

INVESTIGATING NOVEL METHYLOTROPHIC ARCHAEA IN YELLOWSTONE HOT
SPRINGS THROUGH META-OMICS AND TARGETED CULTIVATION

by

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ABSTRACT

In the past two decades, metagenomic sequencing of environmental samples has led to a substantial expansion in our understanding of the domain Archaea. In particular, several newly discovered lineages encode the potential for anaerobic methylotrophy, in either a non-methanogenic or methanogenic fashion. However, due to the absence of cultures, many lineages are only hypothesized to perform methylotrophy based on metagenomic analysis and lack experimental support of their proposed physiology. Furthermore, given the large diversity of uncultivated taxa, there are likely additional groups of methylotrophic archaea that are not yet characterized.

Methyl groups are ubiquitous in nature and thus are important in fueling many metabolic lifestyles and impacting global carbon cycling. Non-methanogenic methylotrophs can convert methyl groups to carbon dioxide and may compete with methanogens for substrates. Methylotrophic methanogens are capable of producing methane, a climate active gas, from methyl groups. For decades methanogens were thought to be restricted to a single lineage “Euryarchaeota”. However, in the last decade, culture-independent metagenomic sequencing has revealed the presence of marker genes for methanogenesis in several uncultivated groups within another archaeal phylum, Thermoproteota.

This thesis presents results on three class-level lineages of methylotrophic archaea within the Thermoproteota phylum identified in Yellowstone National Park hot springs. The first is a metagenomic description of a new uncultivated class-level lineage, termed Culexarchaeia, that encodes a versatile metabolism, including the capacity for non-methanogenic anaerobic methylotrophy. The second lineage discussed is “Methanomethylicia”, whose members are predicted methylotrophic methanogens. Using hot spring sediments, we obtained an enrichment culture containing *Candidatus Methanosuratincola verstraetei* and demonstrated that it produces methane from methanol and hydrogen. The third lineage discussed is Korarchaeia, an enigmatic lineage commonly found in terrestrial hot springs. We obtained an enrichment culture containing *Candidatus Methanodesulfokora washburnensis*, an organism that also makes methane from methanol and hydrogen. These represent the first cultured methanogens outside the Euryarchaeota, and set the stage for future detailed studies on the physiology and biochemistry of these organisms. Together, these results highlight Yellowstone as a natural laboratory for the investigation of poorly understood archaeal lineages through metagenomics and cultivation.

CHAPTER ONE

INTRODUCTION

Methanogens and Archaeal Diversity

Methanogenesis, the production of methane coupled to energy conservation for growth, is one of the most ancient metabolisms on Earth and is performed by specialized Archaea called methanogens (Ueno, Yamada et al. 2006, Thauer, Kaster et al. 2008, Weiss, Sousa et al. 2016). Methanogenesis is a uniquely archaeal metabolism that has not been found in the other domains of life, Bacteria and Eukarya. Some of the first work on methanogens took place in the early 1900s and led to isolation of methanogens in the genera we now know as *Methanosarcina* and *Methanobacterium*. However, at the time, these organisms were classified as ‘methanogenic bacteria’ instead of Archaea, as microscopic observations alone were not enough to distinguish them as a separate domain of life (Barker 1936). It wasn’t until the pioneering work of Carl Woese on the comparison of 16S rRNA gene sequences that these organisms were finally recognized to belong to a domain of life that is distinct from Bacteria and Eukarya, the Archaea (Woese and Fox 1977, Woese, Kandler et al. 1990). From the 1990s to early 2000s, Archaea were only known to contain two phyla: Euryarchaeota, which contained methanogens and halophiles, and Crenarchaeota, which contained mostly extremophilic and thermophilic species. However, with massive community efforts in metagenomic sequencing, the archaeal tree of life has rapidly expanded and today four major groups, each containing several phylum-level lineages, are recognized: the Euryarchaeota, Thermoproteota/‘TACK’, ‘DPANN’, and Asgard superphyla (Adam, Borrel et al. 2017, Spang, Caceres et al. 2017).

Methanogens are widespread in diverse anoxic environments including rice paddies, hot springs, soils, marine and freshwater sediments, oil reservoirs, and the gastrointestinal tracts of animals, where they play key roles in the final steps complete degradation of organic matter to methane (Thauer, Kaster et al. 2008, Garcia, Gribaldo et al. 2022). Methanogens are extremely productive organisms and collectively they are responsible for approximately 70% of global methane emissions, which are on the order of 500-600 Teragrams per year (Conrad 2009). Methane is a potent greenhouse gas, and its accumulation in the atmosphere has contributed roughly 30% to the rise in global temperatures since the industrial revolution (Conrad 2009, IPCC & Masson-Delmotte 2021). Despite these negative impacts on the climate, methanogens are also capable of converting biological and industrial wastes to methane, which could offer a route to renewable energy sources and a more circular carbon economy. Therefore, understanding the diversity, biochemistry, and ecology of methanogens has substantial implications across the fields of climate science, microbiology, biogeochemistry, and biotechnology.

Methanogens are metabolically diverse in the compounds that they can convert to methane. They have long been understood to convert simple compounds such as acetate, CO₂, methanol, methylamines, and methylsulfides to methane (Kurth, Op den Camp et al. 2020). The metabolism of these compounds forms the basis of four major modes of methanogenesis, (i) acetoclastic (acetate), (ii) hydrogenotrophic (or CO₂-reducing), (iii) methyl-dismutating, and (iv) methyl-reducing (with iii and iv both using compounds like methanol, methylamines, or methylsulfides) (Kulkarni, Mand et al. 2018). Recently, it has also been shown that some specialized groups of methanogens can convert an immense array of methoxylated or alkane

compounds into methane, with the description of methoxydotrophic and alkanotrophic methanogenesis, respectively (Mayumi, Mochimaru et al. 2016, Borrel, Adam et al. 2019, Kurth, Nobu et al. 2021, Zhou, Zhang et al. 2022). While enzymes associated with each type of pathway differ, the common enzyme complex in all methanogenic pathways is methyl-coenzyme M reductase (MCR) that produces methane. Furthermore, MCR is also used by methanotrophic or alkanotrophic organisms in the first step of alkane oxidation (e.g. methane, ethane, propane, butane) to CO₂ (Laso-Pérez, Wegener et al. 2016, Chen, Musat et al. 2019, Hahn, Laso-Perez et al. 2020, Garcia, Gribaldo et al. 2022, Zehnle, Laso-Pérez et al. 2023). Therefore, MCR is typically used as a marker gene in metagenomic or amplicon studies for methane and alkane metabolism (Evans, Boyd et al. 2019, Lynes, Krukenberg et al. 2023).

New Players in Methane Metabolism

Importantly, all methanogens that have been cultivated in the last century have been from the lineage Euryarchaeota (Evans, Boyd et al. 2019). This was challenged in 2015 when metagenomic sequencing identified marker genes, including the key MCR complex, for methane metabolism in a lineage within the Thermoproteota, the Bathyarchaeia (Evans, Parks et al. 2015). In the following years multiple new lineages in the Thermoproteota were also found to encode marker genes for methane metabolism, including Methanomethylicia (Verstraetearchaeota), Nezharchaeia, Methylarchaeales, and Korarchaeia (Vanwonterghem, Evans et al. 2016, Borrel, Adam et al. 2019, Hua, Wang et al. 2019, McKay, Dlakić et al. 2019, Wang, Wegener et al. 2019, Ou, Dong et al. 2022). Notably, the majority of these new lineages were predicted to perform methyl-reducing hydrogenotrophic methanogenesis, a methanogenic pathway that was previously thought to have limited environmental and taxonomic breadth relative to the CO₂-

reducing hydrogenotrophic pathway (Vanwonterghem, Evans et al. 2016, Sorokin, Makarova et al. 2017, Borrel, Adam et al. 2019, Söllinger and Urich 2019, Ou, Dong et al. 2022). These discoveries over the last decade have greatly expanded our view on the origin of methanogenesis and the evolution and metabolism of Archaea. The proposed methanogenic Thermoproteota lineages are similarly widespread globally relative to Euryarchaeal methanogens, being found in hot springs, soils, wastewater, and freshwater and marine systems, suggesting that they could also play a large role in methane cycling. However, despite strong metagenomic inferences, the physiology of these organisms could not be experimentally verified, since all new putative methanogenic lineages in the Thermoproteota were uncultured and no culture-independent methods (*e.g.* stable isotope probing, transcriptomics, or proteomics) had been used to test this hypothesis.

Quitting Your Job and Finding a New One, Transitions Between Methanogenic and Non-Methanogenic Metabolisms

The domain Archaea harbors a substantial amount of metabolic diversity and lifestyles for conserving energy. Some examples include ammonia oxidizers, sulfate reducers, acetogens, non-methanogenic methylotrophs, and peptide fermenters that can impact the nitrogen, sulfur, and carbon cycles (Offre, Spang et al. 2013, Baker, De Anda et al. 2020). In contrast to methanogens, other archaeal lineages can live as methanotrophs that consume methane (Borrel, Adam et al. 2019, Garcia, Gribaldo et al. 2022). These different lifestyles can aid in reconstructing the complex evolutionary history of metabolism within the Archaea. For example, methanogens are observed in a patchwork distribution between the Thermoproteota and Euryarchaeota lineages, indicating that methanogenesis has a complicated evolutionary history

including vertical inheritance, loss, and horizontal transfer (Borrel, Adam et al. 2019, Wang, Wegener et al. 2021, Garcia, Gribaldo et al. 2022). However, in many cases it is not clear whether these were larger gene transfer events or gradual sets of genetic exchanges, and the identification of intermediate lineages are needed to resolve this. Of importance for this are lineages related to extant methanogens that don't encode the key MCR complex (Borrel, Adam et al. 2019, Adam, Kolyfetis et al. 2022). One highlight of this, important in understanding the evolution of traditional Euryarchaeota methanogens, includes the discovery of the class Hikarchaeia. Hikarchaeia are related to Methanomicrobia (anaerobic methanogens) and Haloarchaea (a diverse class of aerobic halophiles), and provide an example of an intermediate stage in the transition from a common anaerobic methanogen ancestor to an aerobic and heterotrophic lifestyle (Martijn, Schön et al. 2020). Inclusion of Hikarchaeia in modeling gene gain and loss events indicates that this metabolic transition was characterized by a gradual loss of methanogenesis genes and gains of genes involved in aerobic respiration, rather than a single massive gene transfer event that had been proposed in absence of Hikarchaeia (Martijn, Schön et al. 2020). Similar studies will likely be possible for the evolution of the newly discovered Thermoproteota methanogens, as more closely related and non-methanogenic lineages are discovered. Additionally, organisms that contain the potential for methanogenesis (i.e. encode MCR) as well as key enzymes involved in other energy conservation pathways (McKay, Dlakić et al. 2019) are important for understanding transitions away from methanogenic lifestyles as well as the possible co-existence of methanogenesis with other energy conservation pathways. An example of this is the case of the korarchaeon *Candidatus Methanodesulfokora washburnensis* that encodes the MCR complex as well as genes for dissimilatory sulfite

reduction (dsrAB) (McKay, Dlakić et al. 2019). Similarly, it has recently been shown that the Euryarchaeotal class Archaeoglobi also contains a complex history of carbon and sulfur metabolism, with some members encoding dsrAB, some with MCR, and some encoding both dsrAB and MCR (Liu, Chen et al. 2020). Improved cultivation and discovery of more genomes with MCR co-existing with enzymes involved in other energy conservation pathways will help to elucidate large scale metabolic transitions and the wider metabolic diversity of MCR-encoding archaea.

Overview of the Following Chapters

To further expand on the themes of archaeal diversity and metabolism discussed above, we performed research encompassing culture-independent and culture-dependent methods with a focus on methylotrophic archaea. The study sites we focused on are hot springs in Yellowstone National Park that were found to harbor novel methylotrophic archaea (Lynes, Krukenberg et al. 2023). The extreme conditions found in many hot springs in Yellowstone typically lead to reduced complexity in the microbial communities found there, when compared to more moderate environments like soils. This reduced complexity can make them tractable for microbial ecology and cultivation-based studies. In particular, Yellowstone has a rich history in the discovery of novel archaeal lineages including Korarchaeia, Odinararchaeia, Geoarchaeia, and Marsarchaeia, amongst others (Elkins, Podar et al. 2008, Kozubal, Romine et al. 2013, Beam, Jay et al. 2016, Zaremba-Niedzwiedzka, Caceres et al. 2017, Jay, Beam et al. 2018).

Chapter 2 is a description of a new lineage of Archaea from hot springs in Yellowstone, the class Culexarchaeia, which have a diverse metabolism, including the potential for anaerobic methylotrophy. Non-methanogenic methylotrophy is currently not well understood in Archaea,

with only a handful of described examples in the Brockarchaeia (De Anda, Chen et al. 2021) and some Asgard lineages (Farag, Zhao et al. 2021, Sun, Evans et al. 2021, Valentin-Alvarado, Appler et al. 2023). Here we further expanded the diversity of non-methanogenic methylotrophs in the Thermoproteota. Additionally, Culexarchaeia encode numerous novel hydrogenases including those that potentially interact with heterodisulfide reductases, which are typically involved in methane or sulfur metabolisms. Finally, non-methanogenic Culexarchaeia are close relatives with the methanogenic Methanomethylica lineage that is the focus of Chapter 3. Given their close phylogenetic relationship and contrasting lifestyles, the Culexarchaeia aid in demonstrating a transition from a methanogenic to non-methanogenic lifestyle, a process that has played a role in leading to a patchwork distribution of methane metabolism in Archaea.

Chapter 3 reports the cultivation of a methanogen in the Thermoproteota, which is outside of the traditional Euryarchaeota. This organism, *Candidatus Methanosuratincola verstraetei*, is the most divergent methanogen ever cultured to date and provides a crucial experimental validation that methanogenesis extends to the Thermoproteota. To demonstrate this we used metagenomics, metatranscriptomics, stable isotope tracing, and activity assays to definitively show they use methanol and hydrogen to produce methane. Additionally, we used cryo-electron tomography to visualize their ultrastructure. This revealed the presence of archaella and chemotaxis receptors that are important for motility and taxis towards or away from stimuli. We also observed that cells can form intercellular connections between two or three cells, providing a connected cytoplasm. These structures are likely to be important for the ecophysiology (e.g. chemotaxis and intercellular exchange) of this organism within its native hot spring environment and offer clear routes for future studies on the cell biology of this lineage.

Within the Thermoproteota, *Ca. M. verstraetei* belongs to the class Methanomethylica, which has a very widespread environmental distribution. Besides hot springs, members of the Methanomethylica are found in soils, wastewater treatment plants, oil reservoirs, the cow rumen, and freshwater and marine sediments. This suggests that they have played largely overlooked roles in methane production across numerous anoxic habitats.

Chapter 4 reports the cultivation of a second lineage of Thermoproteota methanogens, the Korarchaeia. This organism, *Ca. Methanodesulfokora washburnensis*, was first reported via metagenomics and it was unclear whether it was capable of (i) methylotrophic methanogenesis from methanol, (ii) sulfite reduction coupled to hydrogen oxidation, or (iii) methane oxidation coupled to sulfite reduction (Borrel, Adam et al. 2019, McKay, Dlakić et al. 2019, Wang, Wegener et al. 2019). In this study, we obtained a highly enriched culture from a hot spring in Yellowstone and demonstrated that *M. washburnensis* is capable of methylotrophic methanogenesis from methanol. To do this we combined activity assays, metagenomics, metatranscriptomics, and stable isotope tracing. Together with Chapter 3, this report is a substantial advancement in the characterization of non-traditional and non-model methanogens within the phylum Thermoproteota.

In summary, the work presented in this thesis provides an expansion of archaeal diversity in the description of Culexarchaeia and the first-ever cultivation and experimental verification of Thermoproteota methanogens (Methanomethylica and Korarchaeia).

Other Contributions

In addition to the three first-author chapters in this dissertation, I had the opportunity to contribute as a co-author to several other manuscripts focused on archaeal diversity, activity, and

cultivation. I was also involved in more broad topics involving development of a novel workflow for combining different single-cell analyses and contribution to a study on the mobilization of uranium in groundwater.

(1) “Diversity and function of methyl-coenzyme M reductase encoding archaea in Yellowstone hot springs revealed by metagenomics and mesocosm experiments” published in *ISME Communications*.

Authors: Mackenzie M. Lynes, Viola Krukenberg, Zackary J. Jay, Anthony J. Kohtz, Christine A. Gobrogge, Rachel L. Spietz, and Roland Hatzenpichler.

A.J.K. contributions – aided in processing metagenomic data, phylogenetic analysis of genomes, reconstruction of the metabolic potential, and editing the manuscript.

This study used metagenomics and mesocosm experiments to investigate the methanogenic community of hot springs in the Lower Culex Basin thermal complex Yellowstone National Park (Lynes, Krukenberg et al. 2023). This study highlights that methanogens are widespread in Yellowstone hot springs and that, while they typically represent minor fractions of the total community, when provided with methanogenic substrates in mesocosms they become enriched under methanogenic conditions. In addition to showing that methanogens are widespread in Yellowstone, it also shows that methanogens there are diverse. Notably, *mcrA* amplicons from 10 different archaeal orders across 39 geothermal features and metagenome assembled genomes from 9 different archaeal orders from 3 selected geothermal features.

(2) “Methylotrophic methanogenesis in the Archaeoglobi revealed by cultivation of *Ca.*

Methanoglobus hypatia from a Yellowstone hot spring” published in *The ISME Journal*.

Authors: Mackenzie M. Lynes, Zackary J. Jay, Anthony J. Kohtz, and Roland Hatzenpichler.

A.J.K. contributions - designing experiments, conducting field sampling, interpreting metabolic potential, developing GC/GCMS protocols, processing GCMS samples, and editing the manuscript.

This study focused on the cultivation of *Candidatus Methanoglobus hypatia*, a methylotrophic methanogen in the Euryarchaeota class Archaeoglobi that can produce methane from methylamines (Lynes, Jay et al. 2024). Archaeoglobi were long thought to live non-methanogenic lifestyles including sulfate reduction, nitrate reduction, and non-methanogenic alkane oxidation (Boyd, Jungbluth et al. 2019). Yet recent studies were able to find MCR complexes in genomes of uncultivated members of the Archaeoglobi, and it was further shown for some of these genomes that the methanogenic pathway was expressed in their native environment (Boyd, Jungbluth et al. 2019, Liu, Chen et al. 2020, Wang, Wegener et al. 2019). This study, along with another recent study, report the cultivation of the first members of *Ca. Methanoglobus*, allowing for a direct study to demonstrate the methanogenic capability of this genus and to substantially expand the diversity of cultivated methanogens in the Euryarchaeota (Buessecker, Chadwick et al. 2023, Lynes, Jay et al. 2024).

(3) “Correlative SIP-FISH-Raman-SEM-NanoSIMS links identity, morphology, biochemistry, and physiology of environmental microbes” published in *ISME Communications*.

Authors: George A. Schaible, Anthony J. Kohtz, John Cliff and Roland Hatzenpichler

A.J.K. contributions – designing the study, developing and testing the workflow, performed culture incubations, collected Raman spectra for the mock community, and analyzed data.

This study reports a workflow that allows the combination of stable isotope probing, fluorescence in situ hybridization, scanning electron microscopy, confocal Raman microspectroscopy, and nano-scale secondary ion mass spectrometry on the same set of cells (Schaible, Kohtz, et al. 2022). While these techniques are typically applied on different sets of single cells, use of this workflow allows for connecting the activity, morphology, biochemistry, and physiology to the identification of a single cell. And while this manuscript validated the approach on an artificial mock community and multicellular magnetotactic bacteria living in a salt marsh, this approach could help in probing the ecophysiology of methylophilic archaea in their native environment.

(4) “Nitrate-stimulated release of naturally occurring sedimentary uranium” published in *Environmental Science and Technology*.

Authors: Jeffrey P. Westrop, Pooja Yadav, PJ Nolan, Kate M. Campbell, Rajesh Singh, Sharon E. Bone, Alicia H. Chan, Anthony J. Kohtz, Donald Pan, Olivia Healy, John R. Bargar, Daniel D. Snow and Karrie Weber.

A.J.K. contributions – assisted with the collection and analysis of field samples. All authors contributed extensively to the analysis, interpretation of the data, and preparation of the manuscript.

This study investigated the role that nitrate plays in mobilizing uranium, a groundwater contaminant that is elevated in many aquifers in the United States (Westrop, Yadav et al. 2023).

Nitrate had previously been shown to correlate with uranium levels, but it was not known what interactions led to this. This study comprehensively shows that microbial reduction of nitrate to nitrite leads to the mobilization of uranium in groundwater.

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CHAPTER TWO

CULEXARCHAEIA, A NOVEL ARCHAEAL CLASS OF
ANAEROBIC GENERALISTS INHABITING GEOTHERMAL
ENVIRONMENTS

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Contributions: Designed the research, processed metagenome data, evaluated the MAGs, and performed phylogenomic and 16S rRNA analyses.

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Contributions: provided useful discussion and contributed to assessing metabolic potential.

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Contributions: Designed the research, collected molecular and geochemical samples from YNP hot springs, acquired funding, and wrote the manuscript.

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Abstract

Geothermal environments, including terrestrial hot springs and deep-sea hydrothermal sediments, often contain many poorly understood lineages of archaea. Here, we recovered ten metagenome-assembled genomes (MAGs) from geothermal sediments and propose that they constitute a new archaeal class within the TACK superphylum, “*Candidatus* Culexarchaeia”, named after the Culex Basin in Yellowstone National Park. Culexarchaeia harbor distinct sets of proteins involved in key cellular processes that are either phylogenetically divergent or are absent from other closely related TACK lineages, with a particular divergence in cell division and cytoskeletal proteins. Metabolic reconstruction revealed that Culexarchaeia have the potential to metabolize a wide variety of organic and inorganic substrates. Notably, Culexarchaeia encode a unique modular, membrane associated, and energy conserving [NiFe]-hydrogenase complex that potentially interacts with heterodisulfide reductase (Hdr) subunits. Comparison of this [NiFe]-hydrogenase complex with similar complexes from other archaea suggests that interactions between membrane associated [NiFe]-hydrogenases and Hdr may be more widespread than previously appreciated in both methanogenic and non-methanogenic lifestyles. The analysis of Culexarchaeia further expands our understanding of the phylogenetic and functional diversity of lineages within the TACK superphylum and the ecology, physiology, and evolution of these organisms in extreme environments.

Introduction

In the past two decades, massive efforts in recovering metagenome assembled genomes (MAGs) from environmental samples have resulted in the description of many novel high-

ranking archaeal lineages (Adam, Borrel et al. 2017, Baker, De Anda et al. 2020). The current picture of archaeal diversity comprises four superphyla - the Asgard, DPANN, Euryarchaeota, and TACK archaea - and it is likely that high-ranking lineages (phylum, class, order) are yet to be discovered (Adam, Borrel et al. 2017, Baker, De Anda et al. 2020). Many archaeal phyla, including Nanoarchaeota (Huber, Hohn et al. 2002), Ca. Korarchaeota (Barns, Delwiche et al. 1996), Ca. Geoarchaeota (Kozubal, Romine et al. 2013), Ca. Odinararchaeota (Zaremba-Niedzwiedzka, Caceres et al. 2017), Ca. Marsarchaeota (Jay, Beam et al. 2018), Ca. Nezharchaeota (Wang, Wegener et al. 2019), and Ca. Brockarchaeota (De Anda, Chen et al. 2021), were originally discovered in extreme geothermal habitats. While most of these lineages have no cultured representatives, they are often proposed to play important roles in biogeochemical cycles (Offre, Spang et al. 2013, Baker, De Anda et al. 2020).

In lieu of cultures, many lineages are currently best understood via MAGs, which allow for determining their taxonomic placement, inferring their metabolic potential, and generating hypotheses on their ecophysiology that may lead to subsequent *in situ* studies or eventual cultivation (Adam, Borrel et al. 2017, Spang, Caceres et al. 2017). For example, metagenomics revealed that several lineages within the TACK superphylum encode methyl-coenzyme M reductase (MCR), a hallmark enzyme long-thought to be exclusive to alkane-cycling Euryarchaeota. This suggests that the potential for methanogenesis and alkane oxidation are more widespread within the archaeal domain than previously realized (Evans, Parks et al. 2015, Vanwonterghem, Evans et al. 2016, Borrel, Adam et al. 2019, McKay, Dlakić et al. 2019, Wang, Wegener et al. 2019). Additionally, the potential for anaerobic methylotrophy, a process that in archaea had been solely described in methylotrophic methanogens, was recently detected in

MAGs of non-methanogenic Ca. Brockarchaeota (De Anda, Chen et al. 2021). Considering the large diversity of uncultivated taxa revealed by environmental metagenomics, characterizing new archaeal lineages through MAGs is crucial to understanding the phylogenetic and metabolic diversity that exists among archaea and revealing their potential impacts on biogeochemical cycling and ecosystem functioning (Lloyd, Steen et al. 2018).

Here, using ten MAGs from terrestrial hot spring and deep-sea hydrothermal seep sediments, we report on a new archaeal class, “*Candidatus Culexarchaeia*”. We describe their biogeographic distribution across geothermal systems, evaluate their phylogenetic affiliation through marker gene and functional gene analysis, and reveal genes encoding unique protein complexes and versatile metabolic pathways.

Materials and Methods

Sample Collection, DNA Extraction, and Metagenome Sequencing

Two hot springs located in the Lower Culex Basin (LCB) thermal complex of Yellowstone National Park (YNP) were sampled for molecular and geochemical analyses. At the time of sampling in October 2017, YNP site LCB-003 (44.57763, -110.78957) had a temperature of 72.5 °C and a pH of 6.47, while YNP site LCB-024 (44.57347, -110.79504) had a temperature of 69.4 °C and a pH of 7.79. Surface sediments (~1 cm deep) were collected, immediately frozen on dry ice before transfer to the lab, and subsequently stored at -80°C until further processing. DNA was extracted from ~1 g of sediment using the FastDNA Spin Kit for Soil (MP Biomedicals) according to manufacturer’s instructions. DNA extracts were shotgun sequenced at the Joint Genome Institute (JGI). Truseq libraries were prepared using low input (10 ng for LCB-003) or regular

input (100 ng for LCB-024) quantities of DNA. Libraries were sequenced on an Illumina NovaSeq platform using the NovaSeq XP V1 reagent kits, S4 flow cell, following a 2x150 indexed run recipe.

Metagenome Assembly, Binning, and Quality Assessment

Metagenomic reads for YNP sites LCB-003 and LCB-024 metagenomes were quality filtered according to JGI's analysis pipeline and assembled with SPAdes v3.12.0 (Nurk, Meleshko et al. 2017) with the following settings: -k 33,55,77,99,111 -meta. Assemblies for the Great Boiling Spring (GBS) (Thomas, Tamadonfar et al. 2019), Washburn Hot Springs (WB) (McKay, Dlakić et al. 2019), and Guaymas Basin (GB) samples were downloaded from the IMG/M portal (Chen, Chu et al. 2019). Assembled scaffolds $\geq 2,000$ bp for each metagenome were binned using six different approaches with four different programs, Maxbin v2.2.4 (Wu, Tang et al. 2014), Concoct v1.0.0 (Alneberg, Bjarnason et al. 2014), Metabat v2.12.1 (with and without coverage) (Kang, Li et al. 2019), and Autometa v1 (bacterial and archaeal modes, including the machine learning step) (Miller, Rees et al. 2019). Bins produced from each program were refined with DAS_Tool (Sieber, Probst et al. 2018). In addition, the published MAG JZ-Bin-30, recovered from a metagenome obtained from a geothermal well in the Yunnan province (China) (Hua, Qu et al. 2018, Hua, Wang et al. 2019), was downloaded from the IMG/M portal. CheckM (Parks, Imelfort et al. 2015) was used to estimate MAG completeness, redundancy, and relative abundance. tRNAs were identified with tRNAscan-SE (Chan and Lowe 2019), using the archaeal-specific covariance models. Optimal growth temperature was predicted for each MAG using Tome, which analyzes proteome-wide 2-mer amino acid compositions (Li, Rabe et al. 2019).

Geochemical Analyses

Geochemical data for most sites discussed in this study have been described previously: GBS (Thomas, Tamadonfar et al. 2019), WB (McKay, Dlakić et al. 2019), and Jinze Spring (JZ) (Hua, Qu et al. 2018, Hua, Wang et al. 2019). The Guaymas Basin (GB) metagenomes were limited in available geochemical data, but the temperature range was 53-83 °C near the time of sampling, based on the temperature profiles measured close to the position the sediment core was collected (Supplementary Table 1). For YNP sites LCB-003 and LCB-024 samples for aqueous geochemistry, including dissolved oxygen, dissolved sulfide, dissolved iron, and dissolved gasses (H₂, CH₄, and CO₂) were collected as described previously (Inskeep, Ackerman et al. 2005) and are available in Supplementary File 7.

Phylogenetic Analyses

The 16S rRNA gene sequences encoded in Culexarchaeia MAGs were used in BLASTn searches to screen NCBI and IMG databases for related sequences. Culexarchaeia 16S rRNA gene sequences were aligned against reference archaeal 16S rRNA gene sequences and masked using SSU-ALIGN (Nawrocki, Kolbe et al. 2009), which produced a final alignment of 1,376 positions that was used for phylogenetic analyses. Maximum likelihood analysis was performed using IQtree2 (Minh, Schmidt et al. 2020) v2.0.6 with the nucleic acid model GTR+F+G and 1,000 ultrafast bootstraps. A set of 43 single-copy marker proteins (Supplementary File 1) used in a previous phylogenomic study (Jay, Beam et al. 2018), were collected from Culexarchaeia MAGs and archaeal reference genomes. These markers were aligned with MUSCLE (Edgar 2004), trimmed with trimAL (Capella-Gutiérrez, Silla-Martínez et al. 2009) using a 50% gap threshold, and concatenated. The final trimmed and concatenated alignment of 10,578 positions was used for

phylogenetic analysis. Maximum likelihood analysis was performed using IQ-tree2 (Minh, Schmidt et al. 2020) v2.0.6 with the LG+C60+F+G model and 1,000 ultrafast bootstraps. Additionally, a set of 46 ribosomal proteins (listed in Supplementary File 1) found in *Culexarchaeia* MAGs and archaeal reference genomes, was used to construct a second maximum likelihood tree (Supplementary Figure 1). Sequences were aligned with MUSCLE, trimmed with trimAL using a 50% gap threshold, and concatenated. The final trimmed and concatenated alignment of 7,178 positions was used for maximum likelihood tree reconstruction using IQ-tree2 v2.0.6, the LG+C60+F+G model, and 1,000 ultrafast bootstraps.

Catalytic subunits of group 4 [NiFe]-hydrogenases encoded in *Culexarchaeia* MAGs were subjected to phylogenetic analysis along with a set of reference sequences extracted from the HydDB (Søndergaard, Pedersen et al. 2016). Amino acid sequences were aligned using Mafft-LINSi and trimmed using trimAL with a gap threshold of 50%, producing a final alignment of 370 residues. A maximum-likelihood tree was reconstructed using IQ-tree2 v2.0.6 with a best fit model LG+R9 selected according to Bayesian information criterion (BIC), 1,000 ultrafast bootstraps, and option -bnni.

Reference amino acid sequences from within the tubulin superfamily (Makarova and Koonin 2010, Duggin, Aylett et al. 2015, Liao, Ithurbide et al. 2021) were downloaded from NCBI (accessed May 2021), combined with sequences obtained from the *Culexarchaeia* MAGs aligned with Mafft-LINSi, and trimmed using trimAL with a gap threshold of 70%. This produced a final alignment of 313 residues. IQ-tree2 v2.0.6 was used for maximum-likelihood analysis with a best-fit model LG+R5 according to BIC and node support was calculated with 1,000 ultrafast bootstraps.

Annotation and Reconstruction of Metabolic Potential

Initial analysis of the metabolic potential of the *Culexarchaeia* MAGs was performed using the annotations provided by the IMG/M database which uses KEGG, COG, pfam, and enzyme ID databases (Chen, Chu et al. 2019). Manual refinement of the IMG/M annotations was done by inspection of gene neighborhoods, and identification of conserved domains and motifs through submission of genes to the NCBI conserved domain database (Lu, Wang et al. 2020) and Interproscan (v5.48) (Jones, Binns et al. 2014). Catalytic subunits of [NiFe]-hydrogenases (COG3261, COG3262, and COG3529) were submitted to the HydDB web portal for classification (Søndergaard, Pedersen et al. 2016). Putative transmembrane spanning subunits of novel membrane-bound [NiFe]-hydrogenases (Drh, Ehd, Ehe and Ehg complexes) were predicted with TMHMM (Krogh, Larsson et al. 2001). Supplementary File 3 includes a full list of genes used to construct Figure 3, including the associated KEGG, COG, pfam, Enzyme IDs, and IMG locus tags.

Assessment of Key Cellular Machinery Genes and Methanogenesis Marker Proteins

The *Culexarchaeia* MAGs and reference genomes from *Marsarchaeia*, *Methanomethylica*, *Geoarchaeia*, *Nezhaarchaeia*, *Sulfolobales*, *Desulfurococcales*, and *Thermoproteales* were screened for the presence and absence of key cellular machinery proteins, identified in previous work (Spang, Hatzenpichler et al. 2010, Brochier-Armanet, Forterre et al. 2011, Guy and Ettema 2011) using arCOG HMMs (Makarova, Wolf et al. 2015) and HMMER v3.2 (Eddy 2011). A set of previously identified methanogenesis marker proteins (Borrel, Adam et al. 2019) were also assessed using arCOGs and HMMER (v3.2) as previously described (Borrel, Adam et al. 2019). Following the HMMsearch against *Culexarchaeia* MAGs and

reference archaeal genomes, putative hits (E-value threshold of $1e^{-3}$) for each arCOG were manually inspected through BLASTp searches (default settings) against the NCBI non-redundant database and submitted to the NCBI Conserved Domain Database (Lu, Wang et al. 2020). Supplementary File 2 provides the data used to construct Figure 1C and contains the arCOG identifiers used in this analysis along with the presence-absence pattern for each genome.

Results and Discussion

In this manuscript, we largely refer to MAGs using the recently proposed archaeal Genome Taxonomy Database (GTDB, Release 202) nomenclature (Rinke, Chuvochina et al. 2021). However, in the interest of being more accessible in the context of previous work and well-established names in the literature, we also list conventional high-ranking superphylum names (Asgard, DPANN, Euryarchaeota, TACK). Parentheses indicate taxa that have undergone significant name changes in the GTDB, but are used interchangeably here (e.g., Ca. Methanomethylicia (Ca. Verstraetearchaeota); Thermoproteia (Crenarchaeota)). For simplicity we avoid prefixing candidate taxa with the term “Ca.” after they have been introduced.

Recovery of MAGs

Metagenomic analysis of microbial communities from three hot springs sediment samples from Yellowstone National Park (WY, USA), one sediment sample from Great Boiling Spring (NV, USA) (Thomas, Tamadonfar et al. 2019), and two deep-sea hydrothermal seep sediment samples from Guaymas Basin (Gulf of California) resulted in the recovery of nine MAGs representative of a new archaeal lineage (Table 1). Additionally, a single MAG (JZ-Bin-30), originally recovered from Jinze Hot Spring (Yunnan, China) (Hua, Qu et al. 2018, Hua, Wang et

al. 2019), that was related to these newly retrieved MAGs, was identified in the Integrated Microbial Genomes (IMG) database. Completeness estimates for the ten MAGs ranged from 89.2-99.0 % and their redundancy ranged from 0-7.79 %. Most of these MAGs contain scaffolds >100 kbp; notably, YNP-LCB-24-027 and GB-1867-005 contain a 1.08 Mbp and 0.84 Mbp scaffold, respectively (Table 1).

Phylogenetic Placement

Phylogenomic analyses using a concatenated alignment of 43 conserved single copy marker genes (Supplementary File 1) placed the ten MAGs as a monophyletic sister clade to the *Methanomethylica* (Verstraetearchaeota) with 100 % bootstrap support (Figure 1A, Supplementary Figure 1). 16S rRNA gene phylogeny further supported placement as a distinct monophyletic clade within the TACK superphylum (100 % bootstrap support; Figure 1B). The average amino acid identity (AAI) values of this lineage compared to other TACK lineages were below 50%, which is comparable to AAI values recovered when comparing *Methanomethylica* to other TACK lineages (Supplementary Figure 2A).

Together, these results support the designation of these MAGs as representatives of a distinct archaeal lineage, for which we propose the name “*Candidatus Culexarchaeia*”, after the Culex Basin in Yellowstone National Park. When using the rank-normalized GTDB taxonomy, the phylogenies and AAI comparisons presented here indicate that these MAGs constitute a class-level lineage, *Culexarchaeia*, separate from *Methanomethylica* (Verstraetearchaeota). Alternatively, when using NCBI taxonomy, these MAGs could constitute a phylum-level lineage, “*Candidatus Culexarchaeota*”, separate from Verstraetearchaeota (*Methanomethylica*). We support the development of a standardized archaeal taxonomy and from here on refer to this

lineage as the class Culexarchaeia. The JZ-Bin-30 MAG identified on IMG was previously proposed to represent a novel species, “Ca. Methanomedium jinzeense”, within the order Methanomethylicia (Verstraetearchaeota) (Berghuis, Yu et al. 2019). However, based on our expanded phylogenetic analysis, we propose to reclassify this MAG as “Ca. Culexarchaeum jinzeense” within the Culexarchaeia. Based on phylogenomic analyses, AAI, average nucleotide identity, and pairwise 16S rRNA nucleotide sequence identity analyses, Culexarchaeia can be separated into two families, Culexarchaeaceae and Culexmicrobiaceae, that are currently composed exclusively of terrestrial and marine representatives, respectively (Figure 1A, Supplementary Figure 2B and 2C).

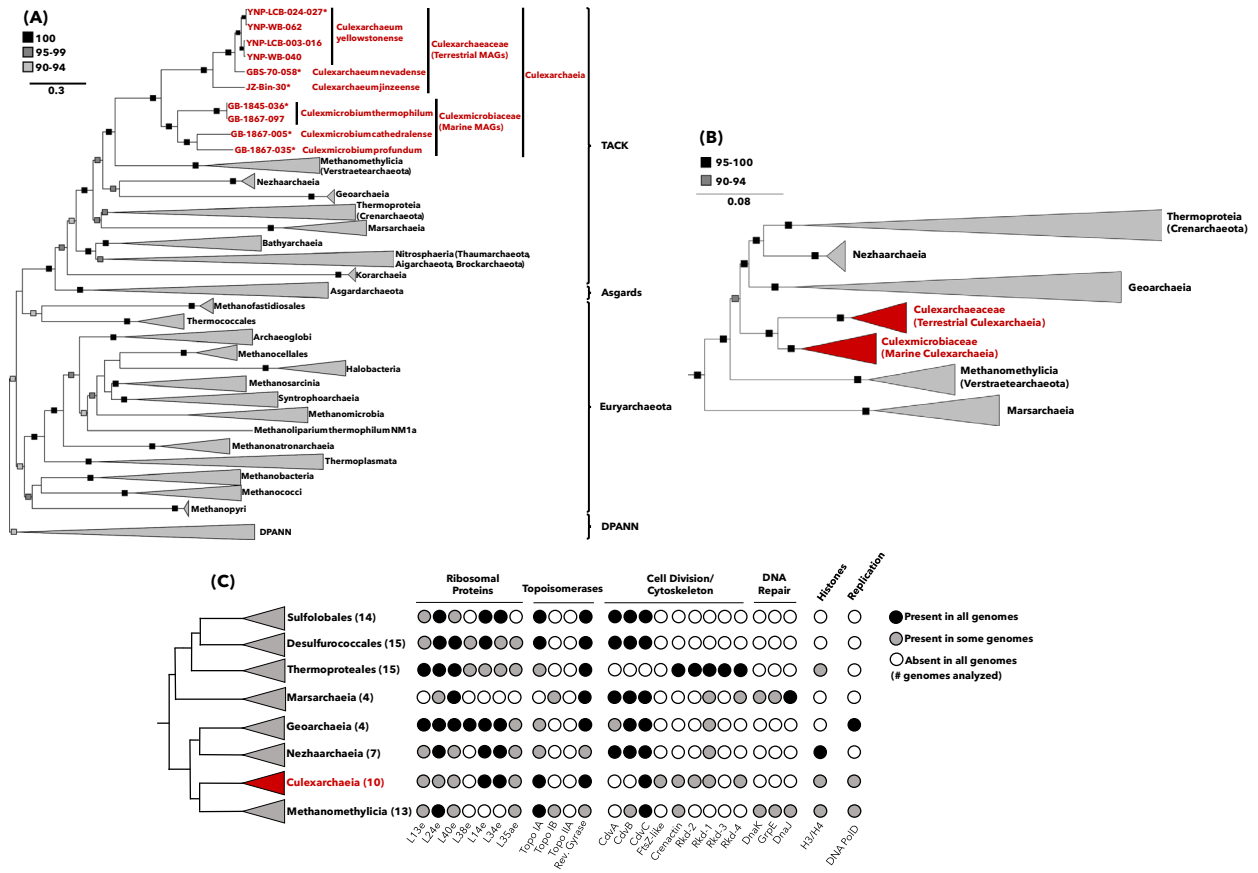


Figure 1 Phylogenetic analysis of Culexarchaeia MAGs and comparison of gene patterns involved in central information-processing machinery. (A) Maximum-likelihood tree, inferred with IQtree and the best-fit LG+C60+F+G model, using a concatenated set of 43 conserved arCOGs (Supplementary File 1). Ultrafast bootstrap support values of 100, 95-99, and 90-94 are indicated. *, indicates MAG ID used to designate type species. (B) Maximum-likelihood tree, inferred with IQtree and best-fit GTR+F+G model, using 16S rRNA gene sequences longer than 1000 bp. Ultrafast bootstrap support values of 95-100 and 90-94 are indicated. (C) Comparison of presence-absence patterns for genes involved in central information-processing machinery among TACK lineages related to Ca. Culexarchaeia. Genes found in all genomes analyzed, some genomes analyzed, and absent from all genomes analyzed are indicated with black, gray, and white circles, respectively. The number of genomes screened in each clade are indicated in parentheses, and a full list of the presence-absence pattern for individual genomes can be found in Supplementary File 2.

Biogeography

To explore the biogeographical distribution of Culexarchaeia, BLASTn searches were used to screen IMG and NCBI non-redundant databases for related 16S rRNA genes

(Supplementary File 8). This revealed that *Culexarchaeia* are globally distributed and are found in circumneutral and slightly acidic pH (5.4-7.8) high temperature (53-83 °C) terrestrial hot spring and deep-sea hydrothermal sediment environments (Figure 2). A small number of 16S rRNA gene sequences were retrieved from deep-sea hydrothermal seep sediment samples in Guaymas Basin with lower temperatures (15 °C; Supplementary File 8). However, the Guaymas Basin hydrothermal system experiences rapidly fluctuating temperature gradients (up to 100 °C over 50 cm depth) as hydrothermal waters percolate through the sediment (Teske, De Beer et al. 2016) (Supplementary Table 2). Consistent with the presence of *Culexarchaeia* in geothermal environments, the predicted optimal growth temperature based on amino acid composition (Li, Rabe et al. 2019) is above 80 °C for all MAGs with an average of 85 °C (Table 1). Furthermore, all MAGs encode the hyperthermophile-specific enzyme reverse gyrase (Supplementary File 2) that is important for growth at high temperatures (Forterre 2002, Lipscomb, Hahn et al. 2017).

We attempted to visualize *Culexarchaeia* cells in cell extracts from sediment slurries of YNP sites LCB-003 and LCB-024 via catalyzed reporter deposition fluorescence in situ hybridization (CARD-FISH) using a newly designed oligonucleotide probe but were unsuccessful. The low relative abundance of *Culexarchaeia* in these sites (0.52% and 0.16% abundance, respectively; Table 1) may have contributed to our inability to detect them. Alternatively, the failure to detect *Culexarchaeia* cells could be due to suboptimal probe accessibility, an inability to penetrate the archaeal cell wall under the permeabilization conditions tested, or low ribosome content of relatively inactive cells.

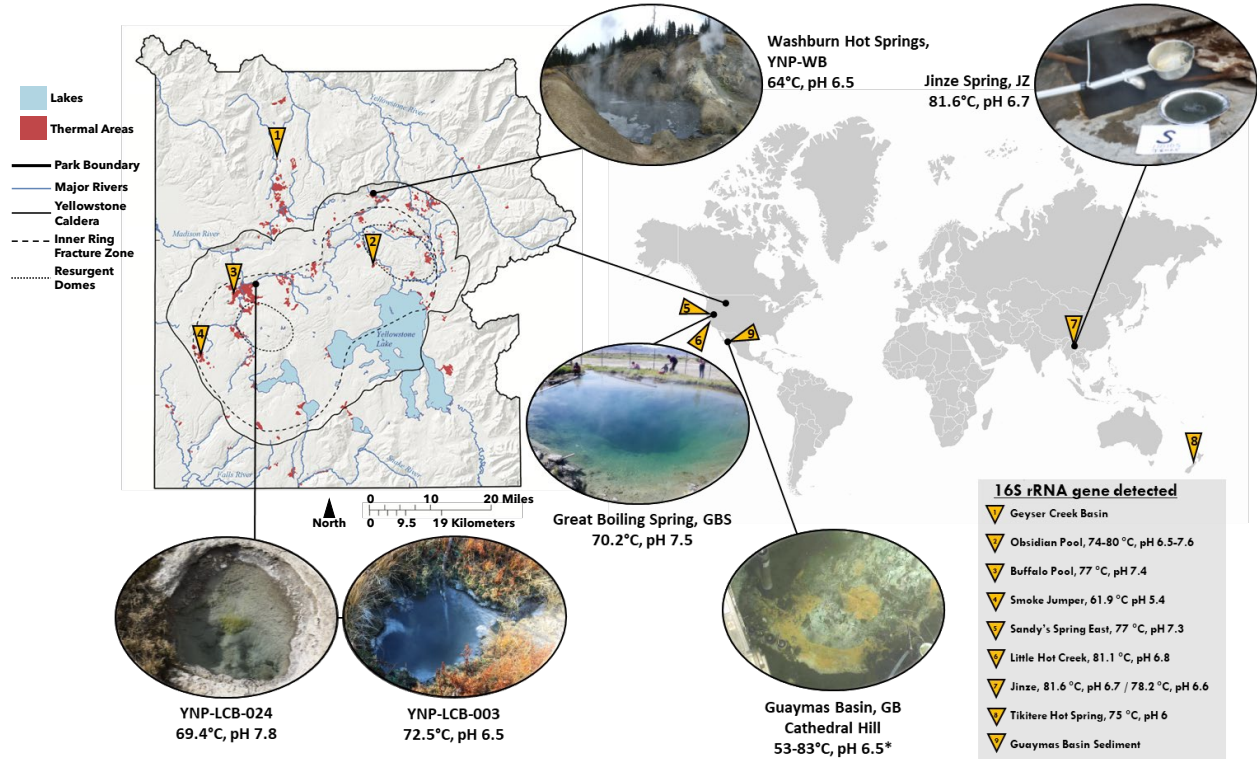


Figure 2 Biogeography of *Culexarchaeia*. Sites from where *Culexarchaeia* MAGs were recovered and where 16S rRNA gene sequences have been detected. A full list of samples and associated metadata can be found in Supplementary File 8. Yellowstone map modified from (Vaughan, Heasler et al. 2014), GBS picture modified from (Thomas, Tamadonfar et al. 2019), and JZ picture modified from (Hua, Qu et al. 2018). Guaymas Basin image courtesy of the Woods Hole Oceanographic Institution.

Comparison of Key Cellular Machinery Proteins Encoded by *Culexarchaeia*

To better understand the differences between *Culexarchaeia* and their closest relatives within the TACK superphylum, we surveyed publicly available genomes within the Geoarchaeia, Marsarchaeia, Methanomethylicia, Nezharchaeia, and Thermoproteia (Crenarchaeota), for the presence or absence of proteins involved in key cellular processes (Figure 1C). These marker proteins have been used in previous studies to delineate differences between high-ranking archaeal taxa (Spang, Hatzepichler et al. 2010, Brochier-Armanet, Forterre et al. 2011, Guy and

Ettema 2011) and include archaeal-specific ribosomal proteins, topoisomerases, histones (H3/H4), proteins involved in DNA replication (DNA PolD), DNA repair, cell division, and cytoskeletal proteins. This analysis revealed a lineage-specific presence-absence pattern for Culexarchaeia that differentiates them from other TACK lineages (Figure 1C). In particular, a unique set of cell division and cytoskeletal proteins was encoded in Culexarchaeia MAGs.

Culexarchaeia Encode a Unique Array of Cell Division and Cytoskeletal Proteins

Many lineages in the TACK superphylum (Bathyarchaeia, Desulfurococcales, Geoarchaeia, Marsarchaeia, Nezharchaeia, Nitrososphaeria (Thaumarchaeota), and Sulfolobales) encode a full complement of the ESCRT-III related cell division proteins (Pende, Sogues et al. 2021) (CdvABC; Figure 1C). Methanomethylica, the closest relative to Culexarchaeia, has previously been noted to only encode CdvB and CdvC. This is consistent with our expanded analysis of recently discovered Methanomethylica representatives (Adam, Borrel et al. 2017, Hua, Wang et al. 2019, Wang, Wegener et al. 2019). In contrast, all Culexarchaeia MAGs only encoded CdvC homologs, which suggests Culexarchaeia may use an alternative mechanism for cell division.

In bacteria, the highly conserved FtsZ tubulin protein mediates the formation of a dynamic division ring during cell division (Liao, Ithurbide et al. 2021). Many archaea (including most members of the Euryarchaeota, DPANN, and Asgard, along with a small number of TACK lineages) also encode homologs of the FtsZ tubulin protein, which is critical for cell division (Liao, Ithurbide et al. 2021). Divergent FtsZ homologs encoded by *Haloferax volcanii*, later termed CetZ, were found to not be directly involved with cell division, but instead have roles in maintaining cell shape and motility, which highlights the functional diversity present in this

family of proteins (Duggin, Aylett et al. 2015). Previously, FtsZ homologs had only been noted in other more distantly related TACK lineages including Bathyarchaeia, Caldarchaeles (Aigarchaeota), Korarchaeia, and Nitrososphaeria (Thaumarchaeota) (Makarova, Yutin et al. 2010, Pende, Sogues et al. 2021). Most of these TACK lineages each encode at least one homolog that clusters with canonical FtsZ1 and/or FtsZ2 from other archaea, which suggests that these proteins likely play a similar role in cell division. Furthermore, Korarchaeia, Bathyarchaeia, and Nitrososphaeria (Thaumarchaeota) also encode non-canonical FtsZ-like sequences that have a yet undetermined role in cellular division or cellular shape maintenance (Liao, Ithurbide et al. 2021) (Supplementary Figure 3).

Members of the Culexarchaeia were found to encode a homolog of FtsZ, a protein that is absent in all closely related TACK lineages with the exception of two Sulfolobales representatives, which have previously been noted to encode highly divergent and extended proteins belonging to a cluster termed FtsZL1 (Makarova and Koonin 2010) (Figure 1C, Supplementary Figure 3). Compared to other FtsZ clades, the Culexarchaeia FtsZ-like sequences form a new, deeply branching clade that is distinct from other known tubulin family homologs and branches between the archaeal FtsZ2 group and CetZ group (Supplementary Figure 3). Culexarchaeia FtsZ-like sequences cluster with other uncharacterized sequences from the Thermoplasmata, Bathyarchaeia, Geothermarchaeales, and unclassified Thermoproteia. This novel clade of FtsZ-like proteins represented by Culexarchaeia sequences will require experimental characterization to determine its role in cellular division, cellular shape maintenance, or motility.

In contrast to the Cdv and FtsZ mediated cell division strategies, the cell division and cytoskeletal proteins encoded by the Thermoproteales have long been a curiosity because, unlike closely related orders within the Thermoproteia (Crenarchaeota), they do not encode homologs of the CdvABC or FtsZ proteins (Ettema, Lindås et al. 2011). Instead, cell division and cellular organization in the Thermoproteales have been proposed to rely on homologs of actin (termed crenactin) and several conserved actin-related cytoskeleton proteins (termed arcadins) (Ettema, Lindås et al. 2011) (Figure 1C). Based on immunostaining of *Pyrobaculum caldifontis*, arcadin-1, arcadin-3, and arcadin-4 were suggested to play auxiliary roles in cell division and cellular shape maintenance, while arcadin-2 was localized in the center of cells between segregated nucleoids, which suggests that it may play a more direct role in cytokinesis (Ettema, Lindås et al. 2011). *Culexarchaeia* MAGs recovered from marine environments contained a similar set of proteins (encoding for crenactin, arcadin-1, arcadin-2, and arcadin-4). In contrast, the terrestrial *Culexarchaeia* MAGs only encoded arcadin-1, but not crenactin (Supplementary File 2). A few mesophilic members within the Methanomethylica order Methanomethylicaes also encode crenactin, while all other Methanomethylica MAGs analyzed did not encode crenactin (Supplementary File 2). In addition, no arcadin-related proteins were identified in our arCOG survey of Methanomethylica MAGs. A BLASTp search against the non-redundant database using default settings and the *Culexarchaeia* crenactin sequences as queries revealed that these sequences are more similar to those found in the TACK (e.g., Methanomethylica and Caldarchaeles) than to those in the Asgard archaea, which are more similar to eukaryotic actin sequences (Stairs and Ettema 2020).

The set of cell division, cell shape, and cytoskeleton proteins encoded by the *Culexarchaeia* suggests that they have a unique cell division and maintenance process. Similarly, members of the *Caldarchaeales* (*Aigarchaeota*) have been noted to encode a unique complement of cell division (FtsZ2, CdvBC) and cytoskeletal proteins (Crenactin, arcadin-1, arcadin-2) (Bernander, Lind et al. 2011). Likewise, some Asgard archaea (e.g., *Heimdallarchaeota*, *Odinarchaeota*) have been found to encode a mixture of proteins possibly involved in cell division machinery (CdvBC in addition to FtsZ1 and/or FtsZ2) and actin-like sequences that are closely related to eukaryotic homologs (Adam, Borrel et al. 2017, Pende, Sogues et al. 2021). It is yet unknown if, or how, these diverse systems interact with each other during the cell cycle (Bernander, Lind et al. 2011, Brochier-Armanet, Forterre et al. 2011). All together, these data suggest that *Culexarchaeia*, along with other TACK and Asgard lineages, are critical to understanding the diversity and evolution of cell division and cytoskeletal proteins within the Archaea.

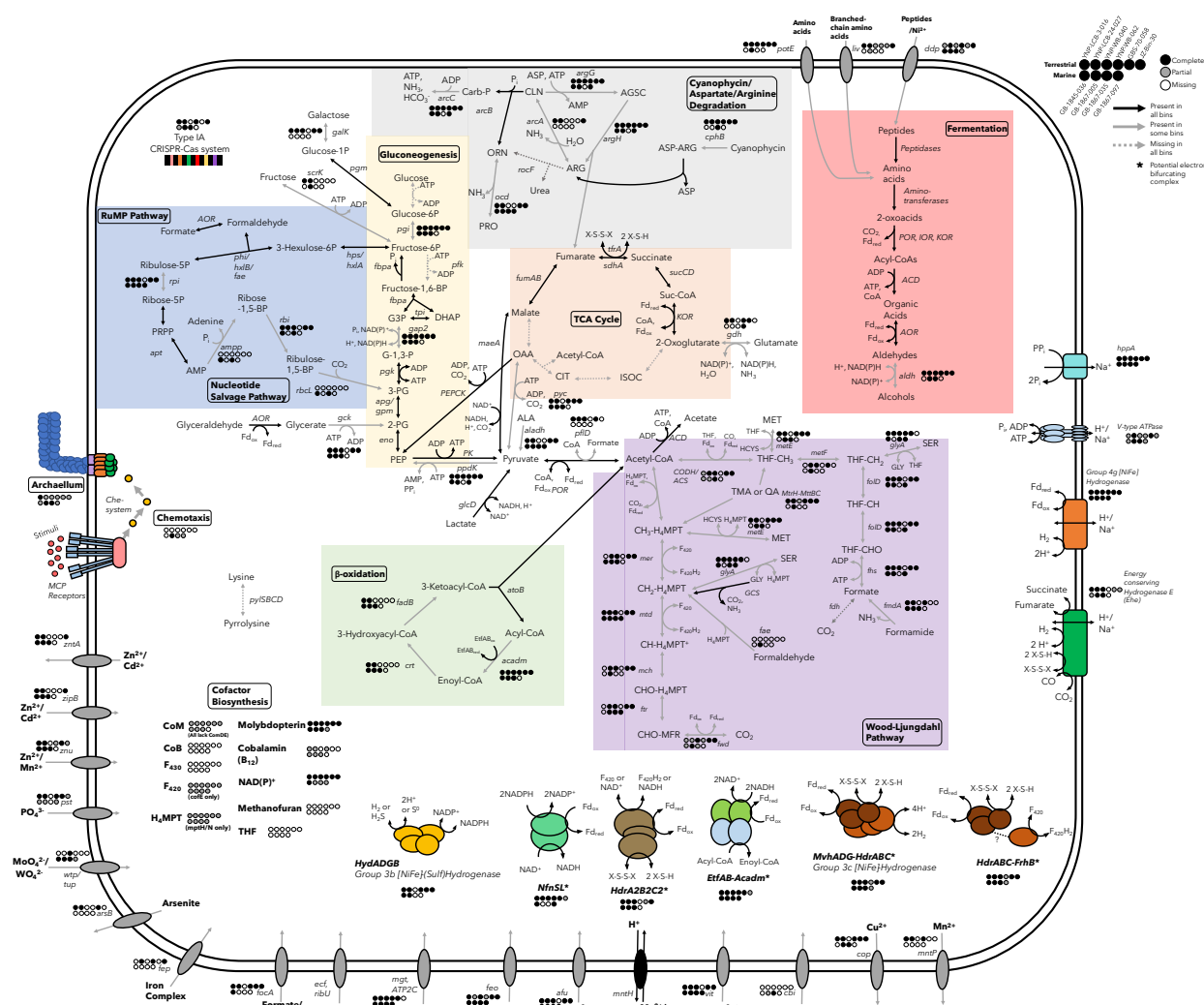


Figure 3 Metabolic potential of *Culexarchaea* MAGs. Solid black arrows indicate presence in all bins, solid gray arrows indicate presence in some bins, and dashed gray arrows indicate absence in all bins. For genes encoding multi-subunit complexes and genes present only in some bins (solid gray arrows) - full presence, partial presence, and absence are shown with solid black circles, solid gray circles, and solid white circles respectively. A full list of genes used to construct this figure, gene IDs for each bin, and abbreviations can be found in Supplementary Files 3 and 4. Enzyme complexes with unclear annotation or association are indicated with '(?)'. Asterisks (*) indicate potential electron-bifurcating complexes.

Metabolic Potential of Culexarchaeia

Culexarchaeia potentially live a generalist lifestyle, with the ability to metabolize a variety of substrates including proteins, amino acids, fatty acids, sugars, and methyl compounds (Figure 3; Supplementary File 3).

Peptide Degradation and Fermentation

Culexarchaeia encode peptide and amino acid transporters that can import exogenous peptides or amino acids that can be catabolized to their respective 2-oxoacids via encoded peptidases and amino-transferases (Kelly and Adams 1994, Musfeldt and Schönheit 2002). Ferredoxin oxidoreductases (POR, IOR, KOR) can then convert these 2-oxoacids to their respective Acyl-CoA substrate and reduce a low-potential ferredoxin (Ma, Hutchins et al. 1997, Musfeldt and Schönheit 2002). Culexarchaeia encode multiple Acyl-CoA synthetases that can convert the Acyl-CoA substrates to organic acids and generate ATP via substrate level phosphorylation (Ma, Hutchins et al. 1997, Musfeldt and Schönheit 2002). Culexarchaeia also encode multiple aldehyde-ferredoxin oxidoreductases (AOR) and alcohol dehydrogenases that could act to interconvert organic acids to aldehydes and aldehydes to alcohols, respectively (Ma, Hutchins et al. 1997, van den Ban, Willemsen et al. 1999, Nissen and Basen 2019). This would remove excess reducing equivalents (NAD(P)H and/or reduced ferredoxin) produced during fermentation. Alternatively, or additionally, reduced ferredoxins produced by ferredoxin oxidoreductases could also be oxidized by a membrane bound group 4g [NiFe]-hydrogenase concomitantly with H₂ production and the transport of ions outside of the cell (Figure 3). This scenario would be akin to the metabolism of *Pyrococcus furiosus*, which uses a membrane bound hydrogenase and ATP synthase to conserve energy during hydrogenogenic fermentation (Sapra,

Bagramyan et al. 2003). Additional fermentative capacity is possible through the conversion of pyruvate to lactate or alanine through the activity of lactate dehydrogenase (GlcD) or alanine dehydrogenase (Aladh), respectively.

Cyanophycin Degradation

All terrestrial MAGs and one marine MAG encode cyanophycinase, a peptidase capable of degrading cyanophycin, a carbon and nitrogen storage polymer (Richter, Hejazi et al. 1999). Cyanophycin is commonly found in cyanobacteria and some heterotrophic bacteria and is composed of repeated aspartate and arginine dipeptides (Füser and Steinbüchel 2007). After degrading cyanophycin polymers, *Culexarchaeia* could further metabolize arginine via the arginine deiminase pathway (Maghnouj, de Sousa Cabral et al. 1998) (*arcA*, *arcB*, *arcC*; Figure 3) to generate ATP, while aspartate could be used in central metabolism via aspartate aminotransferase, converting it to the TCA intermediate oxaloacetate. To the best of our knowledge, cyanophycin degradation by archaea has had extremely limited discussion in the literature and no experimental evidence of archaeal cyanophycin degradation has yet been obtained. Additionally, BLASTp searches against the non-redundant NCBI database with default settings using the *Culexarchaeia* cyanophycinase as a query suggests that a small assortment of Euryarchaea and Asgardarchaea also encode potential cyanophycinase homologs and that the role of archaea in cyanophycin cycling is currently underappreciated (Wong, White et al. 2018). Given the apparent limited capacity for other archaea to potentially degrade this substrate, we speculate that this substrate could be used in combination with antibiotics to aid in isolating *Culexarchaeia*, because cyanophycin could act as a sole carbon, nitrogen, and energy source.

Wood-Ljungdahl Pathway and Methyлотrophy

Culexarchaeia encode portions of both the tetrahydrofolate (THF) and tetrahydromethanopterin (H4MPT) methyl branches of the Wood-Ljungdahl pathway (WLP) (Figure 3). None of the 38 marker genes previously identified in methanogens, including methyl coenzyme-M reductase (MCR) and the biosynthesis genes for the MCR cofactor F430 (Borrel, Adam et al. 2019), were identified in any Culexarchaeia MAGs (Supplementary File 6). Furthermore, Culexarchaeia only encode the MtrH subunit of the tetrahydromethanopterin S-methyltransferase (MTR) complex that is involved in the transfer of a methyl group to Coenzyme-M, which forms methyl-CoM during hydrogenotrophic or methyлотrophic methanogenesis (Wagner, Ermler et al. 2016). These mtrH genes were co-located with genes encoding for trimethylamine methyltransferases (mttB; COG5598), corrinoid proteins (COG5012), and a corrinoid activation protein (COG3984) (Supplementary Figure 4). It was recently shown that non-pyrrolysine members of the MttB superfamily, which exhibit similar co-localization of genes (mtrH-mttB-mttC), are involved in the demethylation and degradation of quaternary amines such as glycine betaine or L-carnitine (Ticak, Kountz et al. 2014, Kountz, Behrman et al. 2020). Culexarchaeia lack the genes necessary for biosynthesis of pyrrolysine (pylSBCD), which supports the hypothesis that this complex could be involved in the catabolism of quaternary amine compounds or yet unknown methylated substrates. Methyl groups transferred by methyltransferase complexes could potentially enter either the THF or H4MPT branches of the WLP, producing THF-CH₃ or CH₃-H4MPT, respectively (Figure 3). Furthermore, Culexarchaeia may compete for methylated compounds with other methyлотrophic

organisms found in geothermal environments, including methylotrophic methanogens, methylotrophic bacteria, or the recently identified Brockarchaeota (De Anda, Chen et al. 2021).

Together, these findings suggest that Culexarchaeia are incapable of methanogenesis, and that the WLP would confer metabolic flexibility by acting as a major hub for the entry and exit of multiple substrates. Specifically, if operating in the reductive direction, acetogenesis could occur from CO₂, formate, or methylated compounds (potentially trimethylamine or quaternary amines) (Figure 3). Formate could be produced through the activity of pyruvate-formate lyase (PflD), formamidase (FmdA), or through interconversion of formaldehyde and formate by aldehyde-ferredoxin oxidoreductases (AOR; Figure 3). Alternatively, the WLP could be used in the oxidative direction to oxidize Acetyl-CoA or methylated compounds to CO₂ or formate. Culexarchaeia may also use the WLP for protein degradation because they encode serine hydroxymethyltransferase, which can produce a methylene-H₄MPT and glycine. The glycine cleavage system (GCS) could then convert glycine to methylene-H₄MPT, CO₂, and NH₃ (Adam, Borrel et al. 2019) (Figure 3).

Beta-Oxidation

Culexarchaeia encode genes for the metabolism of fatty acids through beta-oxidation. This includes medium chain Acyl-CoA dehydrogenases (Acadm), electron transfer flavoproteins (EtfAB), Enoyl-CoA hydratase (Crt), 3-hydroxybutyryl-CoA dehydrogenase (FadB), and Acetyl-CoA C-acetyltransferase (AtoB) (Dibrova, Galperin et al. 2014). Acetyl-CoA produced by this pathway could then be metabolized by oxidation through the WLP or by Acyl-CoA synthetases to produce ATP through substrate level phosphorylation (Figure 3). Additionally, electron transfer flavoproteins and Acyl-CoA dehydrogenase may form an electron-bifurcating complex

(EtfAB-Acadm), as was previously suggested for fatty acid-degrading Firmicutes (Garcia Costas, Poudel et al. 2017).

Carboxydotrophy

Culexarchaeia MAGs encode putative aerobic carbon-monoxide dehydrogenases (CoxLMS), which suggests a capacity for carboxydotrophy through this enzyme complex. Culexarchaeia also encode genes involved in molybdopterin cofactor biosynthesis (Figure 3), which could be involved with such a molybdoenzyme. However, consistent with an analysis of Cox proteins in Caldarchaeles (Aigarchaeota) and a broader analysis of form I Cox proteins in bacteria and archaea, all Culexarchaeia CoxL homologs lack conserved active site residues (VAYRCSFR) found in other characterized Cox proteins and do not cluster with other characterized form I Cox proteins. Together, this suggests these enzymes may use an alternative substrate (Dobbek, Gremer et al. 2002, Beam, Jay et al. 2016, Cordero, Bayly et al. 2019). However, carboxydotrophic potential in Culexarchaeia may exist via a [NiFe]-hydrogenase complex that interacts with carbon monoxide dehydrogenase subunit A (CdhA; discussed below).

Gluconeogenesis, RuMP Pathway, TCA Cycle, and Nucleotide Salvage Pathway

Culexarchaeia encode important enzymes involved in gluconeogenesis, including phosphoenolpyruvate carboxykinase (PEPCK) and pyruvate:phosphate dikinase (PpdK) for the production of phosphoenolpyruvate (PEP) from oxaloacetate and pyruvate, respectively (Bräsen, Esser et al. 2014). While they lack a gene for phosphofructokinase, all MAGs encode fructose biphosphate aldolase/phosphatase (Fbpa) (Say and Fuchs 2010). Together, this suggests Culexarchaeia are capable of gluconeogenesis (Bräsen, Esser et al. 2014).

Culexarchaeia have the potential to metabolize a variety of sugars and aldehydes. Several MAGs encode the proteins necessary for using fructose or galactose, which could be converted to fructose-6-phosphate for entry into the ribulose monophosphate (RuMP) pathway (Figure 3). The RuMP pathway could be used for formaldehyde assimilation/detoxification or biosynthesis of nucleotides (Kato, Yurimoto et al. 2006). Alternatively, formaldehyde produced by 6-phospho-3-hexuloisomerase (HxlB) could be converted to formate via an aldehyde:ferredoxin oxidoreductase and then enter the THF branch of the WLP via formate-tetrahydrofolate ligase (Fhs). A single marine MAG, GB-1867-005, encoded a formaldehyde-activating enzyme (Fae) that could incorporate formaldehyde into the H4MPT branch of the WLP. Glyceraldehyde may be scavenged through conversion to glycerate by an aldehyde-ferredoxin oxidoreductases and glycerate could subsequently be converted to 2-phosphoglycerate by glycerate kinase (Gck) (Figure 3).

Culexarchaeia encode an incomplete tricarboxylic acid (TCA) cycle that may function as a hub for protein fermentation and conversion of TCA intermediates (malate and oxaloacetate) to pyruvate for gluconeogenesis or conversion to Acetyl-CoA (Figure 3). Peptide degradation could produce 2-oxoglutarate and glutamate through the activity of aminotransferases and peptidases, respectively. Then, glutamate dehydrogenase (Gdh) could facilitate the interconversion of these substrates. 2-oxoglutarate could be metabolized to produce reduced ferredoxin and succinyl-CoA via 2-oxoglutarate:ferredoxin oxidoreductase (KOR). Succinyl-CoA could then be stepwise converted to succinate, fumarate, and malate through the activities of succinyl-CoA synthetase (SucCD), fumarate reductase (TfrA), and fumarate hydratase (FumAB), respectively (Figure 3). Malate could be converted to pyruvate by the decarboxylating malic enzyme (MaeA) and enter

gluconeogenesis through the conversion to PEP by pyruvate:phosphate dikinase (Ppdk). Similar to *Methanomethylica*, two marine MAGs encode a full nucleotide salvage pathway that includes Ribulose 1,5-bisphosphate carboxylase (RbcL) for conversion of adenosine monophosphate (AMP) to 3-phosphoglycerate (3-PG), which could be metabolized to acetyl-CoA (Vanwonterghem, Evans et al. 2016).

Extensive Potential for H₂-Cycling and Many Potential Electron Bifurcating Complexes

The large number of hydrogenases and putative electron-bifurcating complexes encoded by *Culexarchaeia* MAGs suggest that these enzymes are important in their metabolism. Flavin-based electron bifurcation involves the coupling of an exergonic redox reaction to drive an endergonic redox reaction (Müller, Chowdhury et al. 2018). Electron bifurcation is important in balancing redox carriers within the cell and increasing efficiency of cell metabolism, particularly for anaerobic organisms that grow on low energy-yielding substrates (Buckel and Thauer 2018, Poudel, Dunham et al. 2018). *Culexarchaeia* encode five different potential electron bifurcating complexes, which places them among a small number of genomes found to encode more than three bifurcating complexes. These enzymes may provide advantages in energy efficiency and competition over low energy-yielding substrates in situ (Buckel and Thauer 2018, Müller, Chowdhury et al. 2018, Poudel, Dunham et al. 2018).

Culexarchaeia encode multiple group 3 and 4 [NiFe]-hydrogenases, which indicates that they could either produce or consume H₂. The group 3c [NiFe]-hydrogenase (MvhADG) may form a bifurcating complex with HdrABC, as found in other diverse archaea, which allows for the oxidation of H₂ to be coupled to the reduction of ferredoxin and a disulfide compound (Wagner, Koch et al. 2017). Alternatively, given the lack of FrhAG in *Culexarchaeia* MAGs,

HdrABC could form a confurcating complex with FrhB, thus coupling the oxidation of a disulfide and ferredoxin to the reduction of F420, similar to a scenario proposed for *Ca. Methanoperedens* that don't encode FrhAG (Arshad, Speth et al. 2015). *Culexarchaeia* also encode three other complexes with the potential to perform electron bifurcation: HdrA2B2C2 (discussed below), EtfAB-Acadm (discussed above with beta oxidation), and NADH-dependent ferredoxin:NADP⁺ oxidoreductase (NfnSL; Figure 3). By coupling the oxidation of NAD(P)H with the reduction of NAD(P)⁺, this enzyme could play a key role in cellular redox balancing (Nguyen, Schut et al. 2017).

Culexarchaeia encode a group 3b [NiFe]-hydrogenase that may couple the reversible oxidation of NADPH to the reduction of protons to evolve H₂ (Ma, Schicho et al. 1993). Characterization of the group 3b [NiFe]-hydrogenase in *Pyrococcus furiosus* revealed that it had sulfhydrogenase activity. This would allow the cell to couple the oxidation of NADPH with the reduction of elemental sulfur (S₀) or polysulfide to H₂S (Ma, Schicho et al. 1993, Ma, Weiss et al. 2000). Thus, *Culexarchaeia* may be involved in sulfur cycling through the activity of this potential sulfhydrogenase. Reduced ferredoxin produced through fermentative metabolism could be oxidized by a membrane-bound group 4g [NiFe]-hydrogenase, coupled to the translocation of ions and the production of H₂ (Figure 3). *Culexarchaeia* also encode a novel group 4 [NiFe]-hydrogenase (termed Ehe, discussed below) that may interact with disulfides, CO, or succinate. Furthermore, the production of H₂ (or formate) by *Culexarchaeia* could potentially form the basis of syntrophic interactions with other community members through the exchange of these compounds.

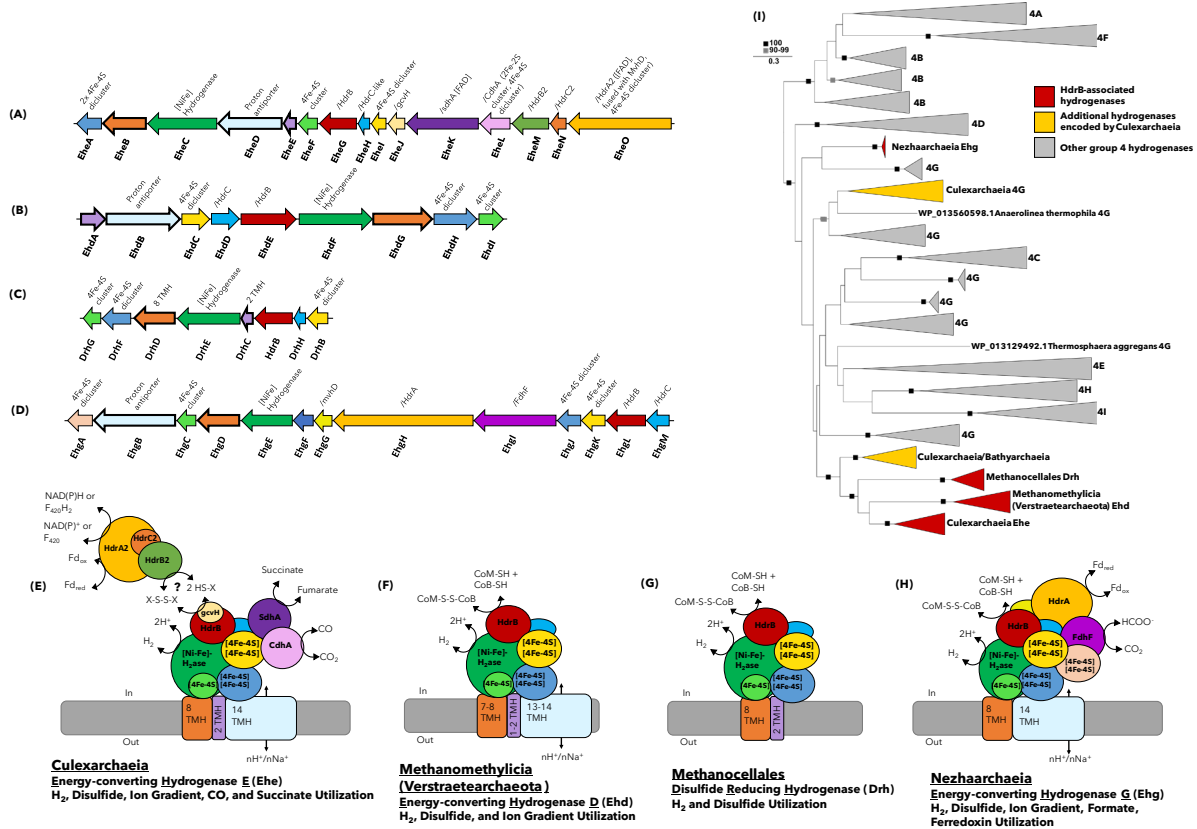


Figure 4 Modular HdrB-interacting [NiFe] Hydrogenases present in Culexarchaeia, Methanomethylica (Verstraetearchaeota), Methanocellales, and Nezhaarchaeia. (A,E) Gene structure and tentative model of the Ehc complex proposed for Culexarchaeia, (B,F) Ehd complex proposed for Methanomethylica (Verstraetearchaeota) (modified after (Adam, Borrel et al. 2019)), (C,G) Drh complex proposed for Methanocellales, and (D,H) Ehg complex proposed for Nezhaarchaeia. Transmembrane helices (TMH) were predicted using TMHMM and sequences in panels A-D with TMHs are indicated with a thick border. Sequences that share a similar structural/functional role in these complexes are indicated with the same color. (I) Maximum-likelihood phylogenetic reconstruction of the catalytic subunit of group 4 [NiFe]-hydrogenases. Ehc, Ehd, Drh, and Ehg clades are displayed in red. Additional Culexarchaeia sequences belonging to group 4 [NiFe]-hydrogenases are labeled in yellow.

An Expanded Diversity of Potential HdrB-Interacting Hydrogenases

A recent evaluation of MAGs affiliated with the Methanomethylica, the closest relatives to Culexarchaeia, revealed genes encoding a novel membrane bound [NiFe]-hydrogenase complex (termed Ehd) that were co-located with genes encoding heterodisulfide reductase

subunits (HdrBC) and an ion antiporter subunit (Borrel, Adam et al. 2019) (Figure 4B, 4F). This Ehd complex was suggested as an alternative pathway to couple the oxidation of H₂ to the reduction of the heterodisulfide CoM-S-S-CoB and translocation of ions outside the cell, which would allow for energy conservation during methylotrophic methanogenesis (Borrel, Adam et al. 2019). A similar membrane-bound hydrogenase complex (termed Ehe here) is encoded in *Culexarchaea* MAGs (Figure 4A, 4E). The Ehd and Ehe gene clusters have the same core structure containing genes for a [NiFe]-hydrogenase complex, HdrBC, and an ion antiporter subunit. However, the genes encoding the Ehe complex are co-located with genes encoding succinate dehydrogenase subunit A (SdhA), carbon monoxide dehydrogenase subunit A (CdhA), a predicted bifurcating heterodisulfide reductase complex (HdrA2B2C2) (Yan, Wang et al. 2017), and a glycine cleavage protein H (GcvH). This suggests that carbon monoxide or succinate oxidation could be coupled to the reduction of protons and the translocation of ions to the outside of the cell. Thermodynamics of succinate oxidation coupled to H₂ production indicate that this reaction becomes favorable at temperatures >65 °C (LaRowe and Amend 2019), which aligns with some of the high-temperature environments that *Culexarchaea* inhabit (50-83°C). Additionally, CO is a common trace gas in geothermal systems and could potentially be oxidized by this complex and coupled to the reduction of protons to H₂ to fuel carboxydrotrophic growth (Shock, Holland et al. 2005).

Alternatively, H₂ oxidation could also be coupled to the reduction of an unknown disulfide (via HdrB) that could be generated through the activity of the HdrA2B2C2 or HdrABC-MvhADG bifurcating complexes. The identity of this disulfide is unknown, but *Culexarchaea* lack a full biosynthetic pathway for CoM and CoB, which suggests that an alternative disulfide

may be used. The GcvH protein, encoded within the Ehc gene cluster, typically functions in the glycine cleavage pathway to coordinate a disulfide containing lipoyl moiety (Tezuka and Ohnishi 2014, Cao, Zhu et al. 2018). This suggests GcvH could play a similar role in coordinating a disulfide containing moiety during potential interactions with HdrB and/or the HdrA2B2C2 bifurcating complex. Determining directionality of the reaction catalyzed by this complex (oxidation or reduction of H₂) and how the diverse set of associated modules (HdrB, CdhA, SdhA, GcvH, and ion transporter) are regulated will require experimental work.

BLASTp searches against the non-redundant NCBI database (default settings), using the genes encoding HdrB and [NiFe]-hydrogenase catalytic subunit revealed gene clusters with similar synteny to the ones encoding the Ehd and Ehc complexes in the methanogenic Methanocellales (Figure 4C, 4G; denoted Drh) and in the predicted methanogenic Nezharchaeia (Figure 4D, 4H; termed here Ehg) (Lyu and Lu 2015, Wang, Wegener et al. 2019). Both gene clusters that encode the Drh and Ehg complexes retain the core structure of the [NiFe]-hydrogenase and HdrB genes, but co-located genes differ. Specifically, the Drh complex in Methanocellales lacks an ion antiporter subunit, which suggests that it is not involved in energy conservation via the production of an ion gradient, but instead could be directly involved in the reduction of CoM-S-S-CoB coupled to H₂ oxidation (Lyu and Lu 2015). The proposed Ehg gene cluster in Nezharchaeia MAGs is co-located with genes for a formate dehydrogenase subunit (FdhF), HdrA subunit, MvhD subunit, and an ion antiporter subunit. This suggests that oxidation of H₂, formate, or ferredoxin might be coupled to the direct reduction of CoM-S-S-CoB and the translocation of protons outside of the cell, thus conserving energy during their proposed hydrogenotrophic methanogenesis.

Phylogenetic analysis of the [NiFe]-hydrogenase catalytic subunit revealed that the Ehd, Ehe, and Drh complexes form a distinct cluster relative to other classified group 4 [NiFe]-hydrogenases. In contrast, the Ehg complex in *Nezhaarchaeia* forms a separate clade near a set of group 4g [NiFe]-hydrogenases (Figure 4I). Additionally, JZ-Bin-30 encodes a second putative HdrB and membrane associated [NiFe]-hydrogenase complex that clusters with *Bathyarchaeia* and *Methanomethylica* sequences (Figure 4I). This cluster was termed Ehd-like, due to the similarity in synteny to the Ehd complex in *Methanomethylica*, which lacks associated SdhA, CdhA, and GcvH subunits.

Importantly, these observations suggest that the strategy of coupling disulfide reductase activity to membrane bound [NiFe]-hydrogenases and ion translocation may be common in both methanogenic and non-methanogenic archaeal lineages. The apparent modular nature of these complexes may have allowed for diversification in substrate usage in the case of *Culexarchaeia* (succinate, CO) and *Nezhaarchaeia* (formate) for their respective metabolisms. Culturing representatives from the *Culexarchaeia*, *Methanomethylica*, or *Nezhaarchaeia* lineages would enable investigations into the function of these disulfide-interacting group 4 [NiFe]-hydrogenases in the metabolism of these organisms.

Conclusion

Here we report on a new archaeal class within the TACK superphylum, “*Candidatus Culexarchaeia*”, that is most closely related to the proposed methylotrophic and methanogenic lineage “*Ca. Methanomethylica*” (*Verstraetearchaeota*) (Vanwonterghem, Evans et al. 2016). In contrast to their closest relatives, *Culexarchaeia* encode the potential for anaerobic methylotrophy but not methanogenesis. Furthermore, they encode the potential for a generalist

lifestyle, with the capacity to use a diverse set of organic (sugars, lipids, proteins) and inorganic (H₂, CO, S₀) substrates. Notably, the potential for anaerobic non-methanogenic methylotrophy and cyanophycin degradation, which to our current knowledge are not widespread among archaea, suggests that Culexarchaeia may be important in cycling these compounds in extreme environments and that these putative functions could be exploited in future cultivation attempts. The biogeographic distribution of terrestrial and marine Culexarchaeia MAGs and 16S rRNA gene sequences indicates that they inhabit high temperature (>50° C) and circumneutral to slightly acidic (pH 5.4-7.8) environments. The potential to metabolize many organic and inorganic substrates suggests that they could be important in the biogeochemical cycles of hydrogen, carbon, and sulfur within diverse geothermal habitats, even at low relative abundances. The metabolic versatility encoded by Culexarchaeia could imply adaptations to changing nutrient conditions present in dynamic geothermal systems, allowing them to access a wide variety of compounds for their carbon and energy needs. Additionally, Culexarchaeia expand the diversity of cell division and cytoskeletal proteins in the TACK archaea, as they encode FtsZ-like, CdvBC, crenactin, and arcadin proteins. Future studies will need to determine how these diverse cell division and cytoskeletal proteins contribute to cell cycle and maintenance processes in Culexarchaeia, and could aid in understanding other lineages that similarly encode multiple division and cytoskeletal systems (e.g., Caldarchaeles (Aigarchaeota), Heimdallarchaeota, and Odinararchaeota).

The phylogenetic placement of Culexarchaeia aids in reconstructing the evolutionary history of TACK archaea. Particularly, their relatedness to the proposed methanogenic Methanomethylicia and Nezhaarchaeia, may provide insights into the evolutionary transitions

between methanogenic and non-methanogenic lineages. Currently, the evolution of the WLP as well as the MCR and MTR complexes in the TACK archaea are not well understood, which precludes our understanding of the evolutionary history of methane metabolism and whether the last ancestral methanogen used methyl compounds or H₂/CO₂ (Berghuis, Yu et al. 2019, Borrel, Adam et al. 2019, Wang, Wegener et al. 2021). Culexarchaea encode the potential for anaerobic non-methanogenic methylotrophy via the WLP, but do not encode MCR or MTR, which provides a stark contrast between the methyl-utilizing Methanomethylia (which encode MCR, but don't encode the WLP and MTR) and the H₂/CO₂ utilizing Nezharchaea (which encode the WLP, MCR, and MTR). If methanogenesis was the metabolism present in the last common ancestor of these lineages, Culexarchaea will aid in understanding shifts from methanogenic to non-methanogenic lifestyles in the TACK archaea. This type of transition is considered to have been an important process in archaeal evolution and is partially responsible for the observed patchwork distribution of MCR-encoding lineages (Borrel, Adam et al. 2019, Evans, Boyd et al. 2019, Zinke, Evans et al. 2021). We expect that the recovery of more deeply branching lineages in the TACK superphylum will help resolve the complicated history of these metabolic transitions and further our understanding of the metabolic capabilities encoded by this diverse superphylum.

Etymology and Proposal of Type Material

Following the recommendations by Chuvochina et al. 2019 (Chuvochina, Rinke et al. 2019), we assign type genomes and propose the following provisional taxonomic assignments. For a tabular list see Supplementary Table 2.

Candidatus Culexarchaeia class nov.

Cu.lex.ar.chae'ia. N.L. neut. n. Culexarchaeales, type order of the class; L. -ia, ending to designate a class; N.L. fem. pl. n. Culexarchaeia, the Culexarchaeum class. The description is the same as for Candidatus Culexarchaeum gen. nov.

Candidatus Culexarchaeales order nov.

Cu.lex.ar.chae'ales. N.L. neut. n. Culexarchaeaceae, type family of the order; L. -ales, ending to designate an order; N.L. fem. pl. n. Culexarchaeales, the Culexarchaeum order. The description is the same as for Candidatus Culexarchaeum gen. nov.

Candidatus Culexarchaeaceae fam. nov.

Cu.lex.ar.chae.ace'ae. N.L. neut. n. Culexarchaeum, type genus of the family; L. -aceae ending to designate a family; N.L. fem. pl. n. Culexarchaeaceae, the Culexarchaeum family. The description is the same as for Candidatus Culexarchaeum gen. nov.

Candidatus Culexarchaeum gen. nov.

Cu.lex.ar.chae'um N.L. neut. n. Culex, referring to the Culex Basin; N.L. neut. n. archaeum, an archaeon; N.L. neut. n. Culexarchaeum, archaeon of Culex, referring to the Culex Basin of Yellowstone National Park, where this lineage was discovered. The type species is Candidatus Culexarchaeum yellowstonense.

Candidatus Culexarchaeum yellowstonense sp. nov.

yel.low.ston.en'se N.L. neut. adj. yellowstonense, from Yellowstone National Park. This uncultured lineage is represented by bin YNP-LCB-024-027, recovered from an unnamed hot spring in the Culex Basin of Yellowstone National Park. The bin has an estimated completeness of 97.2% and a contamination of 0.93% and contains 16S rRNA, 23S rRNA and 5S rRNA genes.

Candidatus Culexarchaeum nevadense sp. nov.

ne.va.den'se N.L. neut. adj. nevadense, from Nevada. This uncultured lineage is represented by bin GBS-70-058, recovered from Great Boiling Springs in Nevada. The bin has an estimated completeness of 96.7% and a contamination of 0.07% and contains 16S rRNA, 23S rRNA, and 5S rRNA genes.

Candidatus Culexarchaeum jinzeense sp. nov.

jin.ze.en'se N.L. neut. adj. jinzeense, from Jinze. Represented by bin JZ-bin-30, recovered from a geothermal well in Jinze, Yunnan Province, China). The bin has an estimated completeness of 96.7%, a contamination of 0%, and contains 16S rRNA, 23S rRNA, and 5S rRNA genes. For a justification for the reclassification of this previously published bin (Berghuis, Yu et al. 2019), please see Supplemental Text.

Candidatus Culexmicrobiaceae fam. nov.

Cu.lex.mi.cro.bi.ace'ae. N.L. neut. n. Culexmicrobium, type genus of the family; L. -aceae ending to designate a family; N.L. fem. pl. n. Culexmicrobiaceae, the Culexmicrobium family. The description is the same as for Candidatus Culexmicrobium gen. nov.

Candidatus Culexmicrobium gen. nov.

*Cu.lex.mi.cro.bi.ace'*ae. N.L. neut. n. *Culex*, referring to the Culex Basin; N.L. neut. n. *microbium*, a microbe; N.L. neut. n. *Culexmicrobium*, microbe of *Culex*, referring to the Culex Basin of Yellowstone National Park, where this lineage was discovered. The type species is *Candidatus Culexmicrobium cathedralensis*.

Candidatus Culexmicrobium cathedralense sp. nov.

ca.the.dra.len'se N.L. neut. adj. *cathedralense*, from Cathedral Hill, a deep-sea hydrothermal vent in Guaymas Basin. This uncultured lineage is represented by bin GB-1867-05, recovered from deep-sea hydrothermal sediment in Guaymas Basin in the Gulf of California (i.e., Sea of Cortés). The bin has an estimated completeness of 99%, a contamination of 3.74%, and contains 16S rRNA, 23S rRNA and 5S rRNA genes.

Candidatus Culexmicrobium thermophilum sp. nov.

ther.mo.phil'um N.L. neut. adj. *thermophilum*, heat-loving. This uncultured lineage is represented by bin GB-1845-036, recovered from deep-sea hydrothermal sediment in Guaymas Basin in the Gulf of California (i.e., Sea of Cortés). The bin has an estimated completeness of 99%, a contamination of 3.74%, and contains 16S rRNA, 23S rRNA and 5S rRNA genes.

Candidatus Culexmicrobium profundum sp. nov.

pro.fund'um N.L. neut. adj. *profundum*, deep. Represented by bin GB-1867-035, recovered from deep-sea hydrothermal sediment in Guaymas Basin in the Gulf of California (i.e., Sea of Cortés).

The bin has an estimated completeness of 96.7%, a contamination of 7.79%, and contains a 5S rRNA gene.

Extended Materials and Methods

Cell Extractions and Catalyzed Reporter Deposition Fluorescence In Situ Hybridization (CARD-FISH)

Sediment samples from YNP sites LCB-003 and LCB-024 were collected in July 2019, fixed with paraformaldehyde at a final concentration of 2% for 8 h at 4°C, washed twice with 1x phosphate buffered saline (PBS) and stored in 1x PBS at 4 °C until cells were extracted. Extractions were performed by diluting aliquots of the sediment slurries 1:1 in PBS, adding methanol to a final concentration of 10%, Tween-20 to a final concentration of 0.1%, and vortexing the solution horizontally (Vortex-Genie2, Scientific Industries, Inc., Bohemia, NY) for five minutes to detach cells from particles. After vortexing, an equal volume of 80% (w/v) Nycodenz was layered under the sample and centrifuged at 16,000 g for 30 min at 4°C. Following centrifugation, the supernatant and interphase layers were transferred to a new tube, diluted 1:1 in PBS, and centrifuged at 16,000 g for 5 min to pellet the cells. The supernatant was removed, and the cells were resuspended in 1x PBS and stored at 4°C. Aliquots of the cell suspension were immobilized onto glass slides and CARD-FISH was performed as described in (Hatzenpichler, Lebedeva et al. 2008) with the following modifications. Cell permeabilization was attempted with either proteinase K (15 µg/mL) for 10 minutes or with 0.1 M HCl for 1 minute at room temperature. Endogenous peroxidases were inactivated by incubating in 0.01 M HCl for 15 minutes. A *Culex* archaeae-specific probe, Culex824 (5'-TCCACCTAACACCTAGCC-3'), targeted at the 16S rRNA of *Culex* archaeae found in

terrestrial hot springs, was designed using the probe design tool within the Arb software and the Silva 132 reference database (Ludwig, Strunk et al. 2004, Quast, Pruesse et al. 2012). Hybridization of probe Culex824 was attempted at formamide concentrations 0, 10, 20, and 35 %. Positive and negative control hybridization reactions were performed with the general archaeal probe Arc915 (5'-GTGCTCCCCCGCCAATTCCT-3') and NON338 (5'-ACTCCTACGGGAGGCAGC-3'), respectively (Ravenschlag, Sahm et al. 2001). All probes were labeled with horseradish peroxidase (HRP) and were purchased from Biomers (Ulm, Germany). Tyramide signal amplification was performed with Alexa Fluor 594 (Thermo Fisher). Samples were stained with DAPI, embedded in citifluor, and visualized under an epifluorescence microscope (Leica DM4B).

Extended Results and Discussion

Reclassification of JZ-bin-30

Based on our analyses, we propose to reclassify bin JZ-bin-30, originally given the provisional taxonomic assignment *Candidatus Methanomedium jinzeense* (Berghuis, Yu et al. 2019), as *Candidatus Culexarchaeum jinzeense* sp. nov. (etymology below). We base this reassignment on the observation in 16S rRNA gene and phylogenomic analyses that JZ-bin-30 does not fall within the candidate class *Methanomethylicia*, but rather within the new candidate class *Culexarchaeia*. Additionally, in contrast to what the name *Methanomedium* implies, all currently available *Culexarchaeia* MAGs, including JZ-bin-30, lack the key enzyme for methanogenesis (methyl-coenzyme M reductase; Mcr) and lack methanogenesis marker genes that have been identified in both bona fide Euryarchaeotal methanogens and newly proposed MCR-encoding archaea outside the Euryarchaeota (Borrel, Adam et al. 2019).

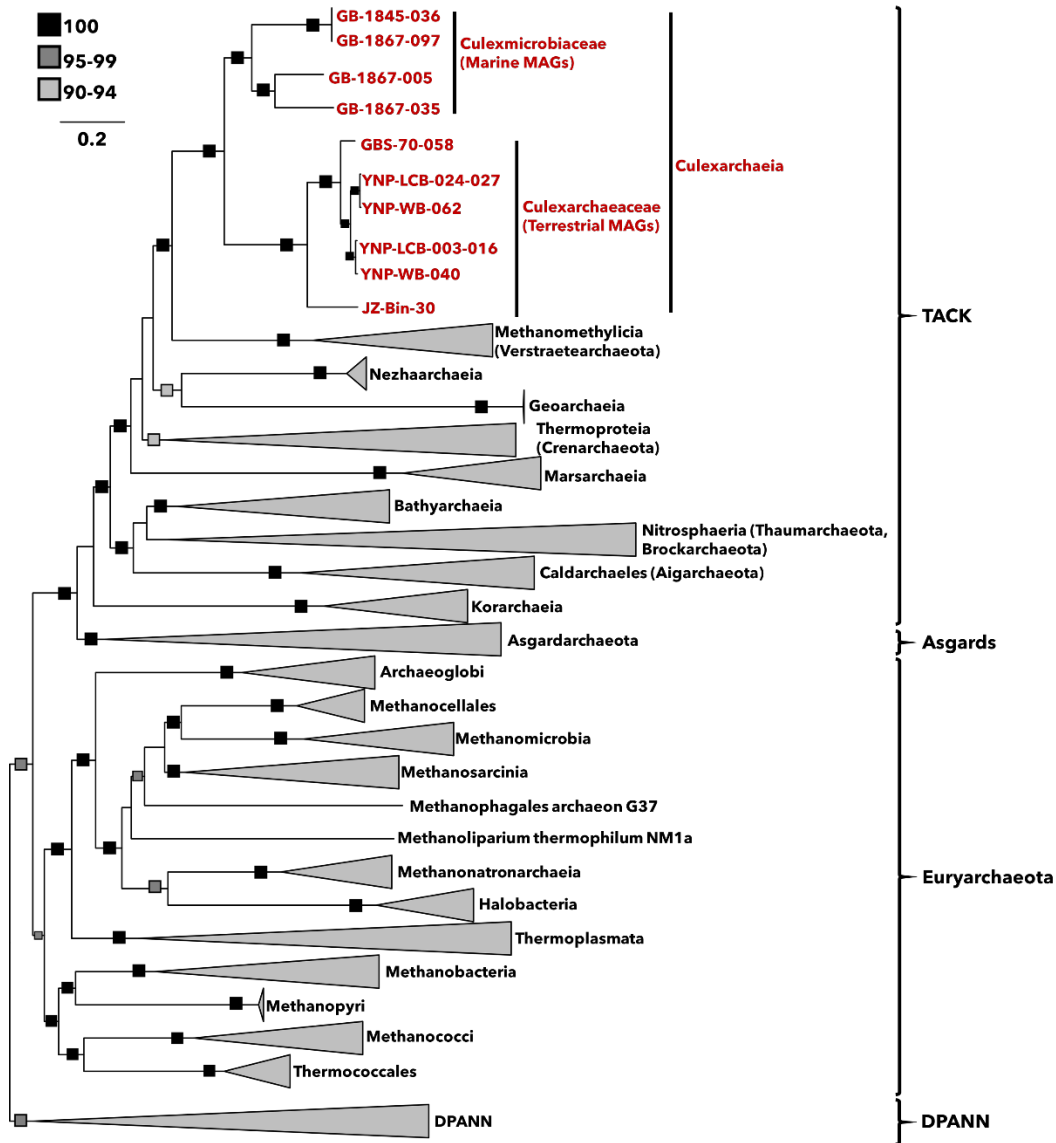
Supporting Information

	Heat-flow probe location 1		Heat-flow probe location 2		
	Time 16:33	Time 16:36	Time 16:44	Time 16:47	Time 16:50
Temp. 1 (7-8 cm)	76.4 °C	83.2 °C	38.6 °C	50.7 °C	53.2 °C
Temp. 2 (~17 cm)	98.3 °C	101.8 °C	58.4 °C	71.1 °C	73.3 °C
Temp. 3 (~27 cm)	104.2 °C	105.6 °C	74.9 °C	81.8 °C	82.8 °C
Temp. 4 (~37 cm)	107.4 °C	108.1 °C	88.2 °C	92.1 °C	93.1 °C
Temp. 5 (~47 cm)	107.2 °C	107.7 °C	98.1 °C	100.1 °C	101.3 °C

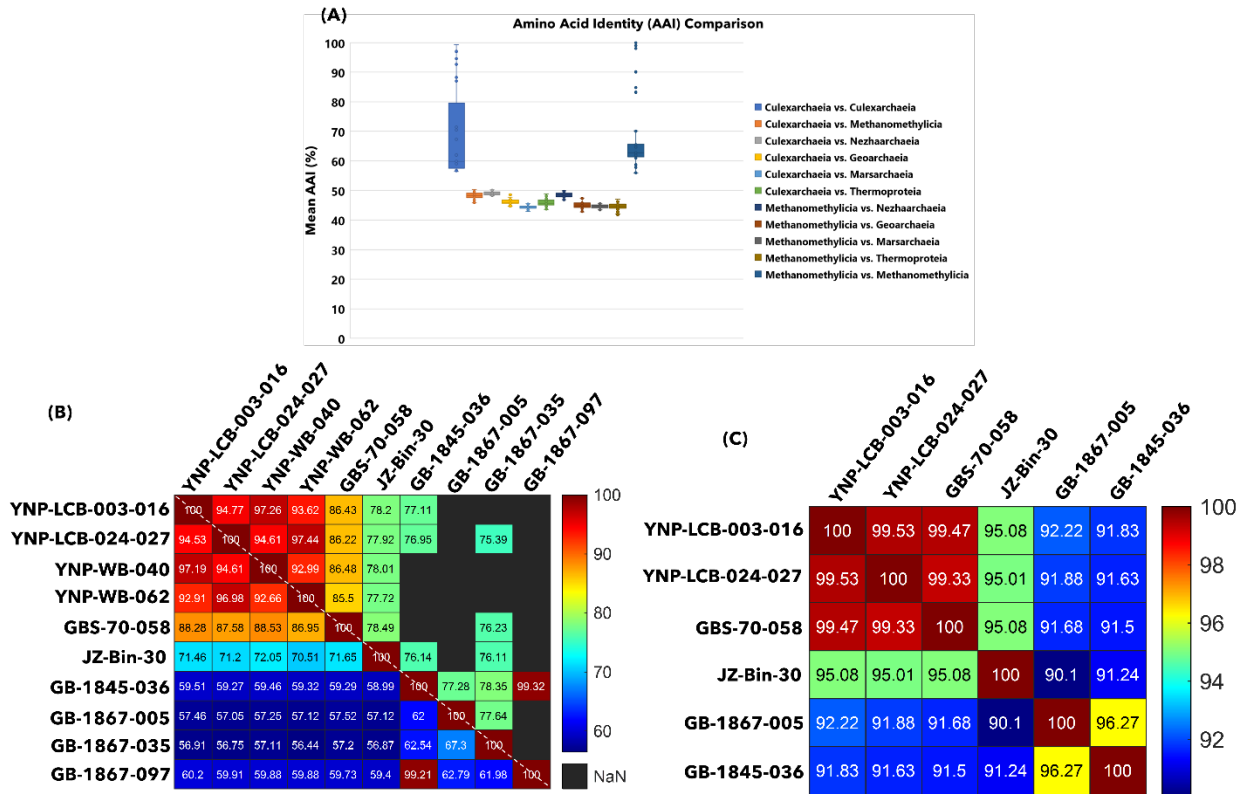
SI Table 1. Temperature profiles recorded by Alvin's heat flow probe on December 24th, 2016, during dive 4872.

Phylum	Class	Order	Family	Genus	Species	MAG ID
Culexarchaeota (NCBI) / Thermoproteota (GTDB)	Culexarchaeia	Culexarchaeles	Culexarchaeaceae	Culexarchaeum		YNP-LCB-24-027 *
					yellowstonense	YNP-LCB-3-016
						YNP-WB-040
						YNP-WB-062
					jinzeense	JZ-Bin-30 *
					nevadense	GBS-70-058 *
			Culexmicrobiaceae	Culexmicrobium	cathedralense	GB-1867-005 *
					profundum	GB-1867-035 *
					thermophilum	GB-1845-036 *
						GB-1867-097

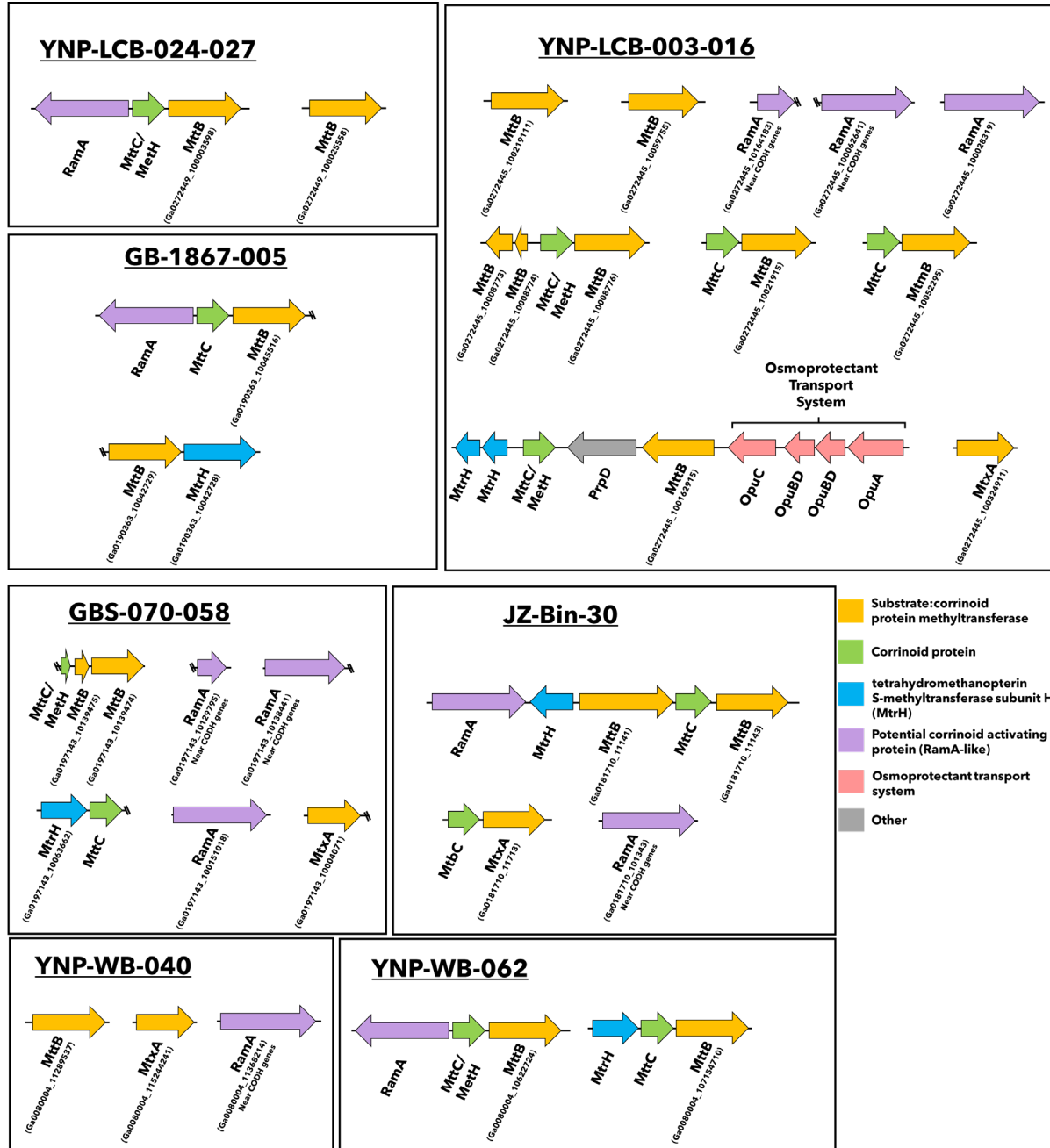
SI Table 2. Proposed naming scheme for the Culexarchaeia MAGs used in this study. Family, genus, and species designations were decided by considering our phylogenomic tree (Figure 1A), pairwise 16S rRNA gene nucleotide identities, and pairwise MAG AAI values. *, indicates MAG ID used to designate type species.



SI Figure 1. Phylogenomic tree of Culexarchaeia MAGs and reference archaeal genomes. Maximum-likelihood tree, inferred with IQtree and the best-fit LG+C60+F+G model, using a concatenated set of 46 conserved ribosomal proteins (Supplementary File 1). Ultrafast bootstrap support values of 100, 95-99, and 90-94 are indicated with black, dark gray, and light gray squares, respectively.



SI Figure 2. Amino acid identities (AAI) of Culexarchaeia and major TACK lineages. Intra-clade AAI, ANI, and 16S rRNA gene percent identity of Culexarchaeia. (A) Analysis shows that the average AAI between TACK classes is below 50%, consistent with the designation of Culexarchaeia as a class level lineage within the TACK superphylum. Thick bar, median; first and third quartile, upper and lower bounds of the box, respectively. Upper and lower whiskers extend to the highest and lowest values within 1.5x of the interquartile range, respectively. (B) Intra-clade AAI and ANI of Culexarchaeia. Values above and below the dashed white line represent ANI and AAI values, respectively. AAI values were calculated with compareM and ANI values were calculated with fastANI. (C) Pairwise % identity of Culexarchaeia 16S rRNA gene sequences calculated by BLASTn.



SI Figure 4. Organization of *Culexarchaeia* methyltransferase genes. The color of the arrows corresponds to the predicted gene function listed. Some proteins in the family COG3984 (RamA-like) were found located next to carbon monoxide dehydrogenase (CODH) genes, suggesting they may play a role in the function of this complex instead. MAGs not listed did not have any methyltransferase genes encoded. Double slashes indicate the gene is truncated at the end of a scaffold. Each cluster of arrows depicted represents a separate scaffold. Locus tag(s) for genes on IMG are indicated in parentheses for each scaffold.

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Data Availability

The raw metagenomes used to recover MAGs of *Culexarchaeia* representatives are publicly available through the JGI IMG-MER database under accessions numbers 3300029977 (YNP-

LCB-024), 3300028675 (YNP-LCB-003), 3300005860 (YNP-WB), 3300020139 (GBS), 3300021469 (GB-1845), and 3300021472 (GB-1867). The nine Culexarchaeia MAGs newly recovered in this study are available in NCBI under BioProject ID PRJNA819097. The JZ-Bin-30 MAG is publicly available through the JGI IMG-MER database accession number Ga0181710.

Competing Interests

The authors declare no competing interests.

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CHAPTER THREE

CULTIVATION AND VISUALIZATION OF A METHANOGEN
OF THE PHYLUM THERMOPROTEOTAContribution of Authors and Co-Authors

Author: Anthony J. Kohtz

Contributions: Developed the research project, designed experiments, and conducted field sampling. Conducted cultivation, BONCAT, SIP, FISH, Raman, and transcriptomic experiments. Performed culture DNA/RNA extractions and phylogenetic analysis of hydrogenases. Analyzed metagenomic data and phylogenetic analysis of genomes, performed the metabolic reconstruction. Gave input on cryoET data analysis. Wrote the manuscript.

Co-Author: Nikolai Petrosian

Contributions: Designed experiments, analyzed cryoET data.

Co-Author: Viola Krukenberg

Contributions: Designed experiments, conducted field sampling, extracted DNA from LCB070 sediment, gave advice on cultivation, performed phylogenetic analysis of MCR sequences, and gave input on metabolic reconstruction.

Co-Author: Zackary Jay

Contributions: Conducted field sampling, analyzed metagenomic data, performed phylogenetic analysis of genomes, and gave input on metabolic reconstruction.

Co-Author: Martin Pilhofer

Contributions: Designed experiments, gave input on cryoET data analysis, acquired funding.

Co-Author: Roland Hatzenpichler

Contributions: Developed and supervised the research project, designed experiments, acquired funding, wrote the manuscript.

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Abstract

Methane is the second most abundant climate-active gas and understanding its sources and sinks is a crucial endeavor in microbiology, biogeochemistry, and climate sciences (Thauer, Kaster et al. 2008, Garcia, Gribaldo et al. 2022). For decades, it was thought that methanogenesis, the ability to conserve energy coupled to methane production, was restricted to a taxonomically restricted and metabolically specialized group of archaea, the Euryarchaeota (Thauer, Kaster et al. 2008). The discovery of marker genes for anaerobic alkane cycling in metagenome-assembled genomes obtained from diverse habitats has led to the hypothesis that archaeal lineages outside the Euryarchaeota are also involved in methanogenesis (Evans, Parks et al. 2015, Vanwonterghem, Evans et al. 2016, Borrel, Adam et al. 2019, Wang, Wegener et al. 2019). Here, we cultured *Candidatus Methanosuratincola verstraetei* strain LCB70, a member of the archaeal class Methanomethylicia (formerly Verstraetearchaeota) within the phylum Thermoproteota, from a terrestrial hot spring. Growth experiments combined with activity assays, stable isotope tracing, and genomic and transcriptomic analyses demonstrated that this thermophilic archaeon grows via methyl-reducing hydrogenotrophic methanogenesis. Cryo-electron tomography revealed that *Ca. M. verstraetei* are coccoid cells with archaella and chemoreceptor arrays, and that they can form intercellular bridges connecting two to three cells with continuous cytoplasm and S-layer. The wide environmental distribution of *Ca. M. verstraetei* suggests that they might play important and hitherto overlooked roles in carbon cycling within diverse anoxic habitats.

Introduction

Methanogens, strictly anaerobic archaea that conserve energy coupled to methane production, are responsible for approximately two-thirds (576 Tg year⁻¹) of global methane emissions (Conrad 2009, Saunio, Staver et al. 2020). Methanogens can exploit CO₂/H₂ and a variety of organic compounds, such as, formate, acetate, or methylated or methoxylated molecules, to fuel their growth (Garcia, Gribaldo et al. 2022). All methanogenic pathways require methyl-coenzyme M reductase (MCR), the enzyme complex catalyzing the conversion of methyl-coenzyme M (CH₃-CoM) and coenzyme B (CoB) into methane and a heterodisulfide (CoM-S-S-CoB) (Thauer 2019). Because of its crucial importance in all known methanogenic pathways as well as the anaerobic oxidation of hydrocarbons, MCR-encoding genes are commonly used to identify potential alkane cycling archaea (Evans, Boyd et al. 2019, Garcia, Gribaldo et al. 2022). Methanogens have been cultured since the early 1900s (Stephenson and Stickland 1933) but to date all belong to the superphylum Euryarchaeota (Rinke, Chuvochina et al. 2021).

The discovery of *mcr* and other methanogenesis marker genes in diverse metagenome-assembled genomes (MAGs) has led to the proposal that several lineages within the archaeal phyla Asgardarchaeota, Hadarchaeota, and Thermoproteota (formerly the TACK superphylum) might engage in anaerobic alkane cycling (Evans, Parks et al. 2015, Vanwonterghem, Evans et al. 2016, Borrel, Adam et al. 2019, McKay, Dlakic et al. 2019, Seitz, Dombrowski et al. 2019, Wang, Wegener et al. 2019). In contrast to most cultured methanogens that conserve energy exclusively via methanogenesis, many of these MCR-encoding MAGs encode alternative energy conserving pathways, such as dissimilatory sulfite reduction (Borrel, Adam et al. 2019, McKay,

Dlakic et al. 2019) or fermentation (Evans, Parks et al. 2015, Vanwonterghem, Evans et al. 2016). The potential of these MCR-encoding archaea to switch their growth modality depending on environmental conditions might provide them an advantage over obligate methanogens. However, so far, no methanogen from outside the Euryarchaeota has been cultured or visualized, and no experimental evidence exists to support metagenome-based metabolic predictions.

Here we combined cultivation, microscopy, metagenomics and metatranscriptomics, growth experiments, stable isotope tracing, and single cell activity assays to demonstrate that a member of the phylum Thermoproteota is a methyl-reducing hydrogenotrophic methanogen. Furthermore, we for the first time visualize the morphology and unique ultrastructure of a methanogen outside the Euryarchaeota by fluorescence microscopy and cryo-electron tomography (cryoET).

Results and Discussion

Cultivation of a Novel Methanogen

Geothermal features, such as hot springs, have recently been shown to harbor a wide diversity of MCR-encoding archaea (McKay, Hatzenpichler et al. 2017, Borrel, Adam et al. 2019, Hua, Wang et al. 2019, McKay, Dlakic et al. 2019, Wang, Wegener et al. 2019, Buessecker, Chadwick et al. 2023, Lynes, Jay et al. 2024, Lynes, Krukenberg et al. 2023, Wang, Qu et al. 2023), which makes them promising sources for targeted cultivation of these microorganisms. In a recent survey of geothermal features in Yellowstone National Park (YNP), we identified the Lower Culex Basin (LCB) as an area with strong potential for methanogenesis (Lynes, Krukenberg et al. 2023). From the sediment metagenome of hot spring LCB070, we reconstructed two MAGs affiliated with the class Methanomethylicia (formerly

Verstraetearchaeota (Vanwonterghem, Evans et al. 2016)) within the phylum Thermoproteota (Rinke, Chuvochina et al. 2021) (Extended Data Fig. 1). Consistent with Methanomethylica MAGs previously obtained from other habitats (Vanwonterghem, Evans et al. 2016, Borrel, Adam et al. 2019, Hua, Wang et al. 2019, Liu, Chen et al. 2022), the two MAGs from LCB070 encode the potential for methyl-reducing hydrogenotrophic methanogenesis (Garcia, Gribaldo et al. 2022).

Using a sediment-water slurry from hot spring LCB070 as inoculum, we initiated a methanogenic culture supplied with methanol and H₂ in anoxic media (64°C, pH 6.5). After one transfer, fluorescence in situ hybridization (FISH) with a newly designed Methanomethyliaceae-specific 16S rRNA-targeted probe detected an abundant population of coccoid cells.

Metagenomic sequencing revealed that the most abundant organism was an MCR-encoding member of the Methanomethylica (31.4% relative abundance; Supplementary Table 1).

Via a combination of Illumina short-read and Nanopore long-read sequencing, we assembled the complete circular genome of this archaeon, which had a size of 1.52 Mb and a GC content of 54.6% (Supplementary Table 1). 16S rRNA gene and single copy marker protein phylogenies placed the archaeon within the phylum Thermoproteota (Fig. 1A). Comparative amino acid, nucleotide, and 16S rRNA gene sequence identity analyses further classified the genome within the genus *Ca. Methanosuratincola* (Vanwonterghem, Evans et al. 2016, Oren, Garrity et al. 2020) (Extended Data Fig. 2). We propose to name this archaeon *Ca.*

Methanosuratincola verstraetii strain LCB70 (see etymology in SI Results and Discussion).

Interestingly, LCB70 was not closely related to the two MAGs recovered from the original hot spring metagenome (Extended Data Fig 1 and 2), suggesting that it represented a low abundance

population in feature LCB070 at the time of sampling. Analysis of *Ca. M. verstraetei* related 16S rRNA gene sequences in public databases revealed that they are present in a variety of anoxic habitats, including bioreactors, freshwater and marine sediments, hot springs, hydrocarbon reservoirs, soils, rumen, and wastewater sludge (Extended Data Fig. 3; Supplementary Table 2). This demonstrates that methanogens within this group might contribute to methane cycling in ecosystems other than hot springs.

By the seventh transfer we had obtained a sediment-free methanogenic culture, in which LCB70 (32.8% relative abundance) and *Methanothermobacter* spp. (9.7% relative abundance) were the only archaeal populations detectable by 16S rRNA gene amplicon sequencing (Fig. 1B). Based on FISH with the LCB70-specific and general archaeal probes, the two populations were distinguished by their coccoid (LCB70) and filamentous (*Methanothermobacter* spp.) morphologies (Fig. 1C). Thus, we established a stable mixed culture of LCB70, a proposed methyl-reducing methanogen, and *Methanothermobacter* spp., obligate CO₂-reducing methanogens (Zeikus, Ben-Bassat et al. 1980, Cheng, Dai et al. 2011, McKay, Klingelsmith et al. 2021). Furthermore, we found that methane production and growth of the culture improved when supplemented with an additional carbon source (lactate, acetate, or pyruvate; Extended Data Fig. 4).

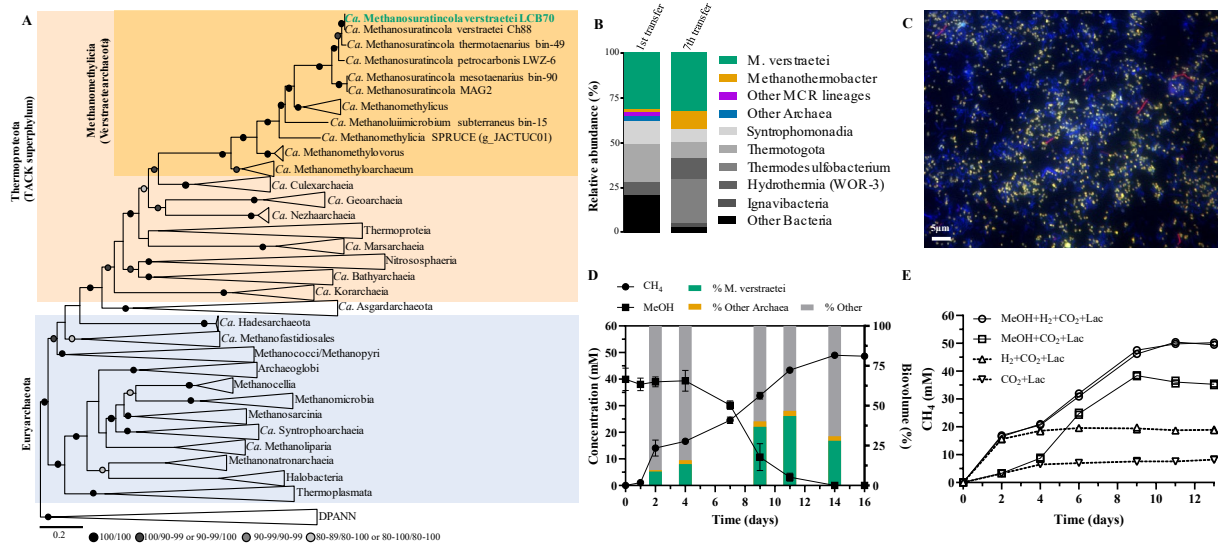


Figure 1. Characterization of the methanogenic enrichment culture. A. Phylogenomic tree of archaea based on a set of 33 single copy marker genes, with *Ca. M. verstraetii* LCB70 highlighted in green. Circles at the nodes indicate the ranges for the SH-like approximate-likelihood ratio test and ultrafast bootstrap support, respectively, and only values above 80 are shown. B. Community composition of the culture at the 1st transfer based on the relative abundance of the LCB70 genome and other MAGs recovered from metagenome sequencing and at the 7th transfer based on 16S rRNA gene amplicon sequencing. C. Visualization of the culture by 16S rRNA-targeted FISH. *M. verstraetii* cells in yellow (dual labeled by the Methanomethylia-specific probe Msur657 and the general archaeal probe Arch915), other archaeal cells in red (labeled by Arch915). DAPI-stained cells not hybridized with either probe in blue. Scale bar, 5 μm . A representative image of $n = 3$ independent samples from one culture is shown. D. Growth curve depicting methane production, methanol depletion, and relative abundance of LCB70 and other archaea (%) over time as determined with FISH. Data for methane and methanol (MeOH) are the mean ($n = 4$ biological replicates) $\pm$ s.d. and % biovolume is the mean ($n = 4$ biological replicates). Where not visible, the error bars are smaller than the symbol. E. Methane production by the culture under different substrate amendments. The line represents the mean and symbols show values for each replicate ($n = 2$ biological replicates). Methane production in incubations without methanol is due to the activity of Methanothermobacter. When methanol, a substrate that Methanothermobacter cannot use, is present LCB70 converts it into methane via methyl-reducing methanogenesis.

LCB70 Converts Methanol to Methane

In cultures supplied with methanol, H₂, and lactate, the relative abundance of LCB70 increased alongside methane production and methanol depletion (Fig. 1D). The biovolume

fraction of LCB70 in the culture determined with FISH increased from an average of 8.7% in the lag phase to up to 43.5% during the log phase of methane production (Fig. 1D). In the stationary phase, when methanol had been depleted and methane production ceased, the biovolume fraction of LCB70 decreased to 28%, likely due to substrate starvation (Fig. 1D). When methanol was omitted, methane production was strongly reduced and growth of LCB70 could not be sustained (Fig. 1E). The biovolume of *Methanothermobacter* ranged from 0.9% to 3.4% between day 2 to 11 (Fig. 1D). In the first two to four days of the culture, methane production is not coupled to methanol depletion and LCB70 is of comparatively low abundance. In contrast, during later stages of the culture, when methane production is coupled to methanol depletion, LCB70 is most abundant, further indicating the reliance of LCB70 on methanol (Fig. 1D).

The two methanogen populations, LCB70 and *Methanothermobacter*, employ different pathways for methanogenesis. LCB70 encodes genes for methyl-reducing hydrogenotrophic methanogenesis but lacks genes for CO₂-reducing hydrogenotrophic or acetoclastic methanogenesis. In contrast, *Methanothermobacter* are obligate H₂ and CO₂ utilizing methanogens which lack methyltransferase genes (Zeikus, Ben-Bassat et al. 1980, McKay, Klingel-Smith et al. 2021). To determine the contribution to methane production by the two methanogens, we further incubated the culture under conditions with and without methanol and hydrogen (Figure 1E). Total methane production in cultures without methanol was greatly reduced and due to the activity of the CO₂-utilizing *Methanothermobacter*. When methanol, a substrate that *Methanothermobacter* cannot use, was present, LCB70 contributed to methane production by converting methanol into methane via methyl-reducing hydrogenotrophic methanogenesis. When H₂ was not supplied, methane production occurred, which indicates that

H₂ producing organisms co-exist with methanogens in the culture (Fig. 1E). We conducted a stable isotope tracer experiment, in which a culture grown in the absence of H₂ was incubated in the presence of ¹³C-methanol. We observed that 52% of the added ¹³C-methanol was converted to ¹³C-methane and that 40% was converted to ¹³C-carbon dioxide (Fig. 2C). The production of ¹³C-carbon dioxide suggests that other organisms in the culture also metabolize methanol. Consistently, methanol-oxidizing bacteria affiliated with *Syntrophomonadia* (7-12% relative abundance) and *Thermotogota* (8-21% relative abundance) that expressed methanol methyltransferases and methanol dehydrogenases, respectively, were co-enriched in the culture (Fig. 1B; Supplementary Table 3). Further, we hypothesize that these methanol-degraders in the culture likely form CO₂, H₂, and acetate and engage in a syntrophic interaction with consumers of these byproducts, including LCB70 and *Methanothermobacter*, as observed in other thermophilic, methanogenic co-cultures and bioreactors (Balk, Weijma et al. 2002, Paulo, Stams et al. 2003). In future studies, ¹³C- and ²H-labeling experiments performed under different physicochemical conditions might help to reconcile these ideas.

To study the metabolic activity of LCB70 under methanogenic and methanogenesis-inhibited conditions, we performed bioorthogonal non-canonical amino acid tagging (BONCAT) incubations to label translationally active cells (Hatzenpichler, Connon et al. 2016, Hatzenpichler, Krukenberg et al. 2020). For this, replicate cultures grown to the exponential phase of methane production were spiked with 2-bromoethanesulfonate (BES), an inhibitor of the MCR complex (Fig. 2B). After a 48-hour incubation, L-homopropargylglycine (HPG), a tracer of translational activity, was added to BES amended cultures and to controls without BES. After 24-hours of incubation with HPG, we analyzed cells by BONCAT-FISH to directly link

taxonomic identity and translational activity at a single cell level. We observed active LCB70 cells under methanogenic conditions, but not under methanogenesis-inhibited conditions (absence of BES; Fig. 2C). This indicates that translational activity and subsequent energy conservation in LCB70 relies on the functional operation of the MCR complex, and thus methanogenesis.

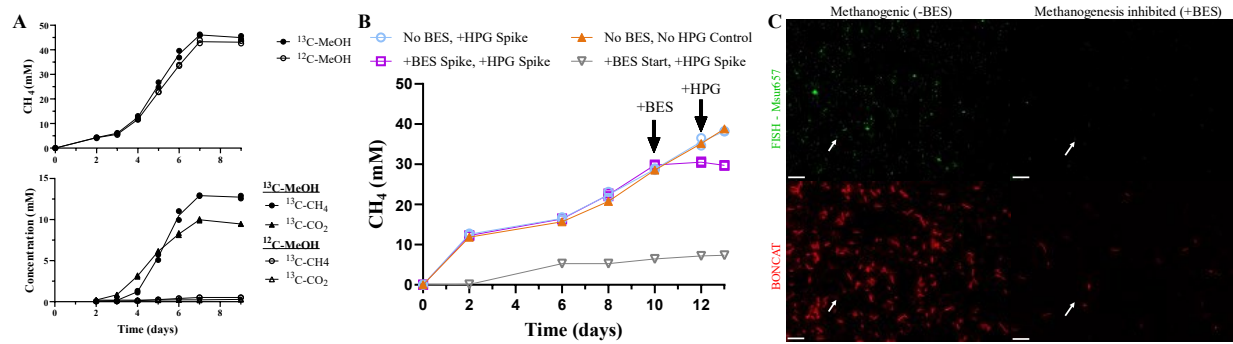


Figure 2. Translational activity and conversion of stable isotope tracers. A. Incubation experiments with ^{13}C -methanol (25 mM ^{13}C -methanol and 25 mM ^{12}C -methanol; closed symbols) and ^{12}C -methanol (50 mM added) as control (open symbols). Top, total methane produced over time. Bottom, ^{13}C -methane (circles) and ^{13}C -carbon dioxide (triangles) produced over time. The line represents the mean and symbols show values for each replicate ($n = 2$ biological replicates). B. Methane production in incubations for BONCAT-FISH experiments ($n = 2$ biological replicates for No BES +HPG Spike and +BES Spike +HPG Spike; $n = 1$ biological replicate for No HPG control and +BES Start). The line represents the mean and symbols show values for each replicate. Arrows indicate BES and HPG addition. Methane production stops upon BES addition on day 10. C. LCB70 cells detected via FISH (green) and translationally active cells detected via BONCAT (red). Under methanogenic conditions, LCB70 cells were translationally active. When methanogenesis was inhibited by BES, LCB70 cells were inactive. White arrows indicate examples of LCB70 cells. Note that the relative fluorescence intensity of LCB70 as compared to other cells is non-informative in BONCAT-experiments (Hatzenpichler, Krukenberg et al. 2020). Representative images from $n = 3$ independent samples from $n = 2$ biological replicate cultures are shown.

Metabolic Potential and Gene Expression

The recovery of the complete circular genome of strain LCB70 enabled us to reconstruct its metabolic potential and analyze its gene expression when grown on methanol, hydrogen, and lactate. Consistent with methanol-dependent methane production in the culture, strain LCB70 encodes the potential for methyl-reducing hydrogenotrophic methanogenesis. Strain LCB70 highly expressed the methanol-specific methyltransferase system (MtaABC) and the MCR complex, enabling the conversion of methanol to CoM-S-S-CoB and methane (Fig. 3 A,B). Strain LCB70 also encodes two copies of another methyltransferase, MtmB, that could be involved in converting monomethylamine to methane, and these genes were expressed at ca. 1% of the rate of MtaB (Fig. 3B). However, in contrast to the close relative *Ca. M. petrocarbonis* LWZ-6, which uses monomethylamine (Wu, Zhou et al. 2023), attempts to grow strain LCB70 on monomethylamine have so far been unsuccessful. Furthermore, strain LCB70 lacks the tetrahydromethanopterin S-methyltransferase (MTR) complex and methyl branch of the Wood-Ljungdahl pathway, indicating it is not able to oxidize methanol to CO₂, which would be required for a methyl-dismutating methanogenesis pathway (Garcia, Gribaldo et al. 2022). Additionally, we observed that expression of the methanogenesis and energy conservation pathways was largely similar across cultures grown with lactate as compared to those grown with pyruvate or acetate as carbon sources (Extended Data Fig. 4; Supplementary Table 4).

Beyond methyltransferases and the MCR complex, LCB70 also encodes several complexes involved in the reduction of CoM-S-S-CoB, energy conservation, and hydrogen metabolism. The proposed HdrBC-interacting [NiFe]-hydrogenase complex, Ehd, was the highest expressed hydrogenase and could couple the oxidation of H₂ to the reduction of CoM-S-S-CoB and translocation of H⁺ or Na⁺ across the membrane (Borrel, Adam et al. 2019, Kohtz,

Jay et al. 2022). Strain LCB70 also expressed a group 4i [NiFe]-hydrogenase, Ehb. In other methanogens, Ehb has been implicated in several processes, including energy conservation and H₂ production in *Methanosphaera stadtmanae*, and providing reduced ferredoxins for anabolism in *Methanococcus maripaludis* (Thauer, Kaster et al. 2008, Major, Liu et al. 2010). Thus, additional physiology studies are required to deduce the function and regulation of Ehb in strain LCB70. Additionally, a cytoplasmic group 3b [NiFe]-hydrogenase that belongs to the group of potential sulfhydrogenases was lowly expressed. This enzyme catalyzes the reversible oxidation of NADPH coupled to the reduction of protons or sulfur (S₀) to H₂ or H₂S and may be important under yet-unknown growth conditions (Ma, Schicho et al. 1993).

Notably, in contrast to previous findings based on *Methanomethylicia* MAGs (Vanwonterghem, Evans et al. 2016, Hua, Wang et al. 2019), we did not identify genes encoding an Fpo-like complex in the LCB70 genome. However, LCB70 expresses a novel group 4g [NiFe] hydrogenase, termed Ehi here, which is also present in diverse Thermoproteota lineages (Fig. 3; Extended Data Fig. 5). This Ehi complex shares similarities to Fpo-like complexes in archaea, including the same 11-subunit organization. However, the catalytic subunit has conserved motifs (CxxC) at the N and C-termini for coordination of a [NiFe] cofactor, which are absent in Fpo/Nuo complexes (Lang, Schuldes et al. 2015, Loh, Hervé et al. 2021). Therefore, we suggest that Ehi could functionally substitute for a Fpo-like complex in LCB70. If Ehi formed a complex with HdrD, in a similar fashion to the Fpo/HdrD complex in *Methanomassiliicoccales* (Lang, Schuldes et al. 2015, Kröninger, Berger et al. 2016, Kröninger, Steiniger et al. 2019), it might constitute another group of potential heterodisulfide reductase associated hydrogenases (Fig.

3A). Alternatively, if Ehi did not form a complex with HdrD, it would be functionally more similar to the Ehb-type hydrogenase.

In LCB70, as in the first description of *Methanomethylicia* MAGs (Vanwonterghem, Evans et al. 2016), a gene with similarity to lactate dehydrogenase (GlcD) was co-located with a heterodisulfide reductase (HdrD) gene (Vanwonterghem, Evans et al. 2016). Notably, both genes were highly expressed by LCB70 during methanogenic growth (Fig. 3B and Extended Data Figure 4C), indicating their importance to its metabolism. Lactate has been observed to be metabolized by syntrophic co-cultures, in which H₂ produced by lactate fermentation is used by H₂-consuming methanogens (Bryant, Campbell et al. 1977, McInerney and Bryant 1981). Alternatively, as suggested previously (Vanwonterghem, Evans et al. 2016, Evans, Boyd et al. 2019, Hua, Wang et al. 2019), these genes may enable lactate to be used as an electron donor to a Ehi/HdrD complex for the reduction of CoM-S-S-CoB, a property that has not been observed in any cultured Euryarchaeal methanogen. Additionally, we identified a second cluster of genes with two additional HdrD copies located adjacent to a gene containing a lactate utilization domain (LUD) (Hwang, Bakolitsa et al. 2013) and a [4Fe-4S] dicluster domain (Fig. 3A; LldG-like; Extended Data Fig. 4D). Interestingly, prior analyses of *Methanomethylicia* MAGs had identified GlcD-like, but not LldG-like genes. However, during methanogenic growth, LCB70 expressed these genes at low levels compared to GlcD, suggesting that they might play an alternative metabolic role (Figure 3B). For additional discussion on the potential for lactate metabolism see SI Results and Discussion. In summary, genomics and transcriptomics demonstrated growth of LCB70 by methyl-reducing hydrogenotrophic methanogenesis from methanol and hydrogen.

Ultrastructure of Strain LCB70

Cultivation of LCB70 allowed us, for the first time, to visualize the ultrastructure of a methanogen outside the Euryarchaeota. We initially imaged a set of plunge-frozen cells of an active LCB70 culture via cryo-electron tomography (cryoET) (Young and Villa 2023). According to FISH, LCB70 cells were the only coccoid archaeal cells in the enrichment (Fig. 1C). Their unique shape, high relative abundance, and presence of an S-layer (a gene encoding an S-layer protein was highly expressed in our transcriptomic datasets; Supplementary Table 4) enabled the identification of LCB70 cells in tomograms. LCB70 cells were surrounded by a cytoplasmic membrane and an S-layer and had an average diameter of 845 ± 163 nm ($n = 55$ cells; Fig. 4, Extended Data Fig. 7). We also visualized archaella and chemoreceptor arrays in tomograms of LCB70 (Figure 4A, Extended Data Fig. 7). Consistently, LCB70 expressed a cluster of archaella

and chemotaxis genes, with the structural archaellin protein ArlB being the highest expressed within this gene cluster (Extended Data Fig. 6). This indicates that LCB70 is motile and capable of sensing and responding to environmental stimuli (Briegel, Ortega et al. 2015, Albers and Jarrell 2018, Quax, Albers et al. 2018). Other structures observed include putative filamentous and spindle shaped virus-like particles, unidentified intracellular semi-ordered filamentous structures, and putative storage granules (Fig. 4, Extended Data Figs. 7 and 8, Supplementary Movie 1). Confocal Raman microspectroscopy of single LCB70 cells identified by FISH (n = 18) indicated that these granules are likely glycogen. This is consistent with the expression of genes involved in glycogen synthesis and metabolism (Extended Data Figure 7; Supplementary Table 4).

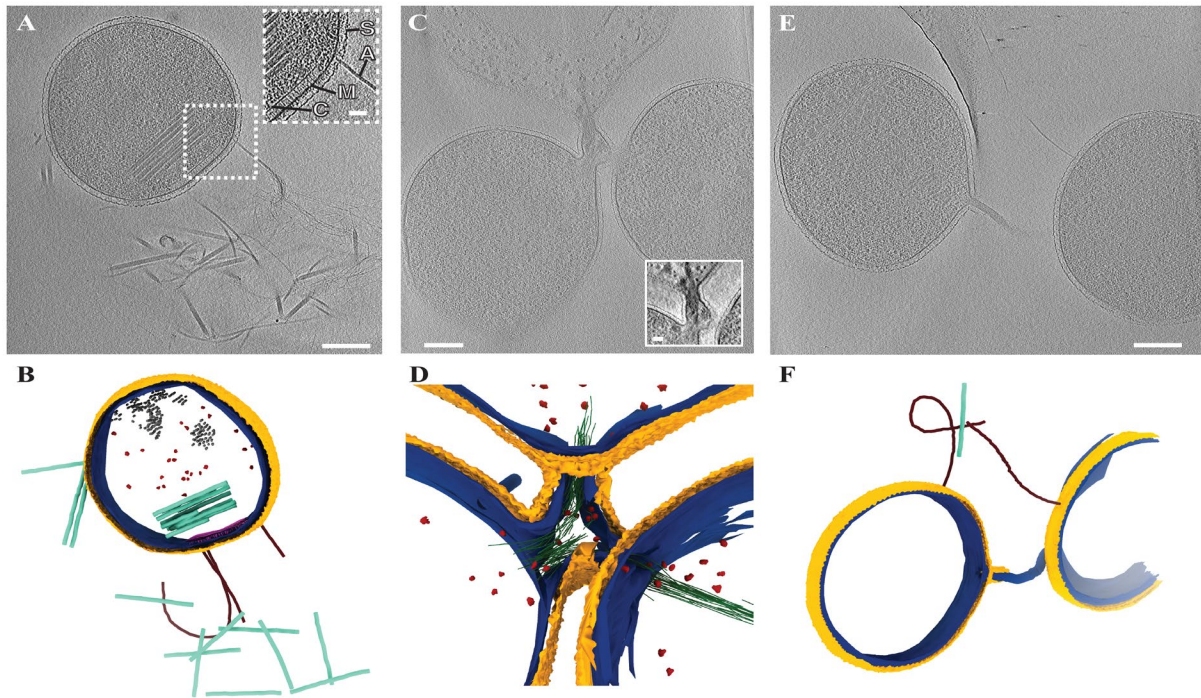


Figure 4. Cryo-electron tomography of LCB70 cells. A, B. Tomographic slice (A) and corresponding model (B) of an LCB70 cell. The S-layer is shown in yellow, the cytoplasmic membrane in blue, putative ribosomes in red, archaella in brown, putative virus-like particles in cyan, chemoreceptor arrays in purple, and unidentified semi-ordered filamentous structures in gray. The latter structures were not observed in tomograms of a later culture. Inset in A shows a magnified view (but different Z slice) of tomogram; M; cytoplasmic membrane, S, S-layer; C, chemoreceptor; A, archaellum. C, D. Tomographic slice (C) and corresponding model (D) of a three-way cell junction (inset in C shows higher contrast image) with a continuous cytoplasmic membrane and S-layer that connects three cells. Putative ribosomes (red), and thin unidentified filaments (green) are located inside the cell junction. Some of these filaments are contained within the volume connecting the three cells, while others span from one cell to another. E, F. Tomographic slice (E) and corresponding model (F) of two LCB70 cells connected by a cell-cell bridge that is only partially covered by an S-layer. For all tomographic slices the thickness is 18.04 nm. Scale bars, 200 nm; 50 nm for insets.

Additional tomograms of LCB70 cells acquired from cultures grown in the presence of lactate, pyruvate, or acetate, approximately a year after the first dataset had been collected, yielded consistent results for the morphology and presence of archaella, chemoreceptor arrays, and storage granules across treatments (Extended Data Figs. 6C and 8). However, LCB70 cells

lacked the abundant semi-ordered filamentous structures observed in the earlier culture, suggesting that these structures are likely not involved in the central metabolism of LCB70 (Extended Data Fig. 8).

During FISH analyses we observed LCB70 cells sometimes forming aggregates (Fig. 1C and Extended Data Fig. 9), suggesting that they might be able to form direct cell-cell interactions. Indeed, via cryoET, we observed cell-cell bridges between LCB70 cells with a continuous cytoplasmic membrane ($n = 4$) and in some cases a continuous ($n = 1$) or semi-continuous ($n = 2$) S-layer (Fig. 4, Extended Data Fig 10). We did not observe cell-cell bridges between LCB70 and other cell types, suggesting that either these interactions are LCB70-specific or that direct inter-species cellular connections are rare. We also visualized cell-cell bridging structures with larger compartments, which resembled vesicles (Fig. 4, Extended Data Fig. 10). We interpret these structures to be potential intermediate forms of the same cell-cell bridges surrounded by an S-layer that connect LCB70 cells. In one tomogram, the volume of a cell-cell bridge (120-140 nm inner diameter, 160-220 nm outer diameter) shared by three cells contained both putative ribosomes and thin filaments of unknown composition (Fig. 4 C,D; Extended Data Fig. 10; Supplementary Movie 2). At this point, we can only speculate about the nature of these interactions and how they form.

In bacteria, cell-cell bridges, including nanotubes, have been shown to be involved in diverse intra- and inter-species interactions, including the exchange of metabolites, electrons, and DNA (Baidya, Bhattacharya et al. 2018). While their function in archaea is not well understood, cell-cell bridges and other cell-cell connections have been observed in at least three different archaeal phyla (Liu, Soler et al. 2021). Cell-cell bridges of LCB70 cells (29-38 nm inner

diameter; 71-73 nm outer diameter) most closely resemble those observed in *Haloferax volcanii* (57-162 nm outer diameter). Similar to LCB070, *H. volcanii* nanotubes are covered by a continuous S-layer and occasionally contain ribosomes and filaments that are speculated to enable cytoplasmic exchange between cells (Sivabalasarma, Wetzel et al. 2021).

Conclusions

In summary, we demonstrate methyl-reducing hydrogenotrophic methanogenesis by *Ca. M. verstraetei* strain LCB70, a methanogen outside the Euryarchaeota, through enrichment cultivation, growth experiments, single cell activity measurements, conversion of isotopically labeled methanol, metabolic reconstructions, and transcriptomics. Additionally, we provide the first insight into the ultrastructure of a non-euryarchaeal methanogen, revealing structures related to motility, chemotaxis, storage granules, and cell-cell connections that may enable exchange of cytoplasmic material. Given the global distribution of these methanogens in diverse anoxic habitats, *Ca. M. verstraetei* and its close relatives (Wu, Zhou et al. 2023) likely contribute to anaerobic carbon cycling dynamics in both natural and engineered environments. Future culture-dependent studies of *Ca. M. verstraetei* will focus on the biochemistry and regulation of methanogenesis as well as structural and functional aspects of their cell biology.

Methods

Chemicals

Unless otherwise specified, all chemicals were obtained from Sigma.

Source of Inoculum and Cultivation

Sediments from hot spring LCB070 (44°34'01.8"N 110°48'20.2"W) located in the Lower Culex Basin (LCB) thermal complex of Yellowstone National Park, WY, USA (YNP) were obtained in October 2019. A mixture of surface sediments (~1 cm deep) and water (62 °C, pH 8.2) were collected into a sterile glass bottle that was sealed with a thick butyl-rubber stopper without headspace. After transport to the lab, sediments were stored at room temperature. Enrichment cultures were established using anoxic medium containing a base of (per liter): KH₂PO₄, 0.5 g; MgSO₄·7H₂O, 0.4 g; NaCl, 0.5 g; NH₄Cl, 0.4 g; CaCl₂·2H₂O, 0.05 g; 2-N-morpholino-ethanesulfonic acid (MES), 2.17 g; yeast extract, 0.1 g; and 0.002% (w/v) (NH₄)₂Fe(SO₄)₂·6H₂O, 5 mM NaHCO₃, 1 mL trace element solution SL-10, 1 mL Selenite-Tungstate solution, 1 mL CCM vitamins (Brandis and Thauer 1981), 0.0005% (w/v) resazurin, 10 mg of coenzyme-M, 2 mg sodium dithionite, 1 mM dithiothreitol, 1 mM Na₂S·9H₂O. The pH was adjusted to 6.5. Sediment slurry was added (10% v/v) and vials were sealed with thick butyl-rubber stoppers and aluminum crimps in an anoxic glove box (Coy). The headspace of the enrichments was sparged with N₂:CO₂ (90:10) for 5 minutes and set to 200 kPa. Methanol (MeOH) and H₂ were added to a final concentration of 10 mM and 50%, respectively. The cultures were incubated at 64 °C in the dark without shaking. Batch cultures were maintained by transfer of 10% v/v inoculum into fresh medium. A sediment-free culture was obtained after three transfers, after which the culture was maintained on increased MeOH concentrations of 40-50 mM and the addition of 10 mM sodium L-lactate as an additional carbon source. Replicate cultures for comparing methane production with or without H₂ and MeOH were performed in duplicate in 30 mL incubation volumes in a 60 mL serum bottle.

Fluorescence In Situ Hybridization and Biovolume Calculation

Subsamples were chemically fixed with paraformaldehyde (PFA, 2% final concentration) for 1 hour at room temperature, washed, and stored in 1x phosphate buffered saline (PBS) at 4°C. An oligonucleotide probe targeting most Methanomethyliciaceae 16S rRNA sequence contained in the Silva(Quast, Pruesse et al. 2012) database (version 132) was designed using the probe design tool in ARB(Ludwig, Strunk et al. 2004) (Msur657, 5'-CCCTCAACCTCTCCCGCC-3'). According to TestProbe(Quast, Pruesse et al. 2012) using the Silva138 database, this probe detects 71% of 16S rRNA sequences within the family Methanomethyliciaceae, and only binds two non-target sequences outside that group. Both sequences are found in the Geoarchaeales, which are absent from our enrichment culture. DOPE-FISH was performed according to Stoecker et al. 2010(Stoecker, Dorninger et al. 2010) using the Msur657 probe doubly labeled with FAM (obtained from IDT-DNA). In order to track Ca. Methanosuratincola verstraetei LCB70 and other archaeal cells, a general archaea-targeted probe Arch915(Stahl 1991), double labeled with Alexa Fluor 647, was used in combination with the FAM-labeled Msur657 probe and the DNA-stain 4',6-diamidino-2-phenylindole (DAPI). All hybridization reactions were carried out at a formamide concentration of 35% (Supplementary Table 6). Negative controls employed double-labeled NONEUB338(Wallner, Amann et al. 1993) and were performed in parallel at a formamide concentration of 35%.

Because the enrichment contained cell aggregates that could not be disassociated (e.g., by sonication or detergent addition) quantitative FISH on a per-cell basis was not possible. Instead, biovolume fractions of LCB70 (cells that bound Msur657 and Arch915) and other archaea (cells that bound to Arch915) in the culture was performed according to Daims 2009(Daims 2009).

Briefly, PFA-fixed biomass was applied to a slide and DOPE-FISH was performed with the Msur657 and Arch915 as described above, followed by counter-staining with DAPI and embedding in Citifluor. Cells were imaged with a Leica DM4B epifluorescence microscope. Images were segmented and biovolumes calculated with the daime software package (Daims, Lückner et al. 2006).

DNA Extraction and Metagenome Sequencing

Hot spring sediment from site LCB070 was collected in October 2019 using a sterile metal cup and was immediately frozen in a dry ice-ethanol bath. DNA was extracted from 10 mL of sediment following the protocol by Zhou et al. 1996 (Zhou, Bruns et al. 1996). Truseq libraries were prepared and sequenced at the DOE Joint Genome Institute (JGI) on the Illumina NovaSeq S4 platform following a 2 x 150 bp indexed run recipe.

15 mL of the first transfer of the LCB70 culture was collected under anoxic conditions and centrifuged at 16,000 x g for 5 minutes to pellet cells, the supernatant was removed, and the pellet stored at -80 °C. Genomic DNA was extracted from the cell pellet using the protocol by Zhou et al. (Zhou, Bruns et al. 1996), with the following modifications. The proteinase K step was extended to 1 hour and the precipitation was performed in the presence of 0.7x volumes isopropanol and 0.1x volume of 3M sodium acetate. Crude extracts were purified using a Zymo clean and concentrator kit (DCC-10, Zymo Research) according to the manufacturer's instructions. Purified genomic DNA was shipped to the Michigan State University genomics core for library preparation and sequencing. A metagenomic library was constructed with the TruSeq Nano DNA kit according to the manufacturer's recommendations. The library was sequenced on

an Illumina NovaSeq 6000 platform using the NovaSeq XP reagent kits, SP flow cell, and followed a 2 x 150 bp indexed run recipe.

Nanopore long-read sequencing was performed using a MinION platform and a R9.4.1 flow cell (Oxford Nanopore Technologies). Library preparation was performed using the rapid kit (SQK-RBK004) according to the manufacturer's instructions. Flow cells were run for 72 hours, resulting in 1.3 Gbp of total sequence data. Base-calling was performed with Guppy (v5.0.16+b9fcd7b) using model dna_r9.4.1_450bps_hac and parameters, --trim_barcodes, --detect_mid_strand_adapter, and --detect_mid_strand_barcodes.

Metagenome Assembly, Binning, and Quality Assessment

For the sediment metagenome from site LCB070, raw metagenomic reads were processed according to JGI's standard workflow, and quality controlled reads were assembled using SPAdes(Nurk, Meleshko et al. 2017) 3.14.1 with options -m 2000 -k 33,55,77,99,127 and -meta. For the enrichment culture metagenome, Illumina reads were evaluated with FastQC v0.11.5 to determine best parameters before trimming (quality, linker, and adapter). Artifact and common contaminant removal was done with rqcfilter2 (maxns=3, maq=3, minlen=51) followed by error correction with bbcms (mincount=2, hcf=0.6) from the BBTools suite v38.94(Bushnell 2014). Resulting reads were assembled with SPAdes v3.15.3 (-k 33,55,77,99,111 -meta -only-assembler) and coverage was determined by mapping the cleaned and corrected reads to the assembled sequences with bbmap (ambiguous=random). Assembled sequences $\geq 2,000$ bp in length were binned with four programs: Maxbin v2.2.7(Wu, Tang et al. 2014), Concoct v1.0.0(Alneberg, Bjarnason et al. 2014), Metabat v2.12.1 (with and without coverage)(Kang, Li et al. 2019), and Autometa v1 (bacterial and archaeal modes, including the machine learning

step) (Miller, Rees et al. 2019) followed by bin refinement with DAS_Tool v1.1.2 (Sieber, Probst et al. 2018), as previously described (Kohtz, Jay et al. 2022). CheckM v1.1.3 (Parks, Imelfort et al. 2015) was used to assess MAG completeness and redundancy.

Assembly of the Circular Genome of LCB70

A long-read assembly was created using nanopore reads with metaFlye (v. 2.8.2), specifying three polishing iterations (Kolmogorov, Bickhart et al. 2020). This step produced a circular assembly of the LCB70 genome. Long-read polishing was done with Medaka (v. 1.5.0), consisting of mini_align, medaka_consensus, the model 941_min_hac_g507, and the medaka stitch commands. Following this, Polypolish (Wick and Holt 2022) was used to polish the assembly three times with the Illumina reads. Finally, three additional rounds of polishing using the Illumina reads were carried out with Polca (Zimin and Salzberg 2020), producing a final polished assembly.

Amplicon Sequencing and Analysis

A 1 mL aliquot of culture was pelleted by centrifugation (16,000 x g; 5 minutes) and DNA from the cell pellet was extracted using the FastDNA Spin Kit for Soil (MP Biomedicals) following the manufacturer's guidelines. Archaeal and bacterial 16S rRNA genes were amplified with the Earth Microbiome Project primer set 515F and 806R (Apprill, McNally et al. 2015, Parada, Needham et al. 2016), amplicon libraries were prepared as described previously (Reichart, Jay et al. 2020), and sequencing was performed at the Molecular Research Core Facility at Idaho State University (Pocatello, ID) using an Illumina MiSeq platform with 2 x 250 bp paired end read chemistry. Reads were processed as described previously (Reichart, Jay et al. 2020) with QIIME 2 (version 2020.2) (Bolyen, Rideout et al. 2019). Briefly, barcode

sequences were removed with cutadapt and then reads were truncated (209 forward, 215 reverse). Reads were denoised, merged, and chimera-checked with DADA2 (Callahan, McMurdie et al. 2016) using default settings. Taxonomic assignment of amplicon sequence variants was done using the sklearn method and the Silva SSU database (Quast, Pruesse et al. 2012) release 132.

Phylogenetic Analyses

The 16S rRNA sequences encoded in publicly available *Methanomethylicia* MAGs were aligned and masked against reference archaeal 16S rRNA sequences using SSU-ALIGN v0.1.1 (Nawrocki, Kolbe et al. 2009), which produced a final alignment of 1,376 positions. Maximum likelihood analysis was performed using FastTree v2.1.10 and default parameters.

A set of 33 single-copy marker proteins (Supplementary Table 5) were collected from *Methanomethylicia* MAGs and reference archaeal genomes. These markers were aligned with MUSCLE (Edgar 2004), trimmed with trimAL (Capella-Gutiérrez, Silla-Martínez et al. 2009) using a 50% gap threshold, and concatenated to produce a final alignment of 7,118 positions. IQtree2 was used to reconstruct a maximum likelihood phylogenetic tree and ModelFinder (Kalyaanamoorthy, Minh et al. 2017) was used to select the best fit model with the additional option --madd LG+C60, LG+C60+F+G, LG+C60+F+R. The best fit LG+F+R10 model was selected according to Bayesian inference criterion and branch support was evaluated with 1,000 ultrafast bootstraps and 1,000 iterations of the SH-like approximate-likelihood ratio test (Guindon, Dufayard et al. 2010).

The McrA sequence of LCB70 was aligned against a set of publicly available reference sequences using MAFFT-linsi (Katoh and Standley 2013), trimmed with trimAL using a 50%

gap threshold. A maximum likelihood phylogenetic tree was reconstructed using IQtree2, using the LG+C60+F+G model and 1,000 ultrafast bootstraps.

Sequences of catalytic subunits of group 4 [NiFe]-hydrogenases encoded by LCB70 were aligned against the HydDB reference sequences and reference FpoD/NuoD subunits. Additional Ehi sequences were collected through Blastp searches of the NCBI nr and IMG databases using the strain LCB70 sequence as a query. References were aligned with MAFFT-linsi and trimmed with trimAL using a 50% gap threshold, producing a final alignment of 365 residues. Maximum likelihood phylogenetic trees were constructed with IQtree2 with the model LG+C60+R+F and 1000 ultrafast bootstraps.

Annotation and Reconstruction of Metabolic Potential

MAGs were annotated using Prokka v1.14.6(Seemann 2014) with both default and custom/in-house annotation databases. Refinement and confirmation of the initial annotation was done by submission of protein sequences to the NCBI conserved domain database (Lu, Wang et al. 2020) and HHpred (Zimmermann, Stephens et al. 2018) using default settings. Furthermore, the strain LCB70 genome was uploaded to the IMG/M database for annotation (Chen, Chu et al. 2019). The catalytic subunits of [NiFe] hydrogenases were submitted to HydDB (Søndergaard, Pedersen et al. 2016) for classification and further confirmed through phylogenetic analysis (above).

RNA Extraction, Sequencing, and Analysis

Enrichment cultures of LCB70 were grown in triplicate in 30 mL of media with 100 mM methanol and hydrogen (50%) and supplemented with 10 mM of either lactate, pyruvate, or acetate. During the exponential phase of methane production, culture bottles were rapidly cooled

from 64 °C by putting them in an ice bath which was placed at -20 °C for 15 minutes; vials were subsequently kept on ice during the procedure. Cells were concentrated by centrifugation at 12,000 x g for 10 minutes and the cell pellet was resuspended in 800 µL of Zymo DNA/RNA Shield and stored at 4 °C overnight. The following day, cells were mixed 1:1 with Zymo RNA lysis buffer and added to Zymo BashingBead lysis tubes (Zymo Research, Irvine, CA) and vortexed at maximum speed for 10 minutes. The tubes were centrifuged at 14,000 x g for 15 minutes and RNA was purified from the supernatant with a Zymo Quick-RNA miniprep kit according to the manufacturer's instructions and including the DNase I step. Extracted RNA was sent to SeqCenter (Pittsburgh, PA) which provided an additional DNase I treatment (Invitrogen), library preparation, and sequencing. Libraries were prepared with Illumina's Stranded Total RNA Prep Ligation with Ribo-Zero Plus kit and 10 bp unique dual indices. Sequencing of the libraries was performed on a NovaSeq X Plus platform producing 2x150 bp reads. bcl-convert (v.4.1.5) was used for demultiplexing, quality control, and adapter trimming. Reads were then processed with rqcfilter 2 (rna=t, trimrnaadapter=t, qtrim=rl, trimq=10, maq=20, maxn=0, minlen=50, mlf=0.33, removeribo=f) and mapped to the genes of all MAGs obtained from the culture using salmon (v.1.10.2; --validateMappings --seqBias) (Patro, Duggal et al. 2017). Reads mapping to rRNA genes were excluded before normalization. Reads mapping to each gene were normalized to gene length and expressed as transcripts per kilobase million (TPM).

Chemical Analyses of Methane and Methanol

During cultivation, 250 µL subsamples of the headspace were taken with a gas tight syringe (Hamilton) and injected into 10 mL autosampler vials that had been sealed with grey chlorobutyl septa. Samples were taken from the autosampler vials and injected into a Shimadzu

2020-GC gas chromatograph equipped with a GS-CarbonPLOT column (30 m x 0.32 mm; 1.5 μ m film thickness; Agilent) and a Rt-Q-BOND column (30 m x 0.32 mm; 1.5 μ m film thickness; Restek) using Helium as a carrier gas. The injector, column, and flame ionization detector (FID) were maintained at 200 °C, 50 °C, and 240 °C, respectively. Methane concentrations were calculated based on injection of a standard curve. For methanol quantification, 500 μ L liquid subsamples were taken with a needle and syringe and placed immediately on ice. The liquid was cleared by centrifugation (16,000 x g; 5 minutes; 4 °C). Then, 400 μ L of supernatant was placed in a sealed, gas-tight 10 mL vial and stored at -20 °C. For analysis, the liquid was heated to 80 °C for 5 minutes and then 500 μ L of headspace was injected into the Shimadzu 2020-GC gas chromatograph. Methanol concentrations were calculated based on injection of a standard curve. All injections on the Shimadzu 2020-GC were performed with a robotic autosampler (AOC-6000).

BONCAT-FISH and Inhibition Experiments

Experiments were carried out in 30 mL culture volumes in 60 mL serum bottles. A control bottle contained 10 mM BES inhibitor at the start of the experiment. After 10 days of growth one set of replicate cultures was spiked with 10 mM BES and the cultures were incubated for 48 hours, after which 100 μ M of the amino acid analog L-homopropargylglycine (HPG) was added to all cultures, except a no HPG control bottle. After HPG addition, the cultures were incubated for 24 hours, and subsamples were fixed in PFA for bioorthogonal non-canonical amino acid tagging and fluorescence in situ hybridization (BONCAT-FISH) analyses. The azide-alkyne click chemistry reaction was performed as described in Hatzenpichler et al. 2016 (Hatzenpichler, Connon et al. 2016) and used azide-labeled TexasRed (Click Chemistry Tools).

Following the click chemistry reaction, DOPE-FISH was performed as described above with a 2xFAM labeled Msur657 probe.

Stable Isotope Tracing

For tracking the conversion of ^{13}C -MeOH to ^{13}C -CH₄, active cultures were incubated in the presence of ^{12}C -MeOH or ^{13}C -MeOH under a 90% N₂, 10% CO₂ headspace. During the incubation, headspace samples were injected into a Shimadzu QP2020 NX GCMS equipped with a GS-CarbonPLOT column (30 m x 0.35 mm; 1.5 μm film thickness; Agilent) using the method described in Ai et al. 2013 (Ai, Zhu et al. 2013) and Helium as a carrier gas. The collection was run in Selected Ion Monitoring mode and peak areas corresponding to m/z of 16 for $^{12}\text{CH}_4$, 17 for $^{13}\text{CH}_4$, 44 for $^{12}\text{CO}_2$, and 45 for $^{13}\text{CO}_2$ were used for quantification. All injections on the Shimadzu QP2020 NX GCMS were performed with a robotic autosampler (AOC-6000).

Habitat Distribution

The 16S rRNA gene sequence for strain LCB70 was submitted to the IMNGS webserver (Lagkourdos, Joseph et al. 2016) to search the NCBI Sequence Read Archive (SRA) for sequences with a sequence similarity cutoff of 97% and a minimum size of 200 bp. Metadata on the sample collection source was collected from each SRA sample that had sequence hits.

Plunge Freezing for Cryo-Electron Tomography

Enrichment cultures containing LCB70 grown at 64 °C with different nutrition sources (lactate, acetate, pyruvate) or treated with BES (with lactate) were plunge frozen. For the initial data collection, the following steps were performed inside an anoxic glove box: 500 μL of the enrichment culture grown with lactate were centrifuged at 16,000 x g for 10 min at 20 °C and the

resulting pellet was resuspended in 100 μ L of the supernatant. All other sample preparations were performed without the concentration step to keep the samples at 64 °C and to avoid potential artifacts. All samples were mixed with Protein A-conjugated 10 nm colloidal gold before plunge freezing. Afterwards, the culture was removed from the glove box and, within a few seconds during which the culture was exposed to lab air, 3.5 μ L of the enrichment culture were then applied to glow-discharged copper EM grids (R2/1, Quantifoil), automatically blotted for 4, 8 or 10 seconds and plunged into liquid ethane using a Vitrobot Mark IV (Thermo Fisher Scientific) (Iancu, Tivol et al. 2006). The plunge frozen grids were clipped into autogrids, stored in liquid nitrogen, and shipped to ETH Zurich in a liquid nitrogen cooled dry shipper.

Cryo-Electron Tomography

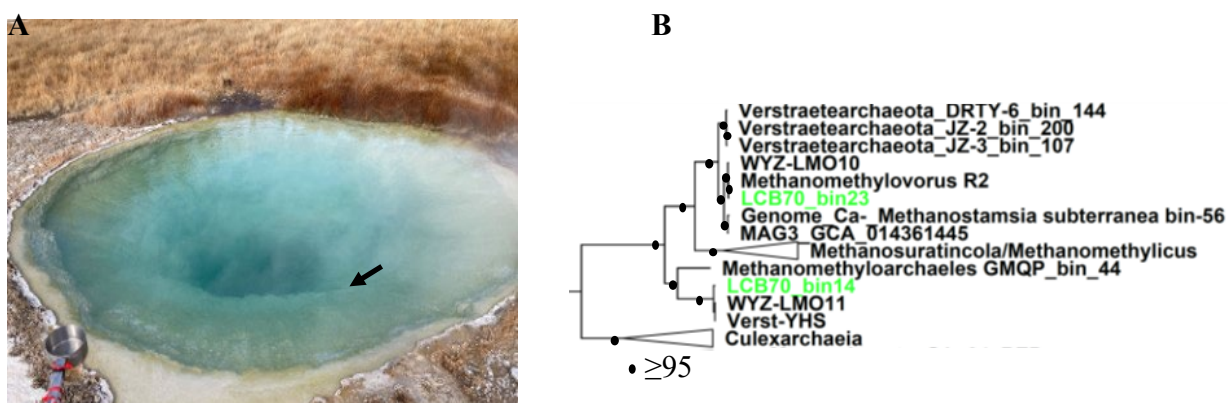
Cryo-electron tomography datasets were collected on a Titan Krios G4 EM (ThermoFisher) operating at 300 kV and equipped with BioContinuum imaging energy filter (slit width 20 eV) and a K3 Summit camera (Gatan). The tilt series were collected at a magnification of 19,500 (effective pixel size 4.51 Å) with a defocus of -8 μ m using a bidirectional scheme from -60° to +60° in 2° incremental steps using SerialEM (Mastronarde 2005). The total dose of a tilt series was 165 to 180e⁻/Å².

Confocal Raman Microspectroscopy

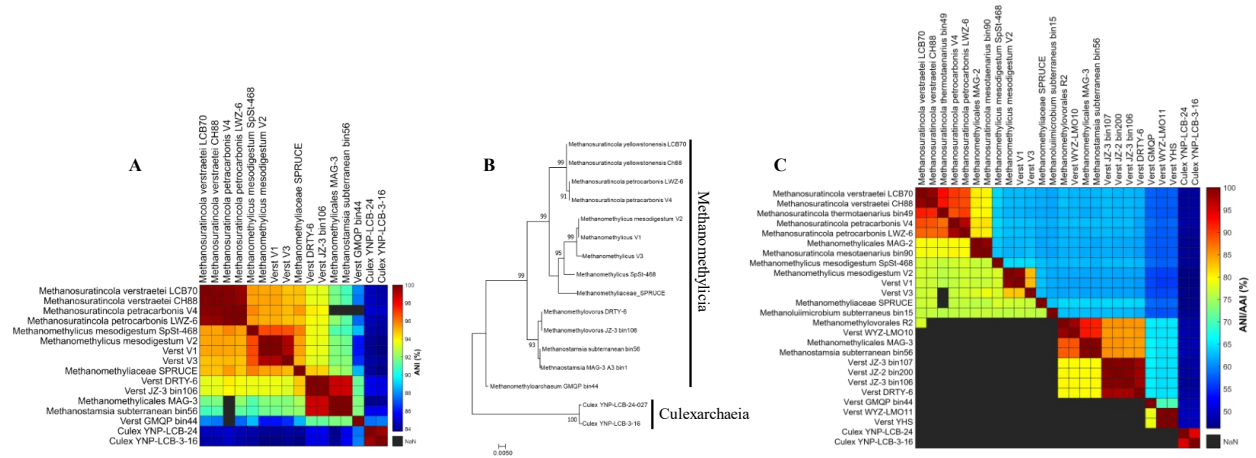
Correlative FISH-Raman microscopy was performed as described in Schaible et al. (Schaible, Kohtz et al. 2022). Succinctly, single LCB70 cells were identified on stainless-steel slides by dual hybridization with the Msur657 and Arch915 probes as described above. Single cell Raman spectra were acquired using the 532 nm laser on a LabRAM HR Evolution Confocal Raman microscope (Horiba Jobin-Yvon) equipped with 300 grooves/mm diffraction grating and

a 100x dry objective. For each cell ($n = 18$), four acquisitions of ten seconds each were collected from 400 to 3,200 cm^{-1} at a laser power of 4.5 mW. Spectra were processed using the LabSpec software package (v.6.5.1.24, Horiba), baselined, normalized to the maximum intensity within the 2,800-3,100 cm^{-1} region. Raman spectra were analyzed for the presence of indicator peaks of glycogen (478-484, 840-860, and 930 cm^{-1}), polyhydroxyalkanoates (432, 840, and 1726 cm^{-1}), and poly-phosphate (690 and 1170 cm^{-1}) (Majed and Gu 2010, Fernando, McIlroy et al. 2019).

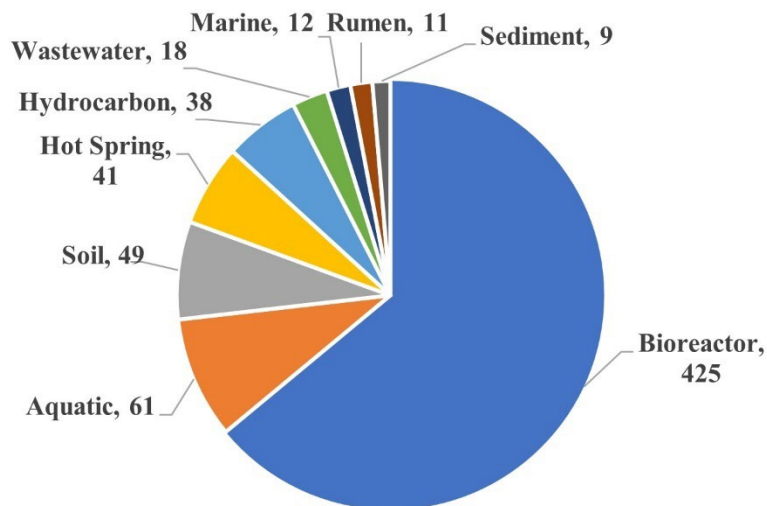
Supporting Information



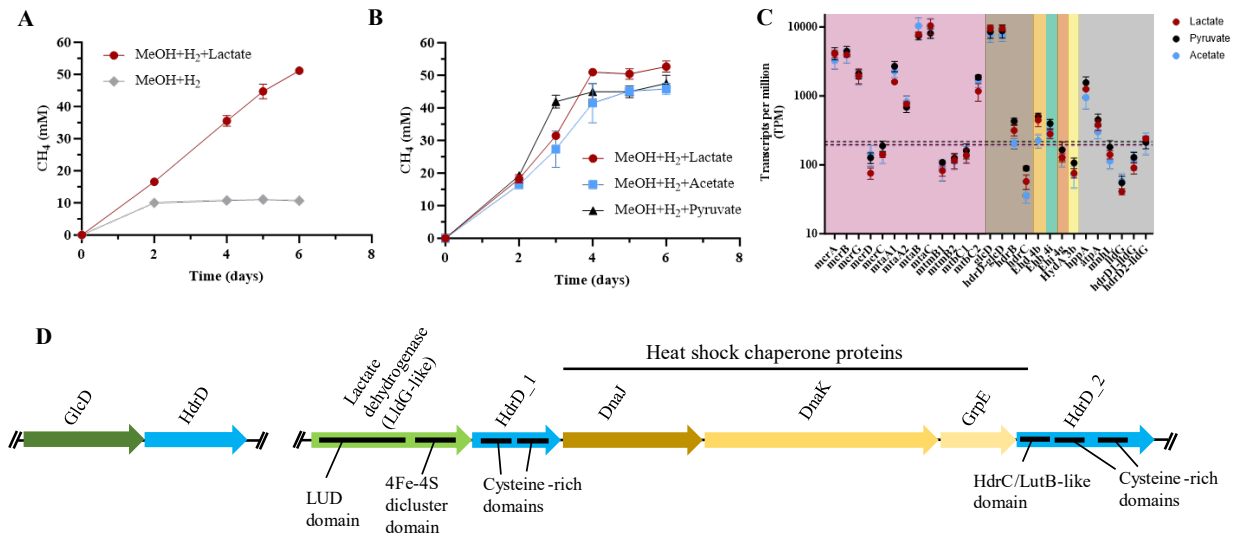
Extended Data Figure 1. Image of hot spring LCB070 and diversity of Methanomethylica MAGs recovered from the hot spring sediment metagenome. A. Hot spring LCB070 in Yellowstone National Park. The arrow indicates the location where sediment samples were taken. B. Phylogenomic tree of Methanomethylica, rooted with MAGs from the closely related lineage, Culexarchaeia (Kohtz, Jay et al. 2022). MAGs recovered from the sediment metagenome are highlighted in green.



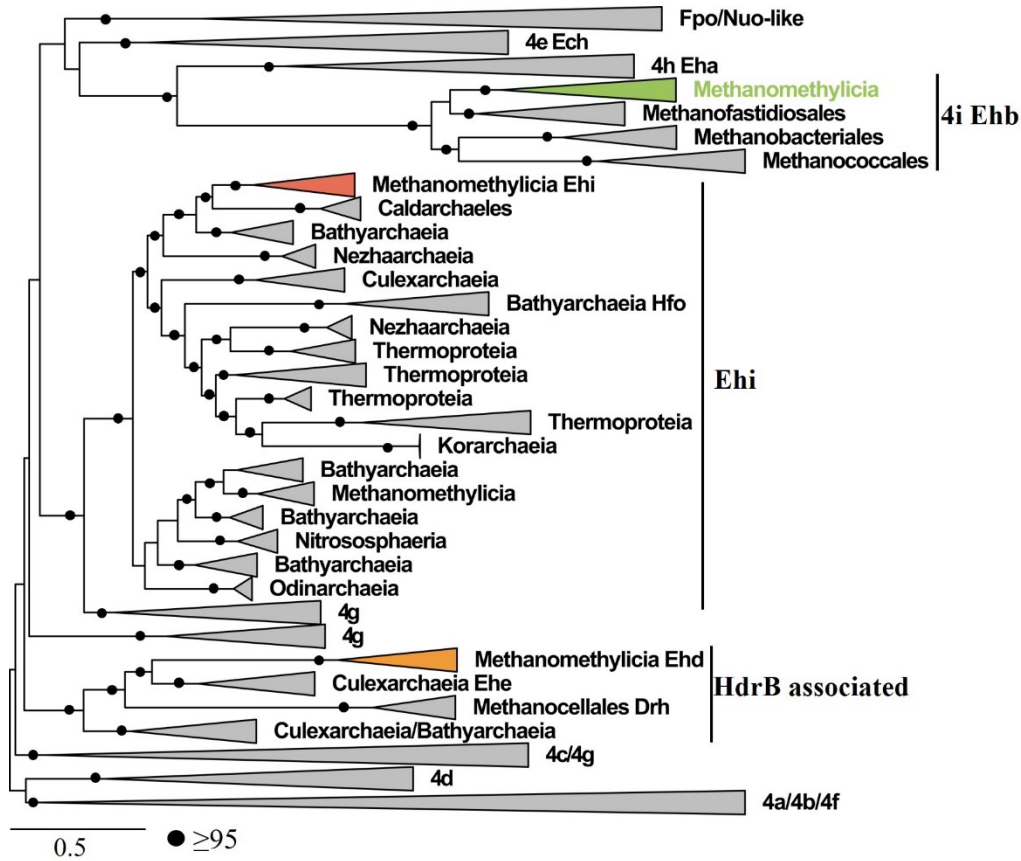
Extended Data Figure 2. Pairwise comparison of 16S rRNA sequence identity and phylogeny and pairwise comparison of *Methanomethylicia* MAGs. A. Pairwise percent sequence identity was calculated with BLASTn. B. Maximum likelihood phylogenetic tree of full length and partial 16S rRNA genes encoded in strain LCB70 and representative *Methanomethylicia* MAGs. Numbers at the nodes indicate bootstrap support, only values above 90 are shown. C. Pairwise average amino acid identity of MAGs (AAI, top) and average nucleotide identity (ANI, bottom) values (in %) were calculated by compareM and FastANI, respectively. Two MAGs from the closely related class *Culexarchaeia* (Kohtz, Jay et al. 2022), were used as an outgroup. The two *Verstraetearchaeota*-affiliated MAGs reconstructed from the original hot spring (LCB070 bin14 and bin 23) have less than 75% ANI similarity to the genome of *Ca. M. verstraetii* strain LCB70.



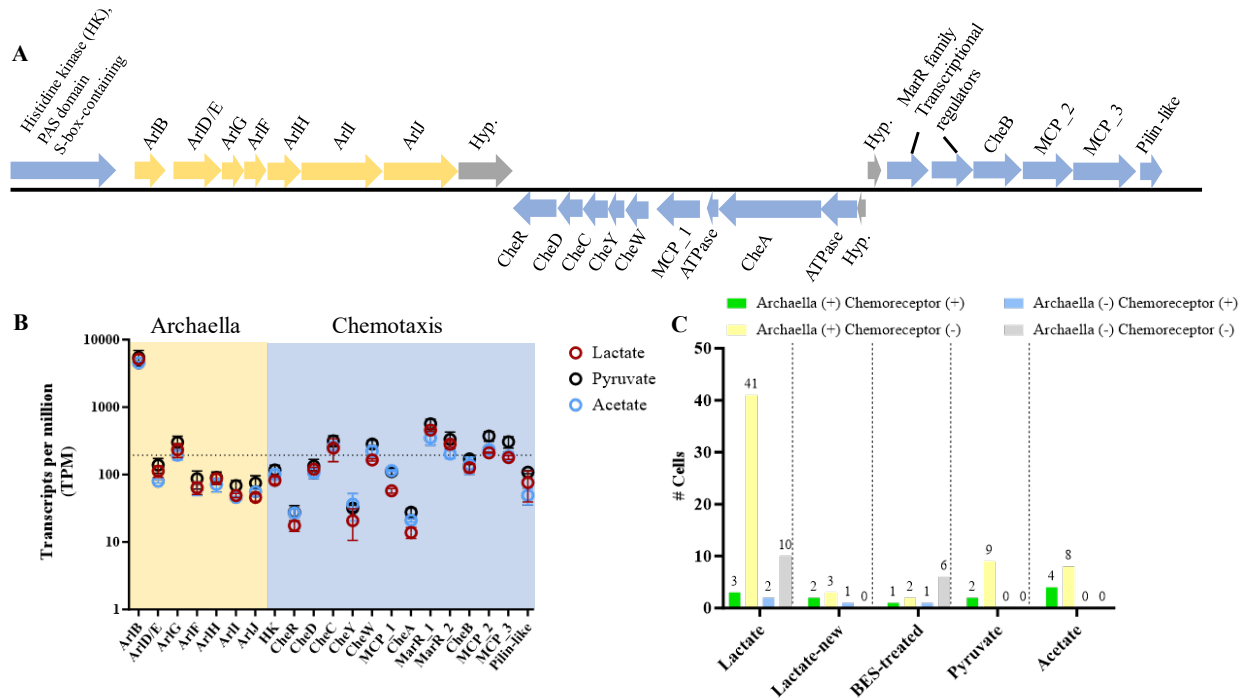
Extended Data Figure 3. Habitat distribution of *Ca. M. verstraetei* LCB70 and related species. Sequences were identified by searching the NCBI SRA database using the IMNGS webserver with default settings. Graph is based on the presence of 16S rRNA gene sequences with >97% sequence identity to strain LCB70 across 664 public samples.



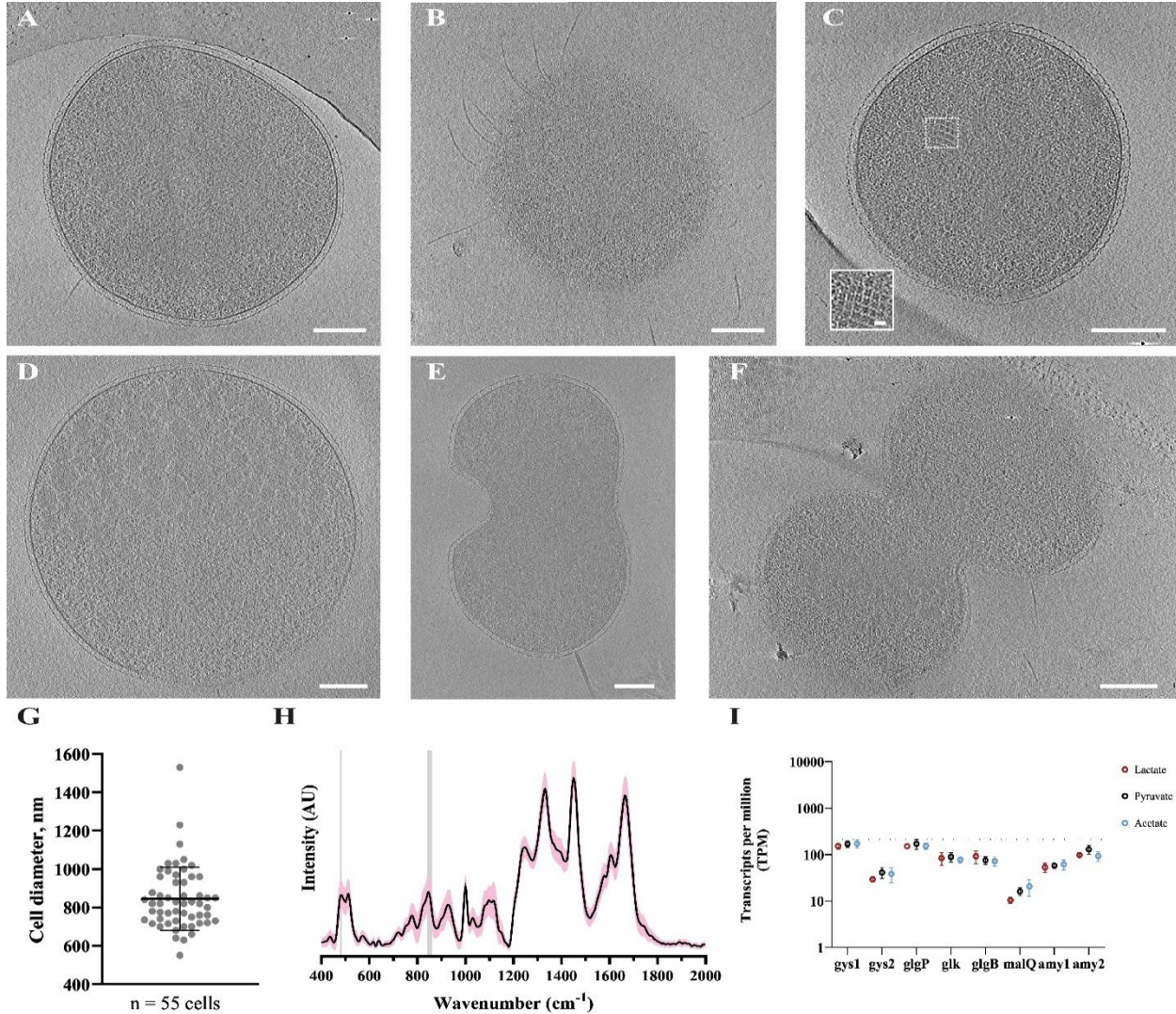
Extended Data Figure 4. Growth of culture LCB70 on different carbon substrates and genomic context of putative lactate dehydrogenase and heterodisulfide reductase (HdrD) subunits. **A**. Growth of culture LCB70 on 50 mM methanol (MeOH) and 50% hydrogen (H₂) in the presence or absence of L-lactate (10 mM). Data symbols for methane are the mean ($n = 3$ biological replicates) $\pm$ s.d. Where not visible, the error bars are smaller than the symbol. **B**. Growth of culture LCB70 on 50 mM MeOH and 50% H₂ with 10 mM of either lactate, pyruvate, or acetate. Data symbols for methane are the mean ($n = 3$ biological replicates) and error bars are one standard deviation. **C**. Transcriptional activity of LCB70 during growth on methanol and hydrogen with lactate, pyruvate, or acetate. Plotted are the mean $\pm$ s.d. ($n = 3$ biological replicates). Where not visible, the error bar is smaller than the symbol. Dashed lines represents the average TPM of all genes. **D**. Organization of putative lactate dehydrogenase genes, double slashes indicate the GlcD and LldG clusters are in different sections of the strain LCB70 genome. Sequences are indicated by arrows and distinct domains within the protein coding sequence are indicated by black bars. LUD, Lactate Utilization Domain.



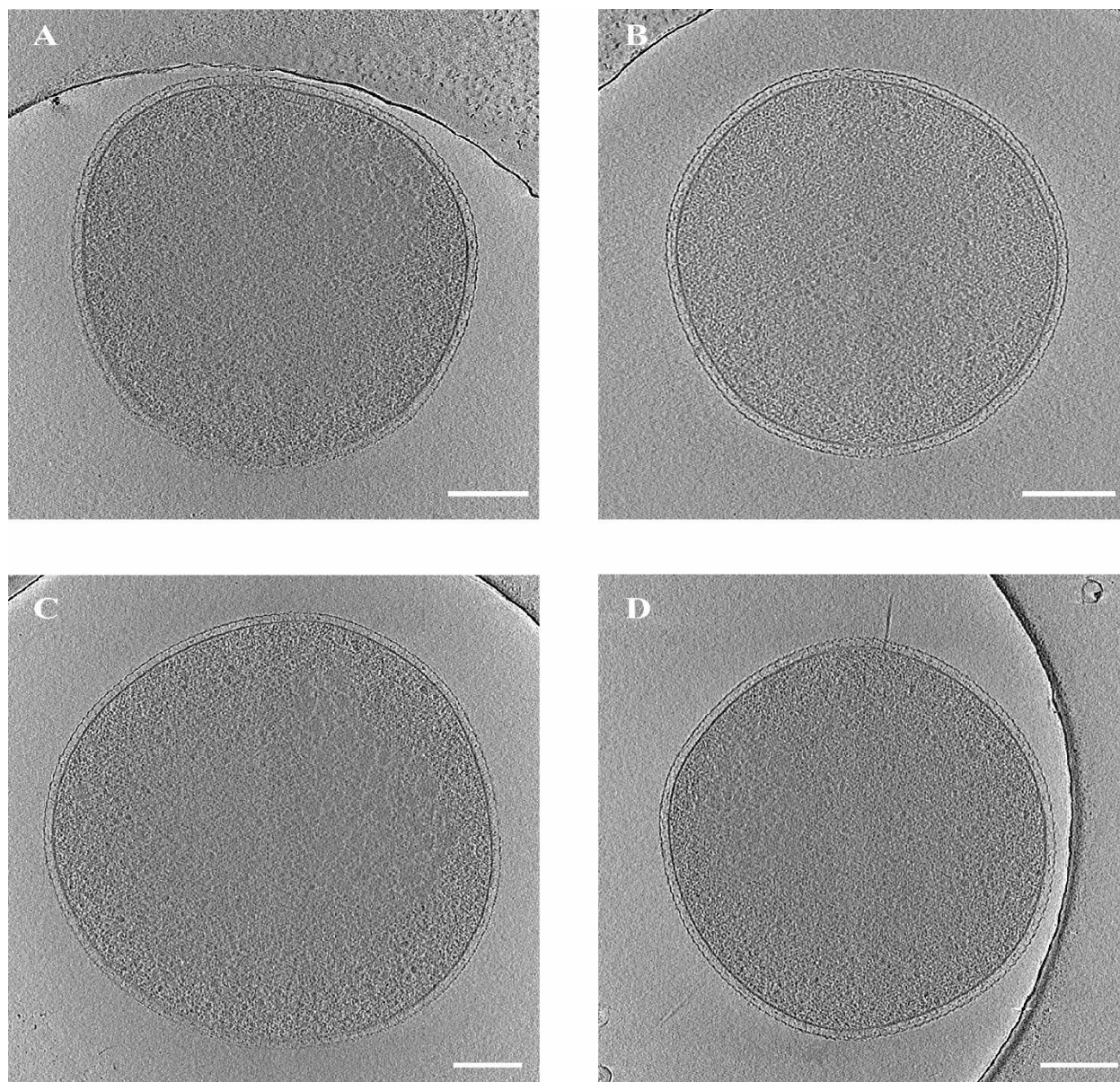
Extended Data Figure 5. Diversity of Group 4 [NiFe] hydrogenases encoded by strain LCB70. Maximum likelihood phylogenetic tree of group 4 [NiFe]-hydrogenases. The colors for the Ehb, Ehd, and Ehi clades match those in Figure 3A. Circles at nodes indicate ultrafast bootstrap values higher than 95.



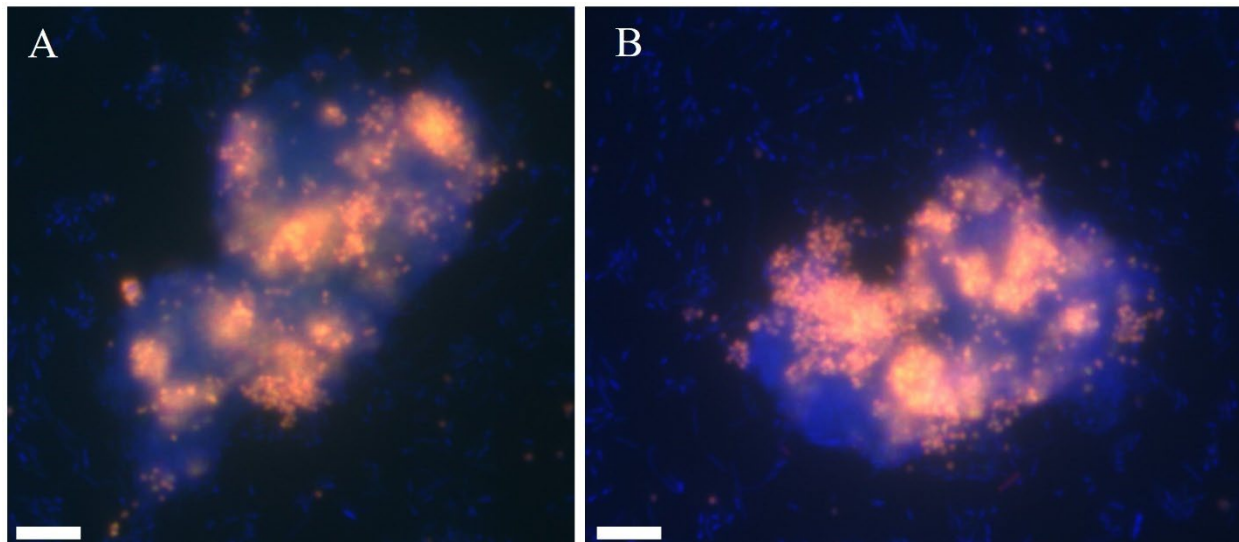
Extended Data Figure 6. Genomic organization, expression, and occurrence of archaeella and chemoreceptors within tomograms. **A**. Organization of archaeella (purple) and chemotaxis gene clusters (pink). Hypothetical proteins (Hyp.) are shown in gray. MCP, Methyl-accepting chemotaxis proteins. PAS domain, Per-Arnt-Sim domain. **B**. Expression profile of archaeella and chemotaxis genes for cultures grown on methanol and hydrogen and either lactate, pyruvate, or acetate as the carbon source ($n = 3$ biological replicates). Plotted are the mean values $\pm$ SD. Where not visible, the error bar is smaller than the symbol. Dashed line represents the average TPM of all genes. **C**. Occurrence of archaeella and chemoreceptor arrays in tomograms of strain LCB70. Note that due to differences in contrast between the tomograms, estimates of chemoreceptor arrays may be slightly underestimated.



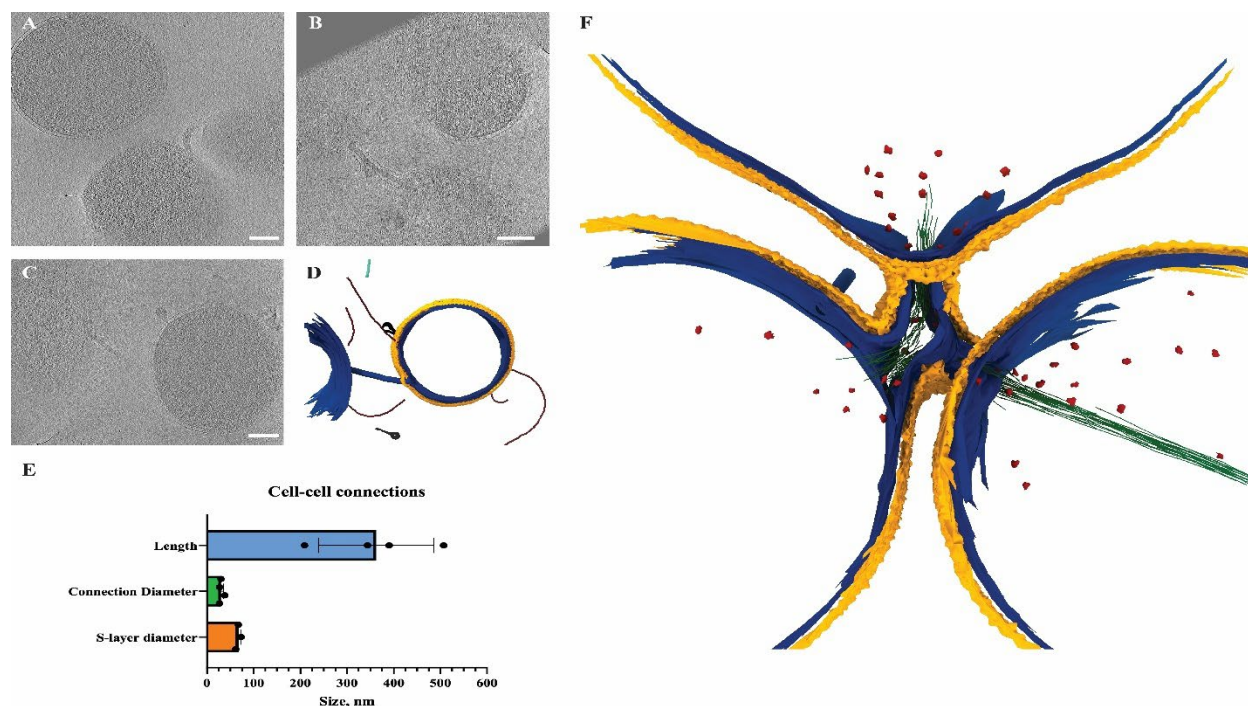
Extended Data Figure 7. Cell biology of strain LCB70. A, B. Two tomographic slices of the same archaeallated LCB70 cell. C. A LCB70 cell with semi-ordered unidentified filamentous structures. A zoomed-in view is shown in the inset. D. A tomographic slice of a cell with putative storage granules. E, F. Examples of potentially dividing LCB70 cells. G. Variation in LCB70 cell diameters (n = 55 cells). The center line represents the mean cell diameter and whiskers represent $\pm$ SD. For all tomographic slices the thickness is 18.04 nm. Scale bars, 200 nm; 25 nm for the inset. H. Raman spectra of the fingerprint region (400-2000 cm^{-1}) of 18 LCB70 cells. Black line shows the average and pink shaded area is one standard deviation. Gray shaded regions are marker regions for glycogen (478-484, and 840-860 cm^{-1}) (Fernando, McIlroy et al. 2019). I. Transcriptional activity of genes involved in glycogen metabolism for cells grown on methanol and hydrogen with either lactate, pyruvate, or acetate as carbon sources (n = 3 biological replicates each). Data are presented as mean values $\pm$ SD. Where not visible, the error bar is smaller than the symbol. Dashed lines indicate the average TPM for each treatment. For full gene names see Supplementary Table 4.



Extended Data Figure 8. Representative tomograms of LCB70 cells grown with different carbon sources. A. Tomographic slices of LCB70 cells grown on 50 mM methanol and 50% hydrogen with 10 mM lactate; B. 10 mM lactate with BES treatment; C. 10 mM pyruvate; D. 10 mM acetate. Scale bar, 200 nm. The thickness of tomographic slices is 18.04 nm.



Data Figure 9. A, B Two examples of cell aggregates in the culture. Cells are stained with the general nucleic acid stain DAPI (blue), and the Methanomethylicaceae-specific FISH probe Msur657 (green), and the general archaeal probe Arch915 (red). LCB70 cells bind to both the Msur657 and Arch915 probes, appearing orange. Scale bars, 5 μ m. Shown are representative micrographs from 3 independent biological replicates.



Extended Data Figure 10. Cell-cell connections between LCB70 cells. A, B. Two examples of interactions between LCB70 cells. Cell-cell bridges without (panel A) or with S-layer (panel B) formed from the cytoplasmic membrane connecting two cells. C, D. Tomographic slice (C) and corresponding model (D) of a cell-cell bridge without a surrounding S-layer. Structures in black are interpreted to either represent virus-like particles or protrusions of the cell membrane. E. Barplot represents the variation in length ($n = 4$ tomograms), diameter ($n = 4$ tomograms), and S-layer diameter of the tube connection between cells ($n = 3$ tomograms), from which only one tube is fully covered by S-layer, two are covered only in area of the cell contact, and one is not covered at all in S-layer. The number in the bar is the mean and error bars represent the standard deviation. For all tomographic slices, the scale bar is 200 nm and the thickness is 18.04 nm. F. A zoomed out view of the model of a junction between the three cells shown in Fig. 4C. S-layer colored in yellow, cytoplasmic membrane in blue, putative ribosomes in red, and thin filaments in green. Scale bars, 200 nm.

Etymology

Strain LCB70 is a methanogen affiliated with the candidate genus *Methanosuratincola*, class *Methanomethylicia*, phylum *Thermoproteota*, for which we propose the name *Ca. Methanosuratincola verstraetei* sp. nov.

Me.tha.no.su.rat.in'co.la. N.L. pref. methano- pertaining to methane; L. masc. or fem. n. incola, inhabitant; N.L. masc. n. Methanosuratincola, methanogen inhabiting the Surat Basin, where this lineage was discovered via metagenomics. ver.strae.te'i N.L. gen. n. verstraetei, in honor of the contributions of Professor Willy Verstraete (Ghent University, Belgium) to anaerobic microbiology and biotechnology.

Locality. Sediment from a hot spring, identified as feature LCB070 in a recent survey of Yellowstone National Park (WY, USA) geothermal features.

Diagnosis. A thermophilic methyl-reducing hydrogenotrophic methanogen of the phylum Thermoproteota that grows as coccoid cells with a diameter of 845 ± 163 nm. Its genome has a length of 1.52 Mb and a GC content of 54.6%.

Supplemental Discussion

The potential for direct use of lactate as an electron donor in CoM-S-S-CoB reduction presents some unique metabolic scenarios for LCB70. Lactate is a common byproduct of fermentative organisms and, during competition for H₂, it might serve as an alternative or additional electron donor for methanogenesis by LCB70. Pyruvate formed from lactate oxidation could provide a carbon source for anabolism via the gluconeogenesis pathway or further oxidized to acetyl-CoA and reduced ferredoxin by pyruvate:ferredoxin oxidoreductase. Acetyl-CoA synthetase could then convert acetyl-CoA to acetate and ATP. Given the high expression of glcD and HdrD, revealing the connection between lactate and methanogenesis in the metabolism of LCB70 will require further studies on the substrates and roles of these enzymes.

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Marika Palmer (University of Manitoba) for input on taxonomy. We thank Professor Willy Verstraete (University of Ghent, Belgium) for allowing us to name this newly cultured methanogen after him.

Data Availability

The LCB70 enrichment culture metagenomic, metatranscriptomic, and amplicon data are deposited under NCBI BioProject ID PRJNA916083. The LCB070 hot spring metagenome is publicly available on the IMG database under the taxon ID 3300043544 and the strain LCB70 genome under taxon ID 8012933652. Cryo-tomograms are deposited at EMDB/EMPIAR under accession numbers EMD-50298 to EMD-50305, EMD-50308 to EMD-50313 and EMD-50315.. Raman spectra are available in the MicrobioRaman database (<https://www.ebi.ac.uk/biostudies/MicrobioRaman/studies>) with accession number S-MBRS7.

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CHAPTER FOUR

METHYL-REDUCING METHANOGENESIS BY A
THERMOPHILIC CULTURE OF KORARCHAEIAContribution of Authors and Co-Authors

Co-First Author: Anthony J. Kohtz

Contributions: Designed experiments, conducted field work, reconstructed the metabolic potential of strain LCB3, performed phylogenetic analyses, performed cultivation, analyzed gene expression data, processed metagenomic data and performed phylogenomic analyses, extracted DNA for metagenome sequencing, performed FISH, stable isotope tracing, substrate tests, transcriptomic experiments, developed GC/GCMS protocols, wrote the manuscript.

Co-First Author: Viola Krukenberg

Contributions: Designed experiments, conducted field work, reconstructed the metabolic potential of strain LCB3, performed phylogenetic analyses, performed cultivation, analyzed gene expression data, performed amplicon sequencing, BONCAT and CARD-FISH experiments, wrote the manuscript.

Co-Author: Zackary J. Jay

Contributions: Conducted field work, reconstructed the metabolic potential of strain LCB3, performed phylogenetic analyses, processed metagenomic data and performed phylogenomic analyses, performed whole genome comparisons and determined environmental distributions, wrote the manuscript.

Co-Author: Roland Hatzenpichler

Contributions: Designed experiments, responsible for funding and supervision of the project, wrote the manuscript.

Manuscript Information

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Abstract

Methanogenesis mediated by archaea is the major source of methane, a strong greenhouse gas, and thus is critical for understanding Earth's climate dynamics. Recently, genes encoding diverse methanogenesis pathways were discovered in metagenome-assembled genomes affiliated with multiple archaeal phyla (Evans, Parks et al. 2015, Vanwonterghem, Evans et al. 2016, Borrel, Adam et al. 2019, Hua, Wang et al. 2019, McKay, Dlakić et al. 2019, Seitz, Dombrowski et al. 2019, Wang, Wegener et al. 2019). However, all experimental studies on methanogens are currently restricted to cultured representatives of the Euryarchaeota. Here we show for the first time methanogenic growth by a member of the lineage Korarchaeia within the phylum Thermoproteota (TACK superphylum) (Borrel, Adam et al. 2019, McKay, Dlakić et al. 2019, Wang, Wegener et al. 2019). Following enrichment cultivation of *Candidatus Methanodesulfokora washburnensis* strain LCB3 we used measurements of metabolic activity and isotope tracer conversion to demonstrate methanol reduction to methane using hydrogen as electron donor. Analysis of the archaeon's circular genome and transcriptome revealed unique modifications in the energy conservation pathways linked to methanogenesis, including enzyme complexes involved in hydrogen and sulfur metabolism. The cultivation and characterization of this novel group of archaea is critical for a deeper evaluation of the diversity, physiology, and biochemistry of methanogens.

Introduction

Methane is a potent greenhouse gas, and its atmospheric levels contribute to regulating Earth's climate. The majority of methane (approximately 58-64%) is generated in anoxic

environments by methanogenic archaea (Conrad 2009, Saunio, Stavert et al. 2019) in a strictly anaerobic process called methanogenesis (Garcia, Gribaldo et al. 2022). These microorganisms play a critical role in the global carbon cycle by catalyzing the final step in organic matter degradation and are of high interest for biotechnological applications including the production of methane as an energy source (Enzmann, Mayer et al. 2018). Methanogens use compounds such as CO₂/H₂, acetate or methanol and the enzymatic pathways leading to methane formation differ depending on the substrate (Kurth, Op den Camp et al. 2020, Garcia, Gribaldo et al. 2022). However, all methanogens employ the methyl-coenzyme M reductase (MCR) complex for the final conversion of methyl-coenzyme M and coenzyme B into methane and the CoM-S-S-CoB heterodisulfide (Thauer 2019). This key enzyme also catalyzes the reversible reaction in the anaerobic oxidation of methane and other alkanes in alkanotrophic archaea (Scheller, Goenrich et al. 2010, Thauer 2019). The isolation of methanogens into axenic cultures has been fundamental to decades of research on their physiology and biochemistry (Thauer, Kaster et al. 2008). To date, methanogenesis has exclusively been studied in lineages of the Euryarchaeota, and no methanogen from outside this superphylum has ever been cultured for experimental investigation. Only recently, environmental metagenomic studies have identified genes of the methanogenesis pathway, including those in the MCR complex, to be encoded by archaea across multiple new lineages (Evans, Parks et al. 2015, Vanwonterghem, Evans et al. 2016, Borrel, Adam et al. 2019, Hua, Wang et al. 2019, McKay, Dlakić et al. 2019, Seitz, Dombrowski et al. 2019, Wang, Wegener et al. 2019). These newly proposed groups of methanogenic archaea could have important but so far unrecognized environmental impacts or hold undiscovered biotechnological potential.

Candidatus *Methanodesulfokora washburnensis*, formerly *Ca. Methanodesulfokores washburnensis* (McKay, Dlakić et al. 2019, Oren and Garrity 2021) was first identified as a potential methanogen by metagenome-assembled genomes (MAGs) obtained from Washburn Hot Springs in Yellowstone National Park (YNP, WY, USA) (Borrel, Adam et al. 2019, McKay, Dlakić et al. 2019, Wang, Wegener et al. 2019). Metabolic reconstruction predicted methyl-reducing hydrogen-dependent methanogenesis among other possible respiratory pathways, including anaerobic oxidation of methane and sulfite reduction (Borrel, Adam et al. 2019, McKay, Dlakić et al. 2019, Wang, Wegener et al. 2019). Its unique metabolic potential at the intersection of carbon and sulfur cycling (Borrel, Adam et al. 2019, McKay, Dlakić et al. 2019, Wang, Wegener et al. 2019), together with its deeply branching phylogenetic position within the phylum Thermoproteota (formerly the TACK superphylum) make this archaeon a prime candidate for culture-dependent research into the physiology, biochemistry, and metabolic versatility of methanogens.

Here, we combined selective cultivation with fluorescence microscopy, growth experiments, and metagenome and metatranscriptome sequencing to demonstrate methyl-reducing methanogenesis by a thermophilic culture of this korarchaeon.

Results and Discussion

Methanogenic Enrichments From Hot Springs

To date, *Ca. Methanodesulfokora* related 16S rRNA genes have almost exclusively been detected in terrestrial geothermal systems, including in hot spring metagenomes from YNP (n=7), New Zealand (n=1) and Kamchatka (n=1) (SI Table 1, 2). Consistently, *Ca. Methanodesulfokora mcrA* gene sequences have only been detected in metagenome assemblies

from geothermal environments in YNP (McKay, Hatzenpichler et al. 2017, Borrel, Adam et al. 2019, McKay, Dlakić et al. 2019, Lynes, Krukenberg et al. 2023) (SI Table 3). We selected Washburn Hot Springs (WHS) and two hot springs in the Lower Culex Basin (LCB003 and LCB058) of YNP as source materials for cultivation. These hot springs ranged in temperature (64-77°C) and pH (6.1-6.5) and varied in the relative abundance of Korarchaeales-related 16S rRNA gene amplicons (0.4-3.6%; Fig. 1A, Extended Data Fig. 1, SI Table 4). From each hot spring anoxic incubations of sediment slurries were conducted at in situ temperature (WHS, 64°C; LCB003, 77°C; LCB058, 70°C) and supplied with methanol and hydrogen in the presence or absence of antibiotics (SI Table 5). Within 64 to 67 days of incubation we detected a strong increase in the relative abundance of both 16S rRNA and *mcrA* gene amplicons identified as *Ca. M. washburnensis* (SI Table 4, 5). *Ca. M. washburnensis* dominated the microbial community of all incubations, constituting up to 86% relative abundance while other MCR-encoding archaea affiliated with Methanomethyliales, Archaeoglobales and Methanobacteriales were also detected (>1% relative abundance). All incubations showed methane production (Fig. 1A, SI Table 5), with incubation LCB003-1B containing near-exclusively *mcrA* gene amplicons related to *Ca. M. washburnensis* (Korarchaeales, >99.9% relative abundance). The highest methane levels were observed in incubations WHS-1B and LCB003-1A, in which, based on 16S rRNA gene amplicon sequencing, both *Ca. M. washburnensis* and a *Methanothermobacter* sp. were detected. *Methanothermobacter* spp. are thermophilic, obligate CO₂-reducing hydrogenotrophic methanogens that previously had been isolated from geothermal environments of YNP (Zeikus, Ben-Bassat et al. 1980, McKay, Klingelsmith et al. 2021).

As inoculum for continued cultivation of *Ca. M. washburnensis* we selected a replicate of the methanogenic enrichment LCB003-1B (SI Table 4, 5). The microbial community of this enrichment lacked previously cultured Euryarchaeotal methanogens (<0.1% relative abundance of 16S rRNA gene amplicons) and was dominated by *Ca. M. washburnensis* (66.6% and 99.9% relative abundance of 16S rRNA and *mcrA* gene amplicons, respectively).

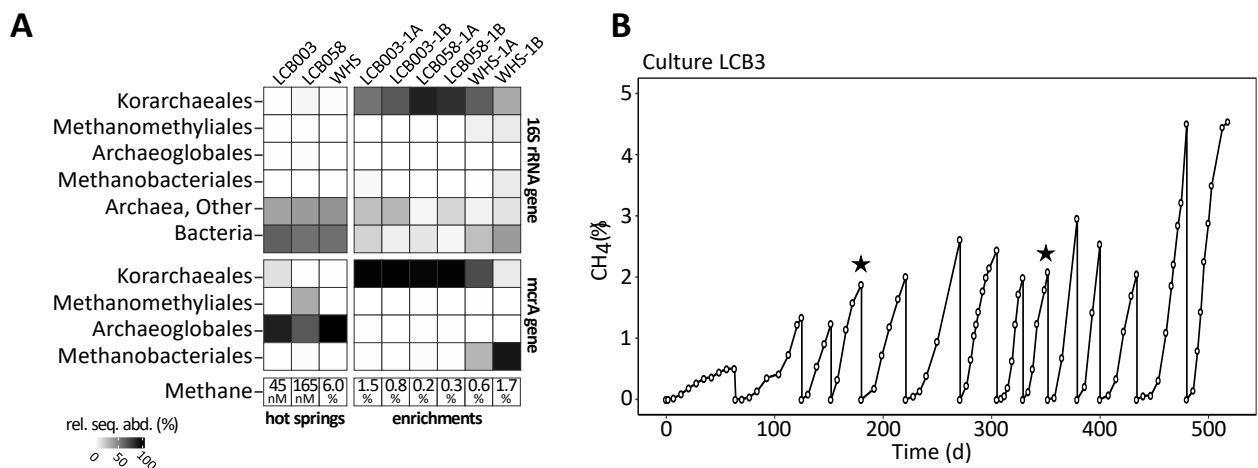


Figure 1. Methane-producing enrichment cultures from hot spring sediments. A. Relative abundance of Korarchaeales in hot spring sediments and in enrichments after 64-67 days of incubation as inferred by amplicon sequencing of *mcrA* and 16S rRNA genes. Enrichments were established from hot springs in the Lower Culex Basin (LCB003, LCB058) and Washburn Hot Springs (WHS). Enrichments were supplied with methanol and hydrogen in the absence (1A) or presence (1B) of antibiotics. Shading is proportional to relative sequence abundance (%). Lineages containing MCR-encoding archaea are depicted on order level, relative abundances >1% are shown. Methane values for hot springs LCB003 and LCB058 from (Lynes, Krukenberg et al. 2023) refer to dissolved methane; methane value for WHS from (McKay, Dlakić et al. 2019); and methane values for enrichments refer to % methane measured in the headspace. B. Headspace methane development of culture LCB3 initiated with sediment material from hot spring LCB003 (day 0-64) and maintained in anoxic media (day 65-518) amended with methanol plus hydrogen and antibiotics. See SI Table 5 for details. Stars indicate timepoints at which metagenome sequencing was performed.

Cultivation and Complete Genome Recovery of *Ca. M. washburnensis*

For selective cultivation, we designed a medium according to the growth requirements for hydrogen-dependent methyl-reducing methanogenesis predicted from metabolic reconstructions of *Ca. M. washburnensis* MAGs (Borrel, Adam et al. 2019, McKay, Dlakić et al. 2019, Wang, Wegener et al. 2019). Methanol (10 mM) and hydrogen (50%) were supplied as methanogenic substrates, antibiotics were amended to reduce bacterial growth, and incubation was conducted at in situ temperature of 77°C. Activity was monitored by measuring methane concentrations in the headspace and cultures were transferred at late exponential phase of methane production. Continuous transfers of the active methanogenic culture into fresh media resulted in a sediment-free culture, designated culture LCB3. During 13 continuous cultivation cycles (i.e., transfers; over 500 days total) the final methane concentration and methane production rate increased steadily from a maximum of 2% to 5% headspace gas, equivalent to about 2 mM of methane and from approximately 63 to 38 days, respectively (Fig. 1B). Importantly, the growth temperature of culture LCB3 is well above that of other cultured methanol-utilizing methanogens (Cheng, Qiu et al. 2007, Sorokin, Makarova et al. 2017) and near the boiling point of methanol (84°C at 200 kPa), which likely reduced the amount of methanol effectively available in the liquid media.

Metagenome long-read sequencing (PacBio) performed on culture LCB3 (day 352, Fig., 1B) resulted in the recovery of a complete, closed chromosome that was highly related to previously obtained *Ca. M. washburnensis* MAGs (NM4, LMO9, and MDKW; SI Table 6) (Borrel, Adam et al. 2019, McKay, Dlakić et al. 2019, Wang, Wegener et al. 2019) based on average nucleotide and amino acid identities (97-98%; Extended Data Fig. 2A), phylogenomic

reconstruction (Fig. 2A, Extended Data Fig. 3A, SI Table 7), and 16S rRNA gene identity and phylogeny (99%; Extended Data Fig. 3B). We thus designate the archaeon enriched here as *Ca. M. washburnensis* strain LCB3. The genome of strain LCB3 is similar in size and number of coding sequences to the MAG of *Ca. M. washburnensis* NM4 and the genome of *Ca. K. cryptofilum* OPF8, but notably smaller than the MAGs of both *Ca. M. washburnensis* MDKW and LMO9 (SI Table 6). MAGs NM4, MDKW, and LMO9 were independently recovered from the same metagenome dataset of Washburn Hot Springs (WHS) but processed by different bioinformatic methods (Borrel, Adam et al. 2019, McKay, Dlakić et al. 2019, Wang, Wegener et al. 2019). The variation in MAGs from WHS together with the genome size of strain LCB3, suggests that the *Ca. M. washburnensis* population in WHS could consist of several strains. We estimate the abundance of strain LCB3 in its native hot spring environments of WHS, LCB003, and LCB058 between 0.1 to 2.9% based on mapping of metagenomic short-reads to its genome. Metagenome short-read sequencing (Illumina) performed on culture LCB3 at two time points (day 180 and 352, Fig. 1B) revealed an enrichment of 62-82% of *Ca. M. washburnensis* (Fig. 2B, Extended Data Fig. 2B). Other community members in culture LCB3 included Archaeoglobaceae, Thermofilum, Fervidicoccaceae, Ignisphaera, and Desulfurococcaceae (Fig. 2B, Extended Data Fig. 3B), for which circular genomes were also recovered (SI Table 8). Consistently, application of a general archaeal CARD-FISH probe revealed filamentous and cocci shaped cells, while no cells were identified with general bacterial probes (Fig. 2B, Extended Data Fig. 4, SI Table 9).

Importantly, the only *mcr* genes (*mcrABG*) identified in the metagenomes from culture LCB3 at both time points belong to the genome of strain LCB3. Phylogenetic analysis of the

McrA revealed that it is highly related to previously recovered McrA from Korarchaeia MAGs and an McrA from a sediment metagenome of hot spring LCB003 (Fig. 2C), which had served as source material for culture LCB3. Together, this is strong evidence that strain LCB3 is the only methanogen in the culture.

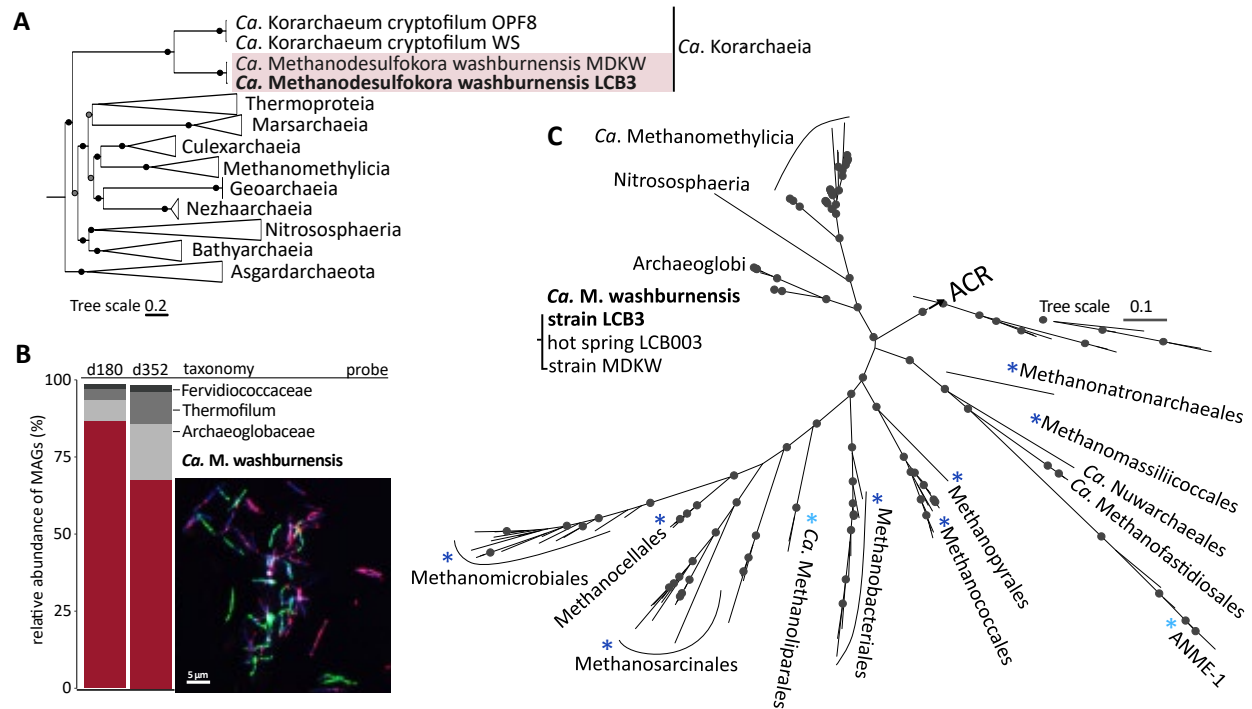


Figure 2. Phylogenetic affiliation of *Ca. Methanodesulfokora washburnensis* strain LCB3. A. Phylogenetic reconstruction based on 33 single copy marker genes showing affiliation of strain LCB3 with representative Korarchaeia genomes and MAGs. B. Composition of culture LCB3 based on relative abundance of recovered genomes (relative abundance >1%), and visualization of korarchaeal (red) and other archaeal cells (green) by CARD-FISH. Note that due to two central mismatches between the 16S rRNA of *Ca. M. washburnensis* and the archaea-specific probe (Arch915), no dual labelling of *Ca. M. washburnensis* was observed. C. Phylogenetic tree of McrA showing the close affiliation of McrA from *Ca. M. washburnensis* strain LCB3 with McrA from strain MDKW and McrA from the metagenome of hot spring LCB003. Asterisks indicate lineages with availability of methanogenic or methanotrophic isolates (dark blue) or enrichment cultures (light blue), which all belong to the superphylum Euryarchaeota. Circles represent bootstrap support values >95. ACR, alkyl-coenzyme M reductase.

Metabolic Activity on Methanol and Hydrogen

Fluorescence in situ hybridization (FISH) on culture LCB3 using a probe specific for the 16S rRNA of *Ca. M. washburnensis* revealed abundant filamentous cells (Fig. 3A, Extended Data Fig. 4, SI Table 9), similar in morphology to the previously described non-methanogenic *Ca. Korarchaeum cryptofilum* OPF8 (Elkins, Podar et al. 2008). Filaments varied in lengths between approximately 3 μm to $>20 \mu\text{m}$, and filaments were observed both individually and in cell aggregates. We tracked the growth of strain LCB3 by catalyzed reporter deposition (CARD)-FISH. This showed that its relative cell abundance increased with methane production from approximately 30% during lag phase to 70% during log phase (Fig. 3A). Based on cell counts, the total cell density of culture LCB3 reached 2×10^7 cells mL^{-1} with an estimated doubling time of six days. By growing culture LCB3 in the presence of ^{13}C -methanol and measuring the transfer of isotopic label to ^{13}C -methane, we confirmed that methanol was converted to methane (Fig. 3B, Extended Data Fig. 5A). Methane production ceased upon addition of bromoethanesulfonate (BES), an inhibitor of the MCR complex. Methane production and growth of strain LCB3 was not sustained without amendment of methanol. Mono-, di- and trimethylamine as well as higher chain alcohols (ethanol and isopropanol) did not support methanogenic growth of strain LCB3 (Extended Data Fig. 5C).

To demonstrate that translational activity is coupled to methanogenesis in strain LCB3, we evaluated its metabolic activity under methanogenic and methanogenesis-inhibited conditions using a combination of bioorthogonal non-canonical amino acid tagging (BONCAT) and FISH. For this, two replicate culture sets were grown to exponential phase, at which point one set was inhibited with BES before both sets were spiked with the amino acid analog L-homopropargylglycine (HPG) to trace translationally active cells (Extended Data Fig. 5B). We

visualized cells of *Ca. M. washburnensis* via CARD-FISH and identified anabolically active cells via click chemistry mediated dye-staining. Based on cell counts, *Ca. M. washburnensis* accounted for approximately 80% of the cells in both culture sets, while BONCAT-labelling revealed anabolic activity of *Ca. M. washburnensis* cells only under methanogenic conditions (Fig. 3C). This indicated that translational activity is dependent on the activity of the MCR complex. Taken together, we present the first experimental evidence that a representative of the Korarchaeia grows by methanogenesis.

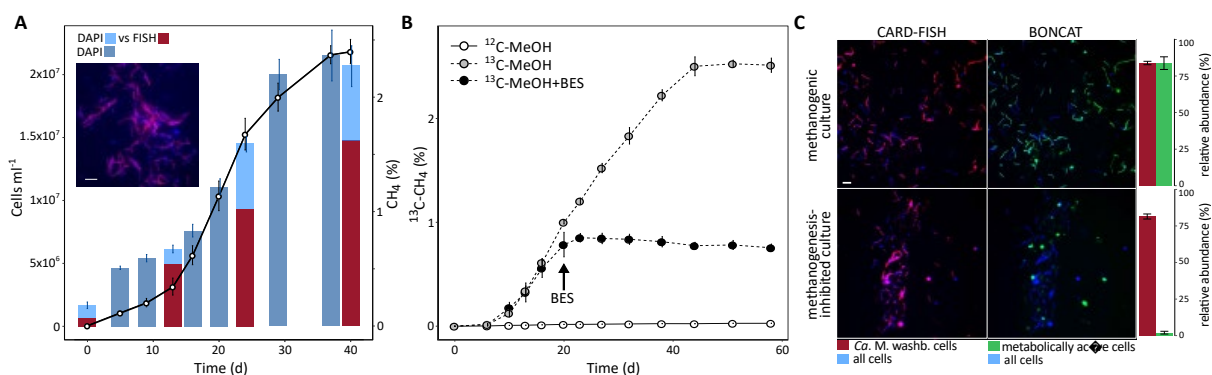


Figure 3. Growth and translational activity of culture LCB3. **A.** Development of methane concentration and cell number. Relative abundance of *Ca. M. washburnensis* cells was determined at four time points (day 0, 12, 24, 40) based on the fraction of *Ca. M. washburnensis* specific FISH counts (red) versus total counts of DAPI stained cells (blue). Error bars indicate the standard deviation of four biological replicates. **B.** Production of ^{13}C -methane in cultures amended with ^{12}C -methanol (open symbols, solid line) or ^{13}C -methanol (grey symbols, dashed line). No ^{13}C enrichment of methane was detected in cultures amended with ^{12}C -methanol. BES addition to active cultures resulted in the inhibition of methanogenesis (black circles, dashed line). Error bars indicate the standard deviation of three biological replicates. **C.** Anabolic activity of cells in culture LCB3 visualized via BONCAT (green), combined with CARD-FISH (red) to identify *Ca. M. washburnensis* cells. *Ca. M. washburnensis* cells were translationally active in a methane-producing culture but not in a culture in which methanogenesis was inhibited with BES. In methanogenic cultures BONCAT-labelled cells (green bar) and CARD-FISH-labelled cells (red bar) each accounted for 84% of all DAPI stained cells. In methanogenesis-inhibited cultures BONCAT-labelled cells accounted for 2% while CARD-FISH-labelled cells accounted for 82%. This suggests that anabolic activity of *Ca. M. washburnensis* cells ceased when methanogenesis is inhibited. Error bars indicate the standard deviation of three biological replicates. Scale bars 5 μm .

Gene Expression During Methyl-Reducing Methanogenesis

To reveal the mechanisms involved in methyl-reducing methanogenesis in strain LCB3 we analyzed its genome and studied its gene expression during growth with methanol and hydrogen (Fig. 4, Extended Data Fig. 6, SI Table 10, 11). Notably, metatranscriptome sequencing conducted on culture LCB3 showed that 98% of the reads mapped to the genome of strain LCB3. Strain LCB3 highly expressed the Mcr complex (mcrABGCD) and the methanol:coenzyme M methyltransferase system (mtaABC) that are both required for the conversion of methanol to methane and a heterodisulfide (CoM-S-S-CoB) (Kurth, Op den Camp et al. 2020, Garcia, Gribaldo et al. 2022). However, the genome did not encode the methyltetrahydromethanopterin:coenzyme M methyltransferase (Mtr) complex and the methyl-branch of the Wood-Ljungdahl pathway (Fig. 4A, SI Table 10). This supports that methanogenesis in strain LCB3 is based on methyl-reduction rather than methyl-disproportionation into methane and carbon dioxide. Consequently, strain LCB3 relies on the oxidation of an electron donor, such as hydrogen, for the reduction of CoM-S-S-CoB, as previously discussed for related MAGs (Borrel, Adam et al. 2019, McKay, Dlakić et al. 2019, Wang, Wegener et al. 2019). Importantly, all previous metabolic inferences suggested an energy conservation mechanism via a soluble electron-bifurcating hydrogenase/heterodisulfide reductase complex (Mvh/Hdr) coupled to a Fpo-like/HdrD complex (Borrel, Adam et al. 2019, McKay, Dlakić et al. 2019, Wang, Wegener et al. 2019). While strain LCB3 expressed the Fpo-like complex (Fig. 4, Extended Data Fig. 7B, SI Table 11) and HdrD subunits, no genes encoding an Mvh/Hdr complex were identified, which suggests an alternative route for linking hydrogen oxidation and CoM-S-S-CoB reduction. Metabolic reconstruction supported by transcriptional activity suggest that in strain LCB3 hydrogen is oxidized by a membrane-bound [NiFe]-

hydrogenase of group 1g (Søndergaard, Pedersen et al. 2016), and electrons are transferred via a membrane-bound electron carrier to an integral membrane b-type cytochrome containing heterodisulfide reductase complex (HdrDE) that catalyzes the cytoplasmic reduction of CoM-S-S-CoB (Fig. 4B, Extended Data Fig. 7A, 8, SI Table 11). Different from previously characterized group 1 [NiFe]-hydrogenases of the 1k/1j type (or Vht/Vho, respectively) in methanogens, the group 1g [NiFe]-hydrogenase complex encoded in the LCB3 genome does not contain a cytochrome b subunit (Extended Data Fig. 7A). Instead, it includes two proteins with similarity to NrfC and NrfD families (Extended Data Fig. 8A), which could substitute for the function of cytochrome b in reducing a membrane-bound electron carrier (Calisto and Pereira 2021). Group 1g [NiFe]-hydrogenases have not been previously linked to methanogenesis and may present a unique Korarchaeia-specific differentiation in the enzymatic machinery of methanogens. Energy conservation during hydrogen-dependent methyl-reducing methanogenesis could thus rely on a simple respiratory chain of membrane-bound group 1g [NiFe]-hydrogenase and b-type cytochrome (HdrE), in which both enzyme complexes would contribute to establishing an ion gradient used for the synthesis of ATP via a V-type ATP synthase (Fig. 4). Strain LCB3 also expressed a long-chain geranylgeranyl diphosphate synthase related to sequences from *Methanosarcina*, *Archaeoglobus*, and *Methylarchaeles* (Extended Data Fig. 9, SI Table 11), suggesting strain LCB3 may synthesize methanophenazine (Ogawa, Yoshimura et al. 2010). Additionally, variable expression levels of enzymes putatively involved in menaquinone biosynthesis (SI Table 11), indicate a potential to produce diverse membrane-bound electron carriers. Notably, c-type cytochromes were not encoded in the LCB3 genome.

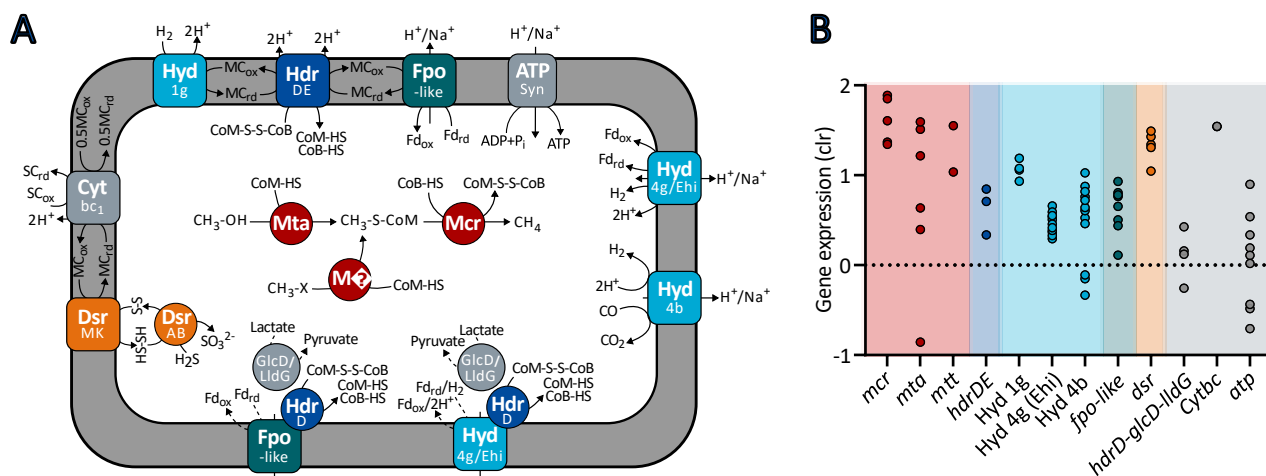


Figure 4. Metabolic reconstruction and gene expression of *Ca. M. washburnensis* strain LCB3. A. Methanogenesis pathway and possible alternative energy conserving processes with linkage to methanogenesis. Dashed lines represent alternative reactions, color-coding differentiates proteins/protein complexes. B. Gene expression presented as center log ratio (CLR) during methanogenic growth on methanol and hydrogen. Dotted line represents the geometric mean expression of all genes. Each dot represents the expression of a subunit in a multi-subunit enzyme complex. Abbreviations: ATP Syn, ATP Synthase; Dsr, Dissimilatory sulfite reductase; Fd, Ferredoxin; Fpo, F420:Methanophenazine oxidoreductase; Glc, Lactate dehydrogenase; Hdr, Heterodisulfide reductase; Hyd, [NiFe]-Hydrogenase; Mcr, Methyl-coenzyme M reductase; Mta, Methanol:coenzyme M methyltransferase; MC, Membrane-bound electron carrier; SC, Soluble electron carrier; ox, oxidized form; rd, reduced form. See SI Table 10, 11 for details on gene annotation and gene expression.

Along with the pathway for hydrogen-dependent methanol-reducing methanogenesis, the transcriptional profile of strain LCB3 revealed several other enzymes possibly linked to methanogenesis. In addition to the methanol:coenzyme M methyltransferase system (mtaABC) for methanol reduction, homologs of a trimethylamine methyltransferase, MttB, and an associated corrinoid protein, MttC, were highly expressed. Proteins in the MttB family have been shown to catalyze the demethylation of a variety of substrates (Ticak, Kountz et al. 2014, Kountz, Behrman et al. 2020, Ellenbogen, Jiang et al. 2021). Low sequence similarity to characterized MttBC homologs along with the absence of growth on trimethylamine, suggest that

this represents a new, yet uncharacterized methyltransferase system. Strain LCB3 also expressed a novel group 4 [NiFe]-hydrogenase, termed here Ehi. This complex could catalyze the reversible oxidation of hydrogen at the expenditure of an ion gradient to produce reduced ferredoxin (Kulkarni, Mand et al. 2018) for CoM-S-S-CoB reduction, akin to the functional role of Ech or Ehb-type hydrogenases in Euryarchaeotal methanogens (Thauer, Kaster et al. 2008). Similar to Fpo, Ehi may form a complex with HdrD, allowing for coupling the direct oxidation of hydrogen to the reduction of CoM-S-S-CoB and ion translocation. Additionally, a membrane-bound group 4b [NiFe]-hydrogenase/carbon monoxide dehydrogenase complex provides the potential to couple the oxidation of carbon monoxide to the internal production of hydrogen and ion translocation (Kim, Bae et al. 2013, Schut, Lipscomb et al. 2016). However, three subunits of the hydrogenase module (including the catalytic subunit) showed low expression relative to the subunits of the carbon monoxide dehydrogenase and ion transporter modules, suggesting these genes may be differentially regulated as has been shown in *Thermococcus* (Kim, Bae et al. 2013, Kim, Choi et al. 2015) (Fig. 4B). Similar to other MCR-encoding archaea within the Thermoproteota, strain LCB3 encoded genes with homology to lactate dehydrogenases, GlcD and LldG, that were each co-located with an HdrD and showed low to moderate expression levels. Interestingly, when supplemented with lactate, methane production of culture LCB3 improved, even without added hydrogen (Extended Data Fig. 5C). This could indicate that lactate oxidation by GlcD may contribute electrons to HdrD for the reduction of CoM-S-S-CoB (Evans, Parks et al. 2015, Vanwonterghem, Evans et al. 2016) coupled to the translocation of ions at either a Fpo-like or Ehi complex, a mechanism previously suggested but not described in any cultured methanogens (Evans, Parks et al. 2015, Vanwonterghem, Evans et al. 2016).

However, methane production also occurred when culture LCB3 was supplemented only with methanol, with lower yields than with methanol plus hydrogen (Extended Data Fig. 5C). Therefore we cannot exclude that trace hydrogen was produced by strain LCB3 or co-enriched archaea, or that hydrogen played an intermediate role in lactate metabolism. Notably, a cluster of genes encoding cytoplasmic and membrane-bound components of the (dissimilatory) sulfite reductase system (DsrABCMK) and a cytochrome bc₁-like complex was highly expressed. The growth medium of strain LCB3 was supplemented with reduced sulfur species and the dissimilatory sulfite reductase system may function in reverse to couple sulfide oxidation with CoM-S-S-CoB reduction and energy conservation as previously speculated for *Ca. M. washburnensis* MKDW (McKay, Dlakić et al. 2019). Alternatively, a highly active Dsr complex may be crucial for the removal of toxic sulfite to prevent inhibition of the sensitive Mcr complex. Moreover, the Dsr complex together with hydrogenases provide a capacity for non-methanogenic growth via sulfite reduction coupled to hydrogen oxidation; however, our attempts to sustain culture LCB3 on sulfite and hydrogen were unsuccessful.

Conclusions

Here, for the first time, we demonstrate that methanogenesis is not restricted to the Euryarchaeota superphylum by culturing *Ca. M. washburnensis* strain LCB3, a methanogenic representative of the Korarchaeia from a terrestrial hot spring. During growth, strain LCB3 forms methane from methanol, shows translational activity reliant on the function of the MCR complex, and expresses genes involved in energy conservation via methanogenesis. The confirmation that a member of the deepest branching lineage within the Thermoproteota (TACK superphylum) is a methanogen has important implications for our understanding of the evolution

and diversification of methanogens. Notably, growth of strain LCB3 at 77°C extends the upper temperature range of both methyl-reducing and b-type cytochrome-involving methanogenesis. This may impact its niche differentiation, because cytochrome-based energy conservation is more efficient (Thauer, Kaster et al. 2008) however, no such methanogen has yet been studied in this regard. Strain LCB3 appears to be specialized towards using methanol as substrate for methanogenesis and incapable of exploiting methylated amines. However, strain LCB3 highly expressed a diverse set of energy-conserving complexes and the potential to use electron donors other than hydrogen, which is rare in cultured methyl-reducing methanogens (Sorokin, Makarova et al. 2017, Kurth, Op den Camp et al. 2020). Our genomic, transcriptomic, and growth experimental evaluation of strain LCB3 indicated metabolic traits unique among methanogens and suggests that many yet unresolved aspects of its physiology, biochemistry, cell biology, and ecology await discovery.

To date, *Ca. M. washburnensis* LCB3 is the second member of the Korarchaeia obtained in an enrichment culture and it contrasts the non-methanogenic peptide fermenter *Ca. K. cryptofilum* (Elkins, Podar et al. 2008). Cultivation of *Ca. M. washburnensis* opens the possibility for fundamental research on the evolution and biology of Korarchaeia and to expand our understanding of methanogenesis and the transition between methanogenic and non-methanogenic lifestyles.

Methods

Chemicals

All chemicals were purchased from Sigma Aldrich unless otherwise specified.

Sample Collection, Enrichment, and Cultivation

Hot spring sediment was retrieved from three geothermal features in Yellowstone National Park (YNP): LCB003 (44.57763, -110.78957; November 2020; 77°C; pH 6.5), LCB058 (44.57039, -110.80521; October 2020; 70°C; pH 6.1) located in the Lower Culex Basin (LCB), and WHS (44.76493, -110.43030; October 2019; 64°C; pH 6.4) located in the Washburn Hot Springs thermal area. A mixture of surface sediments (~1 cm deep) and spring water were collected into glass bottles sealed headspace-free with a butyl rubber stopper. Slurries were stored at in situ temperature for 24 h before transfer to room temperature for long-term storage. Initial enrichments were initiated within 24 h of material retrieval from LCB003 and LCB058 or within two years of material retrieval for WHS, and were set up in sterile serum vials (50 or 70 ml) under a N₂/CO₂/H₂ (90/5/5%) atmosphere. Slurries from LCB058 and WHS were diluted with anoxic media (1:10). Media was prepared as described previously (Laso-Perez, Krukenberg et al. 2018) 34 and contained KH₂PO₄, 0.5 g L⁻¹; MgSO₄·7H₂O, 0.4 g L⁻¹; NaCl, 0.5 g L⁻¹; NH₄Cl, 0.4 g L⁻¹; CaCl₂·2H₂O, 0.05 g L⁻¹; MES, 2.17 g L⁻¹; yeast extract, 0.1 g L⁻¹; and 0.002% (w/v) (NH₄)₂Fe(SO₄)₂·6H₂O, 5 mM NaHCO₃, 1 ml L⁻¹ trace element solution SL-10, 1 ml L⁻¹ Selenite-Tungstate solution (Widdel and Bak 1992), 1 ml L⁻¹ CCM vitamins (Brandis and Thauer 1981), 0.0005% (w/v) resazurin, 10 ml g L⁻¹ of coenzyme-M, 2 ml L⁻¹ sodium dithionite, 1 mM dithiothreitol, 1 mM Na₂S·9H₂O, with pH adjusted to 6.5 with NaOH. Serum vials were sealed with butyl rubber stoppers and aluminum crimps before the headspace was degassed with N₂/CO₂ (90/10) for 5 minutes and set to 200 kPa. Hydrogen and methanol were added at final concentration of 50% and 10 mM, respectively. Two types of incubation were prepared from the slurry of each hot spring. One was amended with the bacterial antibiotics streptomycin (50 mg L⁻¹; inhibitor of protein synthesis) and vancomycin (50 mg L⁻¹; inhibitor

of peptidoglycan synthesis) and one was not amended with antibiotics. Additional control incubations were supplied with only methanol or only hydrogen. Incubation was conducted at in situ temperatures. All stock solutions were anoxic and sterilized by filtration or autoclavation. After 64 days of incubation an initial enrichment from LCB003 (LCB003-1B) was selected as inoculum for the cultivation of *Ca. M. washburnensis* and transferred into anoxic media (10% v/v), amended with hydrogen, methanol and antibiotics. Cultivation was conducted at 77°C and cultures were maintained by regular transfer into fresh media (10% v/v).

Methane Measurements

Methane concentrations were determined from subsamples of 250 µL headspace gas collected with a gas tight syringe (Hamilton) and injected into 10 mL autosampler vials sealed with grey chlorobutyl septa. Subsamples were injected with a robotic autosampler (AOC-6000) into a Shimadzu 2020-GC equipped with a GS-CarbonPLOT column (30 m x 0.32 mm; 1.5 µm film thickness; Agilent) and a Rt-Q-BOND column (30 m x 0.32 mm; 1.5 µm film thickness; Restek) and operated with injector, column, and flame ionization detector (FID) maintained at 200°C, 50°C, and 240°C, respectively. Methane concentrations were calculated based on injection of a standard curve.

Stable Isotope Incubation

For tracking the conversion of ¹³C-methanol to ¹³C-methane, active enrichment cultures were incubated in the presence of ¹³C-methanol (99% labelled; Cambridge Isotope Laboratories). 30 ml incubations were carried out in 60 ml serum bottles with 20% (v/v) inoculum. Control incubations were performed in the presence of (i) ¹²C-methanol (100%), (ii) ¹³C-methanol (100%) plus bromoethanesulfonate (BES) to inhibit methanogenesis, (iii) ¹³C-

methanol (100%) plus paraformaldehyde to inhibit cellular activity, and (iv) ^{13}C -methanol (100%) without inoculum. Substrate and inhibitor were added at a concentration of 10 mM. Headspace samples were collected regularly as described above and analyzed using a Shimadzu QP2020 NX GCMS equipped with a GS-CarbonPLOT column (30 m x 0.35 mm; 30 μm film thickness; Agilent) and operated in Selected Ion Monitoring mode. The instrument was operated as described in (Ai, Zhu et al. 2013). Peak areas corresponding to m/z ratios of 16 for ^{12}C -methane, 17 for ^{13}C -methane were used for quantification.

Fluorescence In Situ Hybridization and Cell Counts

Aliquots of enrichment cultures were fixed in 2% paraformaldehyde (PFA) for 1 h at room temperature, washed twice by centrifugation at 16,000 $\times g$, removing the supernatant and resuspending the cells in 1x phosphate buffered saline (PBS), before cell suspensions were stored at 4°C. For direct cell counts, aliquots of fixed cell suspensions were filtered onto polycarbonate filters (0.2 μm pore size, 25 mm diameter, GTTP Millipore, Germany) and air dried before filter pieces were cut, stained with DAPI (4,6-diamidino-2-phenylindole), embedded in Citifluor-Vectashield, and enumerated under an epifluorescence microscope (Leica DM4B). Relative abundance of target cells was determined from the fraction of CARD-FISH or DOPE-FISH stained cells compared to total cell counts based on DNA stained cells using DAPI. A 16S rRNA-targeted oligonucleotide probe (KRmw515, 5'-CCA GCC TTG CCC TCC CCT-3') specific for *Ca. Methanodesulfokora washburnensis* was designed using the probe design tool in the ARB software package (Ludwig, Strunk et al. 2004). Probe KRmw515 has one mismatch to the 16S rRNA of non-target organism *Ca. Korarchaeum cryptofilum*, which was absent from the culture. The horseradish peroxidase (HRP)-labelled oligonucleotide probe was synthesized by Biomers

(Ulm, Germany). Stringency was tested in a CARD-FISH experiment by increasing the formamide concentration in the hybridization buffer from 0% to 70%. CARD-FISH was performed as described previously (Pernthaler, Pernthaler et al. 2002). Cell wall permeabilization was achieved with lysozyme treatment for 30 min at 37°C [10 mg ml⁻¹ lysozyme, lyophilized powder in 0.1 M Tris-HCl, 0.05 M EDTA, pH 8] followed by proteinase K digestion for 10 min at RT [4.5 mU mL⁻¹ proteinase K (Merck) in 0.1 M Tris-HCl, 0.05 M EDTA and 0.5 M NaCl, pH 8]. Endogenous peroxidases were inactivated with 0.15% H₂O₂ in methanol (30 min, RT). For hybridization, the following oligonucleotide probes were applied at their respective formamide concentration (35% for all): EUB338I-III (Daims, Brühl et al. 1999), Arch915 (Stahl 1991), NON338 (Wallner, Amann et al. 1993), and KRmw515. Catalyzed reporter deposition was performed using tyramides labelled with the fluorochromes Alexa Fluor 594 or Alexa Fluor 488. When performing double hybridizations, the peroxidase enzymes of the first hybridization were inactivated with 0.3% H₂O₂ in methanol (30 min, RT) prior to the hybridization with the second HRP-labelled probe. DOPE-FISH was performed as previously described (Stoecker, Dorninger et al. 2010). Samples were stained with DAPI, embedded in Citifluor-Vectashield, and visualized using epifluorescence microscopy.

BONCAT-FISH Experiments

The metabolic activity of strain LCB3 under methanogenic versus methanogenesis-inhibited conditions was tested via biorthogonal non-canonical amino acid tagging (BONCAT). Replicate incubations of culture LCB3 were initiated with methanol and hydrogen. Inhibited controls were amended with BES at day 0. When methane production reached exponential phase (day 15), triplicate cultures were either amended with BES (5 mM) or remained unamended.

After 72 h both sets were spiked with the biorthogonal amino acid HPG (100 μ M). Subsamples were retrieved after 24 h, fixed in 2% PFA, washed and stored in 1xPBS at 4°C. Aliquots of fixed samples were spotted on glass slides and BONCAT-FISH was performed as previously described (Hatzenpichler, Connon et al. 2016). The fractions of BONCAT-labelled, FISH-labelled and DAPI-stained cells were enumerated to determine the relative abundance of BONCAT and FISH labelled cells.

Growth Experiments

To test whether substrates other than methanol support methanogenic growth 10 mM of either monomethylamine, dimethylamine, trimethylamine, ethanol, or 2-propanol were added to culture LCB3 in the presence of hydrogen (50%). Additionally, culture LCB3 was supplemented with 2 mM L-lactate plus MeOH (10 mM) or plus MeOH (10 mM) and hydrogen (50%). Methane production was monitored over an incubation time of 85 days.

Amplicon Sequencing and Analysis

DNA was extracted from environmental samples, and from initial enrichment cultures (pellet from 0.5 ml) using the FastDNA Spin Kit for Soil (MP Biomedicals, Irvine, CA) following the manufacturer's guidelines. *mcrA* genes were amplified with primer set mlas-mod-F/*mcrA*-rev-R (Steinberg and Regan 2009, Angel, Claus et al. 2012), and archaeal and bacterial 16S rRNA genes were amplified with the updated Earth Microbiome Project primer set 515F and 806R (Apprill, McNally et al. 2015, Parada, Needham et al. 2016, Thompson, Sanders et al. 2017). Amplicon libraries were prepared as previously described (Krukenberg, Reichart et al. 2021) and sequenced at the Molecular Research Core Facility at Idaho State University (Pocatello, ID) using an Illumina MiSeq platform with 2 x 300 bp (*mcrA* amplicon library) and 2

x 250 bp (16S rRNA amplicon library) paired end read chemistry. Both 16S rRNA and mcrA gene reads were processed using QIIME 2 version 2020.2 (Bolyen, Rideout et al. 2019). Primer sequences were removed from demultiplexed reads using cutadapt (Martin 2011) with error rate 0.12, reads were truncated (145 bp forward, 145 bp reverse and 260 bp forward, 200 bp reverse for 16S rRNA and mcrA datasets, respectively), filtered, denoised and merged in DADA2 with default settings (Callahan, McMurdie et al. 2016). 16S rRNA gene amplicon sequence variants (ASVs) were taxonomically classified with the sklearn method and the SILVA 132 database (Quast, Pruesse et al. 2012). mcrA gene ASVs were assigned a taxonomy using vsearch with a minimum identity of 70% and no consensus classification against a reference database of representative near-full length mcrA genes encompassing the diversity of publicly available mcrA. Contaminants were removed using the R package decontam (Davis, Proctor et al. 2018). The mcrA gene dataset was curated by removing ASVs $\leq$ 400 bp and non-mcrA gene ASVs as identified by evaluating the top hits of a blastx search against the NCBI NR database.

Metagenome Sequencing

A 50 mL aliquot of culture LCB3 was centrifuged at 16,000xg for 10 min to pellet cells, supernatant was removed, and the pellet stored at -80°C . Genomic DNA was extracted from the pellet according to (Zhou, Bruns et al. 1996), with the following modifications: (i) cells were disrupted with a tissue grinder, (ii) proteinase K treatment was extended to 1 h, and (iii) DNA was precipitated in the presence of 0.7x volumes of isopropanol and 0.1x volume of 3 M sodium acetate. DNA extracts were purified using the Zymo clean and concentrator kit (DCC-10, Zymo Research) according to the manufacturer's instructions. Purified genomic DNA was used for metagenomic library construction with the Illumina DNA Prep kit following the manufacturer's

recommendations and was sequenced on an Illumina NextSeq 2000 platform with 2x151 bp paired-end read chemistry performed at SeqCenter (Pittsburgh, PA). Additionally, for PacBio long read sequencing a library was generated with a 6-10kb ultra-low input kit and sequenced on a Sequel IIe platform at the Joint Genome Institute (Berkeley, CA)

Metagenome Assembly, Binning, and Quality Assessment

PacBio HiFi sequences were assembled with hifiasm-meta (v0.3-r063.2; --force-rs) (Feng, Cheng et al. 2022) which resulted in the recovery of complete circularized genomes of *Ca. M. washburnensis* and the other archaeal populations in culture LCB3 (*Thermofilum*, *Archaeoglobaceae*, *Ignisphaera*, *Desulfurococcaeae*, and *Fervidicoccaceae*). The *Thermofilum* population was separated into two strains, one circularized and one near-complete linear contig. Genome completeness and redundancy were assessed using CheckM1 v1.1.3 (Parks, Imelfort et al. 2015) and CheckM2 v1.02 (Chklovski, Parks et al. 2023). AAI and ANI values were computed with CompareM v0.0.23 (--fragLen 2000) (<https://github.com/dparks1134/CompareM>) and pyani v0.2.12 (ANIm) (Pritchard, Glover et al. 2016), respectively for available genomes and MAGs of *Ca. M. washburnensis* (n=4) and selected *Ca. Korarchaeum* (n=2) reference genomes.

Illumina read quality, linker, and adapter trimming, artifact and common contaminate removal, and error correction were performed using the rqcfilter2 pipeline (maxns=3, maq=3) and bbcm (mincount=2, hcf=0.6) from the BBTools suite v38.94 (Bushnell B. 2014. BBMap: a fast, accurate, splice-aware aligner. <https://sourceforge.net/projects/bbmap>). Resulting reads were assembled with SPAdes (Nurk, Meleshko et al. 2017) v3.15.3 (-k 33,55,77,99,127 --meta --only-assembler). Relative abundances were estimated by mapping the quality controlled Illumina reads to the PacBio assemblies with bbmap (ambiguous=random). Additionally, the genome of

strain LCB3 was used to recruit reads from various short-read metagenomes with bbmap (ambiguous=random) to determine the relative abundance of strain LCB3 in different geothermal features of YNP.

RNA Extraction, Sequencing, and Analysis

Six replicate cultures (75 mL each) were grown with methanol plus hydrogen to log phase of methane production. Culture bottles were rapidly cooled from 77 °C in a dry ice-ethanol bath (1 min) and kept on ice. Cells were concentrated onto sterivex filters that were then soaked in Zymo DNA/RNA Shield and immediately processed. The filters were placed into Zymo BashingBead lysis tubes (Zymo Research, Irvine, CA) containing 800 µL of Zymo RNA lysis buffer and vortexed at maximum speed for 20 min. The tubes were centrifuged at 14,000x g for 15 min and the supernatant was used for RNA purification with the Zymo Quick-RNA miniprep kit according to the manufacturer's instructions and including the DNase I step. Due to low biomass, the RNA from all six replicates was pooled and concentrated using the Zymo RNA Clean & Concentrator kit according to the manufacturer's instructions. Purified RNA was used for library preparation and sequencing at the SeqCenter (Pittsburgh, PA). RNA was treated again with DNase I (Invitrogen) before library preparation with Illumina's Stranded Total RNA Prep Ligation with Ribo-Zero Plus kit and 10bp unique dual indices. Libraries were sequenced on a NovaSeq X Plus platform producing 2x150 bp reads. Demultiplexing, quality control, and adapter trimming was performed with bcl-convert (v.4.1.5). Reads were further processed with rqcfilter2 (rna=t, trimrnaadapter=t, qtrim=rl, trimq=10, maq=20, maxns=0, minlen=50, mlf=0.33, removeribo=f) and mapped to reference genomes from the LCB3 culture using bbmap (ambig=random). Reads mapping to rRNA genes were excluded prior to normalization. Reads

mapping to each gene were normalized to gene length before calculating the center log ratio (CLR, $\log_{10}$) and transcripts per kilobase million (TPM).

Annotation and Reconstruction of Metabolic Potential

The genome of strain LCB3 was annotated by the IMG/M database (Chen, Chu et al. 2019). Catalytic subunits of [NiFe] hydrogenases (PF00374) were initially classified with HydDB, checked for conserved CXXC motifs at the N and C terminus, and further classified by phylogenetic analyses (Søndergaard, Pedersen et al. 2016). Proteins encoded in the genome were assessed to identify c-type cytochromes as previously described (Kletzin, Heimerl et al. 2015). Protein sequences were searched for CXXCH motifs, SignalP 6.0 (Teufel, Almagro Armenteros et al. 2022) was used to predict signal peptides and DeepTMHMM (Hallgren, Tsirigos et al. 2022) was used to predict transmembrane helices. 11 proteins contained 1 CXXCH motif but no transmembrane helices or signal peptides, indicating that they are not c-type cytochromes. Genes encoding proteins necessary for the biosynthesis of c-type cytochromes (Ccm machinery) were not identified in the LCB3 genome. Predicted optimal growth temperatures of MAGs and genomes were determined with Tome predOGT (Li, Rabe et al. 2019).

Phylogenetic Analyses and Distribution of Marker Genes

A set of 33 single-copy marker proteins were collected from Korarchaeia MAGs and reference archaeal genomes. These markers were aligned with MUSCLE (Edgar 2004), trimmed with trimAL (Capella-Gutiérrez, Silla-Martínez et al. 2009) using a 50% gap threshold, and concatenated to produce a final alignment of 7,118 positions. Iqtree2 (Minh, Schmidt et al. 2020) was used to reconstruct a maximum likelihood phylogenetic tree, using the LG+F+R10 model,

1000 ultrafast bootstraps, and 1000 iterations of the SH-like approximate-likelihood ratio test (Guindon, Dufayard et al. 2010).

16S rRNA genes from the metagenome of culture LCB3 and references were aligned and masked with ssu-align, and a maximum-likelihood phylogenetic tree was constructed with fasttree (Price, Dehal et al. 2009). McrA from the genome of strain LCB3 and publicly available references were aligned with MAFFT-linsi (Katoh and Standley 2013), trimmed with trimAL (Capella-Gutiérrez, Silla-Martínez et al. 2009) with 50% gap threshold and used for maximum likelihood phylogenetic analysis with Iqtree2 (Minh, Schmidt et al. 2020) with LG+C60+F+G model and 1000 ultrafast bootstraps.

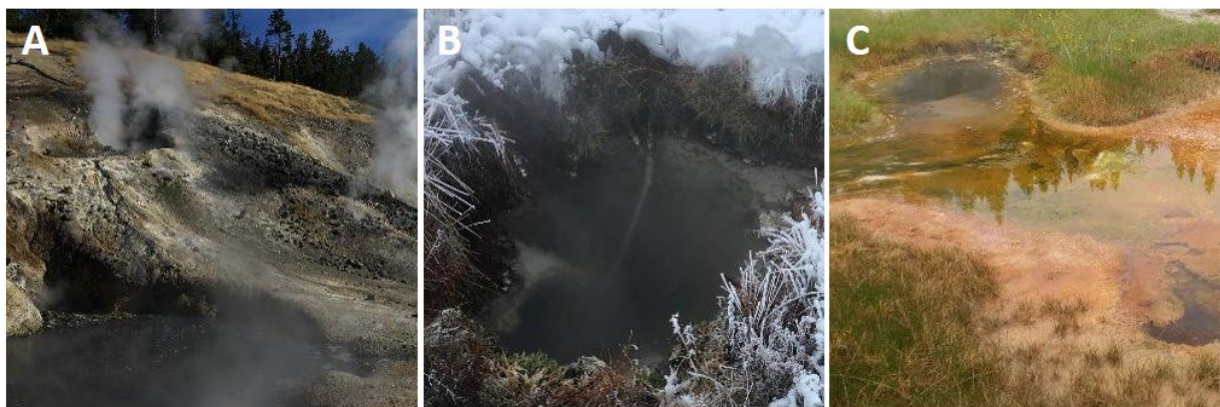
Catalytic subunits of group 1 and group 4 [NiFe]-hydrogenases were extracted from the genome, of strain LCB3 aligned against HydDB (Søndergaard, Pedersen et al. 2016) reference sequences with Mafft-LINSi (Katoh and Standley 2013) and trimmed with trimAL (Capella-Gutiérrez, Silla-Martínez et al. 2009) using a 50% gap threshold. For group 4, additional sequences of subunits FpoD/NuoD belonging to respiratory complexes were collected from (Ou, Dong et al. 2022) and included in the alignments. This produced final alignments of 562 and 365 residues for group 1 and group 4, respectively. Maximum likelihood phylogenetic trees were constructed using Iqtree2 (Minh, Schmidt et al. 2020) with best fit model selected according to Bayesian information criterion and 1000 ultrafast bootstraps. The models used were LG+R10 for group 1 and LG+C60+R+F for group 4.

Geranyl-farnesyl diphosphate synthase homologs were collected by searching for COG0142 and further classified by phylogenetic analysis in comparison with sequences identified in other studies (Ogawa, Yoshimura et al. 2010, Ou, Dong et al. 2022). Sequences were

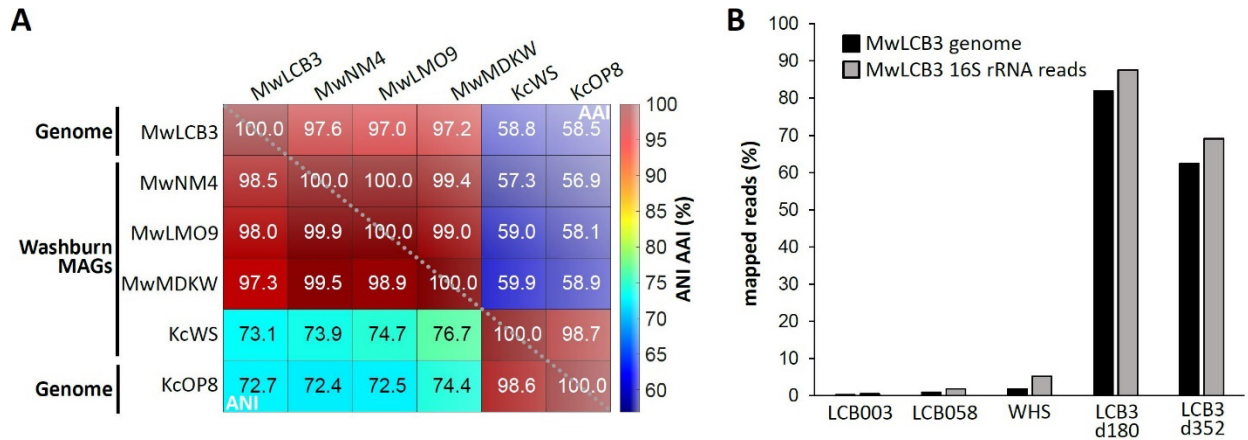
aligned with Mafft-LINSi and trimmed with trimal using a 50% gap threshold, producing a final alignment of 327 residues. A maximum likelihood phylogenetic tree was constructed using Iqtree2 with a best fit model LG+I+G4 selected according to Bayesian information criterion and 1000 ultrafast bootstraps.

The environmental distribution of *Ca. M. washburnensis* was assessed via the IMNGS webserver (Lagkouvardos, Joseph et al. 2016) on Oct 20th 2023. The 16S rRNA gene of strain LCB3 was used to search the NCBI Sequence Read Archive (SRA) with a similarity cutoff of 99% or 97% and a minimum size of 200 bp. The 16S rRNA gene and *McrA* of strain LCB3 were also used to search assembled metagenomes available on the JGI IMG/MER webserver (n=12,114 on Nov. 7, 2023) with blastn and blastp, respectively.

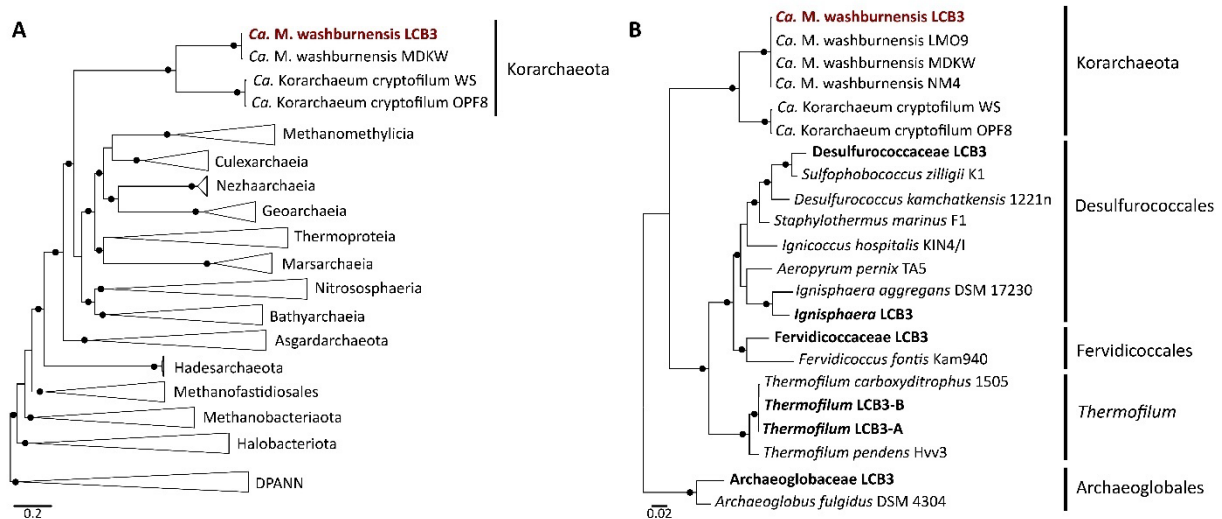
Supporting Information



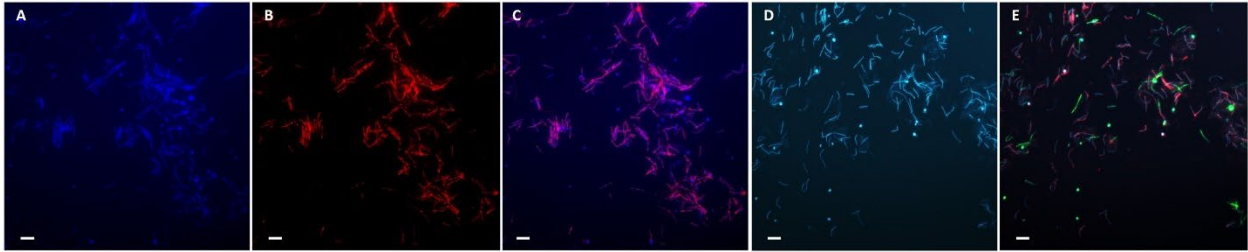
Extended Data Figure 1. Images of hot springs used as source material for cultivation. A. Washburn Hot Springs. B. Hot spring LCB003. C. Hot spring LCB058. All three hot springs are located in Yellowstone National Park (WY, USA).



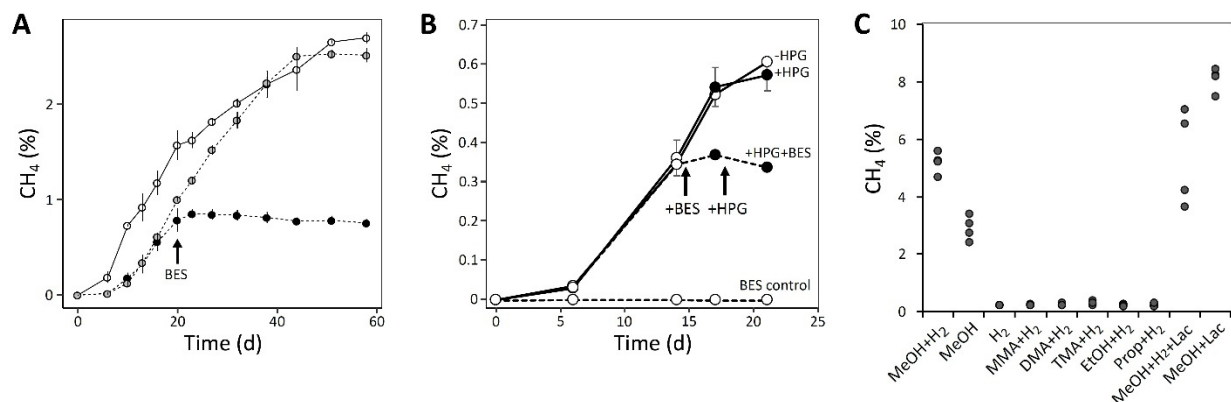
Extended Data Figure 2. Comparison of Korarchaeia genomes and MAGs. A. Pairwise comparisons of average nucleotide (below dotted line) and amino acid (above dotted line) identities (%) of *Ca. Methanodesulfokora* and *Ca. Korarchaeum* genomes and Washburn Hot Spring MAGs. B. *Ca. M. washburnensis* strain LCB3 genome and 16S rRNA gene read abundances in environmental metagenomes LCB003, LCB058, and WHS and in culture LCB3 at day 180 and 352.



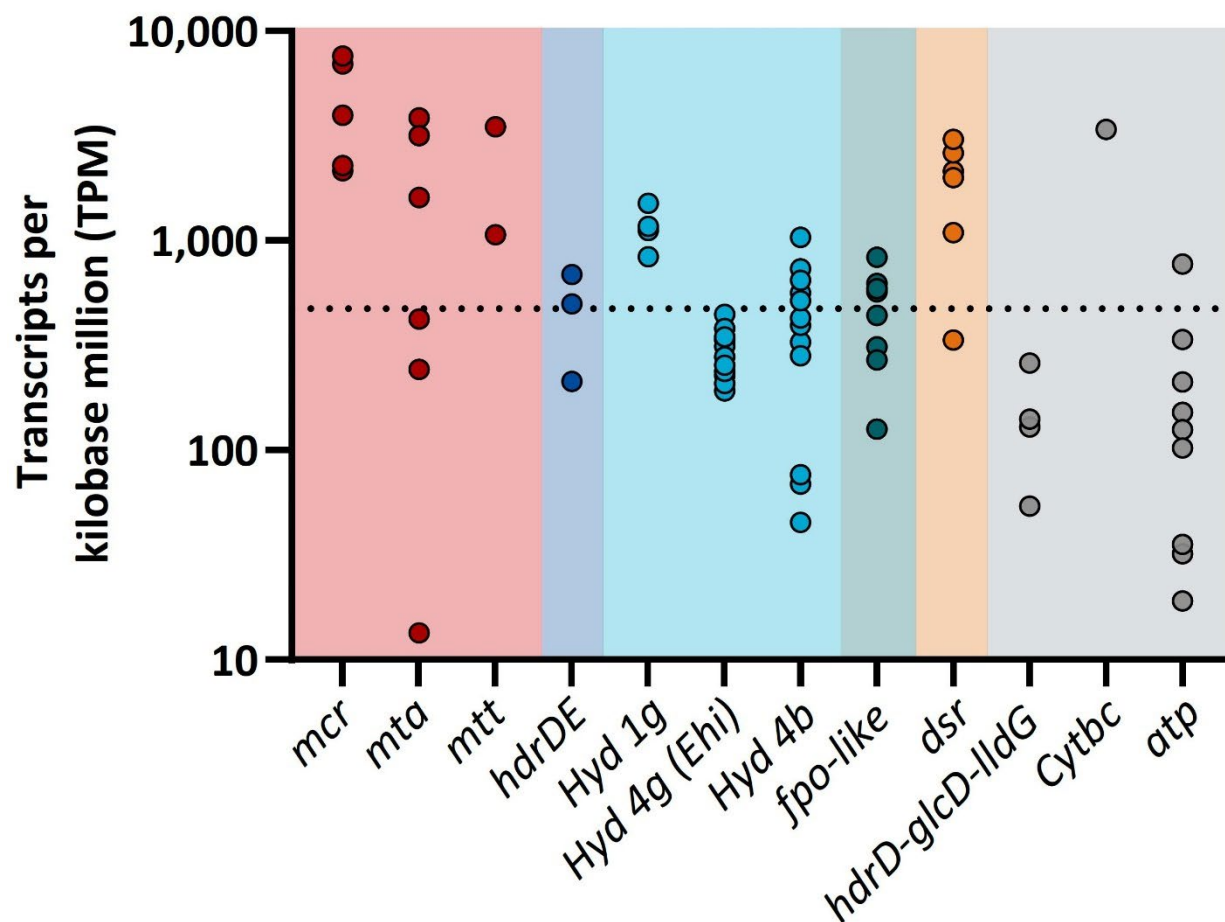
Extended Data Figure 3. Phylogenetic affiliation of *Ca. M. washburnensis* strain LCB3. A. Maximum likelihood phylogenomic tree of archaea based on 33 single copy marker genes. The tree was constructed with IQtree and the best-fit model LG+F+R10, from the concatenated alignment of conserved arCOGs. The *Ca. M. washburnensis* strain LCB3 genome is highlighted in red. Circles indicate ultrafast bootstrap values >95. B. Maximum likelihood phylogenetic tree of 16S rRNA genes from culture LCB3. The tree was constructed with fasttree including the 16S rRNA gene from the LCB3 genome (red), 16S rRNA genes in MAGs from culture LCB3 and reference sequences. Circles indicate bootstrap support values >95.



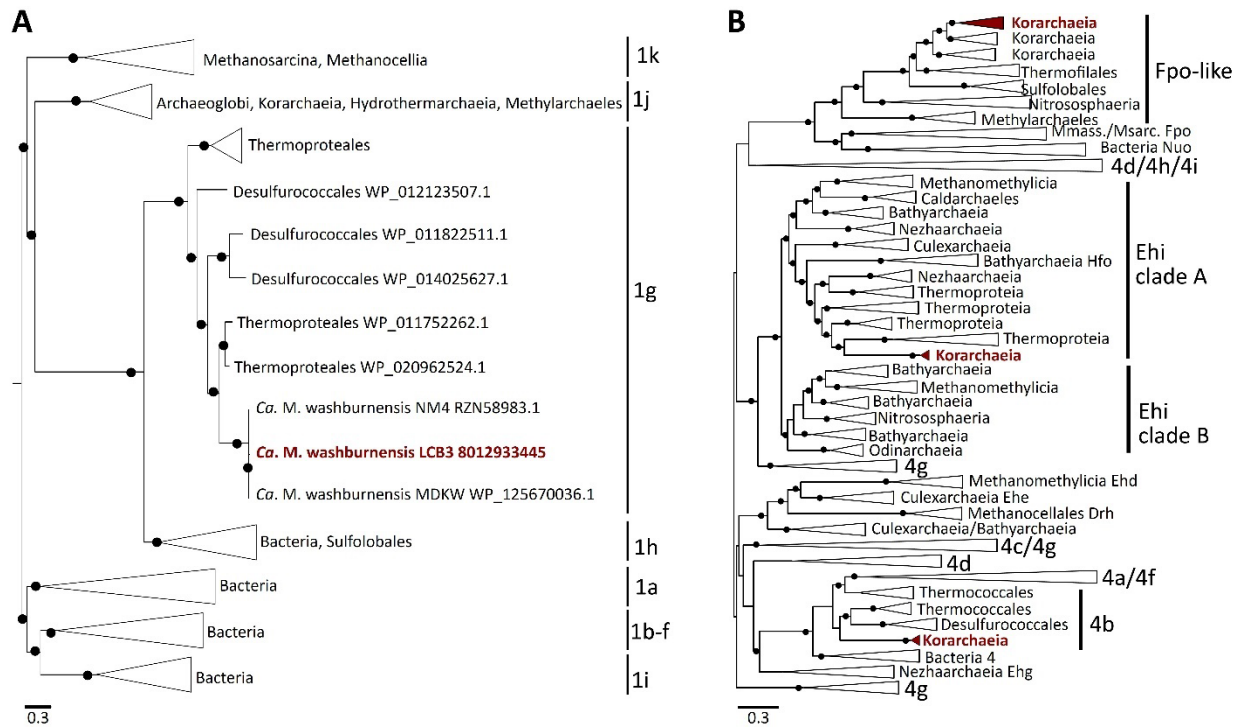
Extended Data Figure 4. Representative fluorescence micrographs of culture LCB3. A. Cells visualized with DAPI (blue). B. Cells visualized by DOPE-FISH using a *Ca. M. washburnensis* specific 16S rRNA-targeted oligonucleotide probe (KRmw515, red). C. Overlay of A and B. D. Cells visualized with DAPI (blue). E. Cells visualized with DAPI (blue, same field of view as in D) combined with dual CARD-FISH using the *Ca. M. washburnensis* specific 16S rRNA-targeted oligonucleotide probe (KRmw515, red) and a general archaea probe (Arch915, green). Note that because of two mismatches of the Arch915 probe to the 16S rRNA of *Ca. M. washburnensis* there is no overlap of signal from probes KRmw515 and Arch915. Scale bars 5 μm .



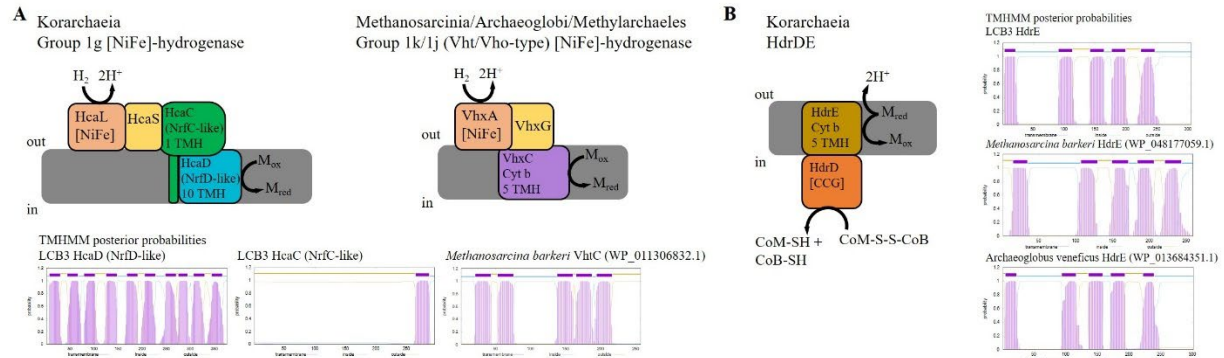
Extended Data Figure 5. Methane production in culture LCB3 under different experimental conditions. A. Methane production curves for SIP incubations (shown in Fig. 1B) amended with 12C-methanol (open white circles), 13C-methanol (gray filled circles), or 13C-methanol with BES (black filled circles). Error bars indicate the standard deviation for three biological replicates. B. Methane production curves for BONCAT-FISH incubations (shown in Fig. 3C) amended with methanol and hydrogen, spiked with BES at day 15 (filled symbols, dashed line) and spiked with HPG at day 18 (filled symbols). Controls were not spiked (open symbols), representing growth under standard cultivation conditions, or were spiked with BES at day 0 (open symbols, dashed line), inhibiting methane production. Incubations were sampled for cell visualization via BONCAT-FISH after 24 h of incubation with HPG (day 19). Error bars indicate standard deviation from three biological replicates. C. Methane produced with different substrates after 85 days of incubation. Error bars indicate the standard deviation for four biological replicates. Concentrations were 10 mM for methanol (MeOH), monomethylamine (MMA), dimethylamine (DMA), trimethylamine (TMA), ethanol (EtOH), and isopropanol (Prop); 2mM for lactate (Lac); and 50% for hydrogen (H₂).



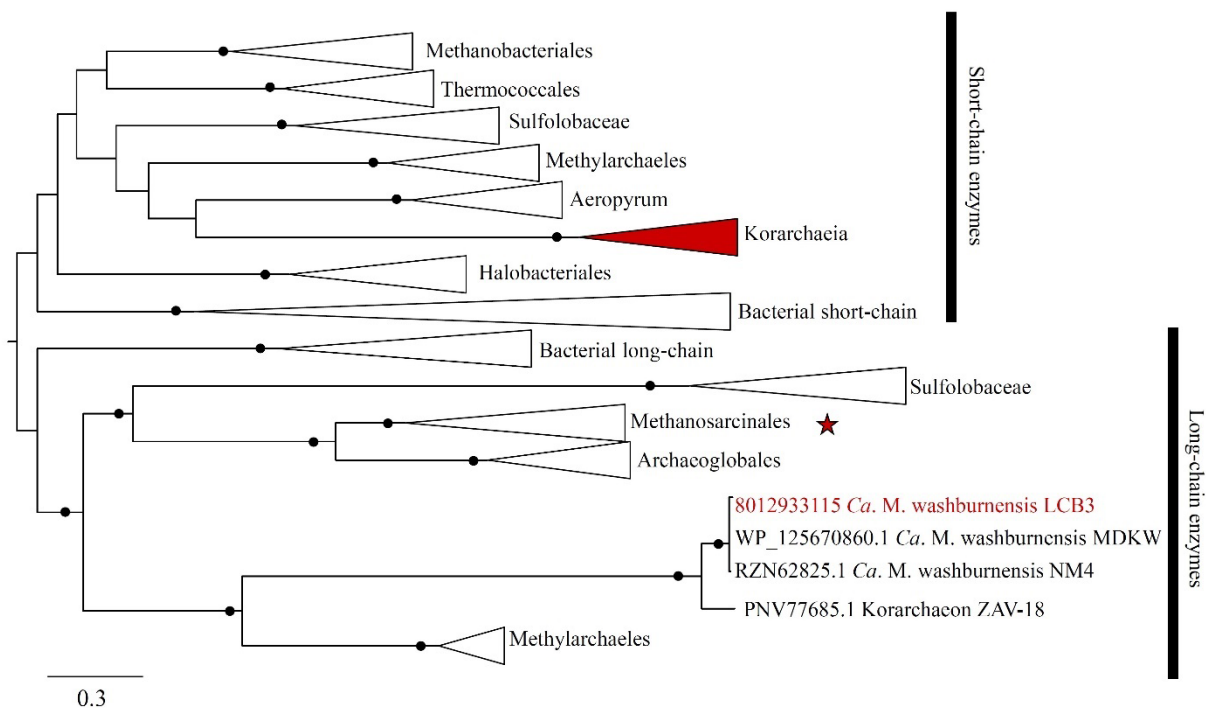
Extended Data Figure 6. Gene expression of *Ca. M. washburnensis* strain LCB3 during methanogenic growth on hydrogen and methanol. Dashed line represents the average transcripts per kilobase million (TPM) of all genes. Each dot represents the expression of a subunit in a multi-subunit enzyme complex. For abbreviations see Figure 4. See SI Table 10, 11 for details on gene annotation and gene expression.



Extended Data Figure 7. Phylogenetic classification of [NiFe]-hydrogenases in *Ca. M. washburnensis* strain LCB3. A. Maximum likelihood phylogenetic tree of group 1 [NiFe]-hydrogenases. The tree was constructed using IQtree2 with the best fit model LG+R10 and 1000 ultrafast bootstraps. Hydrogenase classes were assigned according to the HydDB. The sequence from the LCB3 genome is highlighted in red. Black circles indicate ultrafast bootstrap values >95. B. Maximum likelihood phylogenetic tree of group 4 [NiFe]-hydrogenases. The tree was constructed using IQtree2 with the best fit model LG+R10 and 1000 ultrafast bootstraps. Clades containing sequences from the LCB3 genome are highlighted in red. Black circles indicate ultrafast bootstrap values >95.



Extended Data Figure 8. Annotation of enzyme complexes in *Ca. M. washburnensis* strain LCB3. A. Comparison of group 1 [NiFe]-hydrogenase organization and structure between *Ca. M. washburnensis* strain LCB3 and other MCR-encoding archaeal lineages. Models of the enzyme arrangement in the membrane with matching colors between the models indicating conserved function. Transmembrane helix (TMH) probabilities for intermembrane subunits. Groups 1j/1k have b-type cytochrome containing subunits with five helices, while the Korarchaea group 1g [NiFe]-hydrogenase has an NrfD-like subunit (HcaC) with ten helices and lacks b-type cytochromes. B. Annotation of the *Ca. M. washburnensis* strain LCB3 HdrDE complex. Model representing the arrangement of genes in the membrane. All HdrE subunits analyzed have 5 TMHs. The HdrD subunit in the LCB3 genome has cysteine residues that are conserved in HdrD subunits from other lineages. Transmembrane helices were predicted using the TMHMM 2.0 server.



Extended Data Figure 9. Phylogenetic tree of geranylgeranyl diphosphate synthase homologs in archaea and bacteria. Red clades and text correspond to homologs encoded in *Ca. M. washburnensis* strain LCB3. Red star indicates the clade containing the *Methanosarcina mazei* homolog involved in methanophenazine biosynthesis. Circles represent ultrafast bootstrap values >95. Short-chain and long-chain designations according to (Ou, Dong et al. 2022).

Etymology and Description of Strain LCB3

Etymology. *Methanodesulfokora*, *methanum* (Latin): methane, *de* (Latin): from, *sulfo* (Latin): sulfur, *kore* (Greek): young woman; *washburnensis* (Latin): pertaining to Washburn Hot Springs. The name implies a methane and sulfur metabolizing member of the class Korarchaeia within the phylum Thermoproteota, which metagenome-assembled genome was first obtained from Washburn Hot Springs in Yellowstone National Park. The archaeon cultured in this study represents *Ca. M. washburnensis* strain LCB3.

Locality. Enriched from geothermally heated sediment of an unnamed hot spring, catalogued as feature LCB003, in the Lower Culex Basin of Yellowstone National Park, WY, USA.

Diagnosis. Anaerobic, thermophilic, methyl-reducing methanogen of filamentous morphology.

Acknowledgements

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Data Availability

16S rRNA gene and mcrA gene amplicon data, metagenomic reads, and metatranscriptomic reads are deposited at NCBI under BioProject PRJNA913929. Metagenomes and genomes are

available on IMG/M (JGI) under IMG Genome IDs 3300005860 (WHS), 3300043541 (LCB058), 3300028675 (LCB003), 8012931703 (Ca. *M. washburnensis* strain LCB3), 8015587805 (Archaeoglobaceae archaeon LCB3), 8015589684 (*Thermofilum* sp. LCB3-A), 8015591669 (*Thermofilum* sp. LCB3-B), 8015593670 (Fervidicoccaceae archaeon LCB3), 8015596852 (*Ignisphaera* sp. LCB3), 8015595177 (Desulfurococcaceae archaeon LCB3).

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CHAPTER FIVE

CONCLUSION

The overarching themes of the work presented in this thesis involve archaeal diversity, methanogenesis, methylotrophy, and cultivation. The hot springs we studied in Yellowstone proved to be excellent samples for the cultivation and metagenomics-based exploration of novel lineages of methylotrophic archaea. This conclusion presents a concise overview of the research and potential areas for future research.

In chapter 2 the discovery of *Culexarchaeia* broadened the diversity of archaea, particularly those that have the potential for non-methanogenic methylotrophy. Future work would be drastically enhanced by the development of a FISH probe that specifically targets these organisms. While one probe was designed in our study, hybridization experiments with it were ultimately unsuccessful. This could have been due to several factors, including poor accessibility of the probe to the rRNA, low abundance of *Culexarchaeia* in the hot springs sampled, or low ribosome content of the cells at the time of sampling. *Culexarchaeia* were found to be globally distributed and more abundant in other samples, particularly marine hydrothermal sediments in Guaymas Basin, where in some samples they made up >1% relative abundance. The design of additional probes in different regions of the 16S rRNA gene and their application to more samples would likely result in a successful FISH probe. With the ability to specifically detect *Culexarchaeia* cells via FISH, this would enable stable isotope probing experiments to detect the incorporation of different substrates via Raman microspectroscopy or nanoSIMS experiments. Additionally, FISH would enable tracking the enrichment of *Culexarchaeia* cells in cultivation attempts. While their metabolic potential indicates a generalist lifestyle, we did identify

cyanophycin as a substrate that may be useful in cultivation attempts, given that the metabolism of this compound is rare among Archaea. Cyanophycin may further be combined with antibiotics to reduce the growth of any potential cyanophycin degrading bacteria.

In chapter 3 we cultivated and validated that a member of the Thermoproteota, in the class Methanomethylica, is a methanogen. This organism, *Ca. Methanosuratincola verstraetii* strain LCB70, uses methanol and hydrogen to make methane. The high quality meta-omic data and development of a family-specific FISH probe in this study are likely to enable more studies of this organism, and closely related ones, in their native environments. Additionally, the growth conditions and published media composition are likely to lead to more cultures of Methanomethylica from diverse habitats. Improved studies of the natural gene expression, contributions to methane fluxes, and cultivation of more representatives from diverse environments will allow for more robust statements about the physiology and contributions to global methane emissions of this lineage. Additionally, the strain LCB70 culture is still an enrichment and further attempts to purify the culture to isolation would improve future follow-up studies focused on their biochemistry and physiology.

The wide distribution of Methanomethylica across different environments also opens the possibility for comparative genomics, looking at how different clades within the Methanomethylica differ in gene content and if there are specific adaptations required for inhabiting different niches. Methanomethylica are present in mesophilic and thermophilic habitats, so one approach could study the specific adaptations to high temperature environments. The close phylogenetic relationship between the Methanomethylica and the non-methanogenic Culexarchaeia could also be studied in further detail to determine what genes were specifically

lost or gained along the transition between lifestyles. Then, genes involved in this transition could be contrasted with existing examples in the Euryarchaeota to determine if there are trends or commonalities in the selective pressures that have driven transitions between lifestyles. Additionally, further discovery and description of more deeply branching lineages related to the Methanomethylica and Culexarchaeia will provide more resolution to these metabolic transitions.

In chapter 4 we cultivated and validated that another member of the Thermoproteota, in the class Korarchaeia, is a methanogen. This organism, *Ca. Methanodesulfokora washburnensis* strain LCB3, similarly uses methanol and hydrogen to make methane. However, it differs substantially in its metabolic potential relative to the Methanomethylica culture. In particular, it is more thermophilic and uses a complex membrane bound and cytochrome-b containing electron transport chain that is not found in strain LCB70. Additionally, strain LCB3 expresses genes involved in dissimilatory sulfur metabolism, even under methanogenic conditions. Whether this is a detoxification strategy for sulfite or would enable energy conservation in connection with sulfur metabolism will require further studies. Additionally, cultures of both LCB3 and LCB70 were found to be stimulated by the addition of lactate. The metabolism of this compound by these organisms will require further studies to determine how it specifically affects anabolic or catabolic processes.

In summary, the results in this thesis provide crucial insights into archaeal taxonomic and metabolic diversity. Furthermore, we provide a critically important validation of recently discovered and previously uncultivated methylotrophic methanogens in the lineage Thermoproteota. Additionally, the studies presented here further demonstrate the tractability of

geothermal features like the hot springs in Yellowstone National Park for making discoveries regarding microbial diversity and for the cultivation of novel extremophilic microbes. These important natural resources will undoubtedly continue to provide more insights into microbial diversity, ecophysiology, and biotechnology in the future.

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