect what in fact amounts to a spatially extended CH_4-cycling community, with *mcrA* detected in the upper sulfate reduction zone above the SMTZ, throughout the SMTZ, and in most depth horizons of the methanogenesis zone. Similarly, newly designed methanogen- and methanotroph-specific 16S rRNA primers resulted in detection of methanogen and methanotroph phylotypes, where past surveys with general archaeal primers had yielded exclusively other phylum-level groups, such as Marine Benthic Group B (Deep Sea Archaeal Group; Biddle et al. 2006, Inagaki et al. 2006). We provide a high-resolution depth profile for *mcrA* that enables us to examine distribution patterns of methanogens and anaerobic methanotrophs along porewater chemical gradients.

With novel group-specific *mcrA* and 16S rRNA gene primers, we were able to detect methanogen mRNA and rRNA, in addition to DNA. The detection of *mcrA*-mRNA suggests that ANMG-1 at 44.3 mbsf may be performing methanogenesis under *in situ* conditions, and is part of the active CH_4-cycling community. Amplification of reverse-transcribed 16S rRNA detected sequences *Methanosaeta*-related methanogen in several sediment horizons

(Table 4.2) that also yielded *Methanosaeta*-related *mcrA* genes; thus, *Methanosaeta*-related phylotypes are likely alive throughout the sediment column.

Community zonation

The distribution of *mcrA* genes only partially reflects the traditional model, according to which sulfate reducers will outcompete methanogens for H_2/CO_2 (formate) and acetate in the presence of sulfate (Phelps et al. 1985, Lovley and Goodwin 1988, Hoehler et al. 1998, Hedderich and Whitman 2006), and AOM is restricted to the SMTZ: as expected, *mcrA* of putative methanogens (ANMG-1 and *Methanosaeta*-relatives) was only found in the methanogenesis zone; however, *mcrA* of putatively methanotrophic ANME-1 was not only found in the SMTZ, but also in the upper ~12 m of the methanogenesis zone. Non-competitive substrates that may allow coexistence at other subsurface sites (Lever et al., in prep), do not sustain detectable methanogen populations in the sulfate reduction zone at ODP Site 1230.

The two phylotypes found at 20 mbsf and below, ANMG-1 and *Methanosaeta* relatives, were detected down to 190 and 230 mbsf, respectively (Fig. 4.3, 4.4). ANMG-1 comprises a sister group of ANME-1 according to *mcrA* phylogeny (Fig. 4.3); its occurrence deep in the methanogenesis zone suggests that ANMG-1 is a methanogenic group. The consistently high ^{13}C-isotopic fractionation, ε_{CH4}, of CH_4 relative to DIC (-80‰; Fig. 4.2B) in the methanogenesis zone suggests H_2/CO_2 as the main methanogenic substrate (Whiticar 1999), and it is hence possible that ANMG-1 are hydrogenotrophic methanogens. The widespread occurrence of sequences in the *Methanosaeta* group in the methanogenesis zone contrasts

with the measured isotopic difference between δ^{13}C-CH_4 and -DIC values, since all *Methanosaeta* strains isolated to date are obligate aceticlasts (Whitman et al. 2006).

Thermodynamic modeling

Calculated *in situ* energy yields based on measured H_2 concentrations suggest that hydrogenotrophic methanogenesis is endergonic in most deep sediment layers, which contradicts interpretations based on the difference in carbon isotopic signatures between CH_4 and DIC (Fig. 4.2). Our calculations show that *in situ* H_2 concentrations would need to be 1-2 nM higher for hydrogenotrophic methanogens to meet a critical free energy requirement of -10 kJ mol^{-1} (Fig. 4.6). This discrepancy suggests that measured H_2 concentrations were

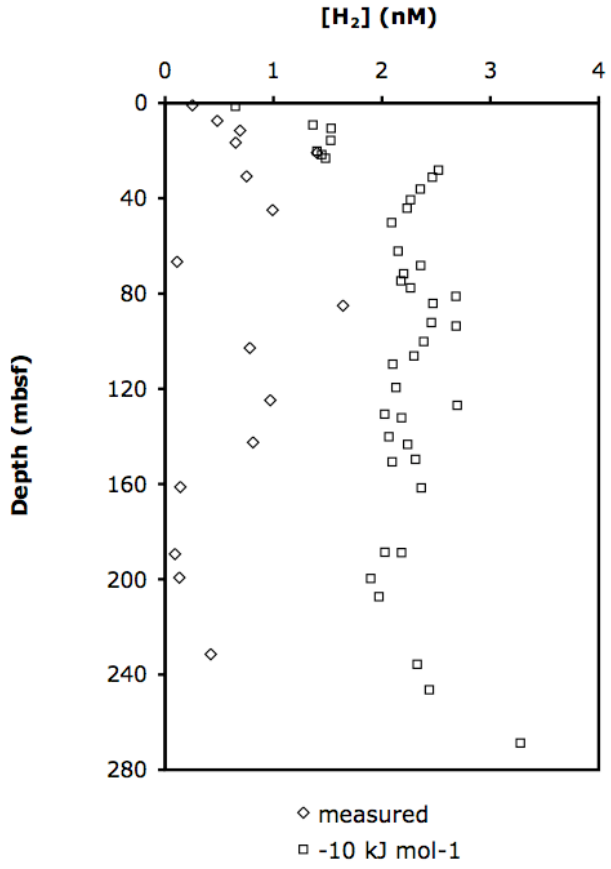


FIGURE 4.6: Measured H_2 concentrations and H_2 concentrations required for $\Delta G' = -10$ kJ mol^{-1}.

subject to artifacts: changes in temperature and pressure over long retrieval periods affect the sensitive microbial turnover of H_2 (Hoehler et al. 1999), and may have offset the balance of H_2 production and consumption, causing measured concentrations to be underestimates of *in situ* concentrations. Moreover, no metabolic pathway is known that would be thermodynamically favorable over methanogenesis in the methanogenesis zone at ODP Site 1230 and could drive H_2 below threshold concentrations for methanogenesis. Autotrophic acetogenesis coupled to aceticlastic methanogenesis could result in the observed difference in carbon isotopic signatures between CH_4 and DIC, but our calculations indicate that *in situ* energy yields for autotrophic acetogenesis are lower than for hydrogenotrophic methanogenesis. Even if autotrophic acetogenesis and aceticlastic methanogenesis reactions were both favorable, the cumulative energy yield of the two reactions would equal that of hydrogenotrophic methanogenesis, which would consequently have higher energy free energy yields than autotrophic acetogens or aceticlastic methanogens.

A plausible explanation for the measured ε_{CH4} is that the *Methanosaeta* relatives detected use enzymatic pathways different from those of isolates in which fractionations were previously measured. A recently isolated marine *Methanosarcina* strain used a different enzymatic pathway than previously isolated *Methanosarcina* (Ferry 2008). Some *Methanosaeta* spp. harbor genes used by *Methanosarcina* in hydrogenotrophic metabolism, suggesting that certain *Methanosaeta* strains might grow as facultative hydrogenotrophs (Smith and Ingram-Smith 2007).

Methanogenesis reactions from formate and acetate are consistently exergonic (Fig. 4.5), but energy yields remain below published critical free energy values of aceticlastic methanogens in coastal marine sediments (Hoehler et al. 1994, 2001). It is possible that

critical free energy requirements in methanogenesis from formate or acetate are lower under extreme energy limitation, as in deep ODP Site 1230 sediments, or that concentrations of reactants and products used in our calculations do not reflect concentrations that methanogens are exposed to *in situ.* While microniches are probably uncommon in deep subsurface sediments, due to dissipation of substrate gradients under the low substrate turnover rates, it is possible that part of the bioavailable acetate and formate remains undetected in the quantification method used during Leg 201 (D'Hondt et al. 2003). We also cannot rule out the possibility that saturating CH_4 concentrations are lower than calculated using the model of Sun and Duan (2007).

Based on extant concentration data, none of the reverse reactions of methanogenesis from H_2/CO_2, formate, or acetate yield energy in the SMTZ or adjacent sediment layers. Yet, the concentration profile of CH_4 combined with the marked changes in δ^{13}C-DIC suggest the occurrence of AOM between ~6 to ≥8 mbsf, and CO_2 reduction between ~≤9-20 mbsf (Fig. 4.4).

CONCLUSIONS

Deep subsurface microorganisms are metabolically active and express the key genes necessary for their metabolism. By detecting *mcrA*-mRNA at 44 mbsf, we have identified a novel archaeal lineage (ANMG-1) that is likely to be metabolically active in the deep subsurface. Recovery of highly unstable mRNA is clearly possible despite the short half-life of mRNA (Rauhut and Klug 1999) and lengthy retrieval periods in ocean drilling (up to several hours; House et al. 2005, Lever et al. 2006). Though we could not detect mRNA in

the deepest horizons examined, 16S rRNA was detected and indicates that even below 200 mbsf the identity of living cells can be determined.

Previous molecular surveys of deep subsurface sediments on the Peru Margin (e.g. Parkes et al. 2005, Biddle et al. 2006, Inagaki et al. 2006) yielded few presumptive methanogenic sequences, no methanotrophic ones, and predominantly uncultured lineages of Archaea with unknown metabolisms. Methanogens and anaerobic methanotrophs probably represent a small portion of the archaeal community at ODP Site 1230. Although we have not performed DNA quantifications, low abundance of methanogens and anaerobic methanotrophs is likely since in most depth horizons the equivalent of 1 g of sediment was required for PCR detection of *mcrA* genes. Small populations of active methanogens may suffice to produce saturating CH_4 concentrations and hydrates over geologic time periods. Similarly, small populations of active methanotrophs distributed over the upper ~6 to ≥8 mbsf may be sufficient to remove all methane produced below.

The co-dominance of the methanogenic community of the deep sediment column by two phylotypes indicates little room for niche differentiation. Examples from macroecology suggest that under extreme resource limitation species diversity is low and biological communities dominated by generalists (Emlen 1966, Pianka 1994). In homogeneous environments with low rates of disturbance diversity also tends to be low, as specialists that are adapted best to the suite of conditions exclude competitors over time (Emlen 1966, Pianka 1994). The deep subsurface sediments at ODP Site 1230 combine high resource (energy) limitation with high habitat homogeneity and stability. In accordance with macroecological examples, we might expect organisms with wide substrate ranges and high degree of adaptation to the environmental conditions to prevail. The widespread occurrence

of a relative of known substrate specialists (*Methanosaeta*) suggests otherwise, however. A fundamental distinction between microbes and macrobiota under energy limitation is that microbes may not only struggle to meet maintenance energy requirements, but also to acquire the critical free energy of a metabolic reaction. Methanogens will compete among themselves and with other metabolic groups for H_2 until the strain with the lowest critical free energy requirement has driven H_2 concentrations below the critical threshold of all others. This form of thermodynamic exclusion, which only occurs in microbes, provides a gateway for substrate specialists, provided they can meet maintenance energy requirements. Accordingly, we would expect to see obligately aceticlastic *Methanosaeta* in the deep subsurface, not the more generalistic *Methanosarcina*, which require higher acetate concentrations to meet critical free energy requirements than *Methanosaeta*. Following the same logic, ANMG-1 would be able to drive concentrations of a different methanogen substrate, most likely H_2 (and formate), below the threshold of other methanogen strains. An alternative explanation is that ANMG-1 and *Methanosaeta* are substrate generalists, which coexist sympatrically in different micro-habitats.

In addition to variables such as temperature, substratum, nutrients/growth factors, and energy source, whose importance is known from cultivation studies, maintenance and critical free energy requirements are likely to shape microbial community composition in energy-limited deep subsurface sediments. The extent to which communities are the outcome of competition for substrates or differential ability to survive starvation requires further investigation and will depend on the metabolic substrate(s) consumed by the organism and whether their concentrations are under thermodynamic control. Deep sedimentary microbial ecosystems may provide good model systems for microbial community analysis integrated

with geochemical habitat study, since they allow the examination of how a limited set of geochemical and physiological variables over time determines microbial ecosystem functioning and diversity.

CHAPTER V

MCRA GENE DIVERSITY IN HYDROTHERMAL SURFACE SEDIMENTS FROM THE GUAYMAS BASIN INVESTIGATED BY GENERAL AND GROUP-SPECIFIC PCR PRIMERS

ABSTRACT

We examined detection sensitivity and phylogenetic breadth of three previously designed *mcrA* primer pairs (mcrI, ME1/ME2, mcrIRD) and 27 mostly newly designed *mcrA* group-specific primer pairs in hydrothermally active sediments from Everest Mound in the Guaymas Basin. The diversity obtained with the mcrI and mcrIRD primer pairs was similar, and higher than that obtained with the ME1/ME2 primer pair. The detection limit was significantly lower with the mcrIRD primer pair, suggesting higher *mcrA* PCR-amplification rates than with the other two general *mcrA* primer pairs. ANME-1-like sequences were the only *mcrA* cluster solely detected with group-specific primers, whereas the remaining 26 group-specific primer pairs yielded no new phylotypes. This suggests that *mcrA* diversity in Guaymas sediments can be covered using the general mcrIRD and group-specific ANME-1 primer pairs.

The mcrA genes detected belong to relatives of known hydrogenotrophs, methylotrophs and anaerobic methanotrophs. No sequences grouping with those of known aceticlasts were detected. Five *mcrA* clusters without cultured members were detected, four of which

possessed no previously detected close relatives. There was no apparent zonation along the temperature gradient, except that all but two groups were absent below 6 cm into the seafloor.

INTRODUCTION

The nucleotide sequence diversity observed in natural samples via PCR assays is largely a function of the organisms present and the PCR probes specificity. Many PCR primer pairs appear to generate clone libraries biased towards certain phylotypes within their ostensible target groups, as has been illustrated recently for the case of the Archaea and "general" archaeal 16S rRNA gene primers (Teske and Sørensen 2007, Hwang et al. 2008). Primer mismatches are a main cause of phylogenetic biases (Diallo et al. 2008) and are often due to the absence of certain sequences in the database when primers were first designed. As sequence data bases of genes continue to grow due to the addition of new genes and genomes, reexamination and, if necessary redesign, of PCR primers is crucial.

Multiple strategies exist to compensate for primer biases. Use of several primer pairs in separate PCR assays is one (Piceno et al. 1999, Rösch et al. 2002, Bürgmann et al. 2004), the underlying argument being that if each individual group is targeted separately, or each primer pair is biased towards a different target group, all phylotypes will be detected eventually. A pitfall of this method is its labor-intensity. Incorporation of degeneracies into PCR primers is an alternative strategy (Zehr and McReynolds 1989, Widmer et al. 1999), but can compromise PCR amplification efficiency and result in a high detection limit (Bürgmann et al. 2004, Juottonen et al. 2006). Which strategy works best is likely to depend on factors

such as template concentration, diversity, and richness, and primer coverage of target genes in a given sample.

Here we examine the performance of several general primer pairs designed to target the alpha subunit of the gene for methyl coenzyme M reductase (*mcrA*). The *mcrA* gene is considered unique to methane-producing Archaea (methanogens) and anaerobic methane-oxidizing Archaea (methanotrophs; Reeve et al. 1997, Hallam et al. 2003), phylogenetically conserved (Friedrich et al. 2005), and hence a suitable marker gene for the study of natural populations of methanogens and anaerobic methanotrophs. We compare the detection limits and diversity detected with three previously designed general *mcrA* primer pairs: mcr*I* (Springer et al. 1995), ME1/ME2 (Hales et al. 1996), and mcr*IRD* (Lever et al. in prep, Chapter III). In addition, we use 27 group-specific *mcrA* primer combinations to test the performance and examine phylogenetic biases of the three general primer pairs. As a study site, we examine surficial sediments of the Everest Mound area in the Guaymas Basin, which was previously shown to host an exceptionally high diversity of methanogens and methanotrophs (Teske et al. 2002, Dhillon et al. 2005), and is hence well-suited for the study of PCR primer sensitivity and biases. We then (1) examine the zonation of mcrA genes in the context of the steep thermal and geochemical gradients (sulfate) of the site, (2) investigate possible metabolic pathways of phylotypes detected based on closest relatives with known metabolisms, and (3) compare overall diversity and community structure to that detected previously detected at a nearby station at Everest Mound (Dhillon et al. 2005).

METHODS

Sampling and Site Characteristics

A 20-cm core of surface sediment was obtained via the manned research submersible *Alvin* (Woods Hole Oceanographic Institution) during a cruise to the Guaymas Basin in April and May 1998. The core came from a site where the temperature increases steeply with depth, from ~2°C at the seafloor to ~160°C at 30 centimeters –below seafloor (cmbsf; Weber and Jørgensen, unpubl.). High productivity of overlying waters results in diatomaceous sediment that accumulates at a rate of >1 mm yr^{-1} (Curray et al. 1979) and total organic carbon contents of 2-4% by weight (Simoneit and Bode 1982). Due to the steep increase in temperature within decimeters of the seafloor, biological activity is believed to be limited to surface sediments. A large portion of the energy utilized by microbial communities at this site derives from relict carbon (Pearson et al. 2005): hydrocarbons (methane, petroleum) and VFAs are produced by magmatic heating of organic matter deeper in the sediment column (Bazylinski et al. 1988, Martens 1990, Pearson et al. 2005), and supplied to the seafloor via upward flow of hydrothermal fluids. Concentrations of sulfate and volatile fatty acids have yet to be determined at this site, but profiles from other, nearby sites suggest high sulfate reduction rates in the uppermost centimeters (Weber and Jørgensen, 2005), and high concentrations of volatile fatty acids that increase with depth (Martens 1990, Dhillon et al. 2005). The coexistence of diverse sulfate reducer and methanogen assemblages in the uppermost centimeters (Dhillon et al. 2003, 2005) suggests that availability of electron donors is not limiting to either group. The detection of putative anaerobic methanotrophs and diverse methanogens suggests co-occurrence of anaerobic oxidation of methane (AOM) and methanogenesis, and the possible presence of microenvironments with highly discrepant porewater chemistry.

DNA Extraction

DNA was extracted following the same protocol as outlined for RNA extraction in Biddle et al. (2006), except that the pH of the extraction buffer and phenol were raised to 8.0, the bead beating time reduced to 15s, and the bead beating speed reduced to 4.0 (Qbiogene, Carlsbad, CA). Moreover, the DNase incubation was omitted, and DNA purified with the PowerClean DNA Clean-Up Kit (MOBIO laboratories, Carlsbad, CA) rather than the RNeasy Mini Kit (Qiagen, Valencia, CA).

PCR Amplification

To target all mcrA genes we used previously designed mcr*I* (Springer et al. 1995), ME1/ME2 (Hales et al. 1996), mcr*IRD* and ANME-1-mcr*I* primer pairs (Chapter III) using the annealing temperatures outlined in Chapter IV. In addition, we designed 26 new group-specific *mcrA* primer combinations using gene alignments (Lever, unpubl.) constructed in the phylogenetic software program ARB (Ludwig et al. 2004; Table 5.1). Annealing temperatures were calculated using the DNA calculator function on the Sigma-Aldrich webpage (Sigma-Aldrich, St. Louis, MO; http://www.sigmaaldrich.com/calc/DNACalc.asp).

PCR assays were performed using the Takara SpeedSTAR HS DNA polymerase kit (TaKaRa Bio USA, Madison, WI) using the following PCR protocol: (1) 1 × 2 min denaturation (98°C), (2) 40 × (a) 10s denaturation (98°C), (b) 30s annealing (see Table 1 for temperatures), (c) 1 min extension (72°C), and (3) 1 × 5 min extension (72°C). DNA bands

TABLE 5.1: Overview of *mcrA* primer pairs designed in this study, their target groups, primer sequences, the # of nucleotides of individual primers, annealing temperatures, amplification of the correct band length (+ = amplification, - = no amplification), and specificity (+ = high, i.e. only target group, - = low, no target group & no *mcrA*, -/+ = amplified *mcrA*, but not target group, (-/+) = amplified *mcrA*, but not only target group).

Name	Target Groups	Primer sequences	# of nucleotides (F; R)	Length of fragment (bp)	Annealing temperature	Amplification[1]	Specificity[2]
mcrMS	*M. sarcina*	F: 5' GACCAGATCTGGCTCGGATC; R: 5' TCGCCCTGGTAGGACAGAAC	20; 20	425	66.0	+	-
mcrAM-2	ANME-2	F: 5' GGATTCACGCAGTACGCAAC; R: 5' CAAGAAGCGTTGGGTAGTCC	20; 20	155	64.0	+	+
mcrMsaeta	*M. saeta*	F: 5' TACACCAACGATGTCCTGGA; R: 5' CACTGATCCTGCAGGTCGTA	20; 20	305	64.0	+	-
mcrMc	*M. coccaceae*	F: 5'AAGAAGAGCAAGAGGTCCAAA; R: 5' TCGTATCCGTAGAATCCTAATCT	21; 23	490	61.0	+	-/+
mcrURFS	Unid. Rice Field Soil McrA	F: 5' GTATGCAACACCAGCATACACC / GTATGCCACAGCAGCATACAC; R: 5' CACCGCACTGATCCTGC	22/ 21; 17	385	64.0	+	-
mcrAM-3 I	ANME-3	F: 5' GATATCATTCAGACAAGCCG; R: 5' AGTTCAAGAGGCTCTCCTTC	20; 20	525	60.0	-	n/a
mcrAM-3 II	ANME-3	F: 5' CCTTGAGGTAGTCGGTGCAG; R: 5' AGTTCAAGAGGCTCTCCTTCGT	20; 22	480	64.5	-	n/a
mcrAM-3etal	ANME-3, *M.coccoides*, -*methylovorans*, -*lobus*, -*halophilus*	F: 5' GATATCATTCAGACAAGCCGT; R: 5' CACCACACTGGTCCTGC	21; 17	480	60.0	+	-
mcrRCI/FC	Rice Cl. I, Fen Cl.	F: 5' TACAAGATGTGCGCCGGT; R: 5' CATGCTTCCTTGTGCAGGTA	18; 20	560	64.0	-	n/a
mcrFCI	Fen Cl.	F: 5' AGCCAGGTGGCATCAAGTT; R: 5' ACTGGTCCTGCAGGTCGTAG	19; 20	510	64.0	-	n/a
mcrFCII	Fen Cl.	F: 5' AGCCAGGTGGCATCAAGTT; R: 5' GACAGGTACCAGCCGTTCA	19; 19	445	64.0	+	-
mcrMcorp	*M. corpusculum*	F: 5' TGTCATCAACATGGCCCAC; R: 5' TCGTAGCCGAAGAAACCAAG	19; 20	550	64.0	+	-
mcrMspir	*M. spirillum*	F: 5' GATGAGTTCACCTACTATGGTAT; R: 5' CTGACAGAGAGTGAGTTGGT	23; 20	335	56.0	+	-
mcrMcul	*M. culleus* & close relatives	F: 5' GGTATGGACTACATCAAGGACAA; R: 5' ACTGGTCCTGGAGGTCGTA	23; 19	290	62.0	+	-
mcrMgen	*M. genium*	F: 5' CACCTACTACGGTATGGACTATAC R: 5' GAGTTTGCTGAACCACACTG	24; 20	310	61.0	+	(-/+)
mcrGMmC	Guaymas M. microbiales Cluster	F: 5' CACCTACTACGGTATGGACTATAC R: 5' AGCTCTCCGAGCAGACCT	24; 18	355	61.0	+	+
mcrMbacA	*M. bacterium aarhusense* group	F: 5' GCAAAACACGCAGAAGTTGT; R: 5' GTCTGGAGTGCTGTTCTTTGTG	20; 22	515	63.0	+	-
mcrMbac	*M. bacteriales* except *aarhusense* and *M. thermobacter*	F: 5'GGTTAGGTTCTTACATGTCTGGTG; R: 5' GCACCACATTGATCTTGTAAATC / TGCTCCACACTGGTCCTG / CACCACACTGGTCCTGGA	24; 23	365	63.0	+	-

mcrMtb	*M. thermobacter*	F: 5' AGCCTACACAGACAACATCCTC; R: 5' CACCACACTGGTCCTGGA	22; 18	315	63.0	+	-
mcrMp	*M. pyrus*	F: 5' CTAGGATCCTACATGTCAGGAGG / R: 5' CCTCACGCTCAGCGAGTT	23; 18	385	64.0	+	+
mcrDBGrI	Deeply-branching Guaymas Group I	F: 5' CGGAGTAGGATTCACGCAGTA; R: 5' GATAGTTTGGACCACGCAGTTC	21; 22	410	64.0	+	+
mcrMlas	Clone mlas, clone DEBITS & relatives	F: 5' ACGACTTCTGCTACTACGGTGC / R: 5' CCTGCCCATCTCCTCCTT	22; 18	255	64.0	+	-
mcrDBM	Deeply-branching Methanogen group from JdF	F: 5' GATGACTTCTGCTACTACGGCAT; R: 5' GCCTACCAGCAGACTCCCT	23; 19	245	64.0	+	-
mcrDBGrII	Deeply-branching Guaymas Group II	F: 5' GCAGTATGCAACCGCTGTT; R: 5' GTCTGCACCTCTGAGCTCAAG	18; 19	395	64.0	+	+
mcrDBGrII I&IV	Deeply-branching Guaymas Groups III & IV	F: 5' GTGTACACGGACAACATCCTGG; R: 5' ACGCTCAGCGAGTTGGC	22; 17	330	64.0	+	-

Cloning and Sequencing

PCR products were purified in a 2.5 % low-melting point agarose gel using 1X Tris acetate- EDTA buffer (40 mM Tris-HCl, 40 mM Na-acetate, 4 mM Na- EDTA). Gel slices containing PCR products of the expected PCR length were excised and purified using S.N.A.P. Mini Kits (Invitrogen, Carlsbad, USA). Purified PCR products were cloned and inserted into TOP10 electrocompetent *E. coli* using the Topo TA Kit following the manufacturer's instructions (Invitrogen, Carlsbad, USA). Plasmid extraction, purification and cycle sequencing were performed at the Josephine Bay Paul Center at MBL (Woods Hole, MA). Sequences were BLAST analyzed using the nucleotide collection in GenBank (www.ncbi.nlm.nih.gov/blast). Phylogenetic trees were created in ARB, using neighbor-joining with Jukes Cantor correction and bootstrap analyses with 1,000 replicates.

RESULTS

We detected a diverse assemblage of 18 different *mcrA* phylotypes belonging to 13 *mcrA* clusters, of which 8 fell into known orders (Methanomicrobiaceae, Methanococcaceae,

Methanopyraceae; Fig. 5.1). *mcrA* sequences were detected only in the upper 10 cmbsf (Fig. 5.2, Table 5.2). Closest known cultivated relatives based on *mcrA* sequence similarity

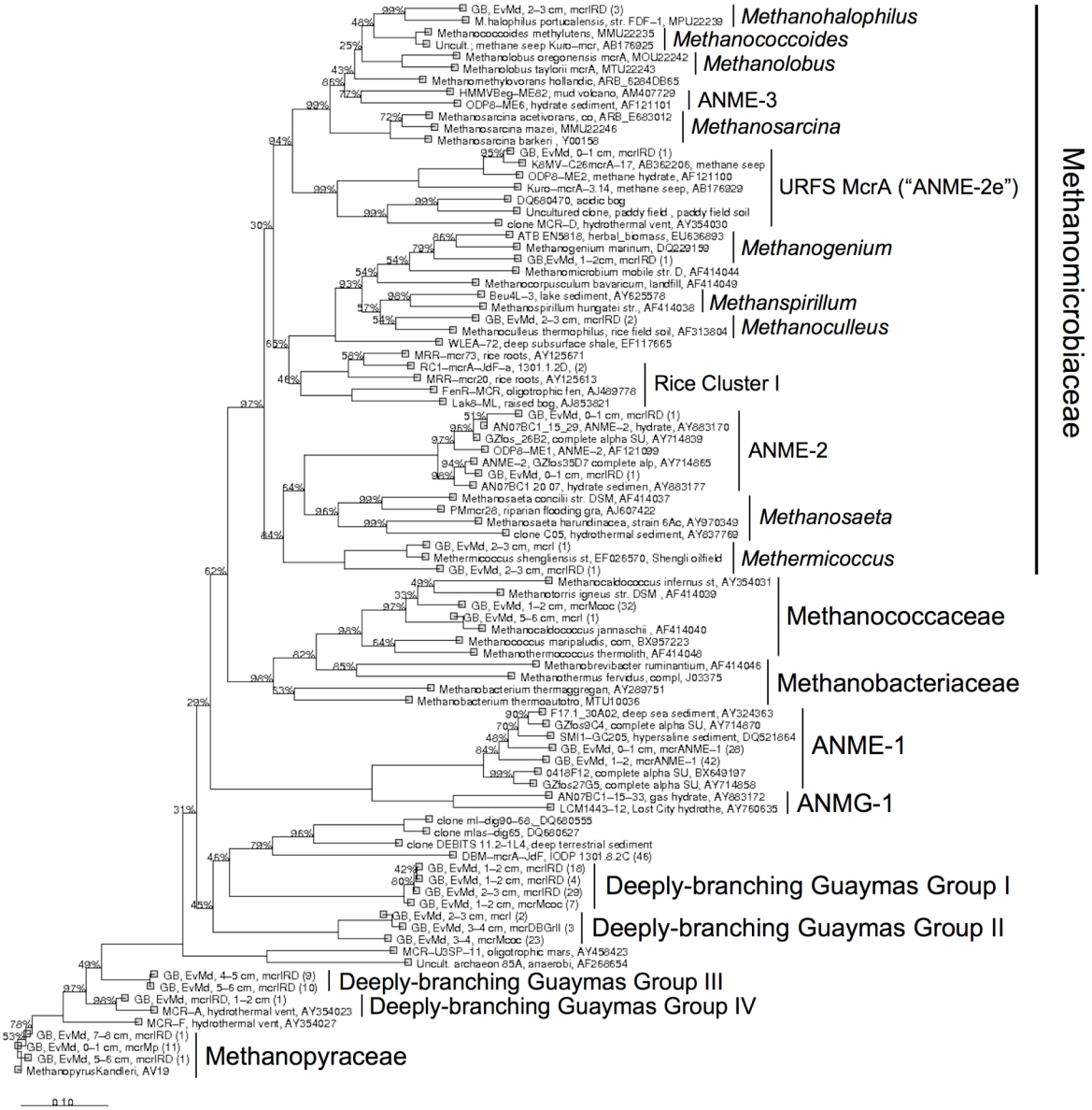


FIGURE 5.1: *McrA* gene phylogeny. Only a small number of representative phylotypes from this study is included. The depth intervals they are from, and the primers used to amplify their *mcrA* sequence are included in the name (GB = Guaymas Basin; EvMd = Everest Mound). Constructed in ARB Neighbor Joining using Jukes-Cantor correction (Ludwig et al. 2003). Bootstrap support (in %, 1,000 replications) is indicated at each branching point, except where bootstrap support was 100%.

TABLE 5.2: Substrate use by closest relatives of the 13 *mcrA* clusters detected, depth intervals and temperature ranges detected in, and number of depths detected in. Includes data obtained with all primer used in this study. [MeOH = methanol, DMS = dimethyl sulfide, TMA = trimethyl amine, + = detected; - = not detected].

		Substrate used by closest relatives													
		H_2/HCO_3^-, formate				MeOH, DMS, TMA		methane		unknown					
Depth (cmbsf)	Temperature (°C)	*Methanogenium*[1]	*Methanoculleus*[1]	*Methanococcus*[1]	*Methanopyrus*[1]	*M.halophilus*[1]	*Methermicoccus*[1]	ANME-1[2]	ANME-2[2]	Unid. Rice Field Soil	DB Guaymas Gr. I	DB Guaymas Gr. II	DB Guaymas Gr. III	DB Guaymas Gr. IV	# of phylotypes (of 13)
0-1	2-8	+	+	-	+	+	+	+	+	+	+	+	-	-	10
1-2	8-14	+	+	+	+	+	+	+	+	-	+	+	-	+	11
2-3	14-20	-	+	-	+	+	+	-	+	-	+	+	-	-	7
3-4	20-25	-	+	-	+	-	+	-	-	-	+	+	-	-	5
4-5	25-31	-	+	-	+	+	-	-	+	-	+	+	+	-	7
5-6	31-37	+	+	+	+	-	+	-	+	-	+	+	+	-	9
7-8	43-49	-	-	-	+	-	-	-	-	-	+	-	-	-	2
9-10	54-60	-	-	-	-	-	-	-	-	-	+	-	-	-	1
12-13	72-78	-	-	-	-	-	-	-	-	-	-	-	-	-	0
15-16	89-95	-	-	-	-	-	-	-	-	-	-	-	-	-	0
19-20	113-118	-	-	-	-	-	-	-	-	-	-	-	-	-	0
# of depths	N/A	3	6	2	7	4	5	2	5	1	8	6	2	1	

belonged to hydrogenotrophs (*Methanogenium*, -*culleus*, -*caldococcus*, -*pyrus*), methylotrophs (*Methanohalophilus*, *Methermicoccus*), and anaerobic methanotrophs (ANME-1, -2). Five clusters without cultured representatives (Unidentified Rice Field Soil *mcrA*, Deeply-branching Guaymas *mcrA* Groups I-IV), and no phylotypes of known aceticlastic genera (*Methanosaeta*, -*sarcina*) were detected. Diversity was highest at the

surface (0-2 cmbsf), and lowest in the lowest two horizons (7-8 and 9-10 cmbsf). There is no clear vertical zonation of phylotypes along the thermal gradients, except that all but two (Deeply-branching Guaymas Group I, *Methanopyrus*) were below detection below 6 cmbsf, certain phylotypes were only detected in surface layers (ANME-1, Unidentified Rice Field Soil *mcrA*, Deeply-branching Guaymas Group IV), and one only at mid-sediment intervals (Deeply-branching Guaymas Group III). Even though we detected relatives of known hyperthermophiles (*Methanopyrus kandleri*, *Methanocaldococcus jannaschii* and *infernus*), their phylotypes were below detection in sediment horizons with temperatures higher than 60°C.

For each of the three general *mcrA* primer pairs, a decrease in *mcrA* diversity occurred with depth (Fig. 5.2). The detection limit and/or phylogenetic composition of clone libraries generated with the three general *mcrA* primer pairs varies greatly; with the ME1/ME2 and mcr*I* primer pairs, we detect mcrA genes in four of the uppermost 8 depth horizons examined, though not in the same depth horizons (Fig. 5.2). Overall *mcrA* richness is higher in clone libraries generated with the mcr*I* primer pair, which allows detection of all 6 groups amplified with the ME1/ME2 primer pair in addition to three further phylotypes (Table 5.3). The mcr*IRD* primer pair amplified mcrA genes in the 8 uppermost sediment intervals examined and yielded the highest diversity of mcrA genes (Fig. 5.2). *McrA* composition of clone libraries obtained with mcr*I* and mcr*IRD* primer pairs is similar, with an overlap in 8 phylotypes.

We detected only one novel phylotype (ANME-1) using the 27 group-specific primer combinations (Table 5.3). Certain phylotypes were detected in depth intervals, where they

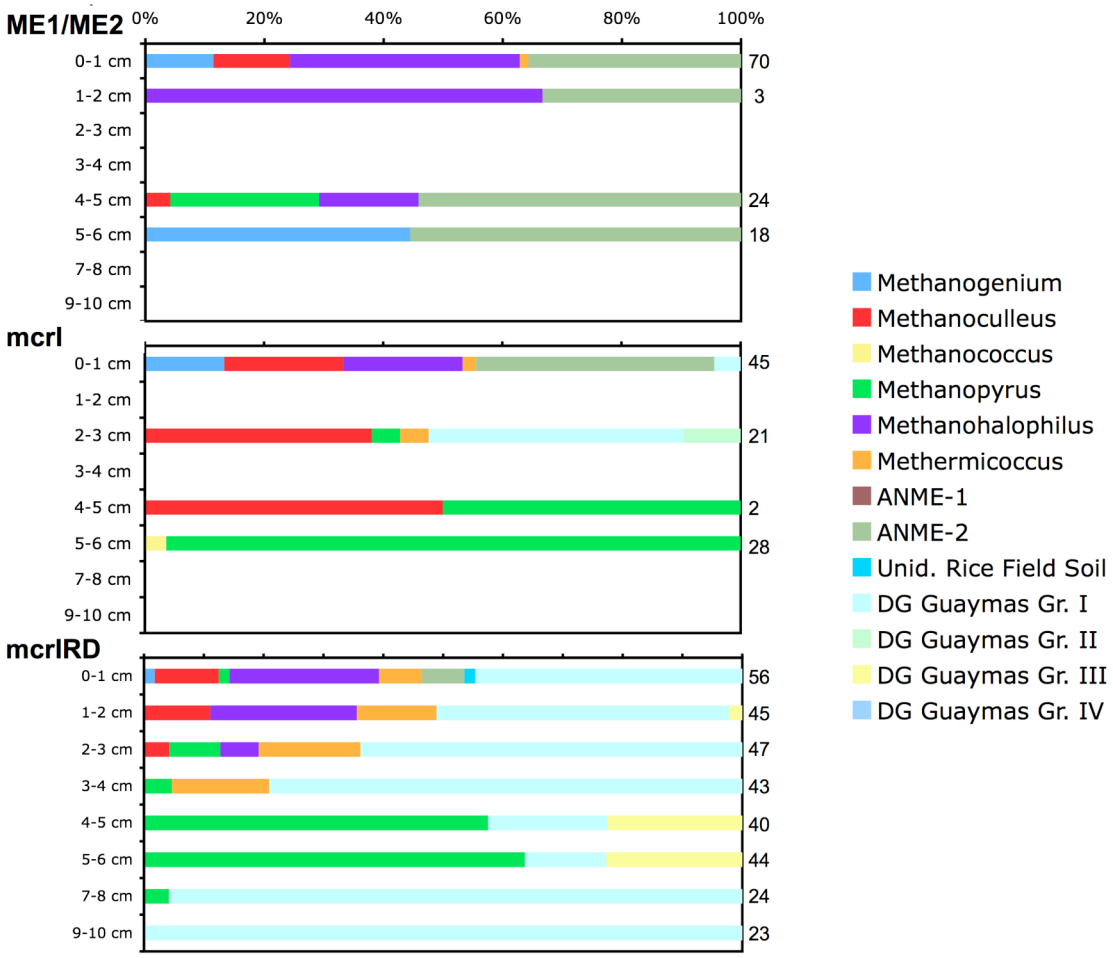


FIGURE 5.2: Depth layers in which mcrA genes were detected, as well as *mcrA* clusters detected within each of these depth layers, using the three general *mcrA* primer pairs (ME1/ME2, mcr*I*, mcr*IRD*).

had not been detected with general primer pairs, e.g. *Methanopyrus* at 1-2 cmbsf (Table 5.3). Primer combinations designed to specifically target groups that had previously not been detected, e.g. mcrMsaeta for *Methanosaeta* or mcrMbac for *Methanobacteriales*, did not result in detection of these groups (Table 5.1, 5.3). Instead they amplified mcrA genes of non-target groups (mcrMbac), or non-mcrA genes (mcrMsaeta). Certain groups detected

TABLE 5.3: *McrA* clusters detected with primers that successfully amplified mcrA genes, depth intervals in which they successfully amplified mcrA genes, and total number of *mcrA* clusters detected with each primer pair.

	Primer Coverage													
Depth (cmbsf)	*Methanogenium*[1]	*Methanoculleus*[1]	*Methanococcus*[1]	*Methanopyrus*[1]	*M.halophilus*[1]	*Methermicoccus*[1]	ANME-1[2]	ANME-2[2]	Unid. Rice Field Soil	DB Guaymas Gr. I	DB Guaymas Gr. II	DB Guaymas Gr. III	DB Guaymas Gr. IV	# *mcrA* clusters (of 13)
ME1/ME2	0-1, 5-6	0-1, 4-5	-	4-5	0-2, 4-5	5-6	-	0-2, 4-6	-	-	-	-	-	6
mcrI	0-1	0-1, 2-3, 4-5	5-6	2-3, 4-6	0-1	0-1, 2-3	-	0-1	-	0-1, 2-3	2-3	-	-	9
mcrIRD	1-2	0-3	1-2	0-1, 2-8	0-3	0-4	-	0-1	0-1	0-10	-	4-6	1-2	11
mcrANME-1	-	-	-	-	-	-	0-2	-	-	-	-	-	-	1
mcrANME-2	-	-	-	-	-	-	-	0-3	-	-	-	-	-	1
mcrMcul	-	-	-	-	-	2-3	-	-	-	-	-	-	-	1
mcrFC	-	1-2	-	-	-	-	-	-	-	-	-	-	-	1
mcrMgen	-	0-4	-	-	-	-	-	-	-	-	-	-	-	1
mcrMm	-	0-3, 5-6	-	-	-	-	-	-	-	-	-	-	-	1
mcrMbac	0-1	-	-	-	0-2	0-1	-	-	-	-	-	-	-	3
mcrMcoc	-	-	-	-	-	-	-	-	-	1-2, 3-4	2-4	-	-	1
mcrDBGrI	-	-	-	-	-	-	-	-	-	0-10	-	-	-	1
mcrDBGrII	-	-	-	-	-	-	-	-	-	-	0-2, 3-6	-	-	2
mcrMp	-	-	-	0-8	-	-	-	-	-	-	-	-	-	1

with general primers, e.g. *Methanogenium*, or *Methanohalophilus*, were not detected at all using group-specific primers (Table 5.1, 5.3).

DISCUSSION

We found no clear zonation along the thermal gradient, except that (1) certain groups occur deeper than others (*Methanopyrus*, Deeply-branching Guaymas *mcrA* Group I), and (2) diversity was highest near the sediment surface (0-2 cmbsf), and lowest in deeper horizons (7-8 and 9-10 cmbsf; Table 5.2, Fig. 5.2). A gradual depth-related decrease in diversity is suggested by individual diversity profiles generated with general primers (Fig. 5.2), but this decrease is only partially reflected in the pooled depth-related diversity (Table 5.2). The fact that several phylotypes, e.g. *Methanogenium*, *Methanocaldococcus*, ANME-2, appear absent from one of the middle layers (2-4 cmbsf), but present above (0-2 cmbsf) and below (4-6 cmbsf) suggests temperature-unrelated changes with depth. Perhaps the sulfate and volatile fatty acid concentration profiles will provide an explanation (to be performed by Dan Albert).

McrA genes were not detected below 10 cmbsf. Given the presence of relatives of hyperthermophiles such as *Methanopyrus kandleri* (temperature maximum: 122˚C; Takai et al. 2008) and *Methanocaldococcus jannaschii* (temperature maximum: 85˚C; Jones et al. 1983), the lack of *mcrA* detection below 10 cmbsf, at temperatures >60°C is curious, and we can only speculate about possible reasons. One possible explanation is that concentrations of volatile fatty acids become toxic below. Very high concentrations of thermogenic acetate (>1 mM) were documented in deeper sediment horizons at a nearby site at Everest Mound (Dhillon et al. 2005). High concentrations of acetate are known to be toxic to methanogens in sewage digestors (Zeikus 1980). Alternatively, thermocatalysis of organic matter in

deeper layers may drive the pH below levels tolerated by methanogens, as has been documented before (Phelps and Zeikus 1984). Upward flow of hydrothermal fluid may, furthermore, cause a constant flux of high concentrations of volatile fatty acids or protons to upper layers of sediment. Methanogens and methanotrophs may be confined to the uppermost 10 cm where diffusive mixing with overlying seawater keeps VFA concentrations and/or pH tolerable. The occurrence of *Methanopyrus* and Deeply-branching Guaymas mcrA Group I in deeper sediment layers may reflect their ability to cope with high VFA concentrations or low pH better than the other groups, and not indicate ability to tolerate higher temperatures. Alternatively, it is possible that there is no zonation at all, and that overall *mcrA* template concentrations decrease with depth into sediments. Accordingly, all but the two most abundant phylotypes, *Methanopyrus* and Deeply-branching Guyamas mcrA Group, would be diluted to extinction in the lowermost layers. This scenario would require all three general primer pairs tested to be biased against *Methanopyrus*, since *Methanopyrus* does not dominate clone libraries in the shallower layers (0-4 cmbsf). We examined this possibility using the ARB phylogenetic software program and found that neither the mcrI nor the mcrIRD primer pairs had any mismatches with published *Methanopyrus* mcrA sequences, while the ME1/ME2 had a total of 5 mismatches (3 forward, 2 reverse). Therefore, at least in the case of *Methanopyrus* mcrA genes, a change in frequency relative to other mcrA phylotypes with depth, i.e. an *mcrA* gene zonation, appears likely. Numbers of mismatches as a source of PCR bias can currently not be examined for the Deeply-branching Guaymas McrA Group, due to the absence of *mcrA* sequences that are inclusive to general and group-specific primer sequences (note: the ME1/ME2 primer pair did not amplify this group).

We detected relatives of known methylotrophic and hydrogenotrophic methanogens, but not of aceticlastic methanogens. Possibly acetate is being utilized by other organisms, e.g. sulfate reducers. Alternatively, acetate may be consumed by one or several of the unknown clusters. Of the latter, the Unidentified Rice Field Soil *mcrA* group (Chin et al. 2004), also termed Cluster M-C (Nercessian et al. 2005) or wrongly grouped with, despite not being monophyletic with ANME-2 (Nunoura et al. 2006) is a genus within the Methanosarcinales (Fig. 5.1) that has previously been found at methane seeps, methane hydrates, hydrothermal vents, deep subsurface sediments and subseafloor basalt (Bidle et al. 1999, Hallam et al. 2003, Nercessian et al. 2005, Nunoura et al. 2006, Lever et al., in prep)., in addition to freshwater environments (Chin et al. 2004). Deeply-branching Guaymas mcrA Groups I-IV unanimously lack similar environmental sequences: the Deeply-branching Guaymas mcrA Group I has a maximum sequence similarity of 73% to its closest relative in GenBank, and the Deeply-branching Guaymas mcrA Group II has a maximum sequence similarity of 70% to its closest relative. The Deeply-branching Guaymas mcrA Group III had 89% sequence similarity to its closest environmental sequence and 85% to its closest cultured relative (*Methanopyrus kandleri*), whereas the Deeply-branching Guaymas mcrA Group IV had 94% similarity to its closest relative among environmental sequences (Mcr-A, hydrothermal vent, AY354023; Fig. 5.1) and 88% sequence similarity to its closest cultured relative (*Methanopyrus kandleri*). Due to the lack of knowledge about these 5 groups, the presence/absence of aceticlastic methanogens can currently not be determined.

Of the three general *mcrA* primer pairs tested, the mcr*IRD* primer had the lowest detection limit, as indicated by successful *mcrA* gene amplifications from 8 compared to 4 depth intervals in the case of the mcr*I* and ME1/ME2 primer pairs. Diversity detected was

also higher in the mcr*IRD* primer pair, which may have been partially a result of the greater number of depth intervals with successful amplifications. The ME1/ME2 and mcrI primer pairs were comparable in sensitivity of *mcrA* detection, but differed in diversity detected, the main difference being that the ME1/ME2 primer pair did not detect any of the groups without cultured representatives. None of the general primer pairs detected ANME-1 *mcrA*. The most likely explanation is the high number of mismatches with ANME-1 *mcrA* sequences (Lever, unpubl.), which are the reason phylogenetic studies using the mcrIRD primer pair have been complemented by the use of the *ANME-1-mcrA* primer pair (Chapters III and IV, Ertefai et al. unpubl., Lin et al. unpubl.).

No new phylotypes were detected with the 26 new group-specific primer pairs designed in this study. In fact, the mcrIRD and mcrI primer pairs resulted in detection of groups not detected with specifically designed group-specific primers (e.g. *Methanogenium*). This could be explained with (1) the higher DNA extract volumes used in PCR assays with general primers (10 μL) compared to group-specific primers (1 μL), and (2) stochastic variation in template occurrence in aliquots of DNA extract used in PCR assays, as discussed previously (von Wintzingerode et al. 1997). Use of the same template volumes in all PCR reactions combined with increased PCR replication may resolve this mystery.

Group-specific primer pairs did, however, lower the detection limit for certain groups: using the mcrIRD primer pair 10 μL were required for PCR detection of mcrA genes in the lowermost sediment intervals (7-8 and 9-10 cmbsf), whereas the *Methanopyrus*-specific mcrMp and the Deeply-branching Guaymas mcrA Group I-specific mcrDBGrI primer pairs yielded PCR detection with 1 μL. Moreover, using group-specific primer pairs we were able to detect *Methanopyrus* and Deeply-branching Guaymas mcrA Group I in shallow layers

where they had not been detected using the mcrIRD primer pair. These examples suggest that, unsurprisingly, amplification efficiency is higher with low-degeneracy group-specific primers. Moreover, amplification biases with the mcrIRD primer pair cannot be precluded, despite it targeting a wide phylogenetic breadth of mcrA genes as shown in this study. The solution to this problem may be as simple as generating larger clone libraries, e.g. with 96 instead of 48 clones per depth horizon.

Our results suggest that the mcrIRD primer pair, when combined with the ANME-1-*mcrA* primer pair, covers a wide diversity of mcrA genes of not only previously cultured groups, but also novel, highly-divergent groups. The group-specific primer pairs were designed to target all known mcrA phylogenetic clusters and a variety of regions within the *mcrA* gene. Yet, with the exception of ANME-1, group-specific primer pairs did not amplify new phylotypes, i.e. phylotypes that had not already been amplified with the mcrIRD primer pair. Though we cannot preclude that certain highly divergent *mcrA* sequences were missed by the mcrIRD or group-specific primer pairs, our results show that the combined use of the mcrIRD and ANME-1-*mcrA* primer pairs covers known *mcrA* sequence diversity in addition to yielding novel phylotypes. The mcrIRD primer pair, therefore, unlike the mcrI or ME1/ME2 primer pairs, combines wide phylogenetic breadth with a relatively low detection limit, and illustrates how reduced primer degeneracy, and hence higher amplification efficiency, does not necessarily compromise the breadth of phylotypes targeted.

CHAPTER VI

CONCLUSIONS

i. MAJOR FINDINGS

Design of new PCR probes allowed construction of the first detailed depth profiles of functional genes in the deep subseafloor. Lack of detection of *mcrA* genes in all but one or a few sediment horizons in previous studies (e.g. Parkes et al. 2005) was probably caused by poor detection sensitivity resulting from high degree of primer degeneracy (Springer et al. 2005) or limited phylogenetic coverage (Hales et al. 2006) of previously published *mcrA* primer pairs. With the aid of previously conducted contamination tests (Lever et al. 2006), we were able to show that mcrA genes persist deep into the basaltic basement underlying a 265-m thick layer of sediment. *Fhs* distributions had not been examined previously in marine sediments. We detect a high diversity of vertically zonated *fhs* phylotypes in several depth horizons of a deep sediment column.

Depth distributions of *mcrA* roughly follow sulfate concentration gradients at the two sites where sulfate concentrations were measured (Juan de Fuca Ridge Flank, Peru Trench). The phylogenetic composition of the methanogen- and methanotroph- assemblages changes is different in methnogenesis zones and SMTZs compared to sulfate reduction zones. We detect *mcrA* in sediments with *in situ* temperatures of 2 to 65°C, and lithologies as diverse as diatomaceous clay, silty clay, sand, breccia, and pillow lava, and observe no related change

in phylogenetic composition. These findings suggest a low degree of adaptation towards temperature or lithology among the methanogens and methanotrophs detected. *Fhs* phylotypes are detected in multiple samples in sulfate reduction zones, including SMTZs, but below detection in the methanogenesis zone at IODP Site 1301. Whether this zonation is indeed related to sulfate concentrations, or other environmental variables, such as lithology or temperature, cannot be determined from our limited data set.

Based on studies of pure cultures of methanogens (compiled in Whiticar et al. 1986 and Whiticar 1999), the high stable carbon isotopic fractionations of methane relative to DIC suggest predominantly methane production from H_2/HCO_3^- in the methanogenesis zones at ODP Site 1230 and IODP Site 1301. Due to the absence of pure culture studies of isotopic fractionations produced by aceticlastic methanogens in marine sediments, the $\delta^{13}C\text{-}CH_4$ relative to DIC does not provide conclusive evidence for a predominantly hydrogenotrophic origin of methane, however. The *mcrA* phylotypes detected, with the exception of those from the SMTZ at IODP Site 1301, provide no evidence for hydrogenotrophic methanogenesis. Thermodynamic calculations argue against hydrogenotrophic methanogenesis at ODP Site 1230, but need to be treated with extreme caution: the H_2 concentrations measured are unlikely to reflect *in situ* concentrations due to the high turnover rates of H_2, and artifacts caused by lengthy core retrieval periods. The detection and even dominance of phylotypes most closely related to obligately aceticlastic methanogens in the methanogenesis zones of IODP Site 1301 and ODP Site 1230 suggests at least a partial contribution of aceticlastic methanogenesis. The potential for methanogenesis reactions from acetate is indicated by calculated *in situ* energy yields of aceticlastic methanogenesis. We detected no close relatives of known methylotrophic methanogens in the deep

methanogenesis zones. This does not mean that methylotrophic methanogens were absent; the metabolisms of the Unidentified Rice Field Soil McrA and ANMG-1 groups, each dominant members of mcrA clone libraries from IODP Site 1301 and ODP Site 1230, respectively, are unknown. Moreover, the high variability and in some cases high kinetic isotope effects associated with methylotrophic methanogenesis, that in some cases render the $\delta^{13}C\text{-}CH_4$ produced methylotrophically indistinguishable from hydrogenotrophically produced methane (Summons et al. 1998, Whiticar 1999), may contribute to the high stable carbon isotopic depletion of methane relative to DIC.

Surface sediments at Everest Mound differ from subsurface horizons at ODP Site 1230 and IODP Site 1301 in that methanogen substrate availability is unlikely to be limiting, as indicated by the occurrence of diverse methanogen assemblages, with close relatives of known hydrogenotrophs, in the presence of high sulfate concentrations (Teske et al. 2002, Dhillon et al. 2005). Thermal degradation of organic matter in deep sediment horizons (Martens 1990), and biological degradation of water column-derived organic matter near the surface, are likely sources of methanogen substrates. High substrate supplies are not the only reason for non-limiting substrate availability, however. Under stable conditions, i.e. in the absence of biological disturbance (e.g. viral predation or microbial consumption), chemical disturbance (e.g. fluctuating pH or redox conditions), or physical disturbance (e.g. temperature fluctuation), one would expect a stable, high-biomass microbial community to establish itself over time, and result in competitive exclusion of hydrogenotrophic methanogens, e.g. by sulfate reducers. Disturbance is hence likely to play a significant role in Guaymas sediments, and facilitate the coexistence of methanogens and sulfate reducers consuming the same substrates. Ecological theory (r/K selection; MacArthur and Wilson

1967, Pianka 1994, Weinbauer and Höfle 1998) predicts that under fluctuating environmental conditions with density-independent mortality, i.e. under high rates of disturbance, fast-growing, opportunistic, i.e. r-selected, species will dominate over slow-growing, competitively superior, K-selected species. The latter we would expect to dominate in stable, i.e. low-disturbance, environments. Indeed we see little overlap in the community composition between the Guaymas site and the deep sediment columns at ODP Site 1230 and IODP Site 1301 (Table 6.1). By comparison, ODP Site 1230 and IODP Site 1301 have in common the detection of ANME-1, and the presence of *mcrA* genes of *Methanosaeta*, a genus comprised of classic K-strategists (Smith and Ingram-Smith 2007). ANME-1 were detected only in the top two centimeters at Guaymas, and hence less dominant members of mcrA clone libraries than in the two deep sediment columns, and *Methanosaeta* were below detection. The higher *mcrA* gene diversity in Guaymas sediments might be a further indicator of decreased competition due to non-limiting substrate concentrations caused by disturbance. Yet, without a better understanding of the source of disturbance in Guaymas sediments, and without experimental evidence, our interpretation of the observed compositional and diversity trends remains speculative.

Our results show that ANME-1 Archaeal sequences are not restricted to SMTZs, but can instead be found in methanogenesis (ODP Site 1230, IODP Site 1301; Tables 3.1 and 4.2) and sulfate reduction zones (IODP Site 1301, probably Everest Mound; Table 3.1). The occurrence in SMTZs is consistent with the widely held notion that ANME-1 are methanotrophic. The co-occurrence with putative methanogens (Unidentified Rice Field Soil *McrA*) in the sulfate-reduction zone of IODP Site 1301 could be explained by the presence of a cryptic methane cycle, where methanogenic Unidentified Rice Field Soil McrA produce

TABLE 6.1: Overview of *mcrA* phylotypes and metabolisms of their closest studied relatives at the three sites.

Substrate	IODP Site 1301	ODP Site 1230	Everest Mound	# of phylotypes
H_2/HCO_3^-, formate	Rice Cluster I	-	*Methanogenium*, -*culleus*, -*coccus*, -*pyrus*	5
Acetate	*Methanosaeta*	*Methanosaeta*	-	1
MeOH, DMS, TMA	-	-	*Methanohalophilus*, *Methermicoccus*	2
Methane	ANME-1, -2, -3	ANME-1	ANME-1, -2	3
Unknown	Deeply-branching Methanogen Group, Fen Cluster, Unidentified Rice Field Soil McrA	ANMG-1	Deeply-branching Guaymas Groups I-IV, Unidentified Rice Field Soil McrA	9
# of phylotypes	8	3	13	20

methane from one substrate, and methanotrophic ANME-1 oxidize the methane produced to a compound different from the substrate of methanogenesis. Our thermodynamic calculations suggest that AOM to H_2/HCO_3^- is thermodynamically favorable in the sulfate reduction zone of IODP Site 1301 if it is assumed that H_2-concentrations are thermodynamically controlled by sulfate reducers. The occurrence of ANME-1 *mcrA* meters below the SMTZ at ODP Site 1230, and tens of meters into the methanogenesis zone at IODP Site 1301, is more difficult to reconcile with methanotrophic activity. It is more easily explained if ANME-1 are facultative methanogens. A similar distribution was recently detected in the sediment column of the Black Sea using the same ANME-1 specific *mcrA* primer pair (Ertefai et al., in prep.). Past failure to detect *mcrA* of ANME-1 in methanogenic zones may be explained by our observation that none of the three general *mcrA* primer pairs examined in this study amplified *mcrA* genes of ANME-1, probably due to a high number of mismatches (Lever, unpubl.). It is likely that *mcrA* of ANME-1 are typically only targeted

successfully with general *mcrA* primers in environments where ANME-1 dominate the *mcrA* community, and ANME-1 *mcrA* copy numbers are high enough for PCR detection despite low PCR amplification rates. These environments, e.g. SMTZs or methane seep environments, may often have measurable sulfate concentrations, which might have led to the notion that ANME-1 are obligately methanotrophic.

Depletions of δ^{13}C-acetate by up to 9‰ relative to TOC suggest an acetogenic contribution to the acetate pool at IODP Site 1301. This depletion could also be explained if fermenters or sulfate reducers were selectively metabolizing lipid-derived organic carbon or other isotopically light compounds of the organic carbon pool; however, this interpretation appears less likely, as lipids have been shown to represent a small portion (0.1-1%) of total organic matter deposited to the seafloor (Wakeham et al. 1997), and the bulk amino acid and carbohydrate pool in sediments is typically similar to or enriched in δ^{13}C compared to bulk TOC (Dauwe and Middelburg 1998, Keil and Fogel 2001, Wang and Druffel 2001). The widespread detection of *fhs* sequences indicates a genetic potential for acetogenesis. Thermodynamic calculations suggest that mixo- and methylotrophic acetogenesis reactions are energetically favorable and might be the source of isotopically light acetate. Autotrophic acetogenesis is also possible, but would require H_2 concentrations to not be thermodynamically controlled by sulfate reducers, as has been shown elsewhere in marine sediments (Hoehler et al. 1998). Our data set is insufficient to attribute with certainty a significant contribution of acetogenesis to total acetate production. However, it is the first data set to indicate a potential niche for acetogenesis, a possibility which deserves further inquiry in the future.

ii. FINAL PERSPECTIVE

Combined knowledge from functional gene profiles, geochemical gradients, stable isotopic signatures, and calculated *in situ* free energy yields provides us with several lines of evidence for methanogenic, methanotrophic, and acetogenic activity in subseafloor environments. The detection of functional genes (*mcrA*, *fhs*) indicates the genetic potential for these processes. The presence of RNA suggests which organisms are alive and active, though it is no proof that they are indeed performing methanogenesis, methanotrophy, or acetogenesis. Concentration gradients and stable carbon isotopic depletions of methane and acetate relative to source compounds are, however, strong evidence for the presence of active methanogenic, methanotrophic, and acetogenic populations. Calculated *in situ* energy yields suggest the thermodynamic potential of certain reactions to serve as energy sources. Yet, there is a discrepancy between calculated *in situ* energy yields, metabolism inferred from stable carbon isotopic signatures and biogeochemical zone of origin, and metabolism of closest known relatives to phylotypes detected. This discrepancy is evidence for the difficulty of determining *in situ* substrate and product concentrations, and our currently insufficient knowledge of the substrates used and the substrate-dependent fractionations associated with methanogenic, methanotrophic and acetogenic reactions. This conundrum is most easily resolved experimentally, and will be a main objective of my postdoctoral studies.

APPENDIX A

OVERVIEW OF SUBSTRATE USE, GENES SEQUENCED (16S, *FHS*), HABITAT ISOLATED FROM, AND REFERENCES USED TO CONSTRUCT TABLE 2.3 ['ND' = NOT DETERMINED].

Scientific name	Substrates							Reference	16S	*fhs*	Habitat isolated from
			aliphatic								
	H_2/CO_2	CO	HCO	MeO	EtOH	Other	o-CH_3				
Acetitomaculum ruminis	+	+	+	?	?	?	+	Greening and Leedle	+	-	Steer rumen
Acetoanaerobium noterae	+	?	-	-	?	-	?	Sleat et al. (1985)	-	-	Swamp sediment
"Acetoanaerobium romashkovii"	+	?	+	+	?	+	?	Davydova-Charakhch'yan et al. (1992)	-	-	Oil field formation water
Acetobacterium bakii	+	+	+	+	-	+	+	Kotsyurbenko et al. (1995)	+	-	Paper-mill waste water
Acetobacterium carbinolicum	+	?	+	+	+	+	+	Eichler and Schink (1984)	+	+	Freshwater sediment, sludge
A. carbinolicum kysingense	+	+	+	+	+	+	+	Paarup et al. (2006)	+	+	Anoxic, fjord sediment
Acetobacterium dehalogenans	+	+	+	+	?	+	+	Traunecker et al. (1991)	-	-	Sewage digester
Acetobacterium fimetarium	+	+	+	-	-	+	+	Kotsyurbenko et al. (1995)	+	-	Digested cattle manure
Acetobacterium malicum	+	?	+	-	-	+	+	Tanaka and Pfennig (1988)	+	-	Freshwater sediment
Acetobacterium paludosum	+	+	+	+	-	+	-	Kotsyurbenko et al. (1995)	+	-	Anaerobic fen sediment
Acetobacterium psammolithicum	+	?	+	+	+	+	+	Krumholz et al. (1999)	+	+	Subsurface sandstone
"Acetobacterium submarinus"	+	?	?	?	?	?	?	Toffin et al. (2004)			?
Acetobacterium tundrae	+	+	+	+	-	+	+	Simankova et al. (2000)	+	-	Tundra soil
Acetobacterium wieringae	+	?	+	-	-	+	?	Braun and Gottschalk (1982)	+	-	Sewage
Acetobacterium woodii	+	?	+	+	-	+	+	Balch et al. (1977), Tschech and Pfennig (1984)	+	+	Estuarine sediment
Acetobacterium sp. AmMan1	+	?	?	?	?	?	+	Dörner and Schink (1991)	-	-	Freshwater sediment
Acetobacterium sp. B10	+	?	?	+	?	?	+	Sembiring and Winter (1989, 1990)	-	-	Waste water pond sediment
Acetobacterium sp. HA1	+	?	+	-	-	+	-	Schramm and Schink (1991)	-	-	Anoxic sewage sludge
Acetobacterium sp.	+	?	+	+	?	?	+	Conrad et al. (1989)	-	-	Lake sediment

HP4											
Acetobacterium sp. KoB58	+	?	+	?	?	+	?	Wagener and Schink (1988)	-	-	Anoxic sewage sludge
Acetobacterium sp. LS1 & LS2	+	?	+	+	+	?	+	Sattley and Madigan (2007)	+	-	Cold Antarctic sediment
Acetobacterium sp. LuPhet1	+	?	-	-	-	+	+	Frings and Schink (1994)	-	-	Sewage sludge
Acetobacterium sp. LuTria3	+	?	+	?	?	+	+	Frings et al. (1994)	-	-	Digested sewage
Acetobacterium sp. MrTac1	+	?	+	+	+	+	+	Emde and Schink (1987)	-	-	Freshwater sediment
Acetobacterium sp. OyTac1	+	+	+	+	+	+	+	Emde and Schink (1987)	-	-	Marine sediment
Acetobacterium sp. RMMac1	+	?	+	+	?	+	+	Schuppert and Schink (1990)	-	-	Marine sediment
Acetobacterium sp. 69	+	?	?	+	+	?	?	Inoue et al. (1992), Bainotti and Nishio (2000)	-	-	Marine sediment
Acetobacterium sp.	+	?	?	?	?	?	?	Kotsyurbenko et al. (1996)	-	-	Tundra wetland soil
Acetohalobium arabaticum	+	+	+	-	?	-	?	Zhilina and Zavarzin (1990)	+	-	Cyanobacterial mat
Acetonema longum	+	?	-	-	-	+	+	Kane and Breznak (1991)	+	-	Termite gut
Archaeoglobus fulgidus	?	+	?	?	?	?	?	Henstra et al. (2007)			Hydrothermal vent
Butyribacterium methylotrophicum	+	?	-	+	?	?	?	Zeikus et al. (1980)	+	-	Sewage
Clostridium aceticum	+	+	+	-	+	?	+	Wieringa (1936, 1940), Braun et al. (1981), Lux and Drake (1992), Gößner et al. (1994)	+	+	Ditch mud
Clostridium autoethanogenum	+	+	-	?	?	?	?	Abrini et al. (1994)	+	-	Rabbit feces
Clostridium coccoides	-	?	+	?	?	+	+	Kaneuchi et al. (1976), Kamlage et al. (1997)	+	-	Mouse feces, human feces
Clostridium difficile AA1	+	?	?	?	?	?	+	Rieu-Lesme et al. (1998)	+	+	Rumen of newborn lamb
*Clostridium formicoaceticum**	-	+	+	?	?	+	+	Andreesen et al. (1970), Lux and Drake (1992), Gößner et al. (1994)	+	+	Mud
Clostridium glycolicum 22	+	?	?	?	?	?	?	Ohwaki and Hungale (1977)	+	-	Sewage sludge
Clostridium glycolicum CIN5	-	?	-	-	-	?	+	Chamkha et al. (2001)			?
Clostridium glycolicum RD-1	+	?	+	?	?	?	?	Küsel et al. (2001)	+	-	Sea grass nodule
Clostridium ljungdahlii	+	+	-	-	+	-	-	Barik et al. (1988), Tanner et al. (1993)	+	-	Chicken waste
Clostridium mayombei	+	?	+	-	-	+	+	Kane et al. (1991)	+	-	Termite gut
Clostridium metho-xybenzovorans	+	?	+	+	?	+	+	Mechichi et al. (1999), Mechichi et al. (2005)	+	-	Sewage
Clostridium scatologenes	+	+	+	-	+	-	+	Weinberg and Ginsbourg (1927), Küsel et al. (2000)	+	-	Coal, coal mine sediment
Clostridium ultunense	+[a)]	-	+	-	-	?	-	Schnürer et al. (1996)	+	-	Swine manure digester
Clostridium sp. CV-	+	?	?	+	?	?	?	Adamse and Velzeboer	-	-	Sludge

AA1								(1982)			
Clostridium sp. M5a3 & F5a15	+	?	+	?	+	?	?	Bernalier et al. (1996b), Leclerc et al. (1997a, b)	-	-	Human feces
Clostridium sp. Ag4f2 & TLN2	+	?	+	?	+	?	?	Bernalier et al. (1996b)	-	-	Human feces
Desulfoarculus baarsii	CO_2/CO-formate			?	?	?	?	Jansen et al. (1984)	+	+	?
Desulfosporomusa polytropa	+	?	+	+	+	+	+	Sass et al. (2004)	+	-	Oligotrophic lake sediment
Desulfosporosinus orientis	+	?	+	+	+	?	+	Campbell and Postgate (1965), Klemps et al. (1985), Hanselmann et al. (1995), Stackebrandt et al. (1997)	+	-	soil
Desulfotomaculum gibsoniae	+	?	+	?	?	?	+	Kuever et al. (1993), Kuever et al. (1999)	-	-	Freshwater mud
Desulfotomaculum thermobenzoicum	?	?	?	?	?	?	+	Tasaki et al. (1992)			?
Eubacterium aggregans	+	-	+	+	?	?	+	Mechichi et al. (1998)	+	-	Sewage
Eubacterium limosum	+	+	-	+	-	?	?	Sharak-Genthner et al. (1981), Chang et al. (1998)	+	+	Sheep rumen, sewage
Holophaga foetida	-	-	-	-	-	-	+	Bak et al. (1992), Liesack et al. (1994)	+	-	anoxic freshwater mud
Homoacetogen strain MC	+	+	+	?	?	+	+	Traunecker et al. (1991)	-	-	Sewage sludge
Homoacetogenic Bacterium strain TMBS 4	-	-	-	-	-	-	+	Bak et al. (1992), Kreft and Schink (1993)	-	-	?
Methanosarcina acetivorans	-	+	?	?	?	?	?	Rother and Metcalf (2004)			Marine sediment
Moorella glycerini	-	-	-	-	-	+	?	Slobodkin et al. (1997)	+	-	Hot spring
Moorella mulderi	+	-	+	+	?	-	?	Balk et al. (2003)	+	-	Thermophilic bioreactor
Moorella thermoacetica	+	+	+	+	+	-	+	Fontaine et al. (1942), Kerby and Zeikus (1983), Drake and Daniel (2004)	+	+	Horse manure, ?
Moorella thermoautotrophica	+	?	+	+	-	-	?	Wiegel et al. (1981)	+	-	Mud and wet soils
Ocobacter pfennigii	-	+	-	-	-	?	+	Krumholz and Bryant (1985)	+	-	Steer rumen fluid
Ruminococcus hydrogenotrophicus	+	?	?	?	?	?	?	Bernalier et al. (1996c)	+	-	Human feces
Ruminococcus productus U1	+	+	-	-	?	+	+	Lorowitz and Bryant (1984), Lux et al. (1990), Misoph et al. (1996)	+	+	Sewage sludge
Ruminococcus productus Marburg	+	+	-	+	?	?	?	Geerligs et al. (1987)	+	-	Sewage sludge
Ruminococcus schinkii	+	?	+	-	-	+	+	Rieu-Lesme et al. (1996b)	+	-	Lamb rumen
Ruminococcus sp. TLF1	+	?	?	?	+	?	?	Bernalier et al. (1996b)	-	-	Human feces
Sporomusa acidovorans	+	?	+	+	-	+	?	Ollivier et al. (1985)	+	-	Distillery effluent
Sporomusa aerivorans	+	?	?	?	?	?	?	Boga and Brune (2003)	+	-	Termite guts
Sporomusa malonica	+	?	+	+	+	+	+	Dehning et al. (1989)	+	-	Freshwater sediment
Sporomusa ovata	+	?	+	+	+	+	?	Möller et al. (1984)	+	+	Freshwater mud, sugar

											beet silage
Sporomusa paucivorans	+	?	+	+	+	+	?	Hermann et al. (1987)	+	-	Freshwater sediment
Sporomusa silvacetica	+	?	+	+	+	+	+	Kuhner et al. (1997)	+	-	Forest soil
Sporomusa sphaeroides	+	?	+	+	+	+	?	Möller et al. (1984)	+	-	Freshwater mud, sludge, soil
Sporomusa termitida	+	+	+	+	+	?	+	Breznak et al. (1988)	+	+	Termite gut
Sporomusa sp. DR5, DR6 & DR1/8	+	?	?	?	+	+	?	Rosencrantz et al. (1999)	+	-	Anoxic flooded soil
Thermacetogenium phaeum	+	?	+	+	+	+	+	Hattori et al. (2000)	-	-	Kraft-pulp waste water
Thermoanaerobacter kivui	+	?	?	-	-	-	?	Leigh et al. (1981)	+	+	Lake sediment
Treponema primitia ZAS-1	+	-	-	-	?	?	-	Graber et al. (2004)	+	+	Termite gut
Treponema primitia ZAS-2	+	-	-	-	?	?	+	Graber et al. (2004)	+	+	Termite gut
Unclassified:											
AOR	+	?	+	?	?	?	?	Zinder and Koch (1984), Lee and Zinder (1988)	-	-	Thermophilic digester
CS1 Van	-	-	-	+	?	-	+	Wolin and Miller (1993)	-	-	Human feces
CS3Glu	+	-	-	+	?	-	-	Wolin and Miller (1993)	-	-	Human feces
D	+	?	?	+	-	+	?	Rieu-Lesme et al. (1995)	-	-	Deer rumen
DMG58	+	?	+	+	+	+	?	Möller et al. (1984)	-	-	River mud
HA	+	?	?	?	?	?	?	Miller and Wolin (1995)	-	-	Horse feces
I52	-	?	+[b]	?	?	?	?	Wolin and Miller (1994)	-	-	Human feces
S5a2	+	?	?	?	+	?	?	Bernalier et al. (1996b), Leclerc et al. (1997a, b)	-	-	Human colon
Ser 8	+	?	?	?	?	?	?	Chaucheyras et al. (1995)	-	-	Sheep rumen
SS1	+	?	?	?	?	?	+	Liu and Suflita (1993)	-	-	Coastal plain sediment
TH-001	-	?	-	?	?	?	+	Frazer and Young (1985)	-	-	Sewage sludge
VK64	+	?	+	?	+	?	?	Bernalier et al. (1996b)	-	-	Human colon
X-8	+	?	?	?	-	?	?	Samain et al. (1982)	-	-	Digester
ZB	+	-	+	+	?	-	-	Nozhevnikova et al. (1994)	-	-	Pond sediment
ZM	+	+	+	-	?	+	+	Nozhevnikova et al. (1994)	-	-	Pond sediment
ZS	+	+	+	+	?	-	+	Nozhevnikova et al. (1994)	-	-	Pond sediment
ZT	+	+	+	+	?	-	?	Nozhevnikova et al. (1994)	-	-	Pond sediment
417/2	+	?	+	+	?	?	?	Davydova-Charakhchyan et al. (1992)	-	-	Oil field
417/5	+	?	+	+	?	?	?	Davydova-Charakhchyan et al. (1992)	-	-	Oil field
"New acetogenic bacterium"	+	-	?	?	?	+	+	Rieu-Lesme et al. (1996a)	+	-	Ungulate rumens

a) resting cells; no growth observed.
b) requires CO_2 in addition to formate.

APPENDIX B

OVERVIEW OF GENBANK ACCESSION NUMBERS (ACC. NO.), DOCUMENTED SUBSTRATE USE, AND REFERENCES OF PHYLOTYPES DISPLAYED IN FIG. 2.5. [ABBREVIATIONS: MEOH = METHANOL; ETOH = ETHANOL; ALIPHATIC = ALIPHATIC COMPOUNDS OTHER THAN ETHANOL (MOST NOTABLY LACTATE); O-CH_3 = METHOXYLATED AROMATIC COMPOUNDS.]

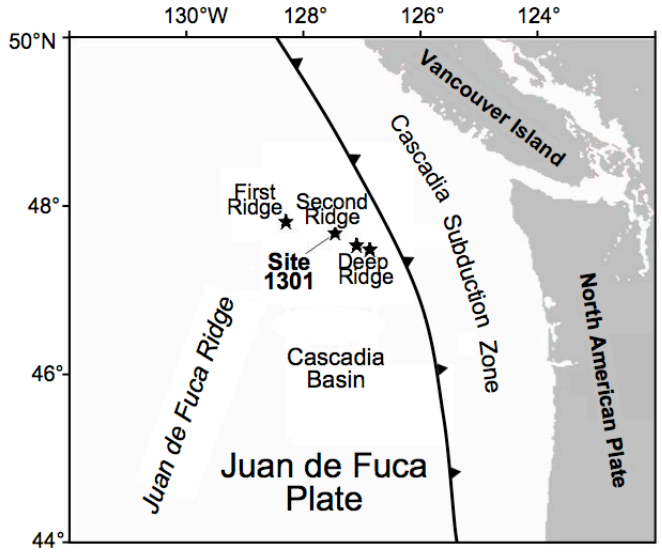
		Substrate							
Acetogens	Acc. No.	**H_2/CO_2**	**CO**	**$HCOO^-$**	**MeOH**	**EtOH**	**aliphatic**	**o-CH_3**	References
Acetobacterium carbinolicum	DQ152902	+	?	+	+	+	+	+	Paarup et al. 2006
Acetobacterium woodii	AF295701	+	?	+	+	-	+	+	Balch et al. 1977
Clostridium aceticum	AF295705	+	+	+	-	+	?	+	Wieringa (1936, 1940), Braun et al. (1981), Lux and Drake (1992), Gößner et al. (1994)
Desulfoarculus baarsii	AJ494749	CO_2/CO-formate			-	-	-	?	Rabus et al. (2006); Leaphart et al. (2003)
Moorella thermoacetica	J02911	+	+	+	+	+	-	+	Fontaine et al. (1942), Kerby and Zeikus (1983), Lovell et al. (1990), Drake and Daniel (2004)
Ruminococcus productus	AF295707	+	+	-	+	-	?	?	Leaphart and Lovell (2001)
Sporomusa ovata	AF295708	+	?	+	+	+	+	?	Moller et al. (1984), Leaphart and Lovell (2001)
Thermoanaerobacter kivui	AF295704	+	?	?	-	-	-	?	Leigh et al. (1981), Leaphart and Lovell (2001)
Treponema primitia, ZAS-1A	AY162313	+	-	-	-	?	?	-	Leadbetter et al. (1999), Graber et al. (2004)
Non-Acetogens									
Candidatus pelagibacter ubique	CP000084	Aerobic chemoorganotroph.							Giovannoni et al. 2005
Clostridium magnum	AF295703	Ferments to acetate.							Leaphart and Lovell (2001)
Corynebacterium diphtheriae gravis	BX248357	Chemoorganotroph; facultative anaerobe.							Cerdeno-Tarraga et al. (2003)
Corynebacterium jeikeium	CR931997	Aerobic chemoorganotroph.							Jackman et al. (1987), Tauch et al. (2005)
Desulfomicrobium baculatum	AJ494755	SRB that oxidizes to acetate.							Leaphart et al. (2003)
Desulfovibrio piger	AJ494750	SRB that oxidizes to acetate.							Moore et al. (1976), Loubinoux et al. (2002)
Desulfotomaculum reducens	CP000612	SRB and metal reducer.							Tebo et al. (1998)
Desulfovibrio desulfuricans	AJ494753	SRB; oxidizes C_{org} to acetate							Hayward et al. (1959), Postgate et al. (1966)
Desulfovibrio salexigens	AJ494751	SRB; oxidizes C_{org} to acetate.							Postgate et al. (1966)
Granulibacter bethesdensis	CP000394	Aerobic chemoorganotroph.							Greenberg et al. (2006)
Haloarcula marismortui	AY596296	Chemoorganotroph w/							Oren et al. (1990), Baliga et al. (2004)

		O_2 and NO_3^- as e^--acceptors.	
Mesorhizobium loti	BA000012	Ferments carbohydrates.	Jarvis et al. (1982), Kaneko et al. (2000)
Methanocorpusculum labreanum	CP000559	H_2-CO_2 and formate utilizing MG.	Zhao et al. (1989)
Methylibium petroleiphilum	CP000555	Aerobic methylotroph.	Hanson et al. (1999), Kane et al. (2007)
Methylobacterium extorquens	AY279316	Aerobic facultative methylotroph.	Bousfield et al. 1985, Vorholt et al. (2000), Kato et al. (2005)
Proteus vulgaris	AF295710	Ferments carbohydrates.	Rustigian and Stuart (1943), Brenner et al. (1995)
Rhodobacter sphaeroides	CP000577	Anoxygenic phototroph.	Imhoff et al. (1984), Choudhari et al. (2007)
Roseovarius nubinhibens	AALY010 00001	Aerobic chemoorganotroph.	Gonzalez et al. (1999), Gonzalez et al. 2003)
Sphingomonas paucimobilis SYK-6	AB186750	Aerobic chemoorganotroph.	Holmes et al. (1977), Nishikawa et al. (1998)
Thermoplasma acidophilum	AL445067	Facultatively anaerobic chemoorganotroph.	Darland et al. (1970), Ruepp et al. (2000), Huber and Stetter (2006)
Treponema azotonutricium, ZAS-9	AY162316	Fermenter.	Leadbetter et al. (1999), Graber et al. (2004)
Treponema denticola	NC_00296 7	Amino acid fermentation	Chan et al. (1993), Seshadri et al. (2004)
Unknowns			
clone G 61	AB353094	?	Hori et al., unpubl.
clone JI5G	AJ494763	?	Leaphart et al. (2003)
clone JI35G	AJ494772	?	Leaphart et al. (2003)
clone JI38G	AJ494773	?	Leaphart et al. (2003)
clone T	AY162309	?	Leadbetter et al. (1999)
clone SAL11	AJ494800	?	Leaphart et al. (2003)
Cs18	DQ278253	Presumably non-AG.	Pester and Brune (2006)
Marinomonas sp. MED 121	AANE010 00003	Unknown; other Marinomonads are aerobic chemoorganotrophs	Other Marinomonads: Solano et al. (1999), Romanenko et al. (2003), Ivanova et al. (2005), Macian et al. (2005), Prabagaran et al. (2005), Yoon et al. (2005), Gupta et al. (2006)
Reinekea sp. MED297	AAOE010 00018	Unknown; aerobic chemoorganotroph?.	Similar species: Romanenko et al. (2004), Pinhassi et al. (2007)
Rs249	DQ278204	Presumably autotrophic AG.	Pester and Brune (2006)
Sulfate-reducing bacterium BG14	AJ494756	?	Leaphart et al. (2003)
Sulfitobacter sp., NAS-14.1	AALZ010 00005	?; cultured *Sulfitobacter* are aerobic chemoorganotrophs.	Sorokin (1995), Pukall et al. (1999), Labrenz et al. (2000), Ivanova et al. (2004)

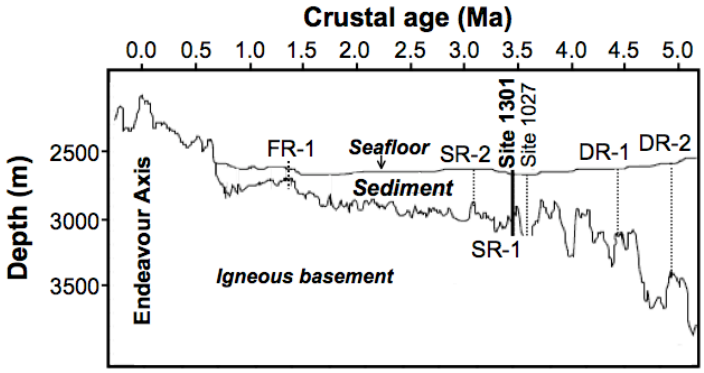
APPENDIX C

(A) MAP OF THE IODP SITE 1301 STUDY AREA. (B) WATER DEPTH, CRUSTAL AGE, SEDIMENT AND BASEMENT TOPOGRAPHY OF THE EASTERN FLANK OF THE JUAN DE FUCA RIDGE (FR = FIRST RIDGE, SR = SECOND RIDGE, DR = DEEP RIDGE). (C) CROSS-SECTIONS OF TYPICAL SEDIMENT AND BASALT CORES RETRIEVED FROM IODP SITE U1301.

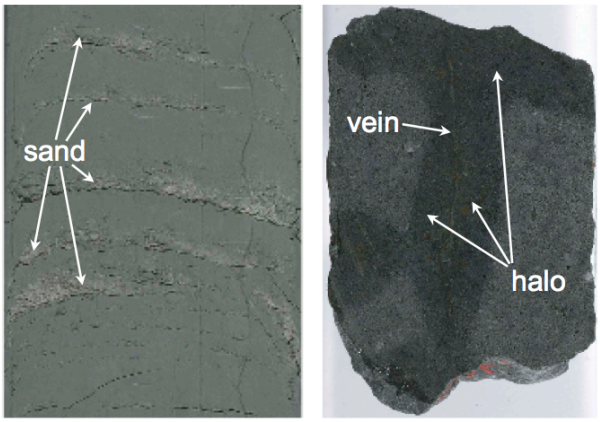
A



B

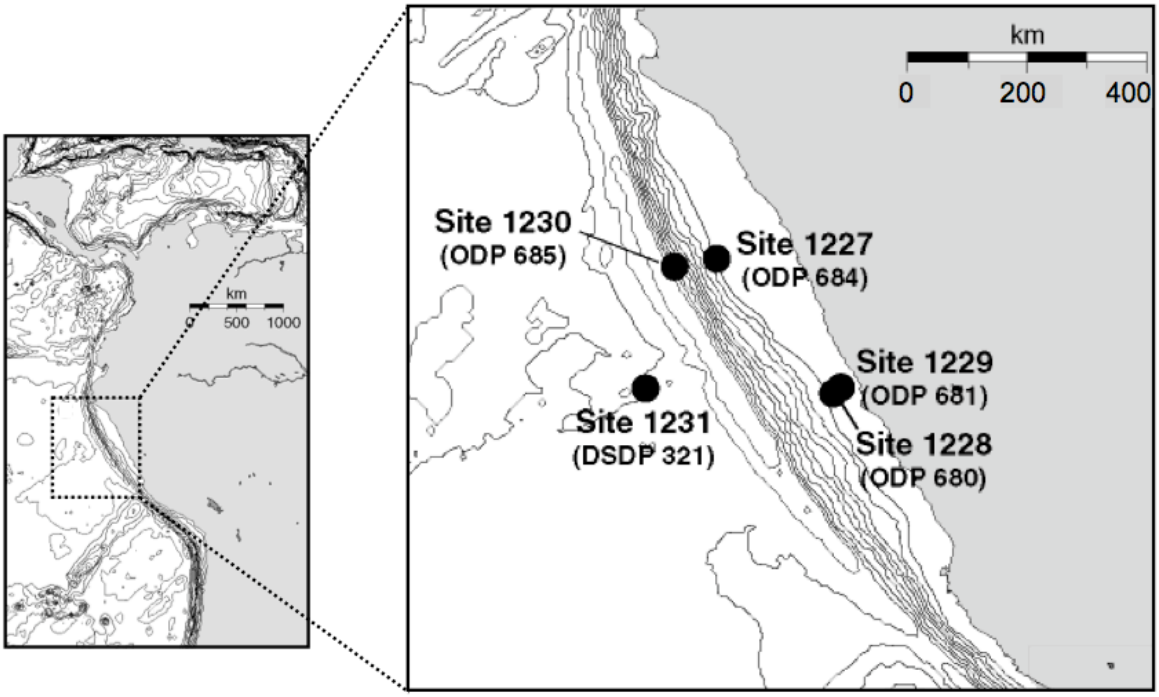


C



APPENDIX D

MAP OF STUDY PERU MARGIN AND SITES SAMPLED DURING ODP LEG 201 AND ODP LEG 112 (IN PARENTHESES) (ADAPTED FROM D'HONDT ET AL. 2003).



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