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2024

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UNIVERSITY OF CALIFORNIA SAN DIEGO
SAN DIEGO STATE UNIVERSITY

Methane Cycling in Arid Soils: Methanotrophic Microbiomes and Associations with Plants

A Dissertation submitted in partial satisfaction of the requirements
for the degree Doctor of Philosophy

in

Biology

by

Nathalie Andree Delherbe Martínez

Committee in charge:

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Professor Eric E. Allen
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San Diego State University
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Professor Nicholas Barber
Professor Elizabeth R. Waters

2024

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Chair

University of California San Diego
San Diego State University

2024

DEDICATION

To everyone who made it possible for girls to dream of being women in science.

To all the women in science who walked the path and kept it open for everyone.

EPIGRAPH

“Probablemente de todos nuestros sentimientos el único que no es verdaderamente nuestro es la esperanza. La esperanza le pertenece a la vida, es la vida misma defendiéndose.”

*“Probably of all our feelings the only one that is not truly ours is hope.
Hope belongs to life; it is life itself defending herself.”*

— Julio Cortázar, Rayuela, capítulo 28

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ACKNOWLEDGEMENTS

Thanks to my family, hermanitas, papá, mamá, abuelos, and tío abuelos, to my friends in Chile and the US, thank you for your support, empowerment, and love, I couldn't make it without each of you, and thanks for being there in the difficult moments and in the celebrations. To my loved partner, you were there before during, and after this Ph.D. and endured closely in the challenging moments and celebrated every accomplishment, in addition to cooking delicious food and having fun every possible moment, you have been the best fan and a huge source of inspiration; I can't imagine writing this without you on my side. I would have named you all here, but this is going to be public, and you are so important that many times you might be an answer to a security question in some U.S. government questionnaire.

Thanks to the ECODIM family, who made it possible to even think about seeking a Ph.D. abroad, speak English, and have fun at the same time. Thank you, Nicole and Rod, a.k.a Dr. Nicole Treafult, and Dr. Rodrigo De La Iglesia, pretty much my putative older sister and brother, thanks for always being there, since I had the fortune to meet you while I was an undergrad, you had been a huge example, inspiration and support, and I'm sure you both represent that for many other lucky students. Thank you to Kurt (Dr. Kurt Hanselmann), Ger (Dr. Ger Van den Engh), Daniel (Dr. Daniel Vaultot), Jake (Dr. Jake Bailey), and Bev (Dr. Beverly Flood), your support before and during the program had been precious, and what you built on the Summer Schools changed for good the scientific future of so many. Thank you, Eric, Dr. Eric E. Allen, for casually inviting us to visit you in California during the 2012 ECODIM, your enthusiasm and support opened the doors for the beginning of this adventure, and I will be everlastingly grateful. Thank you for receiving me (us) and also being my Master advisor.

A huge thank you to my dear mentor Marina, Dr. Marina Kalyuzhnaya, who received, guided, and supported me through one of the most difficult academic situations that can be faced during a Ph.D., I will be forever grateful. You also casually invited me to visit you at SDSU, and it turned out to be a long-term stay, thank you! Thank you for teaching all of us with your actions how to navigate academia (and humanity) as a strong, knowledgeable, creative, and passionate woman in STEM. Thank you for supporting my formation and the one of many others, hopefully, you know the impact you have had on our careers, I'm forever grateful and feel lucky to have crossed our paths. A big thanks to my great lab mates, especially Ricky and Chynna, thanks for the support during this thesis and the fun moments.

Thanks to the members of my dissertation committee, Dr. Elizabeth R. Waters, Dr. Nicholas Barber, Dr. Jack Gilbert, and Dr. Eric E. Allen for your guidance and feedback (and patience!).

Chapter 1, in part, will soon be submitted for publication of the material as it may appear in ISME journal 2024, as "Reverse Chimney: Atmospheric Methane-Munching Biomes of the Southern California Desert (Anza-Borrego National Park)". Nathalie A. Delherbe, Oscar Gomez, Alvaro M. Plominsky, Aaron Oliver, Maximino Manzanera, and Marina G. Kalyuzhnaya. The dissertation author was the primary researcher and author of this paper.

Founding sources: U.S. Department of Energy (DOE) Joint Genome Institute (JGI), award 10.46936/10.25585/60001056 to Dr. Kalyuzhnaya for the project Systems level insights into methane cycling in arid and semi-arid ecosystems via community metagenomics and metatranscriptomics

U.S. Department of Energy under the DOE Office of Science (SC) RENEW contract DE-SC0024289.

SDSU Completion of Research and Creative Activity (CORE) Fellowship 2023.

Department of Energy Systems Biology Knowledgebase (KBase) Data Management Plan. Dr. A. Murat Eren (Meren) and his team for developing, improving, and maintaining their software, and for all their amazing virtual support while learning to use it. And Dr. Wilfred Vermerris for providing advice and seeds to grow *Sorghum*.

Chapter 2, in full, is a reprint of the material as it appears in "Genomic Insights into Moderately Thermophilic Methanotrophs of the Genus *Methylocaldum*." Microorganisms 12, no. 3 (2024): 469. Nathalie Delherbe, David Pearce, Sergey Y But, J Colin Murrell, Valentina N Khmelenina, and Marina G Kalyuzhnaya. The dissertation author was the primary investigator and author of this paper.

Founding sources: *M. szegediense* O-12 genome sequencing and assembly were carried out by the Joint Genome Institute (JGI) under the Office of Science of the U.S. Department of Energy contract DE-AC02-05CH11231. This work was funded by the EnvEast DTP (UKRI grant NE/L002582/1) and Norfolk County Council and by the U.S. Department of Energy under the DOE Office of Science (SC) RENEW contract DE-SC0024289.

Chapter 3 is coauthored with Chynna Bowman, John McNamara, Daisy Ulloa, Uduak George, Elizabeth R. Waters, and Marina G. Kalyuzhnaya. The dissertation author was the primary researcher and author of this chapter.

Founding sources: Founding sources: U.S. Department of Energy (DOE) Joint Genome Institute (JGI), award 10.46936/10.25585/60001056 to Dr. Kalyuzhnaya for the project Systems level insights into methane cycling in arid and semi-arid ecosystems via community metagenomics and metatranscriptomics

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PUBLICATIONS

Nathalie Delherbe, David Pearce, Sergey Y But, J Colin Murrell, Valentina N Khmelenina, and Marina G Kalyuzhnaya. "Genomic Insights into Moderately Thermophilic Methanotrophs of the Genus *Methylocaldum*." *Microorganisms* 12, no. 3 (2024): 469.

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FIELD OF STUDY

Major Field: Microbiology
Studies in Methanotrophic Bacteria
Professor Marina Kalyuzhnaya

ABSTRACT OF THE DISSERTATION

Methane Cycling in Arid Soils: Methanotrophic Microbiomes and Associations with Plants

by

Nathalie Andree Delherbe Martínez

Doctor of Philosophy in Biology

University of California San Diego, 2024
San Diego State University, 2024

Professor Marina G. Kalyuzhnaya Chair

This work takes the first steps towards linking symbiotic associations of microbes to desert plants with global methane cycling. Drylands occupy 37.1% of the Earth's terrestrial surface and are increasingly being converted into agricultural fields. Sustainable and productive use of arid land requires a better understanding of the functional traits of microbes inhabiting native arid environments. To improve our knowledge of native arid ecosystem functioning, we performed

preliminary studies with soil samples collected from Anza-Borrego Desert State Park. The following discoveries were made: 1) Metagenomic data identified the methanotrophic bacteria *Methylocaldum* sp. as the predominant genus within the methanotrophic community in vegetated and unvegetated soil of the Anza-Borrego desert. 2) The consumption rate of atmospheric methane positively correlated with the presence of vegetation; 3) Genome annotations of methanotrophs from the rhizosphere of desert plants highlight several functions that can contribute to interactions with plants; 4) Methanotrophs have the potential to improve plant survival during drought stress. Based on these data we propose that in arid environments plants facilitate methane transport to the ground and cooperate with methane-consuming microbes to capture methane efficiently evolved in deeper soil layers and atmospheric methane (i.e. reverse chimney), which is then metabolized to provide supplemental resources for survival in water-limiting conditions. The results suggest that the plant-methanotroph associations represent an overlooked sink of atmospheric methane in dryland ecosystems. We show that supplementation with native *Methylocaldum* isolates from the Anza-Borrego Desert can potentially improve crop drought tolerance (e.g. *Sorghum*). Furthermore, this work provides essential evidence that targeted engineering of association between crops and methanotrophs in dryland soils can enhance atmospheric methane sink, ultimately influencing the global climate.

INTRODUCTION

The climate is changing faster than previously predicted, and greenhouse gases (GHG) are the chemical footprint of human activities accelerating it. After CO₂, methane is the second most abundant GHG, and the most abundant reduced compound (Dean et al., 2018) and hydrocarbon (Katzenstein et al., 2003) in our atmosphere. Methane (CH₄), a single carbon gas, has a global warming potential 84 times higher than CO₂ over 20 years. It is generated by a myriad of natural and anthropogenic processes. Current estimations indicate that human-induced emissions exceed natural sources (Myhre, Shindell, & Pongratz, 2014; Reay et al., 2018), and many efforts are seeking to address its mitigation worldwide (Duren et al., 2019).

The biological component of these emissions comes from the presence of two groups of organisms, having producers of CH₄ (methanogens) and consumers of it (methanotrophs), being the final balance between their metabolic activities, in addition to the chemical production of CH₄ in each environment, what determines if they have a net emission (source) or uptake (sink) of CH₄ from the Earth's surface to the atmosphere (net flux)(Reay et al., 2018).

Recent studies revealed that in addition to anaerobic archaea as well-known methanogens, members of the Eukarya domain had been reported to produce CH₄ in the presence of oxygen, including cyanobacteria, fungi, microalgae, plants, and animals (Bižić et al., 2020; Keppler et al., 2006; Lenhart et al., 2012; Lenhart et al., 2016; Tuboly et al., 2013). On the other hand, comparatively, very few bacteria and archaea have the metabolic capacity to consume CH₄ and be considered as methanotrophs.

Methanotrophs are microorganisms that utilize CH₄ as their sole carbon source for energy and biomass. While previously described as exclusively aerobic and belonging uniquely to Pseudomonadota (Proteobacteria) (Oren et al., 2021; Oren & Garrity, 2021), recently, modern techniques allowed the discovery of methanotrophs as members of the phylum Verrucomicrobiota (Verrucomicrobia) (Oren et al., 2021; Oren & Garrity, 2021) and the novel Methyloirabilota phylum (NC10), both phylogenetically distant of Pseudomonadota (Proteobacteria) (Bodelier et al., 2019; Chistoserdova, 2018; Dedysh & Knief, 2018).

Furthermore, two decades ago, anaerobic methanotrophic archaea (ANME) were described as performing anaerobic oxidation of CH₄, inhabiting the sediments of marine methane seeps; to them is attributed being one of the major consumers of methane on the planet before it reaches the atmosphere (Boetius et al., 2000; Hinrichs et al., 1999; Knittel et al., 2005; Lloyd, Alperin, & Teske, 2011; Timmers et al., 2017).

Although relying on an exclusive metabolism, considering how phylogenetically distant methanotrophic microorganisms are, it is not surprising they can be found in an extensive range of habitats (Bodelier et al., 2019; Dedysh & Knief, 2018). One extreme ecosystem on which methanotrophs are present is in deserts. As well as other types of aerated soils, drylands have been identified as methane sinks, turning them into an excellent environment to exploit to mitigate CH₄ from the atmosphere. Although the mechanisms and the extent of the impact of biological methane oxidation in deserts over the global carbon budget require a comprehensive understanding, the research in the area counts with a limited number of studies including the Negev Desert, Israel (Angel & Conrad, 2009).

Through a combination of models and observations, it has been indicated that semi-arid ecosystems account for the largest fraction, 39% of the net biome production interannual variability (NBP IAV) of the planet, which takes into account the global gross primary production, ecosystem respiration, and wildfires, and therefore account for most of the variability because of its balance being highly influenced by precipitation and temperature (Ahlström et al., 2015).

Even though semiarid and arid regions (drylands) cover approximately 37.1% of the land surface of the planet (Safriel et al., 2005) and their expansion through desertification processes is a major environmental issue (Tolba & El-Kholy, 1992), there is still a lack of consensus around the speed and mechanisms of the process (Goudie, 2013).

Considering the above-mentioned characteristics of drylands, it is not only interesting but urgent to increase the biogeochemical understanding of these ecosystems from the micro to global scale to protect and enhance their natural methane-sink capacity. All of this motivates this thesis which aims to contribute to the understanding of the functioning of dryland ecosystems as a methane sink. Furthermore, the goal is to apply this extended knowledge to improving the use of soil for agriculture.

With this purpose in mind, becomes essential to consider the capacity of the soil as a living system that sustains biological productivity, maintains environment quality, and promotes plant health (Doran & Zeiss, 2000). The microbial component of the soil is the protagonist of those features, which to be understood requires to delve into the metabolism, growth, and evolution of their members (Gentry, Fuhrmann, & Zuberer, 2021) starting at the individual level, and attempt to understand it at the population and community levels.

Specifically, this work will study the methanotrophs inhabiting the soil of the Anza-Borrego Desert, State Park in California, with a particular focus on the genus *Methylocaldum*. This genus, as is indicated in its name, is a heat-loving microorganism, that can be found in a diversity of environments including aquatic and soils (Takeuchi, 2016), although its adaptations for thriving under high temperatures make it well fit for inhabiting drylands soils, and a promising methanotrophic model for this research.

Considering the unique characteristics of deserts and their impact on mitigating GHG, it is urgent to increase the biogeochemical understanding of these ecosystems. The main goal of this dissertation is to improve the understanding of the microbial communities that enable biological methane cycling and sinking in arid ecosystems. Additionally, the impact of their interactions with plants on the desert's function as a sink for GHG will be explored. Furthermore, in future research, the applicability of desert microbiomes to improve arid/marginal soil health in agricultural settings will be evaluated.

This dissertation includes the following chapters:

Chapter 1: Metagenomics, methane fluxes, and microbial communities in Anza Borrego as a model arid ecosystem.

Chapter 2: Cultivation approach and genome of methanotrophic isolates.

Chapter 3: Integrative ecology, plant-methanotroph interactions in arid microcosms.

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Chapter 1 Metagenomics, Methane Fluxes, and Microbial Communities in Anza-Borrego as a Model Arid Ecosystem.

Chapter 1, in part, will be submitted for publication of the material as it may appear in ISME journal 2024, as “Reverse Chimney: Atmospheric Methane-Munching Biomes of the Southern California Desert (Anza-Borrego National Park)”. Nathalie A. Delherbe, Oscar Gomez, Alvaro M. Plominsky, Aaron Oliver, Maximino Manzanera, and Marina G. Kalyuzhnaya. The dissertation author was the primary researcher and author of this paper.

1.1 Abstract

Drylands, including semiarid, arid, and hyper-arid regions, cover one-third of the Earth's surface and are one of the largest terrestrial sinks for methane. Understanding the structure-function interplay between members of arid biomes can provide critical insights into resilience mechanisms toward anthropogenic and climate-change-driven environmental stressors—water scarcity, heatwaves, and increased atmospheric greenhouse gases (GHGs). This study integrates *in situ* measurements with culture-independent and enrichment-based investigations of methane-consuming microbiomes inhabiting soil in the Anza-Borrego Desert, a model arid ecosystem in Southern California (US). The atmospheric methane consumption ranged between 2.26 to 12.73 $\mu\text{mol CH}_4 \text{ m}^{-2} \text{ h}^{-1}$, peaking during the daytime at vegetated sites. Metagenomic studies revealed similar soil-microbiome compositions at vegetated and unvegetated sites, with *Methylocaldum* spp being the major methanotrophic clade. Eighty-four metagenome-assembled genomes (MAGs) were recovered, with six corresponding to methanotrophic bacteria (three *Methylocaldum*, two

Methylobacter, and one member of uncultivable *Methylococcaceae*). The prevalence of copper-containing methane monooxygenases (CuMOs) in metagenomic datasets suggests a diverse potential for methane oxidation. Signatures of methanotrophic functions (including *pmoAB* and *pmoC* genes) were found in MAGs affiliated with Acidimicrobia and Pedosphaerales. Parallel enrichment studies recovered five pure cultures of methanotrophic bacteria, including four *Methylocaldum* species. Genomic analysis of the *Methylocaldum* isolates and MAGs revealed unique adaptations, including multiple stand-alone *pmoC* paralogs that may have functions beyond methane oxidation. In addition, only vegetative sites harbored methanotrophs with additional genetic signatures typically linked to symbiotic interactions with plants, including tryptophan synthesis and indole-3-acetic acid (IAA) production. Based on *in situ* fluxes and soil microbiome compositions, we propose the existence of arid-soil reverse chimneys, an empowered methane sink represented by yet-to-be-defined cooperation between desert vegetation and methane-consuming microbiomes.

1.2 Introduction

Greenhouse gases (GHG) are the chemical footprint of human activities that accelerate climate change. Methane (CH₄) is the second most abundant GHG, after carbon dioxide (CO₂), constituting the most abundant reduced compound (Dean et al., 2018) and hydrocarbon (Katzenstein et al., 2003) in our atmosphere. Notably, CH₄ has a global warming potential 84 times higher than CO₂ over a period of 20 years (Pachauri et al., 2014). Approximately 500-600 Tg (1 Tg = 10¹² g) of CH₄ are emitted globally into the atmosphere every year (Kirschke et al., 2013; Menon et al., 2007) originated from different natural and anthropogenic processes (Bridgham et al., 2013; Ciais et al., 2014; IPCC, 2014; Kirschke et al., 2013; Myhre, Shindell, & Pongratz, 2014;

Reay et al., 2018), posing the urgent need for worldwide mitigation efforts (Duren et al., 2019). Biological CH₄ emissions are driven by the interplay of two groups of organisms: CH₄ producers (mostly methanogens) and consumers (often described as methanotrophs). The metabolic activities of these two functional microbial groups determine the net CH₄ flux of ecosystems as a CH₄ source (presenting net emissions) or sink (presenting a net uptake from the atmosphere) (Reay et al., 2018). Soils are the major sinks of atmospheric CH₄, consuming 30-42 Tg per year through the activity of methanotrophic bacteria (Beck et al., 2012; Bousquet et al., 2011; Curry, 2007; Ito & Penner, 2004; Spahni et al., 2011). The CH₄ uptake rates in soils—which vary significantly depending on the ecosystem—have declined in the past decades, mostly due to anthropogenic disturbances (Aronson & Helliker, 2010; Ni & Groffman, 2018; Schaefer et al., 2016). The CH₄ consumption rates in dryland areas (which include semiarid, arid, and hyper-arid regions) have been calculated to have an average annual consumption rate as high as 0.66 mg CH₄ m⁻² d⁻¹ (Striegl et al., 1992), which is comparable to that of grassland and forest soils (0.65 mg CH₄ m⁻² d⁻¹ and 0.74-1.26 mg CH₄ m⁻² d⁻¹, respectively) (Henckel et al., 2000; Le Mer & Roger, 2001). Still, the fact that dryland environments comprise roughly one-third of the land surface on Earth (Middleton & Thomas, 1997; Safriel et al., 2005), denotes the importance of studying these regions for the correct modeling of the global CH₄ budget. As dryland microbes contribute to global climate regulation through CO₂, reactive N, and CH₄ emissions, these processes will also likely alter the rate of GHG release and impact the rate of climate change (Coleine et al., 2024). Considering that dryland ecosystems are predicted to expand due to climate change and land-use shifts (Burrell, Evans, & De Kauwe, 2020), characterization of microbial communities, or soil microbiomes, responsible for biogeochemical fluxes from pristine arid soils is essential for understanding the community dynamics across organizational scales and its effects on global carbon fluxes.

Globally, drylands are dominated by bacteria members of the phyla Actinomycetota (Actinobacteria), Chloroflexota (Chloroflexi), and the Pseudomonadota (Proteobacteria) (i.e., class Alphaproteobacteria) (Coleine et al., 2024). Less is known about microbial groups that control the local flux of CH₄, including CH₄-oxidizing bacteria, anaerobic methanotrophic archaea, and methanogenic archaea (Guerrero-Cruz et al., 2021). Different microbial groups, including members of the genera *Methylocapsa*, *Methylococcus*, and the family *Methylocystaceae* have been identified as possible players in methane cycling in these environments (Lafuente et al., 2019). A study assessing >3,400 metagenomes to examine the global patterns of CH₄ metabolism marker gene abundances in soil (a proxy for the distribution of CH₄-metabolizing microorganisms), revealed the existence of latitudinal trends in the global abundances of these microbes (Ou et al., 2024). The variations in global abundances of CH₄-metabolizing microorganisms were primarily governed by vegetation cover type (Ou et al., 2024). Still, the dominant methanotrophs were highly variable across the vegetation types and aridity classes studied (Lafuente et al., 2019).

In this study, we selected the Anza-Borrego Desert State Park (mentioned hereafter as ‘Anza-Borrego’) as a model system for investigating methanotrophic biomes. The Anza-Borrego Desert ecosystem lies within the Colorado Desert of southern California, United States. Once a tropical forest, then a wetland and savanna, and finally one of the hottest deserts in the western US, this area was assessed to determine the impact of microbiota from semiarid regions regarding their impact on the methane cycle. This study integrates *in situ* CH₄ fluxes measurements, microbial metagenomics, and the metabolic potential of methanotrophic bacteria isolates, thus, providing insights into how the soil microbiota, with and without vegetation, consumes methane.

1.3 Materials and Methods

1.3.1 Methane Flux measurements

Methane flux measurements were performed on March 20th, 2020, and March 9th, 2023, at the Anza-Borrego Desert State Park, (33.305667, -116.254626; at 208 m over sea level; Supplementary Fig. S1.1A) using an ultra-portable greenhouse gas analyzer (UPGGA) LGR model 915-001 (ABB Inc., Quebec, Canada). The initial data were collected in 2016 and 2018 using a 3 L chamber. We returned in March 2020 and 2023 to remeasure methane fluxes using a larger 12.87 L acrylic chamber. The chamber was tightly placed over either vegetated (e.g., with the endemic desert verbena *Abronia villosa*) or unvegetated patches while measuring methane fluxes *in situ* (Supplementary Fig. S1.1 B-C). Measurements taken every 1 s were collected during >15 min on six sites spaced >3 m apart. On each site, vegetated and their corresponding unvegetated patches were located <0.5 m from each other (Supplementary Fig. S1.1 D-E).

1.3.2 Soil Samples for Metagenomics

Two different sampling campaigns took place in 2016 and 2023. In 2016, samples were collected on May 30th from three depths of 5, 10, and 20 cm in both vegetated (e.g., brittlebush, desert willow) and unvegetated soil patches. Soil samples from 2016 were placed in sterile bags and kept at ambient temperature. Samples (25g) for DNA and RNA extraction were collected into 50 ml tubes containing 25 ml of TE buffer with 10% phenol:ethanol (5:95 v/v) stop solution, placed on ice, and transported to the laboratory for processing. Enrichment cultures were started within 24 h of sample collection. The 2023 soil samples were collected on March 9th from five of the six sites from which methane flux measurements were obtained. On each subsite (vegetated, unvegetated), six technical replicates equivalent to 1 ml volume of soil enclosed in a 2 ml cryovial

were collected at 10 cm of depth (Supplementary Fig. S1.1 F). Samples were preserved immediately in dry ice and subsequently stored at -80°C until processed for DNA extraction.

1.3.3 Enrichment Studies, Pure Culture and Cultivation

Methanotrophic cultures were isolated from soil samples collected in Spring 2015 and 2016, using the previously described enrichment strategy (Kalyuzhnaya, 2016) and cultivated using P₀% medium (Collins & Kalyuzhnaya, 2018). One culture, the strain RMAD was isolated from the roots of *Abronia villosa* from Anza-Borrego. Roots of *A.villosa* were air-dried under sterile conditions for 24 h, and then recovered in 250 µl of P₀% media and transferred to 250 ml borosilicate bottles with rubber-stop caps, and diluted 1:100 in P₀% media, and supplemented with methane (10% of headspace)(Narváez-Reinaldo et al., 2010), and maintained at 37°C at constant agitation at 200 rpm. Cultures were transferred once before plating in solid media for further colony isolation.

Methylotrophic and non-methylotrophic satellite cultures from methanotrophic enrichments or consortia were obtained by serial plating on minimal medium (Hypho) (Harder, Attwood, & Quayle, 1973) supplemented with 0.1% methanol or P₀% media supplemented with 0.1% (v/v) methanol (P_M) and/or 5% Nutrient Broth (P_{NB}, Thermo Scientific™ Oxoid™).

1.3.4 DNA Preparation, Sequencing, Assembly, and Annotation for Metagenomics

Microbiome DNA was extracted using two different methods between samples from 2016 and 2023. Samples from 2016 were extracted using a modified 25:24:1 phenol/chloroform/isoamyl alcohol protocol (Kalyuzhnaya, Beck, & Chistoserdova, 2011). RNA was eliminated using a

QIAGEN RNeasy mini kit and verified by gel electrophoresis. Samples were submitted to the Joint Genome Institute (JGI) for genome sequencing using 2X250 chemistry on a HiSeq 2500 instrument (Illumina, San Diego, USA). All metagenomes from this study were uploaded and annotated through the Integrated Microbial Genomes (IMG) annotation pipelines (v.4.15.1, v.4.15.2, or v.4.16.1 (Huntemann et al., 2015)) following JGI protocols (Supplementary Table S1.1). Samples collected in 2023, were extracted with a phosphate buffer and high pH lysis buffer protocol adapted from Povedano-Prieto (Povedano-Priego et al., 2021) and Zeugin and Hartley (Zeugin & Hartley, 1985) aiming to recover DNA from high-sand content samples that were adsorbing DNA (Naviaux et al., 2005) (Supplementary Material). Samples were sequenced using 2x250 chemistry on NovaSeq (Illumina, San Diego, USA). Metagenomic reads were processed in Kbase (Arkin et al., 2018) following their pipeline for processing metagenomic samples (Chivian et al., 2023), using MetaSpades for assembly (Nurk et al., 2017). Assembled metagenomes were submitted to the Integrated Microbial Genomes & Microbiomes (IMG/M) system (Supplementary Table S1.1) and annotated with the IMG Pipeline (v.5.1.17 (Huntemann et al., 2015)).

1.3.5 Taxonomic Assignment of Reads

Quality filtered pair end metagenomic reads were classified using Kaiju (v1.9.0 (Menzel, Ng, & Krogh, 2016)) with NCBI BLAST nr+euk (Sayers et al., 2022; Wheeler et al., 2007) as a reference database, allowing mismatches (Greedy mode) with 3 maximum mismatches, 65 of minimum Bitscore, and a maximum E-value of 0.01. Kaiju outputs were converted to summary tables agglomerated at the family taxonomic level using the Kaiju2table program (nested inside Kaiju). Individual metagenome tables were merged using R Studio (v2023.12.1+402).

1.3.6 Alpha and Beta Diversity

The resulting merged count, metadata, and taxonomy tables from 2023 metagenomes were further processed using PhyloSeq (McMurdie & Holmes, 2013). The alpha-diversity of taxonomically assigned metagenomic reads without any normalization was determined using Breakaway (Willis & Bunge, 2015). Count data was rarefied to the smallest read set (using "rarefy_even_depth") and used to calculate various measures of alpha-diversity (using "estimate_richness") within PhyloSeq. Additionally, community similarity/dissimilarity metrics were computed using a Bray-Curtis dissimilarity distance matrix with the rarefied datasets and plotted as an NMDS (using "ordinate" and "plot_ordination") with PhyloSeq. Graphs were visualized using ggplot2 (v3.3.6 (Wilkinson, 2011)) and all processing was done in R (v3.6.1).

1.3.7 Recovery and phylogeny of copper monooxygenases (CuMOs) from desert metagenomes

Translated sequences assigned to KEGGs IDs K10946 (*pmoC/amoC*), K10944 (*pmoA/amoA*), K10945 (*pmoB/amoB*) encoding for the three subunits for two copper monooxygenases (CuMOs), particulate methane monooxygenase (*pmoCAB*) and ammonia monooxygenase (*amoCAB*) were retrieved from the Anza-Borrego 2023 metagenomes annotated in IMG/M. All sequences from publicly available prokaryote genomes on the IMG/M database (v2023-10) for these genes were also retrieved, excluding MAGs without detailed source information. Available MAGs for NC10 and endosymbionts were included in the analysis. A phylogenetic tree for each subunit was generated including both sets of genes (Anza-Borrego 2023 metagenomes, and publicly available). All phylogenetic analyses of this study were performed by making an initial alignment using the multiple alignment program MAFFT (v7.511), choosing the

E-INS-i method (Henckel et al., 2000; Le Mer & Roger, 2001). Phylogenetic relationships were inferred with the MEGA X software (v10.2.6) using the maximum likelihood method. The model of each gene sequence set with the lowest Bayesian information criterion (BIC) scores was calculated utilizing partial deletion with a site coverage cutoff of 95%, and corresponded to LG plus gamma distribution with invariant sites (LG+G+I) (Kumar et al., 2018; Le & Gascuel, 2008; Nei & Kumar, 2000; Stecher, Tamura, & Kumar, 2020). A total of 500 bootstrap replications were computed for each phylogenetic tree of this study. The Newick output of the phylogeny was further processed in iTol (Letunic & Bork, 2021) to color-code the position of each metagenome-retrieved sequence while distinguishing between vegetated or unvegetated origin. Additionally, three types of CuMOs (methane, ammonium, or hydrocarbon monooxygenases) were color-coded based on previous reports for the substrate each organism utilized with their corresponding CuMOs.

1.3.8 Generation of metagenome-assembled genomes (MAGs)

For the 2016 metagenomes, MAGs were generated on IMG/M with MetaBAT (v0.32.5 (Kang et al., 2015)), CheckM (v1.0.11 (Parks et al., 2015)), and GTDB database release 86, GTDB-tk (v0.1.6 (Chivian et al., 2023)). For the 2023 metagenomes, MAGs were generated in Kbase with MetaBAT2 (v1.7 (Kang et al., 2019)) with a minimum contig length set at 1500 bp. Bins were filtered by quality using CheckM (v1.0.18 (Parks et al., 2015)), and then taxonomically classified with the GTDB-Tk (v2.3.2 (Chivian et al., 2023)) overwriting taxonomy and selecting the r214 GTDB version.

A comparison based on the annotated predicted coding sequences for MAGs from 2016 and 2023 metagenomes was obtained in Kbase using DRAM (Shaffer et al., 2023).

1.3.9 DNA preparation, sequencing, assembling, and annotation for bacteria isolates

Genomic DNA was extracted from the isolated bacteria and co-cultures utilizing the GeneJET Genomic DNA purification kit (Thermo Scientific #K0721) or DNeasy PowerLyzer Microbial Kit (Qiagen), following the manufacturer's standard protocol. Whole genome sequencing and assembling was executed at JGI and uploaded into the IMG/MER platform (Chen et al., 2021). The sequencing methods utilized either 2X250 chemistry on a Novaseq S4 or Miseq instruments (Illumina, San Diego, USA). All isolate genomes from this study were uploaded and annotated through the Integrated Microbial Genomes (IMG) annotation pipelines (v.5.0.20; v.4.16.2 (Huntemann et al., 2015)) following JGI protocols (Supplementary Table S1.2).

1.3.10 *Methylocaldum* stand-alone *pmoC* homologs

A maximum likelihood tree representing the phylogenetic relationship of the multiple particulate methane monooxygenase subunit C (PmoC) sequences of *Methylocaldum* was generated based on their amino acid sequences. The model with the lowest BIC scores for the PmoC gene set corresponded to LG with a discrete Gamma distribution with 5 rate categories (Kumar et al., 2018; Le & Gascuel, 2008; Nei & Kumar, 2000; Stecher, Tamura, & Kumar, 2020). This analysis involved 40 amino acid sequences. There was a total of 315 positions in the final dataset. A total of 500 bootstrap replications were computed. The genomic contexts were obtained from NCBI GenBank annotations and manually curated and re-annotated using the software Geneious Prime 2023. Exported gene cluster images were manually matched with the corresponding sequences on the PmoC phylogenetic tree.

1.3.11 *In-silico* reconstruction of *Methylocaldum* PmoC folds

Predicted 3D structures for *Methylocaldum* PmoC proteins were generated using ColabFold (v5.1.2 (Mirdita et al., 2022)), and its models from AlphaFold (v2.3.1 (Jumper et al., 2021)). Structure predictions were evaluated based on similarity to the nearest relative with a characterized structure, a pMMO enzyme from *Methylococcus capsulatus* strain Bath determined using cryo-electron microscopy (PDB: 7S4J) (Koo et al., 2022). Predicted structures were visualized with the software ChimeraX (v1.6.1 (Meng et al., 2023)), and distances between *Methylocaldum* PmoC predicted structures and *M. capsulatus* pMMO were determined using the ChimeraX command matchmaker.

1.3.12 *In-silico* evaluation of the metabolic potential of *Methylocaldum* isolates and MAGs

An overall comparison of the metabolic potential for the isolate genomes was performed based on the percentage of genes assigned to each of the 24 COG categories from the IMG/MER annotations (Chen et al., 2021; Mukherjee et al., 2021). A detailed cross-comparison between the genomes of the isolated strains was done following the established pangenome analysis (Delmont & Eren, 2018) using the Anvi'o software (v7 (Eren et al., 2015)). Genome clustering was generated based on the presence/absence of predicted genes (utilizing their amino acid sequence), and grouping by similarity using the MCL algorithm (inflation of 8 (van Dongen & Abreu-Goodger, 2012), and a Minbit score of 0.8). The organization of the clusters was based on Euclidean distance and Ward linkage methods. The predicted genes were annotated with DIAMOND using the fast parameter (Buchfink, Xie, & Huson, 2015). Additionally, KEGG annotation was obtained for each gene call utilizing the KEGG KOfam database as described by Aramaki et al (Aramaki et al.,

2020). Additionally, the Average Nucleotide Identity multiplied by the Aligned Coverage (ANI x AC) values, called full percent identity in the Anvi'o software, were computed with *anvi-compute-genome-similarity* utilizing PyANI (Pritchard et al., 2016) across the ten *Methylocaldum* genomes. This parameter was chosen because it reflects the genomic relatedness among genomes better than ANI alone (Varghese et al., 2015).

1.4 Results and Discussion

1.4.1 *In situ* methane consumption rates are accelerated by vegetation and linked to daylight intensity

Methane (CH₄) consumption rates of Anza-Borrego soil from vegetated sites were observed to vary during the day, reaching their peak during the hours of most intense sunlight with up to 12.73 $\mu\text{mol m}^{-2} \text{h}^{-1}$ at noon (Fig. 1.1A). The average CH₄ consumption rate in vegetated sites between 10:00 am (i.e., 3 h after sunrise) and 2:00 pm (9.07 $\mu\text{mol m}^{-2} \text{h}^{-1} \pm 2.2$) was 2.4 times higher than the rates measured 3 h before sunset (3.78 $\mu\text{mol m}^{-2} \text{h}^{-1} \pm 0.81$; Fig. 1.1B). In contrast, unvegetated sites from the immediate vicinity constantly consumed CH₄ at a rate between 2.26 to 3.74 $\mu\text{mol m}^{-2} \text{h}^{-1} \pm 0.46$ (Fig. 1.1A). Moreover, the peak CH₄ consumption rates in vegetated were significantly different than the average rates for unvegetated sites measured throughout the day (p-value <0.0001; Fig. 1.1B). This serendipitous observation suggests that Anza-Borrego soil CH₄ consumption rates on vegetated sites are linked to sunlight intensity, while the consumption rates of unvegetated patches are constant during the day (Fig. 1.1A). This suggests that when plants are exposed to higher sunlight intensities, they may transport CH₄ from the atmosphere into the

inner layers of the soil where it becomes more accessible to the methanotrophic microbes. This is the foundation for the reverse chimney hypothesis proposed here (Fig. 1.1C), which could partially explain why deserts constitute natural CH₄ sinks. This hypothesis was coined using as a reference an opposed phenomenon: the chimney effect (Bhullar, Edwards, & Olde Venterink, 2013; Bringel & Couée, 2015; Joabsson, Christensen, & Wallén, 1999; Zhang et al., 2019), in which plants transport CH₄ from the anoxic soil layers from their roots through their system to their leaves and release it to the atmosphere (Fig. 1.1D). The reverse chimney hypothesis proposes that in dryland ecosystems (i.e., low organic matter and limited water availability) plants transport CH₄ and oxygen from the atmosphere through their vascular system down to the soil, where it is consumed and converted by methanotrophic microbiota (Fig. 1.1C). Research on how plants contribute to the modulation of CH₄ fluxes has only recently started to be explored. These results hopefully encourage future studies to further address how the CH₄ concentration, macronutrients (i.e., N, S or P), and moisture levels of the soil determine if a system functions as a sink or source of CH₄.

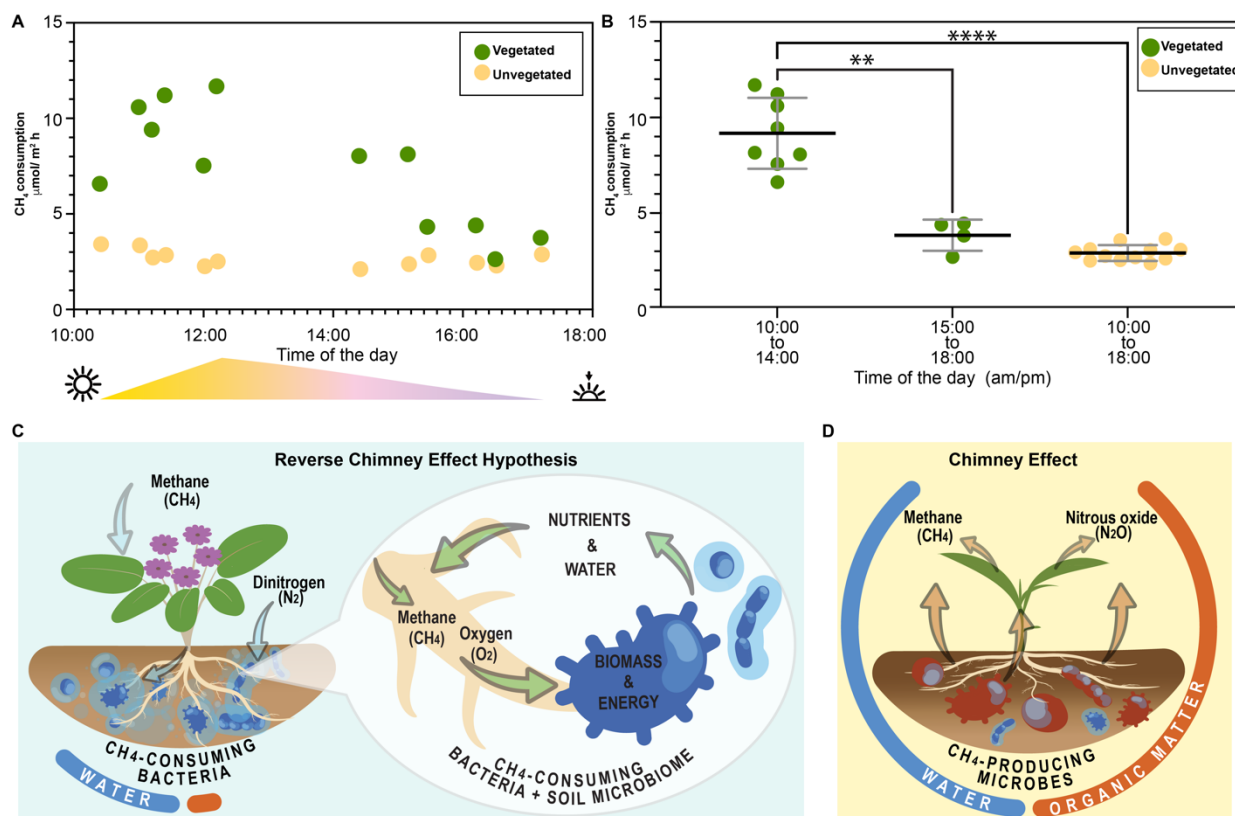


Figure 1.1. Consumption rate of methane comparing vegetated and unvegetated patches in the Anza Borrego Desert State Park.

(A) The presence of vegetation and methanotrophs correlated positively with an increased consumption rate of methane. (B) Comparison of methane consumption between mid-day and evening. Significance with P-value 0.004 (**) and <0.0001 (****) obtained with the non-parametric Mann-Whitney test. (C) Graphical summary of the proposed reverse chimney effect in arid ecosystems. (D) Graphical summary of the chimney effect.

1.4.2 Taxonomic composition of the Anza-Borrego soil microbiome.

The taxonomic assignment of the Anza-Borrego metagenomic reads (microbiome) from 2023 to a (non-viral) family using Kaiju resulted in <45% of the total sequences being assigned, which is within the output range reported for this approach with similar sample types (Menzel, Ng, & Krogh, 2016). This assessment revealed that the most abundant members of these microbiomes were similarly distributed among both vegetated and unvegetated sites (Fig. 1.2A). The most abundant bacteria in these microbiomes belonged to the phyla Actinomycetota (12 to 19%),

Pseudomonadota (7 to 12%), and Acidobacteriota (2 to 3%); and to a lesser extent Chloroflexota (1.8 to 2.5%), Planctomycetota (1.0 to 1.2%), Bacilliota (0.5 to 1.1%), Gemmatimonadota (0.8 to 1.2%), Bacteroidota (0.5 to 2.0%), and Nitrososphaerota (0.4 to 0.9%). The most prominent Actinomycetota families corresponded to Streptomycetaceae, Solirubrobacteraceae, Rubrobacteraceae, Pseudonocardiaceae, Nocardiodaceae, Micromonosporaceae, and Geodermatophilaceae, all with abundances ranging between 0.3 to 3% of all taxonomically assigned reads in these microbiomes (Fig.1.2A). Also, the families Methylobacteriaceae (0.3 to 2.4%) and Bradyrhizobiaceae (0.3 to 0.5%) were the most abundant Pseudomonadota in these microbiomes (Fig. 1.2A). Reads assigned to known methanotroph families were found in all vegetated and unvegetated Anza-Borrego 2023 samples. Methylococcaceae had the highest relative abundance of all the methanotrophs detected (representing between 0.06 to 0.3% of all the taxonomically assigned reads) followed by Methylocystaceae (with 0.04 to 0.06%), and Methylophilaceae (with 0.01 to 0.04%). Methylothermaceae and Methylacidiphilaceae were the less relatively abundant ranging between 0.002 to 0.006% (Figure 1.2B). Taxonomic assignment at genus level revealed *Methylocaldum* (Methylococcaceae) as the relatively more abundant methanotroph in both vegetated and unvegetated samples, between 0.01 to 0.04%. Followed by *Methylocystis* (Methylocystaceae), *Methylocapsa* (Beijerinckiaceae) and *Methylobacter* (Methylococcaceae) with 0.09 to 0.03%. Reads assigned to methanotrophs were also found in Anza-Borrego 2016 metagenomes, with an average of 0.5% of relative abundance of Methylococcaceae, 0.3% Methylophilaceae, 0.09% of Methylocystaceae, 0.007% Methylothermaceae, and 0.003% Methylacidiphilaceae. In agreement with the similar taxonomic composition observed among all the 2023 samples (Fig. 1.2A), both normalized and non-normalized approaches, using the taxonomically assignable metagenomic reads, showed no

significant differences between the alpha-diversity among the Anza-Borrego samples with and without vegetation (Fig. 1.2C, Supplementary Figure S1.2). This trend was also confirmed when assessing the beta diversity using their normalized taxonomically assignable metagenomic read counts (Fig. 1.2D).

These results contradict a previously proposed hypothesis that vegetation can increase plant litter and root exudates that can serve as a source of organic matter for microbes, and lead to distinctive shifts in soil microbial diversity (Coleine et al., 2024). Since this study focused specifically on sites covered by the seasonal vascular plant *Abronia villosa* (desert verbena), which has a lifespan of only 2 to 3 months between late autumn and early spring, it might not have the capacity to produce the levels of exudates to change its surrounding soil microbiota to detectable levels. Furthermore, it is tempting to speculate that in nutrient-poor and arid environment, plants do not waste resources on supporting a broader microbiome community, but rather tend to control nutrient exchange directly with microbes that colonize roots. The mechanisms of such interactions are yet to be established.

The results presented here agree with a previous study reporting no significant changes in the total abundance and richness of key marker genes for methanotrophic microbes when assessing different vegetation and climate types from 80 dryland ecosystems (Lafuente et al., 2019). Based on the recovered soil microbiome structures from non-vegetative and vegetative sites, we conclude that the observed enhancement of the methane flux is plant-driven and might be achieved in two ways: close colonization of plant roots by *Methylocaldum* spp. or enhanced activity of yet-to-be-discovered methanotrophic functions or organisms.

1.4.3 Numerous Copper monooxygenases (CuMOs) are identified in the Anza-Borrego soil metagenomes

Considering the low representation of genomes for dryland soil methanotrophs in current databases and its consequent limitations for their identification in metagenomic datasets, the prevalence of methanotrophs in Anza-Borrego was further assessed using functional metabolic marker genes, such as those coding for the key enzymes for CH₄ oxidation: methane monooxygenases (MMO). This enzyme has two types: a soluble MMO (sMMO) and a particulate MMO (pMMO). However, since only a single sMMO sequence was recovered from all Anza-Borrego 2023 metagenomes, this assessment used the genes encoding for the three subunits of the pMMO: *pmoA*, *pmoB*, and *pmoC*. The search for pMMO subunits using KEGG and TIGR databases additionally retrieved genes for two other copper monooxygenases (CuMOs), representing ammonia and hydrocarbon (short-chain alkane) monooxygenases. The phylogenetic relationship of all CuMOs retrieved in this functional screening was generated to distinguish which taxa had each of the three possible CuMOs. A total of 148 CuMOs A, 166 CuMOs B, and 146 CuMOs C gene sequences were recovered from the assembled Anza-Borrego 2023 metagenomes

(Fig. 1.3). Thirty-seven and forty-three CuMO sequences were associated with canonical methanotrophic taxa (putative methanotrophs) were recovered from vegetated and unvegetated sites, respectively, with the majority corresponding to *pmoB* subunits (25 in vegetated and 27 in unvegetated metagenomes). In addition, CuMOs from ammonia oxidizers were retrieved from both vegetated and unvegetated sites (97 and 116 genes, respectively), as well as CuMOs related to putative hydrocarbon (alkane) oxidizers (58 from vegetated sites and 66 from unvegetated). These results denote the extensive diversity of potential CH₄ oxidizers in the Anza-Borrego soil microbiomes and suggest that the prevalence of all three types of CuMOs may be independent of the presence of vegetation (Fig. 1.3).

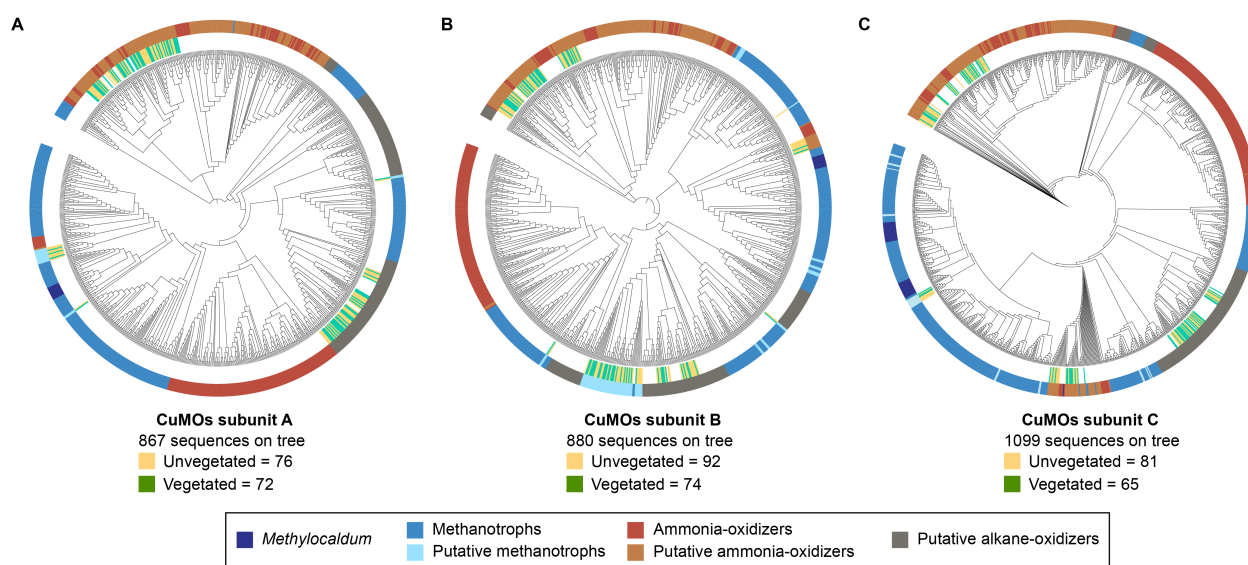


Figure 1.3. Maximum likelihood trees representing the phylogenetic relationship between copper monooxygenases (CuMOs) across bacteria and archaea.

The functional richness for the different subunits from the Anza-Borrego metagenomes is represented by green (vegetated) and yellow (unvegetated) lines at the tips of their corresponding leaves. The outside color-coded ring guides the function of CuMOs, which was assigned based on the taxon at the corresponding leaf.

1.4.4 MAGs and isolate genomes indicate methanotrophy potential in the Anza-Borrego soil microbiome beyond canonical species

The 2016 and 2023 Anza-Borrego soil metagenomes were used to generate 84 metagenome-assembled genomes (MAGs) (Fig. 1.4), of which 10 were high-quality and 53 medium-quality drafts (Bowers et al., 2017). Among them, eight MAGs had methane monooxygenase and methanol dehydrogenase genes. These included three *Methylocaldum*, two *Methylobacter*, one MAG from the family *Methylococcaceae*, and two other MAGs that could only be assigned to the class Gammaproteobacteria, with their *pmoB* subunits having a 36% coverage with 74% identity to *pmoB* of *Methylococcus* and the other a 36% coverage with 75% identity to *Methylocaldum pmoB*.

Five pure cultures of methanotrophic bacteria were obtained from the Anza-Borrego cultivation efforts (Supplementary Material and Supplementary Table S1.1). Among these isolates, four corresponded to *Methylocaldum* (strains 0917, S3V3, YM2, and RMAD-M, all with a genome size of 5.2 to 5.4 Mbp), and one corresponded to *Methylosinus* sp. sav-2 (4.7 Mbp). Additionally, ten isolates corresponded to non-methanotrophic bacteria, including *Bradyrhizobium* (strains W, R2.2-H, BM-T, Y-H1, all with a genome size of 8.0 Mb), *Neorhizobium* sp. R1-B (5.5 Mb) and S3V5DH (5.8 Mb), *Caulobacter* sp. H1 (3.7 Mb) and the methylotroph *Methylobacterium* sp. R2-1 (5.8 Mb).

The overall metabolic potential of Anza-Borrego *Methylocaldum* MAGs was completely captured by the genomes of isolated strains obtained in this study (Fig. 1.4, Supplementary Fig. S1.3). Therefore, since the genus *Methylocaldum* (from the family *Methylococcaceae*) was the most dominant methanotrophic bacterial group detected in the Anza-Borrego metagenomes (Fig. 1.2E), further analysis focused on the metabolic potential of the *Methylocaldum* isolates. A

genomic comparison between the Anza-Borrego *Methylocadum* and all the other ten *Methylocaldum* genomes (Supplementary Table S1.3, Supplementary Fig. S1.5) that were publicly available at the time of this analysis was performed to assess the distinguishing metabolic features of the dryland isolates. For example, all the *Methylocaldum* genomes had the metabolic potential for nitrogen fixation, and both the large (K01601) and small (K01602) subunit of a Type-IA/B Ribulose 1,5-bisphosphate carboxylase (Supplementary Information and Supplementary Fig. S1.6).

Notably, the Anza-Borrego *Methylocaldum* isolates presented more lanthanide-dependent *xoxF3*-type methanol dehydrogenase paralogs compared to the genomes of isolates from other environments following previous *xoxF* assignments (Chistoserdova & Lidstrom, 1997; Huang, Yu, & Chistoserdova, 2018; Keltjens et al., 2014) (Supplementary Fig. S1.7). Further details on key features of the Anza-Borrego genomes, as unveiled by assessing the distribution of the 8,668 groups of orthologous genes comprising the *Methylocaldum* pangenome, are exposed below (Supplementary Tables S1.4 and S1.5).

1.4.5 *Methylocaldum* has four conserved types of *pmoC* paralogs in addition to the canonical gene involved in methane oxidation

As mentioned above, the pMMO encoded by *pmoC*, *pmoA* and *pmoB* genes was the predominant methane oxidizing CuMO in Anza-Borrego metagenomes and isolates. The *Methylocaldum* pangenome revealed the presence of several paralogs among the 39 *pmoC* retrieved (K10946), of which only nine formed parts of the canonical pMMO gene cluster (Fig. 1.5). Notably, the remaining stand-alone *pmoC* paralogs (that included neither *pmoA* nor *pmoB*) could be grouped into four distinctive gene contexts that were conserved even among representatives isolated from different continents (Fig. 1.5A and Supplementary Fig. S1.5). These stand-alone *pmoC* genes clades were named ‘Type 2 to 5’ considering their conserved genomic context and phylogenetic placement to the canonical ‘Type 1’ pMMO gene cluster (Fig. 1.5B and Supplementary Information). The immediate vicinity of the conserved genomic context of Type 2 *Methylocaldum* *pmoC* had genes involved with nucleoside modification or the biosynthesis of GMP, and the generation of radical species by reductive cleavage of S-adenosylmethionine. The genes near Type

3 *pmoC* are involved with the degradation of polyhydroxybutyrate (for energy storage) (Roohi, Zaheer, & Kuddus, 2018) and the Na-translocating NADH-quinone reductase respiratory complex operon (Dibrov, Dibrov, & Pierce, 2017). All Type 4 *pmoC* are located between two genes, one of which has a fumarate/nitrate reduction transcriptional regulator domain, and both contain helix-turn-helix domains. Thus, these genes likely have a role in either transcriptional regulation or DNA binding processes (Aravind et al., 2005), that in *Escherichia coli* have been linked to an oxygen-responsive transcriptional regulation to switch from aerobic to anaerobic metabolism (Uden & Schirawski, 1997). Finally, the *rsbU* gene near the Type 5 *pmoC* has been involved with sensing and responding to general stress, or oxygen starvation in bacteria (Delumeau et al., 2004; Makino et al., 2009). Further analysis of the predicted tertiary structure of *Methylocaldum* PmoC types, showed their differences while still preserving their transmembrane domains (Supplementary Figure 1.8A.). Additionally, the search for the presence of the key amino acid residues forming the Cu D binding site, which recent structural studies had indicated as the methane oxidation catalytic center in PmoC (Tucci et al., 2023) was conserved across all *Methylocaldum* PmoC types (Supplementary Figure 1.8B.). Overall, the functional diversity of the genes in the immediate vicinity and the same orientation as the stand-alone *pmoC* suggests that in *Methylocaldum* these paralogs are likely involved in other functions not related to CH₄-oxidation (Fig. 1.5B and Supplementary Information).

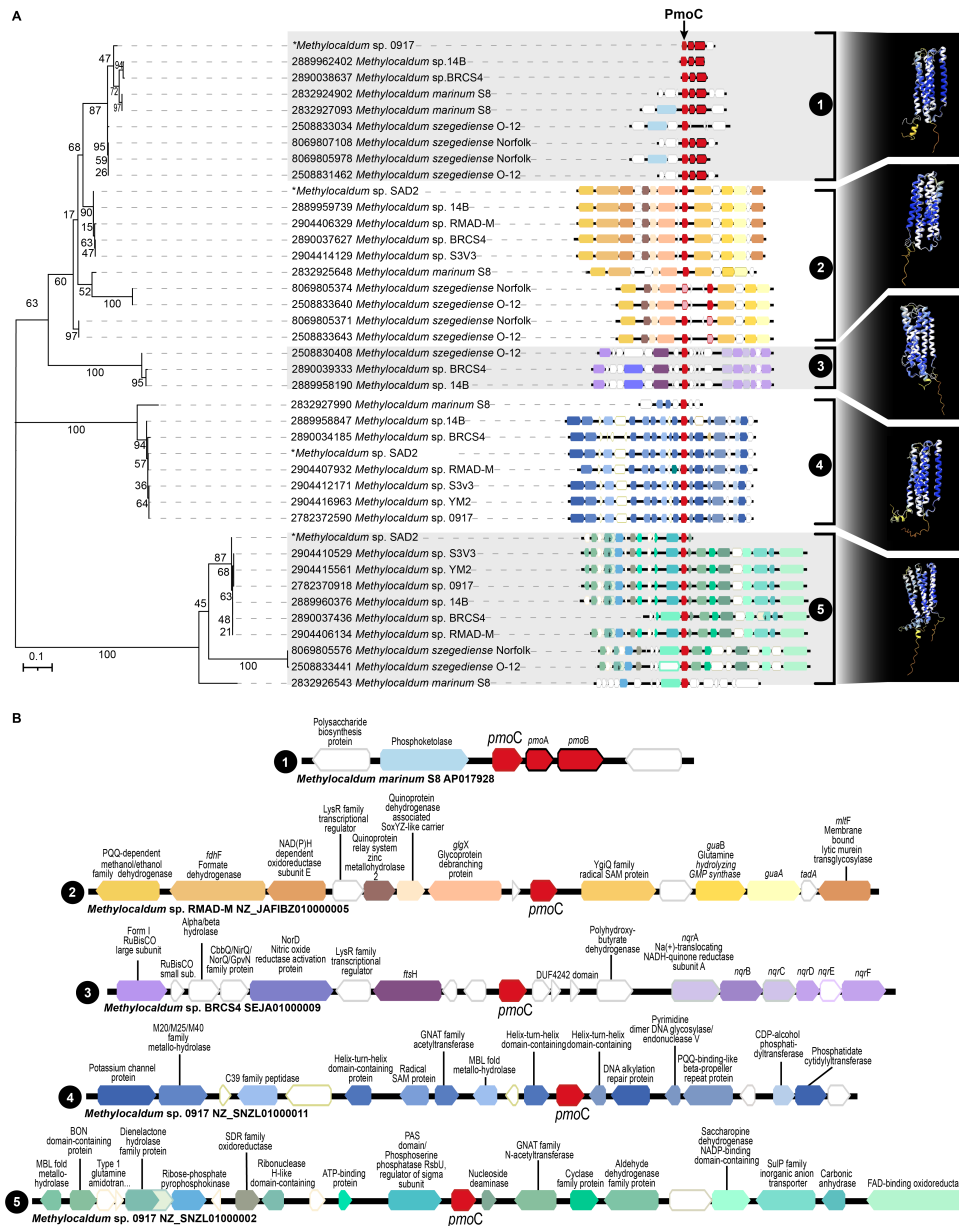


Figure 1.5. Particulate methane monooxygenase subunit C phylogeny and genomic context.

(A) Maximum likelihood tree representing the phylogenetic relationship of *Methylocaldum* particulate methane monooxygenase pMMO, based on the amino acid sequences of the *pmoC* gene. Next to each branch, the projection has a color-coded representation of the predicted functions for the genes in the vicinity of each *pmoC*. Next to each clade, brackets indicate with a number the type of PmoC corresponding to each clade, followed by the AlphaFold models predicted for each of them based on the prediction obtained from the CryoEM structure of *Methylococcus capsulatus* (Bath) pMMO in a native lipid nanodisc at 2.16 Angstrom resolution (ID 7S4J). (B) Gene function prediction for genes in the vicinity of each of the five types of *pmoC* from *Methylocaldum* strains. The canonical *pmoC* from 0917 was recovered from a metatranscriptome.

1.4.6 Anza-Borrego *Methylocaldum* has a TRAP transporter system

A Tripartite ATP-independent Periplasmic (TRAP) type periplasmic transport system (Kelly & Thomas, 2001) was found exclusively in the Anza-Borrego *Methylocaldum* isolates (Fig. 1.4). TRAP transporters, which are one of the three known solute binding-protein dependent systems which are characterized by their high-affinity on the uptake of substrates (being the other two the well-studied ATP-binding cassette (ABC), and the more recently studied tripartite tricarboxylate transporters (Rosa et al., 2018)). TRAP transporters do not require ATP hydrolysis and instead use transmembrane electrochemical gradients (usually sodium or other cations) to transport various molecules including C4-dicarboxylates, sulfonate, and carboxyl-containing substrates (Davies et al., 2021; Rosa et al., 2018).

The putative TRAP-type transport system in the Anza-Borrego *Methylocaldum* isolates was found to be encoded by three genes, a TRAP transporter component, a periplasmic component, and the fused version of the large and small permease component. The putative TRAP-type gene cluster in the Anza-Borrego *Methylocaldum* isolates resembles an evolved variant of the TRAP transporters from *Treponema pallidum* (Radolf et al., 2016; Rosa et al., 2018), where it has been predicted to transport hydrophobic nutrients through the periplasm (Brautigam et al., 2012; Deka et al., 2012; Hawley et al., 2021). Notably, the involvement of this system in transporting substrates in response to reduced water availability was shown in a metaproteomic study reporting that the TRAP-type protein abundances produced by populations of Pseudomonadota (from the genus *Acidithrix*, *Aureimonas*, *Niastella*, and *Pedobacter*) and Actinomycetota (from the genus *Jiangella*) were higher in soils subjected to a regulated irrigation-deficit (Starke et al., 2017). Therefore, TRAP transport mechanisms might be involved in transporting extracellular molecules that improve *Methylocaldum* resilience in the desert environment.

1.4.7 Potential interaction within methanotrophic microbiomes

Methylocaldum strains were initially co-isolated with *Bradyrhizobium* and the challenges for their separation suggested some level of dependency for the methanotroph. The genomic comparison revealed that *Methylocaldum* possesses only the genes necessary for salvaging cobalamin (vitamin B₁₂), though *Bradyrhizobium* has all the necessary genes for the *de novo* synthesis of the essential cofactor (Fig. 1.6.). However, *Methylocaldum* has both methionine synthesis pathways, dependent and independent of vitamin B₁₂, and it can grow without vitamin B₁₂ supplementation in the culture media, yet, a growth limitation is suspected, making the association with *Bradyrhizobium* advantageous. Considering that metagenomic studies indicate that <10% of soil prokaryotes encode the genetic potential for *de novo* synthesis (Lu et al., 2020), hence *Methylocaldum* reliance on external sources of the cofactor is likely fulfilled by symbiosis with *Bradyrhizobium*. Our findings further support a previous study demonstrating that rhizobia can stimulate the growth of methanotrophs via excreted cobalamin (Iguchi, Yurimoto, & Sakai, 2011).

Both rhizobia and methanotrophs have been shown as key for N₂ fixation in non-leguminous plants (Yoneyama et al., 2019). Even though expected for *Bradyrhizobium*, additionally all Anza-Borrego *Methylocaldum* strains have the genetic potential for dinitrogen (N₂) fixation, including *nifH* (K02588) and *nifK* (K02591), in addition to *nifB* (K02585), *nifD* (K02586), *nifQ* (K15790), *nifU* (K04488), and *nifZ* (K02597) (Supplementary Figure 7). Therefore, *Methylocaldum* can potentially fix nitrogen, providing an advantage for both, bacteria and its host plant in the low-nutrient desert environment.

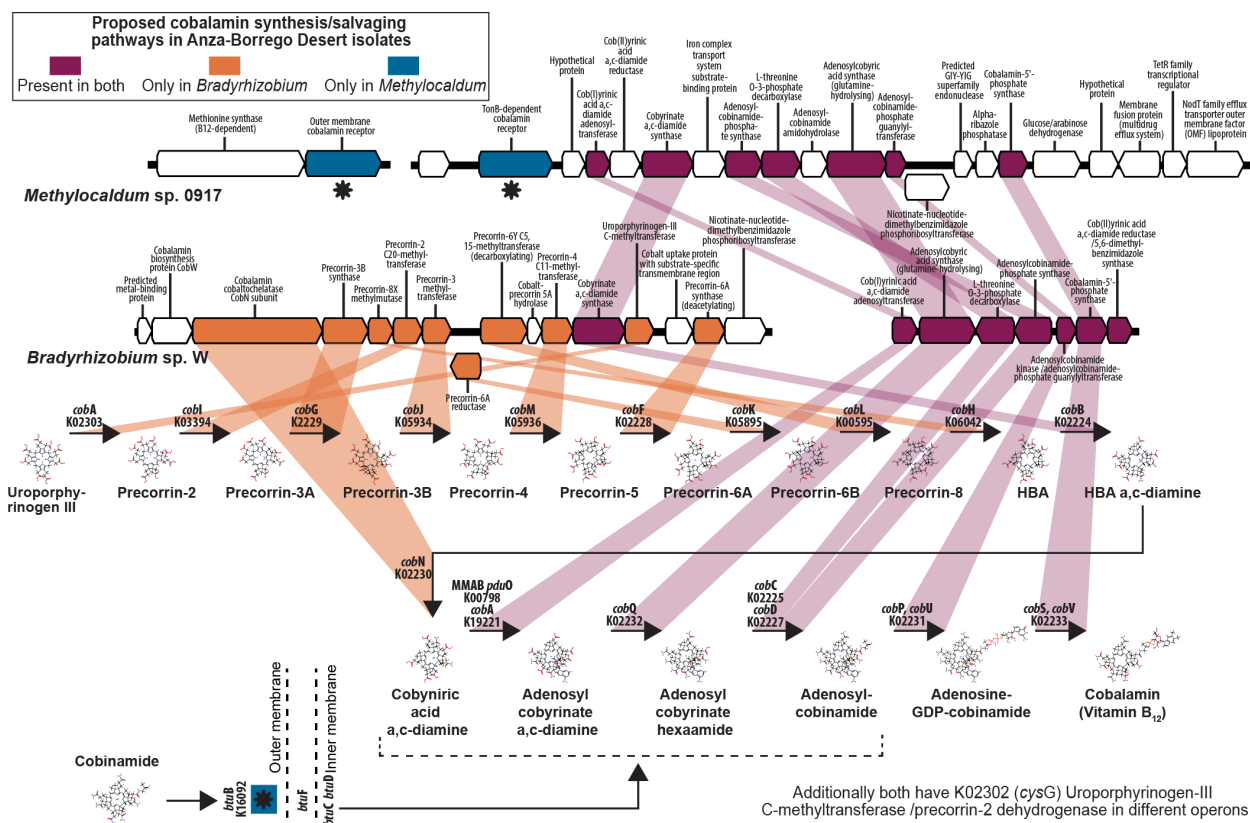


Figure 1.6. Genomic potential for cobalamin (vitamin B12) de novo synthesis and salvaging by Anza-Borrego isolates of *Bradyrhizobium* and *Methylocaldum* respectively.

The required genes for de novo cobalamin synthesis are present in the *Bradyrhizobium* genome. The absence of the initial required genes for de novo synthesis and the presence of two paralogs of the cobalamin outer membrane cobalamin receptor and transporter gene, *btuB*, indicates that *Methylocaldum* relies on a salvaging pathway of cobalamin.

Searches for metabolisms favoring the interaction of the *Methylocaldum-Bradyrhizobium* consortia with plants, pointed to the different pathways for tryptophan synthesis and its subsequent metabolism to indole-3-acetic acid (IAA) production as relevant. *Methylocaldum* and *Bradyrhizobium* both have the necessary genomic potential for tryptophan synthesis (Fig. 1.7). IAA is an important auxin in plants, which acts as a phytohormone regulating plant growth and also mediates bacterial physiology (Duca & Glick, 2020). *Methylocaldum* and *Bradyrhizobium* have the complete gene set necessary for IAA production via tryptamine and indole-3-

Conclusions

This study was inspired by a high methane sink observed in arid ecosystems via *in situ* measurements almost three decades ago (Striegl et al., 1992) and a more recent remote-sensor-based demonstration of arid ecosystems as “black holes” methane, i.e. environments with methane levels significantly below the average (Song et al., 2024). Here we aimed to better understand underlying biological means of arid methane sinks. We investigated methane cycling in the Anza-Borrego State Park, starting with enrichments in 2016, followed with additional experiments set in 2018, 2020 and 2023. Each time we were able to isolate microbes from vegetative sites, which inspired a deeper investigation of methanotrophic biome structure via metagenomic studies and *in situ* methane flux measurements. Integrated, the data highlights the importance of interaction among arid biomes, especially soil microbes and arid vegetation, for atmospheric methane consumption. Plant-supported methane flux shows daily dynamics, suggesting yet-to-be-determined links with the plant’s photosynthetic activity. This observation became the foundation for the reverse chimney hypothesis proposed here (Fig. 1C), which could partially explain why deserts constitute natural CH₄ sinks. This hypothesis was coined as the opposite of the chimney effect (Bhullar, Edwards, & Olde Venterink, 2013; Bringel & Couée, 2015; Joabsson, Christensen, & Wallén, 1999; Zhang et al., 2019), in which plants transport CH₄ from the anoxic soil layers from their roots through their circulatory system to their leaves and release it to the atmosphere (Fig. 1D). The reverse chimney hypothesis proposes that in dryland ecosystems (i.e., low organic matter and limited water availability) plants transport CH₄ and oxygen from the atmosphere through their vascular system down to the soil, where it is consumed and converted by methanotrophic microbiota (Fig. 1C). It is tempting to speculate that plants accelerate desert methane sinks by providing unique ecosystem support for methanotrophic microbes. Research on

how plants contribute to the modulation of CH₄ fluxes has only recently started to be explored. The results presented here encourage future studies aimed at understanding how the CH₄ concentration, macronutrients (i.e., N, S or P), and moisture levels of the soil determine whether a system functions as a sink or source of CH₄. The plant-promoting properties of the desert *Methylocaldum* species will be described in a separate study (Cameron et al., submitted).

The *Methylocaldum* clade is the most prominent methanotroph in the studied ecosystem. Genomic analysis of *Methylocaldum* isolates provides insights into the unique metabolic features of the dominant methanotroph in Anza-Borrego soil, contributing to the understanding of microbial adaptation in arid environments. These adaptations include the presence of multiple stand-alone *pmoC* paralogs in *Methylocaldum*, which may have functions beyond methane oxidation. Furthermore, the TRAP found in Anza-Borrego *Methylocaldum* isolates might play an important role in supporting substrate transport under reduced water availability.

Genomic evidence suggests that *Methylocaldum* may rely on a symbiotic relationship with *Bradyrhizobium* for cobalamin (vitamin B₁₂) due to its lack of *de novo* synthesis genes. Additionally, both *Methylocaldum* and *Bradyrhizobium* possess the necessary genes for tryptophan synthesis and IAA production, indicating a potential interaction that benefits plant growth and bacterial adaptation in arid environments. Establishing beneficial rhizosphere microbiomes in drylands could enhance soil stability by producing an extracellular matrix (Maestre et al., 2021), promoting vascular plant growth, or generating a positive feedback loop that supports the proposed reverse chimney effect.

These findings are the starting point for further research on how CH₄ concentrations, macronutrients, and soil moisture levels influence the role of plant-microbiome biomes in modulating CH₄ fluxes at different scales across seasons in arid ecosystems. Further research is

needed to explore the impact of different vegetation types, environmental conditions, and soil microbiomes on terrestrial CH₄ fluxes.

Supplementary Information

Supplementary Materials and Methods

Enrichment Cultures

Methanotrophic cultures were isolated from soil samples collected in Spring 2015 and 2016, using an enrichment strategy described in (Kalyuzhnaya, 2016) and were cultivated using P₀% medium (Collins & Kalyuzhnaya, 2018), consisting of 1 g L⁻¹ of KNO₃, 0.2 g L⁻¹ of MgSO₄ x 7H₂O, 0.02 g L⁻¹ of CaCl₂ x 2H₂O and 1 mL of 1000x trace solution. After autoclaving (at 121°C for 45 minutes), a sterile phosphate solution (1/20 v/v), containing 5.44 g L⁻¹ of KH₂PO₄ and 5.68 g L⁻¹ Na₂HPO₄ (pH of 6.8), was added. Trace solution (1000x) consisted of 5 g L⁻¹ of Na₂EDTA, 2g L⁻¹ of FeSO₄ x 7 H₂O, 0.3g L⁻¹ of ZnSO₄ x7 H₂O, 0.03 g L⁻¹ of MnCl₂ x 4 H₂O, 0.2 g L⁻¹ of CoCl₂ x 6 H₂O, 1.2 g L⁻¹ of CuSO₄ x 5 H₂O, 0.3 g L⁻¹ of Na₂O₄W x 2H₂O, 0.05 g L⁻¹ of NiCl₂ x 6 H₂O, 0.05 g L⁻¹ of Na₂MoO₄ x 2 H₂O, and 0.03 g L⁻¹ of H₃BO₃.

A subset of soil samples collected on May 30th, 2016, were used for culture enrichment. Soil (1 to 2 g) was added to 25 ml of 1:3 diluted P₀% media in 125 ml borosilicate bottles with rubber stop septa lids and supplemented with 2 ml of 100% methane. After 24 h, 5 ml of methane were added, and samples were incubated for 3-4 days at 30°C with agitation (200rpm). Cultures exhibiting increased turbidity were transferred into 50 ml P₀% media in 125 ml bottles and supplemented with methane (10% of headspace). For each successful enrichment (i.e., culture turbidity increased after each passage at least for three passages), serial dilutions were performed and then spread-plated on solid P₀% media to produce isolated colonies.

Pure culture isolation via co-plating method

Individual clones were selected and re-plated. However, in many cases, only a stable co-culture was obtained. The separation of methanotrophic isolates from a rhizobia-consortium was only achieved by the co-plating technique. Briefly, a petri dish was separated into two zones; co-culture was plated on one side and incubated for 1-2 days. Then single colonies were streaked on the second half of each plate and incubated for an additional 4-5 days. This method helped separate pure methanotrophic cultures from stable co-cultures. Isolation of heterotrophic satellite cultures was carried out by plating on P_M and/or P_{NB} media.

DNA preparation, sequencing, assembly, and annotation for metagenomics.

Samples collected in 2023, were extracted with a phosphate buffer and high pH lysis buffer protocol adapted from Povedano-Prieto et al.(Povedano-Priego et al., 2021) and Zeugin and Hartley(Zeugin & Hartley, 1985) aiming to recover DNA from high-sand content samples that were adsorbing DNA (Naviaux et al., 2005). Briefly, 0.25 g of each sample was placed on 2 mL screw-cap tube with DNA-free 3mm glass beads and 400 μ L of Na_2HPO_4 (120 mM pH 8.5), plus 600 μ L of lysis buffer (100 mM Tris-HCl [pH 9.5]; 100 mM EDTA [pH 8.5]; 100 mM NaCl), 20 μ L SDS 10%, 24 μ L freshly thawed lysozyme (10 mg/mL) and 2 μ L proteinase K (20 mg/mL). Cells were mechanically broken by bead beating on a vortexGenie2 (Scientific Industries) at maximum speed for 40 min in a cold room (4°C). The supernatant was processed through an organic extraction and isopropanol precipitation (Zeugin & Hartley, 1985) (Naviaux et al., 2005). to obtain the DNA. The integrity of the DNA was verified by 1% agarose gel electrophoresis and quantified on a Qubit utilizing high sensitivity kit.

***Methylocaldum* phylogeny**

The 16S rRNA gene and formate dehydrogenase gene phylogeny of the new and previously characterized *Methylocaldum* strains were reconstructed as previously described (Delherbe et al., 2024). Maximum likelihood trees for the RuBisCO were generated based on the amino acid sequences of the large subunit gene (*rbcL*) from representative species of the three domains of life. The model calculated to have the lowest BIC scores for this set of sequences corresponded to LG+G+I.

Supplementary Results and Discussion

Formaldehyde oxidation.

All the analyzed genomes have the potential for C₁ interconversion from tetrahydrofolate (THF) to 10-formyl-THF, but only present a pair of genes for the reductive acetyl-CoA pathway (i.e., the methylene-THF reductase and formate-THF ligase) which is required for the complete THF methylene tetrahydrofolate pathway (Supplementary Table S1.4). Although all *Methylocaldum* genomes lacked the genes to synthesize Tetra-hydromethanopterin (H4MPT), they present genes involved on the reversible transformation of formylmethanofuran to 5-Formyl-5,6,7,8-H4MPT (methenyl-H4MPT cyclohydrolase, formylmethanofuran dehydrogenase, and formyl-methanofuran-H4MPT N-formyltransferase). Therefore, potentially having some capacity to use methanopterin to transfer C₁ fragments between formyl and methyl groups.

Formate.

Formate can be transformed to CO₂ and NADH by the enzyme formate dehydrogenase (FDH). Interestingly, all the *Methylocaldum* genomes have three copies of this gene, but strains O-12 and S8 have only one and two copies, respectively. A previously generated phylogenetic analysis revealed that each one of the copies found in the *M. gracile* group corresponded to the

different types of this enzyme reported in *Methylobacterium extorquens* AM1: *fdh1A*, *fdh2A*, and *fdh3A*. The *fdh* genes of strains S8 and O-12 formed a sister group to the *fdh3A* cluster (Delherbe et al., 2024).

Carbon assimilation: ribulose monophosphate pathway.

All the genomes analyzed had the potential for the assimilation of formaldehyde via ribulose monophosphate (RuMP) pathway. Proposed as one of the most efficient formaldehyde assimilation routes (considering ATP consumption and biomass yield)(He et al., 2018). All *Methylocaldum* genomes have two copies for the 3-hexulose-6-phosphate synthase gene (K08093), but each genome has only one copy for the 6-phospho-3-hexuloisomerase (K08094).

Carbon assimilation: Serine pathway.

Formaldehyde can alternatively be assimilated through the serine pathway after its transformation to methylene-THF (Pham, Nguyen, & Lee, 2022). Although they had some of the components, such as: one copy for serine-glyoxylate transaminase (K00830) in all genomes, the hydroxypyruvate reductase (K12972) found only on *Methylocaldum* sp. SAD2, and one copy for D-3-phosphoglycerate dehydrogenase (K00058) found in all genomes.

Carbon assimilation: Calvin cycle.

Previously, the presence of the key enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCo) on some methanotrophs suggested the possibility of CO₂ fixation through the Calvin–Benson–Bassham (CBB) pathway in these microorganisms (Pham, Nguyen, & Lee, 2022). A single copy for each subunit of the Ribulose 1,5-bisphosphate carboxylase, (RuBisCO-like protein), large subunit RbcL (K01601), and small subunit RbcS (K01602) were found for each *Methylocaldum* genome. Thus, further supporting the potential for members of this genus to utilize CO₂ as carbon source through the CBB cycle, as it has been shown

in *Methylococcus capsulatus* Bath (Henard et al., 2021). A phylogenetic analysis revealed that the large subunit *RbcL* of the Anza-Borrego *Methylocaldum* strains form a sub-clade within the Type I RuBisCO found in *Pseudomonadota* (*Proteobacteria*), *Cyanobacteria*, and higher plants (Supplementary Figure S1.7). This suggests an evolutionary history of the *RbcL* of *Methylocaldum* and *Methylococcus*, related to Type I-A and I-B rubisco forms, which first appeared under the primitive low O₂ atmospheric conditions, and developed alternative strategies to compensate the increasing concentrations of O₂ of the post-Great Oxidation Event through carbon concentrating mechanisms (CCM), such as carboxysomes in cyanobacteria (Poudel et al., 2020) and pyrenoid-based in *Chlamydomonas* (Heureux et al., 2017), even though similar structures had been only described on *Verrucomicrobiota* (*Verrucomicrobia*) members (Semrau, DiSpirito, & Yoon, 2010).

Nitrogen metabolism, nitrogen fixation.

All the analyzed strains have the genetic potential for dinitrogen (N₂) fixation, including *nifH* (K02588) and *nifK* (K02591), in addition to *nifB* (K02585), *nifD* (K02586), *nifQ* (K15790), *nifU* (K04488), and *nifZ* (K02597). Additionally, all *Methylocaldum* isolate genomes in this study have a single copy for the two-component system, NtrC family, nitrogen regulation sensor histidine kinase NtrY (K13598) (Supplementary Table S1.3).

Anza-Borrego *Methylocaldum* have additional *xoxF*-type methanol dehydrogenases

All ten *Methylocaldum* genomes had a single copy for each subunit (*mdh1* and *mdh2*) of the pyrroloquinoline-quinone-dependent methanol dehydrogenase (MDH). This enzyme is unique to methanotrophic/methylotrophic bacteria and donates two electrons to cytochrome c_L to produce formaldehyde (Ghosh et al., 2020). Previously our group described that, in addition to MDH, the

genomes of *Methylocaldum marinum* S8, *Methylocaldum szegediense* O-12, and *Methylocaldum szegediense* Norfolk present the lanthanide-dependent *xoxF*-type methanol dehydrogenase types *xoxF3*, and *xoxF5* (Delherbe et al., 2024). Notably, all the Anza-Borrego *Methylocaldum* isolates from this study presented additional *xoxF3*-type methanol dehydrogenase paralogs (Supplementary figure S1.7). Moreover, one of the *xoxF3* paralogs formed a sister group with only Anza-Borrego genes (Supplementary figure S1.7). Since each *xoxF* clade has been reported to have variable catalytic efficiencies (Huang, Yu, & Chistoserdova, 2018), this might also be true for the various *Methylocaldum*'s *xoxF3* subtypes.

All the *Methylocaldum* genomes had both the large (K01601) and small (K01602) subunit of a Type-IA/B Ribulose 1,5-bisphosphate carboxylase.

Previously, the presence of the key enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCo) on some methanotrophs suggested the possibility of CO₂ fixation through the Calvin–Benson–Bassham (CBB) pathway in these microorganisms (Pham, Nguyen, & Lee, 2022). A single copy for each subunit of the Ribulose 1,5-bisphosphate carboxylase, (RuBisCO-like protein), large subunit RbcL (K01601), and small subunit RbcS (K01602) were found for each *Methylocaldum* genome. Thus, further supporting the potential for members of this genus to utilize CO₂ as carbon source through the CBB cycle, as it has been shown in *Methylococcus capsulatus* Bath (Henard et al., 2021). A phylogenetic analysis revealed that the large subunit RbcL of the Anza-Borrego *Methylocaldum* strains form a sub-clade within the Type I RuBisCO found in *Pseudomonadota* (Proteobacteria), Cyanobacteria, and higher plants (Supplementary Fig. S1.6). This suggests an evolutionary history of the RbcL of *Methylocaldum* and *Methylococcus*, related to Type I-A and I-B rubisco forms, which first appeared under the

primitive low O₂ atmospheric conditions, and developed alternative strategies to compensate the increasing concentrations of O₂ of the post-Great Oxidation Event through carbon concentrating mechanisms (CCM), such as carboxysomes in cyanobacteria (Poudel et al., 2020) and pyrenoid-based in *Chlamydomonas* (Heureux et al., 2017), even though similar structures had been only described on *Verrucomicrobiota* (*Verrucomicrobia*) members (Semrau, DiSpirito, & Yoon, 2010).

All the *Methylocaldum* genomes had the potential for nitrogen fixation

All the analyzed strains have the genetic potential for dinitrogen (N₂) fixation, including *nifH* (K02588) and *nifK* (K02591), in addition to *nifB* (K02585), *nifD* (K02586), *nifQ* (K15790), *nifU* (K04488), and *nifZ* (K02597). Additionally, all *Methylocaldum* isolate genomes in this study have a single copy for the two-component system, NtrC family, nitrogen regulation sensor histidine kinase NtrY (K13598) (Supplementary Table S1.3, supplementary Figure S1.8).

Novel stand-alone *pmoC* reveal a conserved functional diversity among *Methylocaldum* genomes.

While the canonical *pmoC*, *pmoA* and *pmoB* genes comprising the particulate methane monooxygenase were denoted here as the ‘Type 1’ *pmo*-cluster, an additional 4 stand-alone *pmoC* genes that did not have neither *pmoA* nor *pmoB* in their genomic neighborhood were found (Fig. 1.5A). These stand-alone *pmoC* genes were named ‘Type 2 to 5’ considering their genomic context and phylogenetic placement (Fig. 1.5A).

The immediate vicinity of the genomic context conserved for Type 2 *pmoC* of included the presence of radical S-adenosylmethionine (SAM) protein YgiQ, as well as genes coding for a guanosine monophosphate (GMP) synthesis, *guaA* and *guaB*, followed by a tRNA (adenine-34)

deaminase. The YbiQ enzyme has been reported to generate radical species by reductive cleavage of SAM through an unusual Fe-S center, a two-step process that produces/consumes NADH and consumes/produces ATP (Sofia et al., 2001). Notably, cobalamin-dependent radical SAM enzymes with glutamine C-methyltransferase activity have been reported for some methanotrophs (Gagsteiger et al., 2022). More information is available for the archaeal radical SAM protein, which has been reported to synthesize a structurally complex modified nucleoside, called archaeosine (Yokogawa et al., 2019). This archaeosine is similar to the commonly found queuosine, a structurally complex, non-canonical RNA nucleoside present in bacteria, part of the anticodon loop of certain tRNA (Hillmeier et al., 2021). Modified tRNAs have been shown to play a crucial role in offering stable tRNA structures at high temperatures in the extremely-thermophilic bacterium *Thermus thermophilus* (Hori, 2019). Moreover, sequence similarity network analysis indicated that most available radical SAM sequences belong to bacteria, followed by archaea (Holliday et al., 2018). Interestingly, comparatively more predicted radical SAM proteins had been found on methanogenic archaea genomes than in other archaea and bacteria, however, the function of half of them remains elusive (Boswinkle, McKinney, & Allen, 2022; Holliday et al., 2018).

Type 3 *pmoC* stand-alone genes were only found in strains O-12, BRCS4 and 14B. The immediate vicinity of its conserved genomic context has two small hypothetical proteins, polyhydroxy butyrate depolymerase, which catalyzes the degradation of the biopolymer polyhydroxybutyrate (PHB) utilized as nutrient storage (Roohi, Zaheer, & Kuddus, 2018), and the *nqr* Na-translocating NADH-quinone reductase operon, which is a respiratory complex that couples the oxidation of NADH to the translocation of sodium ions across the membrane (Dibrov,

Dibrov, & Pierce, 2017). Suggesting a role of PmoC type 3 in balancing catabolic and anabolic functions.

Type 4 *pmoC* is in between two helix-turn-helix domains, suggesting either a role in transcriptional regulation, DNA binding processes, or being the catalytic domain of another enzyme of a yet unknown function (Aravind et al., 2005). The helix-turn-helix behind *pmoC* has a fumarate/nitrate reduction transcriptional regulator Fnr domain, which in *Escherichia coli* is an oxygen-responsive transcriptional regulator, necessary for switching from aerobic to anaerobic metabolism (Unden & Schirawski, 1997). Additionally, the Type 5 *pmoC* conserved genomic context, contains a DNA alkylation repair protein, for repairing damage by alkylating agents (Mielecki & Grzesiuk, 2014). Followed by a pyrimidine dimer DNA glycosylase/endonuclease V, essential for repairing UV-induced pyrimidine dimers (DNA damage) (Karmanova, Nikulin, & Zimin, 2022).

The immediate vicinity of the Type 5 *pmoC* has genes with a RsbU-like domain with a fused phosphatase and signaling PAS-domain module. PAS-domain S-box proteins are crucial in signal transduction mechanisms in bacteria, and a known sensor domain for complex behavioral pathways in bacteria including motility and quorum sensing (Xing, Gumerov, & Zhulin, 2023). The sigma-B regulation protein RsbU is involved in regulating the general stress-response in bacteria (Delumeau et al., 2004). The PAS-domain within RsbU has a reported role in sensing stresses (i.e., carbon, phosphate, or oxygen starvation) (Makino et al., 2009), and have been implicated in sensing redox potential changes (Jaiswal, Manjeera, & Gopal, 2010). This suggests a possible role for *pmoC* type 4 in sensing or responding to environmental cues/stressors.

Supplementary Figures

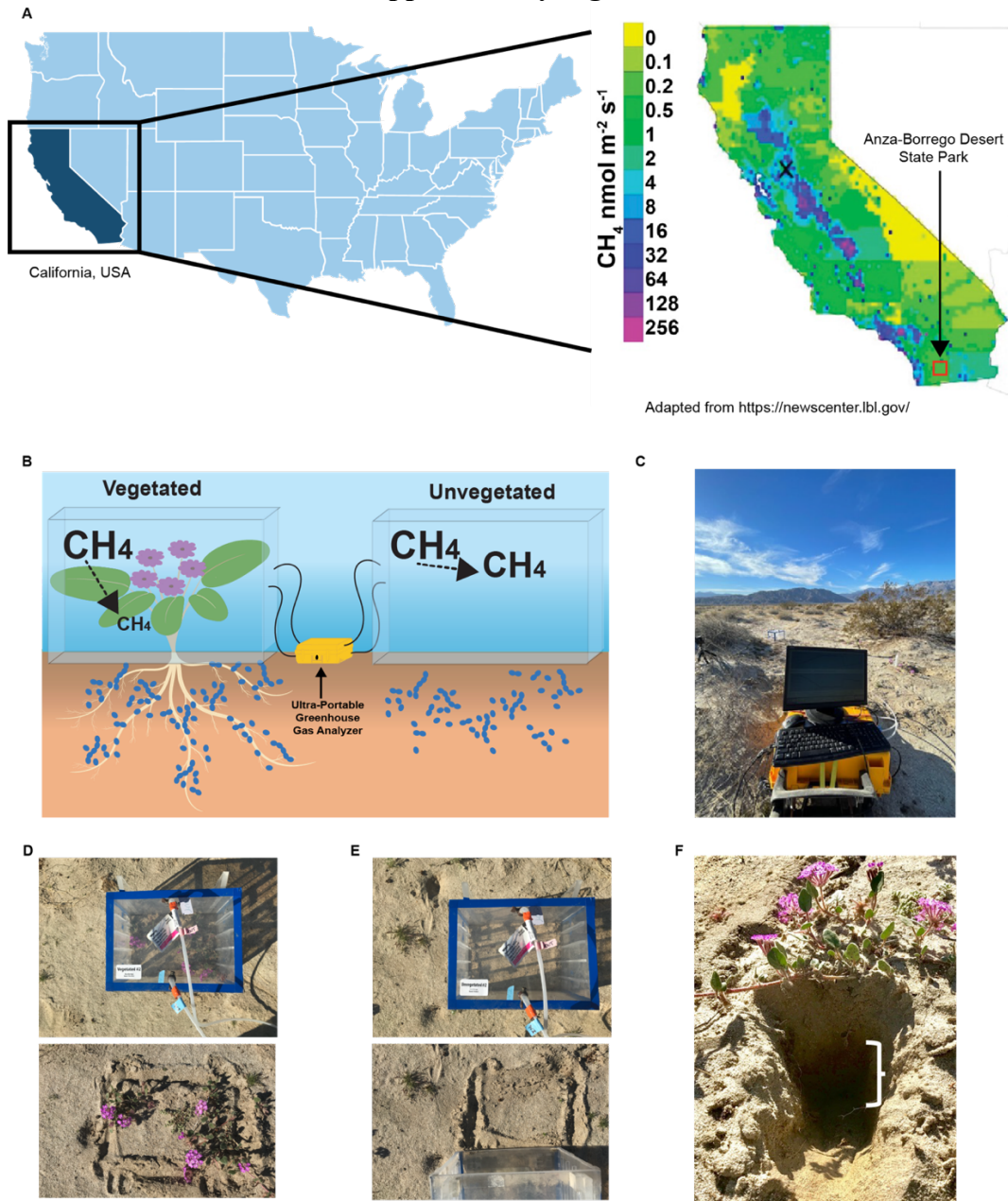


Figure S 1.1. Sampling site and experimental setup.

(A) Methane emissions in California. Location of the Anza Borrego State Park in California, USA. Image adapted from <https://newscenter.lbl.gov/>. (B) Draw of the experimental setup for *in situ* measurement of methane fluxes in Anza-Borrego. (C) Photographs of the sampling site and equipment (ultra-portable greenhouse gas analyzer). (D) Photograph of vegetated patch enclosing the endemic plan *Abronia villosa* (desert verbena) with and without acrylic chamber (E) Photographs of unvegetated patch with and without acrylic chamber. (F) The white bracket depicts the depth (10 cm) at which samples were taken under plants. Soil samples for metagenomes, from unvegetated patches were collected at the same depth.

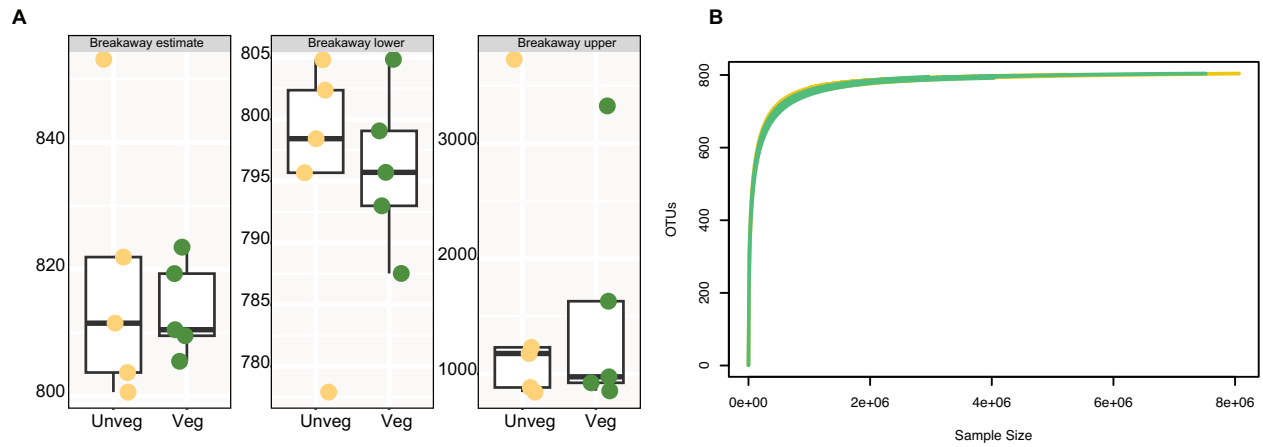


Figure S 1.2. Alpha diversity and rarefaction curve

(A) Alpha-diversity of raw taxonomically assignable metagenomic reads of vegetated and unvegetated samples. (B) Rarefaction curve. Normalized data was randomly rarefied to 1,721,047 reads.

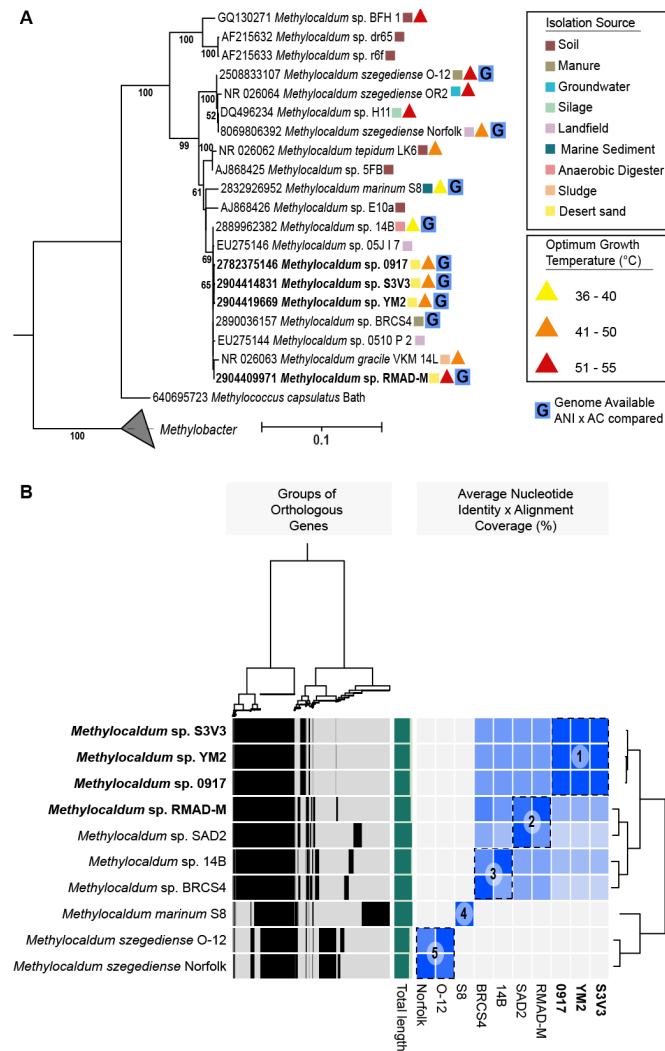
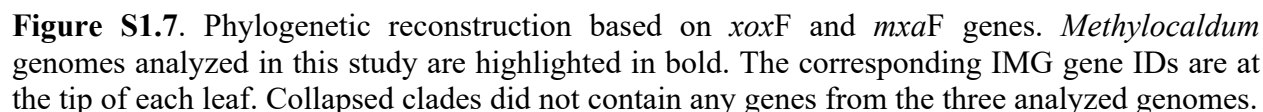


Figure S 1.5. *Methylocaldum* phylogeny and Average Nucleotide Identity times the Alignment Coverage.

(A) Maximum likelihood tree representing the phylogenetic relationship of *Methylocaldum* based on 16S rRNA gene sequences. The topology of the phylogeny, guided by the presence of type strains, indicates that at least 6 clades are formed within the analyzed strains. Iconography at the end of each leaf indicates the environment from which the strain was isolated, when available, the optimum temperature growth range in laboratory conditions is indicated. (B) Pangenome analysis of the ten available *Methylocaldum* genomes at the time was performed. The examination was based on the distribution of 8,668 groups of orthologous genes (black bars) obtained with Anvi'o. The horizontal bars under General Genome Parameters, represent the values for each of the different parameters measured for each genome. A whole-genome comparison to determine the similitude degree among the strains. For this, a heatmap for Average Nucleotide Identity times the Alignment Coverage (ANI x AC) was generated on which four major clades can be distinguished.



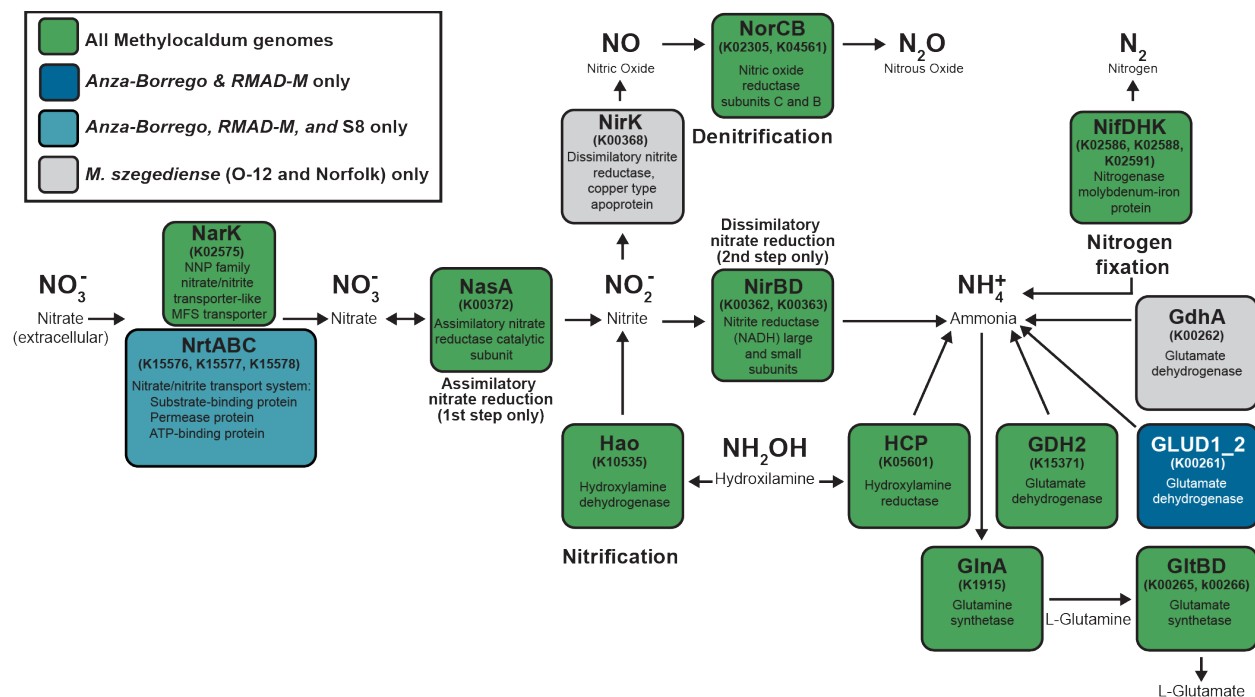


Figure S 1.7. Nitrogen metabolism genes in *Methylocaldum* strains. Diagram representing the presence and absence of genes involved in nitrogen cycling.

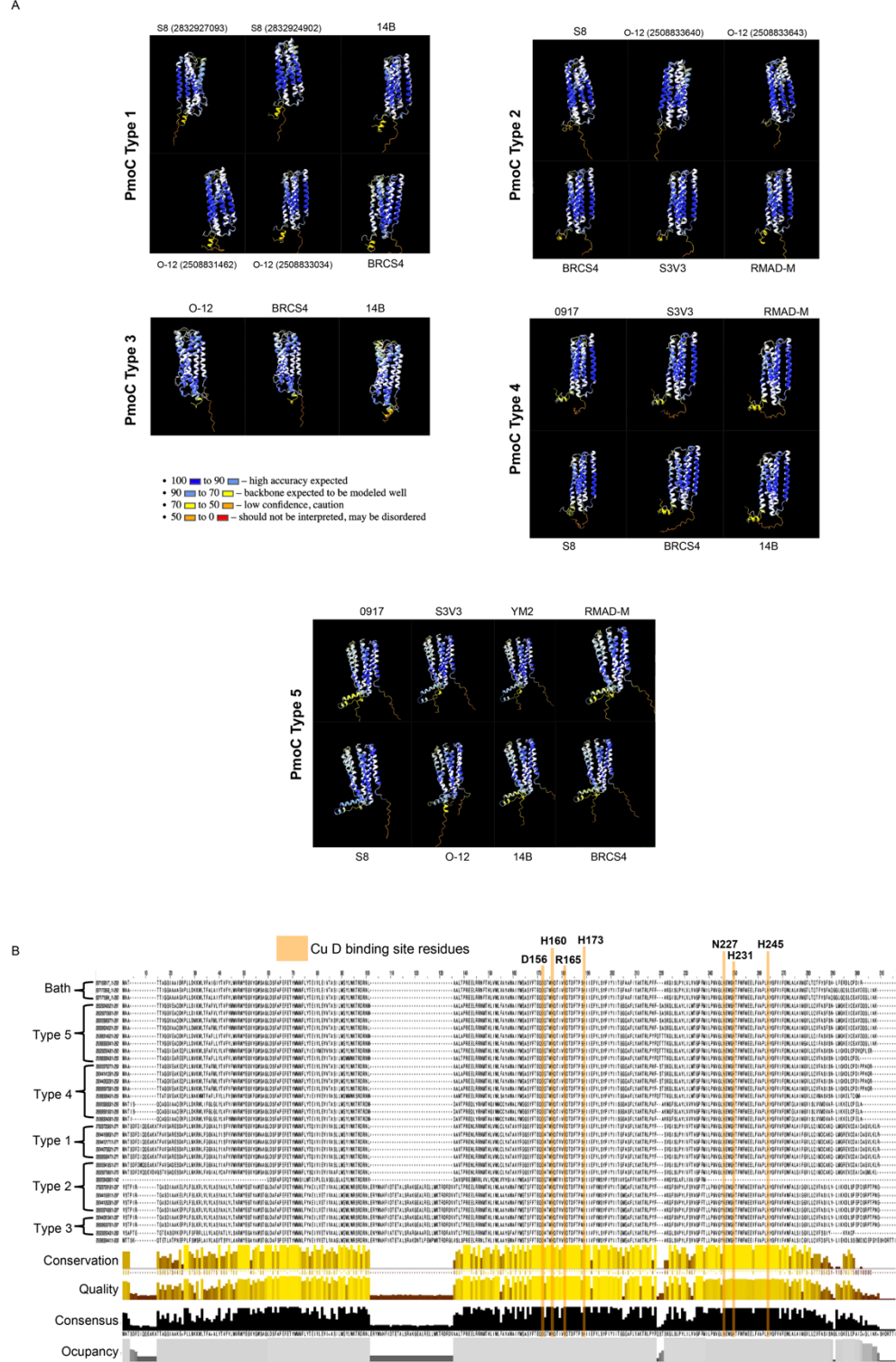


Figure S 1.8. (A) AlphaFold-predicted tertiary structure of *Methylocaldum*'s PmoC types based on *Methylococcus capsulatus* Bath PmoC crystallography. (B)MAFFT-L-INS-i Alignment for PmoC *Methylocaldum* using MAFFT (v7.511). The amino acid residues forming the Cu D binding site described on Tucci et al., 2023 are highlighted and labeled

Supplementary Tables

Table S 1.1. Anza-Borrego soil metagenomes generated in this study.

Metagenome ID	Sequencing Center	IMG Genome ID	Genome Size (assembled)	Gene Count (assembled)
S1+v 20-13C	DOE JGI	3300020131	187019236	409296
S3+v 10-13C	DOE JGI	3300020146	317105193	650436
S3+v 5-13C	DOE JGI	3300020147	312907534	653428
S1+v 10-13C	DOE JGI	3300020152	269467853	523210
S1 20	DOE JGI	3300020154	415175613	915500
S1+v 5-13C	DOE JGI	3300020156	287312560	512354
S1+v 10	DOE JGI	3300020181	882401169	2245240
S1 10	DOE JGI	3300020202	1340989938	3214547
S1 5	DOE JGI	3300020215	1224300329	2884978
S3 20-13C	DOE JGI	3300021057	204709815	476996
S1 5-13C	DOE JGI	3300021061	266934236	639860
S1 10-13C	DOE JGI	3300021062	213311580	526868
S3 10-13C	DOE JGI	3300021066	227159040	579838
S3+v 20-13C	DOE JGI	3300021067	278253160	604522
S1+v 20	DOE JGI	3300021184	596000187	1723296
S3+v 5	DOE JGI	3300024426	800577437	2127945
S3+v 20	DOE JGI	3300024430	705051987	1739013
ABUNV3 2023	UC Davis	3300069200	1267035382	3012337
ABUNV2 2023	UC Davis	3300069201	348486353	931977
ABUNV1 2023	UC Davis	3300069202	1461045267	3838297
ABUNV4 2023	UC Davis	3300069302	812910625	2094070
ABUNV5 2023	UC Davis	3300069303	562751058	1462859
ABVEG1 2023	UC Davis	3300069304	1243370612	3241385
ABVEG2 2023	UC Davis	3300069305	409272842	1141261
ABVEG3 2023	UC Davis	3300069306	623143531	1677139
ABVEG4 2023	UC Davis	3300069307	914596593	2333217
ABVEG5 2023	UC Davis	3300069308	1036166019	2560090

Table S 1.2. Pure methanotrophic and satellite cultures obtained in this study.

Culture	IMG Genome ID	Soil sample (Year collected)	Description	Plant (soil or material)
<i>Methylocaldum</i> sp. 0917	2781126057	2015	Methanotroph, axenic culture	Brittlebush (<i>Encelia farinosa</i>) Rhizosphere 6-10 cm
<i>Methylocaldum</i> sp. S3V3	2904410029	2016	Methanotroph, axenic culture	Desert Willow <i>Chilopsis linearis</i>, Rhizosphere 5cm deep
<i>Methylocaldum</i> RMAD-M	2904404936	2017	Methanotroph, axenic culture	Brittlebush (<i>Encelia farinosa</i>) root
<i>Methylocaldum</i> YM2	2904414868	2017	Methanotroph, axenic culture	Brittlebush (<i>Encelia farinosa</i>) flower
<i>Methylosinus</i> sp. SAV-B	N/A	2015	Methanotroph, axenic culture	Brittlebush(<i>Encelia farinosa</i>) Rhizosphere 6-10 cm
<i>Methylosinus</i> sp. SAV-2	2784132047	2015	Methanotroph, axenic culture	Desert Willow <i>Chilopsis linearis</i>, Rhizosphere 5cm deep
<i>Bradyrhizobium</i> sp. W	2784132048	2015	Satellite culture	Brittlebush (<i>Encelia farinosa</i>) Rhizosphere 6-10 cm
<i>Bradyrhizobium</i> sp. R2.2-H	2781126058	2017	Satellite culture	Brittlebush (<i>Encelia farinosa</i>) root
<i>Bradyrhizobium</i> sp. BM-T	2784132071	2017	Satellite culture	
<i>Bradyrhizobium</i> sp. Y-H1	2781126056	2017	Satellite culture	Desert Sunflower (<i>Geraea canescens</i>)
<i>Neorhizobium</i> sp. R1B	2784132052	2017	Satellite culture	Desert Sunflower (<i>Geraea canescens</i>)
<i>Neorhizobium</i> sp. S3V5DH	2784132046	2016	Satellite culture	Desert Willow <i>Chilopsis linearis</i>, Rhizosphere 5cm deep
<i>Caulobacter</i> sp. H1	2824593709	2016	Satellite culture	NR
<i>Hanschlegelia</i> sp. R3	N/A	2017	Satellite culture	Desert Sunflower (<i>Geraea canescens</i>) roots
<i>Methylobacterium</i> sp. R2-1	2824205890	2017	Satellite culture	Brittlebush(<i>Encelia farinosa</i>) root
<i>Sphingopyxis</i> sp. NOR-H1-2	N/A	2017	Satellite culture	<i>Nerium oleander</i>

NR: plant species was not recorded or identified

Table S 1.3. Overview of *Methylocaldum* strains included in this study. Type strains are marked with *.

Genus/Species	Strain	Physiological Highlights	Temp. range (°C)	C ₁ utilization	Isolation Source/ Country	GC (%)	Genome Length (Mb)	Genome NCBI accession or /and IMG ID	Gene sequence available			Reference
									16S	<i>mmoX</i>	<i>pqq</i> <i>vox mxa</i>	
<i>Methylocaldum</i> sp.	0917				Soil-sand / USA, Anza Borrego Desert, CA	57.9	5.19	2781126057	yes	IMG	IMG	This study
<i>Methylocaldum</i> sp.	YM2				Soil-sand / USA, Anza Borrego Desert, CA	57.9	5.19	2904414868	yes	IMG	IMG	This study
<i>Methylocaldum</i> sp.	S3V3				Soil / Anza-Borrego Desert, CA	57.9	5.19	2904410029	yes	IMG	IMG	This study
<i>Methylocaldum</i> sp.	RMAD-M				Soil-sand / USA, Anza Borrego Desert, CA	58.0	5.45	SAMN17693 797 / 2904404936	yes	IMG	IMG	This study
<i>Methylocaldum gra-cile</i> *	NCIMB-1 1912, VK M: 14L ^T	(Moderate) Thermophilic	20 to 47		Fresh water mud, Active sludge / Russia	n/a	n/a	n/a	yes	NCBI	no	Ex (Bodrossy et al., 1997; Romanovskaya, Malashenko, & Bogachenko, 1978; Takeuchi, 2016)

Table S 1.3. Overview of *Methylocaldum* strains included in this study. Type strains are marked with *, continued.

Genus/Specie	Strain	Physiological Highlights	Temp. range (°C)	C ₁ utilization	Isolation Source/ Country	GC (%)	Genome Length (Mb)	Genome NCBI accession or /and IMG ID	Gene sequence available			Reference
									16S	<i>mmoX</i>	<i>pqq xox mxa</i>	
<i>Methylocaldum ma ritimum</i> *	personal: S8 ^T ,											
	NBRC:	Thermotolerant,	20 to	Methane	Marine			NZ_AP0179				(Takeuchi, 2016;
	109686,	Marine	47	& methanol	sediment / Japan,	58.7	6.09	28.1 /	yes	IMG	IMG	Takeuchi et al.,
	DSM:273 92				Kagoshima Bay			2832923104				2014)
<i>Methylocaldum szegediense</i> *	OR2 ^T	Moderate thermophilic	37 to 62	Methane only	Underground hot spring connected to a natural gas field near Szeged / Hungary	n/a	n/a	n/a	yes	yes	no	(Bodrossy et al., 1997; Bodrossy et al., 1995; Takeuchi, 2016)
<i>Methylocaldum tepidum</i> *	LK6 ^T	Thermotolerant	30 to 47	Methane, can use methanol, formate, formami- de or methyla- mine	Agricultural soil from Rothamsted Experimental Station / England (Harpenden, Hertfordshire)	n/a	n/a	n/a	yes	NCBI		(Bodrossy et al., 1997; Takeuchi, 2016)

Table S 1.3. Overview of *Methylocaldum* strains included in this study. Type strains are marked with *, continued.

Genus/Species	Strain	Physiological Highlights	Temp. range (°C)	C _i utilization	Isolation Source/ Country	GC (%)	Genome Length (Mb)	Genome NCBI accession or /and IMG ID	Gene sequence available				Reference
									16S	<i>mmoX</i>	<i>pqq</i>	<i>xox</i>	
<i>Methylocaldum szegediense</i>	O-12	Moderate											
		thermophilic,	30 to	Methane	Manure /Russia	57.1	5.02	NZ_ATXX0	yes	IMG	IMG		(Eshiniinaev et al., 2004; Takenuchi, 2016)
		Obligate Methanotroph	61	Only	Pushchino Moscow			0000000.1 / 2508501066					
<i>Methylocaldum</i> sp.	14B	methane oxidation in the presence of H ₂ S, fast growth		Obligate mesophilic methanotroph	Solid state anaerobic digester fed corn stover / USA, Wooster, OH								(Reddy et al., 2019; Sheets et al., 2016; Su et al., 2017; Wei et al., 2017b)
			37 to 42			58.2	5.62	SAMN05933 571 / 2889957184	yes	IMG	IMG		
<i>Methylocaldum</i> sp.	BRCS4				Cow manure / United Kingdom	58.1	5.9	SAMN10845 862 / 2890033825		IMG	IMG		none?
<i>Methylocaldum</i> sp.	SAD2	methane oxidation in the presence of H ₂ S	30 to 37		Hydrogen sulfide-rich anaerobic digester / USA, Wooster, OH	58.2	5.91	SAMN05933 458					(Reddy et al., 2019; Su et al., 2017; Wei et al., 2017a)

Table S 1.3. Overview of *Methylocaldum* strains included in this study. Type strains are marked with *, continued.

Genus/Species	Strain	Physiological Highlights	Temp. range (°C)	C ₁ utilization	Isolation Source/ Country	GC (%)	Genome Length (Mb)	Genome NCBI accession or /and IMG ID	Gene sequence available			Reference	
									16S	<i>mmoX</i>	<i>pqq xox mxa</i>		
<i>Methylocaldum</i> sp.	0510-P-2	resilience to a high level of pyrene			Landfill upland								(Chang et al., 2010; Deng, Guo, & Zhu, 2011)
					soil / Taiwan								
					landfill had been completed for 10 years and has planted trees								
<i>Methylocaldum</i> sp.	05J-I-7	resilience to a high level of pyrene			Landfill upland								(Chang et al., 2010; Deng, Guo, & Zhu, 2011; Islam et al., 2016)
					soil / Taiwan								
					landfill had been completed for 10 years and has planted trees								

Table S 1.3. Overview of *Methylocaldum* strains included in this study. Type strains are marked with *, continued.

Genus/Specie	Strain	Physiological Highlights	Temp. range (°C)	C ₁ utilization	Isolation Source/ Country	GC (%)	Genome Length (Mb)	Genome NCBI accession or /and IMG ID	Gene sequence available			Reference
									16S	mmoX	pqq xox mxa	
Methylocaldum sp.	5FB				Soil / Germany, Hünfeld,	n/a	n/a	n/a	yes	yes	yes	(Knief & Dunfield, 2005)
					Rendzina							
					(Leptosol),							
					Mixed forest (Fagus sylvatica, Quercus sp., scattered Pinus sylvestris)							
Methylocaldum sp.	BFH1	Moderately thermophilic and acid-tolerant obligate methanotrophs	30 to 60	Methane and methanol	Soil, Tropical topsoil (5 cm depth) / Bangladesh, small cave heated by a flame of natural methane gas leakage or surface gas blowout	n/a	n/a	n/a	yes	no	no	(Islam et al., 2016)

Table S 1.3. Overview of *Methylocaldum* strains included in this study. Type strains are marked with *, continued.

Genus/Species	Strain	Physiological Highlights	Temp. range (°C)	C ₁ utilization	Isolation Source/ Country	GC (%)	Genome Length (Mb)	Genome NCBI accession or /and IMG ID	Gene sequence available				Reference
									16S	<i>mmoX</i>	<i>pqq</i>	<i>cox mxa</i>	
<i>Methylocaldum</i> sp.	dr65				landfill cover soil	n/a	n/a	n/a	yes	no	no	no	none?
<i>Methylocaldum</i> sp.	E10_A				Soil / Germany, Eiterfeld, Parabraunerde (Luvisol), Farmland (wintergrain)	n/a	n/a	n/a	yes	yes	yes	yes	(Tchawa Yinga et al., 2003)
<i>Methylocaldum</i> sp.	H-11	Moderately thermophilic, Obligate Methanotroph	30 to 61	Methane Only	Silage / Russia Puschino Moscow	n/a	n/a	n/a	yes	no	no	no	(Eshiminaev et al., 2004; Islam et al., 2016; Takeuchi, 2016)
<i>Methylocaldum</i> sp.	r6f	Thermophilic			Landfill cover soil	n/a	n/a	n/a	yes	no	no	no	

Table S 1.4. Nitrogen metabolism key genes presence in *Methylocaldum* strains.

	Assimilatory nitrate reduction (1st step only)					Denitrification			Nitrification		Dissimilatory nitrate reduction (2n step only)				Nitrogen fixation		
	<i>nir</i>	<i>nirA</i>	<i>nirB</i>	<i>nirC</i>	<i>nasA</i>	<i>nirK</i>	<i>norC</i>	<i>norB</i>	<i>hao</i>	<i>hep</i>	<i>nirB</i>	<i>nirD</i>	<i>nirD</i>	<i>nirH</i>	<i>nirK</i>		
<i>Methylocaldum</i> sp. 0917	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+		
<i>Methylocaldum szegediense</i> O-12	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+		
<i>Methylocaldum</i> sp. RMAD-M	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+		
<i>Methylocaldum</i> sp. BRCS4	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+		
<i>Methylocaldum</i> sp. YM2	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+		
<i>Methylocaldum marinum</i> S8	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+		
<i>Methylocaldum</i> sp. S3v3	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+		
<i>Methylocaldum</i> sp. 14B	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+		
<i>Methylocaldum</i> sp. SAD2	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+		

Table S 1.5. Presence of key pathways/genes involved in methanol, formaldehyde oxidation and assimilation in *Methylocaldum* species with available genomes.

H4MPT Tetrahydromethanopterin pathway			H4F methylene tetrahydrofolate pathway							
	Formaldehyde assimilation RuMP pathway M00345	Formaldehyde assimilation Serine Pathway M00346	Methanofuran synthesis M00935	Methanogenesis acetate => methane M00357	Methanogenesis CO2 => methane M00567	Cl-unit interconv. THF => 10- Formyl- THF M00140	Reductive acetyl-CoA pathway (Wood- Ljungdahl pathway) M00377	<i>mdh1</i> K14028	<i>mdh2</i> K14029	<i>cox F</i> K23995
<i>Methylocaldum</i> sp. 0917	✓*	Incomplete (1)	✓ ❖	Incomplete #	Incomplete	✓	Incomplete	✓	✓	✓ (5 copies)
<i>Methylocaldum szegediense</i> O-12	✓*	Incomplete (1)	Incomplete (3)	Incomplete #	Incomplete	✓	Incomplete	✓	✓	✓ (3 copies)
<i>Methylocaldum</i> sp. RMAD-M	✓*	Incomplete (2)	✓ ❖	Incomplete #	Incomplete	✓	Incomplete	✓	✓	✓ (5 copies)
<i>Methylocaldum</i> sp. BRCS4	✓*	Incomplete (1)	✓ ❖	Incomplete #	Incomplete	✓	Incomplete	✓	✓	✓ (5 copies)
<i>Methylocaldum</i> sp. YM2	✓*	Incomplete (1)	✓ ❖	Incomplete #	Incomplete	✓	Incomplete	✓	✓	✓ (5 copies)
<i>Methylocaldum marinum</i> S8	✓	Incomplete (1)	Incomplete (3)	Incomplete #	Incomplete	✓	Incomplete	✓	✓	✓ (5 copies)
<i>Methylocaldum</i> sp. S3v3	✓*	Incomplete (1)	✓ ❖	Incomplete #	Incomplete	✓	Incomplete	✓	✓	✓ (4 copies)
<i>Methylocaldum</i> sp. 14B	✓*	Incomplete (2)	✓ ❖	Incomplete #	Incomplete	✓	Incomplete	✓	✓	✓ (5 copies)
<i>Methylocaldum</i> sp. SAD2	✓*	Incomplete (2)	✓ ❖	Incomplete #	Incomplete	✓	Incomplete	✓	✓	✓ (5 copies)
* only missing K16370 pfkB 6-phosphofructokinase 2										
❖ only missing K00600, K01689, K00830, K08691										
# only have K01895, K00925, K01499, K00201, K00200, K00202, K00672										
❖ only have K00297, K01938										

Table S 1.6. Root-mean-square deviation (RMSD) calculated for *Methylocaldum* PmoC.

Measures the RMSD between two sets of atoms in their current positions, without performing any fitting. The RMSD is a commonly used measure of similarity between two protein structures. The smaller the RMSD is between two structures, the more similar are these two structures

Strain	IMG ID (gene)	PmoC Type	RMSD (angstroms Å)	RMSD (angstroms Å)
<i>Methylocaldum</i> S8	2832924902	1	207 pruned atom pairs = 0.749	All 235 pairs = 1.940
<i>Methylocaldum</i> S8	2832927093	1	207 pruned atom pairs = 0.749	All 235 pairs = 1.940
<i>Methylocaldum</i> BRCS4	2890038637	1	210 pruned atom pairs = 0.809	All 235 pairs = 1.795
<i>Methylocaldum</i> 14B	2889962402	1	213 pruned atom pairs = 0.727	All 235 pairs = 1.856
<i>Methylocaldum</i> O12	2508831462	1	212 pruned atom pairs = 0.729	All 235 pairs = 1.877
<i>Methylocaldum</i> O12	2508833034	1	207 pruned atom pairs = 0.735	All 235 pairs = 1.913
<i>Methylocaldum</i> S8	2832925648	2	211 pruned atom pairs = 0.679	All 235 pairs = 1.864
<i>Methylocaldum</i> BRCS4	2890037627	2	216 pruned atom pairs = 0.753	All 235 pairs = 1.472
<i>Methylocaldum</i> RMADM	2904406329	2	212 pruned atom pairs = 0.704	All 235 pairs = 1.822
<i>Methylocaldum</i> 14B	2889959739	2	212 pruned atom pairs = 0.688	All 235 pairs = 1.746
<i>Methylocaldum</i> O12	2508833640	2	213 pruned atom pairs = 0.694	All 235 pairs = 1.859
<i>Methylocaldum</i> O12	2508833643	2	213 pruned atom pairs = 0.732	All 235 pairs = 1.267
<i>Methylocaldum</i> S3V3	2904414129	2	216 pruned atom pairs = 0.753	All 235 pairs = 1.472
<i>Methylocaldum</i> BRCS4	2890039333	3	210 pruned atom pairs = 0.685	All 226 pairs = 1.280
<i>Methylocaldum</i> 14B	2889958190	3	198 pruned atom pairs = 0.690	All 231 pairs = 2.715
<i>Methylocaldum</i> O12	2508830408	3	210 pruned atom pairs = 0.716	All 231 pairs = 1.985
<i>Methylocaldum</i> 0917	2782372590	4	219 pruned atom pairs = 0.823	All 235 pairs = 1.440
<i>Methylocaldum</i> S8	2832927990	4	218 pruned atom pairs = 0.794	All 235 pairs = 1.817
<i>Methylocaldum</i> BRCS4	2890034185	4	221 pruned atom pairs = 0.778	All 235 pairs = 1.311
<i>Methylocaldum</i> RMADM	2904407932	4	216 pruned atom pairs = 0.797	All 235 pairs = 1.396
<i>Methylocaldum</i> 14B	2889958847	4	217 pruned atom pairs = 0.752	All 235 pairs = 1.408
<i>Methylocaldum</i> YM2	2904416963	4	219 pruned atom pairs = 0.821	All 235 pairs = 1.434
<i>Methylocaldum</i> S3V3	2904412171	4	219 pruned atom pairs = 0.821	All 235 pairs = 1.434
<i>Methylocaldum</i> 0917	2782370918	5	205 pruned atom pairs = 0.711	All 236 pairs = 4.370
<i>Methylocaldum</i> S8	2832926543	5	206 pruned atom pairs = 0.731	All 227 pairs = 1.263
<i>Methylocaldum</i> BRCS4	2890037436	5	209 pruned atom pairs = 0.733	All 236 pairs = 4.630
<i>Methylocaldum</i> RMADM	2904406134	5	209 pruned atom pairs = 0.733	All 236 pairs = 4.630
<i>Methylocaldum</i> 14B	2889960376	5	209 pruned atom pairs = 0.733	All 236 pairs = 4.630
<i>Methylocaldum</i> YM2	2904415561	5	204 pruned atom pairs = 0.692	All 236 pairs = 4.356
<i>Methylocaldum</i> O12	2508833441	5	211 pruned atom pairs = 0.710	All 236 pairs = 6.750
<i>Methylocaldum</i> S3V3	2904410529	5	204 pruned atom pairs = 0.692	All 236 pairs = 4.356
<i>Methylocaldum</i> BRCS4	2890034396		138 pruned atom pairs = 0.834	All 142 pairs = 0.948

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Chapter 2 Cultivation Approach and Genome of Methanotrophic Isolates

Chapter 2, in full, is a reprint of the material as it appears in *Microorganisms*, Nathalie Delherbe, David Pearce, Sergey Y But, J Colin Murrell, Valentina N Khmelenina, and Marina G Kalyuzhnaya. "Genomic Insights into Moderately Thermophilic Methanotrophs of the Genus *Methylocaldum*." *Microorganisms* 12, no. 3 (2024): 469.



Article

Genomic Insights into Moderately Thermophilic Methanotrophs of the Genus *Methylocaldum*

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Abstract: Considering the increasing interest in understanding the biotic component of methane removal from our atmosphere, it becomes essential to study the physiological characteristics and genomic potential of methanotroph isolates, especially their traits allowing them to adapt to elevated growth temperatures. The genetic signatures of *Methylocaldum* species have been detected in many terrestrial and aquatic ecosystems. A small set of representatives of this genus has been isolated and maintained in culture. The genus is commonly described as moderately thermophilic, with the growth optimum reaching 50 °C for some strains. Here, we present a comparative analysis of genomes of three *Methylocaldum* strains—two terrestrial *M. szegediense* strains (O-12 and Norfolk) and one marine strain, *Methylocaldum marinum* (S8). The examination of the core genome inventory of this genus uncovers significant redundancy in primary metabolic pathways, including the machinery for methane oxidation (numerous copies of *pmo* genes) and methanol oxidation (duplications of *mxsA*, *mxsF*-5 genes), three pathways for one-carbon (C1) assimilation, and two methods of carbon storage (glycogen and polyhydroxyalkanoates). We also investigate the genetics of melanin production pathways as a key feature of the genus.

Keywords: thermophilic methanotrophic bacteria; *Methylocaldum*; methane monooxygenase; pyromelanin; methanol dehydrogenase; extremophiles



Citation: Delherbe, N.A.; Pearce, D.; But, S.Y.; Murrell, J.C.; Khmelenina, V.N.; Kalyuzhnaya, M.G. Genomic Insights into Moderately Thermophilic Methanotrophs of the Genus *Methylocaldum*. *Microorganisms* **2024**, *12*, 469. <https://doi.org/10.3390/microorganisms12030469>

Academic Editor: Juan M. Gonzalez

Received: 23 January 2024

Revised: 19 February 2024

Accepted: 19 February 2024

Published: 26 February 2024



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1. Introduction

Microbial methane oxidation is a key process in the carbon cycle at local and global scales. Methane-utilizing bacteria (methanotrophs) inhabiting high-temperature ecosystems include members of the genera *Methylococcus*, *Methylothermus*, and *Methylocaldum* from the phylum Pseudomonadota (Proteobacteria) and the genus *Methylococcoides* in the phylum Verrucomicrobia [1–3]. Thermophilic and thermotolerant species of the *Methylocaldum* genus (Gammaproteobacteria class in the family *Methylococcales*) are commonly identified as important members of the bacterial communities in soils from a range of geographical locations [4], including hot springs [5], landfill cover soils [6,7], tin-mining ponds [8], oil sands [9], flooded rice fields [10], and marine sediments [11]. The genus *Methylocaldum* was introduced by Bodrossy et al. (1997) [5]. The genus currently includes four species: *M. szegediense*, *M. tepidum*, *M. gracile* [5], and *M. marinum* [11]. The isolate *M. szegediense* O-12 was obtained from a sample of cow manure from a farm near Pushchino in the Moscow region in Russia. The pure culture was obtained from an initial enrichment, followed by a serial dilution to extinction technique [12]. *M. szegediense* O-12 grows at a temperature range of 37–59 °C, with optimal growth at 55 °C. *M. szegediense* Norfolk was isolated from biofilter soil enrichments from a landfill in Norfolk County Council, UK, and

grows between 37 °C and 62 °C, with an optimal growth at 50 °C [13]. Here we conduct a comparative analysis between thermotolerant methanotrophs to identify key genes potentially involved in their capability to grow at elevated temperatures. The genomic features of the two terrestrial, moderate thermophilic *M. szegediense* O-12 and Norfolk strains are compared with the thermotolerant *M. marinus* S8 (=NBRC 109686^T = DSM 27392^T) isolated from marine sediments in Kagoshima Bay, Japan, which grows optimally at 36 °C and a salinity of up to 5% NaCl [11].

2. Materials and Methods

Genome sequencing, assembly, and annotation. *M. szegediense* O-12 (taxon ID: 2508501066 (IMG)/675524 (NCBI)) genome sequencing and assembly was conducted in collaboration with U.S. Department of Energy Joint Genome Institute (NCBI assembly ASM42738v1). The genome and assembly of *M. szegediense* Norfolk (taxon ID: 8069803063 (IMG)/73780 (NCBI)) was generated by the laboratory of Dr. J. Colin Murrell, at University of East Anglia, Norwich, UK, utilizing an enhanced genome sequencing coupling short Illumina and long Nanopore reads (NCBI assembly strumpshaw02) [13]. The genome sequence of *M. marinus* S8 (taxon ID: 2832923104 (IMG)/1432792 (NCBI)) was generated by National Institute of Advanced Industrial Science and Technology (AIST), Japan (NCBI assembly ASM358464v1). In addition to being available in NCBI, the three genomes were uploaded and annotated in IMG/MER [14,15].

Methylocaldum phylogenetic analysis was carried out using all (20) complete sequences available for the 16S rRNA gene assigned to *Methylocaldum* strains on NCBI at the time of the analysis (2022). The alignment was generated using the multiple alignment program for amino acid or nucleotide sequences MAFFT v7.511, choosing the E-INS-i method [16–18]. The phylogenetic relationship was inferred using the MEGA X software v10.2.6 using the maximum likelihood method and the model with the lowest Bayesian information criterion (BIC) scores for the set, which corresponded to Tamura 3-parameter (T92) with a discrete Gamma distribution (+G) with 5 rate categories and by assuming that a certain fraction of sites is evolutionarily invariable (+I) [19–22]. The analysis involved 32 nucleotide sequences (including *Methylococcus capsulatus* Bath and *Methylobacter* species as outgroups), and all positions with less than 95% site coverage were eliminated.

pMMO synteny, functional genomic comparison, and ANI × AC. The synteny and functional genomic comparison of the genomes was based on the information obtained using Anvi'o v7 software [23]. The visual representation of the pMMO operon synteny was generated by combining the outputs of Circa plot (v.1.2.1) utilizing the corresponding nucleotide sequence coordinate data and combining them with the gene cluster visualization output from Geneious software v.2023.2.1. The generated data included a list of all coding sequences (CDS) from the detected open reading frames identified by Prodigal v.2.6.3 [24]. The CDS predicted functionality was annotated using the NCBI's Clusters of Orthologous Groups (COG) database [25–27] and the KEGG KOfamKOALA database [28]. The pangenome analysis, based on the pangenome pipeline of Anvi'o v7 [29], generated orthologous groups (OG) of genes based on the homology of their amino acid sequences. A >70% of combined homogeneity index (derived from geometrical and functional homology) was the cut-off to consider a gene as part of an OG. Each OG contained at least one gene. Additionally, the OGs were organized by presence/absence through Euclidean distance and Ward linkage, which was used to categorize them as *shared* by the three genomes or *unique* for each of them. Then, within each of the shared and unique subsets, all CDS with COG assigned to them were used to determine which COG categories were the most represented (>5%). In addition to the top of the pangenome analysis, a whole genome comparison was generated using the program *anvi-compute-genome-similarity* utilizing the PyANI [22] similarity metric to calculate the average nucleotide identity (ANI). Additionally, the full percent identity was calculated for the ANI, which includes the regions that did not align, by multiplying the percent identity by the alignment coverage (ANI × AC).

***pmoC* homologs.** Genomes of *Methylocaldum*, *Methylobacter*, and *Methylococcus* available on IMG/MER were used to search for the range of copies of *pmoC*. The search was based on IMG annotations using the different available identifiers for *pmoC*: TIGR03078, KO10946, and gene product name. A table containing the Anvi'o v7 results for synteny and functional genomic comparison of the genomes can be found in Supplementary Table S1.

XoxF phylogeny. The alignment was generated using the multiple alignment program for amino acid or nucleotide sequences v7.511 [17,18], choosing the E-INS-i method [16–18]. The tree topology was obtained after 100 bootstrap replications using the maximum likelihood method and general time reversible model [20] using MEGA X software v10.2.6 [19,21]. The tree with the highest log likelihood (−175,992.24) [22] was used to generate the summarized version of it.

Fdh phylogeny. The alignment was generated using the multiple alignment program for amino acid or nucleotide sequences MAFFT v7.511 [17,18], choosing the E-INS-i method [16–18]. The tree topology was obtained after 100 bootstrap replications using the maximum likelihood method and the Whelan and Goldman + Freq. model [30]. The tree with the highest log likelihood (−50,331.00) (Supplementary Figure S1) was used to generate the cartoon version of it. The percentage of trees in which the associated taxa clustered together is shown next to the branches. This analysis of 193 amino acid sequences was conducted in MEGA X v10.2.6 [19,21]. There were a total of 1638 positions in the final dataset.

3. Results

3.1. Genome Overview

The *M. szegediense* O-12 genome (taxon ID: 2508501066 (IMG)/675524 (NCBI)) includes two scaffolds of 2.37 Mb and 2.65 Mb, resulting in a genome of 5.02 Mb with an average GC content of 57.03%. It includes two rRNA operons, 22 types of tRNAs (47 total), and 4693 predicted protein-coding genes (3448 predicted functions). The genome of *M. szegediense* sp. Norfolk (taxon ID: 8069803063 (IMG)/73780 (NCBI)) includes a closed genome comprising one chromosome of 4.87 Mb and a 25.7 kb plasmid, resulting in a genome of 4.89 Mb with an average GC content of 57.17%. It contains two rRNA operons, 24 types of tRNAs (53 total), and 4491 predicted protein-coding genes (3378 predicted functions). *Methylocaldum marinum* S8 (taxon ID: 2832923104 (IMG)/1432792 (NCBI)) has a complete genome of 6.08 Mb with a 58.75% GC content, including two rRNA operons, 24 types of tRNAs (51 total), and 5596 predicted protein-coding genes (3846 predicted functions).

3.2. *Methylocaldum* Phylogeny

The phylogeny of *Methylocaldum* strains based on available 16S rRNA sequences shows five clades (Figure 1A). Four of these clades correspond to currently characterized species within the *Methylocaldum* genera (*Methylocaldum tepidum* LK6^T, *Methylocaldum szegediense* OR2^T, *Methylocaldum gracile* VKM 14L^T, and *Methylocaldum marinum* S8^T), while the fifth clade formed by strains BFH1, dr65, and r6f does not have a type strain. A comparison based on the presence or absence of orthologous genes between the three strains analyzed in this study revealed that the *M. szegediense* O-12 and Norfolk strains share 90% and 93%, respectively, of their predicted protein sequences as orthologous, while only 47% of the predicted protein sequences in *M. marinum* S8 were orthologous to genes in the *M. szegediense* O-12 and Norfolk strains (Figure 1B). The average nucleotide identity times the alignment coverage (ANI × AC) percentage between the three strains confirmed that *M. szegediense* O-12 and Norfolk are members of the same species (≥90%), while *M. marinum* S8 shared only 38% with the *M. szegediense* strains (Figure 1B).

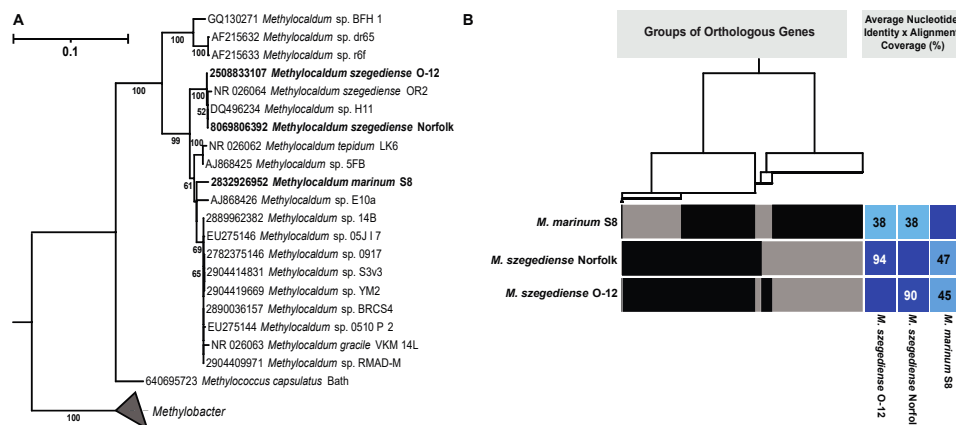


Figure 1. (A) Maximum likelihood tree representing the phylogenetic relationship of *Methylocaldum* based on 16S rRNA gene sequences. Leaf names with the strains analyzed in this article are in bold. Identifiers for each IMG gene ID are at the tip of each leaf. (B) Dendrogram showing the association between cluster of groups of orthologous genes, represented by thin vertical black lines, which, when grouped, form the rectangular blocks depicted on each genome. The clustering is based on the presence or absence of the orthologous genes on each genome. Average nucleotide identity (ANI) times the alignment coverage (AC) percentage of each genome when compared with one of the other *Methylocaldum* strains are shown inside of blue or light blue squares.

3.3. Synteny of Methane Monooxygenase (MMO) Gene Clusters

The identification of key genes necessary for methane oxidation in bacteria revealed the presence of several copies of the *pmoC* gene, which is usually part of the *pmoCAB* operon for the particulate methane monooxygenase (pMMO). Five copies were found in *M. marinum* S8, six were found in *M. szegediense* O-12, and five were found in *M. szegediense* Norfolk (Figure 2). In the *M. marinum* S8 and *M. szegediense* Norfolk strains, two *pmoC* genes formed part of the complete gene cluster for the synthesis of the pMMO, whereas only one *pmoC* gene formed part of the complete gene cluster in *M. szegediense* O-12 (Figure 2). All *pmoC* genes forming part of pMMO operons had high sequence identity to each other and were denominated as type 1 (purple ribbon, Figure 2). Furthermore, the three *Methylocaldum* genomes contained paralogs of *pmoC* as independent genetic components in different loci of their genomes, which have been previously named as stand-alone copies of *pmoC* [31]. Except for a single stand-alone *pmoC* in *M. szegediense* O-12 assigned to type 1, the rest of the stand-alone copies of *pmoC* have different nucleotide sequences than the one forming the pMMO cluster (type 1) and form two separate groups of paralogs, denominated as type 2 and 5 (pink and magenta ribbons, respectively, Figure 2). Furthermore, both *M. szegediense* strains exhibit two stand-alone *pmoC* type 2 relatively next to each other. Additionally, two stand-alone *pmoC* without high homology to any other *pmoC* sequences within the three genomes were found in *M. marinum* S8 (type 4) and *M. szegediense* O-12 (type 3), shown in black-edged triangles in Figure 2. Only *M. marinum* S8 exhibits a complete gene cluster for the soluble methane monooxygenase (sMMO). When compared with other methanotrophs with genomes annotated on IMG/MER (*Methylobacter* [16] and *Methylococcus* [10]), *Methylocaldum* strains have between five and six coding sequences (CDS) assigned to *pmoC*, while *Methylobacter* strains have between one and two and *Methylococcus* have between two and three *pmoC* in the available genomes.

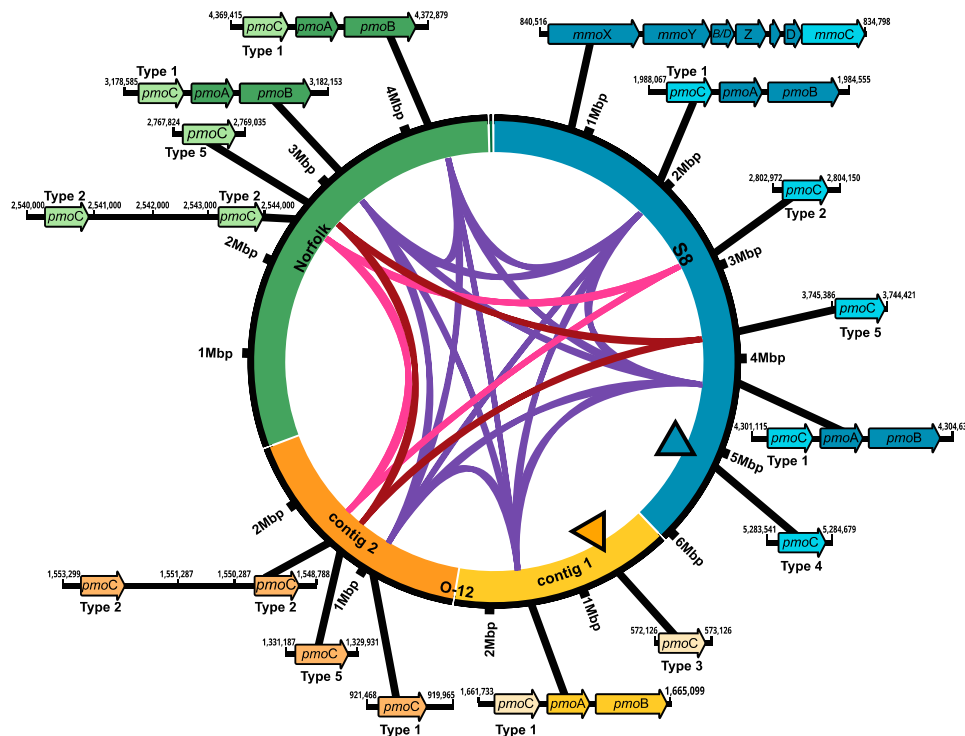


Figure 2. Synteny of methane monooxygenase (MMO) gene clusters in *Methylocaldum* genomes. Schematics for genomes of *M. marinum* S8, *M. szegediense* O-12, and *M. szegediense* Norfolk depicting the locus of MMO gene clusters. Ribbons connecting genomes show *pmoC* genes with high identity as part of pMMO gene cluster (purple) and as stand-alone genetic component (pink and magenta). Black-edge triangles indicate stand-alone copies of *pmoC* without high identity with any other *pmoC* within the three genomes. The gene cluster for soluble methane monooxygenase, sMMO, which is only found in *M. marinum* S8, is also shown.

3.4. Comparative Abundance CDS Assigned to COG Categories

According to the pangenome analysis of the three *Methylocaldum* strains, from a total of 14,284 CDS predicted for the three genomes, 2214 CDS formed orthologous groups (OG) of amino acid sequences based on a $\geq 70\%$ threshold of combined homogeneity index (derived from geometrical and functional homology; Figure 3). Each genome had at least one gene copy as part of each OG assigned as shared among the three strains. Based on their relative abundances, the main shared COG categories (comprising $>5\%$ of the total gene calls that could be categorized) were genes related to categories C (8.58%) involved in energy production and conversion; E (7.36%) amino acid transport and metabolism; H (5.74%) coenzyme transport and metabolism; J (8.04%) translation, ribosomal structure and biogenesis; M (7.09%) cell wall/membrane biogenesis; O (6.23%) post-translational modification; P (6.32%) inorganic ion transport and metabolism; and R (5.42%) classified only as general function.

On the contrary, the CDS found to be unique to each genome, based on a low combined homogeneity index of their amino acidic sequences when compared to their counterparts in the other genomes, were mainly assigned to COG categories M, cell wall/membrane biogenesis (11.57%); X, mobilome including prophages and transposons (10.9%); T, signal

transduction mechanisms (8.15%); P, inorganic ion transport and metabolism (7.70%); R, general function (7.47%); V, defense mechanisms (5.67%); H, coenzyme transport and metabolism (5.17%); and C, energy production and conversion (5%) for *M. marinum* S8. At the same time, the pattern was similar between *M. szegediense* O-12 and Norfolk with V (13.91% and 23.07%), X (15.65% and 7.69%), L, replication, recombination, repair (17.39% and 16.92%), R (8.7% and 13.84), K, and transcription (8.70% and 13.84%), respectively (Figure 3).

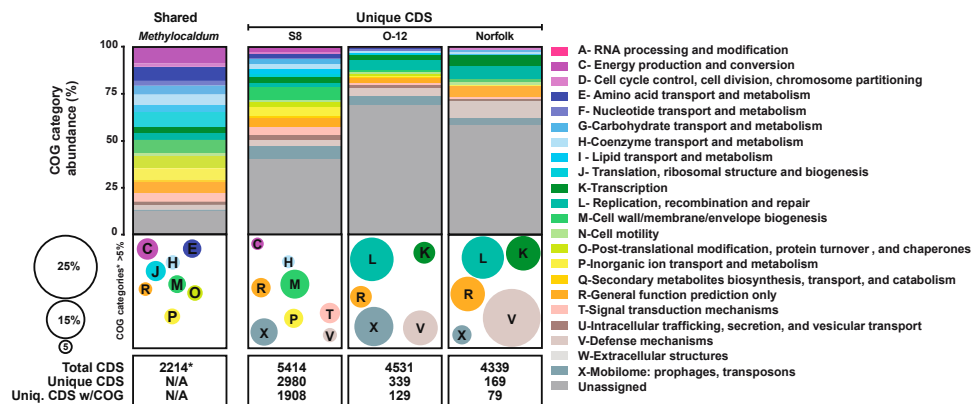


Figure 3. Comparative abundance of COG categories for *Methylocaldum* genomes. The relative percentage of each COG category is depicted by colored horizontal bars and bubbles according to the color-coded legend. Horizontal bars represent the percentage of coding sequences (CDS) assigned to each COG category among the three strains (shared) and the uniqueness of each strain (S8, O-12, and Norfolk). The total number of CDS considered for this comparison is at the bottom of each column. * indicates shared CDS; N/A indicates not applicable for the shared genes column. Bubble sizes and colors depict COG categories that represented >5% of the total when unassigned CDS were not considered.

3.5. Conjugation

Several predicted functions were only found in the two moderate thermophilic *M. szegediense* strains, including K12056, corresponding to the conjugal transfer mating pair stabilization protein TraG, which is one of the pilus assembly proteins in bacterial type IV secretion systems (T4SS) [32]. While *M. szegediense* Norfolk has only two conjugal transfer proteins, TraG and TraN, the *M. szegediense* O-12 genome contains two TraG, as well as TraW, TraF, TraE, and TraL, which participate in pilus assembly; TraA (pilin); TraN and TraU, which are responsible for mating pair stabilization; TraV, TraK, and TraB, which form the core complex; TraD and TraC, which are ATPase proteins reported to be complexed in the cytosol and inner membrane; and also TrbL, which is a subset of proteins described as F-like T4SS [32,33].

3.6. O-Antigen Biosynthesis

Interestingly, two homologs of O-antigen biosynthesis protein RfbC (K20444; COG0438) (COG1216) were found only in the *M. szegediense* strains. RfbC is predicted to catalyze the incorporation of sugars and their products to form the O-antigen polysaccharides in the lipopolysaccharides (LPS) [34]. The enzyme's specificity toward rare sugars enables the production of unusual LPS, thus providing the remarkable diversity of cell envelope recognition patterns observed among bacteria [35–39]. RfbC is a member of the glycosyltransferase family 2 (GT2). A search for other glycosyltransferases belonging to that family revealed the presence of six other OGs shared between the *M. szegediense* strains and absent in *M.*

marinum S8. Additionally, the three *Methylocaldum* strains share three OGs of GT2, and *M. marinum* S8 has four different GT2, which are not shared with the *M. szegediense* strains. Differences between the genetic toolkit for the biosynthesis of the O-antigen among *Methylocaldum* offer a starting point for future studies to elucidate the mechanisms of specialization in their different niches and how they can tolerate a wide range of temperatures. It has been shown that O-antigen structural differences can confer resistance to different types of stress, such as oxidative [40], mechanical [39], and osmotic stresses [41]. Additionally, antigen molecular diversity is a current target of study to understand interdomain symbiosis [42].

3.7. Catalase

Among the distinct functions that were found to be unique for *M. marinum* S8 is catalase KatE (COG 0753, KEGG K03781), which can convert toxic hydrogen peroxide (H_2O_2) into water and oxygen. H_2O_2 is one of the three products of methanethiol (CH_3SH) oxidation ($HCOH$, H_2S , H_2O_2), for which all three genomes have a homolog to the gene *mtOX*, a copper-dependent methanethiol oxidase (MTO) gene characterized in *Hyphomicrobium* sp. VS [43] and annotated in our analysis as K17285. CH_3SH is a volatile organic sulfur compound that can co-occur with methane. It can be consumed by the verrucomicrobial methanotroph *Methylocaldum fumariolicum* SolV, which produces H_2S that is subsequently oxidized for energy [44]. It seems likely that this gene would be beneficial for methanotrophs since it could allow them to flourish in niches that release methane and CH_3SH , an inhibitor of methane oxidation [45].

3.8. Cobalamin

Another unique function of *M. marinum* S8 among the three genomes was a nearly complete de novo cobalamin (B_{12}) biosynthetic pathway. The only missing gene corresponds to *cbiJ-cobK*, whose product, precorrin-6X reductase, converts (Co)Precorrin-6A to (Co)Precorrin-6B. This gene had been previously described as missing in almost all characterized cobalamin-producing archaea [46–48]. This suggests the independence of an external source of the essential cofactor, which would be an advantage, given that most predicted cobalamin producers in the ocean belong to photoautotrophic Cyanobacteria, chemoautotrophic Nitrososphaerota (Thaumarcheota), and a selected group of Pseudomonadota (Proteobacteria), including Rhodobacterales, Rhodospirillales, Deltaproteobacteria, Oceanospirillales, and Pseudomonadales [49]. Both *M. szegediense* strains lacked any recognizable genetic elements required for the corrinoid ring biosynthesis [50]. Nonetheless, all three genomes contain the genes for 5,6-dimethylbenzimidazole (DMB) synthesis, 5,6-dimethylbenzimidazole synthase; *bluB* (COG0778, K04719), which is the lower ligand of coenzyme B_{12} ; all the genes for the final synthesis and repair (K19222 (*cobA*), K00798 (*cobO*), K02232 (*cobQ*), K02225 (*cobC*), K02227 (*cobD*), K02231 (*cobP/cobU*), and K02233 (*cobS*); and, for B_{12} transport, K16092 (*btuB*), which match the previous classification of *M. szegediense* O-12 as potentially unable to do de novo synthesis, but instead to salvage cobamide precursors, assemble the nucleotide loop, and attach a lower ligand, based on its in silico analysis of 11,000 strains [51].

3.9. *M. marinum* S8 Unique Predicted Functions

Additionally, only *M. marinum* S8 has several hydrogenases subunits, including Fe-S-cluster-containing hydrogenase component 2 (HycB) COG1142, hydrogenase-4 membrane subunit HyfE COG4237 (K12140), Ni,Fe-hydrogenase III small subunit (HycG) (PDB:6CFW) COG3260, and the Ni,Fe-hydrogenase III large subunit HycE2 | Ni,Fe-hydrogenase III component G (HycE1) COG3261 | COG3262, which suggests the metabolic potential to obtain energy through membrane-associated H_2 involving hydrogenases. These hydrogenases are in the immediate vicinity of homologs to the formate hydrogenlyase subunit HyfC (COG0650).

Other functions found only in the genome of *M. marinum* S8 are homologs for molybdopterin biosynthesis (COG0303 | 1910, K07219); peroxiredoxin DsrE/DsrF-like

family (COG2044); putative NADH-Flavin reductase YWNB (COG2910); RecA superfamily ATPase KaiC/GvpD/Rad55 (COG0467, K0882); Fe³⁺ permease component FepD, (COG0609|0614) Fe²⁺ transporter FeoB|FeoA (COG0370|1918, K04759); periplasmic components for ABC-type tungstate transport system, TupA (COG4662, K05773) and TupB (COG2998, K05772); heavy metal-binding TRASH/YHS Cu/Ag metallochaperone (COG3350, K16157); nitrous oxide reductase accessory protein NosL (COG4314, K19342); and cobalt transporter membrane subunit CgtA (COG5446).

3.10. C1-Oxidation Pathways Are Highly Redundant

As described above, in addition to having two *pmoCAB* operons in the *M. szegediense* Norfolk and *M. marinum* S8 genomes and the sMMO operon in *M. marinum* S8, the three genomes also contain multiple stand-alone *pmoCs* with unknown functions. They also have multiple methanol dehydrogenases. The *mxhF*|*GIRACKLD* gene clusters in the three *Methylocaldum* strains encode the two subunits of methanol dehydrogenase *mxhF* (K14028) and *mxhI* (K14029) and the natural electron acceptor, cytochrome c_L (*mxhG*). Moreover, the three strains share two OGs of additional methanol dehydrogenases, lanthanide-dependent MDHs, corresponding to *xoxF* types 5 and 3 according to the phylogenetic reconstructions (Figure 4) following previously *xoxF* type assignment [52–54]. *M. marinum* has a third *xoxF* with no homology to genes in the *M. szegediense* strains that also belongs to *xoxF* type 3.

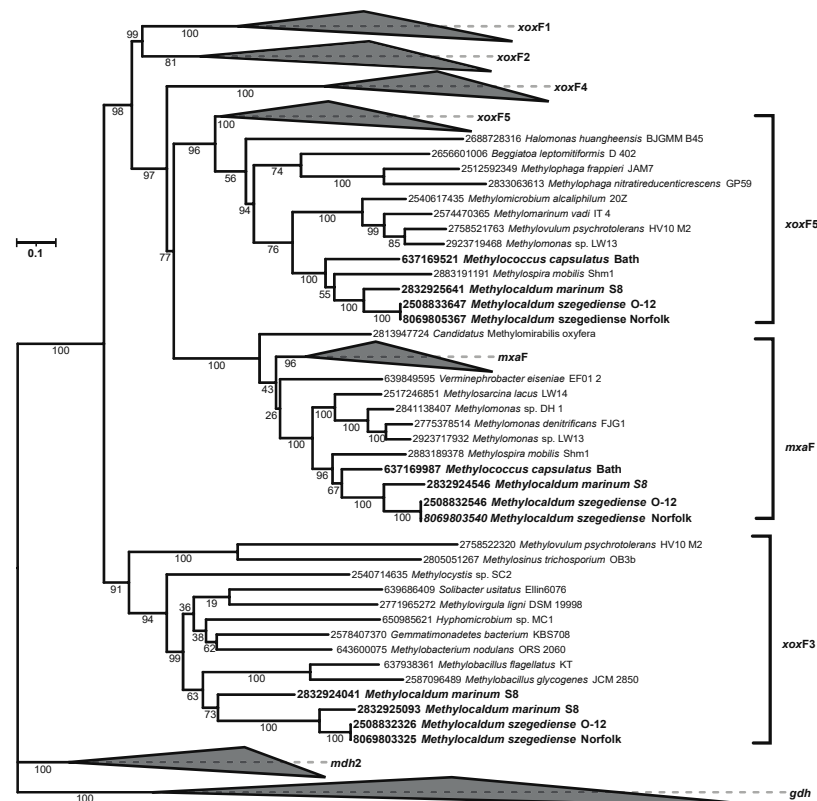


Figure 4. Phylogenetic reconstruction based on all *xoxF* and *mxhF* genes found in the three *Methylocaldum* genomes analyzed (highlighted in bold). The corresponding IMG gene IDs are at the tip of each leaf. Collapsed clades did not contain any genes from the three analyzed genomes.

The three *Methylocaldum* have the set of genes for the tetrahydromethanopterin (HMPT)-dependent pathway required for the oxidation of formaldehyde to formate. Gene redundancy was also found in this pathway. Based on the OG analysis, three different OGs for *fae* (K10713), which encodes the 5,6,7,8-tetrahydromethanopterin hydrolyase, were found among the *Methylocaldum*. One *fae* OG included three CDS from *M. marinum* S8, and two were included for *M. szegediense* O-12 and Norfolk. The other two OGs had only one CDS in each strain. Each strain had two methylene-tetrahydromethanopterin dehydrogenases *mtdB* (K10714) in separate OGs. Only one of the following genes was found in each of the strains: methylenetetrahydrofolate dehydrogenase *mtdA* (K00300), methenyltetrahydromethanopterin cyclohydrolase *mch* (K01499), formylmethanofuran--tetrahydromethanopterin N-formyltransferase *ptr* (K00672), formylmethanofuran dehydrogenase *fdwABC* (K00200, K00201, K00202), glycine hydroxymethyltransferase *glyA* (K00600), and coenzyme F420 hydrogenase subunit beta *frhB* (K00441), for which only *M. marinum* S8 had two CDS.

Interestingly, several formate dehydrogenases encoding genes (*fdh*) were found in the three strains. These enzymes catalyze the oxidation of formate to CO₂, donating electrons to NAD⁺ or cytochromes. The phylogenetic reconstruction based on the amino acid sequence of the major subunits (K00123) of the different formate dehydrogenases found in *Methylocaldum* revealed that they have four types of *fdh* (Figure 5 and Supplementary Figure S1). According to the previously described types of *fdh* in *Methylobacterium extorquens* AM1 [55,56], we detected the presence of three different *fdh* in the three *Methylocaldum* strains, corresponding to *fdh* types 2 and 3, including all their subunits. Additionally, FDH (K00122) was found in the three genomes as well. Moreover, *M. marinum* S8 has the major subunit for *fdh* type 1, although it was previously described to require the subunit B for functioning in the *M. extorquens* AM1 model [56,57].

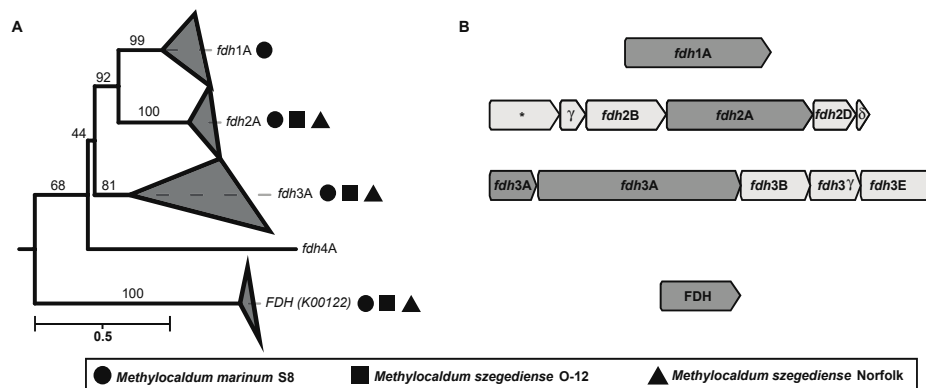


Figure 5. (A) Maximum likelihood tree reconstructing the phylogenetic relationship between the amino acidic sequences of four different types of formate dehydrogenase major subunits (K00123). Geometric figures next to each collapsed clade indicate the presence of a sequence from one of the three *Methylocaldum* strains analyzed in this study. Circles represent *M. marinum* S8, squares represent *M. szegediense* O-12, and triangles represent *M. szegediense* Norfolk. Formate dehydrogenase (FDH) K00122 was used as the outgroup. (B) Gene cluster genomic arrangement found on the three *Methylocaldum* strains analyzed in this study. The asterisk indicates the presence of a predicted protein with unknown function.

3.11. Single-Carbon Assimilation Pathways

These *Methylocaldum* genomes encode three single-carbon assimilation pathways—the ribulose monophosphate (RuMP), serine, and Calvin–Benson–Bassham (CBB) cycle. The genes present in the RuMP pathway include two CDS for 3-hexulose-6-phosphate syn-

thase in each genome, *hxlA* (K08093) and *hps-pi* (K13831), catalyzing the condensation of formaldehyde with ribulose-5-phosphate to D-arabino-hex-3-ulose 6-phosphate, which is converted to D-fructose 6-phosphate by the 6-phospho-3-hexuloisomerase, *hxlB* (K08094) present in the three genomes. D-fructose 6-P can then enter the pentose phosphate pathway.

The large *rbcL/cbbL* (K01601) and small *rbcS/cbbS* (K01602) subunits of the ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO) were found in the three strains, having a single gene difference between *M. szegediense* and *M. marinum*. This includes the presence of *norQ* and *norD* next to RuBisCO genes of the three *Methylocaldum*. *M. marinum* S8 also has pimeloyl-ACP methyl ester carboxylesterase between the RuBisCO and *nor* genes. The presence of *rbc/cbb* genes had been reported in other methanotrophs; however, its activity had not been demonstrated yet [58–60].

Similarly to other gammaproteobacterial methanotrophs, the regeneration of pentose phosphates from hexosephosphates requires fructose-bisphosphate aldolase, for which class I, ALDO (K01623), is present in all three *Methylocaldum* genomes.

As in other gammaproteobacterial methanotrophs, the *Methylocaldum* genomes contain some key genes for the serine pathway of formaldehyde assimilation. These include serine-glyoxylate transaminase, AGXT (K00830), which converts glyoxylate to hydroxypyruvate and glycine hydroxymethyltransferase, *glyA* (K00600), which uses 5,10-methylenetetrahydrofolate and glycine as substrates to produce tetrahydrofolate and L-serine, which can continue to the carbon fixation pathway or sulfur or serine metabolism, respectively. Also, as part of the serine pathway, the three genomes encode malyl-CoA/(S)-citramalyl-CoA lyase *mcl* (K08691), which produces acetyl-CoA and glyoxylate from L-malyl-CoA as a substrate. However, all strains lack phosphoenolpyruvate carboxylase, *ppc* (K01595), which produces oxaloacetate from phosphoenolpyruvate, necessary for the serine pathway. Anaplerotic functions of the serine pathway have been previously proposed in *Methylococcus capsulatus* Bath. Like the strain Bath, all three genomes have the necessary genes to convert malyl-CoA (*mcl*, K08691) to glyoxylate, then to glycine (AGXT, K00830), and subsequently to serine (*glyA*, K00600) as part of the serine pathway. In the same pathway, they also have the genes to convert from D-glycerate to glycerate-2P (*gck*, K11529) to phosphoenolpyruvate (*eno*, K01689).

3.12. Nitrogen Metabolism

All three strains of *Methylocaldum* have the structural genes for nitrogenase (*nifH* (K02588), *nifD* (K02586), and *nifK* (K02591)) in an operon with *nifE*, *nifN*, *nifX*, and *nifQ* genes and also a *nif*-specific ferredoxin III (TIGR02936) (Figure 6B). Two additional homologs of *nifH* and *nifK* genes were also found in the three strains, although their genetic context was different from each other and may not be related to nitrogen fixation. The genetic elements required for nitrate assimilation were found in the three strains, having as a difference the additional presence of the *nrtABC* gene cluster (K15576, K15577, K15578) for the nitrate/nitrite transport system in *M. marinum* S8 only (Figure 6A). The three strains have the *norCB* gene cluster (K02305, K04561) for the reduction of nitric oxide (NO) to nitrous oxide (N₂O); however, only *M. szegediense* O-12 and Norfolk had the *nirK* (K00368) for dissimilatory nitrite reductase for denitrification, and none of them had the alternative *nirS* (K15864) nitrite reductase. Similarly to other methanotrophic species, homologs of hydroxylamine dehydrogenase (K10535) responsible for NH₂OH oxidation to NO₂, as well as hydroxylamine reductase reducing NH₂OH to form NH₄⁺ (K05601), are present in the three genomes. Additionally, the three strains have the genetic potential to assimilate NH₄⁺ through the glutamate cycle, with their genes for glutamine synthetase (GS) *glnA* (K01915) and glutamate synthase (GOGAT) *gltBD* (K00265, K00266) [58]. The three strains have the genetic potential for the reversible conversion of glutamate to 2-oxoglutarate/α-ketoglutarate (which is an intermediate of the Krebs cycle) and ammonia through their glutamate dehydrogenase GDH2 (K15371). Additionally, the three strains also have the gene for alanine dehydrogenase *ald* (K00259), which had been described to participate in the reductive amination of pyruvate in other methanotrophs, under high

NH_4^+ environments [58]. The two *M. szegediense* strains also have the gene for another glutamate dehydrogenase *gdhA* (K00262), which has been demonstrated to be required for *Streptococcus pneumoniae* for adaptation to high temperatures (40 °C) [61].

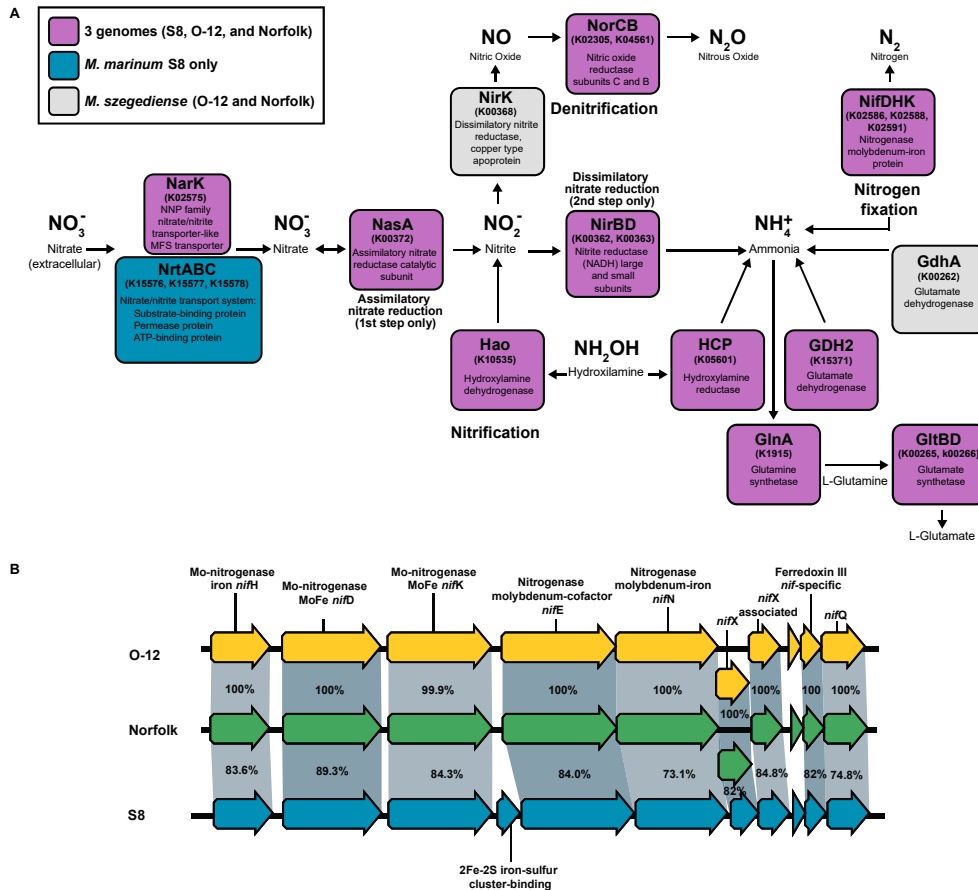


Figure 6. Nitrogen metabolism genes in *Methylocaldum* strains. (A) Diagram representing the presence and absence of genes involved in nitrogen cycling. (B) Synteny of nitrogenase gene cluster present in the three *Methylocaldum* strains. Pairwise identity percentage is indicated inside ribbons connecting homologous genes.

3.13. Carbon Storage

The carbon storage inventory includes glycogen biosynthesis *glgABC* as well as the genes for polyhydroxybutyrate biosynthesis (Supplementary Figure S2). The key genes for polyhydroxyalkanoate synthase *phaC* (K03821), as well as acetyl-CoA C-acetyltransferase *phaA* (K00626) and 3-oxoacyl-[acyl-carrier-protein] reductase *phaB* (K00023), were present in the three *Methylocaldum* strains. Furthermore, additional *phaC* homologs were found in the three strains, indicating possible PHB co-polymer biosynthesis. The ability to produce polyhydroxyalkanoates has been recently observed for axenic cultures of *Methylocaldum*, supporting the genetic observations [62]. In addition to genes for polymer biosynthesis, all three strains contain genes encoding pathways of sucrose biosynthesis.

3.14. Pyomelanin/HGA-Melanin Proposed Production

A signature characteristic of *Methylocaldum* strains is the development of light to dark brown-colored colonies/culture [63]. Previous analyses showed that *M. szegediense* O-12 synthesizes a tyrosine-derived melanin-like pigment upon a decrease in growth temperature (at a suboptimal temperature of 42 °C) [64]. Because of the lack of the canonical *melC1* for tyrosinase (K00505) for the production of eumelanin in *Methylocaldum*, here it is proposed that this color corresponds to a type of melanin, known as pyomelanin or HGA-melanin [65]. The three *Methylocaldum* strains have genes encoding the tyrosine degradation pathway via homogentisic acid (HGA), *hppD* (K00457), and *hmgA* (K00451). In this pathway, the HGA intermediate can continue to be reincorporated into central metabolism as fumarate and acetoacetate [66] or accumulated and, through an spontaneous autooxidation process, converted to benzoquinone, which polymerizes to form pyomelanin [67,68] (Figure 7). The gene encoding histidinol-phosphate aminotransferase *hisC2* (K00817), which produces the HGA precursor 4-hydroxyphenylpyruvate, has four CDS assigned to it in *M. marinum* S8, three CDS in *M. szegediense* O-12 and two CDS in Norfolk.

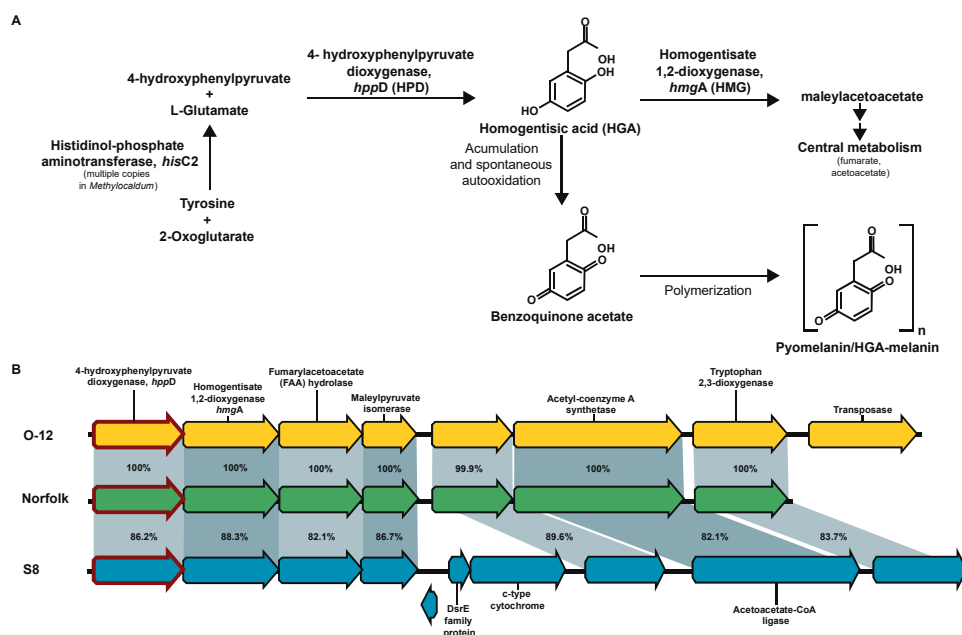


Figure 7. Proposed pyomelanin/HGA-melanin synthesis pathway via accumulation of homogentisic acid (HGA) in *Methylocaldum*. **(A)** Schematic representation of tyrosine degradation pathway having HGA as intermediate synthesized by 4-hydroxyphenylpyruvate dioxygenase (*hppD*, HPD, K00457), which can be degraded via homogentisate 1,2-dioxygenase (*hmgA*, HMG, K00451) to be reincorporated into central metabolism as fumarate and acetoacetate or accumulated and converted to pyomelanin/HGA-melanin via polymerization of the monomer benzoquinone acetate. **(B)** Synteny of gene cluster where the *hppD* gene (red-edge arrows) was present in the three *Methylocaldum* strains. Pairwise identity percentage is indicated inside ribbons connecting homologous genes.

4. Discussion

Here, three genomes of thermophilic/thermotolerant methanotrophs of the genus *Methylocaldum* were analyzed. Most methanotrophs display metabolic plasticity in response to the availability of key nutrients (nitrogen, phosphates) or metals (copper, tungsten,

lanthanides). However, all three members of the *Methylocaldum* genus are superior to other comparable methanotrophs in the number of paralogs for key enzymes as well as the number of metabolic pathways for C₁-carbon conversions and storage. The comparison revealed several key findings regarding their genetic diversity and metabolic potential.

All three strains possess multiple gene clusters for particulate methane monooxygenase (pMMO), suggesting redundancy in methane oxidation capabilities. This redundancy may provide metabolic flexibility, allowing *Methylocaldum* strains to thrive in diverse environmental conditions. The analysis of the genes involved in nitrogen metabolism revealed the presence of nitrogen fixation genes in all three strains, indicating their potential ability to fix atmospheric nitrogen. Additionally, genes involved in nitrate assimilation and nitric oxide reduction are present, suggesting versatility in nitrogen metabolism.

The genome comparison of the three *Methylocaldum* strains revealed insights into their genetic composition and metabolic potential. *M. szegediense* O-12 and Norfolk exhibit similar genomic characteristics, including genome size, GC content, and predicted protein-coding genes. However, *M. marinum* S8 diverges significantly, with a larger genome size and a lower percentage of orthologous genes shared with the *M. szegediense* strains. Phylogenetic analysis based on 16S rRNA sequences and ANI × AC revealed the close relationship between *M. szegediense* O-12 and Norfolk, while *M. marinum* S8 formed a distinct clade. Genome synteny analysis highlighted variations in methane monooxygenase gene clusters among the strains, suggesting potential differences in methane oxidation pathways. Comparative abundance analysis revealed shared and unique functional categories, with genes related to energy production, metabolism, and transport being predominant. In terms of the genetic toolkit for methane oxidation, the *Methylocaldum* strains display redundancy in key enzymes involved in methane metabolism, including multiple copies of *pmoC* genes and methanol dehydrogenases. Additionally, differences in the presence of formate dehydrogenase genes suggest potential variations in formate utilization strategies among the strains. The genomes also encode pathways for single-carbon assimilation, including the ribulose monophosphate (RuMP), serine, and Calvin–Benson–Bassham (CBB) cycles. Nitrogen metabolism genes indicate the capacity for nitrogen fixation and assimilation, with differences in nitrate transport systems among the strains. Carbon storage pathways involve genes for glycogen and polyhydroxybutyrate biosynthesis, suggesting adaptations for carbon storage under varying environmental conditions. Unique functions identified in *M. marinum* S8 include catalase, involved in the detoxification of hydrogen peroxide, and a nearly complete de novo cobalamin biosynthetic pathway, enabling independent synthesis of the essential cofactor. These genomic features may confer advantages for survival and adaptation in specific ecological niches. Furthermore, *M. marinum* S8 harbors additional hydrogenase subunits and genes related to molybdopterin biosynthesis, indicating potential metabolic capabilities for hydrogen metabolism and cofactor biosynthesis. The presence of putative stress response genes and heavy metal-binding proteins suggests adaptation to diverse environmental conditions. O-antigen biosynthesis genes unique to the *M. szegediense* strains offer insights into cell envelope diversity and potential adaptation mechanisms. These differences may contribute to niche specialization and stress resistance in different environments. Lastly, the proposed production of pyomelanin/HGA-melanin in *Methylocaldum* strains suggests a potential mechanism for pigment production and adaptation to suboptimal growth conditions. Genes involved in tyrosine degradation pathways indicate the capacity for melanin synthesis via homogentisic acid accumulation. The significance of preservation of orthologs in organisms inhabiting very different ecological niches remains to be elucidated. However, it is tempting to propose that the evolution of C1 genes was driven by an adaptation to tolerate dramatic changes in temperature.

In summary, genomic analysis of *Methylocaldum* strains reveals extensive genetic diversity and metabolic versatility, with implications for their ecological roles and environmental adaptation strategies. Further research is needed to elucidate the functional significance of these genomic features and their contributions to the ecological success of *Methylocaldum* in diverse habitats.

5. Conclusions

Our research aimed to investigate the genomic characteristics and metabolic potential of well-physiologically characterized *Methylocaldum* strains. Here, we conducted a comparative genomic analysis of three strains: *M. szegediense* O-12 and Norfolk and *M. marinum* S8. Through phylogenetic analysis, genome synteny analysis, and comparative abundance analysis, we identified similarities and differences in their genetic composition and metabolic pathways, particularly focusing on methane oxidation, single-carbon assimilation, nitrogen metabolism, carbon storage, stress response, and pigment production. The results of our study hold broader significance beyond *Methylocaldum* for several reasons. Firstly, the genomic features and metabolic pathways identified in *Methylocaldum* strains provide insights into microbial ecology and biogeochemical cycling. A deeper understanding of the metabolic potential of *Methylocaldum* adds to the current collective knowledge of methanotrophic bacteria, which can be used for biotechnological applications, such as bioconversion and bioenergy production. Additionally, the genetic diversity and metabolic versatility observed in *Methylocaldum* may serve as a model for studying microbial adaptation and evolution in response to changing environmental conditions. Insights gained from studying *Methylocaldum* strains will support future efforts to unveil the ecology and biogeochemical implications of methanotrophic communities in diverse habitats, thus contributing to our broader understanding of microbial ecology and ecosystem functioning.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/microorganisms12030469/s1>, Figure S1: Maximum likelihood tree showing the phylogenetic relationship between the aminoacidic sequences of four different types of formate dehydrogenases major subunits; Figure S2: Gene clusters for *Methylocaldum* carbon storage inventory including glycogen biosynthesis glgABC and polyhydroxybutyrate biosynthesis; Table S1: Table containing Anvi'o v7 outputs for synteny and functional genomic comparison of the *Methylocaldum* genomes.

Author Contributions: N.A.D., D.P. and S.Y.B. contributed to genome sequencing, assembly, and annotation. N.A.D. carried out most of the genomic comparisons presented in this study. M.G.K., J.C.M. and V.N.K. provided conceptual framework for this study. Writing—original draft preparation, N.A.D., M.G.K. and S.Y.B. Writing—review and editing, N.A.D., S.Y.B., D.P., M.G.K., J.C.M. and V.N.K. All authors have read and agreed to the published version of the manuscript.

Funding: *M. szegediense* O-12 genome sequencing and assembly were carried out by the Joint Genome Institute (JGI) under the Office of Science of the U.S. Department of Energy contract DE-AC02-05CH11231. This work was funded by the EnvEast DTP (UKRI grant NE/L002582/1) and Norfolk County Council and by the U.S. Department of Energy under the DOE Office of Science (SC) RENEW contract DE-SC0024289.

Data Availability Statement: Data are contained within the article and Supplementary Materials.

Acknowledgments: We would like to thank Mio Takeuchi from the Biomedical Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Midorigaoka, Ikeda, Osaka, Japan, for providing the genome of *M. marinum* S8 and for her insightful comments on the manuscript. We also thank Alvaro Munoz Plominsky for his bioinformatic support and feedback.

Conflicts of Interest: The authors declare no conflict of interest.

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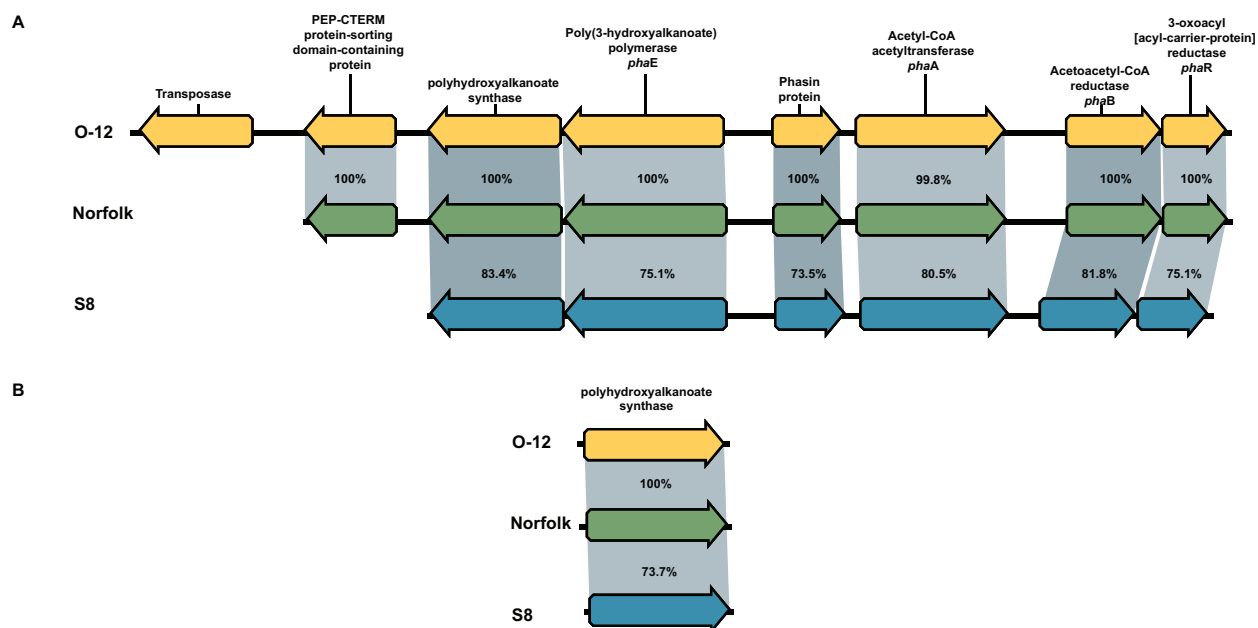
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Supplementary Figures



Supplementary Figure S1 Maximum likelihood tree showing the phylogenetic relationship between the aminoacidic sequences of four different types of formate dehydrogenases major subunits. Formate dehydrogenase K00122 was used as outgroup. Leaf names with the genomes of strains analyzed in this article and the reference sequences to assign different types are in bold. Identifiers for each corresponding gene ID from IMG are at the tip of each leaf, except for Norfolk strain which has NCBI locus tag identifier.



Supplementary Figure S2 Proposed polyhydroxybutyrate biosynthesis pathway. (A) Synteny of gene clusters for PHA/PHB synthesis present in the three *Methylocaldum* strains. (B) Stand-alone polyhydroxyalkanoate synthase found in the three strains. Pairwise identity percentage is indicated inside ribbons connecting homologous genes.

Chapter 3 Integrative Ecology, plant-methanotrophs in arid microcosms

Chapter 3 is coauthored with Chynna Bowman, John McNamara, Daisy Ulloa, Uduak George, Elizabeth R. Waters, and Marina G. Kalyuzhnaya. The dissertation author was the primary researcher and author of this chapter.

3.1 Abstract

Even though the importance of microbial communities associated with plants is recognized in many studies, there is still little knowledge about how specific groups of non-pathogenic bacteria have an impact on the overall health of a plant. Furthermore, even less is known about specific bacteria enhancing the innate capabilities of plants to tolerate a diversity of environmental stresses. Our work takes the first steps towards linking symbiotic associations of microbes to desert plants with global methane cycling. Drylands occupy 37.1% of the Earth's terrestrial surface and are increasingly being converted into agricultural fields. Sustainable and productive use of arid land requires a better understanding of the functional traits of microbes inhabiting native arid environments. To translate the acquired knowledge from studying native arid soil microbiome functioning, Anza-Borrego Desert State Park we performed preliminary studies with the isolated native methanotroph *Methylocaldum* sp. 0917. The addition of methanotrophic bacteria improved plant survival during drought stress. Based on these data we propose that changes in current agricultural practices that enhance the presence and activity of methanotrophic communities can convert crop fields from source to sink of methane. We propose that under environmental conditions of low organic matter and low water input, plants facilitate methane transport and cooperate with methane-consuming microbes to efficiently capture methane evolved in deeper soil layers and atmospheric methane, which is then metabolized to provide supplemental resources for

survival in water-limiting conditions. Our data suggest that the plant-methanotroph associations represent an overlooked sink of atmospheric methane in (semi)arid ecosystems. We show that supplementation with native *Methylocaldum* sp. can improve drought tolerance in crops (e.g. *Sorghum*). Furthermore, our work provides essential evidence that targeted engineering of association between crops and methanotrophs in (semi)arid soils can enhance atmospheric methane sink, ultimately influencing the global climate.

3.2 Introduction

Several anthropogenic activities release methane into the atmosphere, while mostly natural biotic and abiotic factors are responsible for sinking or destroying this greenhouse gas. Methane consumption by native soils represents one of the most prominent methane sinks (Conrad, 2009; Nazaries et al., 2013). On the other hand, agricultural soils often increase methane production in soils, altering the natural equilibrium of methane fluxes on the planet. This negatively impacts the battle against climate change by converting natural methane-sink soils into emitters (Aronson & Helliker, 2010; Aronson, Allison, & Helliker, 2013).

The increase in organic matter through fertilization, and additional water input over agricultural soils, disturbs soil redox balances, inhibiting methane oxidation and thus enhancing GHG emissions (Safriel et al., 2005; Searchinger et al., 2008). In addition, standard agricultural practices (e.g., adding nitrogen fertilizer), induce the release of methane to the atmosphere (Saggar et al., 2008). This does not only occur by diffusion from the anoxic layers, additionally, it is increased by the transport from roots through the plant system and ultimately expelled to the atmosphere. This is known as the chimney effect by plants, coined and described by Joabsson et.

al in 1999 (Joabsson, Christensen, & Wallén, 1999), and characterized in multiple plant species later on (Bhullar, Edwards, & Olde Venterink, 2013; Bringel & Couée, 2015; Milberg et al., 2017; Zhang et al., 2019).

Methanotrophs are methane-consuming microbes, which are the only biotic entities that have been identified as biological methane sinks. They are separated between aerobic and anaerobic ones (Conrad, 2009; Kalyuzhnaya, 2016) based on their dependency on oxygen for the initial methane-oxidation step. Identified aerobic methanotrophs include Alpha- and Gamma-Proteobacteria members and Verrucomicrobia phyla, while anaerobic members belong to the NC10 phylum (Murrell & Jetten, 2009; Semrau, DiSpirito, & Yoon, 2010).

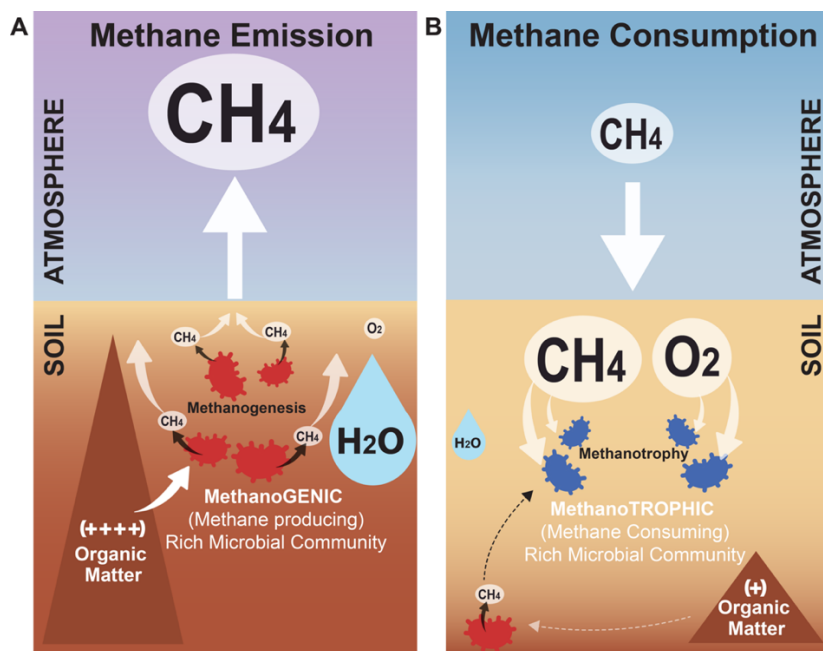


Figure 3.1. Comparison of soil composition and microbial communities as key factors influencing the net flux of methane.

(A) Soils with high organic matter concentrations, and high-water content, tend to be poor in oxygen and harbor communities of methane-producing microorganisms, known as methanogens. (B) Soils with minimum organic matter concentrations, and minimum water content, tend to be well-oxygenated and harbor communities of methane-consuming microorganisms, known as methanotrophs, responsible for the biologic removal of methane from the atmosphere.

Methanotrophs can use methane as a sole source of carbon and energy. Additionally, they can use energy obtained from methane oxidation to fix atmospheric dinitrogen. Moreover, they produce water and CO₂ as by-products and can excrete extracellular polymeric substances (EPS), organic acids, sugars, vitamins, nucleotides, and amino acids (Iguchi et al., 2012; Iguchi, Yurimoto, & Sakai, 2015; Raghoebarsing et al., 2005).

Current research validates and emphasizes the impact of the rhizosphere on plant growth and development, and there is yet an unknown number of new chemical and biochemical interactions involved that have yet to be discovered and their mechanisms understood (Jamil et al., 2022; Korenblum et al., 2020; Nadal & Paszkowski, 2013; O'Banion et al., 2020; Oldroyd, 2013; Schmitz & Harrison, 2014; Singh, Chandra, & Purchase, 2022; Venturi & Keel, 2016). Specifically, little is known about the mutualistic interactions between plants and their methylotrophic microbiomes (Schäfer et al., 2022).

Soil microbial communities and plant microbiomes of both, rhizospheres and phyllospheres, often include methanotrophs, methylotrophs, and other diazotrophic, nitrifying, and denitrifying microbes. Beneficial interactions are well documented for many methylotrophic species, such as plant-growth-promoting rhizobacteria (PGPR), including *Rhizobium*, *Bradyrhizobium*, and pink-pigmented facultative methylotrophic bacteria-phylosymbions (PPMB) (Doronina, Ivanova, & Trotsenko, 2002; Iguchi et al., 2012; Iguchi, Yurimoto, & Sakai, 2015; Raghoebarsing et al., 2005; Schäfer et al., 2022; Zhang et al., 2024)

Plant-methanotroph interactions have been studied mostly in aquatic plants. There, it has been shown that plants support the methane oxidation process by transporting oxygen to their roots, (Iguchi et al., 2012; Iguchi, Yurimoto, & Sakai, 2011, 2015) and in return, the

methanotrophic bacteria produce hormones, such as the auxin indole-3-acetic acid (IAA), suggesting the possibility of symbiotic interactions with plants (Raghoebarsing et al., 2005; Schäfer et al., 2022).

Drought is the second major global disaster of our time, causing natural, and socioeconomic losses annually, with the highest impact on communities sustained primordially by agricultural activities (Rodell & Li, 2023). Understanding the different environmental factors affecting the tolerance of agriculturally significant species has become a priority. One overlooked component until very recently is the microbiome associated with plants, specifically the roots.

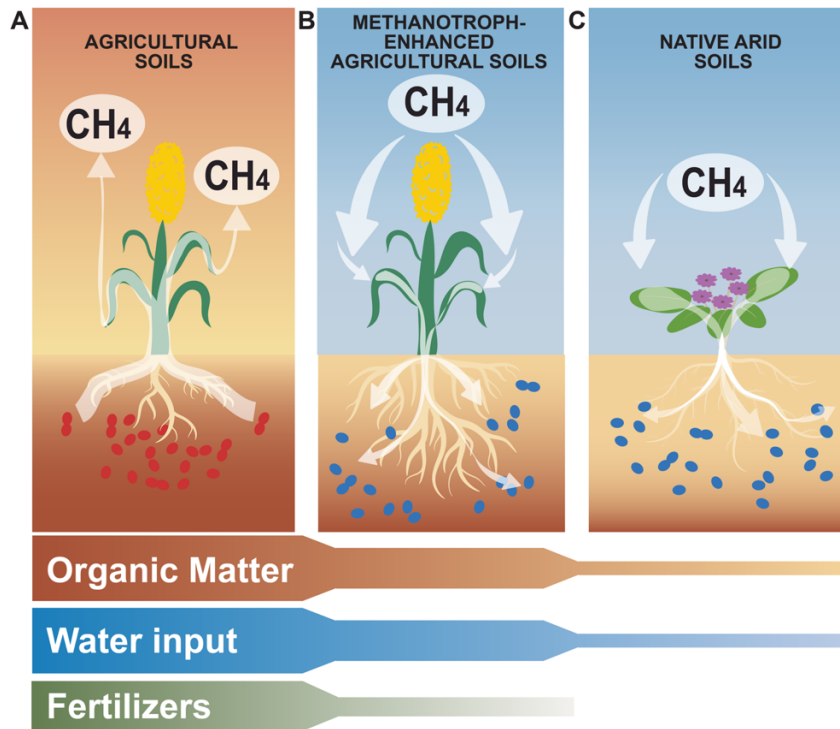


Figure 3.2. Proposed methanotroph-enhanced agricultural soils.

(A) Terrestrial plants can facilitate CH_4 fluxes between the soil and atmosphere through the chimney effect. (B) Proposed agricultural conditions favoring methanotrophic communities by controlling organic matter, and water content of the soil. (C) Under low-organic matter and low-water input conditions, they can transport CH_4 from the atmosphere to the soil where the microorganisms consume them, removing the CH_4 from the atmosphere. This is the proposed *reverse* chimney effect.

Considering the urgency to modulate and modify current agricultural practices targeting a reduction of greenhouse gas emissions while having more resilient crops, this study evaluates the effects of adding methanotrophic bacteria to plants, grown with low organic matter and low-water input, over the methane fluxes. The prediction is it will have the opposite output of the chimney effect. This is because these conditions can favor the methanotrophic communities rather than the methanogenic ones, supporting the proposed *reverse chimney* hypothesis (Chapter 1). The foundational knowledge of this study pursues to contribute to global efforts for removing atmospheric methane while reducing agricultural soil methane emissions it can contribute to slowing climate-change progression (Conrad, 2009; Shindell et al., 2012).

Based on the characteristics of the methanotroph of the genus *Methylocaldum*, beforehand characterized in this dissertation, it was selected as the best candidate for evaluating its effects over the drought-resilient crop *Sorghum bicolor* (Bakari et al., 2023; Jardim et al., 2020). The cultivar BTx623 was chosen because it is a promising bioenergy crop that currently can be grown in semi-arid regions (Deng et al., 2024; Kong et al., 2018). Additionally, there are many *Sorghum* cultivars representing a wide genetic variety (for both cereal and bioenergy purposes), which can be utilized for future studies.

3.3 Materials and Methods

3.3.1 Germination of *Sorghum*

Sorghum bicolor (*S. bicolor*) cultivar BTx623 was used in this study and was gifted to the Waters lab at SDSU from Dr. W. Vermerris of the University of Florida. A single *Sorghum* seed was sown on each 100 mL capacity pot, at approximately 1 cm of depth, in 70g substrate of 1:1 MetroMix (Sun Gro® Horticulture) soil and sand (non-sterile). Additionally, soil-only control pots

were set on the same manner. For experimental purposes, the planting day was considered day 0 (zero). All pots were watered to conserve at least 80% of the moisture for 14 days and were fertilized on day 12 only. All pots were incubated in a growth chamber at 25°C with a diurnal pattern of 16 hours of light with 8 hours of darkness. *S. bicolor* was grown for 14 days before experimental treatment began.

3.3.2 Bacteria Growth

The methanotrophic bacteria *Methylocaldum* sp. 0917 was grown in modified P₀% media (Akberdin et al., 2018), consisting of 1 g L⁻¹ of KNO₃, 0.2 g L⁻¹ of MgSO₄ x 7H₂O, 0.02 g L⁻¹ of CaCl₂ x 2H₂O and 1 mL of 1000x trace solution. After autoclaving (at 121°C for 45 minutes), a sterile phosphate solution (1/20 v/v), containing 5.44 g L⁻¹ of KH₂PO₄ and 5.68 g L⁻¹ Na₂HPO₄ (pH of 6.8), was added. Trace solution (1000x) consisted of 5 g L⁻¹ of Na₂EDTA, 2g L⁻¹ of FeSO₄ x 7 H₂O, 0.3g L⁻¹ of ZnSO₄ x 7 H₂O, 0.03 g L⁻¹ of MnCl₂ x 4 H₂O, 0.2 g L⁻¹ of CoCl₂ x 6 H₂O, 1.2 g L⁻¹ of CuSO₄ x 5 H₂O, 0.3 g L⁻¹ of Na₂O₄W x 2H₂O, 0.05 g L⁻¹ of NiCl₂ x 6 H₂O, 0.05 g L⁻¹ of Na₂MoO₄ x 2 H₂O, and 0.03 g L⁻¹ of H₃BO₃.

Triplicates of batch cultures were maintained in 250 mL borosilicate bottles with rubber stops, by adding 50 ml of P₀% media to provide a 4:1 headspace:medium ratio. Bottles were supplemented with 50 mL of methane (99.9%) by injection through the rubber stop, as carbon and energy source. The inoculum consisted of ~100 mg of *Methylocaldum* sp. 0917 culture streaked from solid media. Cultures were kept in agitation at 200 rpm at 30 °C. Every other day the cultures were opened inside the biosafety cabinet to allow for air equilibrium, then re-capped and 50 mL of methane were injected. Cultures were grown for 10 days, after which the three batches were

combined to be added to the plants. Samples for optical density (OD), and colony-forming units (CFU) were collected to estimate the number of bacteria added.

3.3.3 Addition of Methanotrophs and Methane

On day 14, pots containing plants (42) and no-plant (42) were randomized to either be used as soil control, inoculated with 1 ml at OD 0.3 of *Methylocaldum* sp. 0917, or 1ml of methanotrophic growth media (minimum mineral media) to control for the mineral added, and additionally test if methanotrophs potentially present in the soil/sand mixture could be stimulated. A micropipette was used to inject the culture just above the root crown so microbes would be homogenous across the root system. Additionally, those pots with methanotroph or methanotroph media were supplemented with 5 ml of 5% CH₄ in air (about 0.2 mM), which was injected into the soil at the pot bottom to simulate soil CH₄ flux. Samples were obtained before the inoculations and corresponded to time zero (T0). After day 14, half of the pots were watered, and the other half was not, to mimic extreme drought conditions. On day 19 samples for time one (T1) were collected, and on day 26 the third and last set of samples were collected (T2). A scheme for the experimental design and sampling design can be found in Fig.3.3.

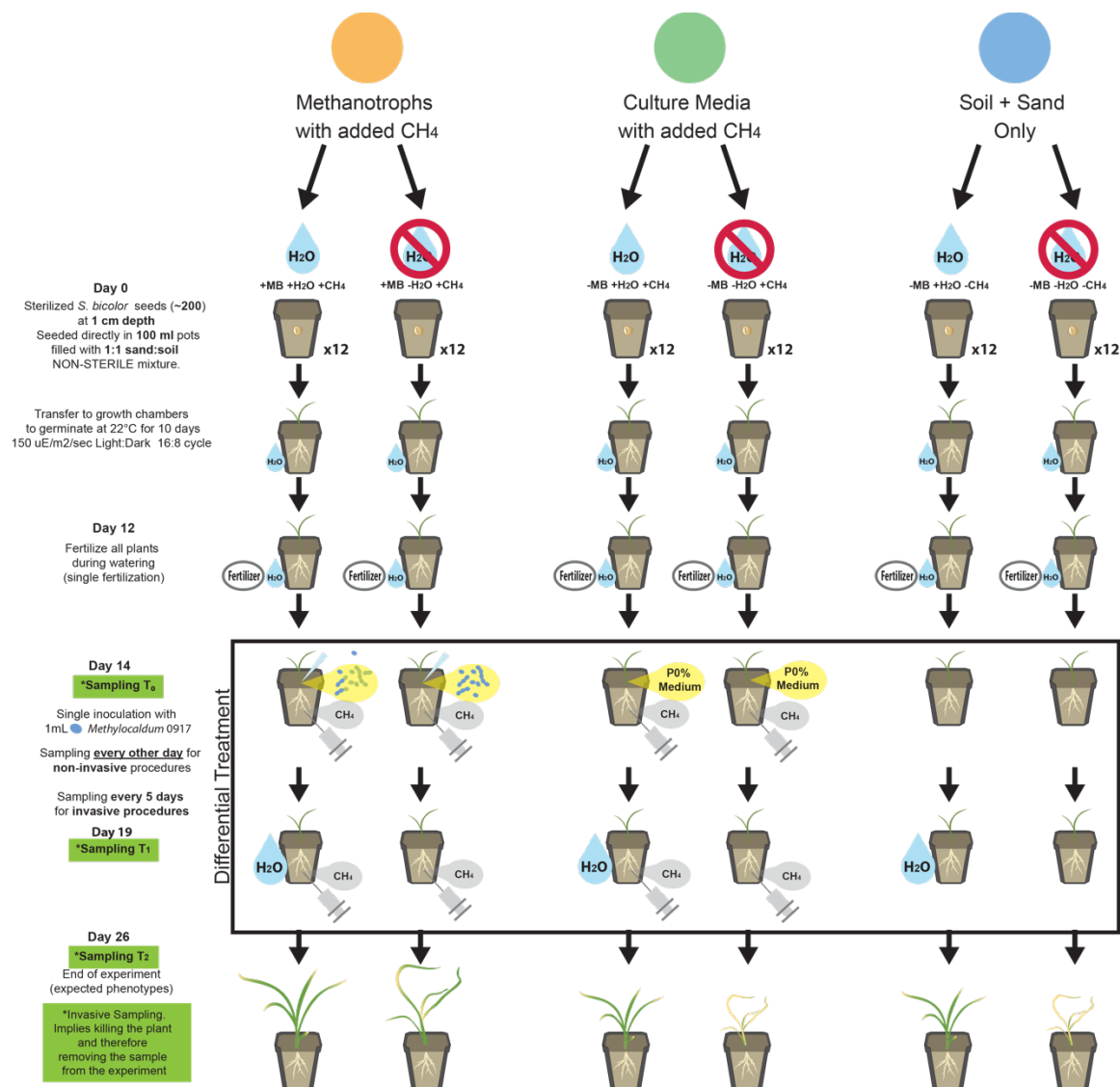


Figure 3.3. Experimental design to evaluate the effect of adding the methanotroph *Methylocaldum* sp. 0917 and methane injections on the crop *Sorghum bicolor* cultivar BTx623.

The experiment included a standard watering strategy and extreme drought simulation.

3.3.4 Capturing of *Sorghum* Vertical Growth

To track shoot growth, plant images were taken every other day for the experimental time, about 15 days. Vertical growth or shoot growth was tracked in *S. bicolor*. Each *S. bicolor* plant to be photographed was placed against a black background and the camera was then positioned 2 feet away. Plant position from the camera and height from the ground were kept constant through the experimental time so that the only independent variable was shoot growth. This was repeated multiple times for all treatments tested.

3.3.5 Chlorophyll fluorescence

Chlorophyll fluorescence was measured as a proxy for plant stress. When photosystem II (PSII) accepts an electron, the chlorophyll goes from a non-excited to an excited state, and then goes back to a non-excited state while emitting light (Maxwell & Johnson, 2000), which can be measured, and utilized as an indicator of the viability of PSII and photosynthetic activity. The instrument utilized corresponded to a pulse-amplitude modulated (PAM) chlorophyll fluorometry, JUNIOR-PAM®. The measurements were taken on the third leaf of each *Sorghum* plant, by gently touching the end of the fiberglass of the fluorometer over the middle part of the blade and giving a pulse of blue light at a wavelength of 460 nm.

3.3.6 *In situ* CH₄, CO₂, H₂O consumption/emission measurements

Four days after the inoculation with the bacteria consortia, six randomly selected plants per each of the treatments were introduced simultaneously in tight-sealed acrylic chamber connected to form a closed system with the Ultra-Portable Greenhouse Gas Analyzer LGR Model 915-0011 to measure consumption/ emission rates of CH₄, CO₂, and H₂O. Measurements were recorded

every second for at least ten minutes for each set of plants, and the corresponding baseline and empty-chamber controls were recorded. These measurements were recorded also on days 6, 8, 10, and 12 post-inoculations. The rates were calculated using the slope from the trend line obtained for each measurement.

3.3.7 Root size and branching analysis

On days 4, 6, 8, 10, and 12 post-inoculation, three plants per treatment were randomly selected to be harvested to measure the root size, and branching pattern. Briefly, each plant root was carefully disentangled, and fully extended over a dark acrylic background. The upper portion of the plant (leaves) was also extended on a white background for better contrast. One picture was obtained for each plant using the same parameters and light source at every sampling point. To describe the root branching system, the box-counting dimension was used. We established that highly branched roots had high fractal dimensions.

3.3.8 Plant area, relative growth rate, and root fractal dimension calculations

To calculate the area of the leaves, the MATLAB® software and the application Color Thresholding together with the image-processing program Fiji were used. From the images, the software obtained the different colors and converted their pixel values to a matrix, which were then analyzed by a program generated at Dr. Uduak George Laboratory at San Diego State University.

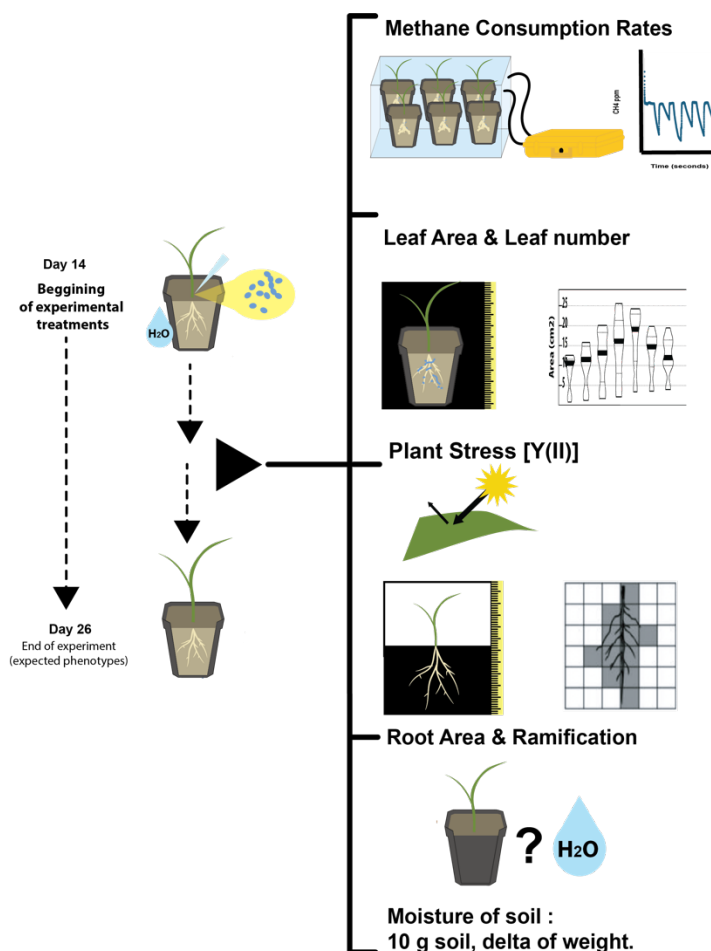


Figure 3.4. Sampling strategy.

Includes non-invasive (methane flux, leaf area, and plant stress), and invasive sample collection (root area and ramification and soil moisture).

3.4 Results and Discussion

3.4.1 Methane Consumption Rates

Regarding the set of watered plants at T1, 19 days after planting all *S. bicolor* seeds, the plants that were inoculated with methanotrophs consumed more methane (0.039 ppm/h $\pm$ 0.012) compared to both the plants with no amendment (0.023 ppm/h $\pm$ 0.004) and those that had only been inoculated with sterile culture media (0.019 ppm/h $\pm$ 0.006; Fig. 3.5 A). Under drought conditions, both plants that had been inoculated with methanotrophs (0.026 ppm/h $\pm$ 0.001) and

sterile culture media (0.029 ppm/h SD $\pm$ 0.004) were found to consume more methane than the plants with no amendment (0.021 nmol/h $\pm$ 0.002) and this difference was statistically significant (p -value <0.05 ; Fig. 3.5 A). Thus, the addition of *Methylocaldum* sp. 0917 and the P₀% culture media were shown to increase the methane-consuming rates of *S. bicolor*, 5 days after their amendment compared to the untreated plants both when they were watered or exposed to drought (Fig. 3.5 A). However, this pattern was not observed at T2, 26 days after planting and after plants had been exposed to extreme drought for 12 days (Fig. 3.5 A). One explanation for the loss of methane-consuming capacities at T2 is the extended time (12 days) since the amendment. After such period the effect of both the addition of *Methylocaldum* sp. 0917 and P₀% culture media likely diluted and whichever methanotrophs that were favored by these amendments could no longer maintain the effect observed at T1 (i.e., 5 days after their amendment). The loss of enhanced methane-consuming capacities of pots with *S. bicolor* at T2 might be due to potential changes in the microbial community favoring microbes that can better utilize the plant exudates instead of atmospheric methane. The amendment of pots without plants with either *Methylocaldum* sp. 0917 and P₀% culture media did not statistically increase their methane consumption rates when exposed to either drought or watered conditions at T1 nor T2 (Fig. 3.5 B).

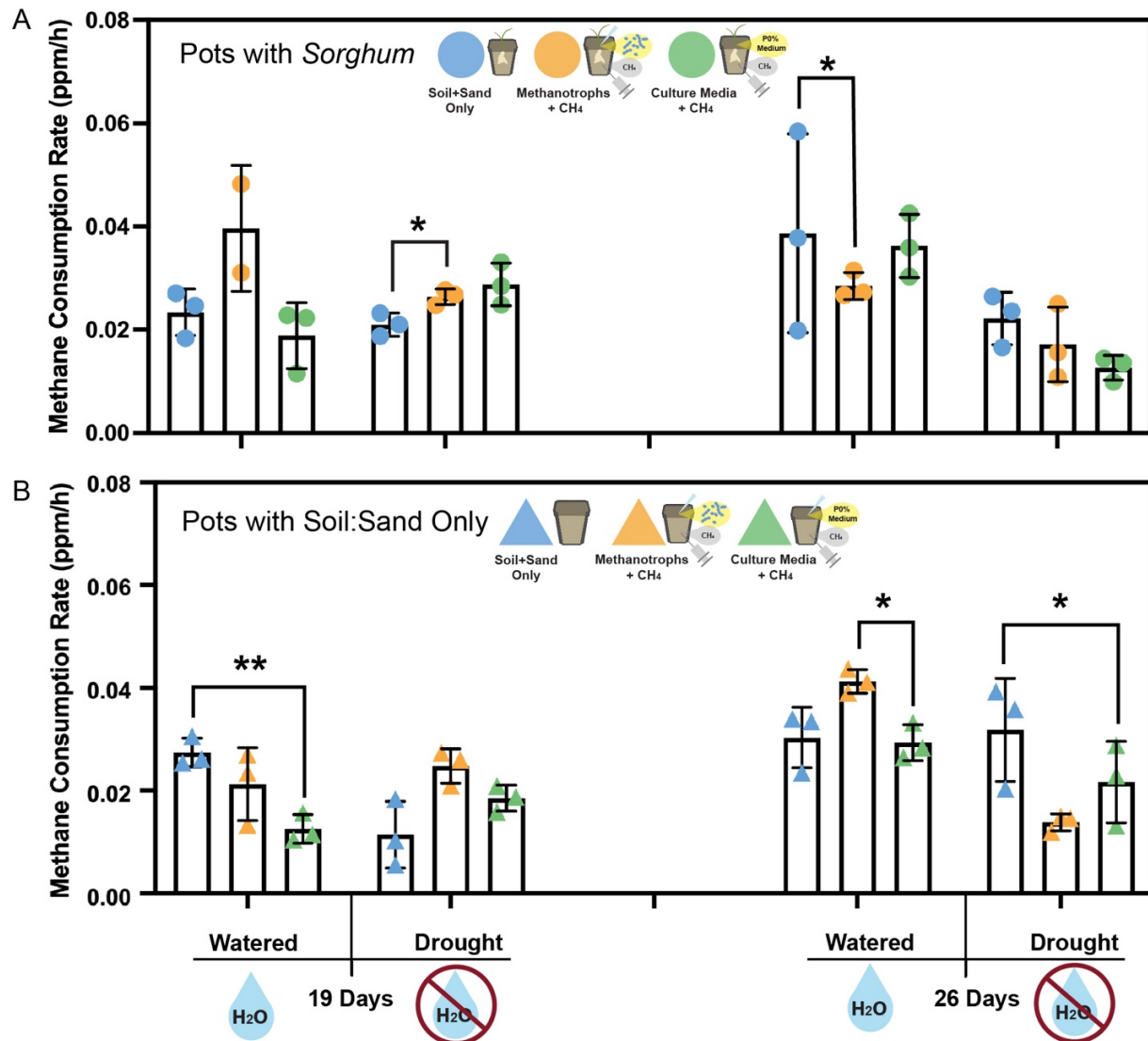


Figure 3.5. Methane consumption rates on days 19 and 26 of the experiment. (A) Pots with *Sorghum* plants. (B) Pots without plants.

3.4.2 Soil Moisture

As expected, for both T1 and T2, the soil of pots that were exposed to drought had half the water content when compared to the pots that had been watered (Fig. 3.6). The differences in moisture content within each treatment (i.e., watered or exposed to drought) and their amendments was found to be statistically significant only for the watered pots with plants that had no amendments at T2 (Fig. 3.6 A).

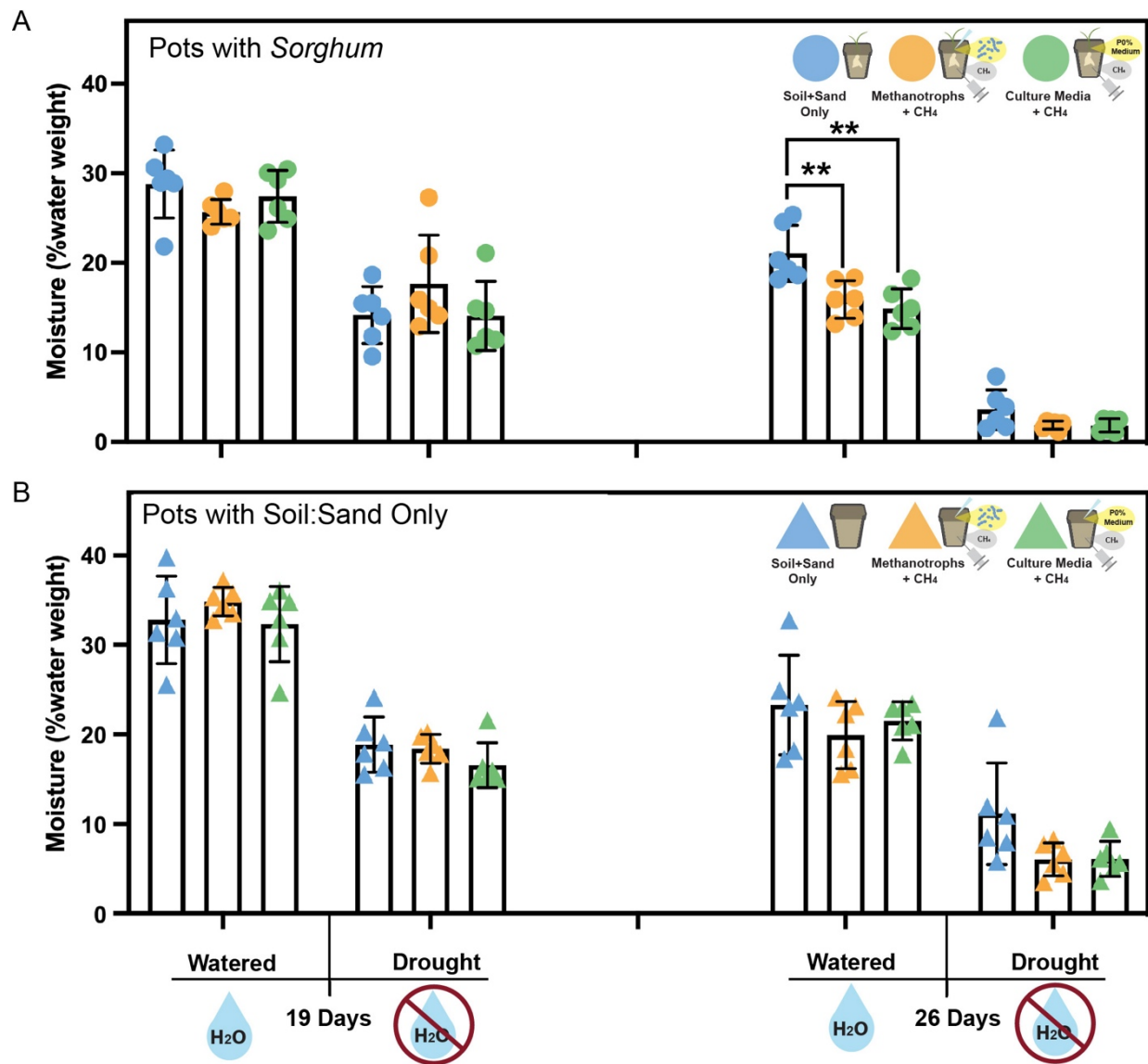


Figure 3.6. Soil moisture as a percentage of water weight on days 19 and 26 of the experiment. (A) Pots with *Sorghum* plants. (B) Pots without plants.

3.4.2 Soil Moisture

The moisture content within groups does not exhibit significant differences after 19 days. However, non-inoculated plant pots exhibit slightly higher moisture after 26 days. Only a tendency of the control group during drought on day 26 to have a slightly higher water content in the soil. Because the moisture content within groups does not exhibit differences it is possible to state that

the differences observed in consumption rates can result from the microbial community and the physiological plant status (Fig. 3.6).

3.4.3 Relative Plant Growth and Stress

Although the differences in the growth of plants were not found to be statistically significant under all treatments (i.e., watered or exposed to drought) and their amendments, the largest *S. bicolor* plants in this study were observed among those amended with *Methylocaldum* sp. 0917 in P₀% culture media at T1 both when watered and when exposed to drought (Fig. 3.7).

Regarding the stress state of the plants, determined by the quantum yield of the photosystem II in their leaves, the plants that were watered presented low-stress levels (i.e., high quantum yield values) at both T1 and T2 (Fig. 3.8). As expected, overall stress levels were higher when plants were exposed to drought (i.e., lower quantum yield values). Notably, in T1 this stress was overcome when plants were amended with *Methylocaldum* sp. 0917 in P₀% culture media or the sterile culture media by itself (Fig. 3.8). However, this protective effect was not observed at T2 among plants exposed to drought (Fig. 3.8). The detection of higher stress levels in the amended plants at T2 is likely due to the shift of the microbial communities present at T1 for populations that do not offer the same protective effect as those favored by the amendments.

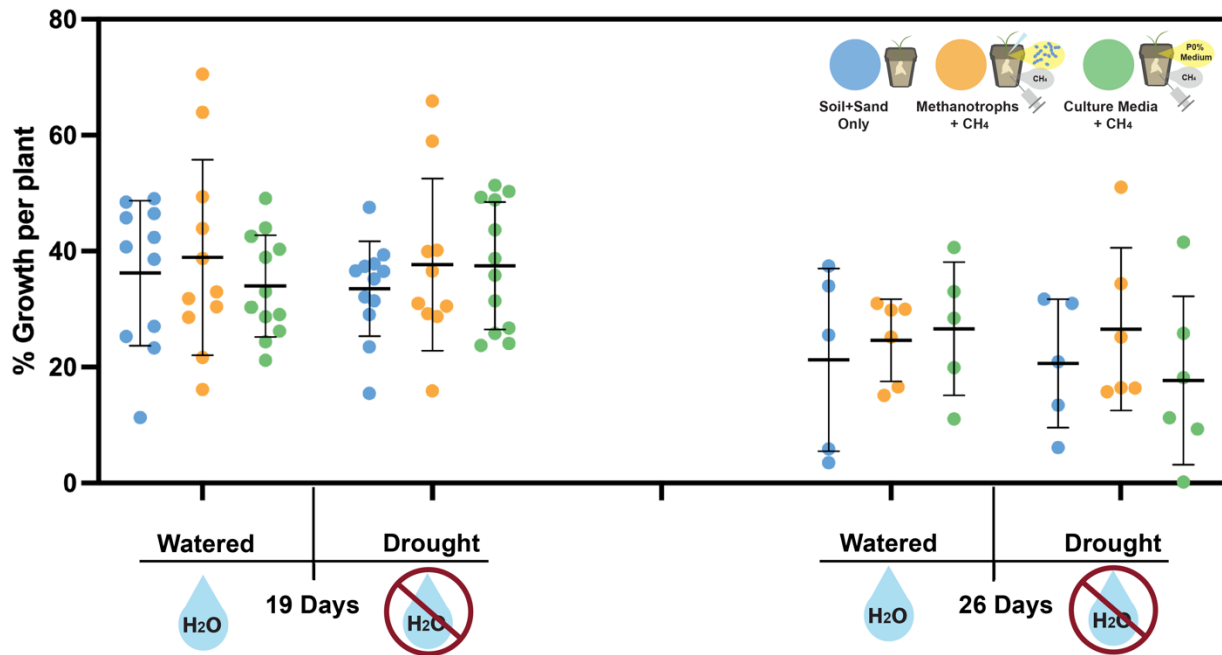


Figure 3.7. Percentage of growth for each plant over time on days 19 and 26 of the experiment based on their leaves' growth.

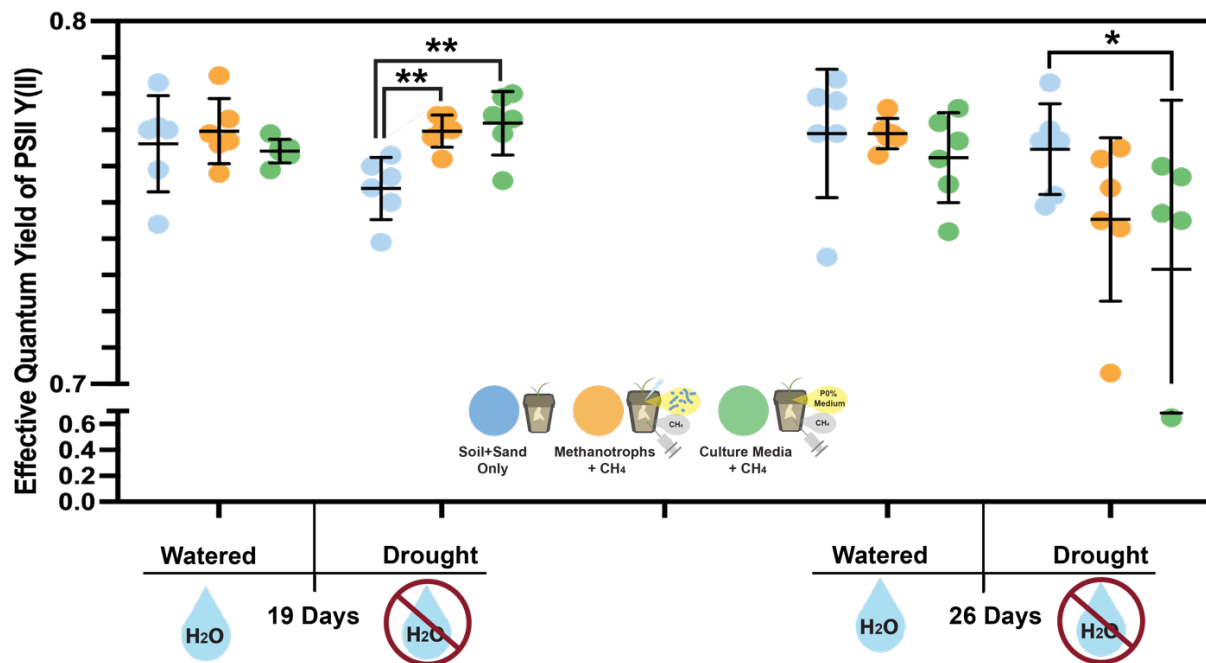


Figure 3.8. Plant stress is inferred through the effective quantum yield of photosystem II. Higher values are indicative of less stress.

3.4.5 Fractal Dimension and Area of the Roots

The amendments with *Methylocaldum* sp. 0917 in P₀% culture media or the sterile culture media by itself did not statistically affect either the fractal index (Fig. 3.9) nor the root area (Fig. 3.10) of *S. bicolor* neither at T1 nor T2. Since *Methylocaldum* sp. 0917 was isolated from a soil sample from the rhizosphere of the desertic *Encelia farinosa* (see Chapter 1, Table S 1.2) it was hypothesized it might have an effect over root development. However, future studies should consider inoculating the seeds of the plant of interest before their planting to better assess if *Methylocaldum* sp. 0917 has any impact on root development.

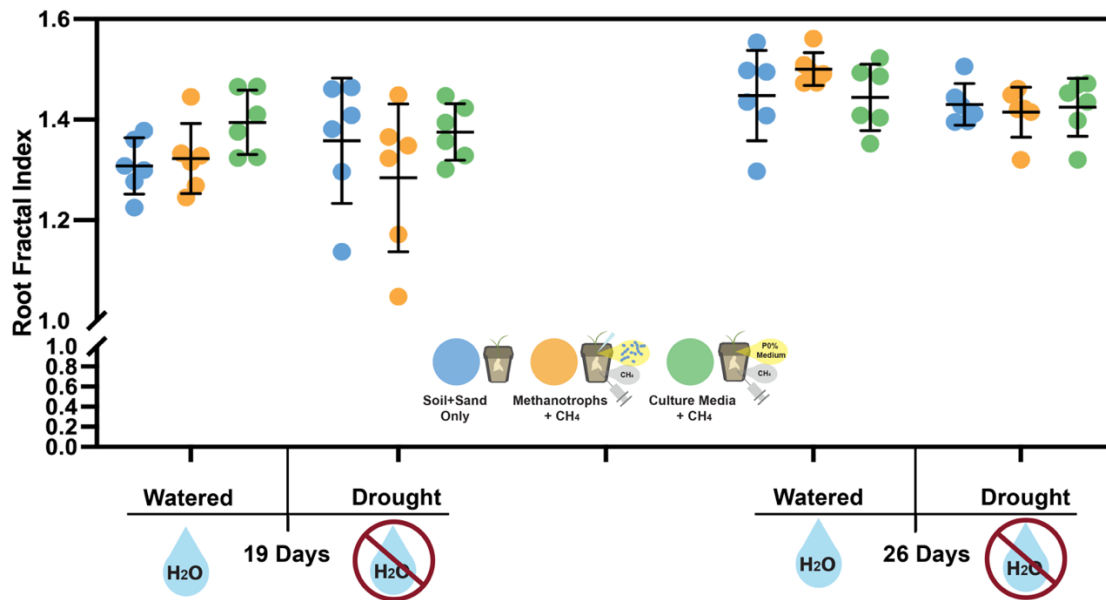


Figure 3.9. Root fractal index of *Sorghum*.

The length and number of ramifications of the roots are expressed as a fractal index. Higher fractal index the root was larger and more ramified.

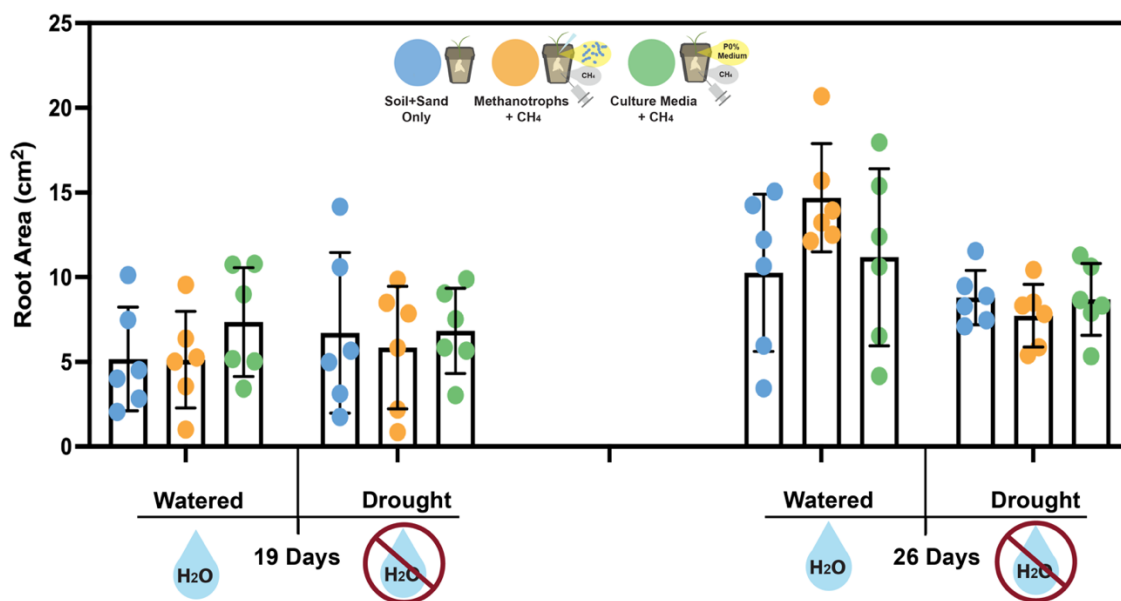


Figure 3.10. Root area of *Sorghum bicolor* quantified total surface in 2D.

3.5 Conclusions

This study showed that, even at the microcosm scale, the inoculation of *S. bicolor* with *Methylocaldum* sp. 0917 in P₀% culture media can increase the methane consumption rate of these small ecosystems (i.e., at T1, 5 days after the amendments; Fig. 3.5 A). Plants might be facilitating methane consumption of methanotrophs, transporting methane and oxygen to the different soil layers, and obtaining additional plant-growth molecules, increasing their growth rates. Moreover, the addition of methanotrophs reduced the stress of the plants when exposed to drought (i.e., at T1 5 days after the amendments; Fig. 3.8). Both of these effects were not observed at T2, but since this time point was 12 days after the amendments, and corresponded to extreme drought conditions, it is likely that the microbial communities shifted compared to those present at T1.

Although these results provide the first insight into which the reverse chimney effect can be stimulated in *Sorghum*, new experiments can provide more understanding of the mechanisms

of methane transport and consumption by the methanotrophic community. Future experiments should include a controlled amount and composition of organic matter content in the soil, remove the addition of fertilizers, and track changes in the microbial community under and without the influence of plant exudates. Also, as it is known for plant research, it is crucial to increase the power of the experiment, by increasing the number of plants utilized and repeating the experiments to provide true biological replicates. Additionally, the results indicate that the positive effects of the amendment with *Methylocaldum* sp. 0917 would require either additional inoculations or developing better strategies to establish stronger recruitment of the bacteria by the plant (e.g. soaking seed into *Methylocaldum* culture). Future investigations should focus on implementing these discoveries in the field, and at the same time delve into the molecular mechanisms governing this interaction.

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