IN SITU AND ENHANCED COAL-BED METHANE PRODUCTION FROM THE POWDER RIVER BASIN

by

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ABSTRACT

The majority of the coal in the Powder River Basin (PRB) is located in formations too deep to be economically mined but microorganisms within some of these deep coal seams generate coal-bed methane (CBM) which can be harvested and utilized as an energy source. However, little is known about the *in situ* microbial community, the environmental conditions conducive to CBM production, or the microbial community interactions that promote CBM production. Several sampling locations within the PRB were identified as methane-producing sites based on geochemical analysis of groundwater. A diffusive microbial sampler (DMS) was utilized for microbial sampling which was loaded with coal and only opened at the bottom of the wells where the coal seam was exposed. Pyrotag analysis of DMS coal identified the predominant *in situ* bacterial and archaeal populations, providing insight into microbes generating CBM within the PRB. Changes in the composition and structure of microbial communities that occur under stimulated conditions were investigated by applying molecular methods in combination with cultivation techniques (with and without nutrient supplementation) to identify conditions which maximize methane production in batch, bench-scale incubations. Results from these studies indicated the addition of yeast extract resulted in an increase in methane production as well as a shift to a microbial population capable of acetate production and/or acetate utilization. Isolation methods targeting coal utilizing *Bacteria* and methanogenic *Archaea* were applied in addition to DNA based methods to infer microbial community members present within coalbeds. The acetoclastic methanogen *Methanosarcina* was isolated which is the only identified methanogen with the high-efficiency acetate kinase (Ack) / phosphotransacetylase (Pta) methane production pathway. This pathway provides increased growth and methane production when acetate concentrations are high which can result from microbial stimulation with nutrients. Genomic analysis revealed Ack evolved through gene duplication and divergence of acetyl CoA synthetase within the methanogenic genome. This research provided novel insight into the evolution of the high-efficiency Ack/Pta pathway. Collectively, this dissertation presents a novel link between the Ack/Pta pathway, stimulated CBM production and genomic insight into the development of this pathway.

CHAPTER 1 - INTRODUCTION

Methane Production

The simplest alkane, one of the most abundant organic compounds on Earth, and the main component of natural gas, methane, has been a byproduct of microbial metabolism for over 3.46 Byr (Kvenvolden and Rogers, 2005; Ueno and others, 2006). Methane production, carried out exclusively by methanogenic Archaea (methanogens), is an essential part of the global carbon cycle (Boone, Whitman, & Rouvere, 1993). Methanogens convert bacterial metabolic waste into methane thereby allowing the bacterial breakdown of complex organic carbon compounds that would otherwise be thermodynamically unfavorable due to build-up of hydrogen and/or other metabolites (Schink, 1997). Methanogens are strict anaerobes, and typically occupy anoxic zones depleted of more common electron acceptors such as iron and sulfate. Methanogens contain enzymes capable of producing methane via CO_2 and H_2 (hydrogenotrophic methanogenesis), acetate (acetoclastic methanogenesis) and/or one-carbon compounds (*e.g.*, formate, methanol, methylamines and methylthiols) (methylotrophic methanogenesis) (Fig. 1) (Gribaldo and Brochier-Armanet, 2006; Ferry and House, 2006). Due to sampling limitations, knowledge about the methanogenic substrates and pathways that favor different environmental conditions has been limited to surface (peatland) environments, which indicate oligotrophic environmental conditions favor hydrogenotrophic methanogenesis while mesotrophic conditions encourage much higher methane production rates and acetoclastic methanogenesis (Galand and others, 2010).

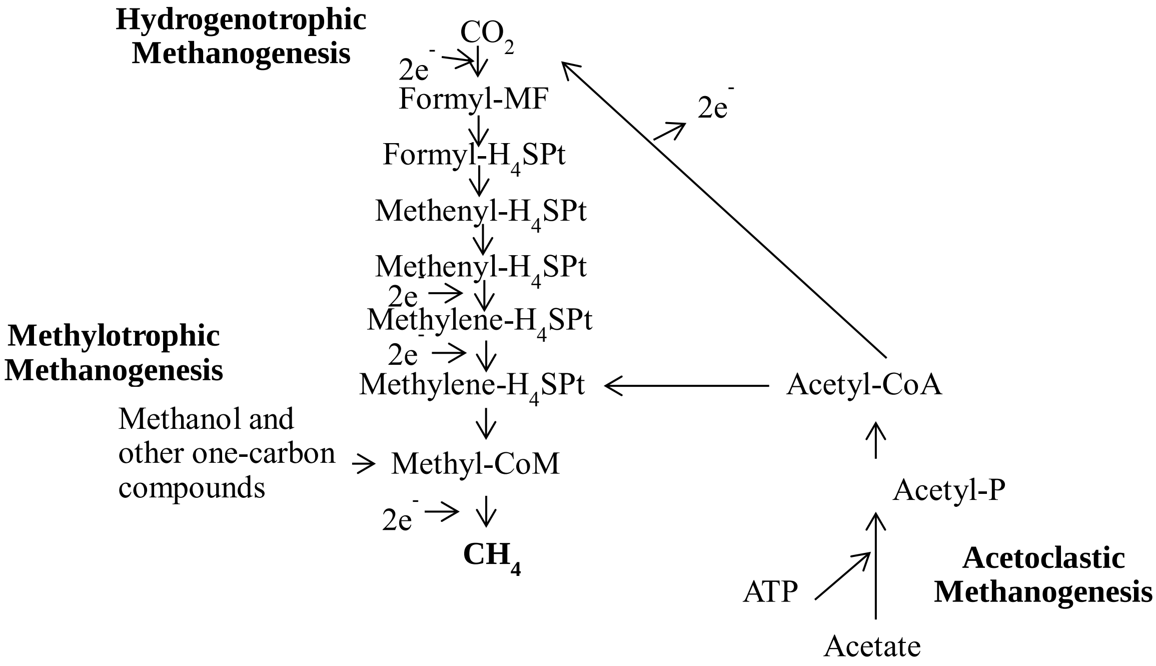

Figure 1: Identified pathways utilized by methanogens to produce methane. Some methanogens only exhibit one pathway while others such as *Methanosarcina* spp. have the genes for all of the pathways but are still generally considered acetoclastic methanogens.

Coalbed Methane Production

Methane within deep underground coal seams called coal-bed methane (CBM) originates from both biogenic and thermogenic processes and is harvested as an energy source in many parts of the world including the United States. The focus of this dissertation is the Powder River Basin (PRB) in Southeastern Montana and Northeastern Wyoming which contains almost entirely biogenic CBM (Strąpoć and others, 2011). Typical estimates for the recoverable CBM from the PRB range from 15-30 trillion cubic feet which is significant considering 1 trillion cubic feet of CBM could power 400,000 households for 20 years (Bauder, 2007). The combustion of CBM produces less nitrogen oxides, carbon dioxide, sulfur dioxide and mercury compounds than burning coal or oil but current CBM production techniques are associated with major pollution issues. The development of CBM involves pumping large volumes of water to the surface through wells that access the coal-bed. Approximately ten years of this type of development resulted in over 4000 produced water ponds in the PRB, causing environmental concerns such as elevated metal and salt concentrations for nearby freshwater (Sowder and others, 2008). An additional limitation with current development is a short production life of the CBM well (<10 yr average) (Meredith and others, 2012). When CBM production was initiated in early 1990s in the PRB very little was known about complex subsurface microbial communities producing CBM. An increased understanding of the functioning *in situ* microbial community producing CBM could identify new sustainable development strategies that support sustained CBM production in the PRB and in other coal-beds worldwide.

Sampling Subsurface Microorganisms

Interest in CBM producing microbial communities has increased dramatically in recent years with the notion that stimulating methane production *in situ* may enhance the life of a CBM well (Strąpoć and others, 2011; Strąpoć and others, 2008; Green and others, 2008; Guo and others, 2012; Jones and others, 2010; Faiz and Hendry, 2006; Klein and others, 2008; Flores and others, 2008; Beckmann and others, 2011; Penner and others, 2010; Unal and others, 2012; Ulrich and Bower, 2008). Many studies investigating the CBM producing microbial community have been restricted due to drilling fluid contamination of cores or an uncertainty of the microbial source when water samples are used as an inoculum (Klein and others, 2008). A novel diffusive microbial sampler (DMS) was utilized in the work presented in Chapters 2, 3 and 4 that was loaded with autoclaved PRB coal and only opened at the bottom of the well where the coal seam was exposed. Our initial investigations indicated inocula from the DMS contained 100 times more microorganisms per milliliter of slurry compared with water samples pumped from the same well and the microbes from the DMS appear to be coal-associated (Fig. 2).

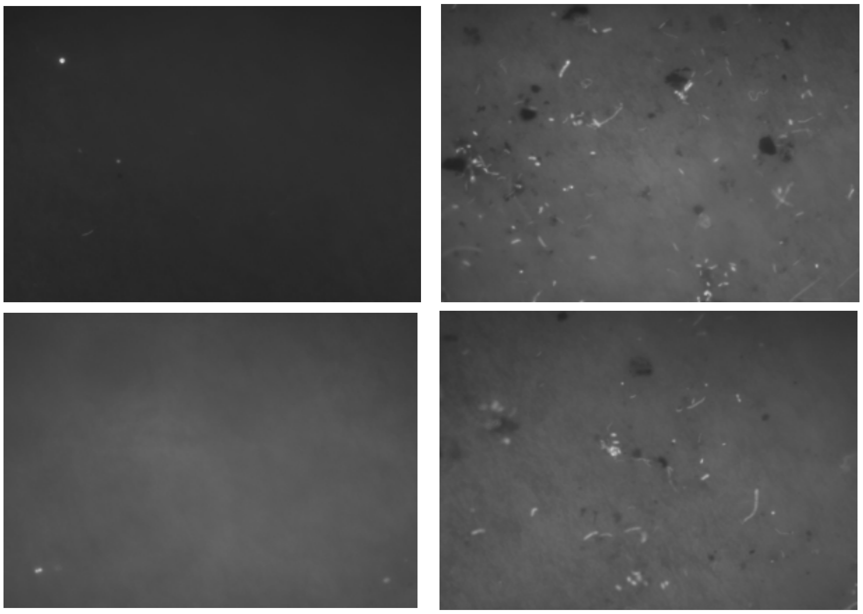

Figure 2: Comparisons between water and DMS sample. The two microscope images on the left are water samples with DAPI stained cells from CBM well water. The two microscope images on the right are DAPI stained samples from the DMS that was placed in the same well. There were 100 times more microbes in the DMS inoculum. The microbes appear to be associated with the black coal in the DMS images.

Stimulated CBM Production

Microbial CBM stimulation involves the addition of nutrients or other amendments to stimulate CBM production from microorganisms already present in a coal seam. This process could occur naturally through nutrients infiltrating a coalbed with groundwater recharge or artificially by pumping nutrients into the coalbed through a well. Several laboratory investigations suggest the addition of nutrients to a coalbed would stimulate CBM production in active and non-producing coalbeds (Jones and others, 2010; Penner and others, 2010; Green and others, 2008). Microbial community changes were previously investigated with real-time polymerase chain reaction (RT-PCR) and results showed that acetoclastic methanogens (*Methanosaeta* and *Methanosarcina*) appeared to increase as methane production increased while the number of hydrogenotrophic methanogens (*Methanomicrobiales*) decreased. (Fig. 3) (Jones and others, 2010). The coal microcosms investigated in Jones and others (2010) were augmented with an inoculum from a wetland environment which could have a different response to nutrient amendments compared to *in situ* microorganisms within a coal-bed. Therefore this study provided insight into a surface microbial community response to nutrient additions when coal is present but did not provide insight into the *in situ* CBM producing microbial community. The DMS inoculum in Chapter 2 provided the ability to inoculate media with *in situ* microorganisms and monitor a response to nutrient additions. Yeast extract and several components of yeast extract stimulated methane production and the microbial community response was investigated using pyrotag analysis (Pilloni and others, 2012). An increase in acetoclastic methanogens and acetogenic *Bacteria* was observed with the addition of yeast extract similarly to the wetland community response observed in Jones and others 2010 (Fig. 3).

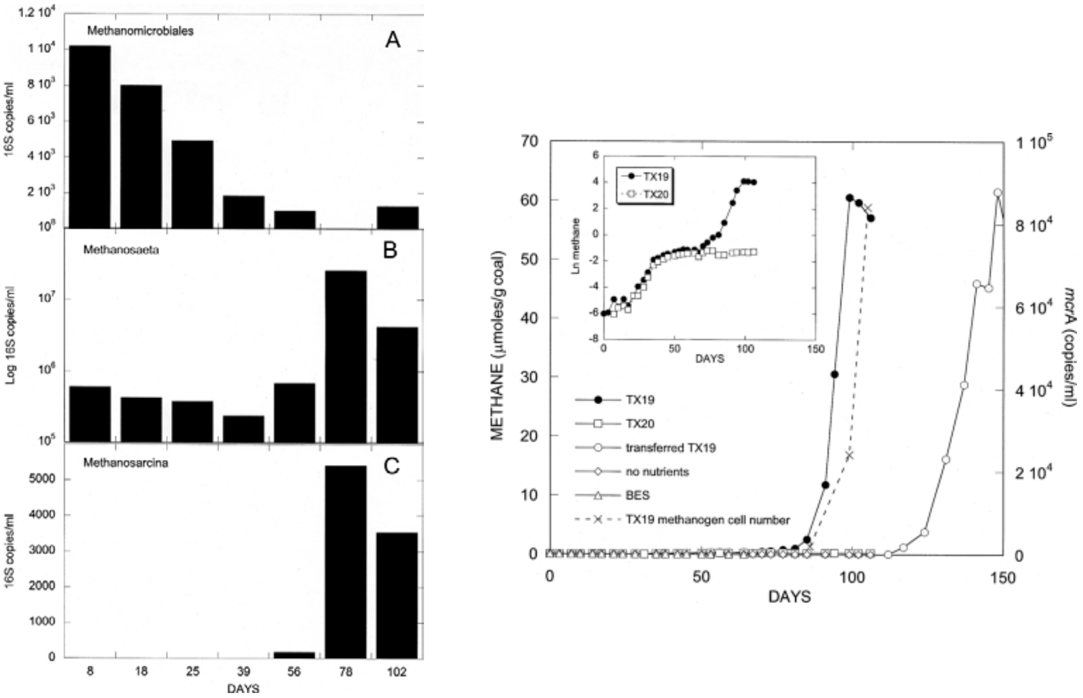

Figure 3. RT-PCR tracking microbial community changes from a methane producing community when stimulated. *Methanosaeta* and *Methanosarcina* are acetoclastic methanogens and appeared to increase dramatically when methane concentrations increased. Figure modified from (Jones and others, 2010)

Connection to *In situ* CBM Production

A microbial shift to acetoclastic methanogenesis has also been documented within the PRB. The proportion of methane originating from acetolclastic versus hydrogenotrophic methanogenesis can be quantified from natural environments with stable isotope probing (Tyler and others, 1997; Gelwicks and others, 1994). A small area of the PRB impacted by major groundwater recharge from the Bighorn mountains exhibits an acetoclastic methane isotopic signature while the majority of the PRB that does not experience the recharge is dominated by hydrogenotrophic methanogenesis (Bates and others, 2011; Rice and others, 2008). CBM well production data within the groundwater recharge area indicate wells closest to the recharge are more productive than wells farther from the recharge suggesting nutrients within the groundwater recharge stimulate CBM production as well as acetoclastic methanogenesis naturally. Initial geochemical investigations from other studies could not identify the stimulating nutrients within the recharge groundwater (Bates and others, 2011). A DMS was placed within a well in a major recharge area of the PRB with an acetoclastic methane signature to further investigate the microbial community and potential stimulating nutrients in Chapter 3. Pyrotag analysis of the DMS slurry indicated a significant proportion of the microbial community was closely related to phototrophic (cyanobacterial and algal) microorganisms. These results indicated phototrophic microorganisms drifting into the coal-beds with groundwater recharge could be stimulating acetoclastic methanogenesis similarly to yeast extract in the laboratory enrichments. A better understanding of nutrients that naturally stimulate CBM could provide insight into novel CBM-production technologies which stimulate acetoclastic methanogenesis in other areas of the PRB.

Acetoclastic Methane Production

Methanosaetaceae and *Methanosarcinaceae* are the only two identified methanogenic families that can utilize acetate as a substrate for methanogenesis. *Methanosaetaceae* contain the deeply rooted genus *Methanosaeta* which uses an adenosine monophosphate-dependent acetyl-CoA synthetase (AMP-Acs) protein that is ubiquitous in nature to activate acetate to acetyl-CoA (Jetten and others, 1992; Lindahl and Chang, 2001). The *Methanosarcinaceae* family includes the most metabolically diverse methanogenic genus *Methanosarcina*, which in addition to being hydrogenotrophic and methylotrophic, can activate acetate to acetyl-CoA with the combined actions of the enzymes acetate kinase (Ack) and phosphotransacetylase (Pta) (Jetten and others, 1992; Lindahl and Chang, 2001; Galagan and others, 2002; Purdy and others, 2002; Winfrey and Ward, 1983). The Ack and Pta enzymes carry out the following reactions providing a methyl group for the central methanogenic pathway (Ferry, 1992).

$CH_3COO^- + ATP \xleftrightarrow{Ack} CH_3COPO_4^{2-} + ADP$

$CH_3COPO_4^{2-} + CoA \xleftrightarrow{Pta} CH_3CO\text{-}CoA + PO_4^{3-}$

In Chapter 4 a *Methanosarcina spp.* was isolated from an enrichment culture that was inoculated with PRB slurry from a DMS. Although *Methanosarcina spp.* have been detected with DNA based methods in two studies investigating CBM production, to the authors knowledge, this was the first time the methanogen has been isolated from a coal-bed (Penner and others, 2010; Strąpoć and others, 2011). The isolation of *Methanosarcina* definitively demonstrated the methanogen is active within the coal-beds. The high-efficiency acetoclastic Ack/Pta provides *Methanosarcina spp.* with increased growth and methane production at higher concentrations of acetate which could result from the bacterial conversion of bioavailable nutrients such as yeast extract or phototrophic biomass to acetate (Jetten, 1990; Liria and others, 1998). These results suggest *in situ* stimulated CBM production could occur because a methanogen with a high-efficiency methane production pathway is active with in the coal-beds.

Evolution of Acetoclastic Methane Production

It is generally thought that energy-conservation was the major force that directed early cell development (Ferry and House, 2006). The Ack/Pta pathway conserves energy by activating acetate at the expense of one high-energy phosphate bond while AMP-*acs* requires two high-energy phosphate bonds (Jetten, 1990; Jetten and others, 1989). This allows *Methanosarcina spp.* to have a higher growth yield compared to other methanogens relying on AMP-Acs when acetate concentrations are high (>1mM) (Min and Zinder, 1989), likely contributing to the large flux of methane from high acetate environments (Lassey, 2008; Sigren and others, 1997). The end-Permian extinction, which was the largest extinction event on Earth, may have occurred due to a large methane release due to the enhanced methane production provided by the Ack/Pta pathway in *Methanosarcina* (Rothman and others, 2014). The purpose of the research in Chapter 4 was to examine the origin of the Ack/Pta pathway in the methanogenic genome.

The research in Chapter 5 identified significant evolutionary relationships between ADP and AMP acetyl coA synthetases and Ack based on shared ATPase motifs that have been previously identified in Ack (Buss and others, 2001; Bork and others, 1992). Therefore this work provides a novel link between the proteins involved in the acetate switch (Wolfe, 2005; Buss and others, 2001). Phylogenetic and genome synteny comparisons provide evidence for the establishment of the first Ack with in the methanogenic genome through gene duplication and divergence of an ADP acetyl coA synthetase (ADP-Acs). A *pta* homolog within *Halobacteriales* provides a mechanism for the acquisition of *pta* within a halophilic environment. Previous research had indicated the Ack/Pta pathway was provided to the ancient methanogen through a horizontal gene transfer of *ack* and *pta* from *Bacteria* but does not provide an origin hypothesis for the Ack/Pta pathway in *Bacteria* and ignores homologs that we identify in Chapter 5 (Fournier, 2009; Fournier and Gogarten, 2008). Therefore this research provides novel insight into the evolution of stimulated methane production as well as the evolution of microbial carbon utilization.

CHAPTER 2 - INVESTIGATION OF COAL-ASSOCIATED BACTERIAL AND ARCHAEAL POPULATIONS FROM A DIFFUSIVE MICROBIAL SAMPLER (DMS)

Contribution of Authors and Co-Authors

Manuscript in Chapter 2

Author: Elliott P. Barnhart

Contributions: Collected field data, conducted most experiments, analyzed data and wrote manuscript

Co-author: Kara Bowen De León

Contributions: Offered advice on experimental design and developed python code for data analysis

Co-author: Bradley D. Ramsay

Contributions: Offered advice on experimental design and conducted data analysis

Co-author: Alfred B. Cunningham

Contributions: Principal Investigator

Corresponding Author: Matthew W. Fields

Contributions: Principal Investigator

Manuscript Information Page

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Errata: the well that was sampled was WR-53 instead of WR-53A but WR-53 was the closest well with associated geochemical data

Abstract

The Powder River Basin (PRB) in southeastern Montana and northeastern Wyoming contains massive coal deposits with biologically generated coal-bed methane (CBM). The microbial ecology of an area within a coal-bed influenced by recent groundwater recharge was sampled with a diffusive microbial sampler (DMS). The DMS contained native coal material and was incubated *in situ* (57 m depth) to allow colonization of the coal particles. Pyrotag sequence analyses of SSU rRNA gene sequences from the coal contained within the post-incubation DMS detected methylotrophic and hydrogenotrophic methanogenic archaea along with diverse bacterial communities. Microbial enrichments (coal or acetate/H_2) were established from the DMS, and the enriched bacterial and archaeal communities were characterized via clone library analysis. The *in situ* bacterial communities were more diverse than the archaeal communities, and the archaeal populations differed between coal incubated *in situ* and in laboratory enrichments. In addition, bacterial diversity was higher for laboratory enrichments with coal compared to enrichments without coal. The elucidation of relationships between microorganisms involved in coal degradation and metabolite (acetate, H_2) utilization within coal-dependent microbial communities is crucial to understanding and improving *in situ* coal-bed methane production.

Introduction

Energy sources with low carbon emissions, such as methane gas, are becoming increasingly important with climate change and rising energy needs worldwide. Subsurface coal, a difficult substrate for microorganisms to utilize due to recalcitrant properties, has been associated with vast methane deposits within coal-beds (Strąpoć and others, 2011). In some of these areas, the methane gas from the coal-beds can be produced and utilized as an industrial energy source. Estimates of recoverable CBM (coal-bed methane) from the Powder River Basin (PRB) in southeastern Montana and northeastern Wyoming vary, but with current extraction techniques an estimated 17.4 trillion cubic feet (TCF) could be recoverable (Meredith and others, 2012). This reserve is substantial considering 1 TCF of methane could supply the natural gas needs of

400,000 households for 20 years (Bauder, 2007). However, little is known about the methanogenic microbial communities responsible for producing the methane, the coal-bed conditions that contribute to methane production, or the key microbial community interactions that contribute to coal-dependent methanogenesis.

Methane production in coal-beds is the consequence of a series of metabolic interactions among various groups of microorganisms (Strąpoć and others, 2011). Molecular techniques have been applied to coal-bed microbial systems in an attempt to detect dominant microbial community members present in produced water and coal core samples (Shimizu and others, 2007; Li and others, 2008; Strąpoć and others, 2008; Jones and others, 2010; Fry and others, 2009; Penner and others, 2010; Midgley and others, 2010). These studies suggest that coal-beds are generally colonized by an array of bacterial populations that commonly include members of the *Proteobacteria* (Li and others, 2008; Shimizu and others, 2007; Midgley and others, 2010; Penner and others, 2010). Archaea have also been detected in several core and water samples originating from coal-beds at a much lower diversity compared to that of bacteria (Green and others, 2008; Li and others, 2008; Shimizu and others, 2007; Strąpoć and others, 2008; Klein and others, 2008; Midgley and others, 2010; Penner and others, 2010). Several models have been proposed which hypothesize the microbial interactions resulting in the microbial breakdown of coal to produce methane. Under low redox conditions, the primary fermentation of polymers and monomers to fatty acids, organic acids (*e.g.*, lactate, succinate, acetate), alcohols (*e.g.*, methanol), hydrogen, and carbon dioxide is carried out by fermentative bacteria (Strąpoć and others, 2011). The primary fermentation products can be utilized by both *Bacteria* and *Archaea* including: secondary fermenting bacteria (syntrophs); homoacetogenic bacteria; and acetoclastic, methylotrophic, and hydrogenotrophic methanogens (Strąpoć and others, 2011). Trace elements have also been shown to impact methane production and methanogen abundance in microcosms inoculated with produced water from the PRB (Unal and others, 2012).

Trace elements and nutrients enhancing the conversion of organic matter within coal-beds to methane are basically derived from two different sources *in situ*: nutrients supplied from the substratum sediments and nutrients from the groundwater recharge. In general, groundwater of the PRB flows from the south towards the northeast and low-transmissivity shale layers common in the PRB isolate the water from other aquifers providing very long flow paths that are dominated by hydrogenotrophic methane production ($CO_2 + 4H_2 \Leftrightarrow CH_4 + 2H_2O$) (Flores and others, 2008; Rice and others, 2008; Bates and others, 2011; Meredith and others, 2012). Several areas of groundwater recharge occur along the western margin of the PRB where the coal seams outcrop, especially near the Montana-Wyoming border (Rice and others, 2008; Bates and others, 2011). Isotopic evidence within this recharge area indicates that acetoclastic or methylotrophic methanogenesis is dominant *in situ* ($CH_3OO^- + H^+ \Leftrightarrow CH_4 + CO_2$) (Flores et al., 2008; Rice et al., 2008; Bates et al., 2011). To further investigate the *in situ* processes mediating acetoclastic or methylotrophic methane production, we utilized a novel sampling technique to gain insight into the microbial ecology present in a well near a recharge area along the western margin of the PRB (GWIC 8430) (Bates and others, 2011).

A significant amount of research has been dedicated to understanding the *in situ* microbial system involved in converting subsurface coal to methane (Midgley and others, 2010; Penner and others, 2010; Strąpoć and others, 2008; Jones and others, 2010; Unal and others, 2012; Guo and others, 2012). Such studies have relied on traditional formation water and/or coal core samples to access the coal-utilizing microbial community. Research suggests that groundwater samples do not reflect microbial densities and activities in the subsurface (Alfreider and others, 1997; Penner and others, 2010). In addition, core samples are often prohibitively expensive to obtain and difficult to collect aseptically. Down-well incubation of sterile substrate has emerged as an alternative method for sampling subsurface microorganisms (Alfreider and others, 1997; Griebler and others, 2002; Peacock and others, 2004; Reardon and others, 2004). A down-well incubation technique was utilized in the present study with a diffusive microbial sampler (DMS) with modifications to only expose the sediment within the inner part of the DMS at the bottom of the well to target coal-utilizing microbial populations.

In this study, we aimed to enhance the fundamental understanding of the ecology and physiology of methane-producing, coal-associated communities, and to identify microbial community responses to laboratory enrichment conditions that included coal and several metabolites (acetate, H_2) suspected to form when coal is degraded by *Bacteria* (Jones et al., 2010; Strąpoć et al., 2011). This analysis allowed new insight into the *in situ* microbial community and populations involved in coal degradation and metabolite utilization.

Methods and Materials

Sampling Site

The study site was located in southeastern Montana in the CX field where significant groundwater recharge occurs from areas several miles away in the Wolf Mountains and further from the BigHorn Mountains with flow moving east at a calculated rate of 80 ft/yr (Bates and others, 2011; Wheaton and Donato, 2004). Well WR-53A is a monitoring well used by the Montana Bureau of Mines and Geology to monitor the effect of methane development on regional groundwater tables. The well was drilled in 1977 to a depth of 57 meters, sealed with a packer from 38.7 meters to 41.8 meters and screened from 42.7 meters to the bottom (57 meters). Complete geochemical analysis, site location and well logs can be obtained at the Montana Bureau of Mines and Geology Groundwater Information Center (GWIC) website: http://mbmggwic.mtech.edu with GWIC ID 8430.

A diffusive microbial sampler (DMS) was designed to colonize native coal material with an indigenous microbial consortium from Powder River Basin (PRB) coal seams. The DMS consisted of a cylinder (2.5 inches in diameter and 5 inches long) with 2 rods extending from the top of the cylinder to a weight 12 inches below the cylinder. The weight sealed the DMS, due to gravity, until contacting the bottom of the well where the rods extending from the weight lifted the coal out of the sealed cylinder allowing colonization by indigenous microorganisms. Sediment within the sampler was composed of approximately 25 grams of sub-bituminous coal particles (>2 mm but <4 mm diameter) from the Decker Coal Mine in the Powder River Basin (PRB) several miles from well WR-53A. The coal particles were encased with #8 stainless steel mesh. The DMS was autoclaved prior to being placed in the well allowing autoclaved coal to act as the negative control in our DNA analysis. The DMS was retrieved from well WR-53A using aseptic techniques after three months of incubation. There was not a noticeable loss of coal from the sampler during the time of incubation.

Microbial Enrichments

Three ml of slurry was immediately added to anaerobic coculture medium (CCM). The CCM contains 30 mM sodium acetate and (per liter) 2.17 g NaCl, 5.5 g $MgCl_2 \cdot 6H_2O$, 0.14 g $CaCl_2 \cdot 2H_2O$, 0.5 g NH_4Cl, and 0.335 g KCl and is buffered with 1.1 mM K_2HPO_4 and 30 mM $NaHCO_3$. One ml per liter of 1000X nonchelated trace elements and 1 ml per liter of 1000X vitamin solution amended with 2.0 g/l choline chloride were added as growth supplements as previously described (Walker and others, 2009). L-cysteine • HCl (1 mM) and sulfide (1 mM as $Na_2S \cdot 9H_2O$) were added as reducing agents. Resazurin (1 mg/l) was added as a redox indicator. Stock solutions of K_2HPO_4 (1 M), $NaHCO_3$ (6.0 M), L-cysteine • HCl (1 M), $Na_2S \cdot 9H_2O$ (1 M), and the nonchelated trace element and vitamin mixtures were prepared under anoxic conditions as previously described (Walker and others, 2009). Coal remaining in the DMS was immediately frozen on dry ice and transported to a -80°C freezer and stored until used for DNA analysis.

Methane production from laboratory cultures was monitored by direct injection on a SRI 8610C gas chromatograph (GC) with a thermal conductivity detector (TCD) and a stainless-steel molecular sieve 13X packed column with helium as the carrier gas. After methane production was detected in the enrichments inoculated from well WR-53A, liquid coal slurry was transferred to fresh CCM medium with 5 g coal added with a CO_2 headspace or 30 mM sodium acetate along with an 80:20 H_2/CO_2 headspace. The enrichments were incubated in the dark at 25°C and methane production was monitored via GC.

DNA Extraction

DNA was extracted from the coal from the DMS sampler, coal cultures and acetate/H_2 cultures. DNA was extracted at the same time point for the coal cultures and acetate/H_2 cultures. Approximately 0.5 g of coal from the DMS was aseptically placed in a 1.5 mL tube with 1 mL of 10% SDS. The samples were heated to 70°C for 15 minutes followed by a vortex pulse to suspend the coal and another 15 minutes at 70°C to wash the microorganisms off the coal. Microbial biomass was retrieved from the cultures by filtering the medium through a 0.2 µm filter and both the filter and the coal were included in the DNA extraction. Total community DNA was extracted using the BIO 101 FAST DNA spin kit (MP Biomedical, Solon, OH) according to the manufacturer's instructions with the following modifications: DNA bound to the silica matrix was allowed to settle for 30 min, and the spin filter was allowed to air-dry in a biosafety cabinet for 30 min following the salt-ethanol wash. In order to account for heterogeneity in community diversity and structure as well as variability in DNA extraction efficiency, the culture was extracted in triplicate. Autoclaved coal was extracted alongside the samples and used as a negative control.

Pyrotag Analysis

Pyrosequencing was used to characterize the bacterial and archaeal populations from the DMS. Nested archaeal and semi-nested bacterial PCR amplification were performed to increase the concentration of DNA for pyrosequencing analysis. The thermal profile used in bacterial PCR reactions included initial denaturation at 94°C (2 min); followed by 25 cycles of denaturation at 94°C (30 sec), annealing at 58°C (1 min), and primer extension at 72°C (1 min); and a final extension at 72°C for 7 min. The final reaction mixture (20 µl) contained 10 µl 2X EconoTaq PLUS (Lucigen, Madison, WI) and 1.0 µM of each forward and reverse primer along with 5-10 µg template. Each sample was amplified with a unique 10-nt barcode to enable multiplexing. The bacterial primers included: barcoded FD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and non-barcoded 1540R (5'-GGAGGTGWTCCARCCGC-3') in the initial amplification and barcoded FD1 and barcoded 529R (5'-GGCAGATTCTTTGCCTTCTG-3') in the second round of amplification (Yakimov and others, 2001; Bowen De León and others, 2012). The archaeal primers included: 21F (5'-TTCYGGTTGATCCYGCCRGA-3') and 1492R (5'-CGGTTACCTTGTTACGACTT-3') and barcoded 751F (5-'CCGACGGTGAGRGRYGAA-3') and 1204R (5-TTMGGGGCATRCIKACCT-3') (Baker and others, 2003). A 0.8% agarose gel in TAE buffer was used to check the PCR products for DNA of the correct size. The gel extracts were cleaned and concentrated using the Wizard SV Gel and PCR Cleanup System® (Promega, Madison, WI), and dsDNA was quantified with a Qubit fluorometer (Life Technologies, Carlsbad, CA, USA). Adaptors for 454 sequencing were ligated to the amplicons and were pyrosequenced in a Roche 454 GS-Junior (454 Life Sciences, Branford, CT, USA). The barcoded sequencing reads were separated by Roche's image analysis and sequence assignment software providing high confidence in assigning sequencing reads to the appropriate sample. Sequences were trimmed to one standard deviation below the mean length (removed if shorter), and subjected to a Phred score filter allowing 15% of the nucleotides to be below Q27, and removed if primer errors or Ns were observed. Forward reads were carried through the analysis. A python script was used for data management and analysis as previously described (Bowen De León and others, 2012). Chimeras were removed using ChimeraSlayer (Haas and others, 2011). The Michigan State University Ribosome Database Project Pyrosequencing Pipeline (http://pyro.cme.msu.edu) was used to align sequences, complete-linkage clustering at 97% similarity, and to calculate Chao1 indices. Sequences were identified with a Basic Local Alignment Search Tool (BLAST) search against the National Center for Biotechnology Information (NCBI) nucleotide (nr/nt) database.

Clone Library Analysis

Clone library analysis was utilized to investigate the archaeal and bacterial populations contained within the enrichment cultures. Approximately 2.0-2.5 ng of DNA was used to perform PCR with universal bacterial primers FD1 and 1540R, universal archaeal primers 21F and 1492R (sequences above) and *mcr*A specific forward primer, 5′-GGTGGTGTMGGATTCACACARTAYGCWACAGC-3′, and a reverse primer, 5′-TTCATTGCRTAGTTWGGRTAGTT-3′ (Luton and others, 2002). The thermal profile used in the bacterial PCR reaction included initial denaturation at 94˚C (2 min); followed by 30 cycles of denaturation at 94˚C (30 sec), annealing at 58˚C (1 min), and primer extension at 72˚C (1 min); and a final extension at 72˚C (7 min). The thermal profile used in the archaeal PCR reaction included: initial denaturation at 94˚C (5 min); followed by 25 cycles of denaturation at 94˚C (30 sec), annealing at 55˚C (30 sec), and primer extension at 72˚C (2 min); and a final extension at 72˚C (7 min). The final reaction mixture (20 µL) contained 10 µL 2X EconoTaq PLUS (Lucigen, Madison, WI) and 1.0 µM of each forward and reverse primer. A 0.8% agarose gel in TAE buffer was used to check the PCR products for DNA of the correct size. Bands were excised and cleaned using the Wizard SV Gel and PCR Cleanup System® (Promega Inc., Madison, WI) according to the manufacturer's instructions (Promega Inc., Madison WI). Cleaned products were cloned using the Invitrogen pCR2.1-TOPO vector according to the manufacturer's instructions (Life Technologies, Carlsbad, CA). Archaeal and bacterial clones (192 each) were picked based on blue/white screening for selection of recombinants and submitted for sequencing to Functional Biosciences (Madison, WI) using Sanger sequencing methods with bacterial primer 529R (sequence above) archaeal primer 519R (5'-ACAATGTCATTGGTCAATTCATT-3') and *mcrA*/M13R, (5′-CAGGAAACAGCTATGACCTTCATTGCRTAGTTWGGRTAGTT-3′). Sequences were extracted from chromatograms using BioEdit (http://www.mbio.ncsu .edu/BioEdit/bioedit.html) and aligned, clustered and dereplicated using the RDP pyrosequencing pipeline as described above. Dereplicated sequences were identified using the BLASTn algorithm by NCBI (http://ncbi.nlm.nih.gov).

Results

Pyrotag Sequence Analysis of In situ Community

A total of 6,144 archaeal sequence reads with 74 operational taxonomic units (OTU) and 5,339 bacterial sequence reads with 407 OTUs were obtained through pyrotag analysis after trimming and quality-checking the sequences. The average read lengths were 380 and 365 bases for archaea and bacteria, respectively. The OTUs were defined with 3% dissimilarity and analyzed with Chao1 diversity estimates (Chao and Lee, 1992; Hughes and others, 2001). Chao1 statistical analysis suggested that further sequencing of the microbial community would lead to additional unique OTUs and reveal more genera/species - although the majority of the estimated diversity was sampled (Table 1). To illustrate in detail the composition of the communities detected with this analysis, each OTU, along with its putative identity and corresponding percent similarity has been provided in Supplementary Table 1.

Microbiota Observed with Pyrotag Analysis

All of the archaeal sequences sampled from the DMS could be classified as closely related to the hydrogenotrophic methanogen, *Methanobacterium* (58.7%), or the methylotrophic methanogen, *Methanolobus* (41.3%) (Mochimaru and others, 2007) (Supplementary Table 1). The detected bacterial community was very diverse and approximately 33% of the bacterial sequence reads belonged to the phylum *Proteobacteria* (32.6%) with the dominant class being β-*Proteobacteria.* The phyla *Actinobacteria* (29.8%), and *Firmicutes* (21.0%) were also predominant and represented a large portion of the sequence reads. Interestingly, a significant portion (almost 16%) of the sequences detected in the analysis were most closely related to phototrophic cyanobacteria and chloroplast sequences (Fig. 4 and Supplementary Table 1). Phototrophic organisms and/or biomass could be carried into the subsurface environment with recharge water and may represent a source of allochthonous carbon and nutrients.

Table 1: Summary of reads, operational taxonomic units (OTUs), diversity and richness estimates of pyrotag analysis

Sample	*Archaea*			*Bacteria*		
	Reads	OTUs[a]	Chao1	Reads	OTUs[a]	Chao1
WR-53A	6144	75	82 (77, 107)[b]	5339	407	587 (524, 684)[b]

[a] The operational taxonomic units (OTU) were defined with 3% dissimilarity.
[b] 95% confidence intervals for Chao1 estimators.

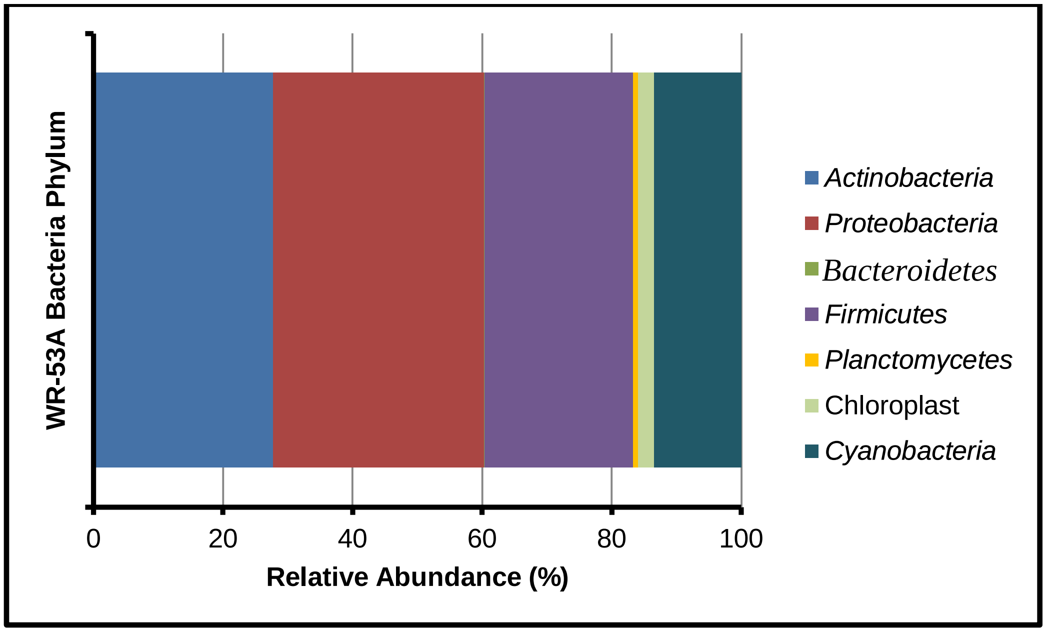

Figure 4. Phylogenetic classification by phylum for the bacterial pyrotag analysis from coal incubated in the DMS at site WR-53A.

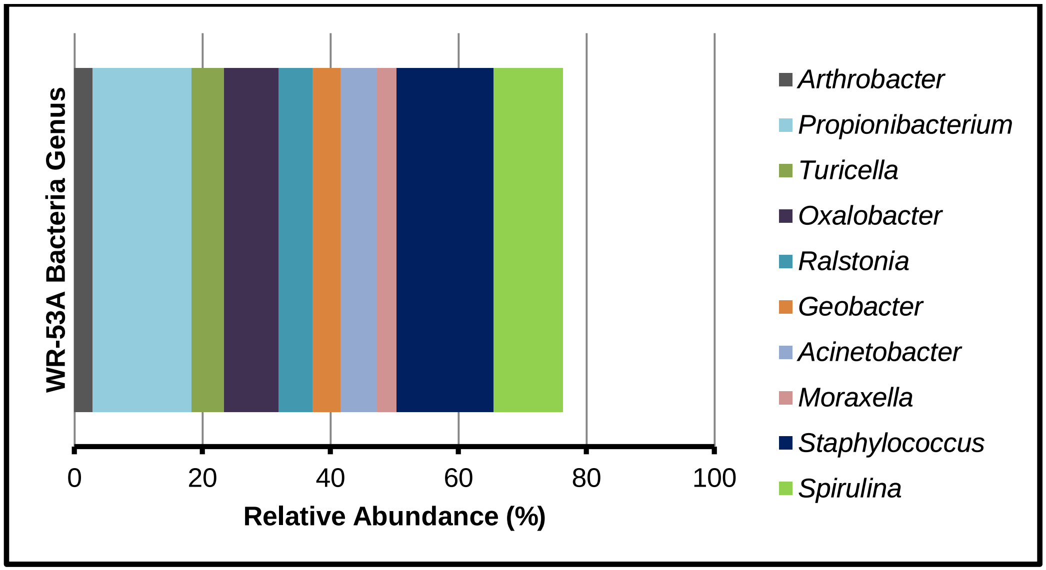

Figure 5: Phylogenetic classification of the top 10 genus for the bacterial pyrotag analysis from coal incubated in the DMS at site WR-53A.

Coal-dependent Microbial Communities

A total of 83 bacterial clones (SSU rDNA gene sequences) from the coal culture and 85 bacterial clones from the acetate/H_2-amended culture (no coal) were sequenced (Fig. 5). The bacterial diversity was greater in the coal enrichment compared to the acetate/H_2 enrichment: 24 versus 8 OTUs at the genus level. An OTU closely related (98-100% sequence identity) to the SSU rRNA gene sequence of *Acetobacterium*, a homoacetogen, comprised approximately 25% of both libraries (Eichler and Schink, 1984). The three next most abundant OTUs in the coal enrichment were most closely related to *Acetivibrio* (97-99% sequence identity), *Clostridium* (84-98% sequence identity), and *Desulfovibrio* (98% sequence identity). Other OTUs detected in the coal but not acetate/H_2 enrichment included *Aminobacterium*, *Geobacter*, *Herbaspirillum*, *Spirochaeta*, and *Synotrophus*.

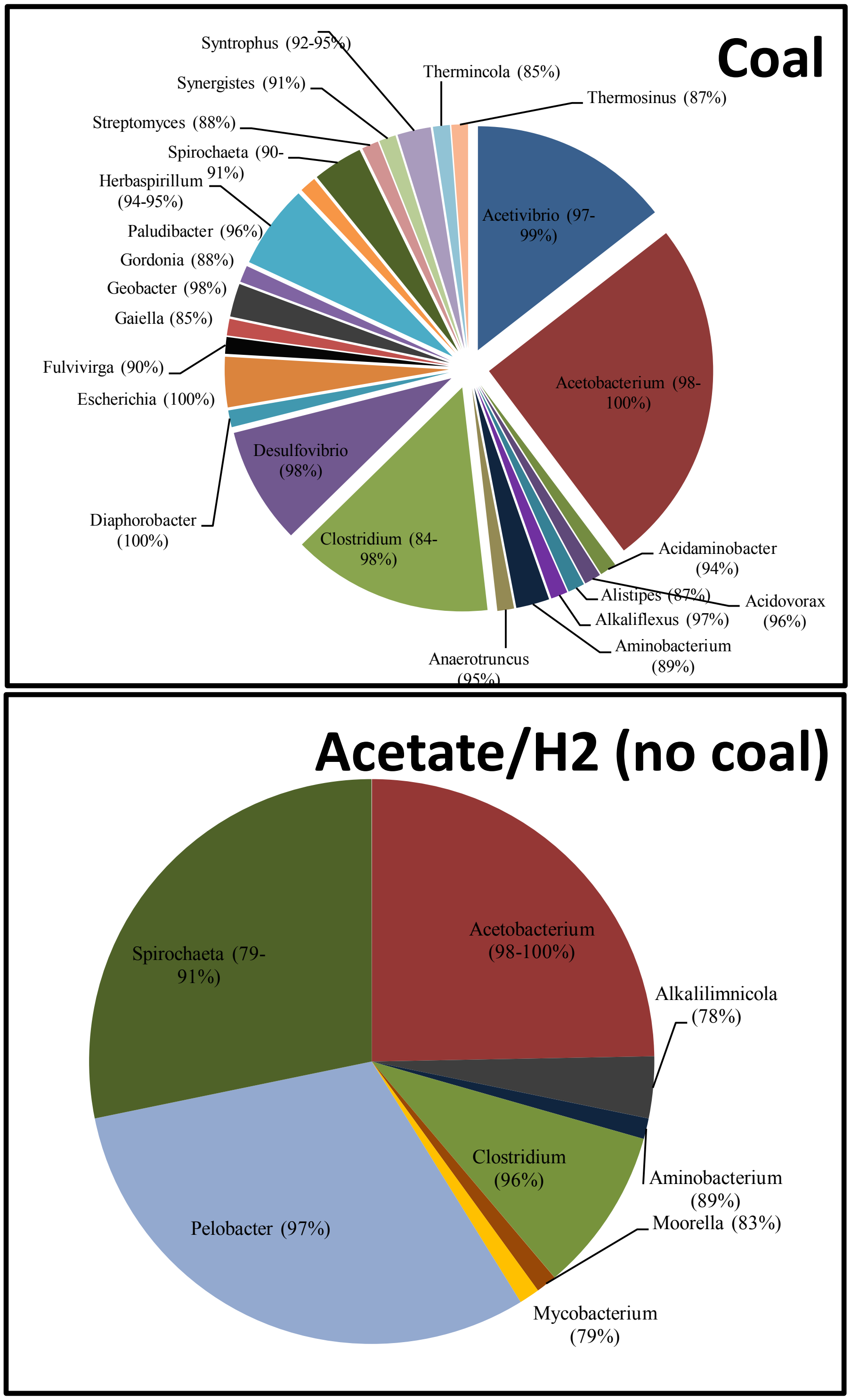

Figure 6: Genus level bacterial population composition for different enrichments from the DMS. Incubated slurry from the DMS was used to inoculate enrichment cultures with coal as the sole carbon source (Top) and compared to enrichments amended with acetate and H_2 (Bottom). The size of the pie section indicates the percentage of the total detected bacterial community represented with the percent identification to the named genus in parentheses.

The archaeal diversity was lower compared to the bacterial diversity and only 3 unique OTUs were detected at the genus level for either enrichment (coal or acetate/H_2) (Fig. 6). The acetate/H_2 enrichment was predominated by *Methanosarcina* (86%), *Methanospirillum* (13%), and *Methanosaeta* (1%). The coal enrichment was predominated by multiple *Methanosarcina* groups (91%) and a *Methanospirillum* group (9%). Similar results were observed when the methanogen specific *mcr*A diversity was compared between the coal- and acetate/H_2- enrichments (data not shown).

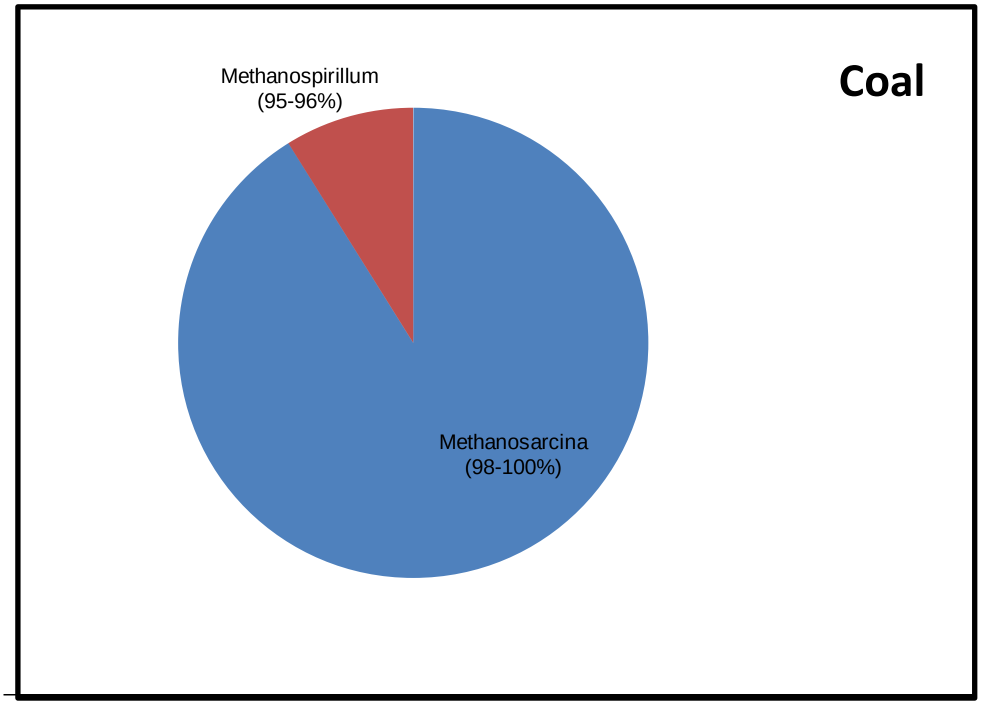

Figure 7 Continued

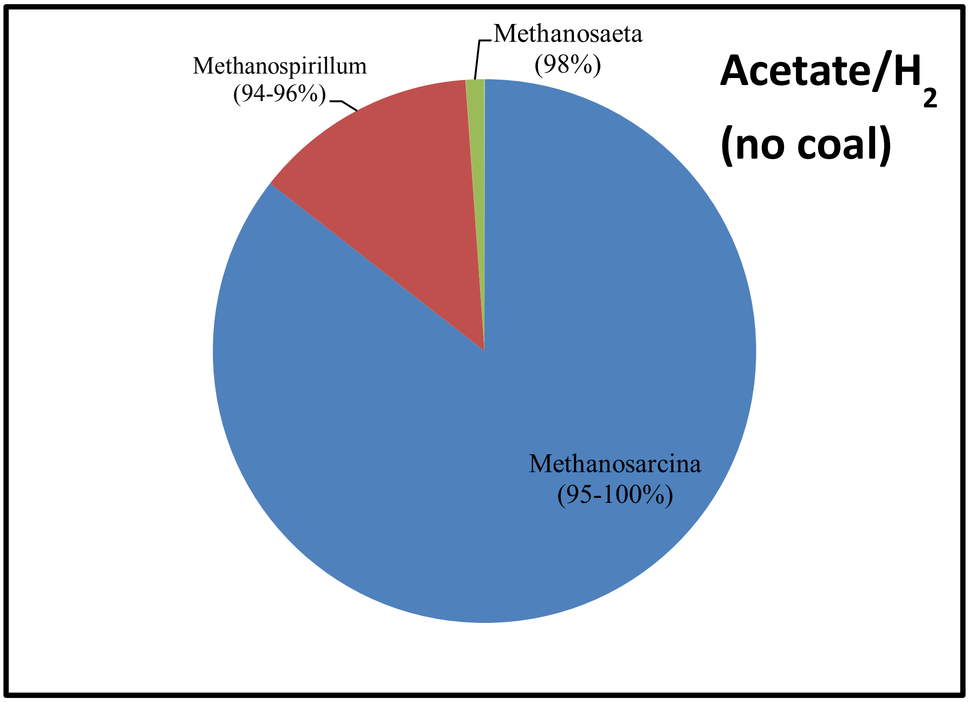

Figure 7: Genus level archaeal population composition for different enrichments from the DMS. Incubated slurry from the DMS was used to inoculate enrichment cultures with coal as the sole carbon source and compared to enrichments amended with acetate and H_2. DNA was extracted from these enrichments and clone libraries were constructed to compare the communities. The size of the pie section indicates the percentage of the total detected bacterial community represented with the percent identification to the named genus in parentheses.

High-throughput 454 pyrotag analysis was utilized to investigate the *in situ* microbial communities. Pyrotag analysis is widely accepted and has been used to investigate the microbial ecology in a vast number of diverse habitats, such as a biogas plant (Schlüter and others, 2008), deep mines (Edwards and others, 2006), the deep marine biosphere (Huber and others, 2007) and subsurface coal-beds in China (Guo and others, 2012). It should be acknowledged that all PCR-based methods are not without potential bias due to differing DNA extraction efficiencies and bias of primer selectivity.

Because of the difficulty of extracting DNA from coal, we used nested and semi-nested PCR in this study, which has also been necessary in other microbial coal-bed investigations (Klein and others, 2008; Guo and others, 2012).

Based on the results from 454 pyrosequencing, both hydrogenotrophic and methylotrophic methanogens were present *in situ*. OTUs related to *Methanolobus* and *Methanobacterium* have been recently detected with pyrotag analysis from subsurface coal-beds in China (Guo and others, 2012). The presence of these methanogenic sequences was not unexpected based on isotopic data indicating that both CO_2-reduction and methyl-type fermentation played major roles in gas generation near this site (Flores and others, 2008). This isotopic data cannot distinguish between acetoclastic methanogenesis and methylotrophic methanogenesis; however, our data indicate methylotrophic instead of acetoclastic methanogenic populations predominate *in situ* in this area of the basin as *Methanolobus* spp. can utilize methanol as a substrate for methanogenesis rather than acetate. Interestingly, sequences indicative of these organisms were not detected in the enrichments from the DMS, and instead sequences indicative of the genera *Methanosarcina, Methanosaeta,* and *Methanospirillum* were detected. *Methanosarcina* spp. are the only identified methanogens which possess all three known pathways for methanogenesis (acetoclastic, methylotrophic and hydrogenotrophic) (Galagan and others, 2002). *Methanosaeta* spp. are limited to acetoclastic methane production while *Methanospirillum* spp. are limited to H_2 and CO_2 for methane production. It is common for molecular-based and cultivation-based methods to detect different populations, and our results highlight the limitations of cultivation-based methods to indicate active populations *in situ*. However, given the right conditions (*i.e.*, higher environmental acetate concentrations), the acetoclastic methanogens identified in the enrichments may dominate *in situ* under stimulated conditions. Further work is needed to accurately assess active microbial populations that contribute to *in situ* methanogenesis under different conditions.

The bacterial community detected with 454 pyrotag analysis was diverse but most of the bacterial sequence reads (32%) belonged to the phylum *Proteobacteria* as previously observed in other CBM basins (Midgley and others, 2010; Penner and others, 2010; Shimizu and others, 2007; Guo and others, 2012). The dominant class of *Proteobacteria* was *β-Proteobacteria* that consists of populations with hydrocarbon-degrading capabilities (Rotaru and others, 2010). *Oxalobacter* which are known for anaerobically breaking down oxalate as a source of energy and carbon for cellular biosynthesis (Jonsson and others, 2004) was a top β-Proteobacterial genus detected in the pyrotag analysis (Fig. 4). Researchers have performed aqueous extractions of different ranks of coals and oxalate was the most prevalent extracted anion from low-rank coals which is the predominant coal in the Powder River Basin (Bou-Raad and others, 2000). Although some inferences can be made about significant microorganisms from pyrotag analysis, molecular biology techniques can be combined with enrichment studies to begin to understand the physiological and biochemical basis of coal-based methanogenesis and the relevant microorganisms involved in the conversion of coal to methane (Strąpoć and others, 2011).

The DMS was raised and used to inoculate anaerobic media with different amendments (*i.e.*, coal or acetate/H_2). Methane production was monitored and clone libraries were used to determine predominant differences in community composition and structure related to coal-dependent methane production. The DNA was extracted from the enrichment cultures with significant methane concentrations at the same time point (150 days), and other researchers have shown that different microbial clades associated with methane production dominate at various time points during batch culturing (Jones and others, 2010). At 150 days the coal-only enrichments had 3-fold higher bacterial SSU rRNA gene sequence diversity compared to the acetate/H_2 culture (24 versus 8 OTUs at the genus level). Based upon enrichment cultures, coal promoted bacterial diversity and the diversity of bacterial populations may play an important role in efficient coal-degradation (i.*e*., due to the heterogeneous nature of coal polymers). The presence of sequences indicative of homoacetagens suggests potential competition between acetogens and hydrogenotrophic methanogens for H_2 and that indirect shuttling of reductants from fermenters to acetoclastic or methylotrophic methanogens may go through homoacetogens.

In order to understand the relationship between communities identified by the enrichment and *in situ* analysis, we compared their community profiles with the pyrotag analysis obtained from the original DMS sample. The Venn diagram (Fig. 7) categorizes the overlap of the detected bacterial phyla in all three samples: WR-53A, coal enrichment and metabolite (acetate, H_2) enrichment. Based on the sampled diversity, the *Actinobacteria*, *Proteobacteria*, *Bacteriodetes*, and *Firmicutes* phyla were observed in all three samples and no phyla were detected in the metabolite enrichment that was not observed in the other samples. Several phyla were only observed in the coal and metabolite enrichments and not in the environmental sample (WR-53A) indicating they may have been rare in the *in situ* community and below the detection limit but could still play an important role in coal degradation or metabolite utilization given the right conditions. The *Spirochaetes* phylum was shared only between the metabolite enrichment and the coal enrichment. *Spirochaetes* have been studied for over 100 years for their role in termite gut biota resulting in methane production (Breznak, 2002). *Spirochaetes* species are capable of obtaining energy for growth by H_2/CO_2-acetogenesis, *i.e.,* $4\ H_2 + 2\ CO_2 \rightarrow CH_3COOH + 2\ H_2O$ ($\Delta G^{\circ\prime} = -105$ kJ per mole acetate) (Leadbetter and others, 1999). *Synergistetes* were only observed in the coal enrichment sample indicating that they also were minor components in the environmental sample (WR-53A) but may also be important players in coal degradation when the right conditions are present. Bacteria within the phylum *Synergistetes* have recently been observed to compete for acetate with acetate-utilizing methanogens (Ito and others, 2011).

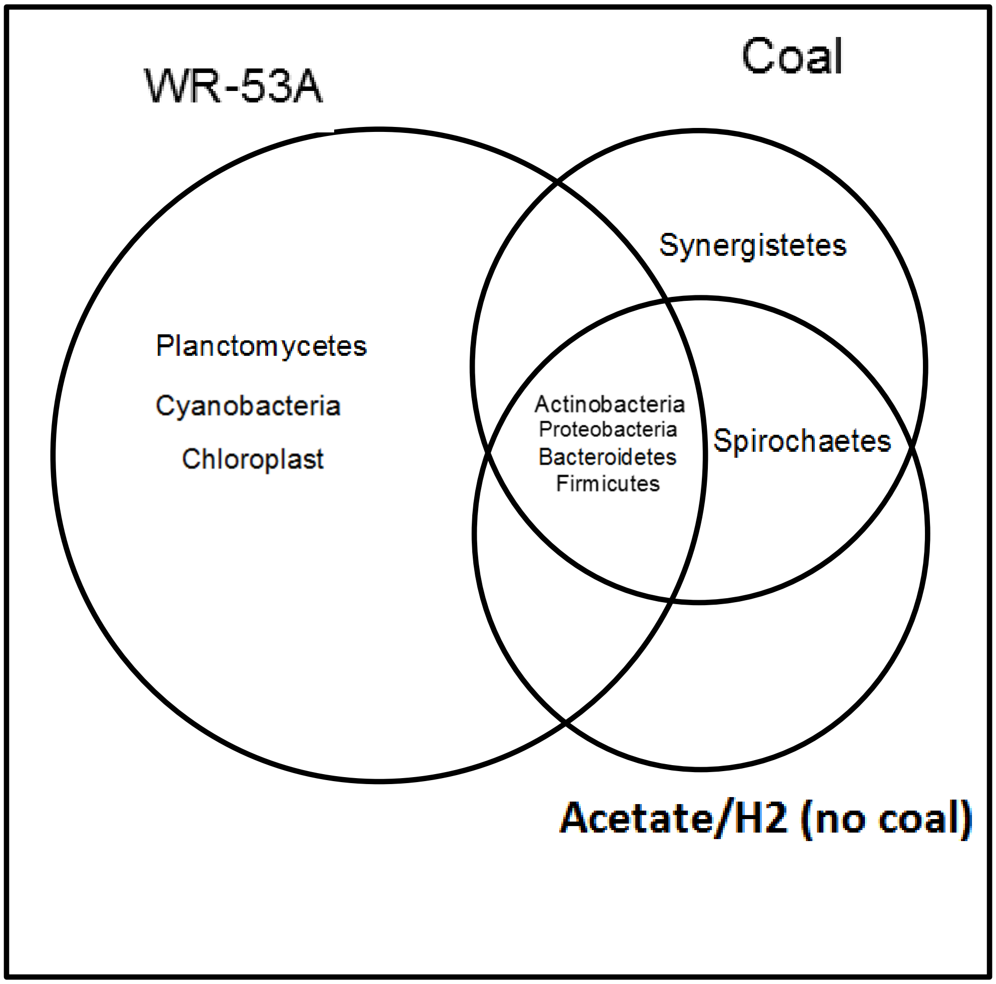

Figure 8: Qualitative Venn diagram showing the bacterial phyla detected within each sample. In this figure the community phylogenetic classification for the analysis of the bacterial phylum composition from the metabolite and coal cultures is compared to the bacterial phylum composition of the coal within the DMS deployed at site WR-53A. Interestingly, there were no phyla unique to the metabolite enrichments. The circle representing the pyrosequencing data was made bigger to highlight the greater number of sequences obtained compared to the clone libraries.

Microorganisms belonging to the phyla *Planctomycetes* and *Cyanobacteria* were only detected in the environmental sample along with microorganisms most closely related to eukaryotic chloroplast sequences indicative of algae. The abundance of the sequences related to *Cyanobacteria* suggests that biomass may have been transported into the subsurface with the groundwater recharge and not a remnant of contamination introduced when the well was drilled in 1977. It is possible that re-charge and/or slow turnover of microbial biomass (e.g., DNA) could explain the observed cyanobacterial sequences. The major recharge areas are miles away and with a calculated flow rate of 80 feet/year, DNA from these phototrophic microorganisms would have to remain in subsurface for many years in order to reach the area where well WR-53A is located (Wheaton and Donato, 2004). Alternatively, clinker ridges and the Anderson coal seam outcrop close to the CX field, which could supply a smaller amount of recharge but allow for a shorter travel distance (Van Voast and Thale, 2001; Wheaton and Donato, 2004). Future research is needed to fully understand the presence of these sequences at the site.

Conclusion

There is currently a great deal of interest as to what extent the *in situ* microbial conversion of coal into methane can contribute towards satisfying the growing energy needs of the United States as more sustainable and renewable sources are developed. The useful life of a CBM well, on average, is currently less than ten years (Meredith and others, 2012). Therefore, stimulation of the methane-producing community contained within the coal-beds is required for sustainable development of domestic resources. We investigated the microbial ecology of an area within the PRB that appears to have been naturally stimulated with groundwater recharge. A novel sampling technique was utilized that was successful in capturing a microbial community consistent with the previous isotopic data retrieved from the area. Pyrotag analysis indicates organisms capable of methylotrophic and hydrogenotrophic methanogenesis dominate the archaeal community within well WR-53A while the bacterial community was diverse. In order to better understand the individual groups of archaea and bacteria involved in coal degradation and metabolite utilization, enrichment techniques were combined with molecular biology methods to infer potentially important organisms involved in producing acetate and/or H_2 from coal and microorganisms that utilize the metabolites. Within the enrichments, coal appeared to maintain bacterial diversity compared to enrichments with H_2 and acetate. Nearly twenty-five percent of the bacterial OTUs from the WR-53A environmental sample had a low similarity (less than 90% identity) to any known cultured bacteria published on the NCBI website (September 2012) and may represent novel species specifically adapted to coal-bed ecosystems. Therefore, further isolation studies from coal-beds are needed to better understand indigenous organisms and function. Obtaining additional samples providing analysis of the microbial community along a coal-bed flow path in several different areas and identifying how the microbial community dynamics change may lead to a better understanding of the differences in the isotopic data observed near the western margin and further into the center of the PRB.

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CHAPTER 3 - STIMULATION OF COAL-DEPENDENT METHANOGENESIS WITH NATIVE MICROBIAL CONSORTIA FROM THE POWDER RIVER BASIN

Contribution of Authors and Co-Authors

Manuscript in Chapter 3

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Contributions: Conducted most experiments, analyzed data and wrote manuscript

Co-author: Bradley D. Ramsay

Contributions: Offered advice on experimental design and conducted data analysis

Co-author: Kara Bowen De León

Contributions: Offered advice on experimental design and developed python code for data analysis

Co-Author: Kristen A. Brileya

Contributions: Helped set up and monitor anaerobic microcosms

Co-Author: Denise M. Akob

Contributions: Helped with data interpretation and manuscript writing

Co-author: Richard E. Macur

Contributions: Provided Algal extract

Co-author: Alfred B. Cunningham

Contributions: Principal Investigator

Corresponding Author: Matthew W. Fields

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Manuscript Information Page

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Abstract

Understanding *in situ* microbial responses to nutrient additions within coal-beds has become increasingly important with the growing interest in enhancing coal-bed methane (CBM) production. Nutrients in the form of yeast extract and several individual components of yeast extract (proteins and amino acids) were added to microcosms containing native microorganisms from Powder River Basin (PRB) coal-beds. CBM production more than doubled when these components were added to coal-containing microcosms. Microbial populations capable of hydrogen production/utilization were detected *in situ* and under non-stimulated conditions in microcosms. Stimulation with yeast extract caused a shift in the detectable community to a higher percentage of acetoclastic methanogens and acetogenic bacteria. Methane isotope analysis from CBM production wells has indicated a similar microbial community shift as observed in stimulation experiments. Hydrogenotrophic methanogenesis signatures dominated most PRB coalbeds but the signature shifted to acetoclastic methanogenesis near major groundwater recharge areas. In conjunction, a high proportion of cyanobacterial and algal SSU rRNA gene sequences were detected in a CBM well within a major recharge area, suggesting that these phototrophic organisms naturally stimulate acetoclastic methane production. In laboratory studies, adding eukaryotic phototrophic (algal) biomass stimulated coal-dependent methanogenesis by PRB enrichments similarly to yeast extract (~30 µmol methane increase per gram of coal after 100 days). These results provide insight into the microbial community shifts that occur when nutrients are added to coal-beds, as well as the processes that may naturally stimulate CBM production in the PRB.

Introduction

An increasing world energy demand is creating unprecedented challenges for recovering power and mitigating the environmental impacts of developing energy resources. Coal-bed methane (CBM), a form of natural gas in many underground coal-beds, has been harvested in the Powder River Basin (PRB) since 1993 (Hower and Jones, 2003). The combustion of CBM produces less nitrogen oxides, carbon dioxide, sulfur dioxide and mercury compounds than coal or oil but major pollution and sustainability issues arise from current production techniques. The most economical and widely utilized technology for CBM development in the PRB involves pumping an average of

16,800 gallons of water/day/well (Rice and Nuccio, 2000) from a CBM-producing coal-bed, recovering the methane and disposing the produced water in holding ponds (Meredith and others, 2012; Bank and Kuuskraa, 2006). This type of production has resulted in a short productive well life averaging less than ten years (Meredith and others, 2012) and over 4000 produced water ponds with elevated sodium and heavy metal concentrations in the PRB (Sowder and others, 2008; Howler and others, 2003).

The *in situ* generation of CBM is microbially driven in the PRB and other sub-bituminous coal-beds around the world, (Strąpoć and others, 2011). Laboratory studies have suggested that microbial CBM production can be stimulated with the addition of yeast extract or tryptone (Green and others, 2008; Penner and others, 2010; Jones and others, 2010). *In situ* enhancement of CBM could create a more sustainable energy source by increasing the life of the CBM wells and provide an incentive for injecting CBM water with nutrients back into the CBM producing coal-bed thereby reducing the amount of polluted CBM water on the ground surface. Although several studies have demonstrated the potential for this technology in the lab (Green and others, 2008; Penner and others, 2010; Jones and others, 2010; Ghosh and others, 2013), very little is known about how these laboratory results relate to *in situ* processes.

Enrichment and Pyrotag Analysis

A diffusive subsurface sampler (DMS) designed to capture the active *in situ* CBM-producing microbial community (Barnhart and others, 2013) was utilized in this study. Laboratory enrichments inoculated with slurry from the DMS indicated yeast extract and individual components of yeast extract (glutamic acid and peptone) enhanced methane production in the presence of coal. The SSU rRNA bacterial sequences from the DMS and the coal enrichment (without yeast extract) were dominated by *Proteobacteria* (80% and 51% of the total library respectively). Sequences most closely related to *Firmicutes* dominated coal with yeast extract and yeast extract only enrichments (67% and 90% respectively). A higher proportion of sequences related to acetoclastic (acetate utilizing) methanogens were detected in YE amended enrichments. This community analysis supports previous microbial coal enrichment studies utilizing RT-PCR which documented an increase in acetoclastic methanogens, in particular *Methanosarcinaceae,* at the same time abundant methane production occurred in the enrichment cultures (Jones and others, 2010).

Connection to *In situ* CBM Production

Isotopes indicative of acetoclastic methanogenesis have been detected in major ground water recharge areas within the PRB (Flores and others, 2008; Galand and others, 2010; Bates and others, 2011). Although many different variables can impact the productivity of a CBM well, production data suggests recharge leads to more productive and sustainable CBM wells near the western margin of the PRB where major recharge occurs (Fig. 8) (Meredith and others, 2011). Recently an unusually high proportion of phototrophic 16S rRNA sequences were obtained from a well within this recharge area (Barnhart et al., 2013). Algae and cyanobacteria percolating into subsurface coal-beds with recharge could provide an allochthonous source of carbon and nutrients which enhances methanogenesis just as yeast cell components stimulate methanogenesis in laboratory enrichments.

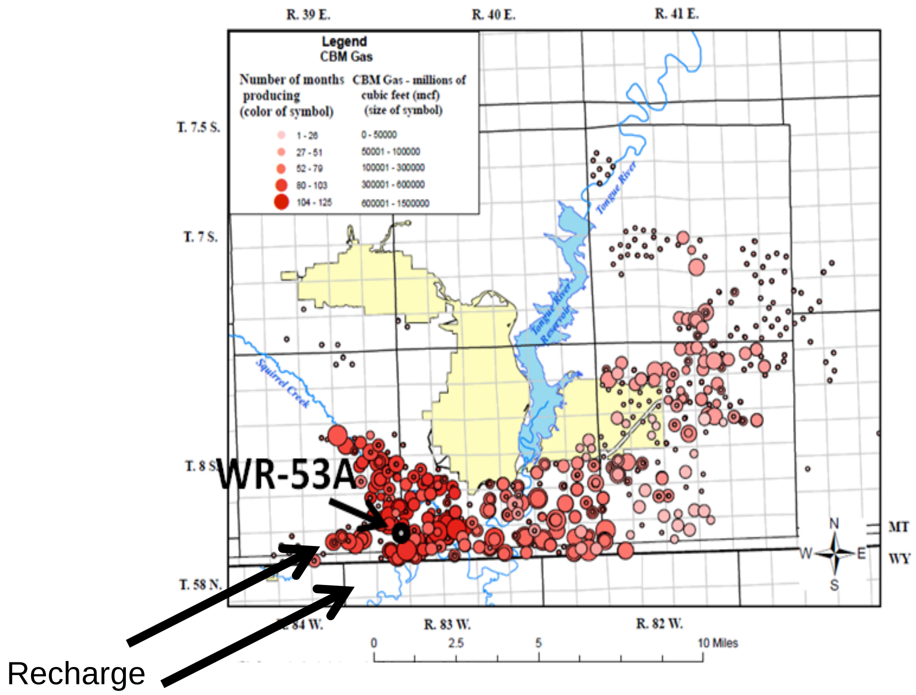

Figure 9: The figure was modified from Meredith et al., (2011) to include recharge information from Bates et al., (2011). This combined information suggests recharge leads to more productive and sustainable CBM wells near the western margin of the PRB. The larger darker red circles indicate wells that produce more CBM and produce CBM longer than smaller pink circles. The arrow pointing to the black circle indicates the well (WR-53A) recently investigated which had a high proportion of phototrophic SSU rRNA sequences (Barnhart et al., 2013).

Phototrophic cells flowing into the coal-beds with recharge water may provide a nutrient delivery mechanism dependent on the turnover of biomass which may be very slow in subsurface environments (Hoehler and Jørgensen, 2013). Inputs of organic matter, such as phototrophic biomass, has been shown to induce a phenomenon known as the priming effect which provides a mechanism for the microbial decomposition of recalcitrant organic matter, such as coal (Guenet and others, 2012; Guenet and others, 2010). The addition of lipid extracted algal biomass to laboratory CBM-producing enrichments greatly enhanced CBM production in this study. These results indicate algae, which grow in 4000 in place ponds due to previous CBM development in the PRB (Sowder and others, 2008), could be used for CBM stimulation after biodiesel-producing lipids have been removed. Photosynthetic microorganisms sequester CO_2, the only byproduct of methane combustion, providing the possibility of a closed-loop energy resource (Fig. 9). This study has identified the potential for a novel energy-generating system based on a process that appears to naturally occur in areas where major groundwater recharge takes place in the PRB. Biomimicry of this process in areas of the PRB that do not receive significant recharge could help sustain CBM production from these areas.

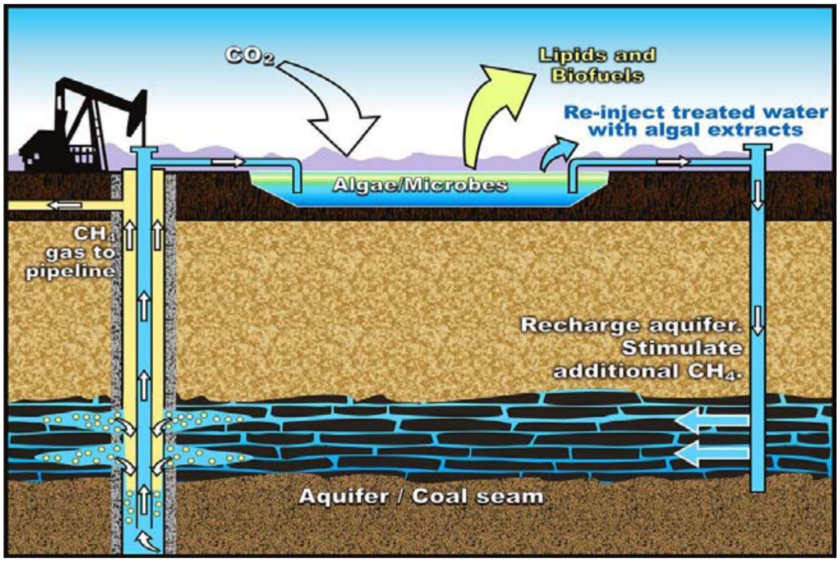

Figure 10: Research has previously demonstrated that CBM producing microbial communities are stimulated with the addition of yeast extract. Algal residues remaining after lipid extraction also stimulate CBM production. The growth of algae for CBM stimulation and lipid production could productively utilize the CBM wastewater ponds already in place in the PRB from previous development.

Methods and Materials

Sampling Site

The study site was located in southeastern Montana in the center of the PRB. A diffusive microbial sampler (DMS) was used to obtain the *in situ* microbial community as previously described (Barnhart and others, 2013) within well HWC-O1. Sediment within the DMS was composed of approximately 25 g of sub-bituminous coal particles (> 2 mm but < 4 mm diameter) from the Decker Coal Mine in the PRB. The well was drilled in to a depth of 70.7 m, sealed with a packer at 63.1 m and screened from 63.7 m to 68.2 m. Water chemistry was analyzed before the DMS was deployed into well. Prior to sampling, the well was flushed by pumping at least five well volumes of water until pH and conductivity were stable. Additional well and water analysis can be obtained at http://mbmggwic.mtech.edu with GWIC ID 8107.

Microbial Enrichments

Three milliliters of slurry from the DMS were added to modified anaerobic coculture medium (CCM). The modified CCM contained (per liter) 3.86 mg $MgCl_2 \cdot 6H_2O$, 5.21 mg $CaCl_2 \cdot 2H_2O$, 0.5 g NH_4Cl, and 5 mg KCl and is buffered with 1.1 mM K_2HPO_4 and 1.04 g/L $NaHCO_3$. One ml per liter of 1000 × nonchelated trace elements and 1 ml per liter of 1000 × vitamin solution amended with 2.0 g/l choline chloride were added as growth supplements as previously described (Walker and others, 2009). L-cysteine·HCl (1 mM) and sulfide (1 mM as $Na_2S \cdot 9H_2O$) were added as reducing agents. Resazurin (1 mg/l) was added as a redox indicator. Stock solutions of K_2HPO_4 (1 M), $NaHCO_3$ (6.0 M), L-cysteine·HCl (1 M), $Na_2S \cdot 9H_2O$ (1 M), and the nonchelated trace element and vitamin mixtures were prepared under anoxic conditions as previously described (Walker and others, 2009). Coal remaining in the DMS was immediately frozen on dry ice and transported to a -80°C freezer and stored for DNA analysis. The coal used in the experiments was obtained from the Decker Coal Mine in the Powder River Basin (MT) by the Montana Bureau of Mines and Geology. The enrichments were incubated in the dark at 25°C and methane production was monitored via a direct injection SRI 8610C gas chromatograph (GC) with a thermal conductivity detector (TCD) and a stainless-steel molecular sieve 13x packed column with helium as the carrier gas. After significant methane production was detected 1 ml of inoculated media was used to inoculate modified CCM media amended with 1 g/l yeast extract along with controls with and without 1 g of coal. When significant methane production was detected 1 ml of inoculated media from a coal only enrichment was used to inoculate modified CCM media containing 1 g coal with either: 1 g/l peptone, 0.629 g/l sodium glutamate, 1 g/l YE or 2 ml/l vitamin solution (Walker and others, 2009) along with controls. The enrichments were incubated in the dark at 25°C and methane production was monitored as previously described.

DNA Extraction and Microbial Community Analysis

DNA was extracted from DMS coal, coal enrichments and yeast extract enrichments with and without coal. DNA was extracted at the same time point for all of the enrichments. Thirty days prior to DNA extraction from yeast extract enrichments 1 g/L yeast extract was added and methane production was enhanced confirming the CBM producing microbial community was viable in the yeast extract enrichments. GC measurements indicated the coal only enrichments steadily produced methane at a very low rate (data not shown). Approximately 0.5 g of coal from the DMS and the enrichments were aseptically placed in a 1.5 ml tube with 1 ml of 10% SDS. The samples were heated to 70˚C to wash the microbes off the coal. Total community DNA was extracted using the BIO 101 FAST DNA spin kit (MP Biomedical, Solon, OH) according to the manufacturer's instructions with modifications as previously described (Barnhart and others, 2013). In order to account for heterogeneity in community diversity and structure as well as variability in DNA extraction efficiency, samples were extracted in triplicate. Autoclaved coal was extracted alongside the samples and used as a negative control.

Pyrosequencing was used to characterize the bacterial and archaeal populations. Nested archaeal and semi-nested bacterial PCR amplification were performed to increase the concentration of DNA for pyrosequencing analysis. The thermal profile used in bacterial PCR reactions included initial denaturation at 94 °C (2 min); followed by 25 cycles of denaturation at 94 °C (30 s), annealing at 58 °C (1 min), and primer extension at 72 °C (1 min); and a final extension at 72 °C for 7 min. The final reaction mixture (20 µl) contained 10 µl 2 × EconoTaq PLUS (Lucigen, Madison, WI) and 1.0 µM of each of the forward and reverse primers along with 5–10 µg template. Each sample was amplified with a unique 10-nt barcode to enable multiplexing. The bacterial primers included: barcoded FD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and non-barcoded 1540R (5′-GGAGGTGWTCCARCCGC-3′) in the initial amplification and barcoded FD1 and barcoded 529R (5′-GGCAGATTCTTTGCCTTCTG-3′) in the second round of amplification (Bowen DeLeón and others, 2012; Yakimov and others, 2001). The archaeal primers included: 21F (5′-TTCYGGTTGATCCYGCCRGA-3′) and 1492R (5′-CGGTTACCTTGTTACGACTT-3′) and barcoded 751F (5-′CCGACGGTGAGRGRYGAA-3′) and 1204R (5-TTMGGGGCATRCIKACCT-3′) (Baker and others, 2003). A 0.8% agarose gel in TAE buffer was used to check the PCR products for DNA of the correct size. The gel extracts were cleaned and concentrated using the Wizard SV Gel and PCR Cleanup System® (Promega Inc., Madison, WI), and dsDNA was quantified with a Qubit fluorometer (Life Technologies, Carlsbad, CA, USA).

Pyrosequencing for the *in situ* DMS sample was done with GS-FLX pyrosequencing technology at the Medical Biofilm Research Institute (Lubbock, TX, USA). The Archaeal and Bacterial amplicons from the enrichments were prepared and sequenced on an in-house GS Junior Titanium™ Pyrosequencer as previously described (Bowen DeLeón and others, 2013). A python script was used for data management and analysis as previously described (Bowen De León and others, 2012). Only the forward reads were carried through the analysis. Chimeras were removed using ChimeraSlayer (Haas and others, 2011). The Michigan State University Ribosome Database Project Pyrosequencing Pipeline (http://pyro.cme.msu.edu) was used to align sequences, complete-linkage clustering at 97% similarity, and to calculate Chao1 indices. Sequences were identified with a Basic Local Alignment Search Tool (BLAST) search against the National Center for Biotechnology Information (NCBI) nucleotide (nr/nt) database.

Preparation of Algal Extract

Scenedesmus sp. str. WC-1 was grown in a low density polyethylene bag reactor (6 mil wall thickness) containing 20 L of Bolds media (Nichols and Bold 1965) under 14/10 h light/dark with ~ 75 mol photons m^{-2} s^{-1}. The reactor was continuously bubbled with air. Cells were harvested after two weeks of growth using centrifugation at 4,000 x g followed by lyophilization. "Lipid-free" biomass was prepared using sonication assisted solvent extraction. Briefly, 100 mg portions of dry cell mass were suspended in 5 mL triple solvent (1:1:1, chloroform:tetrahydrofuran:hexane) and sonicated three times for 20 s with a Branson S-450D sonicator equipped with a microtip probe set to 80 W (Branson, Danbury, CT). The disrupted cell suspension was centrifuged at 3,000 x g for 30 s and the supernatant was removed. Extraction of the remaining biomass was repeated two more times using 5 mL of fresh triple solvent for each cycle. The residual cellular material was air dried and stored at -20 °C prior to use in the growth experiments. 1 mL of media from a coal only enrichment from modified CCM media (Walker and others, 2009) was used to inoculate enrichments containing either 1 g/L YE or 1 g/L algae extract along with controls. The enrichments were incubated in the dark at 25°C and methane production was monitored as previously described.

Results

Bio-stimulation of the Coal-Dependent Community

Yeast extract stimulated the CBM-producing microbial community, as has been previously demonstrated in laboratory enrichments (Green and others, 2008). Short and long-term (31 and 1155 days) methane measurements demonstrated enhanced CBM production with yeast extract was coal-dependent (Fig. 10). Yeast extract contains many nutrients that could possibly stimulate methane production; therefore, different components were tested for stimulation of methane production with the microbial consortium in the presence of coal (e.g., peptone, vitamins, glutamic acid, and yeast extract). Complex proteins and sodium glutamate are major constituents of yeast extract. Aliquots removed from a methane-producing enrichment that had not previously been exposed to yeast extract were added to enrichments that contained individual components of yeast extract with coal. Methane was measured in the headspace of these enrichments after 60 days and 759 days to analyze short and long term enhancement potential of the constituents. Mixed vitamins had a marginal effect on methane production, and while peptone and glutamate increased methane production, yeast extract resulted in the highest increase (Fig. 11).

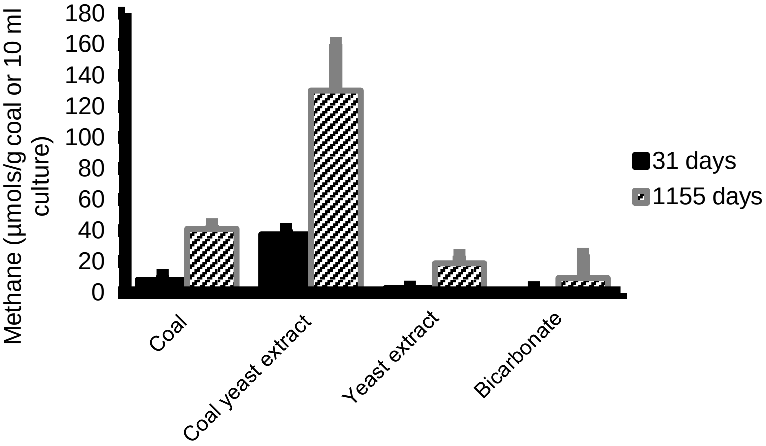

Figure 11: Short and long-term CBM enhancement with yeast extract: Short and long term (31 and 1155 days of incubation) methane measurements from enrichments indicate yeast extract stimulated CBM production methane when coal was also in the media. Coal only enrichments produced more methane than the yeast extract only enrichments. Bicarbonate enrichments contained modified CCM media (without coal or yeast extract). Error bars represent 1 standard deviation for the triplicate enrichments.

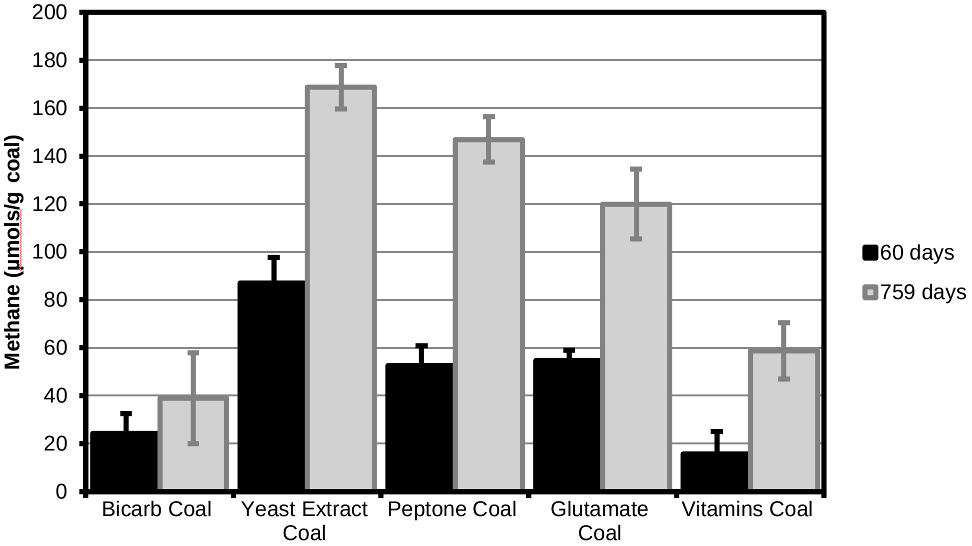

Figure 12: Short and long-term CBM enhancement of yeast extract components: Short and long-term (60 and 759 days) methane measurements indicate individual components of yeast extract stimulate CBM production. Peptone enrichments represent the protein fraction of yeast extract and glutamate enrichments represent the major amino acid in yeast extract. Error bars represent 1 standard deviation for the triplicate enrichments.

Pyrotag Sequence Analysis

The microbial communites within enrichments with coal, coal with yeast extract, and yeast extract without coal were compared with the *in situ* community from the DMS using pyrotag analysis. A total of 183,333 bacterial and 23,981 archaeal sequences were analyzed after trimming. A diverse bacterial community was detected in each enrichment and the DMS but several dominant OTUs were detected. The dominant bacterial genera detected in each library were compared (Fig. 12) and a table with all of the forward reads is provided in the supplementary material (Supplementary Table 2). Our analysis indicated *Geobacter* (29%) and *Collimonas* (25%) dominated the *in situ* bacterial community. These microorganisms have the ability to adapt to oligotrophic conditions through weathering rocks and minerals (Leveau and others, 2010) and the expression of hydrocarbon degredation pathways and when easily degradable compounds are not available (Marozava and others, 2014). *Syntrophus* was the predominant genus detected in the coal enrichment comprising about 15% while *Acetobacterium* (30%) was largest in the yeast extract library and *Fusibacter* (45%) was predominant in the coal and yeast extract library. *Syntrophus* are known to grow in obligate syntrophy with methanogens or other hydrogen-utilizing anaerobes when substrates are not easily fermentable (Stams and Plugge, 2009). *Acetobacterium* and *Fusibacter* have been previously detected in deep hydrocarbon reserviors and are involved in acetate production (Müller and others, 2001; Ravot and others, 1999; Kryachko and others, 2012).

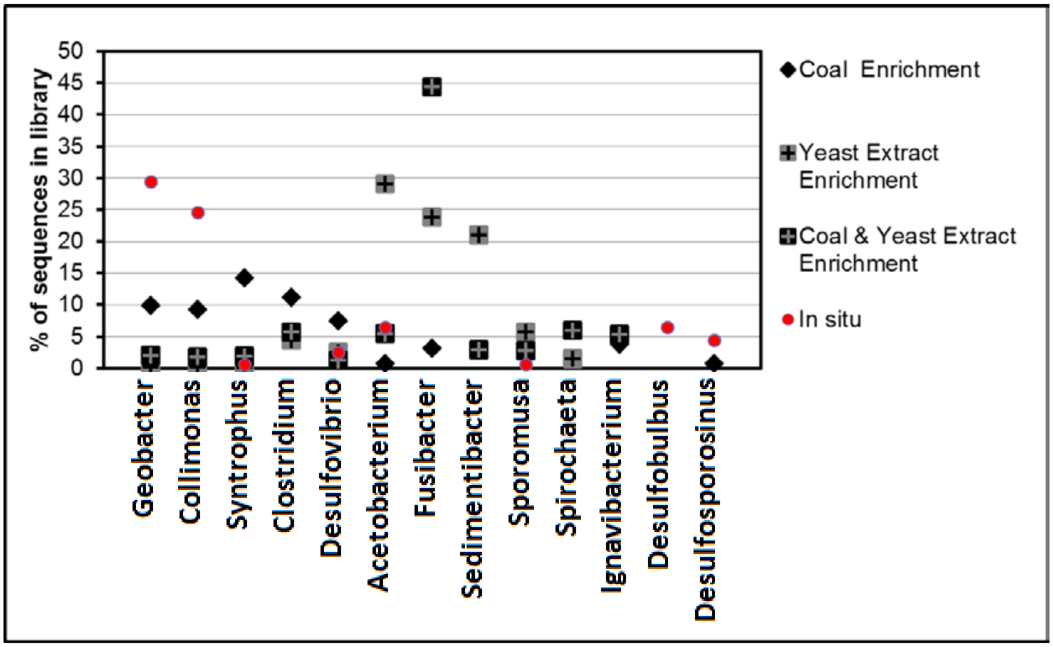

Figure 13: Bacterial pyrotag analysis of the bacterial community from the *in situ* DMS and enriched microcosms. The top 5 dominant bacterial genera detected within each of the libraries are represented. The SSU rDNA bacterial sequences from the DMS and the coal enrichment (without YE) were dominated by *Proteobacteria* (80% and 51% of the total library respectively). Sequences most closely related to *Firmicutes* dominated coal and yeast extract and yeast extract only enrichments (67% and 90% respectively).

The archaeal community was also investigated with pyrotag analysis (Fig. 13 and Supplementary Table 2). The sequences obtained from the DMS were dominated by sequences most closely related to the hydrogenotrophic methanogen *Methanospirillum* (>80%). The sequences obtained from enrichments containing coal and coal + yeast extract were dominated by the hydrogenotrophic methanogen *Methanoregula* (>70% and >85% repectively). The enrichment with yeast extract and no coal was dominated by the metabolically diverse *Methanosarcina* sequences (~75%). Methanogens capable of acetoclastic methanogenesis (*Methanosaeta* and *Methanosarcina*) were detected in the enrichments but not *in situ* indicating these acetoclastic methanogens were below detection limits. All methanogens rely on methane production for energy conservation and type of methanogenesis that predominates (hyrdogenotrophic, methylotrophic or acetoclastic) is very stringently regulated by substrate availability, its concentration, and other substrates present (Ferry, 1992).

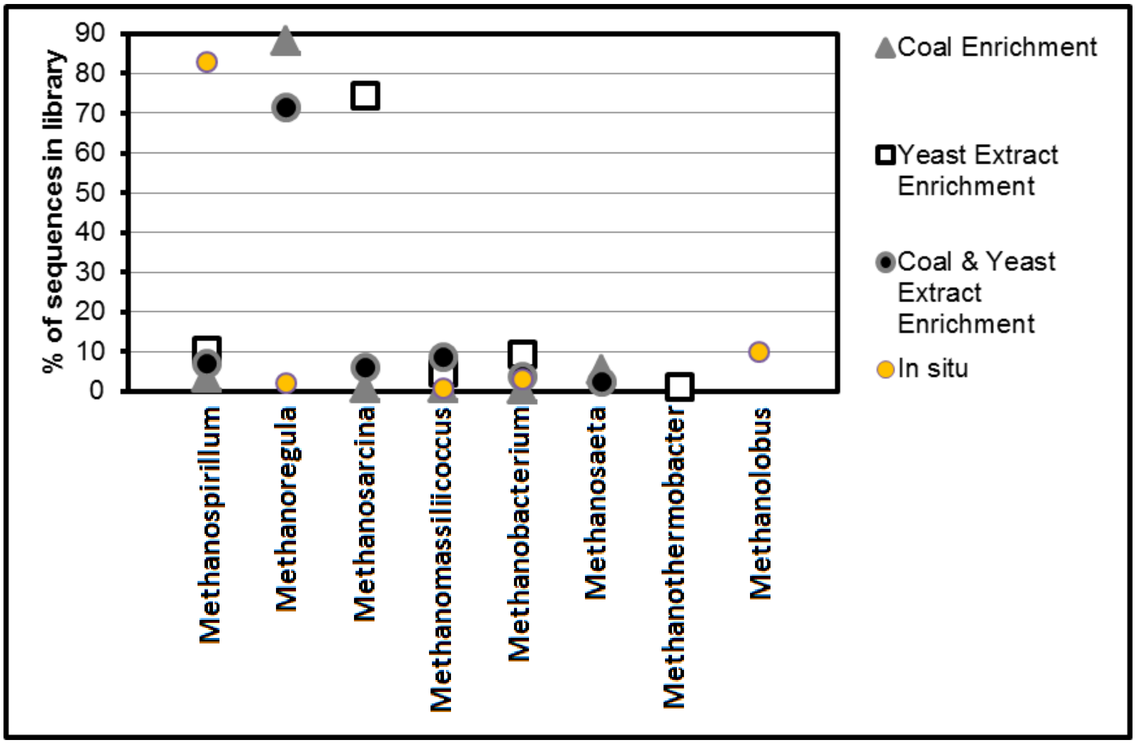

Figure 14: Archaeal Community Structure: Overview of pyrotag analysis of the archaeal community from the *in situ* DMS and enriched microcosms. The top 5 dominant archaeal genera detected within each of the libraries are represented. The dominant members in the *in situ* enrichment more closely represent the *in situ* community than the dominant community members detected in the enrichments with yeast extract. A higher proportion of SSU rRNA sequences closely related to the acetoclastic methanogen *Methanosarcina* were detected in the yeast amended enrichments.

Algae-Enhanced CBM Production

The CBM enhancement potential of algae extract was investigated by adding algal biomass with lipids removed to CBM enrichments (Fig. 14). Algae extract more than doubled the methane production of the CBM-producing microbial community in the presence of coal, compared to enrichments without amendment. The methane production was lower, compared to the yeast extract amended enrichments but exhibited a similar enhancement curve with most of the methane produced early in the enrichment phase.

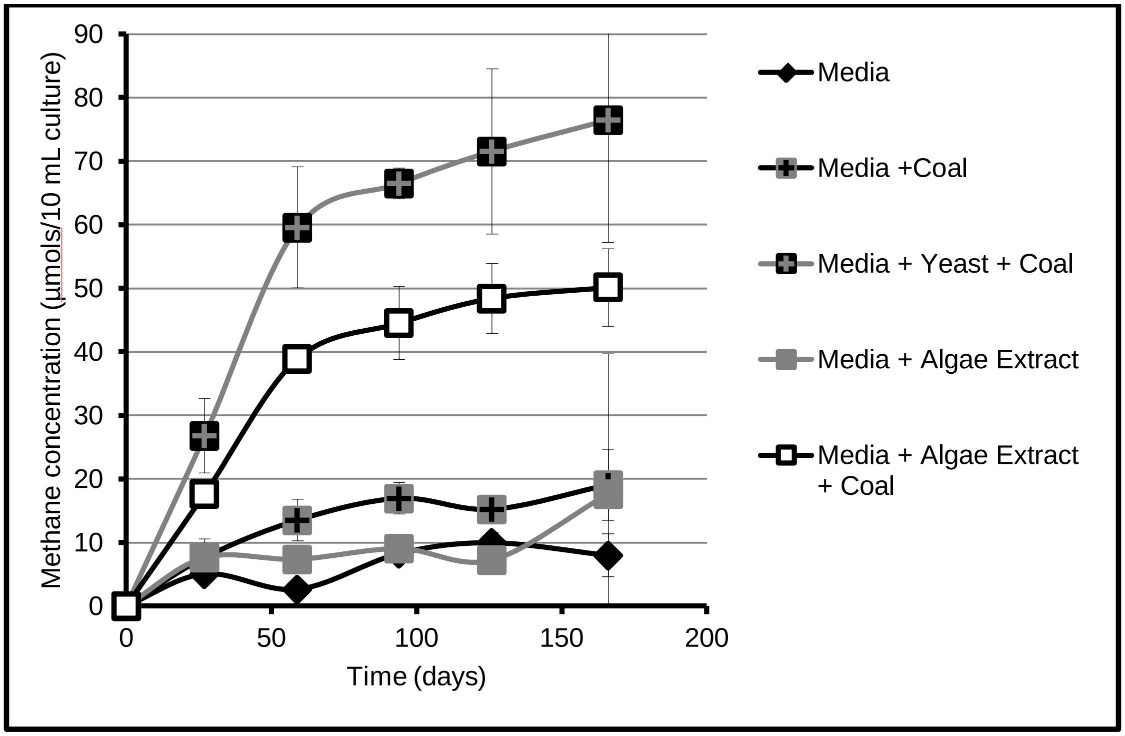

Figure 15: Algae Stimulated CBM: Methane measurements indicate algal biomass with lipids removed increase CBM production. This data supports the hypothesis that algae naturally stimulate CBM production in the PRB. The CBM production was not enhanced as much with algal biomass as with yeast extract.

Discussion

Methane production is an essential part of the global carbon cycle that allows the breakdown of complex organic materials by *Bacteria* that would otherwise be thermodynamically unfavorable (syntrophic degradation) (McInerney and others, 2009).

The anaerobic breakdown of complex carbon molecules is usually very slow but can be stimulated with the addition of certain nutrients (Cunningham and others, 2001). Nutrient additions such as yeast extract can stimulate acetate production by bacteria (Liria and others, 1998). In this study sequences related to *Proteobacteria* (*Geobacter, Collimonas* and *Syntrophus*) dominated the *in situ* DMS and coal enrichment community while *Clostridial* sequences (*Acetobacterium*, *Fusibacter* and *Sedimentibacter*) dominated the communities with yeast extract added (Fig. 12). *Methanosaeta* and *Methanosarcinaceae*, the only two identified methanogenic families that can utilize acetate as a substrate for methanogenesis. These methanogens were detected in enrichments but not *in situ.* These results suggest that these methanogens groups were present *in-situ* but were below the detection limit; however, these groups were stimulated in the batch enrichment cultures. The ability to compare the *in situ* microbial community from the DMS to enrichment communities provided a comparison to previous studies which demonstrated coal-beds are generally colonized by an array of bacterial taxa that commonly include *Proteobacteria* (Li and others, 2008; Midgley and others, 2010; Penner and others, 2010) while *Firmicutes*, mostly from the order *Clostridiales,* dominate enrichment cultures (Green and others, 2008; Strąpoć and others, 2008; Penner and others, 2010). *Methanosarcinaceae* have also been previously detected in enrichment cultures inoculated from coal-beds in the PRB and in Canadian coal-beds (Penner and others, 2010; Green and others, 2008).

The *Methanosarcinaceae* family contains the only identified acetoclastic methanogens (*Methanosaeta* and *Methanosarcina*). *Methanosarcina spp.* are the only identified methanogens with the *acetate kinase*/*phosphotransacetylase* (*ack/pta*) pathway involved in methane production (Ferry, 1992). This pathway allows *Methanosarcina* spp. to activate acetate at the expense of one high-energy phosphate bond while AMP-*acs* utilized by *Methanosaeta* spp. requires two high-energy phosphate bonds (Jetten, 1990; Jetten and others, 1989). The efficiency of the enzymes *Ack/Pta* allow *Methanosarcina* to utilize acetate more rapidly than *Methanosaeta* when concentrations are high (Min and Zinder, 1989) thereby significantly increasing the flux of biogenic methane in high acetate environments. The enrichment cultures with yeast extract but without coal contained the highest proportion of *Methanosarcina* SSU rDNA sequences but did not yield the highest concentrations of methane. Research suggests the presence of hydrogen greatly stimulates the acetoclastic methanogenesis by *Methanosarcina spp*. (Krzycki and Zeikus, 1984). The archaeal community detected in the DMS and the coal enrichment without yeast extract were dominated by hydrogenotrophic methanogens; *Methanospirillum* and *Methanoregula*. SSU rRNA sequences indicative of hydrogenotrophic methanogens also comprised a much larger proportion of the microbial community in the yeast extract enrichment with coal than the yeast extract only enrichment. In addition *Methanospirillum* and *Methanoregula* were the dominant methanogens in water samples from Canadian oil sand reservoirs (Hubert and others, 2011). Although the prediction of function from phylogenetic data can be misleading, the increase in hydrogneotrophic methanogens in association with complex carbon sources such as coal in our pyrotag data is supported by other research. Therefore, *Archaeal* community analysis suggests bacterial degradation of complex carbon sources such as coal or oil allow for the production of hydrogen necessary to support hydrogenotrophic methanogens and possibly stimulate the *Ack/Pta* pathway when bioavailable biomass such as lysed yeast cells are present as a source of acetate.

An acetoclastic methanogenic isotopic signature exists along the western margin of the PRB (Bates and others, 2011; Flores and others, 2008). Previous enrichment studies suggest complex organic nutrients such as yeast extract and tryptone, stimulate CBM production whereas the addition of non-nitrogenous nutrients such as acetate, phosphate, and different anions and cations failed to stimulate CBM production (Penner and others, 2010; Barker and Dallegge, 2006). A thorough investigation of many production wells throughout the PRB provided a positive correlation between nitrogen levels and methane production, but the source of the nitrogen was not determined (Meredith and others, 2011). In addition to providing a bioavailable substrate for acetate production, lysed algal biomass could also provide a nitrogen source based on an average composition of microalgae given by $C_{2.08}H_{3.81}O_{1.0}N_{0.2}P_{0.02}$ (Grobbelaar, 2004; Heaven and others, 2011). A recent study detected a high proportion (>15% of total detected bacterial community) of phototrophic DNA sequences in a well on the western margin of the PRB where significant recharge occurs (Barnhart and others, 2013). This study further suggests that phototrophic biomass in surface waters could naturally impact CBM production *in-situ*.

This research provides a link between green energy development and CBM production while providing a beneficial use for the water produced from CBM development. Large scale production of algae for biofuels is unsustainable with existing technologies (NRC, 2012). Algal biofuel production has been limited, partially due to water access for algal growth (NRC, 2012). The Montana portion of the PRB is much smaller than the Wyoming portion but still could have almost 25,000 CBM production wells at full capacity (BLM, 2007) which would produce more than 1,100,000 ha-m of groundwater (All, 2001; Meyers, 2009). High concentrations of sodium bicarbonate is the geochemical signature of CBM water in the PRB (Van Voast, 2003). Although sodium bicarbonate can negatively impact irrigated soils, it may provide an excellent medium for algal biofuel production because of the recently documented ability of sodium bicarbonate to stimulate lipid production in algae (Gardner and others, 2012). In addition to biofuels, algae also offer a diverse spectrum of valuable products such as food, nutritional compounds, omega-3 fatty acids and animal feed which could provide additional revenue for this energy generating system. Further research is needed to fully understand the applicability of algae-enhanced CBM production.

Conclusion

This analysis suggested stimulation with biomass resulted in a microbial community shift from hydrogen production/utilization to microorganisms capable of acetate production/utilization. Sequences most closely related to *Firmicutes* and acetoclastic methanogens dominated coal with yeast extract and yeast extract only enrichments. A higher proportion of hydrogenotrophic methanogens were detected *in situ* in DMSs and in enrichments with coal only, and these results suggest a hydrogen-based system is important under non-stimulated conditions. Algae biomass with the lipids extracted stimulated methane production similarly to yeast extract (~30µmol methane increase per gram of coal after 100 days). These results help better understand the microbial processes generating an *in situ* acetoclastic isotopic methane signature in a major ground water recharge area of the PRB where a large proportion of phototrophic SSU rRNA sequences were detected (Bates and others, 2011; Barnhart and others, 2013). This research provides a basis for future research into coupled biological systems (surface photosynthesis and subsurface methane production) that could sustainably enhance CBM production and generate algal biofuels while also sequestering carbon dioxide (CO_2).

CHAPTER 4 - ISOLATION OF MICROORGANISMS FROM SUBSURFACE COALBEDS

Contribution of Authors and Co-Authors

Manuscript in Chapter 4

Author: Elliott P. Barnhart

Contributions: Conducted experiments, analyzed data and wrote the chapter

Manuscript Information Page

Elliott P. Barnhart
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Abstract

Coal-bed methane (CBM) has been harvested from the Powder River Basin (PRB) (southeastern Montana and Wyoming) since the early 1990s but little is known about the associated coal-dependent methanogenesis. An initial microbial isolation study from a CBM-producing well in the PRB was conducted to supplement previous phylogenetic analysis. Through several isolation attempts, a facultative anaerobic bacterium (*Pleomorphomonas spp.*) was isolated that could hydrolyze urea. A *Methanosarcina* spp. was also isolated from other *Archaea* in an enrichment culture. Microbial isolation from subsurface environments is important because phylogenetic studies suggest novel microorganisms exist in coal-bed environments. Further isolation studies could provide a better understanding of the ecology and physiology of the methane-producing populations and allow scaffolding for metagenomic studies.

Introduction

Studies from methane producing surface environments (peatlands and wetlands) have determined four functionally diverse microorganisms are necessary for methane production; 1) hydrolytic bacteria, 2) fermentative bacteria, 3) acetogenic bacteria and 4) methanogens (Whiticar, 1999). Hydrolytic bacteria break down higher molecular weight substrates into monomeric subunits such as short-chain fatty acids, monosaccharides and amino acids that can be utilized by fermentative microorganisms to produce short-chain alcohols, acetate, formate, hydrogen and carbon dioxide. While acetate and carbon dioxide can be used directly by methanogens to produce methane, alcohols and acids can be degraded further by syntrophic microbes to produce simple acids, alcohols, and/or H_2 (Whiticar, 1999). Depending on presence of alternative electron acceptors (*e.g.*, sulfate, Fe(III), nitrate), bacteria and archaea will compete for the simple acids, simple alcohols, and H_2. In the absence of alternative electron acceptors, acetogens and methanogens compete for H_2. Each downstream step is important by preventing the buildup of intermediate products and by-products that can become toxic/inhibitory. With coal as the starting material for methane production there are many questions that remain unanswered that include, the types of microorganisms directly breaking down the coal and which substrates are produced for the methanogens.

Studies investigating the community members of coal-beds have mostly relied on the SSU rRNA sequences obtained from either water samples or core samples (Strąpoć and others, 2011; Penner and others, 2010). These studies can result in SSU rRNA sequences that have a low identity to any microorganisms currently in the National Center for Biotechnology Information (NCBI) database or other databases (Barnhart and others, 2013). These results indicate some microorganisms inhabiting the coal-beds have not previously been identified and a better understanding of their physiology might be obtained if these novel microorganisms are isolated from the coal-bed environment and studied in isolation. In addition, isolation studies can help confirm microorganisms are viable and active within the coal-beds which can be very difficult to verify with phylogenetic methods (Klein and others, 2008). Initial isolation studies were conducted in this study to supplement previous phylogenetic methods performed on the same enrichments (Barnhart and others, 2013). Although novel microorganisms were not isolated, several methods were optimized that may help with future isolation studies from coal-bed environments and the presence of several viable microorganisms was confirmed.

Methods

Site Description

The study site (Well WR-53) was located in southeastern Montana in the CX field (Wheaton and Donato, 2004). Well WR-53 is a monitoring well used by the Montana Bureau of Mines and Geology to monitor the effect of CBM development on regional groundwater tables. The well was drilled in 1977 to a depth of 117 meters, sealed with a packer from 88.4 meters to 90.5 meters and screened from 91.44 meters to 116 meters. Site location and well logs can be obtained at the Montana Bureau of Mines and Geology Groundwater Information Center (GWIC) website: http://mbmggwic.mtech.edu with GWIC ID 132907.

Environmental Sampling

A DMS was utilized in this study as previously described (Barnhart and others, 2013). The DMS was filled with sterile coal which is the proposed substrate for methane generation within the PRB. It was deployed to the bottom of methane-producing well WR-53. The DMS was retrieved using aseptic techniques and slurry (3 mL) was immediately added to anaerobic coculture medium (CCM) containing 30mM sodium acetate and (per liter) 2.17 g NaCl, 5.5 g $MgCl_2 \cdot 6H_2O$, 0.14 g $CaCl_2 \cdot 2H_2O$, 0.5 g NH_4Cl, and 0.335 g KCl. The medium was buffered using 1.1 mM K_2HPO_4 and 30 mM $NaHCO_3$ with 1 ml of 1000X per liter nonchelated trace elements and 1 ml of 1000X per liter vitamin solution amended with 2.0 g/L choline chloride added as growth supplements. L-Cysteine • HCl (1 mM) and sulfide (1 mM as $Na_2S \cdot 9H_2O$) were added as reducing agents. Resazurin (1 mg/liter) was added as a redox indicator. Stock solutions of K_2HPO_4 (1 M), $NaHCO_3$ (6.0 M), L-cysteine • HCl (1 M), $Na_2S \cdot 9H_2O$ (1 M), and the nonchelated trace element and vitamin mixtures were prepared under anoxic conditions (Walker and others, 2009). Methane production from laboratory cultures was monitored by direct injection on a SRI 8610C gas chromatograph (GC) with a thermal conductivity detector (TCD) and a stainless-steel molecular sieve 13X packed column with helium as the carrier gas. After significant methane production was detected 1 mL of inoculated media from methane producing cultures was used to inoculate fresh CCM with coal added in place of sodium acetate as a carbon source. Cultures were incubated in the dark at 25°C, methane production was again monitored and phylogenetic and isolation strategies were carried out on positive methane-producing cultures.

Isolation Strategies

Isolation of Archaea

Microbial isolation was initiated by inoculating a new tube of 10 mL sterile CCM with 1 mL of CCM slurry from a methane-producing coal culture anaerobically. This CCM contained all the constituents previously described in addition to methanol 0.05M, yeast extract 0.02%, 30mM sodium acetate and 100mg/L vancomycin but no coal. The headspace was pressurized after inoculation with 80:20 H_2:CO_2. The inoculation was performed in a glove bag to ensure an anaerobic environment was maintained. The cultures were monitored for methane production and the optical density was measured to record growth. The cultures were incubated in the dark at room temperature. After 150 hours significant growth was recorded and methane production was also detected (data not shown). To further isolate methanogens, 1 mL of the methane-producing culture was removed and transferred to a roll tube that contained CCM isolation medium with the addition of 4% agar. The roll tubes were monitored for methane production and examined for colony formation. Methane production was detected but no colonies formed on the inside of the tube. The liquid in the bottom of the roll tube was examined with a phase contrast microscope and microorganisms with the morphology of known methanogens were detected as well as some microorganisms that resembled rod-shaped bacteria (Fig. 15 A). In addition, most of the microorganisms were autofluorescent under 420 nm input light indicative of methanogens (Fig. 15 B).

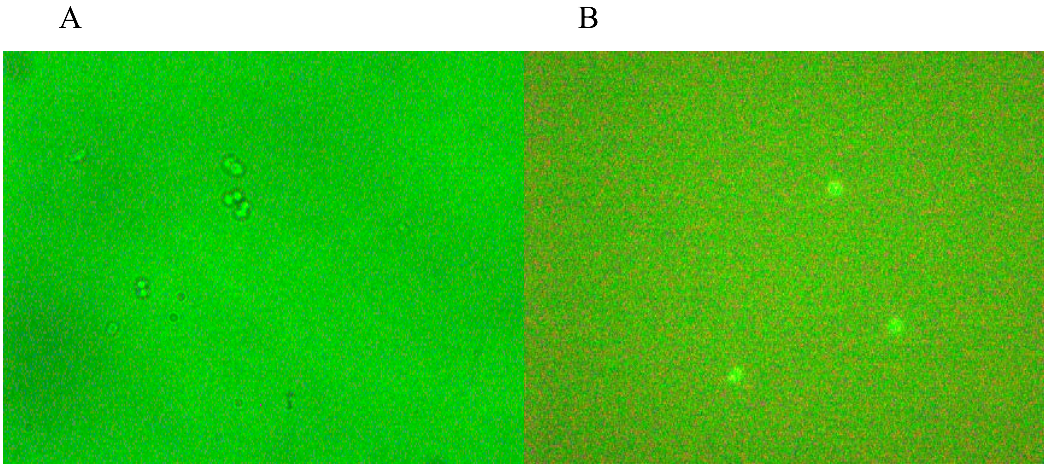

Figure 16: Images of methanogen in bottom of roll tube. (A) Phase contrast image with a green filter at 1000X. (B) Phase contrast image showing autofluorescence of the methanogens.

The detection of non-autofluorescent microorganisms indicated that the methanogens were not fully isolated from bacteria so a dilution to extinction was performed by adding 1 mL of CCM to the roll tube and then extracting 1 mL of liquid back out anaerobically and adding it to sterile CCM. Subsequent dilutions were carried out to further isolate the methanogen. Methane production was monitored for two months, but no methane was detected. Due to time constraints an attempt at sequencing was performed by removing 0.5 mL of liquid from the bottom of the methane positive roll tube and the two lowest dilutions that did not produce methane. The three samples were centrifuged separately and the supernatant was discarded. A toothpick was used to touch the centrifuged pellet and a whole cell PCR was performed using the archaeal primers and thermal profile previously described with an extra 5 minute 95°C lysis step preceding the PCR (Barnhart and others, 2013). A PCR product was only obtained from the PCR reaction using template from the roll tube. The quality of the PCR chromatogram indicated the methanogen was isolated from other *Archaea.* This PCR product was purified and approximately the first 530 base pairs were sequenced as previously described (Barnhart and others, 2013) and added to a phylogenetic tree of clone library sequences from the original enrichment depicting the archaeal community (Fig. 16).

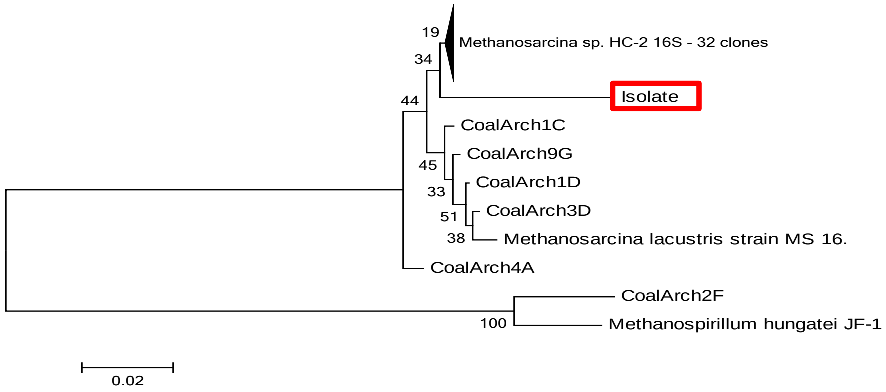

Figure 17: A neighbor -joining distance tree was constructed using the Jukes -Cantor method in MEGA 4 for both *Archaea* to investigate the diversity of microorganisms existing in the original culture with the isolate in the tree identified with the red box.

A scanning electron microscope (SEM) was used to visualize glutaraldehyde-fixed cell suspensions from the liquid from the bottom of the roll tube. The liquid was applied to glass coverslips coated with poly-L-lysine, allowed to adhere for 5 min, rinsed with water and post-fixed with 1% OsO_4 for 30 min. Cells on coverslips were then rinsed with water, dehydrated through a graded ethanol series to absolute ethanol, and then critically point dried with liquid CO_2. Coverslips were mounted on an aluminium stub, sputter-coated with Au/Pd and examined with a scanning electron microscope at an accelerating voltage of 15 kV and magnifications of 20,000–60,000 X (Fig. 17).

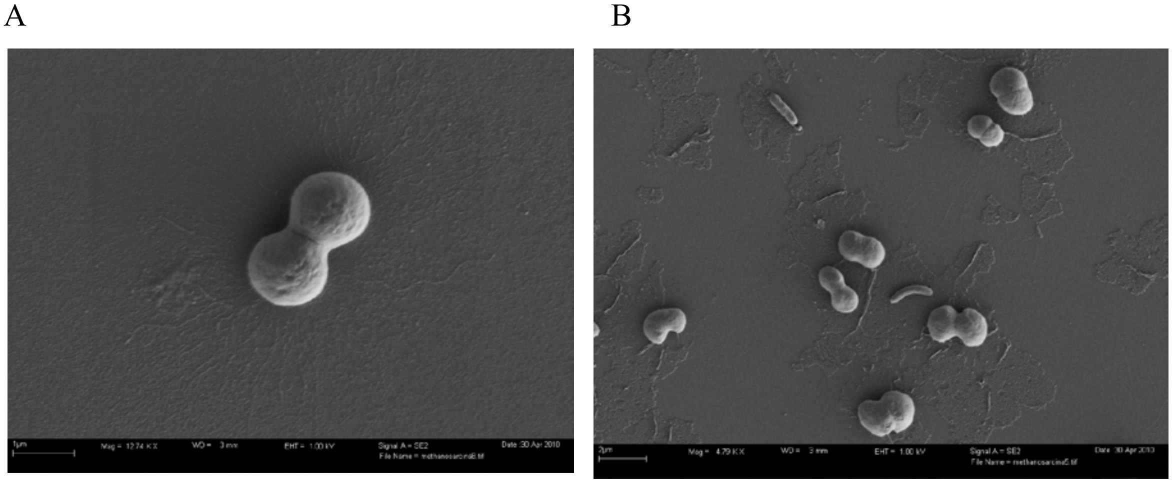

Figure 18: Images of the *Methanosarcina spp.* (A) Scanning electron micrograph of the *Methanosarcina spp.* with the typical barbell shape (B) Scanning electron micrograph of the methanogen and what appear to be several other microorganisms.

Isolation of Bacteria

It had been previously determined through phylogenetic analysis that there was a very diverse bacterial community within the coal enrichments (Barnhart and others, 2013). To reduce the diversity and enrich for spore forming microorganisms, an enrichment was heated at 80°C for ten minutes. Medium (~0.25 mL) from a methane-producing coal culture that had been heated and also one that had not been heated was removed anaerobically with a syringe and transferred to three different solid plates of CCM media that contained 1.5% (w/v) agar and was spread onto the agar medium with a sterile glass plate spreader. The three different CCM plates contained all the constituents of the original CCM described above in addition to: coal extract and 5 g/L yeast extract, coal extract and 5 g/L urea or coal extract only. The coal extract was obtained by adding 1 kg of coal to 1 L of water. The mixture was vortexed, autoclaved and de-gassed with nitrogen. The liquid coal extract was added in place of 50% of the water added to the original CCM. The plates were incubated in the dark in an anaerobic chamber. Colonies only appeared after seven days on yeast extract CCM plates and after ten days on the urea CCM plates that were inoculated from the unheated cultures. No colonies formed on the plates from the heated culture after 2 months of incubation. When significant growth was observed on the yeast extract CCM and urea CCM plates, a colony from each was picked and transferred to a new plate with the same medium composition. This procedure was repeated for two successive transfers but different colony morphologies were observed on each plate, indicating a mixed culture. A new transfer was performed by transferring a colony from a yeast extract CCM plate to a urea CCM plate. This resulted in colonies with uniform colony morphology on the urea CCM plate. A colony from this plate was transferred again to a new urea CCM plate and uniform growth was again observed. A colony was removed, placed in sterile water and removed from the glove bag. This suspension was observed with a phase contrast microscope and a uniform cell morphology appeared (Fig. 18A). A colony was transferred from the urea CCM plate onto a new urea CCM plate and taken out of the glove bag. Growth in the presence of air was observed but at a much slower rate compared to anaerobic growth. The aerobic culture was observed with a phase contrast microscope and the same cell morphology was observed as with the anaerobic suspension (Fig. 18B). Cell suspensions were also visualized with scanning electron microscopy (SEM) (Fig. 18C).

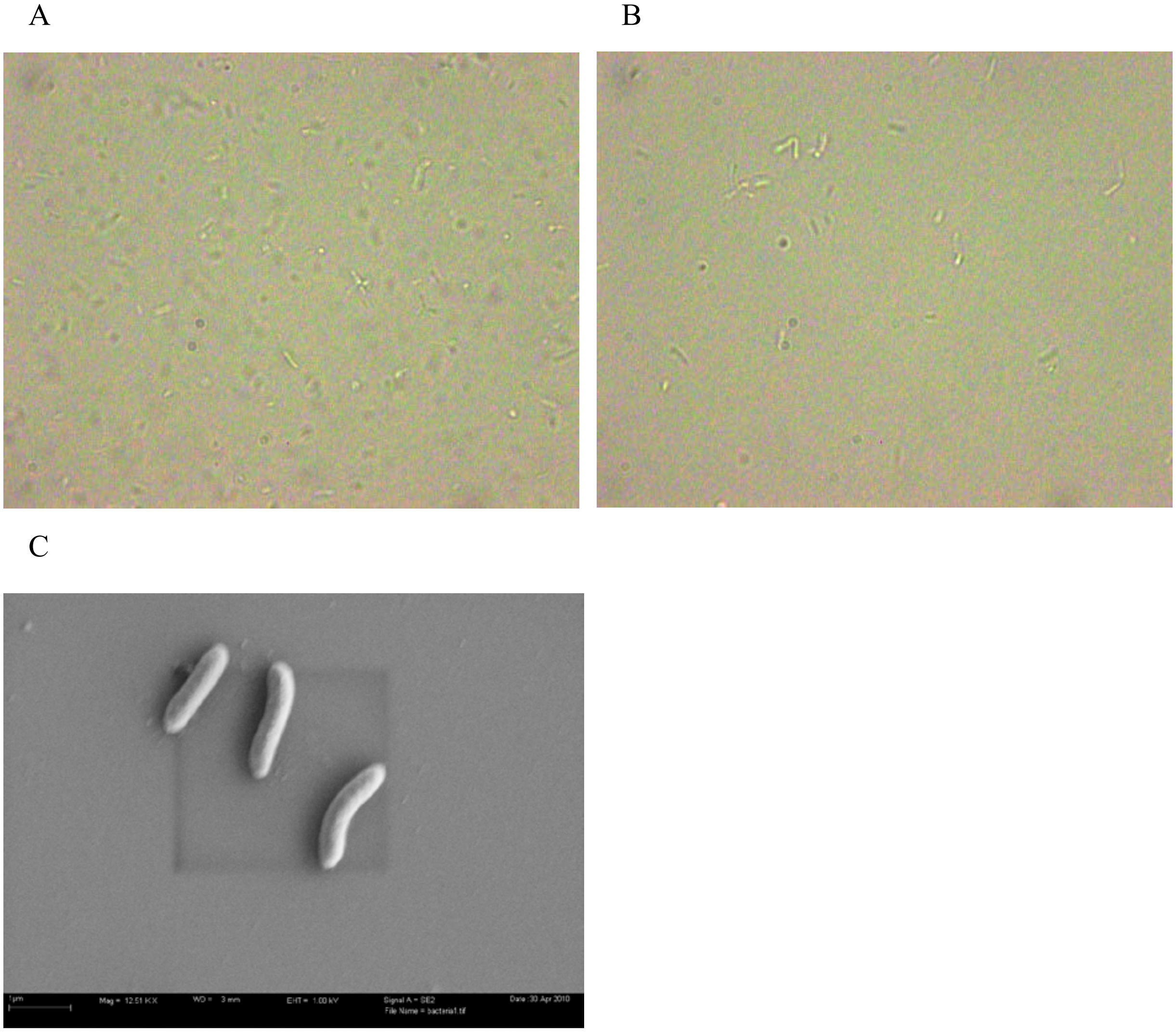

Figure 19: Images of the isolated *Pleomorphomonas* bacteria. (A) Phase contrast image of of the anaerobic culture at 1000X. (B) Phase contrast image of the aerobic culture at 1000X. (C) Scanning electron micrograph of the microorganism.

A toothpick was used to obtain three different colonies on the anaerobic urea CCM plate and a whole cell PCR was performed with an extra 5 minute 95°C lysis step preceding the PCR reaction previously described (Barnhart and others, 2013). A PCR product was obtained from all three PCR reactions. Comparison of the 16S rRNA gene sequence against GenBank using the blastn search tool revealed that the constituents of all three bacterial colonies were most closely related to *Pleomorphomonas oryzae* (100%). A phylogenetic tree constructed using SSU rRNA gene sequences showed that all of the isolates were not closely related to any microorganisms previously found in the enrichment (data not shown). Growth was observed on Christensen agar both anaerobically and aerobically and a color change was observed in the agar indicating the isolate could hydrolyze urea. No growth was observed on the nitrogen-free plates or the nitrogen-free liquid media indicating the isolate could not fix nitrogen. All of the growth experiments were carried out in the dark at room temperature.

Discussion

The involvement of bacteria in the CBM process is not well understood. Coal degrading microorganisms from the deep subsurface have not been previously isolated. Several attempts at targeting different microbial communities were initiated in this study. Isolation strategies targeting spore forming or coal degrading microorganisms were unsuccessful. Colonies only formed on plates that contained yeast extract and urea, inoculated with the non-heat treated culture. Subsequent transfers onto the same plate did not yield isolates, so a cross- transfer of a colony from a yeast extract plate to a urea plate was implemented. This put selective pressure on the mixed colony from the yeast extract plate and enriched for microorganisms that could utilize urea as a nitrogen source. An isolated colony was streaked on a urea and coal extract plate and placed in the dark outside of the glove bag in the presence of oxygen and growth was observed. Although the growth was observed, the colonies did not get as big as when incubated anaerobically, so it was determined that the isolate was a oxygen tolerant. All the cells appeared to be nonmotile pleomorphic rods (Fig. 18). SEM images also showed 1.0 um rod-shaped microorganisms (Fig. 18). Comparison of the SSU rRNA gene sequence against GenBank using the blastn search revealed that the constituents of all three bacterial colonies were most closely related to *Pleomorphomonas oryzae* (100%). *Pleomorphomonas oryzae* has been classified as a nitrogen fixing alpha-proteobacterium that was previously isolated from paddy soil in Japan (Xie and Yokota, 2005). Several physiological tests were performed on the isolated bacteria. Growth was observed on the Christensen agar both anaerobically and aerobically and a color change was also observed in the agar indicating that the organism was producing NH_3 by hydrolyzing the urea which caused the pH to increase in the media. No growth was observed on the nitrogen-free plates or the nitrogen-free liquid media suggesting this isolate could not fix nitrogen on the provided media.

Methanogens were enriched using CCM with methanol, yeast extract, sodium acetate and vancomycin, and an overpressurized headspace of CO_2 and H_2. Vancomycin inhibited bacterial growth by disrupting cell wall synthesis of gram positive bacteria, yet had no effect on the target archaeal populations. The methanol, sodium acetate and H_2:CO_2 were used to enrich all methanogenic populations in the culture because these

Archaea obtain their energy for growth from a limited number of substrates including CO_2-type substrates, methyl substrates, and acetate substrates. Methanogens also utilize different pathways to access these substrates and some pathways function optimally under different concentrations of the substrates. The concentrations of substrates added to these enrichments would select for methanogens that prefer higher concentrations of substrates. The methanogens that can utilize acetate are restricted to the genera *Methanosarcina* and *Methanosaeta* (Boone and others, 1993; Zinder, 1993; Rother, 2010). *Methanosarcina* is the only methanogen with access to the (acetate kinase / phosphotransacetylase) Ack/Pta pathway which can out-compete *Methanosaeta* when acetate concentrations are high (Min and Zinder, 1989). *Methanosarcina spp*. have also been found to utilize $CO_2 + H_2$ and methanol for methane production (Müller and others, 1986). SSU rRNA sequences related to *Methanospirillum* and *Methanosarcina* were found in the original enrichment samples clone library but the number of *Methanosarcina* clones greatly outnumbered the *Methanospirillum* clones (Barnhart and others, 2013). When the liquid from the bottom of the roll tube was visualized with a phase contrast microscope at 1000X the autofluorescent organisms were irregular coccoids 0.5-1.5 um in diameter which indicated that the microorganisms were not a *Methanospirillum* species, which usually grow in groups consisting of chains 10 or 12 cells long (Beveridge and others, 1991). Methanogens contain coenzyme F420 which is autofluorescent at wavelength 420nm. The number of autofluorescent organisms greatly outnumbered the non-autofluorescent small bacteria-like rods that could be in the sample. The non-autofluorescent microorganisms could have been vancomycin-resistant *Bacteria*. The

SEM images of the *Archaea* showed clear 1.0-1.5 um dumbbell shaped cells similar to *Methanosarcina* species isolated in other studies (Zinder, 1993). Comparison of the SSU rRNA gene sequence against GenBank using the Blast search tool revealed the isolate was most closely related to methanogenic species *Methanosarcina* sp. HC-2 (98%). A phylogenetic tree constructed using SSU rRNA gene sequences also showed that this isolate clustered near *Methanosarcina* clones from the original enrichment (Fig. 16). Although *Methanosarcina spp.* have been detected in other coal-bed environments (Penner and others, 2010), this is the first time the methanogen has been isolated from a coal-bed environment. This is the only methanogen known to possess the Ack/Pta pathway which is important in stimulated methane production (Galagan and others, 2002). Isolation of *Methanosarcina* supports phylogenetic analysis and provides further evidence that this methanogen is viable in the subsurface coal-beds which is important if stimulation of microbial CBM production is pursued.

Conclusion

It is important to grow the members of this coal-bed community separately and investigate how they function in isolation to fully understand how coal-bed methane is produced. The results of this study determined that viable bacteria and methanogens exist in the coal-beds and can be isolated. Several isolation strategies were investigated in this study. Through these attempts a facultative anaerobic bacterium was isolated that has the ability to hydrolyze urea while using coal as a carbon source and a *Methanosarcina spp.*, capable of utilizing byproducts from coal degradation was also nearly isolated. Investigation of these microorganisms in isolation could provide a better understanding of the microbial ecology and physiology in coal-beds.

Acknowledgements

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CHAPTER 5 - GENOMIC INSIGHT INTO THE EVOLUTION OF THE ACETATE SWITCH IN ARCHAEA

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Abstract

Understanding how methanogens have evolved to optimize energy conservation is central to understanding the evolution of biological systems, impact on the carbon-cycle, greenhouse gas emissions, and the structuring of bioenergy-production systems. We have identified homology between AMP acetyl coA synthetase (AMP-*acs*), ADP acetyl coA synthetase (ADP-*acs*) and acetate kinase (*ack*) that suggests these proteins are related through gene duplication and divergence. Further evidence is provided by structural relationships shared among the proteins and phylogenetic analysis. Genome synteny comparisons between halophilic methanogens and closely related *Methanosarcina* spp. suggest *ack* originated from an ancient duplicated ADP-*acs* α subunit. A duplicated phosphotransacetylase gene (*pta*) with an active site was identified in halophilic *Euryarchaeota* (*i.e.*, *Halococcus)*, suggesting these sequences also played a role in the development of acetate utilization. Horizontal transfer of the *pta* homolog to a halophilic methanogen that possessed an ancient *ack* could have formed the Ack/Pta pathway. The Ack/Pta pathway would have increased the metabolic diversity of the ancient methanogen and may have led to the speciation of *Methanosarcina*.

Introduction

Acetate is an important metabolite that cells can use to balance carbon and electron flow, and the survival of biological systems requires efficient utilization of resources. The “acetate switch” refers to a metabolic capacity for cells to either excrete or dissimilate acetate and provides an adaptive advantage in dynamic environments that allows an opportunity for rapid growth in the presence of abundant nutrients and enhanced survival when nutrients are depleted (Wolfe, 2005). The AMP acetyl coA synthetase (AMP-acs), phosphotransacetylase (Pta) and acetate kinase (Ack) are central to the acetate switch (Wolfe, 2005; Kuit and others, 2012) and catalyze the overall process of acetate excretion or acetate utilization. The ability to excrete and/or utilize acetate is wide-spread across the three domains, and the acetate kinase is thought to be the early ancestor of the acetate and sugar kinases/Hsc70/actin (ASKHA) protein superfamily (Buss and others, 2001). *Archaea* and several *Eukarya* have an additional enzyme involved in acetate production, ADP acetyl coA synthetase (ADP-acs) that performs a similar biochemical reaction but with a different by-product (Musfeldt and Schönheit, 2002; Sánchez and others, 2000). Gene duplication and divergence, the main source of genome level functional diversity, generates ancestral genes with similar functionality to duplicated genes that diverged within a genome (Kondrashov, 2012; Lynch and Conery, 2000; Conant and Wolfe, 2008). We hypothesize that AMP-acs, ADP-acs and Ack are related through gene duplication and divergence as they share similar substrates and products.

$\mathrm{CH_3CO_2^-} + \mathrm{ATP} + \mathrm{CoA} \rightarrow {}^{\text{AMP-Acs}} \rightarrow \mathrm{CH_3CO\text{-}CoA} + \mathrm{AMP} + 2\mathrm{PO_4^{3-}}$

$\mathrm{CH_3CO_2^-} + \mathrm{ATP} + \mathrm{CoA} \leftarrow {}^{\text{ADP-Acs}} \rightarrow \mathrm{CH_3CO\text{-}CoA} + \mathrm{ADP} + \mathrm{PO_4^{3-}}$

$\mathrm{CH_3CO_2^-} + \mathrm{ATP} \leftarrow {}^{\text{Ack}} \rightarrow \mathrm{CH_3COPO_4^{2-}} + \mathrm{ADP}$

It is generally thought energy-conservation was the major force that directed early cell development (Ferry and House, 2006). Phylogenetic and genome synteny comparisons provide evidence for the establishment of the first Ack within an archaeal, methanogenic genome through gene duplication and divergence of an ADP acetyl coA synthetase (ADP-Acs). A *pta* homolog within *Halobacteriales* provides a mechanism for the acquisition of *pta* within a halophilic environment. Previous research had indicated the Ack/Pta pathway was provided to the ancient methanogen through a horizontal gene transfer of *ack* and *pta* from *Bacteria* but does not provide an origin hypothesis for the Ack/Pta pathway in *Bacteria*. Therefore, the described research provides novel insight into a potential mechanism for the evolution of acetate-dependent methanogenesis.

Methods

Sequence Alignment

Amino acid sequences gi302391242 (6-365), gi301637019 (3-356), gi357173877 (4-356), gi336234763 (7-369), gi197088018 (3-421), gi15802843 (4-400), gi506405859 (3-397), gi17907861 (3-408), gi335930152 (9-505), gi298675798 (9-503), gi294496302 (9-510), gi21226460 (24-468), gi452208966 (11-455), gi19917192 (24-468), gi57160139 (13-469), gi389853130 (17-472), gi452211690 (202-557), gi73669665 (202-559), gi20091733 (205-560), gi493399067 (194-549), gi220928894 (199-559), gi513029392 (256-652), gi330506788 (280-672), gi386001446 (273-662), gi116666161 (267-656) were obtained from the National Center for Biotechnology Information (NCBI) genomic database. The sequences were trimmed in Jalview and aligned with Mafft using a preset default setting followed by the preset settings E-INS-I and finally L-INS-I (Waterhouse and others, 2009; Katoh and Standley, 2013). The sequences were manually adjusted based on secondary structures provided by Jpred (Cole and others, 2008)

HHPred Analysis

Using HHPred (Open source 2.0) a homology detection and structure predictor by HMM-HMM comparison hosted by the Max-Planck Institute for Developmental Biology via the Bioinformatics Toolkit, 6 sequences (2AMPacs, 2ADPacs, 2Ack) from the Jalview alignment were submitted for multiple sequence alignment generation HHblits using 3 iterations and global alignment mode. Comparison to the PDB70 HMM database updated 26 April 2014 was made and structural comparison was made to length of

ADPacs AMPacs and Acetate Kinase and Butyrate Kinase at probability of 100% and Evalues of 9.1E-82, 8.4E-63, 1.3E-23, 1E-12, respectively. This was done without realigning with MAC. This structural evidence supports homology at the superfamily and fold level. When changing the HMM databases by comparing to CDD_19Feb14, Interpro, Pfam but running the same other switches, the outcome was the same but with better E values and higher probability as there are more applicable sequences available in these databases than crystal structures. Both local and global modes modeled homology to the whole protein.

Structure Analysis

A structure of AMP-Acs from *Methanosarcina acetivorans* containing a link between Lys256 and Cys298 identified as 3ETC_A determined from the Protein Data Base was referenced using amino acids 202-517. Amino acids numbered 1-201 in the sequence were removed for alignment purposes as motif A3 (or Phosphate 1 in ASHKA) start at amino acid 208. Structures were compared to Ack, specifically actin, sugar kinase ATPase cleft formed by alpha and beta helixes through Pymol (The PyMOL Molecular Graphics System, Version 1.5.0.4 Schrödinger, LLC).

Phylogenetic Analysis

The BEASTv1.8.0 (Drummond and Rambaut, 2007) tree was created from the manually adjusted sequence alignment using a 10 million step chain with 20% burn-in; run under the WAG amino acid substitution model (Whelan and others, 2001) and rate heterogeneity among sites (gamma distribution with 8 categories) with relaxed exponential clock model and coalescent constant population tree prior. Four Constraint priors were applied for the Buk, Ack, AMP-Acs and ADP-Acs groups from a preliminary UPGMA tree generated from MEGA (data not shown), a randomized tree was used as the starting tree input.

Genome Synteny Analysis

Gene synteny was determined by generating maps which depicted areas of the genome encoding proteins with JGI-IMG Usearch and Nearest Neighbor functions (Markowitz and others, 2006; Markowitz and others, 2008; Markowitz and others, 2012; Edgar, 2010). A gene encoding ADP-Acs β subunit (gi20904871) was observed adjacent to a gene encoding an *ack* protein within the *M. mazei* genome. The amino acid sequence was obtained from Genbank and a tblastn search was performed on the nearest phylogenetic neighbors to *Methanosarcina,* with the ADP-*acs* β subunit (gi20904871) as a query. Homologous genes encoding the ADP-Acs β subunit (gi20904871) within the halophilic methanogens genomes were fused with genes encoding an ADP-*acs* α subunit (gi294496302) and (gi298675798).

Phosphotransacetylase Homolog

A blastp search of completed *Archaeal* genomes in the NCBI and the Joint Genome Institute (JGI) genomic databases (2013) with the corresponding translated *pta* sequence (gi20092407) from *Methanosarcina acetoverans* as a query revealed a *pta* homolog fused to a malic enzyme (*me*) or malate dehydrogenase decarboxylating (*mdh*) gene within every *Halobacteriales* genome as well as a lone *pta* homolog isolated from *mdh* in

Halococcus (Altschul and others, 1990; Benson and others, 2004). The *pta* homologs in *Halobacteriales* have a nearly 100% query coverage and an amino acid identity between 30%-35% (all E values $<2e^{-34}$). Amino acid sequences gi518500513 (726-742), gi378259043 (422-438), gi445789056 (721-737), gi257170109 (722-738), gi495013868 (301-319), gi490155740 (301-319), gi19917662 (303-319), gi452099000 (303-319), gi220929552 (303-319), gi346722880 (304-320) were aligned and trimmed as previously described.

Shared ATPase Motif Conservation

In addition to shared substrates and products, our alignment shows the three proteins (AMP-Acs; ADP-Acs; and Ack) share motif and active site features (Fig. 19). The active site of AMP-Acs, located between the two domains of the protein, is formed by core motifs that include a serine and glycine-rich loop (termed region A3), a threonine-glutamate dipeptide with an invariant glutamate residue (A5), a DX6GXR motif with an invariant glycine (A8), and a region that contains a lysine residue completely conserved in all AMP-forming enzymes (A10) (Gulick and others, 2003; Starai and others, 2002). The ADP-Acs active site has not been as well defined due to being discovered recently and a lack of protein structures. However, active site and motif inferences have been previously reported based on a close relation to succinyl CoA synthetase (SucCoA) proteins (Bräsen and others, 2008; Sánchez and others, 2000; Musfeldt and others, 1999). The α- and β-subunits of ADP-*acs* and SucCoA are often either fused or closely associated ensuring that these subunits are transcribed and work in tandem (Bräsen and Schönheit, 2004; Bapteste and others, 2005; Sánchez and others, 2000). The ADP-Acs and SucCoA α−subunits contain a motif similar to the A3 motif in AMP-Acs and an active site dependent on the phosphorylation of a conserved histidine and glutamate residue which aligns with the A5 region in AMP-Acs (Bräsen and others, 2008). Similarly to AMP-Acs and ADP-Acs, Ack is a two domain protein with an initial serine and glycine rich motif (termed Phosphate 1) (Buss and others, 2001; Thaker and others, 2012). The second motif (Connect 1) aligns with A5 in AMP-Acs, with an invariant negatively charged glutamate in place of aspartate in both ADP-Acs and AMP-Acs. An active site histidine residue is completely conserved in Ack, ADP-Acs, SucCoA and several AMP-Acs analyzed (Fig. 19) (Ingram-Smith, 2000; Sánchez and others, 2000; Fraser and others, 1999). In addition, the phosphate 2 motif in Ack share secondary structural features with the A8 motif from AMP-Acs (Thaker and others, 2012; Gulick and others, 2003). The Connect 2 motif of Ack contains a glutamate residue that is highly conserved and aligns with a conserved aspartate at the C terminus of AMP-Acs and the fused αβ ADP-Acs but is absent in non-fused ADP-Acs and SucCoa indicating the fusion between ADP-Acs α and β subunits is important in relating ADP-Acs to AMP-Acs and Ack through duplication and divergence. A HMM-HMM comparison with our full alignment (supplementary figure 3) identified significant homology between all the sequences. An HHsearch can identify homologs not currently assigned to the same superfamilies within the Structural Classification Of Proteins (SCOP) database (Söding, 2005). HHsearches predicted Ack, AMP-Acs and ADP-Acs as homologs with high confidence based on structures and folds (E-values < $1E^{-}12$) according to both HMM-HMM global and local multiple alignment comparisons.

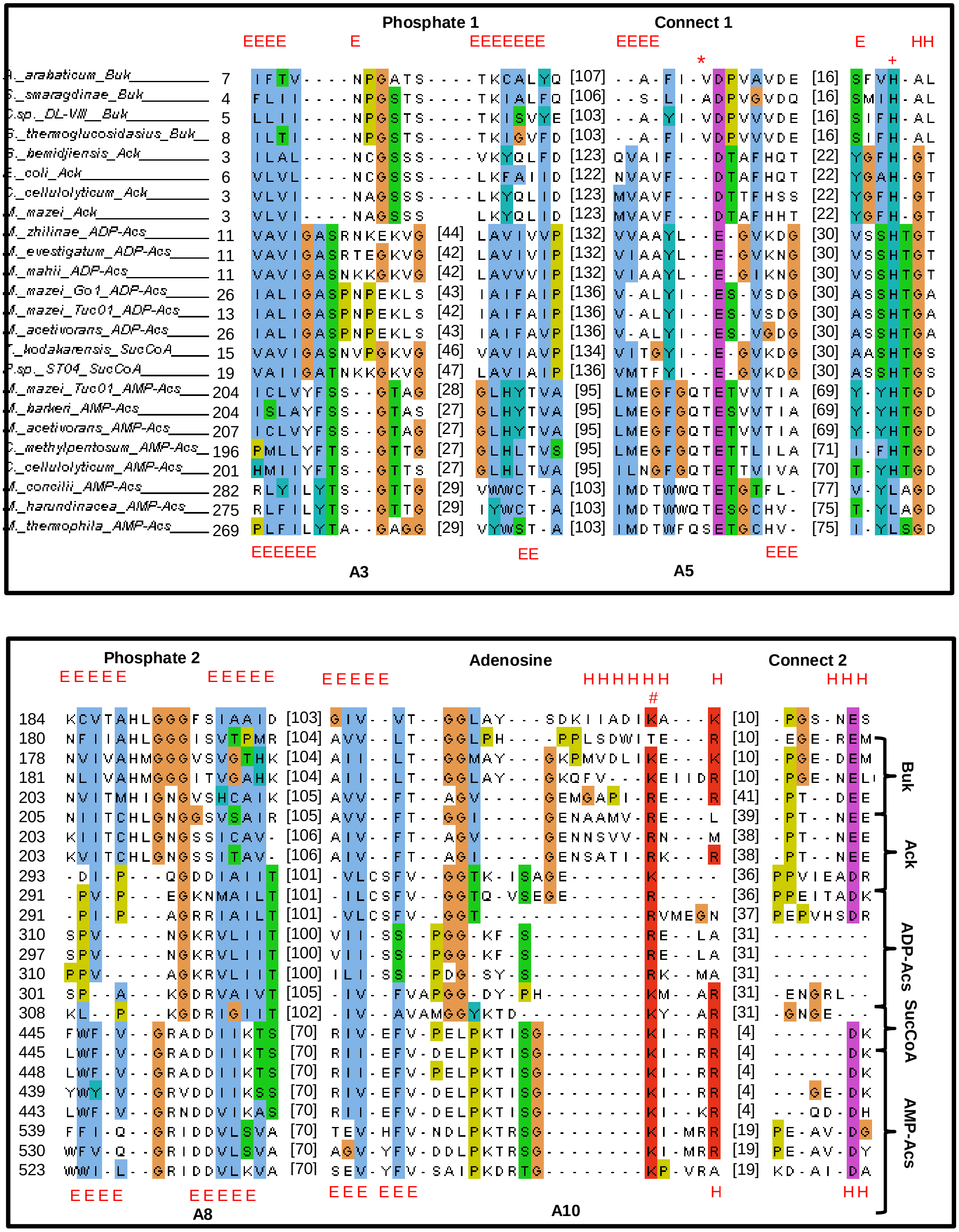

Figure 20: AMP-Acs, ADP-Acs, Ack, SucCoA and Buk multiple sequence alignment highlighting the shared motifs using the ClustalX color scheme (Thompson and others, 1997). The Ack motifs and secondary structures are labeled above the alignment and the shared AMP-Acs motifs and secondary structure is labeled below. The (*), (+) and (#) labels the completely conserved negatively charged residue, the conserved histidine residue in ADP-Acs and SucCoA and the acetylation site of AMP-Acs respectively.

Structural Relationships

The three dimensional structures of AMP-Acs, ADP-Acs and Ack indicate that all three proteins consist of two domains (Gulick and others, 2003; Sánchez and others, 2000; Chittori and others, 2012). The two domains join together forming the active site cleft with an ATP binding site at the bottom of the cleft. Structural considerations indicate Ack is the primogenital protein of the ASKHA superfamily of phosphotransferases and potentially the first kinase protein that evolved (Urkinase) (Cheek and others, 2002; Buss and others, 2001). The ASKHA proteins share a structural feature which involves two domains with similar folds on either side of a large cleft with an ATP binding site at the bottom of the cleft (Bork and others, 1992). This structural feature indicates all of the proteins in the ASKHA superfamily were formed through gene duplication and divergence because this structure is the result of complex internal core folds due to motif conservation involved with ATP binding (Bork and others, 1992; Cheek and others, 2002; Cheek and others, 2005). AMP-Acs, theorized as one of the earliest proteins based on geological evidence, is present in an ancient operon-like cluster of genes in methanogens and acetogens that do not possess Ack (Takami and others, 2012; Poehlein and others, 2012; Russell and Martin, 2010). The identification of ATPase motif conservation and resulting structural features in AMP-Acs allows deeper insight into the evolution of the ASKHA superfamily and an ATPase domain common to proteins in all three domains of life, including actin in eukaryotic cells (Figure 2) (Bork and others, 1992; Schuler and others, 2006).

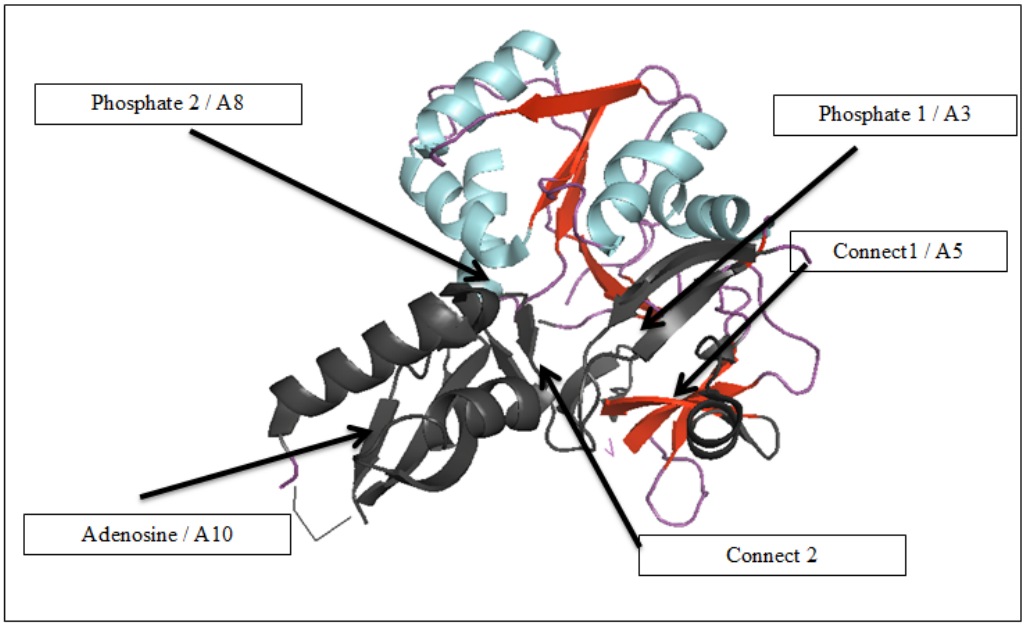

Figure 21: The darkened sub structure is the location of the ASKHA superfamily sequence motifs in *Methanosarcina* AMP-Acs previously described in Bork et al. 1992. This structure comparison showing the binding cleft (amino acid region 202-517) supports combining of two major and diverse superfamilies in an ancient archaeon. PDB structure 3ETC_A, image from Pymol.

Phylogenetic Relationships

Methanosarcina is the only identified *Archaea* with Ack, suggesting *ack* was either horizontally transferred or evolved within the *Methanosarcina* genome. Previous studies investigating the evolution of Ack suggest *ack* was transferred from *Bacteria* because homologs had not been identified within *Archaea* (Fournier and Gogarten, 2008; Fournier, 2009). The identification of novel *ack* homologs within *Methanosarcina* genomes (AMP-*acs* and ADP-*acs*) with such strong motif conservation suggest *ack* evolved through gene duplication and divergence within the ancient *Methanosarcina* genome. A phylogenetic comparison of Ack, AMP-Acs, ADP-Acs and SucCoA shows the predicted cladding of the proteins (Fig. 21). The tree reveals evidence that Ack shared a common sequence ancestor with ADP-Acs from halophilic methanogens (*Methanohalobium evestigatum*, *Methanosalsum zhilinae* and *Methanohalophilus mahii*) with a posterior probability (PP) of 0.85. Previous phylogenetic comparisons suggest halophilic methanogens are closely related to *Methanosarcina* (Singh and others, 2005; Lyimo and others, 2000; Chellapandi, 2011). Branch lengths indicate a large number of substitutions between the Ack proteins and both the ADP-Acs and AMP-Acs proteins. Butyrate kinase (Buk) and Ack appear to share a common ancestor which supports previous research suggesting Buk evolved from an ancient Ack (Buss and others, 2001).

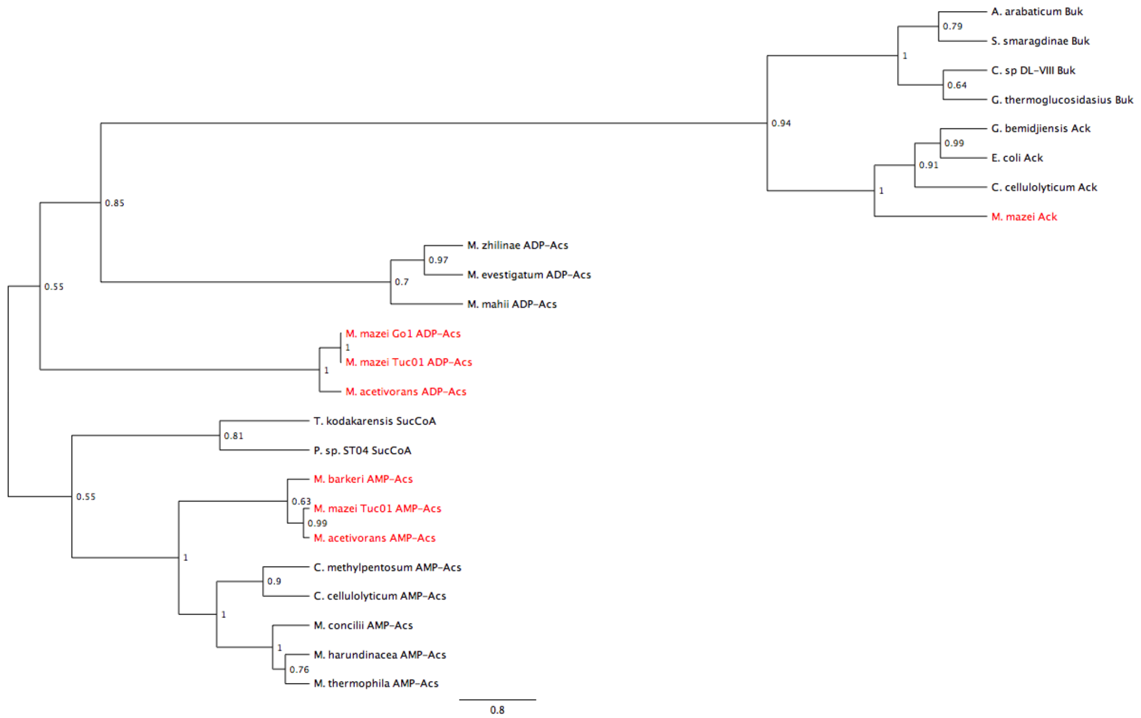

Figure 22: Phylogenetic analysis of Buk, Ack, SucCoA, ADP-Acs and AMP-Acs using BEAST (Drummond and Rambaut, 2007). The posterior probabilities label each node and branch lengths are scaled to expected substitutions per site. The taxa highlighted in red are of the *Methanosarcina* genus illustrating the relationship between the Ack, ADP-Acs and AMP-Acs.

Methanogens ADP-acs and Ack Share Synteny

Genome synteny can be maintained for hundreds of millions of years, therefore genes of close phylogenetic relatives in the same loci are frequently orthologous (Ghedin and others, 2004). Genome synteny comparisons suggest *ack* in *Methanosarcina* is located in the same loci as the *ADP-acs* α subunit in the close phylogenetic relatives *M. mahii* and *M. zhilinae* (Fig. 22). Most archaeal genomes contain homologs to the α- and β-subunits of ADP-*acs* that are either fused or closely associated (Bräsen and Schönheit, 2004; Bapteste and others, 2005). The ADP-*acs* β−subunit from the halophilic methanogens was in the same loci and has a high amino acid sequence identity with the ADP-*acs* β-subunit encoded by *Methanosarcina mazei* (ID% >50%, all E values $<7e^{-84}$). As seen in Figure 3, the ADP-*acs* β−subunit in the *Methanosarcina acetivorans* genome has two genes separating it from *ack,* M. mazei has one small gene, and no homologs of the β−subunit were found within the *Methanosarcina barkeri* genome. Previous comparative genomics of *Methanosarcina* indicate *M. mazei* may represent an ancient genomic state of *Methanosarcina spp.* with a relatively small genome and the *M. acetivorans* genome is the result of uniformly distributed multi-gene scale insertions and duplications, possibly resulting in the extra gene separating *ack* and the ADP-*acs* β−subunit (Maeder and others, 2006). Moreover, the *M. barkeri* genome is characterized by localized inversions associated with the loss of gene content possibly resulting in the loss of the ADP-*acs* β−subunit (Maeder and others, 2006). *M. mazei* and *M. acetivorans* contain a sole gene encoding the ADP-*acs* α−subunit (gi20904723 and gi19919192) in a gene loci different from the halophilic methanogens and separate from the β-subunit of ADP-*acs*. Duplicate copies of the ADP-*acs* α-subunit could have provided the opportunity for alternative selection of function and the divergence into *ack*.

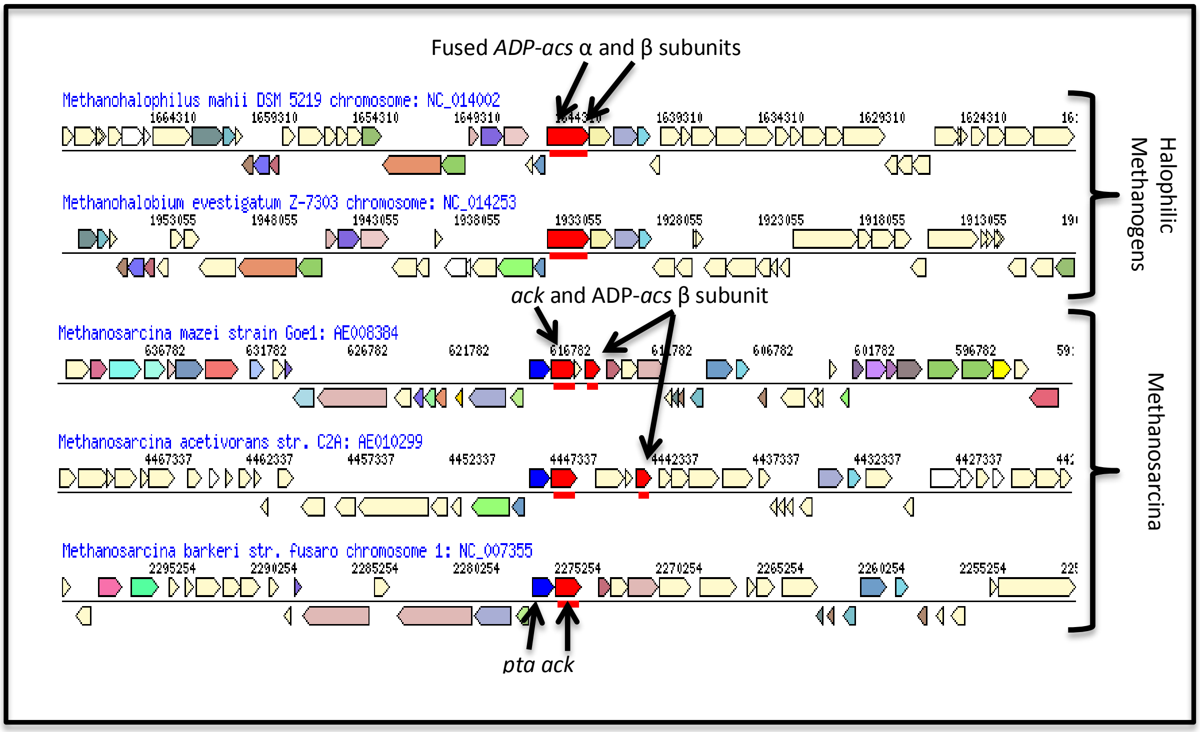

Figure 23: Synteny of ADP-*acs* in halophilic methanogen and *Methanosarcina* genomes. The ADP-acs β subunit exhibits significant homology to all halophilic methanognes fused ADP-acs (red). The *pta* (blue) appears next to *ack* (red) within all the sequenced *Methanosarcina* genomes.

Evolution of a Catalytic Pta in *Halobacteriales*

Following the formation of acetyl-P by Ack, phosphotransacetylase (Pta) catalyzes the reversible transfer of the acetyl group from acetyl-P to CoA forming acetyl-CoA and inorganic phosphate, maintaining a balance between biosynthesis and energy generation (Lawrence and Ferry, 2006; El-Mansi and others, 2006). Our analysis identified two different Pta isoforms (Pta and MaeB) in *Halobacteriales* not previously described in *Archaea* (Fig. 23). MaeB consists of a fusion between malic enzyme and *pta* that has been characterized in *Bacteria*, and results suggest the homologous Pta portion of MaeB enables better metabolic modulation of malic enzyme but lacks Pta activity (Bologna and others, 2007). Many *Halobacteriales* contain duplicate *maeB* genes providing the possibility for one copy to diverge. *Halococcus* contain *maeB* as well as a lone *pta* with necessary catalytic residues (Lawrence and others, 2006), suggesting divergence of a duplicated *maeB* within the ancient *Halococcus* genome formed Pta. Although further analysis is warranted, the evolution of a catalytic Pta in *Halobacteriales* is supported by the presence of a catalytic Pta residue (arginine 310) in all *Halobacteriales maeB* but absent in bacterial *maeB* analyzed in this report (Fig. 23) (Lawrence and others, 2006). In addition, methanogens and *Halobacteriales* have been shown to engage in genetic exchange, similar to conjugation in *Bacteria* (Rosenshine and others, 1989; Allers and Mevarech, 2005; Mevarech and Werczberger, 1985). Horizontally transferring the duplicated *pta* to an ancient halophilic methanogen with access to *ack* could have established the first Ack/Pta pathway.

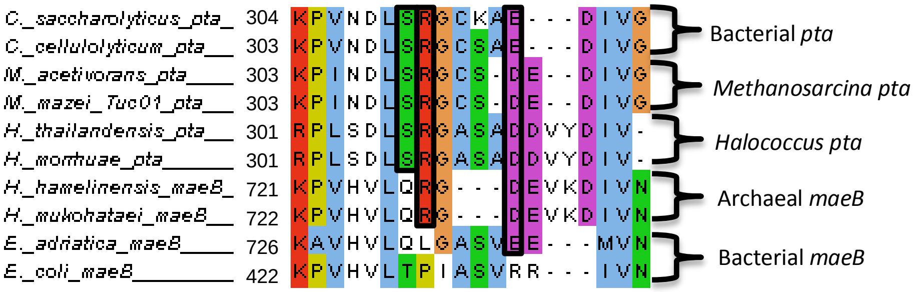

Figure 24: Alignment highlighting the shared active site residues between archaeal and bacterial *maeB* and *pta*. Active site residues are in the black boxes.

Discussion

The abundant genomic information available today has provided novel insight into the establishment of the acetate switch that was a turning point in the evolution of methane production and microbial carbon utilization. We provide sequence motif, structural conservation, phylogenetic, and genome synteny evidence suggesting AMP-Acs, ADP-Acs and Ack are related through gene duplication and divergence. In addition, phylogenetic and genome synteny results suggest the ancient ADP-Acs α subunit duplicated and diverged into Ack. The ADP-Acs α subunit from halophilic methanogens has features including a two domain structure, a glutamate residue which aligns with the magnesium binding aspartate in Ack and a conserved C-terminus glycine residue that was predicted to be part of the ancient Ack by other researchers (Buss and others, 2001; Bork and others, 1992).

The high identity of the *Archaeal* Ack and Pta to homologs in several *Eukarya* and many *Bacteria* suggest ancient origins (Ferry, 2011; Ingram-Smith and others, 2006). Short-chain carboxylic acids and methane production are both associated with early biotic environments (Ueno and others, 2006; Huber and Wächtershäuser, 1997). The ancient *Methanosarcina* spp. with access to the Ack/Pta pathway would have capitalized on the higher acetate concentrations associated with *Bacteria* producing acetate by an ancient Wood-Ljungdahl pathway (Russell and Martin, 2010; Poehlein and others, 2012). Evolutionarily ancient *Clostridia* utilize an ancient Wood-Ljungdhal pathway without Ack, Pta, or cytochromes and rely on sodium as a coupling ion (Poehlein and others, 2012; Mulkidjanian and others, 2008). An early reliance on sodium may have promoted colocalization with halophilic methanogens possessing the ancient *pta* and *ack* genes (Mulkidjanian and others, 2008). Phylogenetic analysis suggests *pta* and *ack* in *Methanosarcina* are most closely related to those in *Clostridium* (Fournier and Gogarten, 2008). The *ack* and *pta* in *Clostridium* are in a loci different from the gene cluster encoded for the ancient Wood-Ljungdahl pathway (Poehlein and others, 2012; Kuit and others, 2012) and in the same orientation as the homologs observed in *Methanosarcina* which implicates horizontal gene transfer of both genes in one transfer event (Fournier and Gogarten, 2008) from the ancient *Methanosarcina* to *Clostridium*. The extensive distribution of *pta* and *ack* throughout *Bacteria* suggests the encoded proteins contributed to the evolutionary fitness of the domain (Orr, 2009).

Today, many organisms take advantage of the reversibility of the Ack/Pta pathway to conserve energy and maintain steady-state levels of free CoA in the cell (Starai and Escalante-Semerena, 2004). This flexibility contributes greatly to survival when alternating between copiotrophic and oligotrophic conditions often experienced *in situ* (Wolfe, 2005). Thermodynamic constraints of acetate activation suggest Ack/Pta is suboptimal for acetate consumers at low acetate concentrations, while these organisms would have an increased energetic yield in high acetate environments. Conversely for acetate producing organisms, Ack/Pta provides a thermodynamic gradient which is functional under the largest range of acetate concentrations while AMP-Acs is often considered irreversible. During direct resource competition, increased rate or alternative substrate have been found to provide a major competitive advantage (Pfeiffer and others, 2001).

Research indicates that a brief period of the Archaean eon coincides with rapid diversification of bacterial lineages that gave rise to 27% of major modern gene families (David and Alm, 2011). Functional analyses of the genes originating during this expansion show they were likely to be involved in electron-transport and respiratory pathways (David and Alm, 2011). This expansion could have contributed to the wide distribution of the Ack/Pta pathway in many bacterial lineages and the evolution of the ASKHA superfamily (Buss and others, 2001). Additionally, eukaryotes have incorporated Ack into at least three different metabolic pathways including the Ack/Pta pathway, the Ack/Xfp pathway and a pathway involving ADP-Acs and Ack (Ingram-Smith and others, 2006). Ack has been observed to be widespread in fungi with putative *ack* sequences identified in three of the four fungal phyla including the *Chytridiomycota*, the earliest branch of true fungi (Ingram-Smith and others, 2006). Although the evolution of the Ack/Pta pathway may have greatly impacted the diversity of life on Earth, the enhanced methane production provided by the pathway under certain environmental conditions may have greatly decreased diversity at least once.

Five major extinction events have been identified that greatly diminished the diversity of life on Earth (Rohde and Muller, 2005). Recent research suggests the largest extinction, the end-Permian extinction which decimated up to 95% of shell-bearing marine species and 80% of land animals, was the result of a large methane release from *Methanosarcina* (Knoll and others, 2007; Rothman and others, 2014). Contrary to previous analysis, our results indicate the Ack/Pta pathway evolved within the methanogenic genome and did not immediately appear as the result of a horizontal transfer event from *Bacteria* (Fournier and Gogarten, 2008; Rothman and others, 2014). Thus, the potential for a large methane release was in place long before the end-Permian extinction, suggesting a change in environmental conditions was the major driving force which promoted the activity of *Methanosarcina*. Research indicates the ancient combustion of coal through an interaction with basalt sills of the Siberian Traps released trace metals that created the conditions which stimulated methane production and instigated the end-Permian extinction (Ogden and Sleep, 2012; Rothman and others, 2014; Glass and Orphan, 2012; Reddy and others, 2005). Today, atmospheric methane concentrations have nearly tripled since pre-industrial times and this increase has been partly attributed to coal combustion (Bousquet and others, 2006; Kirschke and others, 2013). An increased understanding of the evolution of the Ack/Pta pathway and the historical impacts on the biosphere when this pathway is stimulated provides further motivation for the development of sustainable energy generating technologies that do not release methane stimulating metals.

Stimulated methane production from the Ack/Pta pathway could sustain energy production from biologically driven natural gas sources where methane can be trapped and utilized as an energy source such as biogenic coal-bed methane and biogas from waste (Strąpoć and others, 2011; Jones and others, 2010; Ngumah and others, 2013). Increased use of natural gas from stimulated methane production would reduce emissions, including metals, compared to coal and oil combustion (Pacsi and others, 2013; Jaramillo and others, 2007), but steps should be taken to minimize methane leaks from these developments which could offset the environmental benefits (Moore and others, 2014).

Conclusion

The data presented here offers an essential link in the evolutionary history of metabolic pathways by providing the first origin hypothesis of *ack* and *pta* which are observed in all three domains of life and may have contributed to rapid diversification. This research revealed novel motif, active site, and structural conservation between AMP-Acs, ADP-Acs and Ack suggesting the proteins are related through gene duplication and divergence. The data suggested the first Ack evolved from a duplicated ADP-acs α subunit in *Methanosarcina*. A *pta* homolog was shown to be conserved in all the *Halobacteriales* genomes sequenced to date, and the close relation of *Methanosarcina spp*. to halophilic methanogens suggests the *pta* gene could have been acquired via horizontal gene transfer from *Halobacteriales* that existed in the same environment. The formation of the Ack/Pta pathway could have led to the speciation of *Methanosarcina* and the metabolic diversification of *Bacteria*. This event greatly impacted the cycling of carbon via a metabolic node that coordinates cellular catabolism and anabolism, and likely was a turning point in the evolutionary history of microbial life and carbon cycling on a global scale.

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CHAPTER 6 - EPILOGUE

This dissertation investigated the subsurface microbial communities involved with CBM production and the mechanisms by which CBM production could be stimulated. An increased understanding of stimulated CBM production has produced new ideas about how to sustain CBM production from coalbeds and also minimize the water issues that plague current development technologies. This work also reveals a connection between acetoclastic methanogenesis and stimulated CBM production production based upon bench-scale, laboratory experiments. In addition, we provide novel insight into the evolution of the acetate switch within methanogenic *Archaea* which provides an increased understanding of the evolution of methanogenesis as well as microbial carbon utilization for biological systems.

Chapter 2 involved the development and use of a novel diffusive microbial sampler (DMS) to examine the *in situ* microbial communities existing in a coalbed stimulated by groundwater recharge. The region where the DMS was deployed exhibited an acetoclastic methanogenesis isotopic signature which was unique compared to the majority of the PRB which has a hydrogenotrophic methane signature (Bates and others, 2011). A large proportion of the SSU-rRNA sequences from the DMS were most closely related to phototrophic microorganisms (cyanobacteria and algae) which indicated a potential source of stimulating biomass in this region of the PRB. Dead biomass and recent plant photosynthate can stimulate the decomposition of more recalcitrant organic matter through priming effects (Guenet and others, 2010; Basiliko and others, 2012). Traditionally, the model for CBM production involves bacteria breaking down the coal into substrates (acetate, methanol and CO_2 and H_2) that are utilized by methanogens to produce methane (Strąpoć and others, 2011). Carbon and nutrients carried by recharge groundwater infiltrating the coal-beds provides another source of carbon and nutrients that could support or stimulate methanogenesis. This provides support for a more recent view on microbial survival in subsurface environments through the slow turnover of biomass (Hoehler and Jorgensen, 2013).

The DMS was utilized again in Chapter 3 to obtain a CBM-producing microbial community from a coalbed farther from significant groundwater recharge. The microbial community from the DMS was subjected to nutrient additions and CBM production was analyzed in conjunction with the microbial community changes that resulted from the nutrient additions. The results from this work was similar to other studies that have shown that nutrient supplements trigger additional methane production in laboratory coal enrichments (Strąpoć and others, 2011; Strąpoć and others, 2008; Green and others, 2008; Guo and others, 2012; Jones and others, 2010; Faiz and Hendry, 2006; Klein and others, 2008; Flores and others, 2008; Beckmann and others, 2011; Penner and others, 2010; Unal and others, 2012; Ulrich and Bower, 2008). The DMS allowed a novel comparison between the *in situ* coal-associated microbial community and the community changes that occur under enriched laboratory conditions. Sequences related to *Proteobacteria* (*Geobacter, Collimonas* and *Syntrophus*) dominated the *in situ* DMS and coal enrichment community while *Clostridial* sequences (*Acetobacterium*, *Fusibacter* and *Sedimentibacter*) dominated the communities with yeast extract added. *Methanosaeta* and *Methanosarcinaceae* were detected in enrichments but not *in situ*.

These results suggest that these methanogens groups were present *in situ* but were below the detection limit; however, these groups were stimulated in the batch enrichment cultures. The acetoclastic methane isotope signature along the *in situ* margin of the Powder River Basin suggests acetoclastic methanogens (*Methanosaeta* and *Methanosarcinaceae)* are stimulated by recharge water that enters near that part of the Basin. Algae and cyanobacterial SSU-rDNA sequences were detected in a well in this part of the Powder River Basin and we were able to show that phototrophic biomass stimulated CBM production similarly to yeast extract in laboratory-based enrichment cultures. This information provided new insight into *in situ* and laboratory-based, stimulated microbial coal-bed community composition and physiology that may lead to strategies to stimulate *in situ*, microbially-enhanced coal-bed methane production.

Chapter 4 investigated the use of different strategies to isolate microorganisms from subsurface coalbeds. *Methanosarcina* has been detected in other microbial CBM investigations with DNA based methods but to the authors knowledge this was the first time the methanogen has been isolated from a coal seam. *Methanosarcina* is the only methanogen with *ack* and *pta* which allows *Methanosarcina* to thrive in higher acetate concentrations than other acetate utilizing methanogens (Ferry, 1992). The presence of *Methanosarcina* within coalbeds suggests bioaugmentation would not be necessary for stimulated methane production to occur because a microbial community with enhanced CBM producing potential already exists within the coalbeds.

The evolution of acetoclastic methanogenesis within the ancient *Methanosarcina* genome was investigated in Chapter 5. This research provides novel evidence suggesting an ATPase domain common to a major protein superfamily (ASKHA) in *Bacteria* and *Eukaryotes* is present in acetyl coA synthetase proteins in *Archaea.* Genome synteny research suggested the initial *ack* formed from a duplicated ADP-acetyl coA synthetase α subunit gene (Buss and others, 2001; Bork and others, 1992; Schuler and others, 2006). Previous reports investigating the origin of the Ack/Ptaproteins within *Methanosarcina* could not identify *pta* or *ack* homologs within any other archaeal species (Fournier, 2009; Fournier and Gogarten, 2008). Our work reveals a Blast search of current databases can provide a homologous *pta* in archaeal *Halobacteriales.* A more in depth analysis exposed a *pta* with an active site suggesting an active *pta* could have evolved within *Halobacteriales.* This study offered an essential link in the evolutionary history of metabolic pathways by providing the first origin hypothesis of *ack* and *pta* that suggests these genes evolved in *Archaea* through gene duplication and divergence. The evolution of the Ack/Pta pathway greatly impacted the cycling of carbon via a metabolic node that coordinates cellular catabolism and anabolism, and likely was a turning point in the evolutionary history of microbial life and carbon cycling on a global scale.

The work presented in this dissertation provides a better understanding of the microbial communities in the PRB but many questions still remain about CBM production and sustainable methods for the development of this energy resource. Many of the identified OTUs had a low similarity (less than 90% identity) to any known cultured bacteria listed in the NCBI sequence database and may represent novel species specifically adapted to coal-bed ecosystems. Therefore, further isolation studies from coal-beds are needed to better understand indigenous microorganisms and function.

Obtaining additional DMS samples from wells that provide insight into the *in situ* microbial community along a coal-bed flow path in several different areas of the Powder River Basin and identifying how the microbial community dynamics change could lead to a better understanding of the differences in the isotopic data observed. The development of flowing systems in the lab could also increase *in situ* relevance and upscale the experiments which would move this research closer to a final goal of a field demonstration.

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APPENDICES

APPENDIX A

SUPPLEMENTARY MATERIAL FOR CHAPTER 2

Supplementary Table 1

Sequence #, OTU # and % ID from the pyrotag analysis in Chapter 2.

	Sequence No.	OTU No. At 97%	% ID
Archaeal Domain			
Euryarchaotes Phylum			
Methanolobus	2538	29	95-99
Methanobacterium	3606	45	94-100
Bacterial Domain			
Actinobacteria Phylum			
Actinomycetales	3	2	97-98
Arthrobacter	156	11	90-98
Atopobium	50	6	84-88
Cellulomonas	4	1	98
Corynebacterium	40	9	93-99
Dietzia	2	2	93-97
Gordonia	101	7	90-95
Mycobacterium	1	1	92
Olsenella	1	1	85
Propionibacterium	818	37	86-100
Rhodococcus	11	2	83-84
Saccharopolyspora	16	3	82-88
Streptomyces	105	14	84-87
Tessaracoccus	4	1	93
Turicella	276	21	94-99
Bacteriodetes Phylum			
Hymenobacter	1	1	94
Proteobacteria Phylum			

α proteobacteria			
Methylobacterium	5	3	93-99
Methylocapsa	13	3	95-99
Methylosinus	15	3	98-99
Modestobacter	3	1	85
Oceanibaculum	1	1	97
Paracraurococcus	1	1	85
Rhodopila	1	1	83
β Proteobacteria			
Burkholderia	41	10	92-99
Collimonas	26	1	93
Cupriavidus	1	1	99
Herbaspirillum	53	10	89-99
Oxalobacter	453	11	89-95
Propionivibrio	28	4	93-99
Ralstonia	283	22	85-99
Schlegelella	2	1	94
δ proteobacteria			
Desulfobulbus	3	1	80
Desulfuromonas	17	2	92
Geobacter	234	27	89-99
Lawsonia	8	1	83
Syntrophorhabdus	8	1	91
γ proteobacteria			
Acinetobacter	297	28	94-100
Dyella	14	2	95-98
Escherichia	5	1	98
Moraxella	170	17	84-99
Nevskia	23	3	95-99

Pseudomonas	33	5	96-100
Firmicutes Phylum			
Acetobacterium	101	14	91-99
Alloiococcus	27	2	96
Caloramator	4	1	84
Clostridium	17	9	82-99
Dehalobacter	18	4	87-91
Desulfosporosinus	7	2	95-96
Desulfotomaculum	1	1	86
Fusibacter	1	1	90
Quinella	7	1	80
Sporomusa	7	3	84-90
Staphylococcus	811	33	87-100
Streptococcus	91	5	91-100
Thermosinus	20	2	90
Trichococcus	10	1	99
Planctomycetes Phylum			
Planctomyces	41	1	89
Cyanobacteria Phylum			
Cyanobacterium	7	1	88
Cyanothece	22	6	84-87
Gloeobacter	29	8	83-87
Halomicronema	26	3	85-86
Leptolyngbya	23	1	88
Nostoc	37	5	82-85
Phormidium	1	1	85
Spirulina	573	3	80-83
Chloroplast	131	19	89-100

APPENDIX B

SUPPLEMENTARY MATERIAL FOR CHAPTER 3

Supplementary Table 2

Sequence #, (OTU #) and % ID from the pyrotag analysis in Chapter 3.

	In situ (DMS)	Coal	Yeast Extract	Yeast Extract and Coal
Archaeal Domain				
Crenarchaeota Phylum				
Ignicoccus	3 (1) 89%			
Thermofilum	50 (2) 88-89%			
Euryarchaotes Phylum				
Methanobacterium	253 (9) 93-100%	1 (1) 99%	10 (1) 99%	9 (1) 99%
Methanococcus		1 (1) 99%		
Methanoculleus	1 (1) 93%			
Methanofolis	4 (1) 89%			
Methanohalophilus	2 (1) 95%			
Methanolinea				3 (1) 97%
Methanolobus	853 (24) 92-100%			
Methanomassiliicoccus	70 (2) 91-92%	2 (2) 95%	5 (1) 96%	21 (4) 92-99%
Methanomethylovorans	38 (2) 92%			
Methanoplanus	1 (1) 87%			
Methanoregula	181 (12) 94-98%	241 (2) 98%		177 (3) 96-98%
Methanosaeta		15 (1) 100%		6 (1) 100%
Methanosarcina		2 (1) 99%	79 (1) 99%	15 (1) 100%
Methanosphaerula	1 (1) 93%			
Methanospirillum	7158 (69) 89-	9 (1) 98%	11 (1) 98%	17 (2) 97-99%

	99%			
Methanothermobacter			1 (1) 90%	
Methanothermus	8 (1) 88%	2 (1) 87%		
Bacterial Domain				
Acidobacteria Phylum				
Acidobacterium			1 (15) 81%	202 (1) 81%
Geothrix	2 (1) 96%			51 (1) 95%
Gloeobacter	2 (1) 84%			
Solibacter				53 (2) 89-90%
Actinobacteria Phylum				
Acidothermus				2 (1) 82%
Actinobacterium			2 (1) 98%	25 (1) 98%
Actinomadura				1 (1) 79%
Amycolatopsis	1 (1) 83%			
Cellulomonas				1 (1) 98%
Cryobacterium				1 (1) 94%
Frigoribacterium				150 (1) 77%
Propionicimonas			15 (1) 97%	52 (2) 95-97%
Solirubrobacter		1 (1) 83%		9 (1) 86%
Streptomyces		3 (2) 86-87%		
Bacteriodetes Phylum				
Adhaeribacter			1 (1) 84%	
Alistipes		1 (1) 86%		62 (1) 84%
Alkaliflexus				59 (2) 84-86%
Chitinophaga			1 (1) 98%	
Cytophaga		4 (2) 78-85%		1 (1) 86%
Ferruginibacter				1 (1) 95%
Flavobacterium	1 (1) 84%			28 (2) 83-84%
Fulvivirga		2 (1) 88%	257 (2) 86-87%	1192 (2) 86-87%

Hymenobacter				1 (1) 97%
Meniscus			16 (1) 98%	913 (3) 93-98%
Paludibacter			6 (1) 95%	13 (3) 93-96%
Prolixibacter	1 (1) 85%		17 (1) 86%	939 (2) 86-87%
Rhodothermus				8 (1) 81%
Chloroflexi Phylum				
Anaerolinea		1 (1) 81%	13 (3) 76-86%	79 (4) 77-80%
Bellilinea			3 (2) 89-90%	10 (2) 89-90%
Caldilinea				1 (1) 85%
Dehalococcoides			2 (2) 82%	54 (3) 81-82%
Dehalogenimonas				15 (2) 78-85%
Leptolinea		5 (2) 86-96%	63 (4) 85-99%	71 (6) 88-97%
Levilinea			50 (2) 88-90%	178 (3) 86-91%
Longilinea			103 (1) 85%	
Firmicutes Phylum				
Anaerospora			7 (1) 99%	182 (4) 90-98%
Anaerovorax			4 (1) 91%	2 (2) 91-93%
Acetivibrio		5 (1) 99%	59 (3) 95-99%	6 (1) 99%
Acetobacterium	13 (4) 96-98%	1 (1) 97%	3475 (14) 87-99%	2524 (9) 93-99%
Acidaminobacter		3 (1) 93%		
Butyrivibrio			5 (1) 90%	10 (1) 90%
Catabacter			1 (1) 81%	
Caloramator				41 (2) 83%
Clostridium		18 (2) 96%	522 (26) 81-100%	2629 (40) 77-100%
Desulfosporosinus	9 (2) 93-95%	1 (1) 96%		
Desulfitobacterium				1 (1) 95%
Erysipelothrix			215 (1) 86%	

Faecalibacterium			148 (4) 80-84%	395 (4) 79-84%
Fusibacter		5 (2) 87-88%	2842 (8) 88-92%	20590 (19) 87-92%
Guggenheimella	1 (1) 90%		3 (2) 87-88%	5 (2) 80-84%
Ignavibacterium		6 (3) 84-90%		
Mahella	1 (1) 81%		59 (1) 82%	52 (1) 82%
Oscillibacter			59 (1) 87%	33 (2) 86-87%
Oxobacter				1 (1) 87%
Paenibacillus				1 (1) 98%
Papillibacter				10 (1) 84%
Pelosinus				2 (1) 91%
Ruminococcus			12 (3) 81-89%	24 (2) 81-89%
Sedimentibacter			2507 (9) 92-99%	1326 (8) 90-99%
Sporomusa	1 (1) 88%		674 (7) 83-90%	1242 (5) 83-85%
Sporotalea			16 (2) 89-100%	476 (6) 89-100%
Syntrophomonas		3 (2) 88-89%	15 (1) 87%	1329 (6) 82-89%
Thermoactinomyces			144 (3) 82-83%	
Thermosediminibacter		1 (1) 80%		
Thermosinus	6 (1) 86%	2 (1) 85%		88 (2) 84-85%
Fusobacteria Phylum				
Fusobacterium			66 (1) 83%	74 (2) 81-83%
Ignavibacteriae Phylum				
Ignavibacterium		6 (3) 84-90%		2460 (5) 83-89%
Lentisphaerae Phylum				
Victivallis			1 (1) 89%	8 (1) 90%
Proteobacteria Phylum				
α proteobacteria				
Acidocella				1 (1) 99%
Bradyrhizobium				1 (1) 100%
Kaistia	1 (1) 97%			

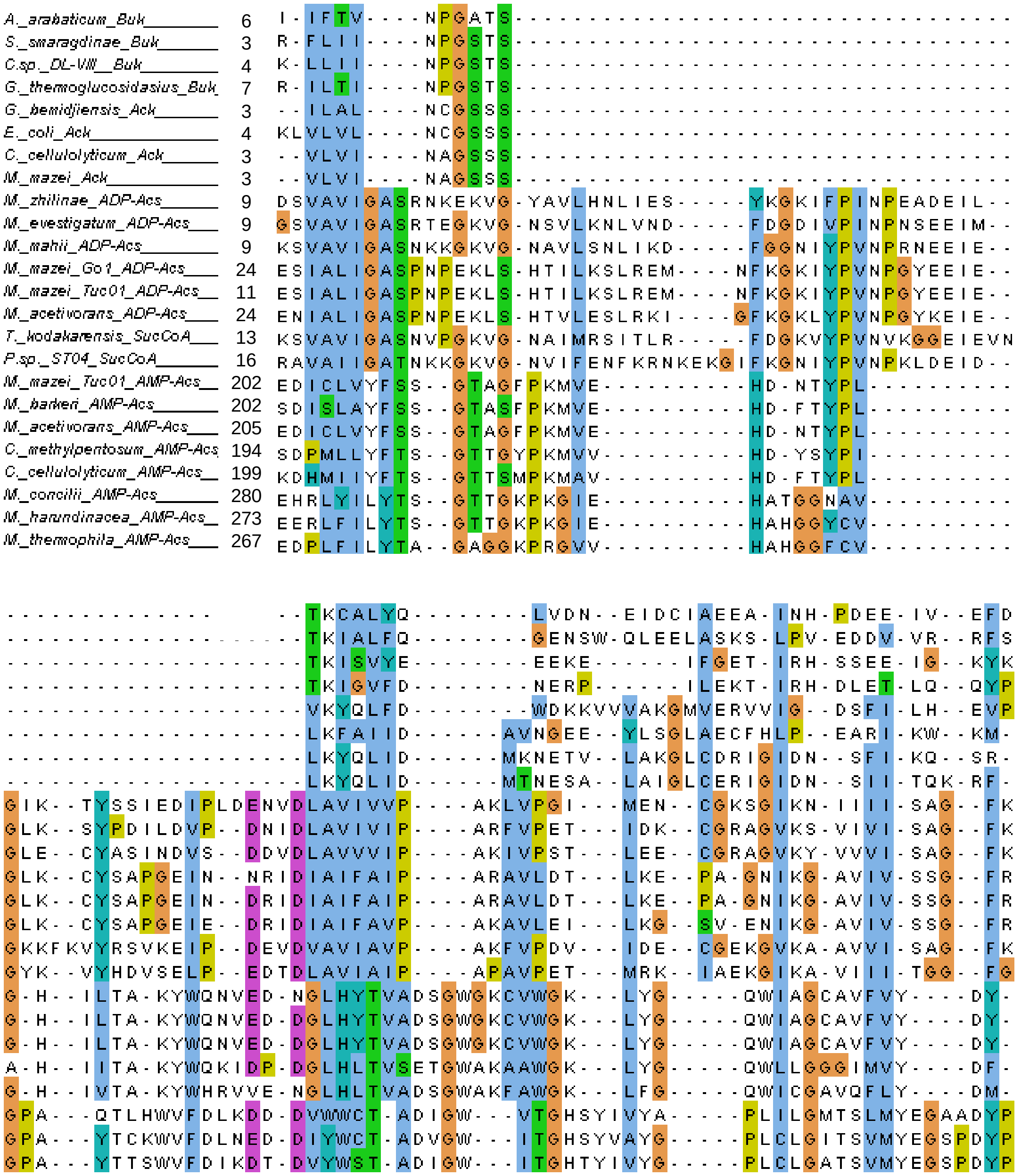
Magnetospirillum		1 (1) 99%		
Methylocystis	1 (6) 100%			
Sphingosinicella			1 (1) 81%	
Sphingomonas				1 (1) 98%
Tistrella				7 (1) 88%
β Proteobacteria				
Alcaligenes			1 (1) 100%	
Azovibrio	7 (1) 93%			
Burkholderia			2 (1) 98%	5 (2) 96-98%
Collimonas	50 (1) 92%	15 (1) 92%	12 (1) 92%	771 (2) 91%
Cupriavidus	1 (1) 93%		2 (1) 85%	
Dechloromonas		1 (1) 97%	28 (1) 97%	394 (2) 96-97%
Ferrovum	1 (1) 92%			
Janthinobacterium			13 (1) 100%	32 (2) 97-100%
Massilia				1 (1) 90%
Oxalobacter	8 (3) 90-95%	6 (1) 93%		
Polaromonas			2 (1) 98%	
Ralstonia	2 (1) 99%			9 (1) 99%
Rhodocyclus	2 (1) 98%			
Rhodoferax	1 (1) 99%		1 (1) 87%	
δ proteobacteria				
Desulfatibacillum				64 (1) 77%
Desulfobulbus	13 (2) 93-97%			
Desulfomonile				8 (1) 96%
Desulfonatronospira				1 (1) 81%
Desulfovibrio	5 (3) 98-99%	12 (3) 98-100%	299 (2) 87-97%	565 (5) 88-98%
Geobacter	60 (8) 91-97%	16 (2) 94-100%	13 (2) 92-93%	920 (9) 92-96%
Pelobacter	2 (1) 96%			8 (1) 79%
Smithella		7 (3) 90-93%		204 (5) 91-92%

Syntrophobacter	1 (1) 88%			
Syntrophorhabdus				3 (1) 92%
Syntrophus	1 (1) 90%	25 (2) 93-95%	6 (1) 92%	835 (4) 91-96%
ἐ proteobacteria				
Sulfurimonas	1 (1) 91%			
γ proteobacteria				
Aeromonas			1 (1) 100%	
Acidithiobacillus	1 (1) 78%			
Legionella	1 (1) 96%			
Vibrio			1 (1) 97%	1 (1) 97%
Planctomycetes Phylum				
Pirellula	1 (1) 88%		2 (1) 87%	108 (1) 82%
Spirochaetes phylum				
Leptospira	1 (1) 84%			
Spirochaeta		2 (2) 88-90%	175 (2) 87-88%	2723 (11) 86-97%
Treponema		9 (1) 89%		2021 (5) 82-88%
Synergistetes				
Thermanaerovibrio		4 (1) 85%		
Verrucomicrobia Phylum				
Verrucomicrobium				1 (1) 96%
Chloroplast				
Spirogyra		1 (1) 78%		1 (1) 100%
Cyanobacteria				
Nostoc			3 (1) 83%	14 (1) 83%
Tolypothrix				2 (1) 94%

APPENDIX C

SUPPLEMENTARY MATERIAL FOR CHAPTER 5

Supplementary Figure 1 Multiple sequence alignment of Buk, Ack, ADP-Acs, SucCoA and AMP-Acs used in the study. The sequences were first aligned with Mafft and then manually adjusted based on secondary structure predictions. The conservation is highlighted using the ClustalX color scheme[66].



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A._arabaticum_Buk              6    I-IFTV----NPGATS----------------------------------
S._smaragdinae_Buk             3    R-FLII----NPGSTS----------------------------------
C.sp._DL-VIII__Buk             4    K-LLII----NPGSTS----------------------------------
G._themoglucosidasius_Buk      7    R-ILTI----NPGSTS----------------------------------
G._bemidjiensis_Ack            3    --ILAL----NCGSSS----------------------------------
E._coli_Ack                    4    KLVLVL----NCGSSS----------------------------------
C._cellulolyticum_Ack          3    --VLVI----NAGSSS----------------------------------
M._mazei_Ack                   3    --VLVI----NAGSSS----------------------------------
M._zhilinae_ADP-Acs            9    DSVAVIGASRNKEKVG-YAVLHNLIES-----YKGKIFPINPEADEIL--
M._evestigatum_ADP-Acs         9    GSVAVIGASRTEGKVG-NSVLKNLVND-----FDGDIVPINPNSEEIM--
M._mahii_ADP-Acs               9    KSVAVIGASNKKGKVG-NAVLSNLIKD-----FGGNIYPVNPRNEEIE--
M._mazei_Go1_ADP-Acs          24    ESIALIGASPNPEKLS-HTILKSLREM----NFKGKIYPVNPGYEEIE--
M._mazei_Tuc01_ADP-Acs        11    ESIALIGASPNPEKLS-HTILKSLREM----NFKGKIYPVNPGYEEIE--
M._acetivorans_ADP-Acs        24    ENIALIGASPNPEKLS-HTVLESLRKI----GFKGKLYPVNPGYKEIE--
T._kodakarensis_SucCoA        13    KSVAVIGASNVPGKVG-NAIMRSITLR-----FDGKVYPVNVKGGEIEVN
P.sp._ST04_SucCoA             16    RAVAIIGATNKKGKVG-NVIFENFKRNKEKGIFKGNIYPVNPKLDEID--
M._mazei_Tuc01_AMP-Acs       202    EDICLVYFSS--GTAGFPKMVE----------HD-NTYPL----------
M._barkeri_AMP-Acs           202    SDISLAYFSS--GTASFPKMVE----------HD-FTYPL----------
M._acetivorans_AMP-Acs       205    EDICLVYFSS--GTAGFPKMVE----------HD-NTYPL----------
C._methylpentosum_AMP-Acs    194    SDPMLLYFTS--GTTGYPKMVV----------HD-YSYPI----------
C._cellulolyticum_AMP-Acs    199    KDHMIIYFTS--GTTSMPKMAV----------HD-FTYPL----------
M._concilii_AMP-Acs          280    EHRLYILYTS--GTTGKPKGIE----------HATGGNAV----------
M._harundinacea_AMP-Acs      273    EERLFILYTS--GTTGKPKGIE----------HAHGGYCV----------
M._themophila_AMP-Acs        267    EDPLFILYTA--GAGGKPRGVV----------HAHGGFCV----------

--------------------TKCALYQ--------LVDN--EIDCIAEEA-INH-PDEE-IV--EFD
--------------------TKIALFQ--------GENSW-QLEELASKS-LPV-EDDV-VR--RFS
--------------------TKISVYE--------EEKE------IFGET-IRH-SSEE-IG--KYK
--------------------TKIGVFD--------NERP------ILEKT-IRH-DLET-LQ--QYP
--------------------VKYQLFD--------WDKKVVVAKGMVERVVIG--DSFI-LH--EVP
--------------------LKFAIID------AVNGEE--YLSGLAECFHLP--EARI-KW--KM-
--------------------LKYQLID------MKNETV--LAKGLCDRIGIDN--SFI-KQ--SR-
--------------------LKYQLID------MTNESA--LAIGLCERIGIDN--SII-TQK-RF-
GIK--TYSSIEDIPLDENVDLAVIVVP----AKLVPGI---MEN--CGKSGIKN-IIII-SAG--FK
GLK--SYPDILDVP--DNIDLAVIVIP----ARFVPET---IDK--CGRAGVKS-VIVI-SAG--FK
GLE--CYASINDVS--DDVDLAVVVIP----AKIVPST---LEE--CGRAGVKY-VVVI-SAG--FK
GLK--CYSAPGEIN--NRIDIAIFAIP----ARAVLDT---LKE--PA-GNIKG-AVIV-SSG--FR
GLK--CYSAPGEIN--DRIDIAIFAIP----ARAVLDT---LKE--PA-GNIKG-AVIV-SSG--FR
GLK--CYSAPGEIE--DRIDIAIFAVP----AKAVLEI---LKG--SV-ENIKG-AVIV-SSG--FR
GKKFKVYRSVKEIP--DEVDVAVIAVP----AKFVPDV---IDE--CGEKGVKA-AVVI-SAG--FK
GYK--VYHDVSELP--EDTDLAVIAIP----APAVPET---MRK--IAEKGIKA-VIII-TGG--FG
G-H--ILTA-KYWQNVED-NGLHYTVADSGWGKCVWGK---LYG-----QWIAGCAVFVY----DY-
G-H--ILTA-KYWQNVED-DGLHYTVADSGWGKCVWGK---LYG-----QWIAGCAVFVY----DY-
G-H--ILTA-KYWQNVED-DGLHYTVADSGWGKCVWGK---LYG-----QWIAGCAVFVY----DY-
A-H--IITA-KYWQKIDP-DGLHLTVSETGWAKAAWGK---LYG-----QWLLGGGIMVY----DF-
G-H--IVTA-KYWHRVVE-NGLHLTVADSGWAKFAWGK---LFG-----QWICGAVQFLY----DM-
GPA---QTLHWVFDLKDD-DVWWCT-ADIGW---VTGHSYIVYA-----PLILGMTSLMYEGAADYP
GPA---YTCKWVFDLNED-DIYWCT-ADVGW---ITGHSYVAYG-----PLCLGITSVMYEGSPDYP
GPA---YTTSWVFDIKDT-DVYWST-ADIGW---ITGHTYIVYG-----PLCLGATSVMYEGSPDYP
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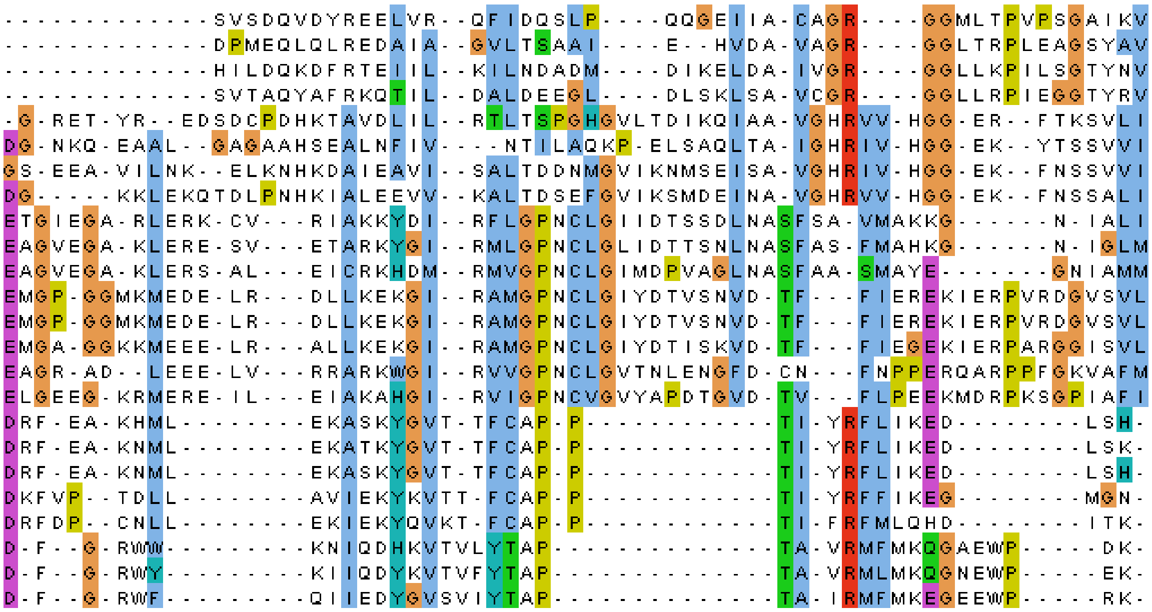

```
-------------SVSDQVDYREELVR--QFIDQSLP----QQGEIIA-CAGR----GGMLTPVPSGAIKV
-------------DPMEQLQLREDAIA--GVLTSAAI----E--HVDA-VAGR----GGLTRPLEAGSYAV
-------------HILDQKDFRTEIIL--KILNDADM----DIKELDA-IVGR----GGLLKPILSGTYNV
-------------SVTAQYAFRKQTIL--DALDEEGL----DLSKLSA-VCGR----GGLLRPIEGGTYRV
-G-RET-YR--EDSDCPDHKTAVDLIL--RTLTSPGHGVLTDIKQIAA-VGHRVV-HGG-ER--FTKSVLI
DG-NKQ-EAAL--GAGAAHSEALNFIV----NTILAQKP-ELSAQLTA-IGHRIV-HGG-EK--YTSSVVI
GS-EEA-VILNK--ELKNHKDAIEAVI--SALTDDNMGVIKNMSEISA-VGHRIV-HGG-EK--FNSSVVI
DG-----KKLEKQTDLPNHKIALEEVV--KALTDSEFGVIKSMDEINA-VGHRVV-HGG-EK--FNSSALI
ETGIEGA-RLERK-CV---RIAKKYDI--RFLGPNCLGIIDTSSDLNASFSA-VMAKKG------N-IALI
EAGVEGA-KLERE-SV---ETARKYGI--RMLGPNCLGLIDTTSNLNASFAS-FMAHKG------N-IGLM
EAGVEGA-KLERS-AL---EICRKHDM--RMVGPNCLGIMDPVAGLNASFAA-SMAYE-------GNIAMM
EMGP-GGMKMEDE-LR---DLLKEKGI--RAMGPNCLGIYDTVSNVD-TF---FIEREKIERPVRDGVSVL
EMGP-GGMKMEDE-LR---DLLKEKGI--RAMGPNCLGIYDTVSNVD-TF---FIEREKIERPVRDGVSVL
EMGA-GGKKMEEE-LR---ALLKEKGI--RAMGPNCLGIYDTISKVD-TF---FIEGEKIERPARGGISVL
EAGR-AD--LEEE-LV---RRARKWGI--RVVGPNCLGVTNLENGFD-CN---FNPPERQARPPFGKVAFM
ELGEEG-KRMERE-IL---EIAKAHGI--RVIGPNCVGVYAPDTGVD-TV---FLPEEKMDRPKSGPIAFI
DRF-EA-KHML--------EKASKYGVT-TFCAP-P------------TI-YRFLIKED--------LSH-
DRF-EA-KNML--------EKATKYGVT-TFCAP-P------------TI-YRFLIKED--------LSK-
DRF-EA-KNML--------EKASKYGVT-TFCAP-P------------TI-YRFLIKED--------LSH-
DKFVP--TDLL--------AVIEKYKVTT-FCAP-P------------TI-YRFFIKEG--------MGN-
DRFDP--CNLL--------EKIEKYQVKT-FCAP-P------------TI-FRFMLQHD--------ITK-
D-F--G-RWW---------KNIQDHKVTVLYTAP--------------TA-VRMFMKQGAEWP-----DK-
D-F--G-RWY---------KIIQDYKVTVFYTAP--------------TA-VRMLMKQGNEWP-----EK-
D-F--G-RWF---------QIIEDYGVSVIYTAP--------------TA-IRMFMKEGEEWP-----RK-
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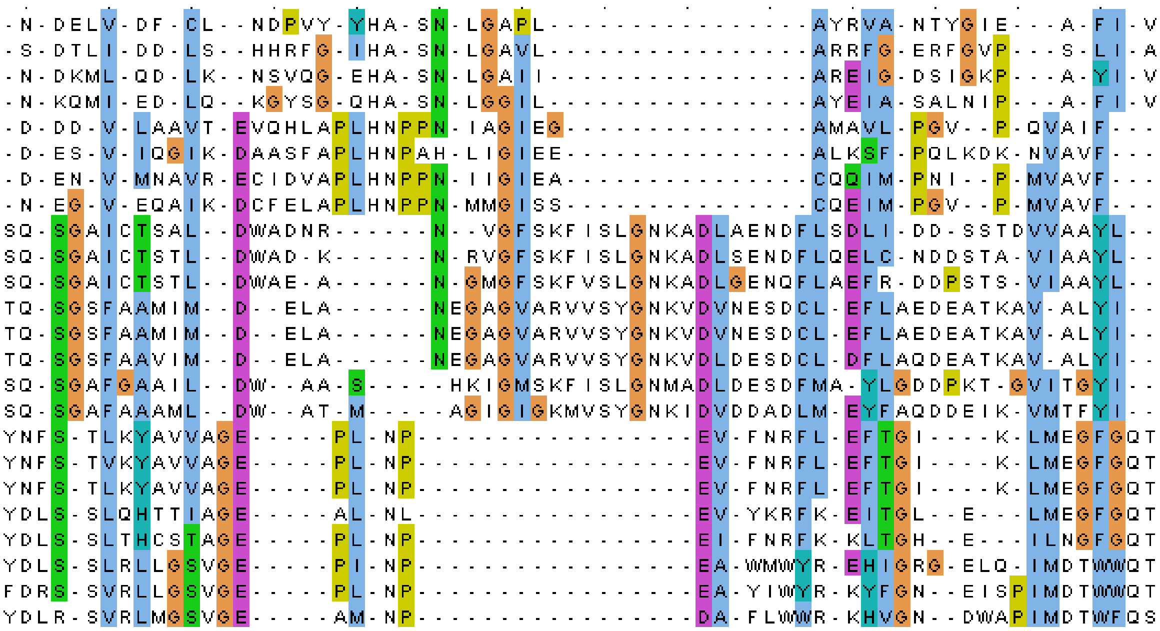

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-N-DELV-DF-CL--NDPVY-YHA-SN-LGAPL----------------AYRVA-NTYGIE---A-FI-V
-S-DTLI-DD-LS--HHRFG-IHA-SN-LGAVL----------------ARRFG-ERFGVP---S-LI-A
-N-DKML-QD-LK--NSVQG-EHA-SN-LGAII----------------AREIG-DSIGKP---A-YI-V
-N-KQMI-ED-LQ--KGYSG-QHA-SN-LGGIL----------------AYEIA-SALNIP---A-FI-V
-D-DD-V-LAAVT-EVQHLAPLHNPPN-IAGIEG---------------AMAVL-PGV--P-QVAIF---
-D-ES-V-IQGIK-DAASFAPLHNPAH-LIGIEE---------------ALKSF-PQLKDK-NVAVF---
-D-EN-V-MNAVR-ECIDVAPLHNPPN-IIGIEA---------------CQQIM-PNI--P-MVAVF---
-N-EG-V-EQAIK-DCFELAPLHNPPN-MMGISS---------------CQEIM-PGV--P-MVAVF---
SQ-SGAICTSAL--DWADNR------N--VGFSKFISLGNKADLAENDFLSDLI-DD-SSTDVVAAYL--
SQ-SGAICTSTL--DWAD-K------N-RVGFSKFISLGNKADLSENDFLQELC-NDDSTA-VIAAYL--
SQ-SGAICTSTL--DWAE-A------N-GMGFSKFVSLGNKADLGENQFLAEFR-DDPSTS-VIAAYL--
TQ-SGSFAAMIM--D--ELA------NEGAGVARVVSYGNKVDVNESDCL-EFLAEDEATKAV-ALYI--
TQ-SGSFAAMIM--D--ELA------NEGAGVARVVSYGNKVDVNESDCL-EFLAEDEATKAV-ALYI--
TQ-SGSFAAVIM--D--ELA------NEGAGVARVVSYGNKVDLDESDCL-DFLAQDEATKAV-ALYI--
SQ-SGAFGAAIL--DW--AA-S-----HKIGMSKFISLGNMADLDESDFMA-YLGDDPKT-GVITGYI--
SQ-SGAFAAAML--DW--AT-M-----AGIGIGKMVSYGNKIDVDDADLM-EYFAQDDEIK-VMTFYI--
YNFS-TLKYAVVAGE-----PL-NP-----------------EV-FNRFL-EFTGI----K-LMEGFGQT
YNFS-TVKYAVVAGE-----PL-NP-----------------EV-FNRFL-EFTGI----K-LMEGFGQT
YNFS-TLKYAVVAGE-----PL-NP-----------------EV-FNRFL-EFTGI----K-LMEGFGQT
YDLS-SLQHTTIAGE-----AL-NL-----------------EV-YKRFK-EITGL--E---LMEGFGQT
YDLS-SLTHCSTAGE-----PL-NP-----------------EI-FNRFK-KLTGH--E---ILNGFGQT
YDLS-SLRLLGSVGE-----PI-NP-----------------EA-WMWYR-EHIGRG-ELQ-IMDTWWQT
FDRS-SVRLLGSVGE-----PL-NP-----------------EA-YIWYR-KYFGN--EISPIMDTWWQT
YDLR-SVRLMGSVGE-----AM-NP-----------------DA-FLWWR-KHVGN--DWAPIMDTWFQS
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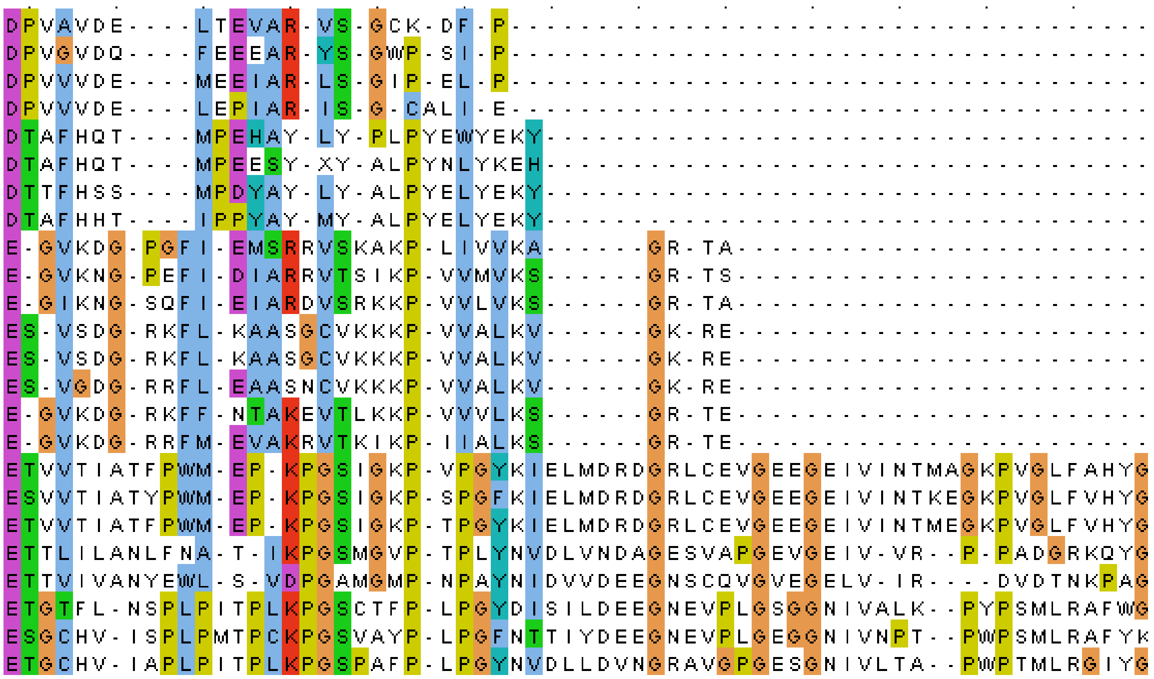

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DPVAVDE----LTEVAR-VS-GCK-DF-P-------------------------------------
DPVGVDQ----FEEEAR-YS-GWP-SI-P-------------------------------------
DPVVVDE----MEEIAR-LS-GIP-EL-P-------------------------------------
DPVVVDE----LEPIAR-IS-G-CALI-E-------------------------------------
DTAFHQT----MPEHAY-LY-PLPYEWYEKY-----------------------------------
DTAFHQT----MPEESY-XY-ALPYNLYKEH-----------------------------------
DTTFHSS----MPDYAY-LY-ALPYELYEKY-----------------------------------
DTAFHHT----IPPYAY-MY-ALPYELYEKY-----------------------------------
E-GVKDG-PGFI-EMSRRVSKAKP-LIVVKA------GR-TA------------------------
E-GVKNG-PEFI-DIARRVTSIKP-VVMVKS------GR-TS------------------------
E-GIKNG-SQFI-EIARDVSRKKP-VVLVKS------GR-TA------------------------
ES-VSDG-RKFL-KAASGCVKKKP-VVALKV------GK-RE------------------------
ES-VSDG-RKFL-KAASGCVKKKP-VVALKV------GK-RE------------------------
ES-VGDG-RRFL-EAASNCVKKKP-VVALKV------GK-RE------------------------
E-GVKDG-RKFF-NTAKEVTLKKP-VVVLKS------GR-TE------------------------
E-GVKDG-RRFM-EVAKRVTKIKP-IIALKS------GR-TE------------------------
ETVVTIATFPWM-EP-KPGSIGKP-VPGYKIELMDRDGRLCEVGEEGEIVINTMAGKPVGLFAHYG
ESVVTIATYPWM-EP-KPGSIGKP-SPGFKIELMDRDGRLCEVGEEGEIVINTKEGKPVGLFVHYG
ETVVTIATFPWM-EP-KPGSIGKP-TPGYKIELMDRDGRLCEVGEEGEIVINTMEGKPVGLFVHYG
ETTLILANLFNA-T-IKPGSMGVP-TPLYNVDLVNDAGESVAPGEVGEIV-VR--P-PADGRKQYG
ETTVIVANYEWL-S-VDPGAMGMP-NPAYNIDVVDEEGNSCQVGVEGELV-IR----DVDTNKPAG
ETGTFL-NSPLPITPLKPGSCTFP-LPGYDISILDEEGNEVPLGSGGNIVALK--PYPSMLRAFWG
ESGCHV-ISPLPMTPCKPGSVAYP-LPGFNTTIYDEEGNEVPLGEGGNIVNPT--PWPSMLRAFYK
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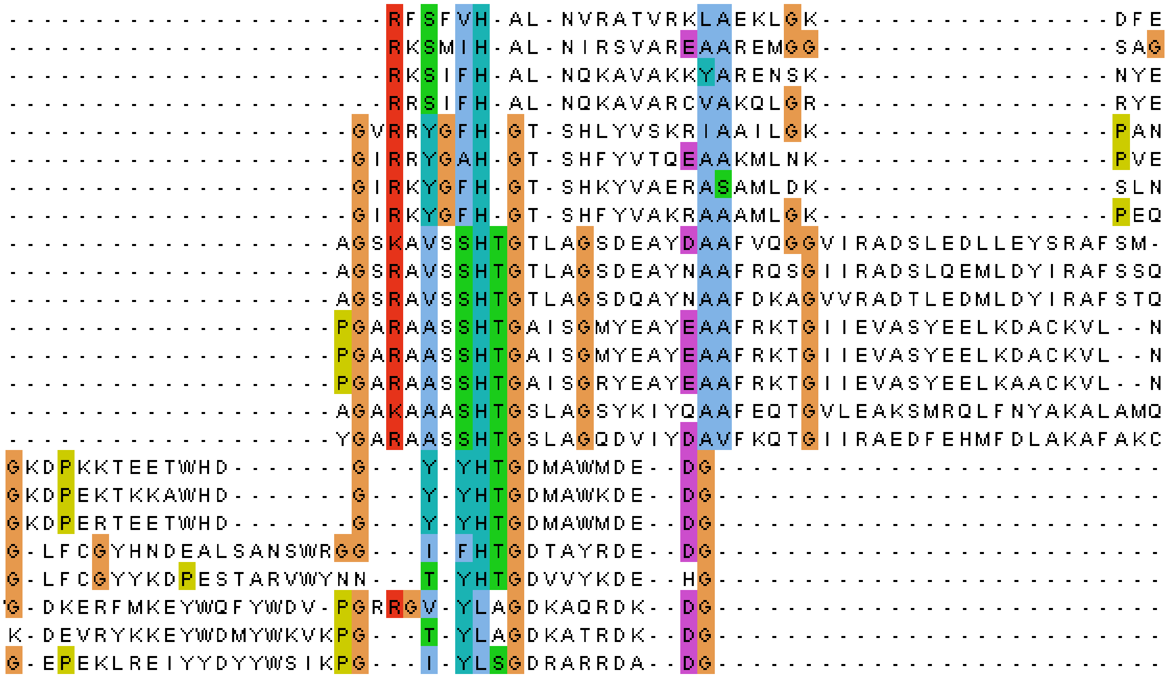

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----------------------RFSFVH-AL-NVRATVRKLAEKLGK-----------------DFE
----------------------RKSMIH-AL-NIRSVAREAAREMGG-----------------SAG
----------------------RKSIFH-AL-NQKAVAKKYARENSK-----------------NYE
----------------------RRSIFH-AL-NQKAVARCVAKQLGR-----------------RYE
--------------------GVRRYGFH-GT-SHLYVSKRIAAILGK-----------------PAN
--------------------GIRRYGAH-GT-SHFYVTQEAAKMLNK-----------------PVE
--------------------GIRKYGFH-GT-SHKYVAERASAMLDK-----------------SLN
--------------------GIRKYGFH-GT-SHFYVAKRAAAMLGK-----------------PEQ
-------------------AGSKAVSSHTGTLAGSDEAYDAAFVQGGVIRADSLEDLLEYSRAFSM-
-------------------AGSRAVSSHTGTLAGSDEAYNAAFRQSGIIRADSLQEMLDYIRAFSSQ
-------------------AGSRAVSSHTGTLAGSDQAYNAAFDKAGVVRADTLEDMLDYIRAFSTQ
-------------------PGARAASSHTGAISGMYEAYEAAFRKTGIIEVASYEELKDACKVL--N
-------------------PGARAASSHTGAISGMYEAYEAAFRKTGIIEVASYEELKDACKVL--N
-------------------PGARAASSHTGAISGRYEAYEAAFRKTGIIEVASYEELKAACKVL--N
-------------------AGAKAAASHTGSLAGSYKIYQAAFEQTGVLEAKSMRQLFNYAKALAMQ
-------------------YGARAASSHTGSLAGQDVIYDAVFKQTGIIRAEDFEHMFDLAKAFAKC
GKDPKKTEETWHD-------G---Y-YHTGDMAWMDE--DG--------------------------
GKDPEKTKKAWHD-------G---Y-YHTGDMAWKDE--DG--------------------------
GKDPERTEETWHD-------G---Y-YHTGDMAWMDE--DG--------------------------
G-LFCGYHNDEALSANSWRGG---I-FHTGDTAYRDE--DG--------------------------
G-LFCGYYKDPESTARVWYNN---T-YHTGDVVYKDE--HG--------------------------
G-DKERFMKEYWQFYWDV-PGRRGV-YLAGDKAQRDK--DG--------------------------
K-DEVRYKKEYWDMYWKVKPG---T-YLAGDKATRDK--DG--------------------------
G-EPEKLREIYYDYYWSIKPG---I-YLSGDRARRDA--DG--------------------------
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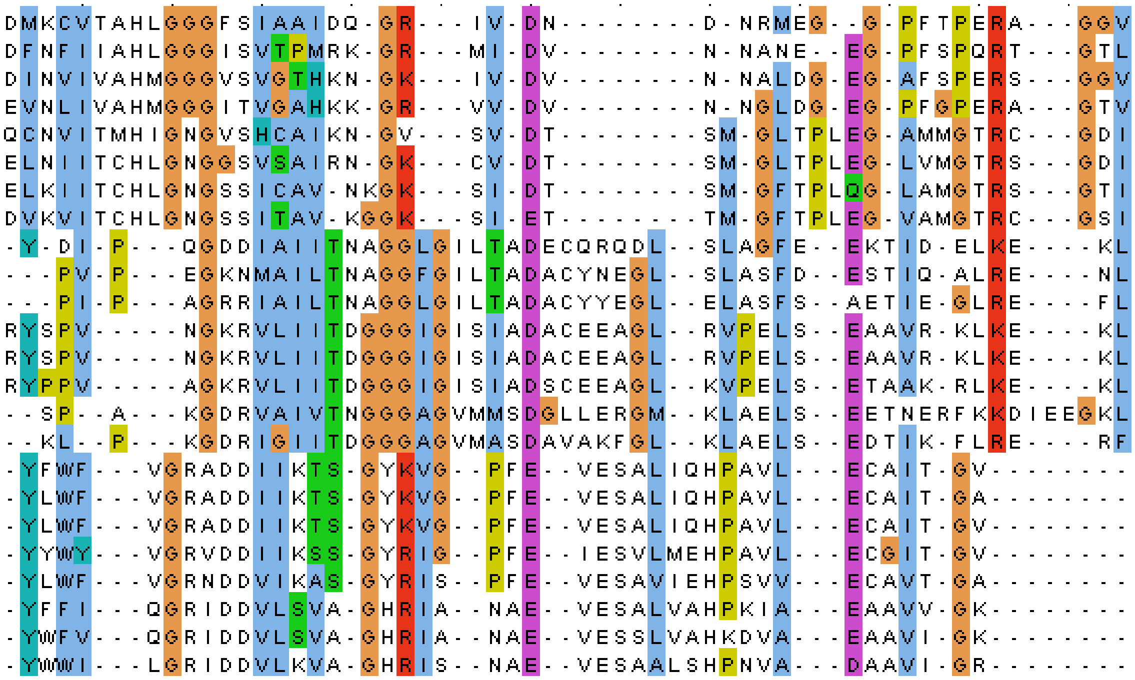

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DMKCVTAHLGGGFSIAAIDQ-GR---IV-DN--------D-NRMEG--G-PFTPERA---GGV
DFNFIIAHLGGGISVTPMRK-GR---MI-DV--------N-NANE--EG-PFSPQRT---GTL
DINVIVAHMGGGVSVGTHKN-GK---IV-DV--------N-NALDG-EG-AFSPERS---GGV
EVNLIVAHMGGGITVGAHKK-GR---VV-DV--------N-NGLDG-EG-PFGPERA---GTV
QCNVITMHIGNGVSHCAIKN-GV---SV-DT--------SM-GLTPLEG-AMMGTRC---GDI
ELNIITCHLGNGGSVSAIRN-GK---CV-DT--------SM-GLTPLEG-LVMGTRS---GDI
ELKIITCHLGNGSSICAV-NKGK---SI-DT--------SM-GFTPLQG-LAMGTRS---GTI
DVKVITCHLGNGSSITAV-KGGK---SI-ET--------TM-GFTPLEG-VAMGTRC---GSI
-Y-DI-P---QGDDIAIITNAGGLGILTADECQRQDL--SLAGFE--EKTID-ELKE----KL
---PV-P---EGKNMAILTNAGGFGILTADACYNEGL--SLASFD--ESTIQ-ALRE----NL
---PI-P---AGRRIAILTNAGGLGILTADACYYEGL--ELASFS--AETIE-GLRE----FL
RYSPV-----NGKRVLIITDGGGIGISIADACEEAGL--RVPELS--EAAVR-KLKE----KL
RYSPV-----NGKRVLIITDGGGIGISIADACEEAGL--RVPELS--EAAVR-KLKE----KL
RYPPV-----AGKRVLIITDGGGIGISIADSCEEAGL--KVPELS--ETAAK-RLKE----KL
--SP--A---KGDRVAIVTNGGGAGVMMSDGLLERGM--KLAELS--EETNERFKKDIEEGKL
--KL--P---KGDRIGIITDGGGAGVMASDAVAKFGL--KLAELS--EDTIK-FLRE----RF
-YFWF---VGRADDIIKTS-GYKVG--PFE--VESALIQHPAVL---ECAIT-GV--------
-YLWF---VGRADDIIKTS-GYKVG--PFE--VESALIQHPAVL---ECAIT-GA--------
-YLWF---VGRADDIIKTS-GYKVG--PFE--VESALIQHPAVL---ECAIT-GV--------
-YYWY---VGRVDDIIKSS-GYRIG--PFE--IESVLMEHPAVL---ECGIT-GV--------
-YLWF---VGRNDDVIKAS-GYRIS--PFE--VESAVIEHPSVV---ECAVT-GA--------
-YFFI---QGRIDDVLSVA-GHRIA--NAE--VESALVAHPKIA---EAAVV-GK--------
-YWFV---QGRIDDVLSVA-GHRIA--NAE--VESSLVAHKDVA---EAAVI-GK--------
-YWWI---LGRIDDVLKVA-GHRIS--NAE--VESAALSHPNVA---DAAVI-GR--------
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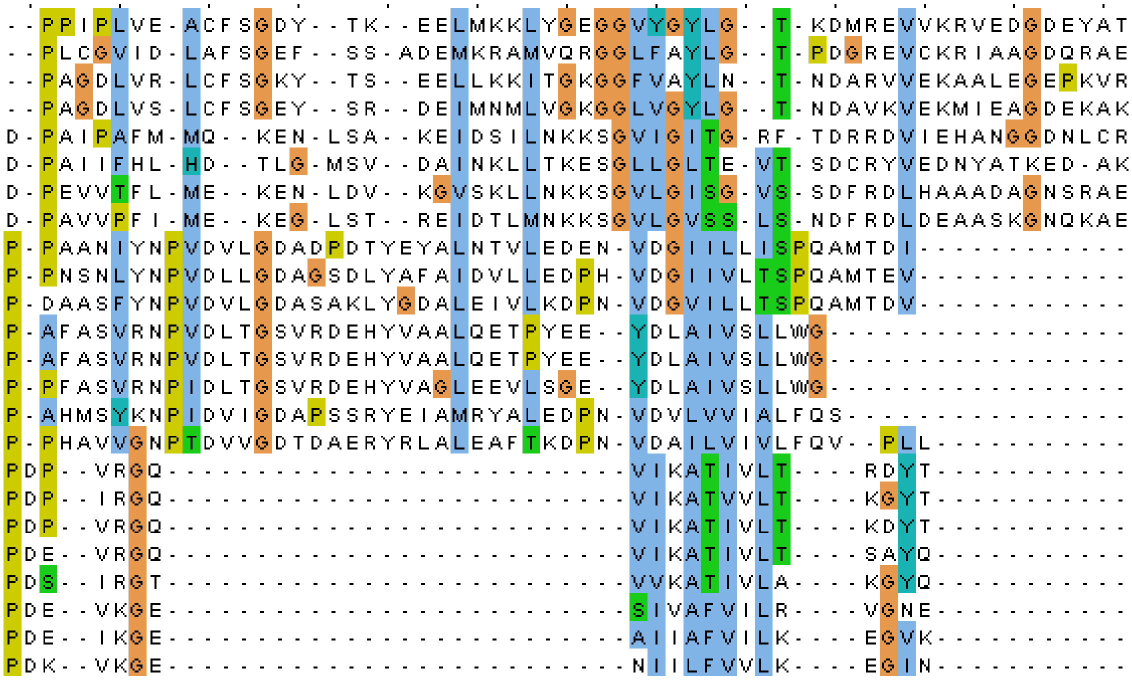

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--PPIPLVE-ACFSGDY--TK--EELMKKLYGEGGVYGYLG--T-KDMREVVKRVEDGDEYAT
--PLCGVID-LAFSGEF--SS-ADEMKRAMVQRGGLFAYLG--T-PDGREVCKRIAAGDQRAE
--PAGDLVR-LCFSGKY--TS--EELLKKITGKGGFVAYLN--T-NDARVVEKAALEGEPKVR
--PAGDLVS-LCFSGEY--SR--DEIMNMLVGKGGLVGYLG--T-NDAVKVEKMIEAGDEKAK
D-PAIPAFM-MQ--KEN-LSA--KEIDSILNKKSGVIGITG-RF-TDRRDVIEHANGGDNLCR
D-PAIIFHL-HD--TLG-MSV--DAINKLLTKESGLLGLTE-VT-SDCRYVEDNYATKED-AK
D-PEVVTFL-ME--KEN-LDV--KGVSKLLNKKSGVLGISG-VS-SDFRDLHAAADAGNSRAE
D-PAVVPFI-ME--KEG-LST--REIDTLMNKKSGVLGVSS-LS-NDFRDLDEAASKGNQKAE
P-PAANIYNPVDVLGDADPDTYEYALNTVLEDEN-VDGIILLISPQAMTDI------------
P-PNSNLYNPVDLLGDAGSDLYAFAIDVLLEDPH-VDGIIVLTSPQAMTEV------------
P-DAASFYNPVDVLGDASAKLYGDALEIVLKDPN-VDGVILLTSPQAMTDV------------
P-AFASVRNPVDLTGSVRDEHYVAALQETPYEE--YDLAIVSLLWG-----------------
P-AFASVRNPVDLTGSVRDEHYVAALQETPYEE--YDLAIVSLLWG-----------------
P-PFASVRNPIDLTGSVRDEHYVAGLEEVLSGE--YDLAIVSLLWG-----------------
P-AHMSYKNPIDVIGDAPSSRYEIAMRYALEDPN-VDVLVVIALFQS----------------
P-PHAVVGNPTDVVGDTDAERYRLALEAFTKDPN-VDAILVIVLFQV--PLL-----------
PDP--VRGQ--------------------------VIKATIVLT----RDYT-----------
PDP--IRGQ--------------------------VIKATVVLT----KGYT-----------
PDP--VRGQ--------------------------VIKATIVLT----KDYT-----------
PDE--VRGQ--------------------------VIKATIVLT----SAYQ-----------
PDS--IRGT--------------------------VVKATIVLA----KGYQ-----------
PDE--VKGE--------------------------SIVAFVILR----VGNE-----------
PDE--IKGE--------------------------AIIAFVILK----EGVK-----------
PDK--VKGE--------------------------NIILFVVLK----EGIN-----------
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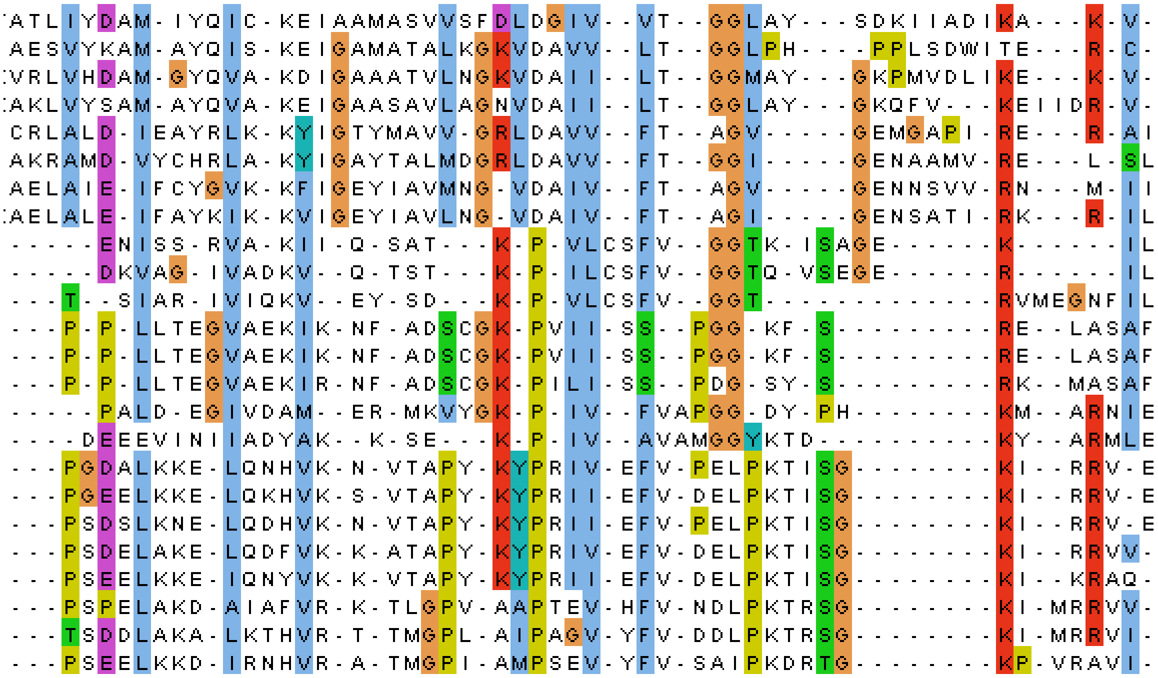

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ATLIYDAM-IYQIC-KEIAAMASVVSFDLDGIV--VT--GGLAY---SDKIIADIKA---K-V-
AESVYKAM-AYQIS-KEIGAMATALKGKVDAVV--LT--GGLPH----PPLSDWITE---R-C-
VRLVHDAM-GYQVA-KDIGAAATVLNGKVDAII--LT--GGMAY---GKPMVDLIKE---K-V-
AKLVYSAM-AYQVA-KEIGAASAVLAGNVDAII--LT--GGLAY---GKQFV---KEIIDR-V-
CRLALD-IEAYRLK-KYIGTYMAVV-GRLDAVV--FT--AGV-----GEMGAPI-RE---R-AI
AKRAMD-VYCHRLA-KYIGAYTALMDGRLDAVV--FT--GGI-----GENAAMV-RE---L-SL
AELAIE-IFCYGVK-KFIGEYIAVMNG-VDAIV--FT--AGV-----GENNSVV-RN---M-II
AELALE-IFAYKIK-KVIGEYIAVLNG-VDAIV--FT--AGI-----GENSATI-RK---R-IL
-----ENISS-RVA-KII-Q-SAT---K-P-VLCSFV--GGTK-ISAGE------K------IL
-----DKVAG-IVADKV--Q-TST---K-P-ILCSFV--GGTQ-VSEGE------R------IL
---T--SIAR-IVIQKV--EY-SD---K-P-VLCSFV--GGT-------------RVMEGNFIL
---P-P-LLTEGVAEKIK-NF-ADSCGK-PVII-SS--PGG-KF-S---------RE--LASAF
---P-P-LLTEGVAEKIK-NF-ADSCGK-PVII-SS--PGG-KF-S---------RE--LASAF
---P-P-LLTEGVAEKIR-NF-ADSCGK-PILI-SS--PDG-SY-S---------RK--MASAF
-----PALD-EGIVDAM--ER-MKVYGK-P-IV--FVAPGG-DY-PH--------KM--ARNIE
----DEEEVINIIADYAK--K-SE---K-P-IV--AVAMGGYKTD----------KY--ARMLE
---PGDALKKE-LQNHVK-N-VTAPY-KYPRIV-EFV-PELPKTISG--------KI--RRV-E
---PGEELKKE-LQKHVK-S-VTAPY-KYPRII-EFV-DELPKTISG--------KI--RRV-E
---PSDSLKNE-LQDHVK-N-VTAPY-KYPRII-EFV-PELPKTISG--------KI--RRV-E
---PSDELAKE-LQDFVK-K-ATAPY-KYPRIV-EFV-DELPKTISG--------KI--RRVV-
---PSEELKKE-IQNYVK-K-VTAPY-KYPRII-EFV-DELPKTISG--------KI--KRAQ-
---PSPELAKD-AIAFVR-K-TLGPV-AAPTEV-HFV-NDLPKTRSG--------KI-MRRVV-
---TSDDLAKA-LKTHVR-T-TMGPL-AIPAGV-YFV-DDLPKTRSG--------KI-MRRVI-
---PSEELKKD-IRNHVR-A-TMGPI-AMPSEV-YFV-SAIPKDRTG--------KP-VRAVI-
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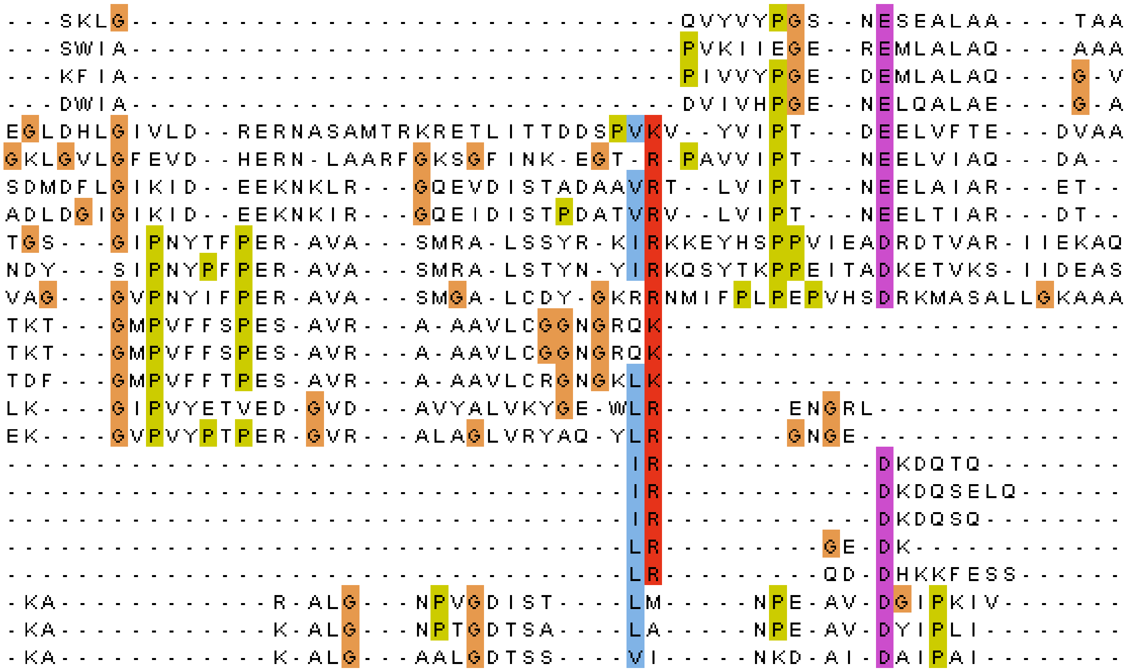

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---SKLG-------------------------------QVYVYPGS--NESEALAA----TAA
---SWIA-------------------------------PVKIIEGE--REMLALAQ----AAA
---KFIA-------------------------------PIVVYPGE--DEMLALAQ----G-V
---DWIA-------------------------------DVIVHPGE--NELQALAE----G-A
EGLDHLGIVLD--RERNASAMTRKRETLITTDDSPVKV--YVIPT---DEELVFTE---DVAA
GKLGVLGFEVD--HERN-LAARFGKSGFINK-EGT-R-PAVVIPT---NEELVIAQ---DA--
SDMDFLGIKID--EEKNKLR---GQEVDISTADAAVRT--LVIPT---NEELAIAR---ET--
ADLDGIGIKID--EEKNKIR---GQEIDISTPDATVRV--LVIPT---NEELTIAR---DT--
TGS---GIPNYTFPER-AVA---SMRA-LSSYR-KIRKKEYHSPPVIEADRDTVAR-IIEKAQ
NDY---SIPNYPFPER-AVA---SMRA-LSTYN-YIRKQSYTKPPEITADKETVKS-IIDEAS
VAG---GVPNYIFPER-AVA---SMGA-LCDY-GKRRNMIFPLPEPVHSDRKMASALLGKAAA
TKT---GMPVFFSPES-AVR---A-AAVLCGGNGRQK--------------------------
TKT---GMPVFFSPES-AVR---A-AAVLCGGNGRQK--------------------------
TDF---GMPVFFTPES-AVR---A-AAVLCRGNGKLK--------------------------
LK----GIPVYETVED-GVD---AVYALVKYGE-WLR-------ENGRL--------------
EK----GVPVYPTPER-GVR---ALAGLVRYAQ-YLR-------GNGE---------------
-----------------------------------IR------------DKDQTQ--------
-----------------------------------IR------------DKDQSELQ------
-----------------------------------IR------------DKDQSQ--------
-----------------------------------LR---------GE-DK------------
-----------------------------------LR---------QD-DHKKFESS------
-KA------------R-ALG---NPVGDIST----LM-----NPE-AV-DGIPKIV-------
-KA------------K-ALG---NPTGDTSA----LA-----NPE-AV-DYIPLI--------
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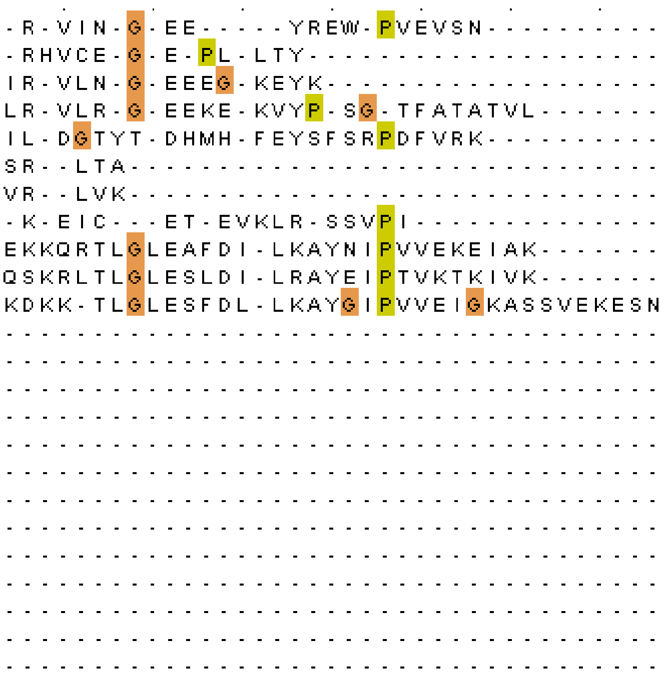

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-R-VIN-G-EE-----YREW-PVEVSN----------
-RHVCE-G-E-PL-LTY--------------------
IR-VLN-G-EEEG-KEYK-------------------
LR-VLR-G-EEKE-KVYP-SG-TFATATVL-------
IL-DGTYT-DHMH-FEYSFSRPDFVRK----------
SR--LTA------------------------------
VR--LVK------------------------------
-K-EIC---ET-EVKLR-SSVPI--------------
EKKQRTLGLEAFDI-LKAYNIPVVEKEIAK-------
QSKRLTLGLESLDI-LRAYEIPTVKTKIVK-------
KDKK-TLGLESFDL-LKAYGIPVVEIGKASSVEKESN
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