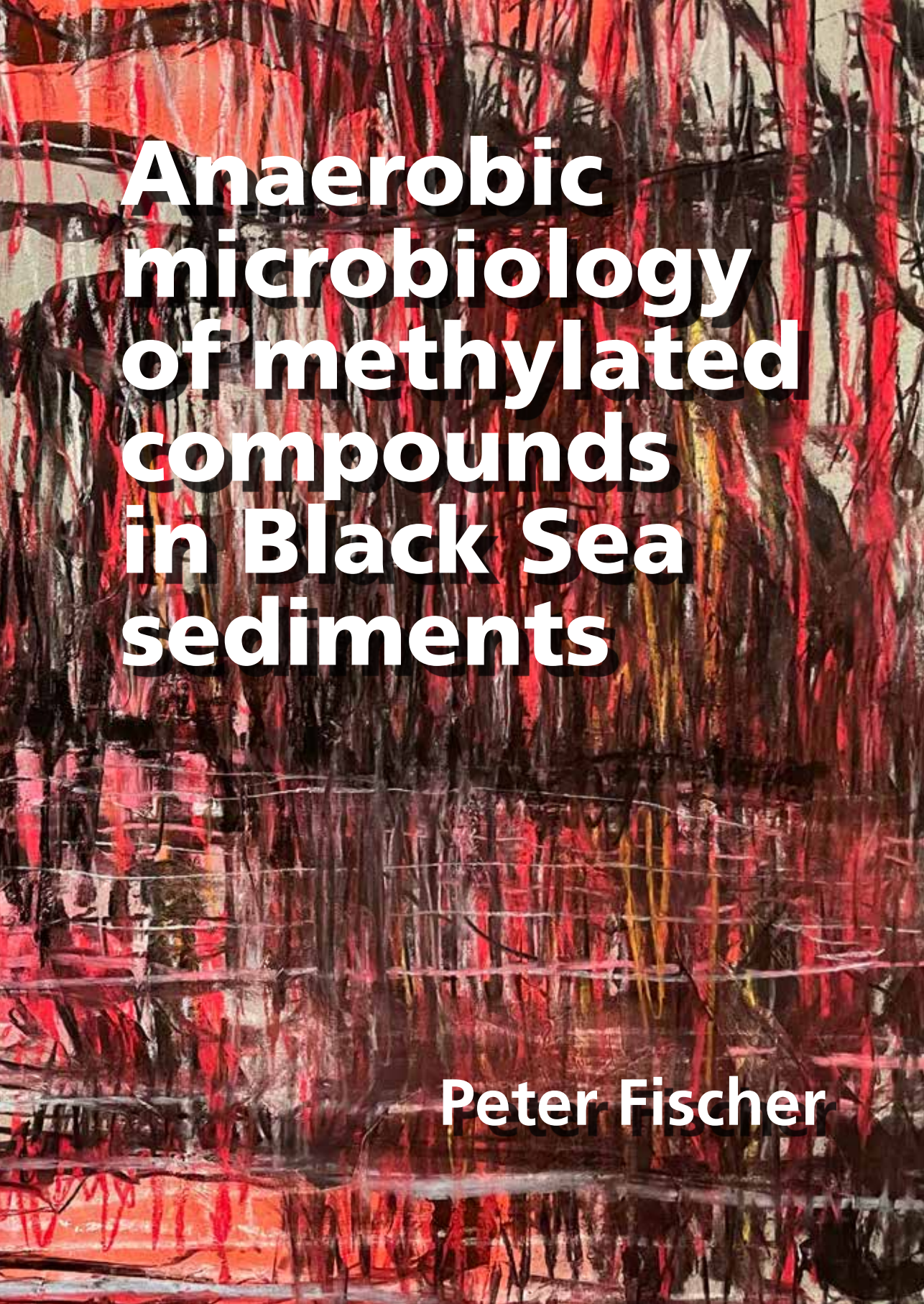

Anaerobic microbiology of methylated compounds in Black Sea sediments

Peter Fischer

Propositions

1. Microbial utilization of methylated compounds in marine sediments is more complex than previously assumed. (this thesis)

2. Methylated quaternary amines are an underestimated source for microbial metabolism. (this thesis)

3. Without a sufficient knowledge of philosophy, the pursuit of science is at best pointless.

4. Scientific progress is slowed down by forcing scientists to apply for grants.

5. Social media algorithms need to be regulated by democratic institutions to retain an open marketplace of ideas.

6. An economic system based on infinite growth in a finite system is an oxymoron.

Propositions belonging to the thesis, entitled

Anaerobic microbiology of methylated compounds in Black Sea sediments

Peter Fischer
Wageningen, 12 May 2023

Anaerobic microbiology of methylated compounds in Black Sea sediments

Peter Fischer

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Peter Fischer

Thesis

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In 't diepste willen van mijn hart,
moest ik mij vaak vergissen

P.J. Troelstra

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General introduction & Thesis outline

1.1 Marine systems

The majority of the world's surface consists of water. Approximately 65% of the surface of our planet consists of oceans. It is therefore no surprise that these marine systems play an important role in the global cycling of all the major elements that constitute life: carbon, sulfur, nitrogen, phosphorous, hydrogen and oxygen. In contrast to surface terrestrial ecosystems, where 95% of all biomass is found in plants and less than 2% is found in microorganisms, over two thirds of the biomass in marine ecosystems is found in unicellular organisms (1). As such, microorganisms play an important role in all constituents of marine ecosystems, from the surface waters down to the sediments on top the of which marine waters rest. Marine sediments are rich in microscopic life, with estimates ranging from 2.9×10^{29} to 5.93×10^{29} microbial cells in sediments worldwide, representing 0.18-3.6% of the total global biomass (2, 3). Microbial ecosystems in marine sediments contribute to the decomposition and degradation of dead organic material, deposited from the water column as flora and fauna die off (4). While only little available dead organic material reaches the sediment, this so-called 'necromass' can drive microbial turnover in sediments for hundreds of thousands of years (5). Earlier reports suggested that only top-layer, relatively young sediments constitute a significant active microbial population, as necromass is quickly depleted. In this scenario, microbes in underlying sediments would be dormant or in endospore form (6). However, measurements on deep sub-surface populations have shown active microbial life (7, 8). Turnover times of these microorganisms was much slower than observed in richer environments, with doubling times measured in geological timescales of hundreds to thousands of years, utilizing highly recalcitrant allochthonous necromass, deposited millions of years prior (9). Regardless, microbial activity in the upper sediments is orders of magnitude greater than in lower sediments (2).

An important part of the available energy pool in marine sediments comes from **methylated compounds**. Although different definitions are possible and valid, in this thesis, methylated compounds are defined as any small organic compound containing a methyl group bound to a non-carbon atom (10) . These compounds serve an important role in marine organisms, specifically in the form of zwitterionic osmoprotectants, namely compounds meant to protect against the salinity of seawater or changes in salinity. The most studied osmoprotectant is glycine betaine, a trimethylated glycine amino acid. This compound was first isolated from the common sugar beet, leading to its common name betaine, from the Linnean species name for beetroot, *Beta vulgaris*. Glycine betaine and other similar methylated compounds such as dimethylsulfoniopropionate, β-alanine betaine or choline are synthesized by most

marine organisms to maintain the low sodium environment needed within cells against the high sodium concentrations of their surrounding environment (11, 12). By synthesizing compatible solutes like glycine betaine, sodium ions cannot enter the cells through osmosis due to their similar charge (13). While large amounts of energy are required to continually synthesize these compounds, this "low salt-in" strategy is still preferred over the "high salt-in" strategy of active import of potassium ions to counteract sodium influx at the relatively moderate salinity of sea water, due to the requirement of a high acid intracellular environment for potassium influx to be effective, and the subsequent proteome adaptations needed to deal with this acidity (13, 14). As a result, tremendous amounts of methylated compounds are produced by organisms in marine environments. In response to fluctuating osmotic pressures or through cell lysis, these compounds are then released to the environment, where they are quickly taken up by other community members for use as osmolyte, but they are also readily available as a metabolic substrate for many organisms (15). During breakdown of these osmolytes, smaller methylated compounds are produced, for example trimethylamine as a product of glycine betaine fermentation. Additionally, major marine methylated compounds that can be produced through breakdown of osmolytes or other larger organic methylated compounds are methanol, dimethylamine, monomethylamine and dimethylsulfide (16–18). These methylated compounds are not used by the fermentative microorganisms themselves, but are released in the environment where they can be further metabolized (19). All of these compounds are produced and utilized by microorganisms in the presence of oxygen, but are also an important driver in anoxic environments. The focus of this thesis is on the anoxic conversion of methylated compounds, with sulfate or carbon dioxide as terminal electron acceptors.

1.2 Marine sediment stratification

In most marine waters, there is a continuous mixing of the water between the complete length of the water column, allowing oxygen diffusion from the atmosphere throughout the entire body of water. In the sediments, much less mixing can occur and as such, depending on a multitude of factors including distance to the shoreline, proximity to continental plate boundaries, water column depth and presence of fauna, oxygen in the sediment is rapidly depleted through aerobic respiration, ranging from the millimeter to meter scale in sediment depth before sediments become anoxic (20). While oxygen is the most commonly known driver of respiration to obtain energy, it is by far not the only one. Any two molecules that differ in potential energy can theoretically react with each other in so-called redox reactions, where electrons are exchanged between a compound high in potential energy (a 'reduced'

molecule), donating electrons, and one low in energy (an 'oxidized' molecule), receiving these electrons. Redox reactions release energy, which can be stored chemically by microorganisms in the form of ATP, the universal energy carrier of life. The theoretical amount of energy that can be obtained through the reduction of common electron acceptors other than oxygen, for example nitrate, sulfate or carbon dioxide, is generally lower, and as such, occur only in environments where all oxygen is depleted.

Once oxygen is depleted in marine sediments, sulfate becomes the dominant electron acceptor for microbial respiration. While compounds such as nitrate or ferric iron are more energetically favorable electron acceptors, concentrations of sulfate are much higher in marine sediments, measured in the millimolar scale, as opposed to nanomolar scale for nitrate and ferric iron (21–23). Thus, the upper layers of anoxic sediments are referred to as the sulfate reduction zone. In this zone, sulfate reduction is the major microbial metabolism, which is carried out by ***sulfate-reducing microorganisms*** (SRM). SRM comprise microorganisms throughout the prokaryotic tree of life, both bacterial and archaeal (24). Best described, however, are SRM from the phylum *Desulfobacterota*, a separate phylum previously known as an order within *Deltaproteobacteria* (25). These micro-organisms can reduce sulfate using a broad range of organic and inorganic electron donors, but usually persist on the fermentation products of heterotrophic organisms in the sediment. An estimated 12 to 29% of all marine carbon remineralization is through sulfate reduction, emphasizing the crucial role this metabolic group plays in the carbon and sulfur cycles (26). Dissimilatory sulfate reduction is mediated in multiple steps. Sulfate must first be activated in an ATP-dependent reaction to adenosine 5' phosphosulfate, after which a reduction to sulfite occurs. Finally, sulfite is reduced to sulfide (24). The key proteins mediating sulfate reduction are dissimilatory sulfite reductases, which are encoded by the genes *dsrA*, *dsrB*, *dsrC* and *dsrD*. DsrABC form the reduction complex, whereas DsrD is an allosteric activator of the DsrABC complex (27). Due to the commonality of *dsrAB* and *dsrD* in all known SRM, they are commonly used as marker genes to detect sulfate reduction in anoxic environments (28).

As sulfate is depleted in the anoxic marine sediment, the major microbial form of respiration becomes methanogenesis and thus, this area is referred to as the methanogenesis zone. Methanogenesis is the biogenic production of methane by methanogenic archaea. Methane is an important driver of climate change, with an estimated 36 times greater potential greenhouse effect than CO_2 (29). Methanogenic archaea, also called ***methanogens***, have so far been associated within the phylum *Euryar-*

chaeota, with all the cultured methanogens belonging to this phylum. However, several metagenomes recovered genomes belonging to other *Candidatus* phyla, e.g. Nezhaarchaeota, Korarchaeota and Verstratearcheota, harbor the key methanogenesis gene cluster coding for methyl-coenzyme M reductase (mcr), indicating that methanogenesis might be more widespread phylogenetically (30–32). Even in the light of this expanded diversity, thus far, methanogens have only been described within Archaea (33). Methanogens classically utilize a narrow range of compounds for their metabolism, and their pathways are divided between acetoclastic (utilizing acetate), hydrogenotrophic (utilizing hydrogen) and methylotrophic (utilizing methylated compounds) methanogenesis (34, 35). Recently these three pathways have been expanded to methanogenesis originating from alkanes and methoxylated compounds, yet the pool of suitable substrates remains restricted (36, 37). The key protein in all three methanogenic pathways is Mcr, which catalyzes the final step forming methane, and as such its subunit A coding gene *mcrA* is commonly used as a marker gene to detect methanogens in environmental studies (35).

Bridging the sulfate reduction and methanogenesis zones is the sulfate-methane transition zone (SMTZ). In this zone, methane produced by methanogens in lower sediments appears in sediments containing the last remnants of sulfate. Here, a syntrophic consortium of methane-oxidizing archaea and SRM are capable of oxidizing methane by coupling it to sulfate reduction, thereby mitigating methane emissions to the atmosphere. These SRM typically employ a reversal of the methanogenesis pathway (38, 39). Figure 1 displays a schematic generalized overview of redox profile in marine sediments.

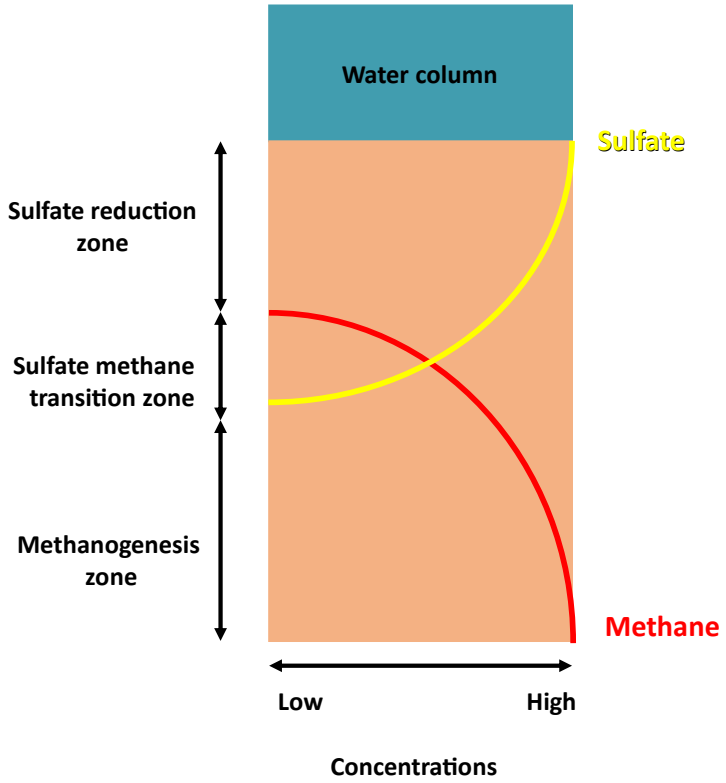

Figure 1: Schematic overview of marine sediments. As sulfate (in yellow) is depleted, methanogenesis takes place with the consequent rise in methane (in red). In the sulfate-methane transition zone (SMTZ), methane is oxidized by a consortium of sulfate-reducing microorganisms and methane-oxidizing archaea.

1.3 Methylated compounds in anoxic sediments

Methanogens and SRM share many common substrates, such as hydrogen/CO_2 or acetate. However, reduction of sulfate offers a higher theoretical energy yield and as such, these two metabolisms are generally thought of as mutually exclusive. Consequentially, SRM rapidly outcompete methanogens for their common substrates, relegating methanogens to sediments where sulfate is depleted (18, 39, 40). An exception to this was long believed to happen with the conversion of methylated compounds.

For a long time, methylated compounds were thought to be non-competitive substrates for methanogens, allowing them to co-exist with SRM in the sulfate reduction zone (41). The presence of methylotrophic methanogens in sulfate-rich environments is evidenced through various ways. Many marine methylotrophic

methanogens from sulfate-rich anoxic sediments have been isolated and community analysis based on presence of the "microbial fingerprint" gene, 16S rRNA, showed the presence of key methanogenic species in sulfate-rich sediments. Additionally, active production of methane from these sediments has been measured in in situ studies. These findings all corroborate the view that methylotrophic methanogens are active in sediments where other methanogens are outcompeted by SRM (42–44). However, the claim that these compounds are indeed fully non-competitive is dubious at best. Already, a wealth of evidence indirectly suggests sulfate-reducing microorganisms and other microorganisms are capable of utilizing methylated compounds in their respiratory rather than fermentative metabolism. For example, enzymes for methylotrophy were found to be abundant in the proteome of the deep sub-surface sulfate reducer *Desulfofundulus kuznetsovii* [1] when growing on methanol (45). Carbon balances on the conversion rate of methylated compound utilization in anoxic sediments do not fully close when accounting for only methanogenesis (46). Furthermore, the leakage of hydrogen by methylotrophic methanogens has been implied to have an influence on the overall thermodynamics of this system (47). Additionally, methanol is routinely added to waste water treatment facilities as an extra electron donor to increase sulfate reduction rates, indicating the known use of methanol for sulfate reduction outside marine environments (48). Therefore, the ecology behind the utilization of these small methylated compounds is likely more complex than has been the long-held assumption.

The list of methylated compounds that can be directly demethylated is ever increasing. This has been described mostly in acetogenic bacteria in the human gut, especially *Eubacterium limosum*, which is capable of directly demethylating proline betaine, carnitine and γ-butyrobetaine and demethylation of glycine betaine and choline has been described in acetogens as well (49–51). Methanogens likewise have been shown to directly utilize organic methylated compounds, with glycine betaine, choline and N,N-dimethylethanolamine serving as substrate for methylotrophic methanogenesis (52–54). Thus far, the key proteins facilitating demethylation in all described organic methylated compounds are encoded by homologs of the same family of genes, the MttB-superfamily protein coding genes. These genes are all related to the gene encoding the key protein in trimethylamine demethylation, trimethylamine methyltransferase, yet do not encode for the characteristic amino acid pyrrolysine that is required for the proper functioning of this protein (54, 55).

1 Desulfofundulus kuznetsovii *is a recent reclassification of* Desulfotomaculum kuznetsovii *(194)*

1.4 Pathways for methylated compound utilization

Sulfate-reducing microorganisms and methanogens are considered to be mutually exclusive metabolisms, yet share many commonalities. These metabolisms often assimilate inorganic carbon in their metabolism through the same pathway: the reductive acetyl-CoA pathway, also known as the Wood-Ljungdahl pathway, named in honor of its discoverers Harland G. Wood and Lars G. Ljungdahl. The Wood-Ljungdahl pathway is considered to be one of the earliest carbon assimilation pathways that has emerged, and may have been utilized by our last universally shared common ancestor (56). This pathway consists of two branches, each of which reduces one molecule of CO_2: the methyl- and carbonyl branch. While the carbonyl branch is a straightforward, one step reduction of CO_2 to CO, the methyl branch consists of five intermediaries where CO_2 is stepwise reduced to a methyl group. The carrier molecule for almost every step in the methyl branch is tetrahydrofolate (THF) in bacteria, and methanopterin (MPT) in archaea. The obtained CO and methyl groups are then connected to co-enzyme A, to form acetyl-CoA. Acetyl-CoA is a central molecule for biosynthesis of many organic molecules microorganisms need to grow and replicate (figure 2). Thus, acetyl-CoA can be incorporated in the biomass of the organism, but it can also be oxidized to acetate, to yield ATP. Rather than reduction from CO_2, methyl-THF or methyl-H_4MPT can be obtained from methylated compounds by certain microorganisms, a process called methylotrophy. To do this, methylotrophs use a class of enzymes called **methyltransferases.** Due to this shared reliance of methanogens and sulfate-reducing microorganisms on the Wood-Ljungdahl pathway and the intricate links between this pathway and methylated compounds, a clearer overview of what happens with methylated compounds in an environment where they all occur is needed.

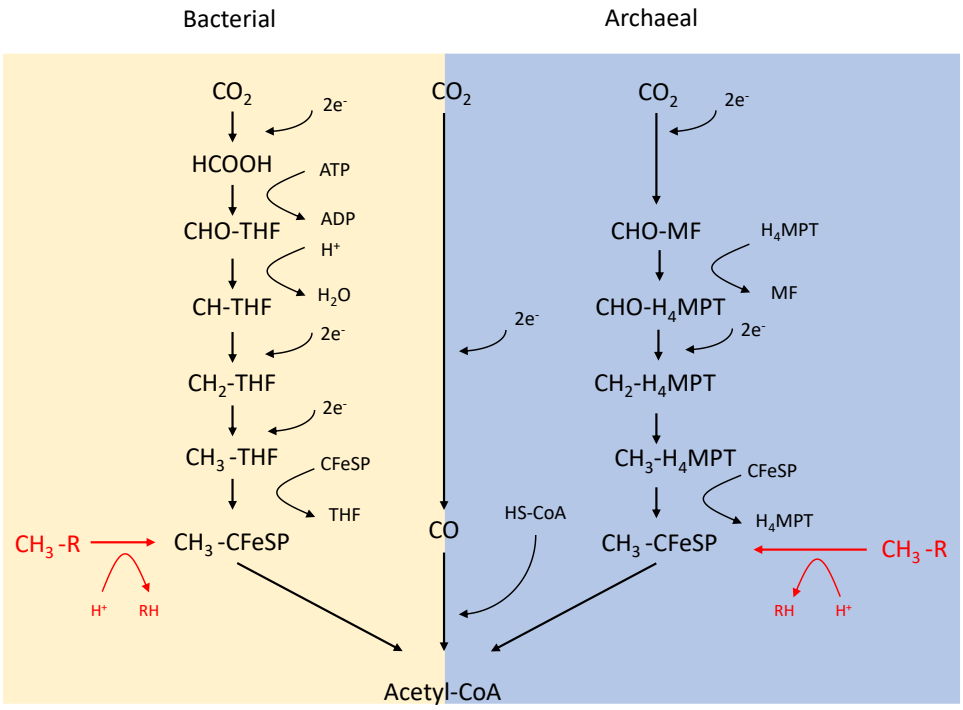

Figure 2: Wood-Ljungdahl pathway, also known as the reductive acetyl-CoA pathway (generalized) for bacteria (left, yellow box) and archaea (right, blue box). In red the alternative methyl-branch utilizing methylated compounds. Abbreviations: CO_2: carbon dioxide; CO: carbon monoxide; HCOOH: formate; CHO: formyl; CH: methenyl; CH_2: methylene; CH_3: methyl; THF: tetrahydrofolate; CFeSP: corrinoid iron-sulfur protein; MF: methanofuran; H_4MPT: tetrahydromethanopterin; CoA: co-enzyme A; CH_3R: methylated compound.

1.5 Knowledge gaps and introduction to the Black Sea

There are still many unknowns regarding the fate of methylated compounds in marine sediments. While it is clear that methanogenesis is not the only possible outcome for these compounds, there is a lack of data on the extent to which these alternatives to methanogenesis occur. Most of the research has focused solely on methanogenesis, and while it is accepted that methylotrophic methanogenesis constitutes the vast majority of methane production in sulfate rich sediments, there is no data on quantifying how much of methylated compound turnover is directed towards methanogenesis, sulfate reduction or other metabolisms (43, 46, 57, 58). Furthermore, while there is evidence outside of marine environments linking sulfate reduction and acetogenesis with methylotrophy, there are many unknowns on other metabolisms capable of utilizing these compounds. Recently uncovered genomes of novel line-

ages of archaea often encode genes linked with methylotrophy, suggesting a broader spread of these genes than previously thought (59–62). However, systematic analysis on the range and diversity of these genes in novel lineages is still lacking. Furthermore, there are currently no cultured representatives of these archaea available to study their physiology. At its core, the main questions that are yet unanswered are as follows:

- Which organisms are involved in the anaerobic conversion of methylated compounds in marine sediments?
- Which pathways do these organisms employ for the conversion of methylated compounds?
- Which specific methylated compounds do these organisms utilize?
- What are the interactions between methylotrophic microorganisms from different trophic groups such as SRM and methanogens?

This thesis focuses on answering these core questions. It does so by zooming in on one particular marine system as a model site: the Black Sea. The Black Sea has been a model site for anaerobic microbiology for decades and its biogeochemistry is well described (63–65). This sea, bridging Europe with Asia is the largest permanent anoxic marine basin. It spans 436,400 square kilometers (not including the Sea of Azov) and has a volume of 547,000 km^3 (66). Most of the basin consists of abyssal plain and consequently the majority of the sea is at a depth of around 2000 meters, with a maximum of 2212 meters. The only connection to other bodies of water is through the narrow Bosporus strait in the southwest, connecting the Black Sea with the Sea of Marmara and the Mediterranean Sea. The Black Sea is supplied with water by many rivers, most importantly the Danube, Don, Dnepr. Combined with precipitation, the total water flux is net positive, meaning more water is gained through river supply and precipitation than is lost through evaporation. As such, there is an asymmetrical balance with the Sea of Marmara and the Mediterranean Sea; more water is exported through the strait of Bosporus than is imported, which leads to the salinity of the upper layer of Black Sea water to to be much lower than other marine bodies of water, at about 18‰ compared with 35‰ of the Mediterranean Sea. Due to the differences in density of water based on their salinity, this leads to a permanent stratification of water layers in the Black Sea, with a top layer of low salinity water with an influx from rivers on top of a much larger layer of higher salinity water supplied through the Sea of Marmara (67). As these layers cannot intermix well, little oxygen is able to diffuse from the atmosphere to the bottom layer, leading to permanent anoxia at depths greater than ~100 meters that

has persisted since the connection between the Black Sea and the Sea of Marmara opened after the Pleistocene ended and sea levels began to rise as a result of melting ice caps, estimated to be between 6,500 and 12,000 years ago (68). The high levels of sulfate in the anoxic zone leads to high levels of microbial sulfate reduction, producing an estimated 30-50 Tg $year^{-1}$ of sulfide (69, 70). Thus, the Black Sea water column is anoxic and highly sulfidic, a condition called euxinia, after the Greek name for the Black Sea: Pontos Euxeinos (Πόντος Εὔξεινος).

Black Sea sediments are high in organic material, with a continuous sapropel layer of ~200 centimeters, which emerged in the postglacial Holocene years up until ~2000 years ago by a continuous deposition of algal necromass (71). The top 40 centimeters of sediment, as well as interspaced throughout various stages of the sapropel, high concentrations of calcium carbonate are present, the result of multiple blooms of the coccolithophore *Emiliana huxleyi* throughout the Holocene that dominated the entire Black Sea (72). At depths greater than 200 centimeters, the lacustrine layer begins, which is depleted of organic carbon and originated during or before the Pleistocene (72). As a result of the high levels or organic material and sulfate present in the upper layers of Black Sea sediments, microbial sulfate reduction rates are high, estimated to be ~38 $nmol \cdot cm \cdot day^{-1}$ (73). Consequentially, sulfide production rates are high, estimated to be between 3.2-5.2 Tg of sulfide $year^{-1}$ in all sediments, or about 10% of all produced sulfide in the Black Sea (70). Based on the relative presence of the sulfate reduction marker genes *drsAB* as a fraction of the general microbial 16S rRNA marker gene, between 5-10% of all microorganisms in the sulfate reduction zone and as much as 30% of all microorganisms in the sulfate-methane transition zone are estimated to be SRM in the Black Sea sediment (74).

In 2018, a sampling expedition, code named 64PE444 onboard of Research Vessel Pelagia, operated by the Royal Netherlands Institute for Sea Research (NIOZ), was undertaken. This cruise sampled sea water and sediments of the Black Sea in the Bulgarian Exclusive Economic Zone for three and a half days. All experimental data obtained and discussed in this thesis is based upon sediments retrieved during this cruise.

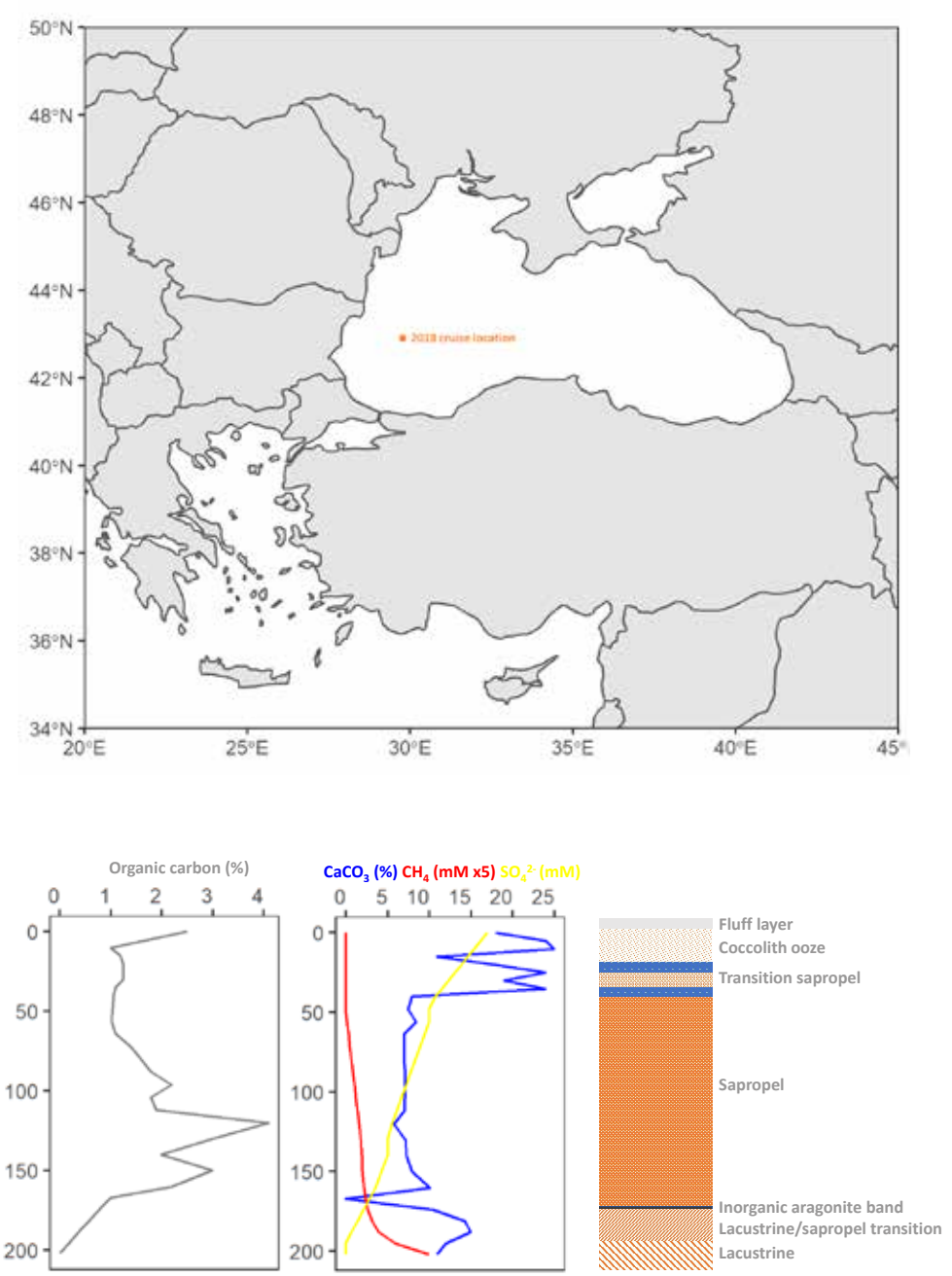

Figure 3: A) Map of the Black Sea with 2018 sampling location. B) Sediment profiles of organic material, calcium carbonate and biogeochemical layers. Combined data from Jorgensen et al (2001); Jones & Gagnon (1993); Calvert, Vogel & Southon (1987) (20, 71, 72)

1.6 Thesis aims & overview

This thesis aims to further unravel the unknowns surrounding the fate of methylated compounds in anoxic marine sediments, using the Black Sea as a model environment. Using a combination of the traditional cultivation based approach with modern (meta)genome and (meta)transcriptome techniques, this body of work addresses some of the known unknowns regarding methylotrophy in a marine context. I also explicitly aim to formulate new research questions by identifying new known unknowns and speculate on different hypotheses regarding possible resolutions of these.

In **Chapter 2** the role of methanol in anoxic marine contexts is described in detail. Methanol is considered to be one of the most important methylated compounds in marine systems. The origin of this compound in marine sediments is explained, as are the biochemical details of methanol conversion by methanogenic, sulfate-reducing and acetogenic microorganisms, which also serves as a proxy for the conversion mechanisms of other methylated compounds. Furthermore, through a data mining of publicly available marine metagenome datasets, the environmental distribution of anoxic methanol metabolism in marine systems is discussed.

Chapter 3 is a cultivation independent investigation on the presence of genes involved in methylotrophy in Black Sea sediments. Through a metagenome sequencing approach, the presence of several genes associated with methylotrophic metabolism in a wide range of microorganisms is detailed. The main conclusion is that, based on the dispersal of these genes, methylotrophy can take place in a much broader range of microorganisms than previously thought.

Chapters 4 and **5** describe the utilization of classical cultivation-dependent techniques to obtain methylotrophic microorganisms suitable for in-depth research under laboratory conditions. First, in **chapter 4**, a highly enriched methylotrophic methanogenic culture is obtained and characterized, which is capable of growth on the methylated compounds methanol, glycine betaine, monomethylamine, dimethylamine, trimethylamine and tetramethylamine. Using metatranscriptomics, a putative gene coding for a protein involved in methanogenic utilization of tetramethylamine is identified. Then, in **chapter 5** a novel methylotrophic strain, strain P130, closely affiliated to the sulfate-reducing microorganism *Desulfosporosinus nitroreducens* is isolated and its methylotrophic metabolism studied. Strain P130 is capable of growth on the methylated compounds methanol, trimethylamine and glycine betaine. The genome of this microorganisms reveals the potential for the utilization of the same set of methyltransferase genes involved in methylotrophic methanogenesis. We further-

more provide evidence that strain P130 is only the second described bacterium capable of incorporating the rare 22nd amino acid, pyrrolysine, in certain proteins.

In **chapter 6**, the hypothesis that sulfate-reducing microorganisms can co-exist with methylotrophic methanogens while growing on common methylated substrates is put to the test. A mixed culture of the obtained methanogenic enrichment and sulfate-reducing organism is grown using glycine betaine as sole substrate. Through a transcriptomic approach, we demonstrate the cascading utilization of glycine betaine by both sulfate reducer and methanogen.

Finally, in **chapter 7**, the obtained results are discussed in a broad scientific context.

Anaerobic microbial methanol conversion in marine sediments

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Abstract

Methanol is an ubiquitous compound that plays a role in microbial processes as a carbon and energy source, intermediate in metabolic processes or as end product in fermentation. In anoxic environments, methanol can act as sole carbon and energy source for several guilds of microorganisms: sulfate-reducing microorganisms, nitrate reducing microorganisms, acetogens and methanogens. In marine sediments, these guilds compete for methanol as their common substrate, employing different biochemical pathways. In this review, we will give an overview of current knowledge of the various ways in which methanol reaches marine sediments, the ecology of microorganisms capable of utilizing methanol and their metabolism. Furthermore, through a metagenomic analysis, we shed light on the unknown diversity of methanol utilizers in marine sediments which is yet to be explored.

2.1 Introduction

Marine sediments are rich in biomass and a source of unknown microbial diversity, with microbial cell densities as high as 109 cells per cubic centimeter (up to five orders of magnitude higher than the water column) (39). Marine sediments consist of deposits of clay, decaying organic matter, calciferous remains and other solids. While oxygen can diffuse in these sediments, it is rapidly consumed by aerobic organisms, in an oxic layer ranging from a few millimeters to several meters in depth, depending on a variety of factors such as organic matter input to sediment surface, sediment permeability, turbation by water currents or macrofauna, water column height, microbial activity or proximity to continental shelves (75, 76). The underlying sediment remains anoxic, where microbial fermentation and anaerobic respiration are the main metabolic processes. Molecules containing no carbon-carbon bounds such as trimethylamine, dimethylsulfide, methane and methanol are suggested to be important energy sources for microorganisms in these environments (19, 77, 78). As the fermentation products of common osmolytes or carbohydrates, these compounds are widely present in marine systems. Besides converted by microbial activity, they can influence the climate as atmospheric aerosols and as such, their role in marine environments is in general well-reviewed. (19, 38, 79, 80). However, there is a lack of information on the microbial utilization of methanol in anoxic marine sediments. In this review, we aim to present what is currently known about the presence and fate of methanol in anoxic marine sediments. To provide insight into anaerobic microbial methanol utilization in diverse marine sediments, we performed a metagenome mining of 246 published metagenomes of anoxic marine sediments for key methanol utilization genes. This effort reveals the ubiquitousness of several genes involved in anaerobic methanol conversion in marine sediments, further supporting the importance of methanol utilizing microorganisms in these environments.

2.2 Sources of methanol in marine sediments

Several studies have quantified methanol concentrations in marine systems, both in water columns and sediments. Concentrations in the water column range from less than 27 nM up to 429 nM in marine systems, although estimates vary widely even between studies on the same site (81–86). It should be noted, however, that most of these studies focused solely on the Atlantic Ocean and mainly on shallow, aerobic sub-surface waters, with only one study investigating a gradient of up to 500 meters depth (84). Estimates of methanol levels in coastal area sediments range between 0.3 μM to over 100 μM in environments as diverse as the Orca Basin in the Gulf Mexico, East Japan Sea and the South China Sea (77, 87, 88). In these studies, methanol concentrations were found to increase with depth, with the lowest concentra-

tions close to the sea floor and the highest concentrations at depths of 10 to 20 meters below the sea floor. This increase in methanol with depth is attributed to higher methanol turnover to CO2 near the sediment-water column interphase (88). Table 1 gives an overview of marine anaerobic sediments where methanol concentrations have been measured.

Table 1: measured levels of marine sediment methanol concentrations

Location	Depth	Methanol concentration	Ref.
Black Sea sediment	0-700 cm bsf[(1)]	6 mM at sea floor 1 mM 100-400 cm bsf 6 mM 500 cm bsf	(87)
Northern Gulf of Mexico	0-30 cm bsf	2 mM at sea floor 65 mM 30 cm bsf	(87)
South China Sea	0-700 cm bsf	4.3 mM at sea floor 111.7 mM at 700 cm bsf	(88)
Umitaka Spur, eastern Japan Sea	0-350 cm bsf	0.3-3.5 mM at 0-3 meters bsf 20 mM at 30 meters bsf	(77)
Intertidal sediment, Lowes Cove, Maine	Sea floor	0.5-3.5 mM	(46)
Guaymas Basin, Gulf of California	0-40 cm bsf	0.2-2 mM at sea floor 36.7 mM at 35 cm bsf	(89)
Western Mediterranean Sea	0-500 cm bsf	0.5-1.5 uM across all depths	(43)

(1) bsf – below sea floor

Methanol sources in marine systems are attributed to both *in situ* production and external depositions from terrestrial origins. Terrestrial methanol mainly originates as a by-product of plant growth and to a lesser degree through fermentation of pectin (90, 91). Because of its volatility, terrestrially produced methanol evaporates into the atmosphere, with an estimated annual emission of 70-350 Tg (92). A large amount of this methanol, estimated between 8-101 Tg year $^{-1}$ is deposited in the oceans through air-sea exchange, diffusion and rainfall (93). It should be noted that higher estimations of methanol deposition in the oceans also take sea to air emissions into account, which is estimated to be 30 to 85 Tg year $^{-1}$, thereby diminishing total methanol deposition in the oceans (83, 84, 86, 93, 94)

Methanol is also produced in situ in the oceans. A study of methanol production in Atlantic waters estimated a net production of around 49 nmol L^{-1} day $^{-1}$ (95). Sources of this methanol are primary production by phytoplankton where methanol is an exudate by-product and through microbial fermentation of algal carbohydrates such as galactins and pectin (95–97). Phytoplankton accounts for almost half of the global primary production and as such, is suspected to be a major contributor to marine methanol production, the same equivalent to terrestrial primary production (98, 99). Interestingly, there have been very few studies quantifying net methanol production by phytoplankton. Mincer and Aicher (2016) assessed methanol production through 13C-labelled bicarbonate addition to axenic phytoplankton cultures (99). As much as 0.3% of all assimilated carbon was sequestered in methanol, and it was implied that the genus *Prochlorococcus* alone could produce 846-1693 Tg of methanol per year, worldwide (99, 100). Furthermore, phytoplankton mobilize between 10-35% of their assimilated carbon into pectin, lignin and galactans, which are methoxylated polysaccharides (101). Thus, through demethoxylation of these carbohydrates by both aerobic microorganisms in the water column and oxic sediment and anaerobic microorganisms in the anoxic sediment, methanol is released (96, 101–103).

Chemolithotrophic microorganisms present in anaerobic sediments are capable of producing methanol. Anaerobic oxidation of methane (AOM) occurs in most anoxic systems where methane is present, by methane oxidizing microorganisms or syntrophic microbial communities capable of using sulfate, nitrate, manganese or iron as terminal electron acceptor (38, 104–107). Canonically, anaerobic methane oxidizers can metabolise methane through a reversal of the methanogenesis process (108). This process, contrary to oxic methanotrophy (which relies on the activity of oxygen-dependent methane monooxygenase), does not involve methanol as intermediate (109, 110). However, in freshwater sediments, methane oxidation by nitrate- and nitrite-dependent facultative anaerobic organisms has been shown to occur via

particulate methane monooxygenase, with methanol as a key intermediate (111). *Ca.* Methylomirabilis oxyfera utilizes a strategy of intracellular production of oxygen from nitrite. This oxygen can then act as electron acceptor for methane oxidation, also utilizing particulate methane monooxygenase (38, 112, 113). These processes with methanol as intermediary are leaky, and diffused methanol can be used by surrounding methylotrophic microorganisms (78, 112). Thus far, these processes have only been described in freshwater environments, which are richer in nitrite and nitrate than marine sediments (111, 114). However, in an enrichment culture of marine origin with methane as substrate, the NC10 phylum to which *Ca.* M. oxyfera belongs was abundant (115). Furthermore, phylogenetic studies on marine oxygen minimum zones report detection of 16S rRNA genes closely related to NC10 bacteria as well as transcripts of particulate methane monooxygenase and nitric oxide reductase genes in oxygen minimum zones off the coast of northern Mexico and Costa Rica (116). These findings suggest an environmental role of the NC10 phylum in marine environments as well.

It is unclear if and how methanol produced in surface water or exchanged with the air reaches the sediment of marine systems. As discussed above, methanol levels in the sediment are substantially higher than in the water column. Gradual deposition of methanol in the water column into the sediment is possible, but relatively high turnover rates of methanol in the water column (<1 day) suggests metabolization before it reaches anoxic sediments (83). This high methanol turnover can also explain its lower concentrations in the water column (117). In marine sediments, methanol turnover is estimated to be between 22 to more than 100 days (118).

2.3 Biochemistry of microbial methanol utilization

In anaerobic environments, methanol can be converted by three distinct processes: oxidation to CO_2 (e.g. by sulfate-reducing microorganisms (SRM), equation 1), conversion with CO_2 to acetate (by acetogens, equation 2), or conversion to methane and CO_2 (by methanogens, equation 3) (77, 119–121). The distinct pathways employed for methanol conversion by these organisms are described below, and are summarized in figure 1.

$4\ CH_3OH + 3\ SO_4^{2-} \rightarrow 4\ HCO_3^- + 3\ HS^- + 4\ H_2O + H^+$ -364 kJ/reaction (1)

$4\ CH_3OH + 2\ HCO_3^- \rightarrow 3\ C_2H_3O_2 + 4\ H_2O + H^+$ -221 kJ/reaction (2)

$4\ CH_3OH \rightarrow 3\ CH_4 + CO_2 + 2\ H_2O$ -311 kJ/reaction (3)

The methanol methyltransferase system is a major pathway for methanol metabolism in anoxic environments. This pathway is catalyzed by the methanol:coenzyme M methyltransferase MtaABC, found in methanogens, acetogens and SRM (45, 122, 123). Subunit MtaB cleaves the C-O bond of the methanol and transfers the methyl group to a second subunit, MtaC, using cobalamin as a cofactor (122). MtaC requires the cobalamin to contain the highly reduced cobalt(I), which is only possible in strict anoxic conditions (124, 125). The methylated group is subsequently transferred to either coenzyme-M (HS-CoM) in methanogens or tetrahydrofolic acid (THF) in acetogens and SRM by MtaA, forming CH_3-CoM or CH_3-THF, respectively (45, 125–128). It has been proposed that MtaA can be replaced with THF-methyltransferase in Sporomusa strain An4 (123).

In methanogenic methanol conversion, one quarter of the substrate is oxidized to CO_2 through a reversal of the hydrogen/CO_2 methanogenesis pathway. This process yields six electrons per molecule of methanol, generating sufficient reducing power to reduce the remaining three quarters of the substrate, which is shuttled to CoM-CH_3. This CoM-CH_3 is further reduced by methyl coenzyme M reductase (Mcr) to methane and HS-CoM ((129, 130). Furthermore, some methanogens of the order *Methanomassiliicoccales* couple H_2 oxidation to methanol reduction, yielding solely methane as product (131–133).

In acetogens and SRM generated CH_3-THF can be integrated in the Wood-Ljungdahl pathway (WLP, figure 1). One in four molecules of CH_3-THF is used in a reversal of the methyl branch of the WLP, oxidizing the methylated group to CO_2 and generating 2 moles of NAD(P)H, one mole of ATP and one mole of H_2. The generated H_2 is then utilized in a bifurcating mechanism to generate 0.5 moles of reduced ferredoxin (Fd2-). The microorganisms invest ATP to produce a proton/sodium gradient to generate an additional 2.5 moles of reduced ferredoxin through an RNF complex. This ferredoxin is subsequently used for the reduction of three molecules of CO_2 to three moles of CO in the carbonyl branch of the WLP. The WLP carbonyl branch then converts this CO and the three remaining moles of CH_3-THF through acetyl-CoA to acetate, generating three moles of ATP (134).

Methanol dehydrogenase pathways are organized in three distinct clusters: MxaFI, Mdh2 and XoxF. Both MxaFI and XoxF methanol dehydrogenase clusters occur in a wide range of microorganisms and environments, including oceans, soils, or the human microbiome (135–138). Mdh2-type is less widespread and has been detected in soil environments only (139, 140). Although functionally similar, MxaFI and

Mdh2 only share about 35% of amino acid identity, while Mdh2 shares up to 80% identity to other alcohol dehydrogenases with a low affinity for (78, 139, 141–143). MxaFI and Mdh2 methanol dehydrogenases catalyze the conversion of methanol to formaldehyde, releasing two electrons which are then shuttled to cytochrome C, whereas XoxF catalyzes the conversion of methanol to formate. Mdh2 utilizes NAD(P) as co-factor to shuttle electrons to cytochrome C (144). Both MxaFI and XoxF enzyme systems utilize pyrroloquinoline quinone (PQQ) as cofactor for electron transport to cytochrome C, but they differ in the active site metal. MxaFI utilizes calcium whereas XoxF utilizes a range of rare earth elements called lanthanides (145). Incorporation of strontium in vitro instead of calcium by MxaFI methanol dehydrogenase has been reported, resulting in increased reaction rates (threefold over calcium) and lower activation energy (by 13.4 kJ/mol) (146). While calcium is abundantly present in seawater, with concentrations measured in the millimolar range, strontium is available with concentrations around 150 μM. Whether all microorganisms containing these methanol dehydrogenases are able to incorporate strontium in vivo and whether this has ecological meaning in regards to methanol competitiveness for microorganisms utilizing this strategy requires further research. XoxF enzymes can work with any element of the lanthanide group, although the specific activity is higher with the two lightest lanthanides, lanthanum and cerium, compared to heavier lanthanides like neodymium or promethium (78, 143, 145, 147). While XoxF is mostly described in aerobic marine microorganisms, *Ca.* Methylomirabilis oxyfera utilizes XoxF in its metabolism, which is intracellular aerobic (78, 148, 149). Under high calcium and low lanthanide concentrations, the methanotroph *Methylomcrobium buryatense*, which contains both methanol dehydrogenase pathways in its genome has a higher expression of XoxF than of MxaFI (150). Interestingly, while metagenomes of anaerobic terrestrial environments revealed multiple XoxF-type alcohol dehydrogenases, PQQ biosynthesis requires molecular oxygen (151–153). Diffusion of PQQ from aerobic marine sediments, where XoxF is the most abundant methanol dehydrogenase could provide anoxic sediments with this cofactor (148, 154). Furthermore, it cannot be excluded that alternative, unknown pathways for PQQ biosynthesis that may not involve oxygen exist.

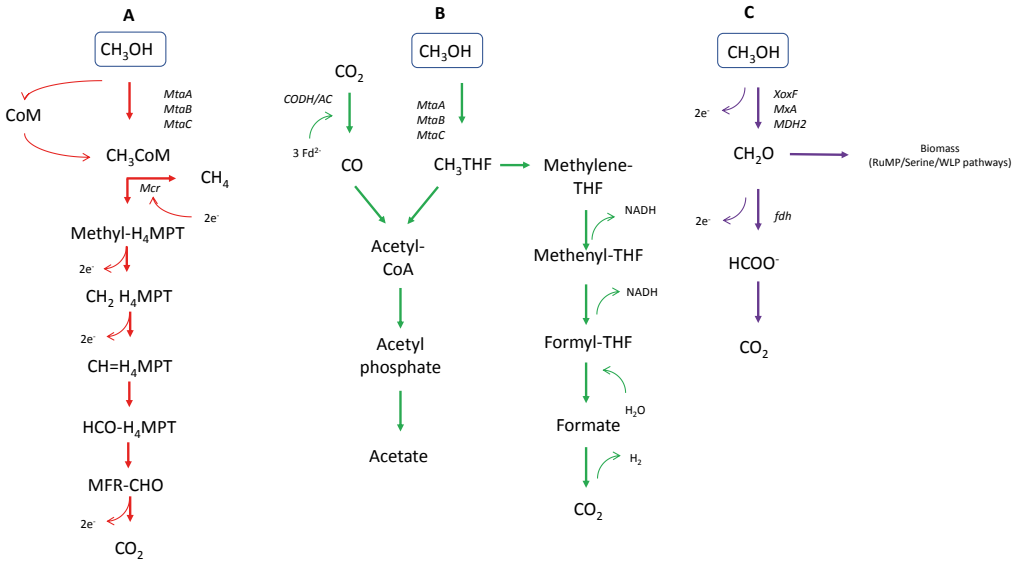

Figure 1: methanol degradation pathways as outlined in this review. A: methanogenesis. Abbreviations abbreviations: MT methyl-transferase 1; CoM co-enzyme M; H4MPT tetrahydromethanopterin; Mcr: methyl-coenzyme M reductase; MFR methanofuran. B: acetogenesis pathway. Abbreviations: THF: tetrahydrofolic acid; CODH/ACs: carbon monoxide dehydrogenase/Acetyl-coA synthetase. C: respiratory methanol oxidation. Abbreviations: xoxF lanthanide-dependent methanol dehyrdogenase; MxA calcium-dependent methanol dehydrogenase; RuMP pathway ribulose monophosphate pathway for carbon fixation; WLP Wood Ljungdahl pathway for carbon fixation.

2.4 Microbial ecology of methanol degradation in marine environments

The processes mentioned in the previous paragraph occur in many phyla of both Bacteria and Archaea, including SRM, nitrate reducing microorganisms, acetogens, and methanogens which all occur in marine sediments. Furthermore, metals such as ferric iron or manganese can be present in these environments and can act as electron acceptor for some methanol oxidizers (107). However, near neutral pH these compounds are insoluble and occur mainly as minerals, severely limiting their use as electron acceptor without specialized extracellular electron transfer (EET) systems, as found in genera like *Geobacter* or *Shewanella* (155). Although microorganisms capable of EET utilizing methanol have been described, such as *Shewanella putrefaciens*, there is very little known on their role in marine sediments (156). Multiple marine species capable of growing on methanol in anaerobic conditions have been isolated and characterized, and these are described in table 2.

Sulfate-reducing microorganisms occur amongst seven lineages, five of which are within the domain Bacteria and two in the domain Archaea, although methanol utilizing SRM have only been described in Bacteria. Bacterial SRM belong to *Deltaproteobacteria*, *Clostridia*, *Nitrospirae*, *Thermodesulfobacteria* and *Thermodesulfobiaceae* (24). However, based on single copy marker gene analysis, the phylum *Deltaproteobacteria* was recently proposed to be divided into the sulfate-reducing phylum *Desulfobacterota* and the non-sulfate-reducing phyla *Myxococcota*, *Bdellovibrionota* and SAR324. Additionally, this assessment places the phylum *Thermodesulfobacteria* within the phylum *Desulfobacterota* (25). Furthermore, based on metagenomic datasets, thirteen bacterial and archaeal phyla were identified to have the genes for sulfate reduction (*dsrABCD*), thereby doubling the number of known taxa (28). Only a few marine sulfate reducers have been proven to grow with methanol as sole electron donor and carbon source (table 2). However, methanol is not conventionally tested as substrate in the characterization of newly isolated marine SRM.

As the most energetically favorable electron acceptor after oxygen that is abundantly available, nitrate reduction is common at the oxic-anoxic interphase, carried out by anaerobes and facultative anaerobes (157). However, there is a lack of investigation on the use of methanol by nitrate reducers in oxygen minimum zones or marine anoxic sediments. While dissolved organic matter is often not limiting in these systems, methanol is available and yields comparable energy as DOM per reducing equivalent (158). While freshwater denitrification with methanol as electron donor is widely studied due to its biotechnological relevance, whether this process occurs in marine sediments is less clear (159, 160).

Acetogens are a diverse group of bacteria, occurring in 22 genera comprising over 100 species (161). Their key defining trait is the utilization of the Wood-Ljungdahl pathway to fix CO_2 for both energy and biomass production. This metabolism is widely dispersed amongst many habitats, and acetogens are metabolically flexible, incorporating a broad range of electron donors besides C1 compounds, such as sugars or organic acids (161). Currently, there are few well-characterized marine acetogens capable of growing on methanol (table 2). *Acetobacterium woodii* was isolated from a former ocean inlet that was closed off, but had characteristics similar to the sea (134, 161). Furthermore, Schuppert and Schink isolated an acetogen from the marine Rio Marin in Venice, Italy (162). However, this strain was never deposited in a culture collection. About 67% of all cultivated acetogens have been shown to grow on methanol (163). Acetogenesis has been described in enriched cultures from marine sedi-

ments, and genetic evidence for genes in the Wood-Lljungdahl pathway, such as the presence of formyltetrahydrofolate synthetase and methenyltetrahydrofolate cyclohydrolase coding genes, have been found in metagenomes from a diverse range of marine systems, for example the Juan de Fuca Ridge, the Guamas Basin, the Baltic Sea and the Arctic Sea (61, 163–168). These findings suggest that acetogenesis and the WL pathway are widely spread in marine systems.

Methanogens were canonically only described within the archaeal phylum Euryarcheaota, comprising seven orders and 29 genera (169, 170). However, recent genomic discoveries have found organisms containing genes coding for methanogenic pathways in the distantly related archaeal phyla *Bathyarchaeota* and *Verstraetearchaeota*, suggesting a much wider phylogenetic range of this metabolism than previously thought. (31, 128, 171, 172). Besides marine systems, methanogens have mainly been isolated from animal rumen, rice paddies, soils and freshwater systems (35). In marine anaerobic systems, methanogens occur in the sulfate-depleted zone of the sediment, usually several meters below the sea floor, as most methanogens are outcompeted for their common substrates hydrogen and acetate by SRM (173). However, methylotrophic methanogenesis has been described to occur in the sulfate zone of the sediment and attributed to the utilization of non-competitive substrates such as methanol (174). Furthermore, through interspecies hydrogen transfer, syntrophic interactions between methanogens and SRM for substrates is possible, leading to methane production in the sulfate reduction zone (175, 176). It is uncertain whether methanol can be utilized in syntrophic interactions in marine environments. A recent study of Mediterranean Sea sediments suggested methanol as being the primary source of methane in sulfate-rich sediments as hydrogenotrophic methanogenesis appeared to be outcompeted by sulfate reduction, with up to 98% of total methane produced in the top sediment deriving from methanol, suggesting methanol to be the main source of methanogenesis (43, 118). Cultured methylotrophic methanogens comprise three orders, *Methanosarcinales*, *Methanobacteriales* and *Methanomassiliicoccales*, although only *Methanosarcinales* have marine representatives. An overview of methanol utilizing methanogens currently isolated is shown in table 2. Based on genomic information, methylotrophic methanogenesis has been found outside of the canonical methanogenic phyla, such as Verstraetearchaeota, which suggests a much broader phylogeny of methylotrophic methanogenesis than previously thought (31).

Table 2: isolated marine microorganisms capable of growing on methanoland their growing conditions

Name organism	Original isolation source	Methylated substratesa	Temperature range (°C)	pH range	Ref.
Methanogens					
Methanococcoides methylutens	Scripps Canyon, California	Methanol, trimethylamine, dimethylamine, methylamine, glycine betaine	30-35	7-7.5	(177, 178)
Methanococcoides burtonii	Salt water lake in Antarctica	Methanol, trimethylamine, dimethylamine, methylamine, glycine betaine	5.6-29.5	6.8-8	(178, 179)
Methanococcoides alaskense	Skan Bay, Alaska	Methanol, trimethylamine, dimethylamine, methylamine, glycine betaine	-2.3-28.4	6.3-7.5	(178, 180)
Methanococcoides vulcani	Napoli mud volcano, Mediterranean Sea	Methanol, trimethylamine, dimethylamine, methylamine, glycine betaine	ND-35	6-7.8	(53, 178)
Methanosarcina acetivorans	Scripps Canyon, California	Methanol, trimethylamine, dimethylamine, methylamine, glycine betaine, methylated thiols	10-50	5.5-8.5	(181, 182)
Methanosarcina baltica	Baltic sea	Methanol, methylamine, trimethylamine	-22.3-27	4.9-8.5	(183)
Methanosarcina semesiae	Dar es Salaam mangrove, Tanzania	Methanol, trimethylamine, Dimethylsulfide	30-35	6.5-7.5	(184)
Methanosarcina siciliae	Scripps canyon, California	Trimethylamine, Dimethylamine, Methylamine, Methanol, Dimethylsulfide	15-40	5-7.5	(185)
Methermicoccus shengliensis	Shengli oilfield, South China Sea	Methanol, trimethylamine, methylamine	50-70	5.5-8	(186)

Methanohalophilus halophilus	Shark Bay, Australia	Methanol, Trimethylamine, Dimethylamine, Methylamine	26-36	6.3-8	(187)
Methanolobus bombayensis	Arabian sea	Trimethylamine, Dimethylamine, Methylamine, Methanol, Dimethylsulfide	15-43	6.2-8.3	(188)
Methanolobus vulcani	San Fransisco Bay, California	Trimethylamine, Dimethylamine, Methylamine, Methanethiol, Dimethylsulfide, Methanol	13-45	6-7.5	(189)
Methanolobus profundi	Deep subsurface sediments, Movara, Japan		9-37	6.1-7.8	(190)
Methanolobus taylorii	San Fransisco Bay, California		5-45	5.7-9.2	(191)
Methanolobus tindarius	Tindari, Sicily		7-50	5.5-8	(192)
Sulfate-reducing microorganisms					
Desulfallas arcticus	Marine surface sediment, Svalbard, Norway	Methanol	26-46.5	7.1-7.5	(193, 194)
Desulfoconvexum algidum	Marine surface sediment, Svalbard, Norway	Methanol	0-20	7.2-7.4	(195)
Desulfospira joergensenii	Marine sediment, Acrcachon Bay, France	Methanolc	8-30	7.4-ND	(196)

Desulfatiglans anilini	Marine sediment, North Sea coast, Germany	Methanol	12-40	6-8	(197)
Desulfosporosinus nitroreducens	Baltic sea sediment,	Methanol	10-30	6.4-8.1	(198)
Acetogens					
Acetobacterium woodii[b]	Oyster pond, Massachusetts	Methanol	2-45	5.9-8.5	(199)
Nitrate reducing microorganisms					
Methylophaga nitratireducenticrescens	Seawater denitrification reactor, Montreal Canada	Methanol	15-37	6-11	(159)

a *Some of the mentioned organisms are also capable of degrading non-methylated compounds such as acetate or CO2:H2 but this is outside of the scope of this review.*

b A. woodii *was isolated from an ocean inlet that was closed off to the sea*

c D. joergensenii *showed sulfide production on methanol, but no growth*

It is assumed that microorganisms containing methanol methyltransferase pathways outcompete microorganisms containing methanol dehydrogenases under optimal conditions for both pathways, due to its higher affinity for methanol (200, 201). This could mean methanogens and acetogens might be able to outcompete SRM for methanol. Several studies on environmental systems have indicated that in anaerobic methanol degradation, cobalt is often the limiting factor (200). As cobalt is the active metal in methyltransferases, this corroborates the theory of competitive advantage of methanogens and acetogens. However, Sousa et al (2018) showed the presence of methanol methyltransferase genes in the genome of the sulfate-reducing bacterium *Desulfotomaculum kuznetsovii* as well as proteomic evidence for an upregulation in the presence of cobalt (45). This would mean that under cobalt-rich conditions, this SRM would be able to compete with methanogens and acetogens for the available methanol. Under cobalt-limiting conditions, it has a competitive advantage as the methanol dehydrogenase is not dependent on cobalt. While *D. kuznetsovii* is not a marine organism, the genes encoding for the methyltransferase pathway in *D. kuznetsovii* have orthologs in many other closely related SRM, for example the marine *Desulfallas arcticus*, *Desulfosporosinus fructosivorans* or the freshwater species *Desulfosporosinus merideii* and *Desulfosporosinus lacus* (193, 198, 202, 203). Anoxic marine sediments have been found to be relatively rich in cobalt, while it is depleted in the oxic sediment and pore waters above (204). This suggest the possi-

bility of rapid consumption in the oxic-anoxic interphase, indicating competitive advantages for microorganisms containing both pathways.

2.5 Metagenomic assessment of key genes involved in methanol metabolism in marine sediments

To assess the dispersion of methanol conversion amongst marine sediments worldwide, we mined core metabolic genes involved in the cycling of methanol in deposited annotated metagenomes in the Integrated Microbial Genomes & Microbiomes (IMG) v.5.0 database following the procedure described by (205). Six genes involved in methanol utilization pathways were selected: three methanol methyltransferase genes: *mtaA*; *mtaB*; *mtaC*), one lanthanide-dependent alcohol dehydrogenase (*xoxF*) one methanol-derived formaldehyde dehydrogenase (*fdhA*) and one pectin methanolesterase (*pesT*) (206). Formaldehyde dehydrogenase was chosen over canonical methanol dehydrogenase due to the high chance of methanol dehydrogenases mistakenly being annotated as other alcohol dehydrogenases, as there is a high similarity between residues between these enzymes and thus increasing the risk of false positives (207). Pectin esterase was included to test the hypothesis whether methanol is produced in sediments from pectin.

Utilizing the search function of IMG and manual curation, all metagenomes of anoxic marine sediment samples were exported. In total, 246 metagenomes were selected for this assay. Of these metagenomes, annotation data and metadata containing information on sampling location and sample site water column depth were also collected (supplementary table S1). Supplementary figure S1 shows a world map with sample locations of all metagenomes grouped by water column height (figure S1). Most of the metagenomes derived from datasets close to shores, based on the metadata supplied by IMG (table S1). This is unsurprising, as deep sea sampling is logistically difficult and expensive (208). Likewise, most datasets originate from either the US coasts or the North Sea in Europe, as they are relatively close to large, well-funded marine institutes. Thus most information on methanol cycling as discussed in this review is biased to these environments. There is only a very limited set of metagenomes from deep ocean samples, for example IMG3300010241-3 obtained from the South China Sea or IMG3300016982 obtained from the Southern Atlantic Ocean. Thus, it is difficult to differentiate between coastal ecosystems, which are generally richer in organic material and have a higher carbon turnover than deeper sea ecosystems (209, 210).

Using a hidden markov model (HMM) based approach, all metagenomes were scanned for the listed genes. Detailed workflow and tools used can be found

in supplementary text 1. Total data output was visualized using the R package pheatmap (211). Figure 2 displays the results of this metagenome mining effort. Figure S2a-f shows world maps with corresponding positive locations for each of the six mapped genes.

Based on the prevalence of genes involved in methanol conversion, the utilization is widespread in diverse anoxic marine sediments. Out of 246 published metagenomes, 78% mapped positively for at least one of the selected marker genes in this study. Over 50% metagenomes mapped positively for at least three selected marker genes, suggesting utilization of methanol by all trophic groups discussed in this review. Interestingly, *mtaB* is much less present than *mtaC*; 117 positive hits in all metagenomes versus 172 for *mtaC*, even though for the utilization of methanol mtaB and mtaC form a complex (182). Whether this represents evolutionary artefacts or alternative pathways involving orthologs of these genes is not known. As MtaC contains a corrinoid center to which the methyl group of methanol is bound, it is possible it acts as a carrier for methyl groups derived from non-methanol origins, for example trimethylamine, which is widely present in marine sediments and utilized by methanogens (19, 212). Lanthanide-dependent methanol dehydrogenase could be detected in 112 of the 246 datasets, whereas formaldehyde dehydrogenase was found in 87 datasets. As XoxF produces formaldehyde, it is possible other, currently unknown types of formaldehyde incorporation are present in these metagenomes. Furthermore, the presence of *xoxF* as artefacts in these metagenomes that are not actively utilized is also possible. Pectin esterase was present in 52 out of 246 metagenomes. While this was the least present of all studied genes, this still supports the hypothesis that complex carbohydrate degradation can occur in some marine sediments, rather than only in the upper water column.

Figure 2: Metagenomic mining heatmap. Dark blue indicates presence, yellow absence. Gene abbreviations: xoxF: lanthanide dependent methanol dehydrogenase F; mtaA: methanol methyltransferase A; mtaB: methanol methylransferase B; mtaC: methanol methyltransferase C; fdhA: formaldehyde dehydrogenase A; pest: pectin methanolesterase. Each cluster denotes 50 metagenomes screened.

2.6 Concluding remarks

Methanol is an important compound in the global biogeochemical cycles, which has received little attention in marine sediments. There are still many gaps in our knowledge on the prevalence of methanol in these environments, how it becomes available and which microorganisms are involved in its cycling. The utilization of non-canonical metals in methanol dehydrogenases requires a rethinking of bio-active metals involved in this process. Cultivation approaches fail to recover the vast majority of microorganisms from marine sediments. The use of metagenomic data provides a reliable indication of the diversity and potential functions of specific microorganisms. In this regard, our approach of data mining marine sediment metagenomes for key methanol metabolism genes indicated this compound is released and utilized in anoxic marine sediments worldwide in a variety of ways. Thus, it can be concluded that methanol utilization is an active force in anoxic marine sediments, based on their genomic presence in these environments. This methodology can aid in the further understanding of the ecology of marine anoxic systems.

2.7 Acknowledgments

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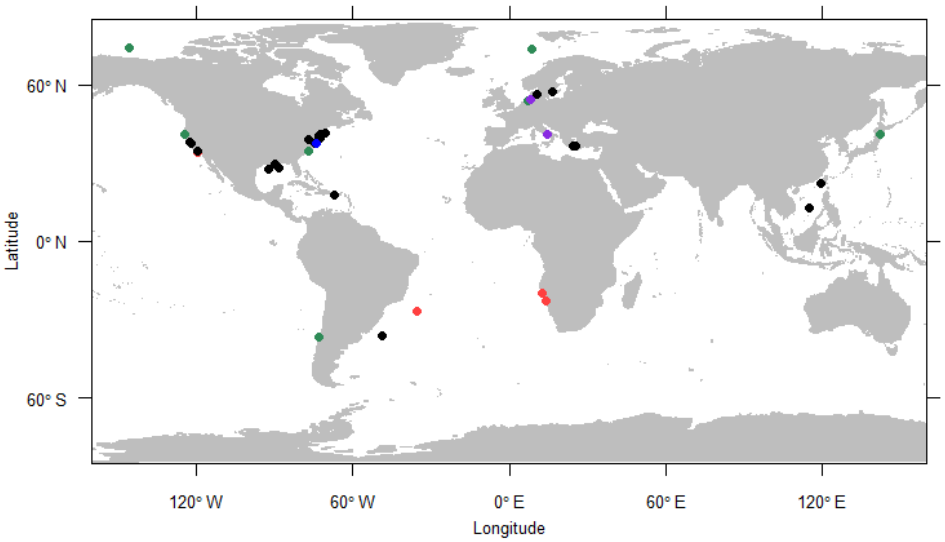
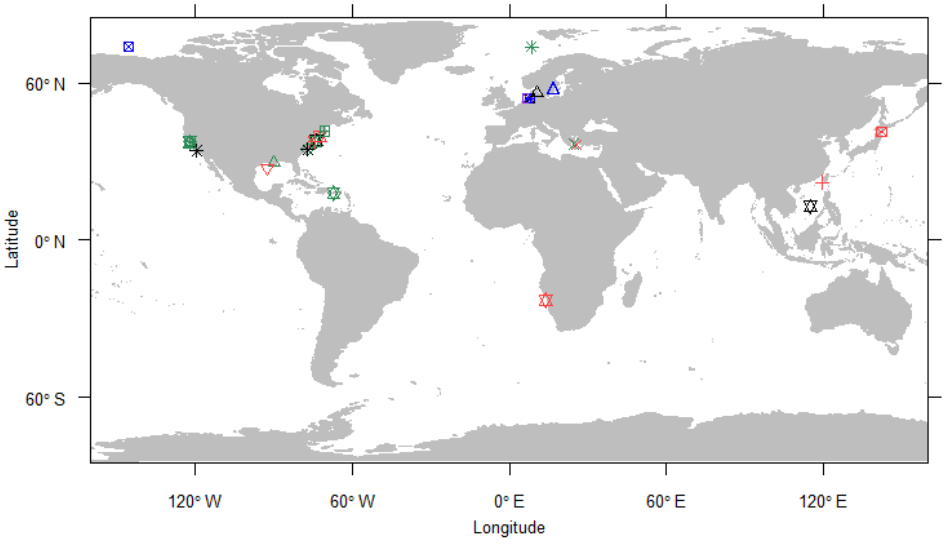
2.8 Supplementary information



Supplementary figure S1: *world map of sampling locations selected for this metagenome study. Green points: water column depth < 1 meter; blue dots: water column depth 1-10 meters; purple dots: 10-100 meters; red dots: water column depth >100 meters; black dots: no data.*
Image created with R package oceanmap (213).



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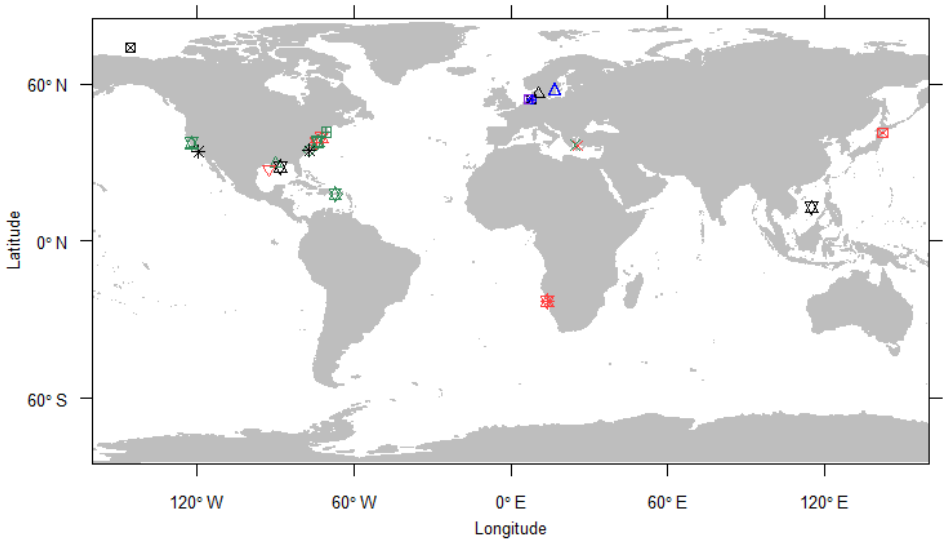
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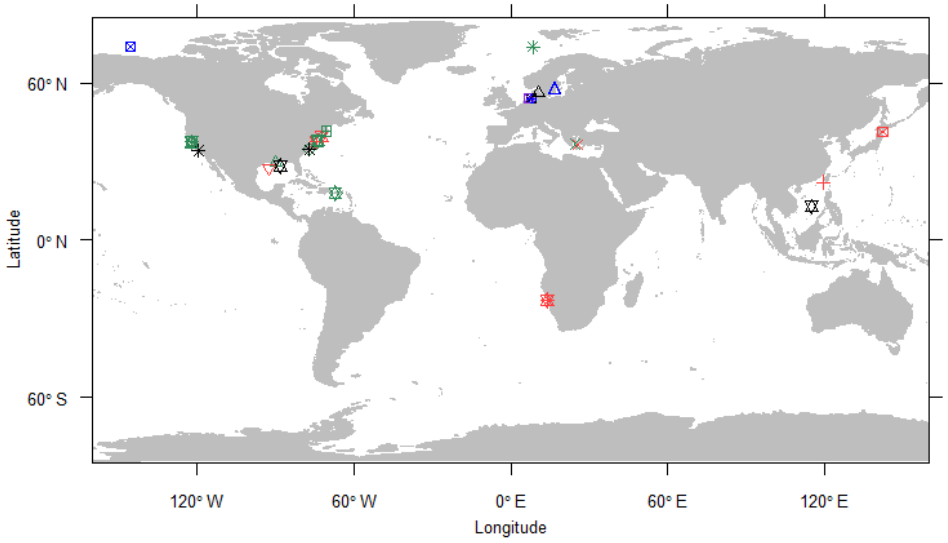
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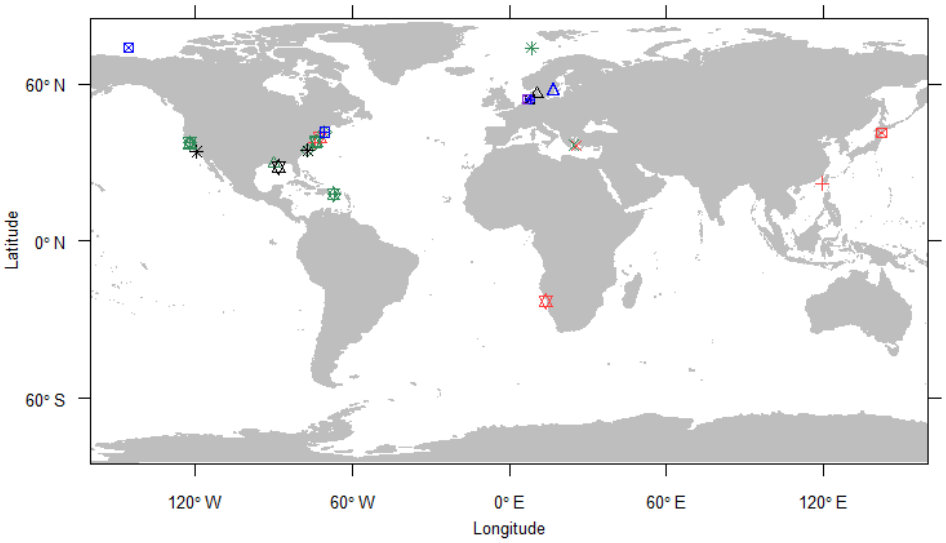
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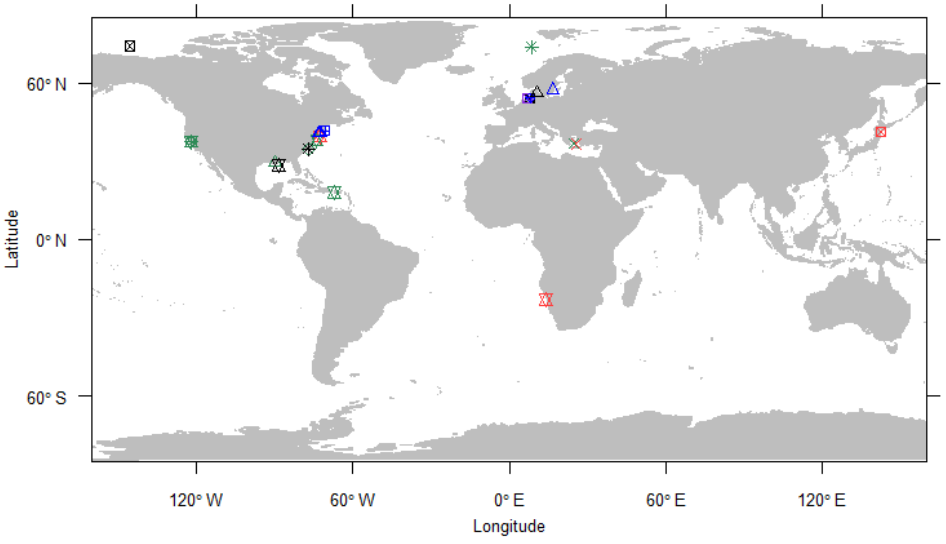
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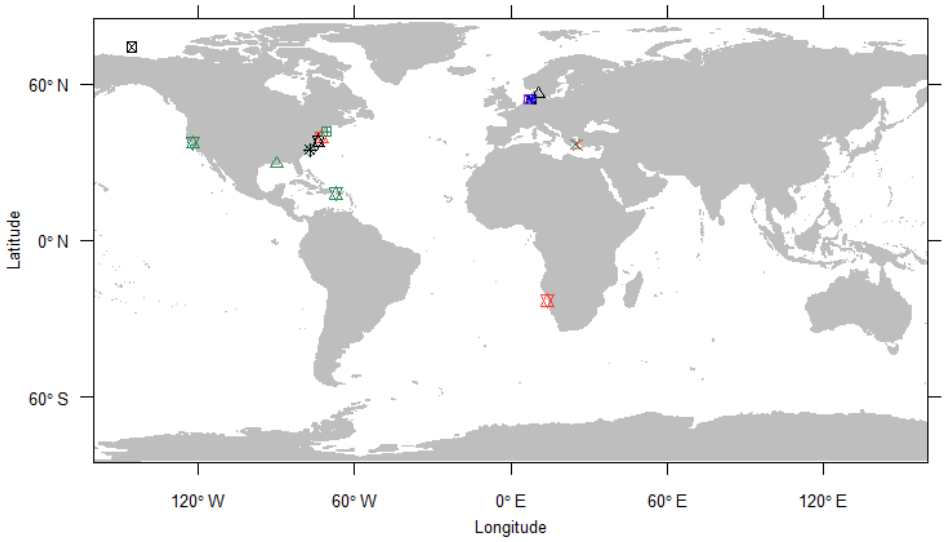

F

Supplementary figure S2: *world map of sampling locations for metagenome study. A: positive* mtaA *datasets; B: positive* mtaB *datasets; C: positive* mtaC *datasets; D: positive* xoxF *datasets; E: positive* fdhA *datasets; F: positive* pest *datasets. Color legend: green points: water column depth < 1 meter; blue dots: water column depth 1-10 meters; purple dots: 10-100 meters; red dots: water column depth >100 meters; black dots: no data. Images created with R package oceanmap (213).*

Supplementary text S1

Metagenome mining workflow:

From IMG, compressed .tar files of all metagenomes were downloaded, containing annotation data. From these files, annotated amino acid profiles were used.

For all selected genes, TIGRFAM HMM profiles were utilized, with the exception of Pest and *mtaB*, where no TIGRFAMs were available. Instead, PFAMs were utilized for these genes. The list of HMMs can be seen in supplementary table S2 (214, 215). Additionally, the *xoxF* TIGRFAM HMM was updated with the *xoxF/mdh* HMM as described in Anantharaman et al (28). To mine the obtained metagenomes, the function hmmsearch of HMMer version 3.3 was utilized (Howard Hughes Medical Institute, 2019) with the selected HMMs and output defined as aligned Stockholm format. Minimum threshold scores for positive hits were selected per gene, with a minimum inclusion bitscore threshold of 75 for *xoxF*, *pest* and *fdhA* and 100 for *mtaA*, *mtaB*, *mtaC*, respectively. Metagenomes with no positive hits yielded empty Stock-

holm files, whereas metagenomes with positive hits yielded Stockholm files with alignments. This characteristic was utilized to create lists with the file sizes of each metagenome for each individual HMM, where a file size of 0 indicates a negative hit and a file size of >0 indicates a positive hit. As all metagenomes were obtained using different methodologies, no quantitative information was used downstream and only a presence/absence matrix was utilized. The resulting results were uploaded in R version 3.6.3 (R core team, 2020). Total data output was visualized using the package pheatmap (211).

Metagenomic analysis of anaerobic methylated quaternary amine metabolism in anoxic marine sediments

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Abstract

Methylated quaternary amines, such as the osmolytes betaine and choline are ubiquitous in marine environments. Degradation of these compounds yields volatile methylated amines, e.g. trimethylamine, which are well known to be substrates for methanogens in anoxic marine sediments. However, other marine benthic microorganisms, such as acetogens or sulfate-reducing microorganisms may be capable of utilizing these compounds. Little is known about the diversity of marine microorganisms metabolize methylated amines. Here, we studied the diversity and potential for microbial methylated amine degradation in anoxic Black Sea sediments through a metagenomic approach. We reconstructed 394 metagenome-assembled genomes, 227 of which contained at least one putative amine-linked corrinoid methyltransferase. Some of the metagenome-assembled genomes with corrinoid methyltransferases additionally contained homologs for genes involved in sulfate reduction and acetogenesis pathways, but not in methanogenesis. However, the majority of recovered MAGs with genes for methyl transfer displayed no evidence for genes belonging to any of these canonically described metabolisms linked with anaerobic use of methylated compounds, suggesting a higher metabolic diversity of metabolisms linked with these compounds than previously thought.

3.1 Introduction

In marine environments, prokaryotes and algae produce osmolytes, such as glycine betaine, choline and dimethylsulfoniopropionate (DMSP), which are methylated compounds that can be used by certain microbes as electron, carbon, sulfur and nitrogen sources (216–219). When these osmolytes are fermented in the sediment, smaller methylated compounds such as trimethylamine (TMA) or dimethylsulfide (DMS) are released in the environment (18).

Anoxic marine sediments occupy vast regions of the ocean, mainly in anoxic basins and continental margins. However, the diversity of microorganisms and their metabolic processes within these environments remain poorly characterized (2). In the absence of oxygen, the most commonly available electron acceptor for microbial metabolism in these sediments is sulfate, which diffuses from the water column to sediments up to 1-4 meters deep, known as the sulfate reduction zone (20). Sulfate is reduced to hydrogen sulfide by sulfate-reducing microorganisms (SRM), that can utilize a broad range of organic compounds and hydrogen as electron donors (24, 220). As sulfate is depleted in deeper sediments, the dominant microbial metabolism becomes methanogenesis, where e.g. acetate, methylated compounds or CO_2 plus hydrogen are converted to methane, in what is called the methanogenesis zone (41). While it is often assumed that in sulfate rich environments, SRM outcompete methanogens for their common substrates, methanogens do exist and actively produce methane in such sediments by utilizing methylated compounds (19, 41, 43, 46, 174). These compounds are assumed to be non-competitive substrates for methanogens, as the methyltransferase pathway utilized by methanogens is kinetically favorable over the common dehydrogenase pathway employed by SRM (221). However, some SRM can also use methyltransferases, as was shown for *Desulfofundulus kuznetsovii*, potentially allowing them to compete with methanogens. Additionally, acetogens, a third important trophic group in anoxic marine sediments, produce acetate from methylated compounds using the methyltransferase pathway (123, 222).

Methylated compounds can be utilized by methanogens through a class of proteins called corrinoid methyltransferases (33, 182). Most research on these proteins has been conducted with reference to small methylated compounds such as TMA or DMS, but recent evidence has shown that larger methylated compounds, for example glycine betaine and proline betaine, can be demethylated in a similar fashion (19, 50, 54, 223). In general, this process is mediated by two methyltransferase proteins, methyltransferase I and II (figure 1). Methyltransferase I (MTI), a two-subunit substrate specific methyl-cleaving module, is encoded by the genes *mtxB* and

mtxC, where x denotes substrate. MtxB cleaves the methyl group from the donor compound and transfers it to the corrinoid methyl binding enzyme MtxC. For the functioning of MtxC, reduction of the neutral cobalt(II) cofactor to cobalt(I) is required, which is mediated by the ATP-dependent RamA (224). Then, methyltransferase II (MTII), encoded by the gene mtxA, transfers the methyl group from corrinoid-CH_3 to an accepting co-factor. In methanogens, this accepting co-factor is the co-enzyme M (CoM), forming methyl-CoM, which is then funneled into the energy conserving methanogenesis pathway (223). Among known acetogens and SRM that use the methyltransferase pathway, this acceptor is tetrahydrofolate (THF) (45, 225). In contrast with the substrate specificity of MTI, MTII is assumed to be less substrate specific and can mediate the transfer of the methyl moieties originating from a range of substrates. Two MTII systems, MTII-M and MTT-A, were described in *Methanosarcina barkeri*. While MTII-M is capable of transferring methyl moieties originating from monomethylamine (MMA), dimethylamine (DMA) and TMA to CoM, MTII-A was specific for methyl moiety transfer for either methanol or TMA, but not MMA or DMA (226). In the acetogen *Eubacterium limosum*, a methylcobamine-THF methyltransferase encoded by *mtqA* was identified to be upregulated during growth with proline betaine and carnitine (49, 227). Transfer of the methyl group to THF in *Sporomusa ovata* strain An4 is mediated by a THF-methyltransferase, involved in the Wood-Ljungdahl (WL) pathway, rather than the more commonly described MtxA in methanogens (123). Methylated compounds metabolized via this pathway in methanogens include TMA (228), DMA (229), MMA (230), methanol (122) and glycine betaine (178). Methanethiol and DMS are utilized similarly, but MTI is encoded by a single gene, rather than two genes (231, 232). The amino acid sequences of the methyltransferase subunits MtmB (EC 2.1.1.248) , MtdB (EC 2.1.1.249) and MttB (EC 2.1.1.250) for MMA, DMA and TMA are three of the few sequences to comprise the 22nd amino acid pyrrolysine, which is crucial to their enzymatic functioning (233).

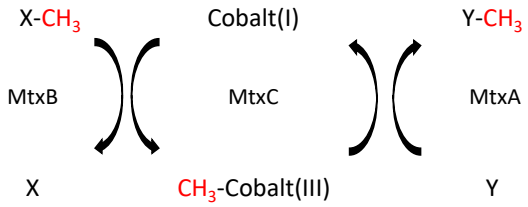

Figure 1: Standardized methyltransferase pathway. X denotes methyl donor rest group, Y denotes final methyl acceptor rest group. MtxB, C and A indicate enzymatic products of the genes methyl:corrinoid methyltransferase complex BC and corrinoid:CoM methyltransferase A, respectively.

The protein family to which pyrrolysine-containing TMA transferases belong, comprises many additional members lacking the characteristic pyrrolysine residue (50). Collectively, this family is referred to as the MttB-superfamily and includes multiple clades of suspected methyltransferases. The best described member of this family is MtgB (EC2.1.1.376), encoded by the gene *mtgB*, a glycine betaine methyltransferase, demethylating one of the three methyl groups of this quaternary amine compound, forming N,N-dimethylglycine (54). Functional homologs of this protein have been described in *Desulfitobacterium hafniense*, isolated from sewage sludge, as well as the methanogen *Methanolobus vulcani* strain B1d, isolated from estuarine sediment (50, 54, 234, 235). Three other proteins in the MttB-superfamily, MtpB, MtyB and MtcB, have been shown to be corrinoid methyltransferases on the quaternary amine compounds proline betaine, γ-butyrobetaine and carnitine in *E. limosum* (49, 51, 227). Proteomic data of *Eubacterium maltosivorans* identified an MttB-family protein likely to be a choline methyltransferase (236). Homologs mapping to genes coding for MttB-superfamily proteins have been found in hundreds of genomes in a wide range of environments, yet to date no in-depth investigation regarding the potential metabolisms of these microorganisms has been performed.

In spite of the long hold assumption that the methyltransferase pathway is mainly found in methanogens, there is wide genetic and physiological evidence for the presence of methyltransferases in SRM and acetogens (45, 46, 123, 237). In SRM and acetogens, methyl-THF is oxidized to CO_2 in a reversal of the methyl branch of the WL pathway, where sulfate acts as terminal electron acceptor for SRM and acetogens use the obtained reducing equivalent to reduce CO_2 to CO in the carbonyl branch of the WL pathway (45, 123, 225, 238). However, it remains unknown to what extent these pathways are characteristic for microorganisms in anoxic marine sediments where methylated compounds are abundant (219). An overview of described genes coding for quaternary amine methyltransferase, including the MttB-superfamily coding genes, can be found in Table 1.

Table 1: Overview of proteins and their coding genes for quaternary amine linked methyltransferases.

Protein	Key gene	EC	Ref.
Methylamine:corrinoid methyltransferase	*mtmB*	2.1.1.248	(230)
Dimethylamine:corrinoid methyltransferase	*mtbB*	2.1.1.249	(229)
Trimethylamine:corrinoid methyltransferase	*mttB*	2.1.1.250	(228)
Glycine betaine:corrinoid methyltransferase	*mtgB*	2.1.1.376	(50)
Proline betaine:corrinoid methyltransferase	*mtpB*	Not yet determined	(227)
Carnitine:corrinoid methyltransferase	*mtcB*	Not yet determined	(49)
γ-Butyrobetaine:corrinoid methyltransferase	*mtyB*	Not yet determined	(51)
Choline:corrinoid methyltransferase	Predicted *mttB*-protein coding gene CPZ25_RS12890	Not yet determined	(236)
Trimethylamine linked corrinoid:CoM methyltransferase	*mttA*	2.1.1.247	(182)
Glycine betaine linked corrinoid:THF methyltransfease	*mtgA*	2.1.1.378	(50)
Proline betaine/carnitine linked corrinoid:THF methyltransferase	*mtqA*	Not yet determined	(49)
Corrinoid co-factor protein	*mttC*	2.1.1.247	(182)

In the present study, we assessed the presence of genes coding for corrinoid methyltransferases for methylated amines in anoxic, sulfate rich Black Sea sediments. The Black Sea is the largest anoxic basin in the world and has been commonly used as a model site for anaerobic marine microbiology due to its permanent and stable euxinia, meaning its conditions are anoxic and highly sulfidic (74, 239–241). Black Sea sediments are rich in organic compounds, including methylated amines such as betaines or choline, and their fermentation products (e.g. TMA and MMA). Microbial community analyses of Black Sea sediments in combination with the cultivation of certain microbial representatives have revealed the presence of heterotrophic bacteria as well as SRM and methanogenic archaea (74, 242). These sediments harbor various metabolically interconnected microorganisms, presumably capable of converting organic matter to methylated compounds, which in turn can be consumed by acetogens, SRM, methanogens or other metabolic groups that are currently unknown.

To obtain a better overview on the microbial potential for the utilization of these compounds in the Black Sea, we sequenced the metagenome of Black Sea seafloor surface sediments (reaching a maximum depth of 30 cm divided into six individual depths, SI Table 1) and screened the recovered metagenome-assembled genomes (MAGs) for the presence of key genes coding for corrinoid methyltransferases and connected metabolic pathways.

3.2 Results & Discussion

3.2.1 Phylogenetic diversity of metagenome-assembled genomes from Black Sea sediments

In total, 394 MAGs of medium to high quality were recovered from the Black Sea sediment, according to the definitions of Bowers et al. (311). The average completeness of the MAGs is 78.6±14.2% with an average contamination of 3.4±3.5%. From this, 34 MAGs were classified as archaeal, while 360 were classified as bacterial. The archaeal MAGs were primarily assigned to *Asgardarchaeota* (37%) and *Thermoproteota* (37%). The bacterial MAGs were primarily assigned to *Chloroflexota* (33%), *Planctomycetota* (21.7%) and *Desulfobacterota* (11.4%) (figure 2). A non-metric dimensional scaling (NMDS) analysis on the diversity on phylum level between the different sampling depths revealed no observable patterns between depth and microbial distribution of the major observed bacterial and archaeal phyla (Supplementary figure S1). The presence or absence of methylated genes of interest did not statistically differ between the different sampling depths (Chi-square test, p-value 0.97). As such, the obtained data will be treated as one large dataset, representing a sampling of the upper 30 centimeters of Black Sea sediment as a whole. The phylogenomic classification and genome statistics of all recovered MAGs can be found in supplementary table S2.

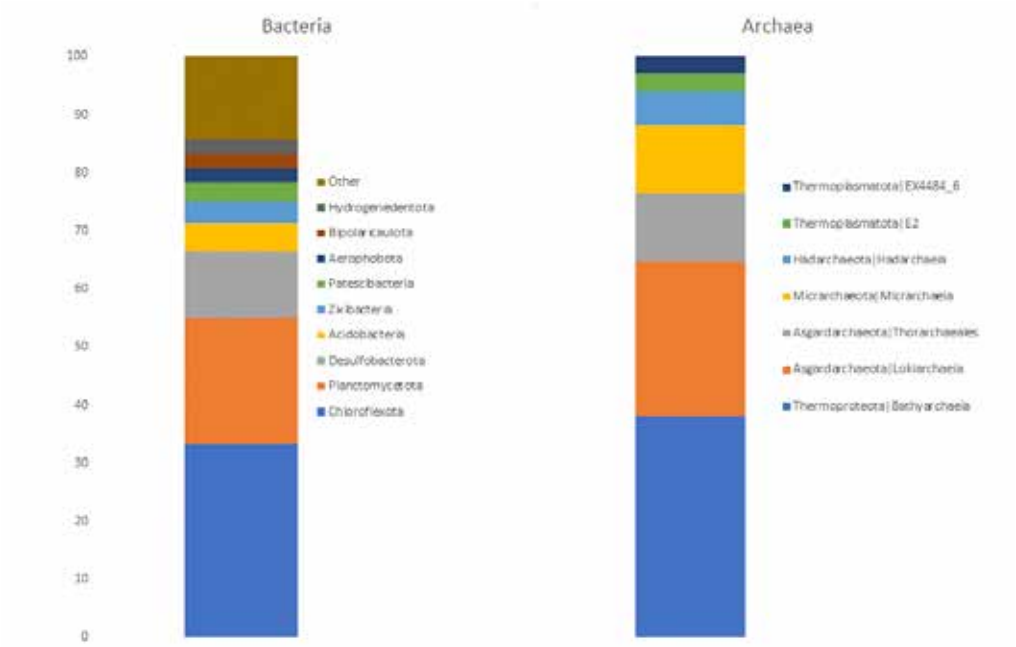

Figure 2: Percentages of total taxonomic classification of all recovered bacterial and archaeal aetagebine assembled genomes to A) phylum level in Bacteria (based on 360 bacterial genomes) and B) class level in Archaea (based on 34 archaeal genomes).

3.2.2 Detection of amine specific methyltransferases in metagenome assembled genomes

From the total recovered MAGs, 227 contained at least one homolog for the core methyltransferase families *mtmB*, *mtdB*. All further analyses were based on these 227 MAGs and detailed information about these MAGs and their key metabolic genes can be found in supplementary data S3. Of these MAGs, 216 contained at least one *mttB*-superfamily coding gene but only 6 MAGs harbored a putative *mttB* gene with an in-frame TAG codon, indicating a pyrrolysine residue in its protein. While only 1 MAG was found with a pyrrolysine coding mtmB gene coding for MMA methyltransferase (although this MAG contained no pyrrolysine biosynthesis genes), 33 MAGs were identified to contain a gene annotated as mtmB yet lacking the pyrrolysine residue. These genes will be referred to as *mtmB*-family coding genes henceforth. Furthermore, 7 MAGs were identified including the pyrrolysine containing DMA methyltransferase *mtdB* (figure 3).

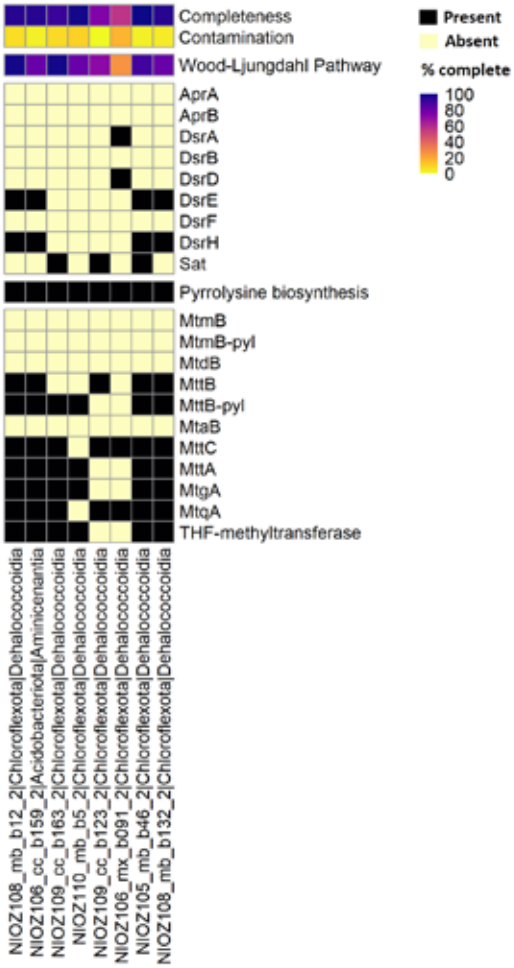

Figure 3: Summary of sulfur and methyltransferase metabolisms of metagenome assembled genomes containing complete pyrrolysine biosynthesis. Top rows indicate genome completeness, genome contamination and Wood-Ljungdahl pathway presence, respectively. Abbreviations: aprAB: APS reductase A and B; dsrABDEFH: dissimilatory sulfite reductase A, B, D, E, F and H; sat: sulfate adenylyltransferase; mtmB: corrinoid:methylamine methyltransferase B; mtmB-pyl: pyrrolysine containing corrinoid:methylamine methyltransferase B; mtmB-pyl: pyrrolysine coding corrinoid:methylamine methyltransferase mtdB: corrinoid:dimethylamine methyltransferase; mttB: corrinoid:trimethylamine methyltransferase B; mttB-pyl: pyrrolysine containing corrinoid:trimethylamine methyltransferase; mtaB: corrinoid:methanol methyltransferase; mttC: trimethylamine:corrinoid methyltransferase C; mtgA: glycine betaine linked corrinoid:methyltransferase A; mtqA: proline betaine/carnitine linked corrinoid:methyltransferase A; mttA: trimethylamine-linked corrinoid:methyltransferase A; THF-methyltransferase: tetrahydrofolate-linked methyltransferase. Full taxonomic affiliation of metagenome assembled genome IDs can be found in supplementary table S1.

Genes coding for proteins containing pyrrolysine coding methyltransferases demethylating MMA, DMA and TMA were rare in the analyzed Black Sea sediment metagenome, with only 6 MAGs containing either *mtmB*, *mtdB*, *mttB* with an in-frame TAG codon and a complete gene operon for pyrrolysine biosynthesis. All MAGs containing *mtmB* or *mtdB* also contained *mttB*. With the exception of one MAG belonging to *Acidobacteriota*, all MAGs with a pyrrolysine containing methyltransferase coding gene were classified as *Dehalococcoidia* within the phylum *Chloroflexota*. Pyrrolysine biosynthesis and its subsequent incorporation in corrinoid methyltransferases in bacteria is not well characterized. While many methanogenic archaea encode this pathway, most genomic descriptions of pyrrolysine biosynthesis in bacteria have been in SRM and acetogens. Only one bacterium, *Acetohalobium arabaticum*, has been shown to synthesize pyrrolysine and utilization of TMA in vivo (243, 244). Notably, none of the six MAGs identified in our study contained any genes for reductive sulfate or sulfite metabolism. However, the WL pathway was near-complete in two MAGs (only one gene missing) and mostly complete (2-3 genes missing) in all but one MAG. In the MAG with the least complete WL pathway, most of the methyl branch of the WL pathway is missing although the carbonyl branch is present (supplementary data S3). This might indicate that a connection between the utilization of methylated amines in these MAGs involves the WL pathway and is linked to acetogenic growth. However, in the genomes of putative sulfate-reducing *Desulfobacterota*, pyrrolysine biosynthesis coding genes were identified in marine sediments contaminated with industrial waste. This indicates that depending on environmental conditions, sulfate reduction may also be linked with the utilization of pyrrolysine (245).

The vast majority of putative corrinoid methyltransferases present in MAGs recovered from the top 30 cm of Black Sea sediment belonged to genes coding for proteins of the MttB-superfamily. In total, 216 MAGs contained at least one such gene, with many MAGs containing multiple homologs. Only four of these proteins in this superfamily have been described in detail: MtpB, MtcB, MtgB and MtyB (table 1). However, coding genes of potential members of the MttB-superfamily with an as yet unknown function have been described in hundreds of genomes from a wide range of environments (50, 54). Phylogenetic analyses and clustering of all MttB-superfamily coding gene homologs in our dataset reveal a potentially complex evolutionary history including horizontal gene transfer (HGT) and low influence of the taxonomy of a certain organism (figure 4). This could suggest a phylogenetic clustering based, at least in part, on function, i.e. based on substrates. Of the four known substrates affected by the proteins encoded by MttB-superfamily genes, the gene encoding

MtgB in *D. hafniense* clustered closely with the MtgB encoding gene in *M. vulcani*, supporting a function-based relationship (54). In contrast, *mtcB*, *mtpB*, and *mtyB*, all originating from *E. limosum*, clustered closely together. As such, enzyme substrate being the primary driver of the diversity of this family of genes seems unlikely but could be one of multiple driving forces in the diversity of the MttB-superfamily. Characterization of a wider range of proteins within this superfamily, including the testing of a broader range of quaternary amine substrates originating from a broader taxonomic range is necessary to resolve the complex history and functional diversification of the MttB-superfamily proteins and should resolve the low bootstrap support observed in the phylogenetic tree in figure 4.

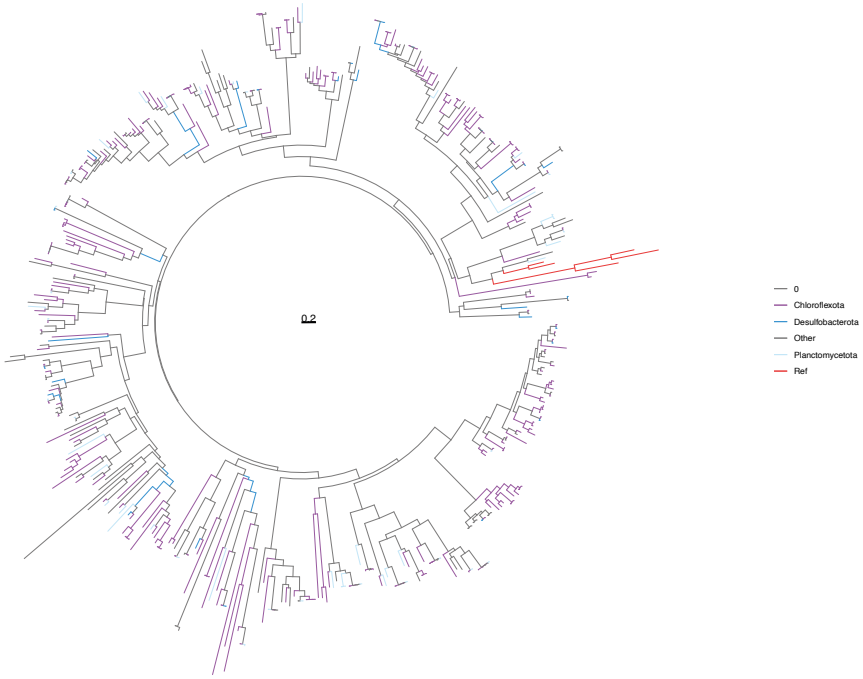

*Figure 4: Midpoint rooted phylogenetic tree of MttB-superfamily genes obtained from Black Sea sediment MAGs, with MtpB, MtcB, MtyB and MtgB coding genes as reference in red and phylogenetic labeling of the MAG of origin in purple (*Chloroflexota*), dark blue (*Desulfobacterota*), light blue (*Planctomycetota*) and dark gray (other phyla). The full tree in long format, with full phylogenetic origin of each gene, can be found in supplementary data S3.*

It is difficult to predict the potential substrates for demethylation used by the MttB-superfamily proteins encoded by the various MAGs in our dataset. Since all currently known MttB-superfamily coding genes are involved in demethylation of

methylated quaternary ammonium compounds, it seems likely that other genes in this family demethylate similar quaternary ammonium compounds. In marine algae, many of such compounds have been described, for example choline, alpha-alaninebetaine, ascophylline, N-acetylamine, hordenine or homarine (12). All of these compounds play a role in osmotic balance similar to glycine betaine. It is possible that a broad range of such compounds present in anoxic sediments serve as substrates for some of the herein described MttB-superfamily proteins, yet further research is needed to verify this hypothesis.

Besides MttB-superfamily gene homologs, we identified 35 putative homologs of MMA methyltransferases lacking pyrrolysine, which we here refer to as MtmB-family (figure 5). Interestingly, we observed no such family of genes for DMA methyltransferase homologs. The distribution of these genes was found in all of the most prominent phyla in our dataset, both archaeal and bacterial. Among previously isolated microorganisms with MttB-superfamily coding genes, similar MtmB-family gene homologs were found in the genomes of the methanogens *Methanomethylovorans hollandica* DSM15978, *Methanimicrococcus blatticola* DSM 13328, the acetogens *Sporomusa ovata* strain H1, *Acetohalobium arabaticum* strain Z—7288 and the SRM *Desulfobacterium vacuolatum* DSM5501 and *Desulfosporosinus orientis* DSM 765, amongst others. Our hypothesis is that genes in the MttB-family encode proteins demethylating quaternary ammonium compounds with both three (glycine betaine, carnitine) and two (proline betaine) attached methyl groups. Quaternary ammonium compounds with one attached methyl group, for example N-methyltaurine or

homarine, could be potential substrates for proteins encoded by members of the mtmB-family of genes.

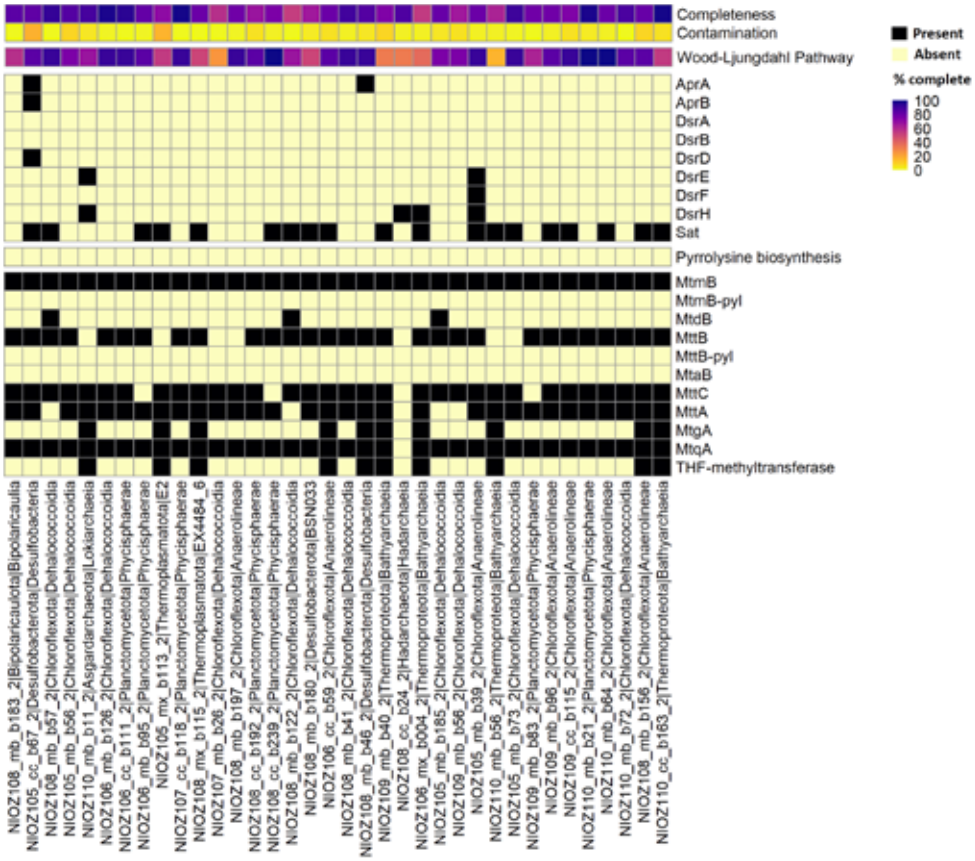

Figure 5: Summary of sulfur and methyltransferase metabolisms of metagenome assembled genomes containing at least one MtmB-family coding gene. Top rows indicate genome completeness, genome contamination and Wood-Ljungdahl pathway presence, respectively. Abbreviations: aprAB: APS reductase A and B; dsrABDEFH: dissimilatory sulfite reductase A, B, D, E, F and H; sat: sulfate adenyltransferase; mtmB: corrinoid:methylamine methyltransferase B; mtmB-pyl: pyrrolysine containing corrinoid:methylamine methyltransferase B; mtmB-pyl: pyrrolysine coding corrinoid:methylamine methyltransferase mtdB: corrinoid:dimethylamine methyltransferase; mttB: corrinoid:trimethylamine methyltransferase B; mttB-pyl: pyrrolysine containing corrinoid:trimethylamine methyltransferase; mtaB: corrinoid:methanol methyltransferase; mttC: trimethylamine:corrinoid methyltransferase C; mtgA: glycine betaine linked corrinoid:methyltransferase A; mtqA: proline betaine/carnitine linked corrinoid:methyltransferase A; mttA: trimethylamine-linked corrinoid:methyltransferase A; THF_methyltransferase: tetrahydrofolate-linked methyltransferase. Full taxonomic affiliation of of metagenome assembled genome IDs can be found in supplementary table S1.

MTI consists of the subunit encoded by *mtxB* as described above and a corrinoid

protein encoded by *mtxC*. This *mtxC* is present in the vast majority (89%) of MAGs described here. Use of methylated compounds relies on transfer of the methyl group bound to the Co(I) co-factor to an acceptor by MT2. This is best described in methanogens, where co-enzyme M (CoM) is the acceptor molecule. In the short list of acetogens and SRM utilizing the methyltransferase pathway, this acceptor is tetrahydrofolate (THF) (45, 225). Our results showed that among all characterized MTII, homologs of the carnitine and proline betaine-linked *mtqA* were the most common in the Black Sea metagenome, which was present in 160 MAGs. Furthermore, the glycine-betaine linked *mtgA* was present in 43 MAGs, *mtxA* (our model cannot not discriminate between MTII-A and MTII-M coding genes) was present in 88 MAGs and methylcobalamine-THF methyltransferase was present in 43 MAGs. Thus, it is likely that in the event of methyl transfer, these moieties can be transferred to the methyl acceptor by a range of substrate unspecific MT2, encoded by *mtqA* or another of the less present MT2 homologs.

In total, out of 227 MAGs containing a core *mtxB* methyltransferase gene, 198 contained both at least one *mtxC* gene and at least one *mtxA* gene, which suggests that the vast majority of the MAGs recovered from our metagenome has the potential to perform the complete methyltransferase pathway as is currently known. A total of 272 mtxB genes present in 134 MAGs are localized directly next to or less than 5 kb adjacent to a corrinoid protein coding gene *mtxC*. This would allow these genes to directly form a putative MTI complex. All other genes were not localized near any known genes in the methyltransferase pathways. In most described methyltransferase gene complexes, *mtxB* and *mtxC* are directly adjacent or close by in the genome, whereas mtxA can be located further in the genome, although it is not uncommon for it to be placed in the same operon. For example, in *Methanosarcina barkeri*, both methanol methyltransferase *mtaB* genes and the trimethylamine methyltransferase *mttB* are flanked directly by their corresponding corrinoid protein coding genes and the dimethylamine methyltransferase mtdB is located around 2 kb downstream. The gene coding for MTII-A is located directly adjacent to the genes coding for MTI, whereas the gene coding for MTII-M is located elsewhere in the genome, not close to any known gene involved in the methyltransferase pathway (226). Likewise, in *Desulfitobacterium hafniense*, *mtgC* and *mtgA* are both located directly adjacent to the glycine betaine methyltransferase coding *mtgB* (50). However, in *Eubacterium limosum*, where the utilization of the quaternary amines proline betaine, carnitine and γ-butyrobetaine was described, their corresponding MttB-superfamily coding genes *mtpB*, *mtcB* and *mtyB* are not localized in the vicinity of either their cognate corrinoid protein coding gene *mtqC* or *mtqA* whose products were highly

present in proteome studies (49, 51, 227). As such, while the observed co-localization of the majority of the *mtxB* genes with corrinoid protein coding *mtxC* genes strongly suggest a role in methyltransferase activity for these genes, the absence of such co-localization in the remainder of the genes does not necessarily negate such activity. Thus far, no functions other than metabolism-driven methyl transfer have been described for homologs of genes coding for MttB-superfamily proteins. Only a small fraction of these homologs have been described in detail and as such, it seems probable that as of yet unknown functions for MttB-superfamily proteins will be uncovered.

3.3.3 Phylogenetic and metabolic diversity of putative methylotrophic MAGs

Of the 227 MAGs containing a core methyltransferase, 16 belong to the domain Archaea, representing 40% of all recovered archaeal genomes and 214 to the domain Bacteria, representing 60% of all recovered bacterial genomes. The phylogeny of all recovered MAGs is summarized in supplementary data S2. Two phyla within archaea encoded potential methyltransferase genes, *Asgardarchaeota* and *Thermoproteota*, based on GTDB taxonomy. All *Asgardarchaeota* MAGs belonged to the class *Lokiarchaeota*, with the exception of one, which belonged to the class Thorarchaeia and all *Thermoproteota* belonged to the class *Bathyarchaeia*. Both *Asgardarchaeota* and *Bathyarchaeia* have been previously linked with the presence of corrinoid methyltransferases (60, 61, 246, 247). The bacterial MAGs encoding potential methyltransferases were dominated by three phyla: *Chloroflexota*, *Plancomycetota* and *Desulfobacterota* with 42%, 15% and 12% of all MAGs mapping to these phyla, respectively. Bacterial MAGs mapping to the phylum *Chloroflexota* represented two classes within this phylum: *Anaerolineae* (34.9% of all *Chloroflexota*) and *Dehalococcoida* (65.1% of all *Chloroflexota*). With the exception of two MAGs, which could not be assigned on a higher level than phylum, all *Planctomycetota* were placed within the class *Phycisphaerae*. Similarly, with the exception of two MAGs, all *Desulfobacterota* were placed within the class *Desulfobacteria*, of which 60% were placed assigned to the order *Desulfatiglandales*. Figure 6 gives an overview of the phylogenetic placement of all recovered MAGs and the presence of key genes involved in methylotrophy within all MAGs and backbone genomes.

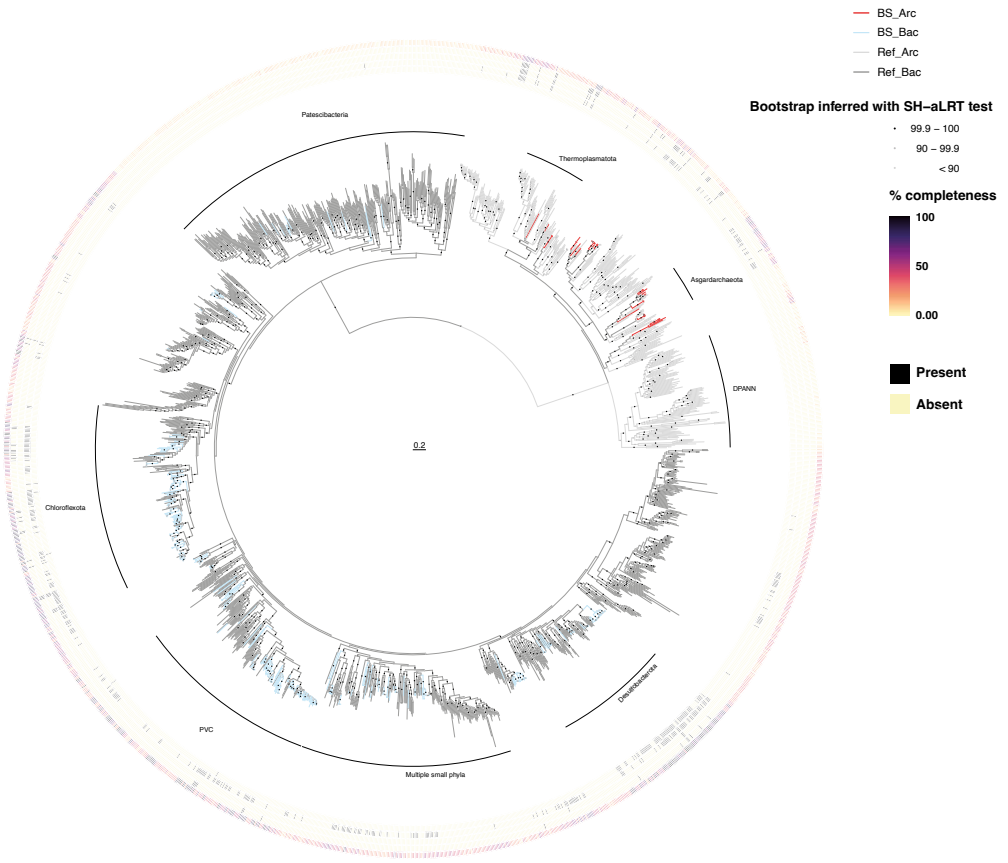

Figure 6: Phylogenetic tree of all recovered of metagenome assembled genomes in reference tree annotated with presence of key genes and pathways mentioned in this study for each individual genome. Key genes included in the heatmap are (from inside to outside): dsrD (presence/absence); mttB (presence/absence); mtdB (presence/absence); mtmB (presence/absence); Wood-Ljungdahl pathway (% complete). BS_Arc: recovered archaeal MAGs; BS_Bac: recovered bacterial MAGs; Ref_Arc: archaeal backbone genomes; Ref_Bac: bacterial backbone genomes. A long format of this tree can be found in supplementary data S4.

The phylum *Planctomycetota* has been previously linked with aerobic methylotrophy (248). In the planctomycete *Gemmata obscuriglobus*, a pathway has been described to utilize methylated compounds using the co-factor tetrahydromethanopterin(H_4MPT), predominantly found in archaea (249). In *G. obscuriglobus*, methyl groups are oxidized to formaldehyde by a dehydrogenase. This formaldehyde is then activated by the formaldehyde activating enzyme Fae (EC4.1.2.43), reacting with H_4MPT to form methylene-H_4MPT, which is then further oxidized to methe-

nyl-H_4MPT and formyl-H_4MPT, respectively, in reactions analogous to a reversed archaeal WL pathway. After this, formyl-H_4MPT is oxidized to formate and finally to CO_2 (249). Horizontal gene transfer is thought to be responsible for the presence of this pathway in aerobic *Planctomycetota* (250). While none of the *Planctomycetota* in the dataset described here contain any of the genes described in this pathway, three closely related MAGs assigned to the *Anaerolines* family within the phylum *Chloroflexota* contained the genes *mtd*, *mch* and the *fhc* complex for methylene-H_4MPT dehydrogenase (EC1.12.98.2), methenyl-H_4MPT cyclohydrolase (EC3.5.4.27) and formyltransferase/hydrolase complex (EC2.3.1.101;EC2.5.4.10), respectively. Only one of these three MAGs contained the gene coding for the formaldehyde activation enzyme FaeA, transferring formaldehyde to methanopterin. As formaldehyde is a toxic intermediate, microorganisms require strategies to mitigate the toxic stress associated with it. Thus, an alternative pathway of methylated compound utilization through methyltransferase to methyl-H_4MPT is plausible. After transfer of the methyl moiety to H_4MPT, the pathway as described *G. obscuriglobus* and other *Planctomycetota* seems a viable alternative in anoxic conditions.

Not all bacterial lineages in our dataset have been linked previously to the utilization of methylated amines. Currently, this physiological trait has mostly been described to occur in members of the *Clostridiales*, *Actinobacteria*, *Spirochaetes* and *Desulfobacterota* (25, 251). Many representatives of these classes of bacteria are either sulfur reducers or acetogens. The class *Dehalococcoidia* has previously been suggested to perform reductive dehalogenation (252, 253). A corrinoid dependent methyltransferase has been described to be utilized in *Methylobacterium* to demethylate chloromethane and cobalamin is a common co-factor in other reductive dechlorination pathways (254–256). This could suggest a role of MttB-superfamily proteins in the reductive dehalogenation of methylated halogens. However, the methylchloride:THF methyltransferase observed in *Methylobacterium* has little homology with other members of the MttB-superfamily.

None of the MAGs belong to canonical methanogens, although several *Bathyarchaeota* were recovered, some of which are known to encode genes from the methanogenesis/reverse methanogenesis pathway (128, 171, 257). However, *Bathyarchaeota* retrieved MAGs in this study did not contain the key methanogenesis gene *mcrA*, coding for methyl-coenzyme M reductase. While the unbinned metagenomic assembly appeared to contain one homolog of *mcrA*, it is unclear to which MAG this belongs and whether it occurs in the context of methanogenic or methanotrophic metabolism. Utilization of methylated compounds in the sulfate reduction zone of

anoxic marine sediments has primarily been linked with methanogenesis rather than with the more dominant sulfate-reducing or acetogenic metabolisms (43, 52, 178, 184, 221). Most environmental studies on methylotrophic marine methanogens have focused on coastal and therefore shallow water sediments (258–260). However, sediments underlying deeper water columns are described to contain methylotrophic methanogens, for example in the South China Sea at 1457 meters below sea level or the Gulf of Mexico at 2400 meters below sea level (42, 88). In these studies, relatively high concentrations of methanol and TMA were measured near the sediment-water column interface, which declined over a depth of 100 centimeters of sediment. Thus, it is possible that methylotrophic methanogens occur in deeper sediments rather than in the direct upper sediments that were investigated in this study. Whether this is in co-occurence with sulfate-reducing and acetogenic bacteria remains to be elucidated.

Acetogens utilize methylated compounds through the WL pathway. Our results showed 17 MAGs with the complete pathway (either bacterial or archaeal) and an additional 42 MAGs contain an almost complete WL pathway, although the specific missing gene differs between MAGs (figure 7, supplementary data S3). MAGs with a complete or near-complete WL pathway belong to the *Chloroflexota*, *Planctomycetota* and *Desulfobacterota*. All but two of these contain at least one gene coding for a member of the MttB-superfamily or MtmB-family. Methyl groups can be incorporated directly into the WL pathway through linkage to THF, forming methyl-THF. While currently, there is no experimental evidence for further incorporation of this methyl-THF group in the WL pathway, it is probable that this is similar to the fate of the well-described methyl-THF originating from methanol in *E. limosum* (261, 262). Therefore, it is likely that the microorganisms represented in our dataset with a (near) complete WL pathway are able to use proteins encoded by corrinoid methyltransferase genes to incorporate methyl groups through THF in the WL pathway. Transfer of the methyl group to THF in putative acetogens from our dataset could be mediated by either proteins encoded by *mtaA* and THF-methyltransferase gene but mainly through proteins encoded by *mtqA* (figure 7).

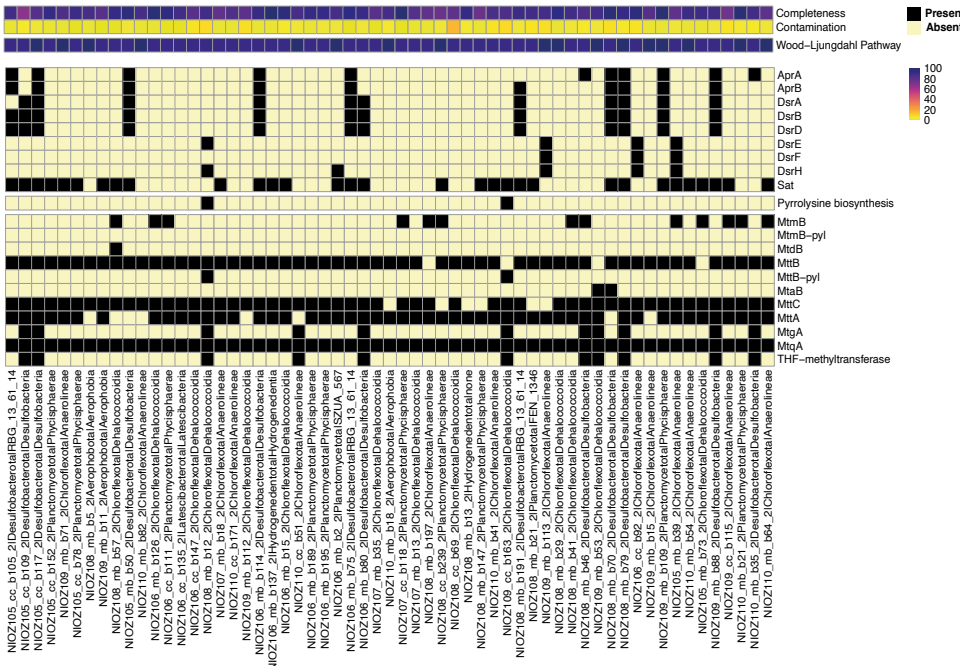

Figure 7: Summary of sulfur and methyltransferase metabolisms of metagenome assembled genomes containing a complete or near-complete Wood-Ljungdahl pathway. Top rows indicate genome completeness, genome contamination and Wood-Ljungdahl pathway completeness, respectively. Abbreviations: aprAB: APS reductase A and B; dsrABDEFH: dissimilatory sulfite reductase A, B, D, E, F and H; sat: sulfate adenylyltransferase; mtmB: corrinoid:methylamine methyltransferase B; mtmB-pyl: pyrrolysine containing corrinoid:methylamine methyltransferase B; mtmB-pyl: pyrrolysine coding corrinoid:methylamine methyltransferase mtdB: corrinoid:dimethylamine methyltransferase; mttB: corrinoid:trimethylamine methyltransferase B; mttB-pyl: pyrrolysine containing corrinoid:trimethylamine methyltransferase; mtaB: corrinoid:methanol methyltransferase; mttC: trimethylamine:corrinoid methyltransferase C; mtgA: glycine betaine linked corrinoid:methyltransferase A; mtqA: proline betaine/carnitine linked corrinoid:methyltransferase A; mttA: trimethylamine-linked corrinoid:methyltransferase A; THF_methyltransferase: tetrahydrofolate-linked methyltransferase. Full taxonomic affiliation of metagenome assembled genome IDs can be found in supplementary table S1.

Fourteen MAGs, most of which classified as *Desulfobacterota*, contained the full gene set for sulfate reduction to sulfide (dissimilatory sulfite reductase AB (*dsrAB*, EC1.8.99.5); dissimilatory sulfite reductase D (*dsrD*); APS reductase AB (*aprAB*); sulfate adenylyltransferase (sat, EC2.7.7.4), supplementary table S3). A further fifteen MAGs contained an incomplete gene set coding for sulfate reduction, but contained the key activator gene *dsrD* (27). While there are no known pathways of methylated amine metabolism in SRM, in *D. kuznetsovii*, proteomic evidence was given for the transfer of a methyl group from methanol to THF, followed with subsequent oxidation through a reversal of the WL pathway in combination with the reduc-

tion of sulfate to sulfide (45). This transfer was mediated by MtaA, rather than the THF-methyltransferase as described in acetogens such as in *S. ovata* strain An4 (123). All but two MAGs with genes for sulfate reduction have a putative *mtxA* gene. Only four MAGs in the full metagenome contain genes for sulfate reduction without an MttB-superfamily coding gene present. Figure 8 shows the metabolic summary of all MAGs involved in sulfur metabolism.

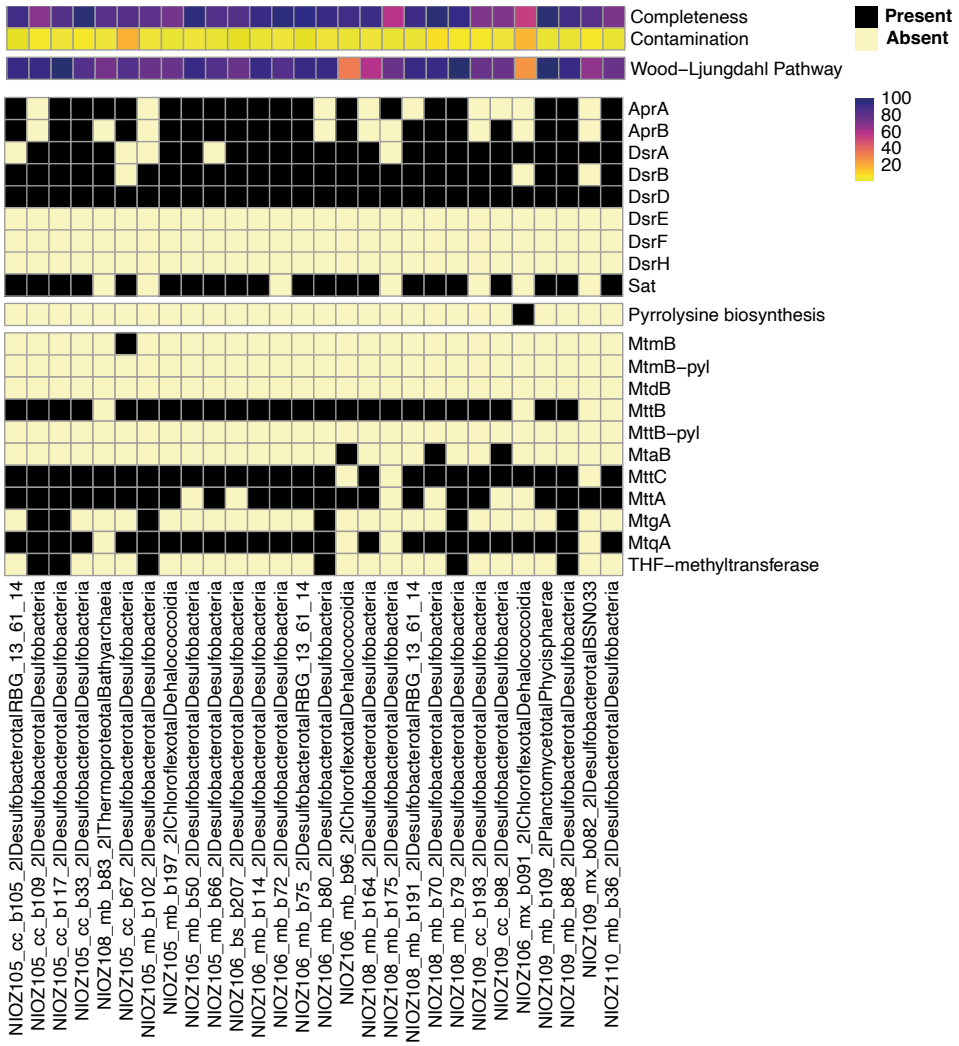

Figure 8: Summary of sulfur and methyltransferase metabolisms of metagenome assembled genomes containing complete or near-complete sulfate reduction pathway and at least one corrinoid methyltransferase coding gene. Abbreviations: dsrAB: dissimilatory sulfite reductase AB; dsrD: dissimilatory

sulfite reductase D; aprAB: APS reductase; sat: sulfate adenylyltransferase; asrABC: anaerobic sulfate reductase ABC; dsrEFH: dissimilatory sulfite reductase EFH; mtmB: corrinoid:methylamine methyltransferase B; mtmB-pyl: pyrrolysine containing corrinoid:methylamine methyltransferase B; mtdB: corrinoid:dimethylamine methyltransferase; mttB: corrinoid:trimethylamine methyltransferase B; mttB-pyl: pyrrolysine containing corrinoid:trimethylamine methyltransferase; mttA: trimethylamine-linked corrinoid:methyltransferase A; mtgA: glycine betaine linked corrinoid:methyltransferase A; mtqA: proline betaine/carnitine linked corrinoid:methyltransferase A; mttC: trimethylamine:corrinoid methyltransferase C. Values indicate the proportion of completeness of the pathyway. Taxonomic affiliation of metagenome assembled genome IDs can be found in supplementary table S1.

Although anaerobic methylotrophy has been canonically linked with methanogenesis, acetogenesis and sulfate reduction, the majority of the MAGs with a putative methyltransferase homolog in our dataset did not contain complete gene sets linked to either of these metabolisms. The utilization of methylated compounds in general, and quaternary amines specifically, has been suggested to be an important aspect of marine sediments and deep-subsurface coal and shale beds communities (263, 264). Thus far, little physiological research has been conducted on the utilization of methylated compounds, while genes for anaerobic methylotrophy have been found in an increasing range of MAGs (60). Members of a reconstructed potential methylotrophic group of archaea, *Brockarchaeota*, was predicted based on the gene content of reconstructed MAGs to convert methyl-THF generated through corrinoid methyltransferases to methenyl-THF. This methenyl-THF can then be incorporated in the reductive glycine pathway (59). Our work indicates that anaerobic methylotrophy is more widespread than previously though. Investigating alternative pathways that allow microorganisms to utilize methylated compounds, such as the pathway described above, will be an interesting avenue for forthcoming research.

3.3.4 Ecological context

The biogeochemical conditions of our study site, Black Sea sediments, are well described, although the microbial composition is not well-known. The upper layer, from the sediment-water interphase up to ~200 cm in depth are rich in organic material and sulfate, while nitrate is absent and any iron and manganese minerals are in highly reduced form due to settling with sulfide in the water column (20). As such, the major microbial metabolism driving organic material remineralization has been considered to be sulfate reduction, which is supported by a wide range of *dsrAB* marker gene amplicons obtained from Black Sea sediments (74). An investigation in active cells using qPCR likewise revealed Black Sea sediments harbor large quantities of *dsrAB* as well as *mcrA* genes while FISH and cell counts revealed the presence of equal amounts of archaeal and bacterial cells, with a high presence of *Chloroflexi*, *Anaerolineae* and *Caldilinaea* (265). Similar patterns in archaeal/bacterial distribution have been observed elsewhere (266). The bacterial MAGs in our dataset follow

similar patterns in taxonomic distribution as observed in earlier Black Sea investigations, as well as global patterns in anoxic marine sediments (267). This suggests that while our dataset is likely representative in bacterial diversity in anoxic marine sediments, it is likely lacking in representation on archaeal diversity. This could furthermore be an explanation about the lacking data on methanogenic archaea in this dataset.

The availability of methylated compounds in Black Sea sediments is not known. To our knowledge, no investigation in the distribution of quaternary amines in sediments has ever been undertaken. However, the distribution of glycine betaine and choline in coastal waters has been studied, which was found to be 9.2 and 0.5 nmol, respectively in the Millbay marina and 0.9 and 0.2 nmol in a nearby coastal sampling station (218). In the North Pacific Transition Zone, glycine betaine was found to constitute up to 17% of labile organic matter (268). These concentrations are similar to observed TMA concentrations in seawater (19). In sediments, TMA concentrations are significantly higher than in seawater, measured in micromolar scale. As quaternary amines are the primary source of TMA, it can be extrapolated that likewise, in marine sediments, quaternary amine concentrations are similar to observed TMA concentrations (19). Thus, a large pool of quaternary amines is likely available for metabolism by methyltransferase-containing microorganisms. However, pore water extractions are needed to fully determine the availability and potential cycling of these compounds.

3.4 Conclusions

Our results suggest that microorganisms from Black Sea marine anoxic sediments have the genetic capability of utilizing a wide range of methylated amines. We present genomic evidence for a wider range of potential genes involved in the canonical corrinoid methyltransferase system. A family of gene homologs to the methylamine methyltransferase yet lacking the characteristic pyrrolysine residue may perform similar functions to the more widely described family of genes homologous to trimethylamine methyltransferase. These genes were found in MAGs connected with the genetic capability for canonical anaerobic methylotrophic metabolism: sulfate reduction and acetogenesis. However, most genomes in our dataset did not encode metabolic pathways known to be linked with the utilization of methylated amines. Thus, describing these metabolisms in greater detail using both genomic and physiological approaches will be an important endeavor to further understand the cycling of methylated amines and the contribution of these processes to global carbon and nitrogen cycles. This metagenomic survey of the microbial genetic capabilities of methylated amine utilization in anoxic marine Black Sea provides a basis for the

identification, isolation and physiological characterization of novel microbial groups metabolizing marine methylated compounds to unravel the relative importance of methylated amine cycling in marine sediments.

3.5 Experimental procedures

3.5.1 Sampling, assembly, binning & taxonomic affiliation

Sediment samples were collected from the western basin of the Black Sea in Summer 2018 during R/V Pelagia cruise number 64PE444 from station 2 (42° 53.8' N 30° 40.7' E) with a multicore and cut into slices of five cm each using a core extruder. Slices were stored in geochemical bags and stored at -80°C until further processing.

Total DNA was isolated using the Qiagen DNAeasy PowerSoil. DNA concentrations were measured using a Qubit™ Fluorometer, and a final concentration of 4 ng/µl of each sample (using a total amount of 200 ng) was used to prepare libraries for Novaseq sequencing. Library preparation and sequencing was performed at the Utrecht Sequencing Facility (USEQ) on an Illumina Novaseq 6000 with a S4 flowcell shared with other samples and with the following specifications: 2x150 bp reads, and shearing of 350bp.

Sequences were trimmed using trimmomatic v0.36 (settings: ILLUMINACLIP:TruSeq3-PE-2.fa:1:30:10 SLIDINGWINDOW:5:22 MINLEN:100) (269) and individually assembled with megahit v1.2.8 using default settings. Contigs shorter than 2500 bp were discarded for subsequent steps. Coverage information was obtained by generating mapping files for each assembly file by mapping all high-quality reads of all samples against the assembly of one sample using the BWA-MEM algorithm in paired-end mode (bwa-0.7.17-r1188 using default settings) (270). The resulting sam files were sorted and converted to bam using samtools version 0.1.19 (271). Depth files required for the binning tools were generated using jgi_summarize_bam_contig_depths (available with the metabat tool) (272)

Metagenomic binning was performed on individual assemblies using the binning tools metabat v2.12.1 (272) concoct v1.1.0 (273), maxbin v2.2.4 (274) and BinSanity v0.2.6.4 (275) using default settings. Results from the four different binning tools were combined using DAS Tool v1.1.0 (276) using default settings. The accuracy of the binning approach was evaluated by calculating the percentage of completeness and contamination using CheckM lineage_wf v1.0.11 (277) and an initial taxonomic assignment was generated using gtdbtk classify_wf v0.3.2 (using GTDB-Tk reference

data version r89)(278). MAGs were included in further analysis at a completeness of >40% and contamination of <20%.

MAGs were cleaned by removing contigs if (a) the contig had a GC difference of 10% compared to the average MAG GC content, (b) the relative abundance of a contig differed by 0.5x compared to the average relative abundance of the MAG or (c) if, for an archaeal MAG, all proteins on a contig were assigned to bacteria (and vice versa). For (c) protein-coding genes were predicted using prodigal (settings: -p meta) (279) and all proteins were used as a query against the NCBI_nr database with diamond and the following settings: diamond blastp -q genomes.faa--more-sensitive --evalue 1e-5 --seq 50 --no-self-hits --db nr.dmnd --taxonmap prot.accession2taxid.gz --outfmt 6 qseqid qtitle qlen sseqid salltitles slen qstart qend sstart send evalue bitscore length pident staxids (280).

3.5.2 Identification of selected genes

After initial genome annotation, additional annotation for genes of interest was performed using HMMer suite 3.3 (281). Hidden Markov models (HMMs) were utilized as follows: for *mttA* (TMA methyltransferase A), *mttB* (TMA methyltransferase B), *mttC* (TMA methyltransferase C). *mtmB* (MMA methyltransferase B) and *mtbB* (DMA methyltransferase B) TIGRFAM HMM profiles were utilized: TIGR01463; TIGR02369; TIGR02370; TIGR02367 and TIGR02368 respectively (215). For *mtaB* (methanol methyltransferase B) the pfam profile PF12176 HMM was used (214). Sequence cutoffs were determined following the recommended thresholds as suggested previously (282). For mtgA, mtqA and THF-linked methyltransferase A custom HMM profiles were created. Sequences for these genes were obtained from NCBI from the genomes of *Desulfitobacterium hafniense*, *Eubacterium limosum* and *Sporomusa* strain An4, respectively (50, 123, 227). High sequence similarity homologs were obtained by querying against the NCBI nr database with diamond. Sequences were aligned with mafft v7.453 (settings –localpair –maxiterate 16) (283). The obtained alignments were then built in an HMM profile using hmmbuild of the HMMer suite at default settings. Noise and trusted cutoffs for all HMMs were manually inspected by mapping profile HMMs to a curated database of sequences and updated when necessary.

Genes coding for putative pyrrolysine biosynthesis were identified in all genomes using TIGRfam hmm profiles for *pylB*, *pylC* and *pylD*; TIGR03910, TIGR03909 and TIGR03911. MAGs with positive hits for all three genes were manually inspected for presence of *pylS* and tRNA-pyl. All ORFs assigned as methyltransferase genes in

MAGs with a complete pyrrolysine biosynthesis pathway were manually inspected for the presence of the TAG codon encoding pyrrolysine.

Sulfur cycle metabolism in all MAGs was identified by screening MAGs for the presence of the genes as proposed by Anantharaman et al (28) for sulfate reduction and sulfur oxidation. TIGRFAM hmm profiles were utilized for all genes, with the exception of dissimilatory sulfite reductase D (*dsrD*) where the HMM profile created by Anantharaman et al (28). was used. The genes constituting the Wood-Ljungdahl were determined through kofamKOALA (282). All utilized HMM profiles can be accessed in our data repository.

3.5.3 Phylogenetic assessment of MttB-superfamily genes

A phylogenetic tree of all ORFs from our dataset assigned as trimethylamine methyltransferase B without pyrrolysine was constructed, and included the sequences for *mtyB*, *mtgB*, *mtpB* and *mtcB* for γ-butyrobetaine, glycine betaine, proline betaine and carnitine methyltransferase, respectively (49–51, 227). All sequences longer than 420 amino acid residues were aligned with mafft-linsi v7.429 (settings: --localpair –maxiterate 16) (283) and trimmed using BMGE v1.12 (settings: -m BLOSUM30 -h 0.55) (284). The output of this was used to construct a Maximum likelihood phylogeny using IQ-tree version 1.6.12 (settings: --mset LG madd LG+C20+R+F --bb 1000) (285). A final figure was generated in R using packages ggtree, phytools, phangorn, tidyr and treeio and finalized using Adobe Illustrator (286–289). All raw sequences, alignment and the files generated by IQ-tree can be accessed in our repository.

3.5.4 Phylogenetic inference of a species tree

To generate a representative archaeal reference set all archaeal genomes were downloaded from NCBI (January 2019). Genomes with a completeness >40% and contamination <25% (determined with CheckM lineage_wf v1.0.11) (277) were used for an initial phylogenetic analysis using the PhyloSift marker set. In brief, homologs of 34 PhyloSift marker proteins were identified in each genome using the PhyloSift v1.0.1 'find' mode (settings: --besthit) (290). Subsequently, marker proteins were individually aligned using MAFFT L-INS-i v7.407 (settings: --reorder) and trimmed using BMGE v1.12 (settings: -m BLOSUM30 -h 0.55) (283, 284). The concatenated protein alignment was used to reconstruct a phylogenetic tree using IQ-TREE v1.6.7 (settings: -m LG -nt AUTO -bb 1000 -alrt 1000). Based on this tree, a sub-selection of 379 archaeal reference genomes was generated, which covers the archaeal phylogenetic diversity and still allows to perform computationally intensive phylogenetic analyses needed.

To generate a bacterial backbone, we downloaded a list of available genomes from NCBI in 2018 [https://ftp.ncbi.nlm.nih.gov/genomes/genbank/bacteria/assembly_summary.txt]. This list was parsed to only include type strains or representative genomes. From this list we randomly selected a representative strain per genus. In a second step, we selected 15-30 genomes from candidate phyla from the unparsed list. This initial set was screened for completeness and contamination in CheckM and genomes with less than 50% completeness and more than 10% contamination were excluded. Initial phylogenies were generated using alignments generated by PhyloSift using the PhyloSift search, align and name algorithms to find marker proteins. Individual genes were aligned with MAFFT L-INS-i (settings: --reorder) and trimmed using BMGE (settings: -m BLOSUM30 -h 0.55). The concatenated protein alignment was used to reconstruct a phylogenetic tree using IQ-TREE v1.6.7 (settings: -m LG -bb 1000 -alrt 1000). The initial alignments were screened for long-branches and genomes that did not fall into the expected position in the tree were removed from the taxa selection resulting in a set of 3020 bacterial genomes. Based on this tree, a sub-selection of 1140 bacterial reference genomes was generated, based on genomes with the highest completeness and lowest contamination scores, covering the bacterial phylogenetic diversity and still allows to perform computationally intensive phylogenetic analyses needed.

For all recovered MAGs and backbone genomes, coding DNA sequences were obtained through de novo annotation using Prokka v. 1.13 (291). Genomes were annotated using the HMMer suite v3.3 (settings: hmmsearch) using the Clusters of Orthologous Genes (COGs) database as reference models (281, 292). For multiple positive COG hits on a single gene, the COG with the highest bitscore was used as annotation and highest e-value if bitscores were identical. From each annotation, 24 conserved marker proteins were extracted and each individual marker protein was aligned with MAFFT L-INS-i (settings: --maxiterate 16 –reorder) and trimmed using BMGE (settings: -m BLOSUM30 -h0.55). Individual phylogenetic trees for each marker gene alignment were created in FastTree version 2.1 (settings: -lg) (293). Initial alignments were screened for long-branches and genes that did not fall within expected positions in the trees were removed from the selection. After initial alignment, all marker genes were concatenated using catfasta2phyml.pl (https://github.com/nylander/catfasta2phyml). A phylogenomic tree was created on the concatenated alignment using IQtree version 2.0.6 (settings: -m LG+C60+F+R -bb 1000 -alrt 1000 -safe) (285).

The phylogenomic tree was analyzed and visualized in R using a combination of the packages ggtree, phytools, phangorn, tidyr and treeio (286–289). Presence of key

methylated genes and carbon metabolism was analyzed on all backbone genomes as above and visualized on the tree using ggplot2 and edited in Adobe Illustrator. All raw gene sequences, trimmed and untrimmed alignments and IQ-tree output, as well as a long form tree can be accessed in our data repository.

3.6 Data availability

All (intermediate) data and supplementary files can be accessed in our data repository: https://doi.org/10.6084/m9.figshare.c.5955198

3.7 Funding

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Comparative transcriptomics of a methylotrophic methanogenic enrichment capable of degrading tetramethylammonium

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Abstract

Methylotrophic methanogenesis, the production of methane from methylated compounds by methanogenic archaea, significantly contributes to the global emissions of the potent greenhouse gas methane. Tetramethylammonium is a methylated compound that can be converted to methane and ammonium by methanogens. However, the physiology of the microorganisms capable of this conversion is not yet clear, and thus far no gene coding for a tetramethylammonium methyltransferase has been identified. In this study, we have enriched from marine sediments a methylotrophic methanogenic culture dominated by an archaeon of the genus *Methanococcoides*, capable of methane production from different methylated compounds, including trimethylamine and tetramethylammonium. Through a transcriptomic approach, we detected upregulation of a non-pyrrolysine coding homolog of the monomethylamine methyltransferase in enrichments growing on tetramethylammonium. We hypothesize this gene codes for a tetramethylammonium:corrinoid methyltransferase. These results expand our knowledge on the utilization of tetramethylamine by methanogenic archaea.

4.1 Introduction

Methanogenesis, the biological production of methane by methanogenic archaea, is a major contributor to global methane emissions, with an estimated 70% of worldwide methane emissions originating from these microorganisms (29). Traditionally, methanogenic archaea are divided in three metabolic groups, hydrogenotrophic methanogens, using hydrogen/CO_2 for growth; acetoclastic methanogens, cleaving acetate and methylotrophic methanogens, utilizing a range of methylated compounds. However, the range of substrates for methanogenesis has been expanded to include compounds such as CO, ethanol or methoxylated aromatic compounds (34). In sulfate rich anoxic marine sediments, only methylotrophic methanogenesis is thought to occur, as sulfate-reducing microorganisms outcompete hydrogenogenic and acetoclastic methanogens (40). Small methylated compounds, such as methanol or trimethylamine (TMA), have been shown to be utilized by methylotrophic methanogens in sulfate rich marine sediments through cultivation, genomics and in-situ measurements (43, 58). Furthermore, common methylated osmolytes such as betaine and choline can serve as direct substrate for methylotrophic methanogenesis (53, 54).

Methylotrophic methanogenesis is distributed in a limited range of archaeal orders: *Methanomassiliicoccales*, *Methanobacteriales*, *Methanonatronarchaeles* and *Methanosarcinales*, respectively (294–297). Additionally, some members of the *Candidatus* phylum *Verstraetearchaeota* are also predicted to perform methylotrophic methanogenesis based on metagenomic data (31). Methylotrophic methanogens utilize a set of substrate specific proteins, i.e. corrinoid methyltransferases. The key enzyme of methanogenesis is methyl co-enzyme M reductase (MCR). This enzyme reduces methyl-CoM to methane and a disulfide of co-enzyme S and CoM (CoM-S-S-CoB). This reduction is then coupled to the generation of a trans-membrane electrochemical ion gradient utilized for energy conservation. The best described methylotrophic methanogenic pathway couples the oxidation of one molecule of the methyl group of the methylated compound to CO_2 with the reduction of three molecules of methyl-CoM (35). Additionally, several methylotrophic methanogens using formate or hydrogen as electron donor have also been described, converting all methylated substrate to methane (296, 298–300). All methylotrophic methanogens produce methyl-CoM through a class of substrate specific enzymes, corrinoid methyltransferases, that transfer the methyl group of the methylated compound to co-enzyme M. For most methylated compounds, the corrinoid methyltransferase system consists of two separate proteins: MTI and MTII. However, dimethylsulfide methyltransferase consists of a fusion of MTI and MTII in one protein (301). MTI comprises two subunits, a substrate-specific methyltransferase, encoded by *mtxB* (where x denotes substrate,

e.g. *mttB* for TMA methyltransferase) and a corrinoid co-factor, encoded by *mtxC*. MTII facilitates the transfer of the methyl moiety from the corrinoid co-factor to CoM and is encoded by *mtxA* (124, 302). Methyltransferases involved in methyl cleavage of the tertiary, secondary and primary amines trimethylamine, dimethylamine (DMA) and methylamine (MMA) are unique due to the incorporation of the 22nd amino acid, pyrrolysine. This amino acid performs a crucial role in the catalytic center of these methyltransferases (55).

Methylated quaternary amines are likewise utilized by methylotrophic methanogens, although these are much less described than simple methylated compounds such as methanol or TMA (52, 178). Interestingly, all quaternary amine methyltransferases currently described are close homologs to trimethylamine:corrinoid methyltransferase coding *mttB*, yet lack the characteristic pyrrolysine residue which is crucial for demethylating TMA (50, 50, 51, 54, 227). A class of genes homologue to monomethylamine:corrinoid methyltransferase *mtmB*, also lacking pyrrolysine residues has been described to occur in a range of genomes of methylotrophic organisms, although their function is currently unknown (chapter 3). Due to the close co-localization of corrinoid protein coding genes in the genomes described to contain *mtmB*-family genes, they are suspected to be involved in methyl:corrinoid transfer. However, the specific compounds on which the proteins encoded by these genes are active are currently unknown.

The simplest quaternary amine, tetramethylammonium, can serve as suitable donor for methanogenesis (235, 303). While a 42 kDa protein was identified to transfer a methyl group from a corrinoid co-factor to the co-enzyme M in *Methanococcoides* strain NaT1, this strain was lost before its genome could be sequenced and thus no tetramethylammonium:corrinoid methyltransferase gene has yet been identified (303, 304). No other studies have been conducted thus far to uncover the genetics and enzymology of methanogenesis from tetramethylamine. As such, it is currently an open question whether tetramethylammonium:corrinoid methyltransferase contains a pyrrolysine residue, or whether it is a different enzyme altogether. In the present study, we have obtained a highly enriched community from deep sea Black Sea sediments, capable of methylotrophic methanogenesis on the quaternary amine tetramethylammonium. This enrichment was dominated by a novel methanogenic archaeon within the genus *Methanococcoides*, for which we propose the name *Candidatus* Methanococcoides chernomoris. Through a transcriptomic approach, we aimed to detect upregulated genes under conditions with tetramethylammonium as sole methanogenic substrate. A non-pyrrolysine coding member of the *mtmB*-family was identified in *Ca*. Mc. chernomoris that we hypothesize is involved in the conver-

sion of tetramethylammonium. These findings further our understanding of methyltransferase families involved in methylotrophic methanogenesis.

4.2 Materials and methods

4.2.1 Sediment sampling and microbial enrichment setup

Sediment was collected from the Black Sea station 1 (42° 53.8' N 30° 40.7' E, Bulgarian exclusive economic zone) in August 2018 during the 64PE444 research cruise of R/V Pelagia at 2000 meters of water depth. Sediment cores of 10 cm in diameter and 30 to 45 cm in length were collected with a multicorer, sliced in 1 cm slices and immediately transferred into a glovebag (Aldrich© AtmosBag) flushed with N_2. Sediment slices of 29-30 cm below sea floor level were transferred to sterile 1L bottles and mixed with 500 mL anoxic reduced basal medium as described previously (240), yielding about 600 mL of sediment slurry. Basal medium consisted of (g L^{-1}) NaCl 17.16; KCl 0.3715; NaBr 0.0557; NH_4Cl 0.1548; K_2HPO_4 0.5653; $MgCl_2$ 3.511; $CaCl_2$ 0.091; sulfide 0.360. Medium was amended with 1 mL L^{-1} of vitamin solution consisting of (mg L^{-1}) biotin 20; nicotinic acid; 200; pyridoxine 300; riboflavin 50; thiamine 200; cyanocobalamin 100; p-aminobenzoic acid 80; pantothenic acid 100; lipoic acid 30 and folic acid 80. Additionally, 1 mL L^{-1} each of acid and base trace element solution was added. Acid trace element solution consisted of (mg L^{-1}): H_3BO3 10; $MnCl_2$ 53; $FeCl_2$ 943; $CoCl_2$ 42; $ZnSO_4$ 70; $NiCl_2$ 25; $CuCl_2$ 20; KI 10 $SrCl_2$ 10; $CeCl_3$ 10 and 13 mL L^{-1} HCl 25% and base element solution consisted of (mg L^{-1}): Na_2MoO_4 36; Na_2WO_4 8; Na_2SeO_3 6; Na_3VO_4 1; NaOH 400. Additionally, 1 mL L^{-1} 0.1% w/v Na-resazurin solution was added as redox indicator. The obtained sediment slurry was used immediately to create enrichment cultures by transferring 5 mL of sediment slurry to 45 mL anoxic reduced basal medium. The enrichment culture was amended with 20 mM sodium molybdate to inhibit sulfate reduction and 10 mM methanol as carbon and energy source from anoxic, separately autoclaved 1 M stocks. Serum bottles of 117 mL were used, sealed with butyl rubber stoppers (Rubber BV, Hilversum, the Netherlands) and capped with aluminum caps using a 100% N_2 gas as headspace at 1.5 atmosphere. Sediment enrichments were stored at Black Sea *in-situ* temperatures of 10°C for 80 days for transport. After arrival, the headspace was replaced with a mixture of 80/20 (v/v) N_2/CO_2 headspace at 1.7 atmosphere and enrichments were incubated at 20°C. Enrichments were monitored for depletion of carbon source, and then transferred to fresh medium using 10% (v/v) inoculum. After 8 subculturing cycles, 50 mg L^{-1} of kanamycin and vancomycin was added to further inhibit bacterial growth. A total of 12 transfers were performed over 2 years of enrichment time, after which a stable enrichment could be maintained.

4.2.2 Physiological assessment

Physiological studies were performed on the stable enrichment culture to assess the growth characteristics of the enrichment. Unless stated otherwise, all physiological tests were performed using the described basal anoxic medium with 80/20 (v/v) N_2/CO2 as headspace at 1.7 atmosphere at 20°C, pH 7, supplemented with 0.1 g L^{-1} yeast extract. Tests were run for a duration of 60 days or until methane production ceased. Growth was confirmed by positive measurement of methane (see analytical techniques).

Methylated compound utilization by the enrichment culture was tested on the following compounds at 10 mM concentration: methanol; monomethylamine (MMA); dimethylamine (DMA); trimethylamine (TMA); tetramethylammonium (QMA); glycine betaine (GB); sarcosine (SAR); N,N-dimethylglycine (DMG) choline (CHO); N,N-dimethylethanolamine (DEA); N-methylethanolamine (MEA); acetate; formate. Additionally, gaseous substrates were also tested using 80/20 (v/v) H_2/CO_2 and 80/20 (v/v) CO/CO_2 as headspace gas at 1.7 atmosphere. Cultures amended with 0.1 g L^{-1} yeast extract under 80/20 (v/v) N_2/CO_2 headspace gas served as negative control.

4.2.3 Analytical techniques

Methylated amines were measured using ion exchange chromatography, using an ICS2100 platform (Thermo Fisher, Waltham, MA). The ICS2100 held a Dionex Ionpac SCS1 column (Thermo Fisher, Waltham, MA), 250 mm x 2 mm, set to a temperature of 30 °C. The eluent consisted of 3 mM methanesulfonic acid in 10% acetronitrile. The flow was set at 0.25 ml/min. Detection was done with a non-suppressed conductivity detector. Due to overlapping peaks with other ions, no reliable measurements of MMA, GB and QMA could be observed. Methane was measured using gas chromatography using a GC2010 system (Shimadzu corporation, Kyoto, Japan). The GC carried a Porabond Q column, 25m x 0.53 mm x 10 um (Agilent Technologies, USA) that was held at 40 °C. Carrier gas was nitrogen, with an applied pressure of 50 kPa. A sample of 0.5 ml gas was injected via a split injector, with a split factor of 25. Detector was a flame ionization detector.

4.2.4 DNA extraction and metagenome sequencing

Enrichments were grown in triplicate as above with TMA as carbon and energy source. Biomass was harvested when ~75% of TMA was depleted. 20 mL of liquid culture was sampled and centrifuged for 20 minutes at 10.000 x g using a Sorvall Legend XTR centrifuge (Thermo Fisher, Waltham, MA). Supernatant was discarded and pellet was resuspended in 0.5 mL PBS buffer. DNA was extracted using the

DNeasy PowerSoil Kit (Qiagen, Hilden, Germany) following supplier's instructions. Obtained DNA was cleaned and concentrated using the Zymo kit (Zymo Research, Irvine, CA) following manufacturer's instructions. DNA concentrations were quantified using Qubit (Thermo Fisher, Waltham, MA). Genome sequencing was performed externally by Novogene using the Illumina HiSeq2500 platform and sequence quality was checked using the internal Novogene pipeline (Novogene, Hong Kong, China). Quality controlled and trimmed reads of maximum 150 basepairs (bp) were obtained. Additional trimming was performed by using bbduk.sh from the bbtools v38.62 suite using functions ktrim=r, k=23, mink=7, hdist=1, qtrim=rl, trimq=30, ftm=5, maq=20, minlen=50 (305). Reads were assembled in contigs using MetaSpades v3.15 at default settings (306). Assembly quality was assessed using Quast v5.0.2 at default settings and reads were mapped back to contigs using Bowtie2 v2.4.2 at default settings. Metagenome assembled genomes (MAGs) were retrieved using the Metawrap v1.2.2 pipeline (307). Briefly, contigs were assigned to MAGs using Metabat2 v2.12.1, Concoct v1.0.0 and Maxbin2 v2.0.0 (272–274). Obtained MAGs were consolidated and refined using the bin_refinement pipeline, allowing for MAGs with >70% completeness and <10% contamination to be included. From the obtained MAGs, genome quality was checked for completeness and contamination using CheckM v1.0.12 with function lineage workflow (277). Taxonomic classification was inferred using gtdb-tk v1.3.0 using function classify_wf (278). Initial genome annotation was performed using PROKKA v.13 at default settings and function -kingdom Archaea for genomes assigned as archaeal and –kingdom Bacteria for bacterial genomes (291). Additional gene annotation for carbohydrate active enzymes was performed using the CAZYmes database and profile HMMs for specific methyltransferase genes of interest as described in chapter 3 (292). Predicted pyrrolysine coding genes were manually curated to include pyrrolysine in amino acid translations.

4.2.5 RNA extraction and transcriptome sequencing

Enrichments were grown in 500 mL serum bottles with in total 250 mL bicarbonate-buffered medium as described above with either tetramethylammonium (QMA) or trimethylamine (TMA) as carbon and energy source. For each compound, triplicate cultures were grown. Biomass was harvested under anoxic conditions after ~2 mM of methane was produced. To each bottle, 200 mL ice cold sterile fresh medium was added to reduce transcriptional activity and bottles were kept on ice. Cultures were centrifuged at 10.000 x g for 20 minutes at 4˚C in a Sorvall LYNX 4000 centrifuge (Thermo Fisher Scientific, Waltham, MA). Supernatant was discarded and pelleted biomass was washed with ice cold PBS buffer and centrifuged using a Sorvall Legend XTR centrifuge at 10.000 x g for 10 minutes at 4˚C. Supernatant was

discarded and pelleted biomass was snap-frozen with liquid nitrogen and stored at -70°C until further use. Cells were lysed through sonication and DNA and RNA was extracted using the Masterpure Gram Positive DNA Purification Kit according to manufacturer's instructions (Biosearch Technologies,Hoddesdon, United Kingdom). β-mercaptoethanol was added to the lysis solution to inhibit RNase. RNA was purified using a Maxwell RSC simplyRNA cells kit, following manufacturer's instructions, utilizing the Maxwell RSC instrument (Promega, Madison, WI). Samples were sent for rRNA depletion and sequencing at Novogene (Hong Kong, China). Sequencing was performed utilizing a NovaSeq 6000 platform which yielded paired-end reads of 150 bp.

4.2.6 Transcriptomic data analysis

Quality of the obtained reads was analyzed with FastQC v0.11.9 (308). Low quality sequences, adapter sequences and PhiX contamination were trimmed using bbduk.sh from the bbtools v38.62 suite using functions ktrim=r, k=23, mink=7, hdist=1, qtrim=rl, trimq=30, ftm=5, maq=20, minlen=50 (305). Trimmed reads were mapped back to the draft enrichment genomes using function bbsplit.sh from the bbtools v38.62 suite at default settings (305). Mapping quality statistics and counts per gene were compiled using function samtools view from the samtools v1.10 suite (271). Reads per gene were calculated to the annotations from Prokka and manual annotation of the obtained MAGs. Expression data was normalized and analyzed for differential expression and statistical significance using DESeq2 v1.34.0 (309). Briefly, differential expression is calculated by fitting normalized count data per gene to a generalized linear model using gamma-Poisson distribution, followed by a Wald significance test between conditions. To account for false discovery rates, p-values were adjusted using the Benjamini-Hochberg method. In samples where no reads were mapped to one or more replicates for both conditions, adjusted p-value could not be calculated and these genes were omitted from the dataset. Count data per gene were normalized to transcripts-per-million (TPM) by dividing normalized counts by the corresponding gene length in kilobasepairs (kbp). Normalized counts per kbp were then divided by the total sample sum and multiplied by $1{\cdot}10^6$. Visualization of results was performed in RStudio using packages pheatmap (211) and EnhancedVolcano (310) with additional editing in Adobe InDesign.

4.3 Results

4.3.1 Methylotrophic activities of Black Sea sediment enrichments

The methylotrophic methanogenic enrichment was capable of methane production in cultures amended with methanol, trimethylamine, dimethylamine, monomethylamine, glycine betaine and tetramethylamine. No methane production was observed in cultures amended with dimethylsulfide, choline, N,N-dimethylethanolamine, N-methylethanolamine, N,N-dimethylglycine, sarcosine, acetate, formate, H_2/CO_2 80/20 (v/v) or CO/CO_2 80/20 (v/v). Growth rates were 0.43±0.092 day $^{-1}$ and 0.38 ±0.025 day $^{-1}$ for TMA and QMA, respectively, based on the average increase in methane produced in triplicate cultures (Figure 1).

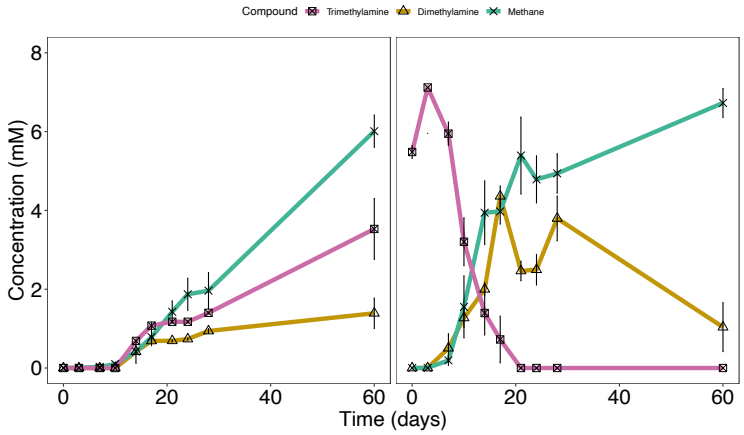

Figure 1: Growth characteristics of the methylotrophic methanogenic enrichment growing on tetramethylamine and trimethylamine.

4.3.2 Genomic potential of the methylotrophic enrichment and identification of genes coding for methylated compound metabolism

Metagenome sequencing of the enrichment culture yielded three high quality metagenome-assembled genomes (MAGs), two bacterial and one archaeal (Table 1), using the criteria as defined by Bowers et al (311). In total, 94% of all reads mapped back to a concatenation of the three obtained MAGs. No additional MAGs of any quality could be constructed from the assembled contigs. Taxonomic affiliation of the bacterial bins with GTDBtk revealed a MAG assigned to the family *Izemoplasmataceae* of the phylum *Tenericutes* (MAG1) and one to the family *Marinifilaceae* of the phylum *Bacteroidota* (MAG2). The sole archaeal MAG was assigned to the family *Methanosarcinaceae* of the phylum *Euryarchaeota* (MAG3). Using average nucleotide identity

(ANI) thresholds as described by Barco *et al.*, MAG1 represents a novel species within the genus *Izemoplasma*, for which we propose the name *Candidatus* Izemoplasma apoleimma, MAG represents a novel species of a novel genus within the family *Marinifilaceae*, for which we propose the name *Candidatus* Pulmentibacterium quisquilia and MAG3 represents a novel archaeal species within the genus *Methanococcoides* for which we propose the name *Candidatus* Methanococcoides chernomoris. Further naming information can be found in the supplementary material.

Table 1: Genome identity and statistics of retrieved metagenome assembled genomes of the methylotrophic methanogenic enrichment.

MAG ID	M32_MAG1	M32_MAG2	M32_MAG3
Taxonomic affiliation	Bacteria; Tenericutes; Bacilli; Izemoplasmatales; Isemoplasmata; Izemoplasma	Bacteria; Bacteroidota; Bacteroidia; Bacteroidales; Marinifilacea; Labilibaculum	Archaea; Halobacteriota; Methanosarcinia; Methanosarcinales; Methanosarcinaceae; *Methano-coccoides*
Proposed name	*Candidatus* Izemoplasma apoleimma	*Candidatus* Pulmentibacterium quisquilia	*Candidatus* Methanococcoides chernomoris
Genome size (MB)	2.12	4.92	2.56
Gene coding sequences	2091	4003	2648
Contigs	2	57	92
Completeness (contamination) (%)	100 (1.33)	98.92 (2.42)	100 (0.65)
Average coverage (reads per basepair)	33.82	50.99	336.94
Closest relative accession	GCF_000755705.1	GCF_002356295.1	GCF_000013725.1
ANI closest placement (%)	77.96	58.91	90.83

Ca. Methanococcoides chernomoris contains genes coding for the complete methanogenesis pathway, including the key gene coding for methyl-CoM reductase subunit A *(mcrA)* (supplementary table S1). *Ca.* Izemoplasma apoleimma and *Ca.* Pulmentibac-

terium quisquilia are predicted to be saccharolytic organisms, capable of utilizing a range of simple and complex sugars (supplementary table S2, S3). While no evidence for dissimilatory reduction of sulfate or nitrate was found in any of the MAGs, *Ca*. P. quisquilia contains a sulfide oxidoreductase gene, suggesting it could be capable of sulfide oxidation.

Of the three retrieved MAGs, only *Ca*. Mc. chernomoris contained genes potentially involved with the utilization of methylated compounds. In total, this MAG contains two genes predicted to encode methanol methyltransferase *(mtaB)*, two genes predicted to encode methylamine methyltransferase *(mtmB)*, two genes predicted to encode dimethylamine methyltransferase *(mtdB)*, and one gene predicted to encode trimethylamine methyltransferase *(mttB)*. Additionally, two non-pyrrolysine coding genes associated with the MttB-superfamily were present and two non-pyrrolysine coding genes associated with the MtmB-family. Both *mtaB* genes are located on the same operon in close proximity to each other, as are both pyrrolysine incorporating *mtmB* genes. With the exception of one MttB-superfamily coding gene and one MtmB-family coding gene, each methyltransferase is flanked directly or is in close proximity to a corrinoid protein coding gene. Two additional corrinoid protein coding genes are present in the genome, not located adjacent to any known methyltransferase. One complete pyrrolysine cassette is present in the genome, with all genes constituting the cassette located directly adjacent to one another. A total of five methylcorrinoid:CoM methyltransferases coding genes are present in the genome. All but one of the methylcorrinoid:CoM methyltransferases are not located on operons containing any known substrate specific methyltransferase coding genes. This one methylcorrinoid:CoM methyltransferase (locus tag METCH_01887) is located closely to a predicted methanol methyltransferase coding gene (METCH_01883). However, a second predicted methylcorrinoid:CoM methyltransferase coding gene (locus tag METCH_00028) is located directly adjacent to the pyrrolysine cassette (locus tags METCH_00023-00027), but no methyl:corrinoid methyltransferase coding gene is located close by. An overview of all methyltransferase coding genes in *Ca*. Mc. chernomoris and associated genes can be found in Table 2.

Table 2: List of genes potentially involved in methyltransferase activity identified in the metagenome-derived genome classified as the euryarchaeon Ca. Methanococcoides chernomoris, found in the enrichment

locus_tag	Gene name	Closest homolog
METCH_00023	*tRNA-pyl*	tRNA-pyl
METCH_00024	*pylS*	Pyrrolysine incorporation protein S
METCH_00025	*pylB*	Pyrrolysine biosynthesis protein B
METCH_00026	*pylC*	Pyrrolysine biosynthesis protein C
METCH_00027	*pylD*	Pyrrolysine biosynthesis protein D
METCH_00028	*mttA*	Methylcorrinoid methyltransferase
METCH_00354	*mttB*	MttB-superfamily protein, non-pyl
METCH_00361	*mtmB*	MtmB-family protein, non-pyl
METCH_00362	*mtmC*	Corrinoid protein
METCH_00436	*mttA*	Methylcorrinoid:tetrahydrofolate methyltransferase
METCH_00689	*mttA*	Methylcorrinoid:tetrahydrofolate methyltransferase
METCH_00882	*mtmB*	MtmB-family protein, non-pyl
METCH_01165	*mtmC*	Corrinoid protein
METCH_01166	*mtmB*	Methylamine methyltransferase, pyl-incorporating
METCH_01175	*mtmC*	Corrinoid protein
METCH_01176	*mtmB*	Methylamine methyltransferase, pyl-incorporating
METCH_01312	*mttC*	Corrinoid protein
METCH_01314	*mttB*	Trimethylamine methyltransferase, pyl-incorporating
METCH_01315	*ramA*	Corrinoid activator gene
METCH_01405	*mtbC*	Corrinoid protein
METCH_01406	*mtbB*	Dimethylamine methyltransferase, pyl-incorporating
METCH_01843	*mttC*	Corrinoid protein
METCH_01881	*mtaB*	Methanol methyltransferase
METCH_01882	*mtbC*	Corrinoid protein
METCH_01883	*mtaB*	Methanol methyltransferase
METCH_01884	*mtaC*	Corrinoid protein
METCH_01886	*ramA*	Corrinoid activator gene
METCH_01887	*mttA*	Methylcorrinoid methyltransferase
METCH_02159	*mttA*	Methylcorrinoid methyltransferase
METCH_02312	*mtbB*	Dimethylamine methyltransferase, pyl-incorporating
METCH_02314	*mtbC*	Corrinoid protein
METCH_02408	*mttC*	Corrinoid protein
METCH_02409	*mttB*	MttB-superfamily gene, non-pyl
METCH_02411	*mttC*	Corrinoid protein

4.3.3 Gene expression of the methylotrophic enrichment

Transcriptomic analyses were performed in RNA extracted from the methylotrophic enrichment growing on tetramethylamine and trimethylamine. For all replicates, over 95% of the trimmed reads mapped back to either of the three genomes recovered in the metagenome sequencing, indicating a high-quality sequencing run. All replicates showed high similarity with the exception of one TMA replicate, which consistently showed lower similarity for all three genomes. However, within-condition similarity of TMA remained much higher than between-similarity of TMA and QMA and as such, the divergent replicate was included in the analysis (supplementary figure 1). In total, 89% of all mapped reads mapped to *Ca*. Mc. chernomoris, 10.5% mapped to *Ca*. P. quisquilia and 0.5% mapped to *Ca*. I. apoleimma. Based on the total expression profiles, the inclusion threshold for gene expression was defined as having a transcript per million (TPM) higher than 10. Expression was defined as low with TPM ranges between 10 and 100, medium with TPM ranges between 101 and 250, high with TPM ranges between 251 and 1000 and very high with TPM ranges of 1001 and above.

In *Ca*. I. apoleimma, a total of 1035 genes were highly of very highly expressed under either TMA or QMA conditions. Of these, 114 had a $\log_2$-fold change of ±2 or more, indicating differential expression. Of these, 37 are of unknown function, 17 are involved with ribosomal function or protein synthesis, 11 with other anabolic processes, 19 are involved in membrane transport, mostly involving sugars, 17 are involved with metabolism. The remainder of the upregulated genes are classified in other functions such as chemotaxis, stress response or nitrogen uptake (supplementary table S4).

In *Ca*. P. quisquilia, a total of 829 genes were highly expressed under either condition. Of these genes, 174 had a $\log_2$fold change of ±2 or more. The majority of these genes, 90 in total, are of unknown function. From all other upregulated genes, 26 are involved with ribosomal function of protein synthesis, 20 are involved in membrane transport, mostly involving sugars, 22 are involved with metabolism or the electron transport chain and 7 are involved in anabolism. The sulfide oxidation gene *cysL* was upregulated under TMA conditions, whereas it was only lowly expressed under QMA conditions. No further trends were observed in the division between upregulation of the described genes in either conditions (supplementary table S5).

In *Ca*. Mc. chernomoris, a total of 601 genes were highly expressed under either condition. Of these genes, 61 had a $\log_2$fold change of ±2 or more. A large cluster

of genes of unknown function, spanning from METCH_00541 to METCH_567 were all upregulated under TMA conditions. Furthermore, 3 genes involved with central carbon metabolism were upregulated under QMA conditions involved in pyruvate synthase (METCH_01932-01934). The majority of all other highly expressed genes with differential expression were either genes involved in methylated compound metabolism or located adjacent to these genes, discussed below.

The pyrrolysine cassette, consisting of tRNA-pyl, the pyrrolysine incorporation gene *pylS* and the pyrrolysine biosynthesis genes *pylBCD* were all expressed at medium levels, with the exception of tRNA-pyl under QMA conditions, which was expressed at low levels. This indicates pyrrolysine is actively synthesized and incorporated in both QMA and TMA conditions. No significant changes in expression were observed between the two conditions, log2fold changes between 0.15 and 0.61 for these genes. A methylcorrinoid:CoM methyltransferase coding gene (locus tag METCH_00028) located directly adjacent to the pyrrolysine cassette was highly expressed under both QMA and TMA conditions, with a non-significant difference (log2fold change -0.11) in expression. Of the four other methylcorrinoid:CoM methyltransferase genes in the genome, one (METCH_01887) was expressed below the inclusion threshold (<10 TPM), two (METCH_00436 and METCH_002159) were medium expressed (TPM between 100 and 250) and one (METCH_00689) was highly expressed under both conditions. Of all five methylcorrinoid:CoM methyltransferase coding genes, only one has a statistically significant differential expression, METCH_02159, which is upregulated under QMA conditions (log_2fold change 1.51, adjusted p-value 0.00021). Neither of the two methanol:corrinoid methyltransferase coding genes were highly expressed. The first, METCH_01881 was expressed under the inclusion threshold for both conditions, as was its corrinoid protein coding gene METCH_01882. The second methanol:methyltransferase coding gene, METCH_01883 and its corrinoid protein coding gene METCH_01884 were lowly expressed, with no significant difference between the two conditions.

The genome of *Ca*. Mc. chernomoris contains one potential pyrrolysine encoding trimethylamine:corrinoid methyltransferase, METCH_01314. This gene was very highly expressed under both conditions, with no statistically significant difference between expression profiles. Its adjacent corrinoid protein coding gene, METCH_01312 was also highly expressed under both conditions with no statistically significant difference between expression profiles between TMA and QMA conditions. Two pyrrolysine encoding dimethylamine:corrinoid methyltransferases are present in the genome of *Ca*. Mc. chernomoris. The first, METCH_01406 is highly

expressed under both conditions. Under the QMA condition this expression is significantly higher than under TMA conditions (log_2fold change 4.98, adjusted p-value $< 1{\cdot}10^{-5}$). Its associated corrinoid protein coding gene, METCH_01405, is highly expressed under QMA conditions, and below the inclusion threshold under TMA conditions (log_2fold change 10.92, adjusted p-value $< 1{\cdot}10^{-5}$). Likewise, the second dimethylamine:corrinoid methyltransferase, METCH_02312, is highly expressed under both conditions. However, this gene is significantly higher expressed under TMA conditions, as is its associated corrinoid protein coding gene METCH_02314 (log_2fold changes -1.39 and -1.5 and adjusted p-values 0.0013 and 0.00037, respectively). Similarly, the two monomethylamine:corrinoid methyltransferase genes present in the genome of *Ca.* Mc. chernomoris were both significantly differentially expressed under both conditions. The first of these genes, METCH_01166 as well as its associated corrinoid protein coding gene METCH_01165 were highly expressed under both conditions, but significantly more so under TMA conditions (log_2fold changes -2.38 and -2.2 and adjusted p-values $3.11{\cdot}10^{-5}$ and 0.0005, respectively). In contrast, the second monomethylamine:corrinoid methyltransferase coding gene, METCH_01176, was highly expressed under two of the three replicate QMA conditions, and only slightly above the inclusion threshold in TMA (log_2fold change 5.62, adjusted p-value $< 1{\cdot}10^{-5}$). Its associated corrinoid protein coding gene, METCH_01175, was statistically significantly upregulated under QMA conditions (log_2fold change 4.6, adjusted p-value $< 1{\cdot}10^{-5}$). However, expression levels of this corrinoid protein coding gene were still much lower than its associated methyltransferase, with low expression under QMA conditions and below threshold expression under TMA conditions (figure 2).

Two MttB-superfamily genes not coding for pyrrolysine are present in the genome. The expression of the first, METCH_00354 was low under both conditions. No corrinoid protein coding gene is adjacent to this gene. The second MttB-superfamily gene, METCH_02409 was highly expressed under both conditions, with no statistically significant difference between the two conditions. This gene is flanked by two corrinoid proteins, METCH_02408 and METCH_02411. METCH_02408 was highly expressed (albeit only just above the threshold) under TMA conditions, and had medium expression under QMA conditions, without a significant difference between the two expression profiles. METCH_02411 had low expression under both conditions.

Two genes annotated as non-pyrrolysine *mtmB* are present in the genome. The first of these genes, METCH_00361 as well as its corrinoid protein coding gene

METCH_00362 were highly upregulated under both conditions. However, expression was significantly higher under QMA conditions (log_2fold changes 5.68 and 5.52 and both adjusted p-values < $1 \cdot 10^{-5}$). With the exception of ribosomal genes, this *mtmB* gene and its corrinoid protein coding gene were the most highly expressed genes in any of the replicates under QMA conditions. The second *mtmB* gene, METCH_00882, which does not have an associated corrinoid protein coding gene, was highly expressed under TMA conditions but lowly expressed under QMA (log_2fold change -2.34 and adjusted p-value $1.7 \cdot 10^{-5}$).

Two corrinoid activation protein coding genes, required to change the oxidation state of the cobalt in corrinoid proteins from I to III, were present in the genome. The first, METCH_01315, which is located next to the trimethylamine:corrinoid methyltransferase METCH_01314, was highly expressed under TMA conditions and lowly expressed under QMA conditions, a significant difference in expression. The second, METCH_01886, located close to the methanol:corrinoid methyltransferase METCH_01883, was expressed slightly above the inclusion threshold under both conditions. An overview of the expression of all genes involved or predicted to be involved in methyltransferase activity is given in figure 2. Complete information on the expression of all genes can be found in supplementary table 5.

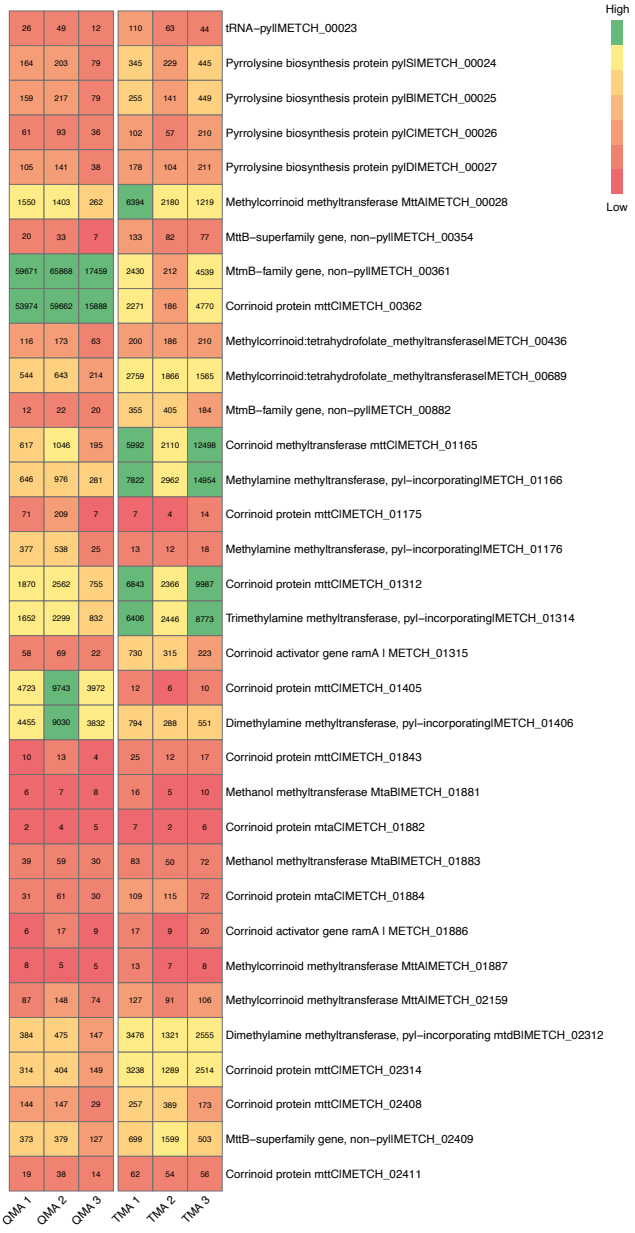

*Figure 2: Overview of expression of gene homologs involved in or hypothesized to be involved in methyltransferase activity of the methanogenic enrichment when growing on tetramethylamine (left) or trimethylamine (right). Numbers in each cell correspond to transcripts per million. Asterisks after each gene name indicate significant upregulation; *: significant upregulation (adjusted p-value <0.005); ***: highly significant upregulation (adjusted p-value <0.00005).*

4.4 Discussion

In this study, we have enriched a methylotrophic community, capable of converting methylated compounds to methane. Through genome sequencing in combination with a transcriptomic approach we have identified and chacaracterized the genome of a novel methanogenic archaeal strain, *Candidatus* Methanococcoides chernomoris, with the potential of methanogenic growth on a range of methylated amines, including tetramethylamine, trimethylamine and glycine betaine. Additionally, two bacterial strains, *Candidatus* Pulmentibacterium quisquilia and *Candidatus* Izimoplasma apoleimma were enriched along with the methanogenic archaeaon. Based on their low relative abundance in the amount of mapped reads in the transcriptome analysis, genome analysis and previous reports on the genus *Izemoplasma* and the family *Marinifilaceae*, these organisms are expected to rely on organic material generated through biomass degradation for their metabolism and/or the yeast extract present in all cultures and are not involved in the degradation of methylated compounds (242, 312). Likewise, our transcriptomic analysis showed much lower divergence between the two experimental conditions compared to *Ca*. Mc. chernomoris. No genes involved in any known methylated pathway were upregulated in the transcriptome of the two bacterial genomes. As such, they are considered as heterotrophic members of the methanogenic culture without any discernable influence on the methylotrophic metabolism of *Ca*. Methanococcoides chernomoris.

Methylotrophic methanogenesis is a major contributor to global methane emissions and is observed in many anoxic environments (33). *Ca*. Mc. chernomoris is likewise capable of methylotrophic methanogenesis, using methanol, glycine betaine, MMA, DMA, TMA and QMA as substrate. While the genetic basis of methanogenesis on most of these substrates is well described, QMA utilization is more elusive. Methanogens growing on TMA are capable of full conversion of TMA to ammonia, with succesive demethylation to DMA, then MMA, then ammonia in the cytoplasm (35). With the production of TMA when growing on QMA and production of DMA under both conditions, It can be assumed that QMA is fully demethylated to ammonia by *Ca*. Mc. chernomoris, with a first demethylation of QMA to TMA, and subsequent demethylations occuring through the well-described pyrrolysine containing methyltransferase genes. However, as we were unable to reliably measure QMA and MMA this remains an assumption. As the growth rate of *Ca*. Mc. chernomoris, as measured by methane production, is higher on TMA than on QMA (0.43 versus 0.38 day^{-1}), demethylation of QMA is likely a rate limiting step. This difference in growth rate was also observed in *Methanococcoides* strain NM1, whereas *Methanococcoides* strains AM1, Q3c and NaT1 showed comparable growth rates when growing on QMA

or TMA (235, 303, 313). Whether this is difference in growth rates due to substrate specificity of the responsible protein or due to other factors remains an open question.

Besides two ribosomal genes, the two most highly expressed genes under QMA conditions were an MtmB-family coding gene and its associated corrinoid protein coding gene, METCH_00361 and METCH_00362, respectively. However, under TMA conditions these genes were also highly expressed, yet significantly less so. MtmB-family coding genes were only recently described and thus far, no enzymatic analysis has been conducted. As such, their specific function is only speculative (chapter 3). Due to their close homology to methylamine methyltransferase and usual co-location with corrinoid protein coding genes, it is hypothesized that, like MttB-superfamily genes, they are involved in methylated compound:corrinoid methyl transfer. However, the methylated compounds these MtmB-homolgs are potentially using as substrate are unknown. Of the strains that have been shown to grow on tetramethylammnium, only two genomes are available, *Methanococcoides* strain AM1 and strain NM1 (313). Both these organisms have *mtmB*-homologs in their genome, as do most publicly available *Methanococcoides* genomes (chapter 3). We thus hypothesize that MtmB-family proteins are responsible for tetramethylammonium:corrinoid methyltransfer, with METCH_00361 the gene encoding for tetramethylamine:corrinoid methyltransferase in *Ca*. Mc. chernomoris and METCH_00362 coding the associated corrinoid protein to which the methyl group from QMA is transfered. We follow the naming conventions from Tanaka et al., 1997, who called the methylcorrinoid:CoM methyltransferase originating from QMA *mtqA*, and propose the name *mtqB* for METCH_00361 and *mtqC* for METCH_00362, despite the name *mtqA* is also claimed for a methylcorrinoid:THF methyltransferase involved in the methyltransferase pathway of proline betaine and carnitine in *Eubacterium limosum* (49, 50, 227, 303). Similar to MttB-superfamily coding genes, it is possible that non-pyrrolysine *mtmB* homologs code for a range of methylated compound methyltransferases. While not as widespread as non-pyrrolysine *mttB* homologs, *mtmB* homologs have been observed in a wider range of organisms than pyrrolysine coding *mtmB* genes (chapter 3). The second MtmB-homolog of *Ca*. Mc. chernomoris, METCH_00882 was highly significantly upregulated under TMA conditions. Regardless, under this condition, total transcripts per million of this gene were still an order of magnitude lower than METCH_00361. However, under QMA conditions transcripts per million were just above the inclusion threshold. This *mtmB*-homolog does not have an associated corrinoid protein, but is flanked by an ATPase associated with diverse cellular functions (AAA family ATPase) and a hydantoinase/oxoprolinase family protein

coding genes which display similar expression profiles as METCH_00882. This could suggest that this homolog of *mtmB* is involved in an ATP-dependent anabolic reaction, rather than a catabolic reaction, which is not required when growing on QMA (supplementary table S6).

Under standard methylotrophic methanogenesis, 75% of the methylated compound is reduced to methane, while 25% of the methylated compound is oxidized to CO_2. When growing on glycine betaine, the ratio of produced methane to N,N-dimethylglycine is close to 75%, indicating that growing on this compound, *Ca*. Mc. chernomoris is likely performing standard methylotrophic methanogenesis with no external electron donor such as hydrogen. However, TMA production under QMA conditions and DMA production under TMA conditions is not in stoichiometric balance if only the starting substrate would be consumed. It can be assumed some of the products TMA and DMA are directly further demethylated in the cytosol of the cells, which is demonstrated by the increase in DMA when growing on QMA. Likewise, it can be expected MMA is further formed through the demethylation of DMA, although due to our inability to accurately measure MMA this remains an assumption.

However, most of the product of each succesive demethylation is released to the environment by the organism. Through our transcriptomic approach, we uncovered two pyrrolysine containing methyltransferase coding genes significantly upregulated when growing on QMA as compared to TMA. These genes are annotated as dimethylamine- and monomethylamine:corrinoid methyltransferases. Two different pyrrolysine containing genes also annotated as dimethylamine- and monomethylamine:corrinoid methyltransferases were significantly upregulated when growing on TMA. The sole pyrrolysine containing gene in the genome annotated as TMA:corrinoid methyltransferase was highly expressed under both conditions. While it was more upregulated under TMA conditions, this was not statistically significantly so. As such, the expression of the genes coding for TMA, DMA and MMA is not surprising, as these substrates become available due to the degradation of QMA to TMA. However, this differential upregulation of genes predicted to perform the same function is surprising.

Paralogs of methylamine:corrinoid methyltransferase proteins in *Methanosarcina acetivorans* have been shown to have distinct functions, with one paralog involved with metabolism and another involved in nitrogen uptake (314). This nitrogen uptake does not appear to be the primary function of either the two *mtmB* genes or the two *mtbB* genes, as evidenced by the presence of 5 mM ammonia under all conditions.

Furthermore, the *mtmB* genes are located on the same operon and appear to be a recent duplication based on the high level of homology, with only 6% amino acids different, all outside of the catalytic centre. While the two *mtbB* homologs are not located in vicinity of another, there is likewise a high level of homology with 7% amino acids different. In *Methanosarcina acetivorans* C2A, the genes coding for three isozymes of methanol:corrinoid methyltransferase were, amongst other factors, differentially expressed based on growth phase. In this work, it was suggested that depending on the methanol concentrations in the medium and consequentially in the cytosol, different methyltransferases with a different enzyme velocity and substrate affinity trade-off can be expressed to optimize energy gain based on the availability or scarcity of the substrate (315, 316). As lower amounts of DMA were measured in the QMA cultures, and therefore likely also lower amounts of MMA, it is possible that all available methyltransferase systems for MMA and DMA are expressed when growing on QMA as a response to lower substrate availability. In this scenario, one of these systems (METCH_1175/1176 for MMA and METCH_1406/1407 for DMA) has a higher affinity for its substrate but lower enzyme velocity and the other (METCH_1165/1166 for MMA and METCH_ 02312/02314 for DMA) has a lower affinity for its substrate but higher velocity. Under TMA, when larger amounts of DMA and therefore likely also MMA are available, there is less need to express the high affinity homologs and are seen to a much lesser degree in our dataset. However, enzyme activity tests of all pyrrolysine containing methyltransferases are needed to confirm this hypothesis. Figure 3 displays the proposed succession of methylated amine demethylation under TMA and QMA conditions based on the transcriptomic results.

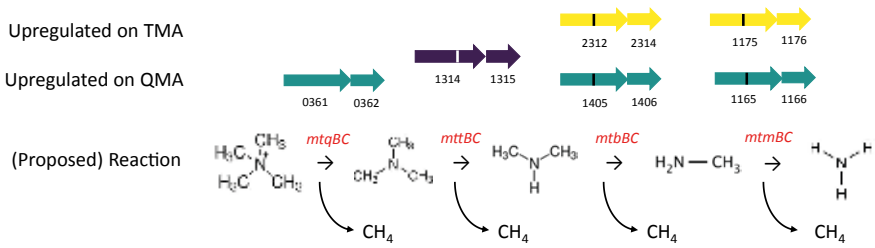

Figure 3: Proposed succession of methylated amine demethylation under TMA and QMA conditions. Yellow arrows depict genes upregulated under TMA conditions, green arrows depict genes upregulated under QMA conditions and blue arrows depict genes not significantly upregulated under either condition. Numbers indicate locus tags and gene names are depicted in red.

No additional pyrrolysine-containing genes were detected in the genome. However, the genome contains two non-pyrrolysine MttB-superfamily coding genes. MttB-family proteins have been linked with the demethylation of the quaternary amines glycine betaine, carnitine, γ-butyrobetaine, proline betaine and choline (50, 51, 54, 227, 236). With the exception of glycine betaine, these methyltransferases have all been described in non-methanogenic bacteria. Homologs of MttB-superfamily genes are widely found in methanogenic archaea and growth on quaternary amines such as choline and N,N-dimethylethanolamine has been described in methanogenic organisms containing these genes (52, 317). Of the two MttB-superfamily genes present in the genome of *Ca.* Mc. chernomoris, METCH_02409, was expressed at medium levels under both conditions, as was its associated corrinoid protein coding gene (figure 2). This gene clusters closely with the glycine betaine-linked *mtgB* from *Methanolobus vulcani* and *Desulfitobacterium hafniense* (50, 235). The second MttB-superfamily gene, METCH_00354, which did not have an associated corrinoid protein coding gene, was expressed at low levels under both conditions, yet still significantly higher expressed under TMA conditions (log2fold change -0,84, adjusted p-value 0.048). As all cultures contained yeast extract, in which glycine betaine occurs, this compound was present in small amounts under all conditions and could therefore be responsible for the expression of the expressed MttB-superfamily coding gene (318). Furthermore, it is possible that under natural conditions, glycine betaine serves as the primary metabolic source and as such, *mtgB* is always expressed as was suggested for the methanol methyltransferase *mtaB* in *Methanococcoides vulcani* (53).

In contrast to the substrate specificity of methylated compound:corrinoid methyltransferases, the methylcorrinoid:CoM methyltransferase system MTII is not neccesarily bound to a single substrate. While methanol specific MTI systems have been described, MTII systems active on both methanol and methylamine are known (226). In *Eubacterium limosum*, quaternary amine methyltransferase MTII systems have been described specifically for glycine betaine, but also a single MTII system active on methyl groups originating from proline betaine, carnitine and y-butyrobetaine (49, 51, 227). In *Ca.* Mc. Chernomoris, of the five MTII coding genes *mtxA*, one is highly expressed under both conditions, METCH_00028. This gene is located directly after the pyrrolysine biosynthesis and incorporation complex *pylSBCD*. One other MTII coding gene, METCH_00689 was highly expressed under TMA conditions, and medium expressed under QMA conditions, although not on a statistically significant differential expression. The other MTII coding genes were all expressed at low levels under both conditions. This does not neccesarily imply the proteins coded by these genes are not active, as low constitute expression can have an effect. However, the

high expression of other MTII coding genes does imply these proteins are preferred. As the high level of expression of the three pyrrolysine containing methylated amine and the putative tetramethylamine methyltransferase I systems infer that at least four MTII systems are active under QMA conditions and three under TMA, this suggests that that methylcorrinoid:CoM methyltransfer is performed by MTII systems that are not substrate specific. To infer which MTII system is related to which methylated amines further investigation is required.

4.5 Conclusions

Here, we demonstrate the methanogenic degradation of tetramethylammonium by a methanogenic enrichment obtained from anoxic Black Sea sediments. Based on genomic and transcriptomic analyses, a methanogenic archaeon, *Ca*. Methanococcoides chernomoris, is responsible for this degradation, while other members of the of the enrichment are saccharolytic heterotrophs. Through a transcriptomic approach, we have identified a gene we hypothesize codes for a tetramethylammonium:corrinoid methyltransferase. This gene is a member of the recently described *mtmB*-family, non-pyrrolysine containing homologs of monomethylamine:corrinoid methyltransferase. While biochemical data is needed to further prove the activity of the protein coded by this gene, the strong upregulation in methanogenic enrichments growing on tetramethylammonium is a strong indicator of the function of this gene. We furthermore infer the complete degradation of QMA and TMA to ammonia based on the expression of the genes coding for of pyrrolysine containing methylated amine methyltransferases. These results further improve our understanding of the genetic basis of methylotrophic methanogenesis by using different methylated amines.

4.6 Protologue

Three novel, uncultured taxa are described in this study. High quality metagenome assembled genome data serves as type material, as outlined by Chuvochina et al., 2019 (319).

Description of *Candidatus* ^{U}Pulmentibacterium, gen. nov.

^{U}*Pulmentibacterium* (Pul.men.ti.bac.te'ri.um. L. f. n. *pulmentus*, side-dish; N.L fem. N. *Pulmentibacterium*, bacterium which consumes secondary metabolites of a culture). Member of the family *Marinifilaceae*. Type species is *Pulmentibacterium quisquilia*.

Description of *Candidatus* [U]Pulmentibacterium quisquilia

[U]*Pulmentibacterium quisquilia* (quis'qui.li.a L. f. n. quisquilia, waste; N.L. fem. N. *Pulmentibacterium quisquilia*, bacterium which eats the waste products of another species). Genome and transcriptome analysis predicts a saccharolytic fermentative metabolism and potentially sulfide oxidation. Type material is M32.bin2

Description of *Candidatus* [U]Izemoplasma apoleimma

[U]*Izemoplasma apoleimma* (a'po.leim.ma Gr. masc. n. *apoleimma*/ἀπόλειμμα, remnants, leftovers; N.L. masc. n. *apoleimma*, remnants, leftovers). Genome and transcriptome analysis predicts a saccharolytic fermentative metabolism as well as degradation of DNA. Type material is M32.bin1

Description of *Candidatus* [U]Methanococcoides chernomoris

Methanococcoides chernomoris (cher.no'mo.ris Bulgarian, masc. adj. *cherno more*/Черно море, from the Black Sea; N.L. masc. adj. *chernomoris*, from the Black Sea). Genome and transcriptome analysis predicts a methylotrophic methanogenic metabolism capable of utilizing methanol, MMA, DMA, TMA, QMA and glycine betaine as growth substrates. Type material is M32.bin3

4.7 Supplementary information

Supplementary information and (intermediate) data can be downloaded from our data repository at https://doi.org/10.6084/m9.figshare.c.6263232.v1

4.8 Acknowledgements

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A novel *Desulfosporosinus nitroreducens* strain P130 capable of anaerobic methylotrophy isolated from Black Sea sediments

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Abstract

Methylated compounds such as methanol and trimethylamine have long been considered non-competitive substrates for methanogens in sulfate rich anoxic marine sediments. Methanogenic archaea utilize substrate-specific methyltransferase that funnel methyl groups into the methanogenic pathway. However, there is increasing genomic and physiological evidence that methyltransferase pathways are present in the genomes of microorganisms from other metabolic groups, such as sulfate reducers. Thus, these microorganisms can potentially utilize methylated compounds and compete with methanogens in marine sediments. However, there is a lack of physiological data from isolated marine strains to further corroborate this. Here, we have enriched and isolated a novel strain P130 from anoxic Black Sea sediments that is closely related to the sulfate reducer *Desulfosporosinus nitroreducens*, capable of growth on methanol, glycine betaine and trimethylamine. Genome analysis revealed the presence of specific methyltransferase-dependent pathways, which likely play a role in the conversion of these compounds. Furthermore, the genes coding for the biosynthesis and incorporation of the 22nd amino acid pyrrolysine, crucial for the functioning of trimethylamine methyltransferase, were present in the genome of strain P130. We provide further physiological and transcriptomic insights in the utilization of these methylated compounds by the new isolate.

5.1 Introduction

Methylated compounds, here considered as compounds with one or more methyl groups bound to a non-carbon atom, can be used as carbon and energy source by a diverse range of microorganisms including acetogens, methanogens and sulfate reducers (320, 321). A wide range of methylated compounds exist, for example methanol, trimethylamine (TMA) and glycine betaine. Many of these compounds are common in many marine organisms where they serve a biological function as osmolytes to counter salt stress (219, 322). Dead algae and fauna in the water column, known as necromass, sinks to sediment, where organic methylated are released to the environment (4). In the sediments, oxygen is readily depleted and sulfate becomes the most commonly available favorable electron acceptor for microbial metabolisms (2). The upper anoxic sediment is thus referred to as the sulfate reduction zone, where sulfate-reducing microorganisms (SRM) are the primary drivers of anaerobic respiration (20). No sulfate is available in deeper sediments, where the major microbial metabolism is methanogenesis. Thus, this zone is called the methanogenesis zone (41).

Due to the more favorable energetics of sulfate reduction and dependence on common substrates (e.g. hydrogen/CO_2 or acetate), methanogenesis and sulfate reduction are considered to be mutually exclusive. However, in the sulfate reduction zone, methanogenesis is thought to occur as well. Here, methanogens utilize methylated compounds as "non-competitive" substrates, thereby releasing methane to the water column, where it can be oxidized by methane-oxidizing micro-organisms or released to the atmosphere (42, 44, 46). While much research has been conducted on the utilization of methylated compounds by methanogens (40, 54, 178), it is unclear to what extent methylated compounds are indeed non-competitive substrates. There is an increasing body of evidence that trophic groups other than methanogens, most importantly sulfate-reducing microorganisms and acetogens, are likewise able to utilize methylated compounds. Substrate-specific methyltransferases, the class of genes coding for proteins that methanogens utilize to grow on methylated compounds, have been found in the genomes of a wide range of bacteria and non-methanogenic archaea. For example, these genes are present in the genomes of the archaeal phylum *Sifarchaeota* and the acetogenic bacteria *Acetobacterium* Woodie and *Sporomusa ovata* (60, 225). A recent metagenomic survey of anoxic Black Sea sediments revealed that corrinoid methyltransferase genes were found in a substantial proportion of all reconstructed metagenome assembled genomes in a diverse range of trophic groups, including sulfate-reducing microorganisms and acetogens (chapter 3). The implication of methyltransferase-dependent methanol

conversion in *Desulfofundulus kuznetsovii* has been shown previously by proteomics and stable isotope fractionation, thereby giving further evidence for the active utilization of this pathway (45).

The corrinoid methyltransferases active on the methylated amines monomethylamine (MMA), dimethylamine (DMA) and TMA in archaea all contain the 22nd amino acid pyrrolysine. In these proteins, pyrrolysine is predicted to form a covalent bond with its substrate, allowing nucleophilic attack to occur (55). As such, the presence of pyrrolysine is crucial for the functioning of these proteins. TMA metabolism utilizing the trimethylamine:corrinoid methyltransferase pathway has been described in detail in the acetogen *Acetohalobium arabaticum*, revealing the biosynthesis and incorporation of pyrrolysine in bacteria (243, 323). Pyrrolysine is encoded in genomes as the amber codon TAG, normally a stop codon. In methanogens, the genetic code is adapted to the inclusion of pyrrolysine by minimizing TAG as stop codon in the genome to ~5%, whereas *Acetohalobium arabaticum* alters the biosynthesis of pyrrolysine and subsequent incorporation in the amino acid alphabet in the presence or absence of TMA (243). Furthermore, the genetic presence of TMA utilization coding genes has been explored in the human gut pathogen genus *Bilophila* and in marine *Desulfobacterota* although physiological data is lacking (245, 324). Homologs of the gene coding for trimethylamine:corrinoid methyltransferase lacking pyrrolysine are common throughout the bacterial and archaeal kingdoms, and have been linked with methyltransferase activity of various methylated quaternary amines (49–51, 227). This all suggests a larger role for methylated compounds in non-methanogenic micro-organisms, marine or otherwise. However, there currently is a lack of data linking genomic and physiological data on the presence of corrinoid methyltransferases in non-methanogenic marine methylotrophic strains.

To bridge this gap, we aimed to enrich and isolate non-methanogenic methylotrophic organisms using methanol as methylated substrate from sediments collected from 2000 meters below sea level in the Black Sea. Due to the stable anoxic conditions and high load of organic matter present in the sediment, it has been the site for many previous investigations (239–241). The community structure and substrate consumption over 24 months of enrichment was followed after which a stable enrichment was achieved. From this enrichment, an axenic culture, strain P130, was obtained which is phylogenetically similar to *Desulfosporosinus nitroreducens* strain 59.4B^{T} within the phylum *Firmicutes* based on genome comparison. This strain was able to utilize a range of methylated compounds, including methanol, glycine betaine and TMA, but not DMA or MMA. Genomic inspection showed the presence of substrate specific

corrinoid methyltransferases for methanol, trimethylamine and glycine betaine. Additionally, genes coding for the reductive glycine betaine pathway (*grd*) were present in the genome of strain P130. Through a transcriptomic approach, we identified the simultaneous expression of both the glycine betaine methyltransferase and grd pathways when growing on glycine betaine. These results further demonstrate the more nuanced fate of diverse methylated compounds in marine sediments than previously thought.

5.2 Results

5.2.1 Enrichment for methanol oxidizing SRM and general microbial diversity

Enrichment cultures inoculated with Black Seas sediment were established with 10 mM of methanol and 10 mM sulfate as sole electron donor and acceptor, respectively, using sediments collected from 29-30 cm and 5-6 cm below sea floor (bsf) (referred to as S29 and S5, respectively). To inhibit methanogenesis, 20 mM of bromo-ethanosulfonate (BrES) was added. In both enrichments, starting from the first inoculation, methanol was depleted and sulfide was produced, with no accumulation of other substrates throughout all enrichments (figure 1a). Over 24 months, successive transfers of 10% v/v of enrichment culture to fresh medium were performed after depletion of substrate. After 12 months of subsequent transfers, DNA was extracted from the established enrichments and 16S rRNA amplicon sequencing was performed to determine the microbial diversity of the enrichments. The microbial diversity of S29 based on 16S rRNA sequences indicated the enrichment was dominated by the families *Marinifiliaceae*, *Halanaerobiaceae* and *Desulfofarcimen*, with 58.3%, 18.5% and 11.3% of total 16S rRNA gene reads, respectively. For S5, the enrichment sequencing revealed the most abundant 16S rRNA gene sequences were assigned to the families *Desulfofarcimen*, *Clostridiaceae* and *Marinifiliaceae*, with 36.1%, 17.7% and 13.8% of total reads mapped, respectively (figure 11).

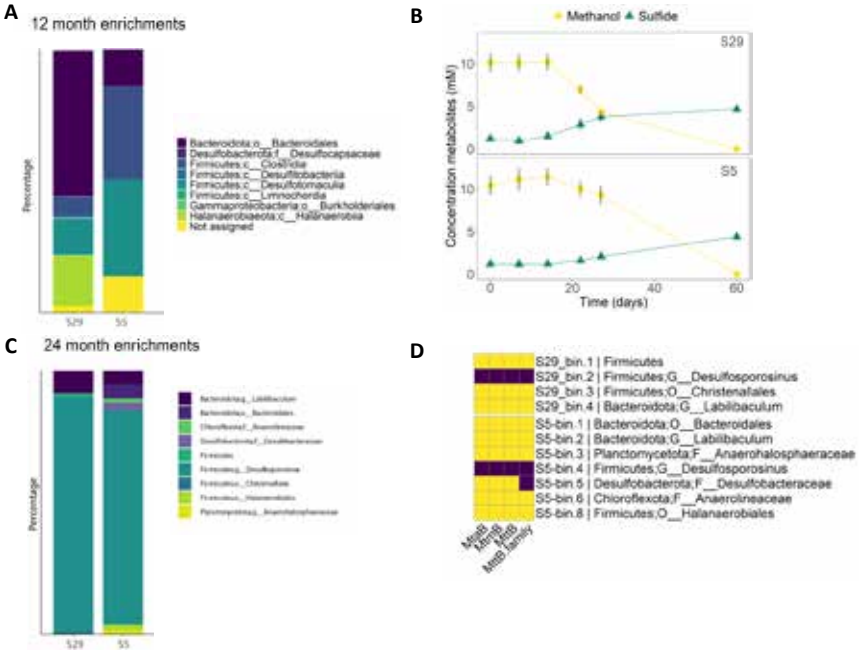

Figure 1: A) Microbial community diversity of methanol converting enrichments, after 12 months (6 successive transfers); results based on assigned reads of 16S rRNA gene amplicon sequencing (average of 3 technical replicates). S29: series inoculated with 29-30cm bsf sediment. S5: series inoculated with 5-6 cm bsf sediment. Legend shows the assignation of the 16S rRNA gene reads to phylum and class level based on the SIVLA non redundant r138 database. B) Depletion of methanol and production of sulfide by enrichment cultures S29 and S5 at 12 months of enrichment (6 successive transfers). C) Microbial community diversity of methanol converting enrichments S29 and S5, after 24 months (12 successive transfers); results based on total assigned reads of metagenome assembled genomes in each enrichment. Legend shows the phylum and lowest recovered taxonomic rank based on classification using GTDBtk. D) Presence (in dark blue) and absence (in yellow) of the key corrinoid methyltransferase genes for methanol (MtaB), monomethylamine (MtmB), trimethylamine (MttB) and putative methylated quaternary amine methyltransferases (MttB-family).

After 24 months of successive transfers, we performed metagenome sequencing on the enrichments to further assess the metabolic capabilities of the members. We retrieved 4 metagenome assembled genomes (MAGs) above inclusion threshold (using minimum genome information thresholds as suggested by Bowers *et al.*) from the S29 enrichment, and 7 MAGs from the S5 enrichment (supplementary table S1) (311). S29_bin.2 and S5_bin.4 showed significant overlap, with an average nucleotide identity of 98.5%, indicating they are strains of the same species in the genus *Desulfosporosinus*. Based on average genome coverage, both enrichments were dominated by strains belonging to this genus (figure 1c). Homologs for putative corrinoid methyltransferases were found in these two MAGs and in one other MAG from the S5 enrichment (figure 1d).

5.2.2 Isolation of two novel strains of *Desulfosporosinus nitroreducens*

Two methylotrophic strains, P130 and P125 were obtained from the stable enrichments S29 and S5, respectively. 16S rRNA gene sequence identity between these strains is 99.9%, both strains are almost identical strains of the same species, with an identity of 98.6% to the 16S rRNA gene of *Desulfosporosinus nitroreducens* strain 59.4B^T. Due to the faster depletion of methanol in enrichment culture S29 (figure 1b), strain P130 was chosen as representative for further analysis. To obtain genomic similarity information between strain P130 and its closest relative, we performed whole genome sequencing of *D. nitroreducens* strain 59.4B^T (198) and strain P130.

5.2.3 Phylogeny and genomic analysis of D. nitroreducens strains P130 and 59.4B^T

The draft genomes of strain P130 and *Desulfosporosinus nitroreducens* strain 59.4B^T consisted of 66 and 95 contigs and were 5.31 Mbp and 5.45 Mbp in length, yielding 5.006 and 5.203 DNA coding sequences, respectively. CheckM analysis determined a completeness of 99.94% for both strain P130 and *D. nitroreducens* 59.4B^T with a contamination of 1.96 and 1.36%, respectively. This contamination is most likely due to gene duplication rather than foreign DNA, as similar low rates of contamination are found throughout isolate sequences (277). Average nucleotide identity between strains P130 and 59.4B^T is 99%, indicating strain P130 is a strain of *D. nitroreducens*.

Strain P130 contained homologs of methyltransferase coding genes linked with the utilization of methanol (*mtaB*) and monomethylamine (*mtmB*), both present once in the genome. Additionally, two trimethylamine:corrinoid methyltransferase coding genes (*mttB*) coding for in-frame pyrrolysine are present in the genome. The pyrrolysine biosynthesis gene cassette, containing genes for the biosynthesis and incorporation of pyrrolysine, is present three times, twice located surrounding the two *mttB* homologs, and once surrounding the *mtmB* homolog (Figure 2a-c). Nine homologs of *mttB* with no in-frame pyrrolysine are coded in the genome. Three homologs of the corrinoid activation protein coding gene *ramA* are also coded in the genome. Two operons coding for complete glycine reductase pathways are present in the genome. One of these contains glycine reductase B and E (*grdBE*), indicating it is a glycine specific reductase gene cluster. The other operon contains *grdHI*, indicating it is a glycine betaine specific reductase gene cluster (figure 2d) (325). Strain P130 contained five putative alcohol dehydrogenase coding genes. While *D. nitroreducens* strain 59.4B^T likewise contained the methyltransferase coding genes *mtaB*, three *mttB* genes and nine putative MttB-superfamily coding genes and five putative alcohol dehydrogenases, the monomethylamine methyltransferase coding *mtmB* gene was

absent in the genome (supplementary tables S2 & S3).

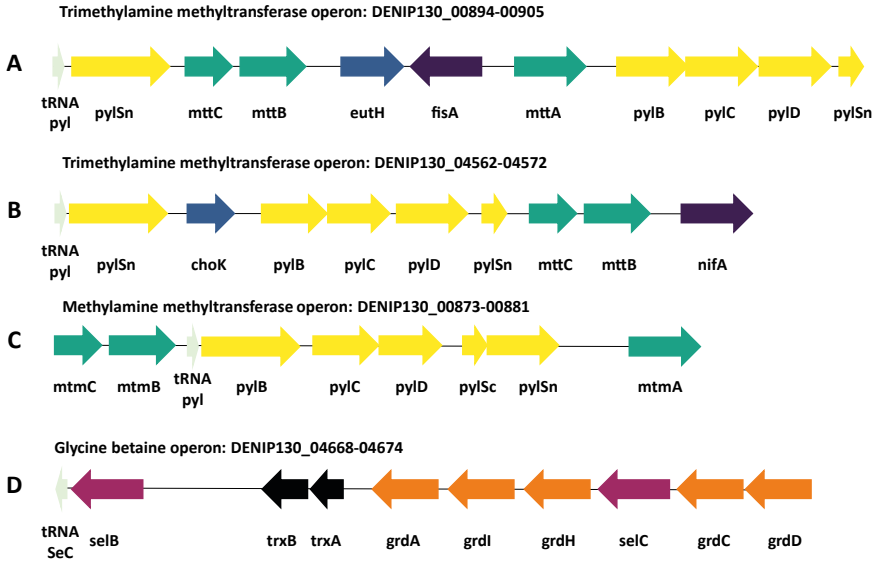

Figure 2: Operons coding for methylated compound metabolism of D. nitroreducens strain P130. A: trimethylamine operon I, B: trimethylamine operon II; C) monomethylamine operon; D) glycine betaine operon. Corrinoid methyltransferase genes are indicated in teal; pyrrolysine biosynthesis and incorporation genes are indicated in yellow; regulatory genes are indicated in dark blue; transfer RNA genes are indicated in light green; glycine reductase genes are indicated in orange; selenocysteine biosynthesis and incorporation genes are indicated in burgundy; electron carriers are indicated in black; genes of unknown functionality are indicated in dark blue.

5.2.4 Physiological characterization of strain P130

Desulfosporosinus nitroreducens strain P130 has similar growth characteristics as described for *D. nitroreducens* strain 59.4B^T with the following exceptions: growth is optimal under salinity of 1.8% and optimum growth temperature is 25°C, compared to a maximum salinity of 1% and optimum growth temperature of 30°C for *D. nitroreducens* strain 59.4B^T. Additionally, strain P130 was tested for growth on methylated compounds, which were not tested for in detail in the characterization of *D. nitroreducens* strain 59.4B^T (198).

Growth was observed utilizing methanol, trimethylamine and glycine betaine as electron donor and both sulfate and thiosulfate as electron acceptor. No growth was observed utilizing dimethylsulfide (DMS), trimethylamineoxide (TMAO), MMA, DMA or tetramethylamine. Methanol was oxidized to CO_2, sulfate and thiosulfate were completely reduced to sulfide. Growth on trimethylamine resulted in the accu-

mulation of dimethylamine.Products formed during growth on glycine betaine were CO_2, acetate, trimethylamine, sulfide and N,N-dimethylglycine (figure 3).

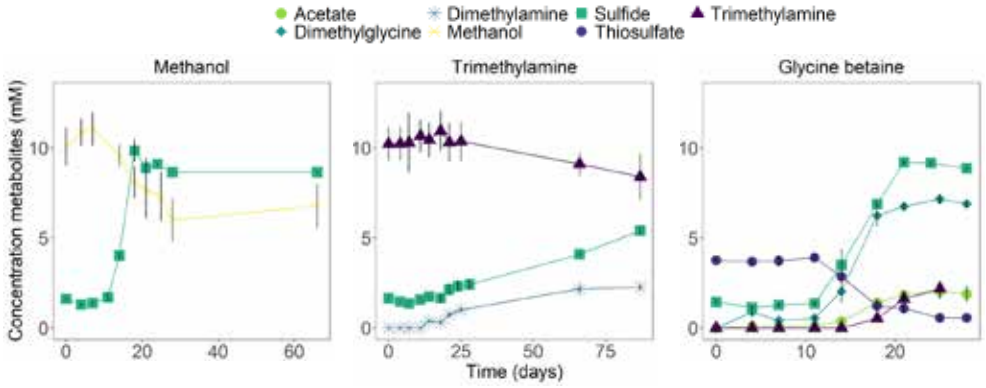

Figure 3: Substrate consumption and product formation by strain P130 grown on A) methanol; B) glycine betaine; C) trimethylamine. Results are averages from three independent biological replicates, error bars indicate standard deviation from the average.

5.2.5 Gene expression analysis of strain P130 growing on glycine betaine

RNA was extracted from strain P130 growing on glycine betaine at mid-exponential growth. Ribosomal RNA depleted RNA was sequenced, quality trimmed and mapped to the genome of strain P130. Over 99.9% of the obtained reads for each replicate were mapped to the genome of *D. nitroreducens* strain P130. Expression profiles were similar throughout the replicates. Based on the total expression profile, genes with a transcript-per million (tpm) rate of 25 or over were included in the analysis. Expression levels are defined as low expression (tpm > 25 < 100), medium (tpm > 100 < 500), high (tpm > 500 < 1000) and very high (tpm > 1000). The expression profile of all genes can be found in supplementary table S4.

With the exception of a stress response protein coding gene (N-acetylmuramoyl L-alanine amidase coding *lytC*, DENIP130_03269) the highest expressed genes belong to the operon coding for glycine betaine reductase, spanning from the open reading frames (ORF) DENIP130_04668 to DENIP130_04674. The associated thioredoxin and thioredoxin reductase coding genes, selenocysteine elongation and tRNA-SeC and the glycine betaine transporter OpuD on the same operon were likewise very highly expressed. Similarly, one MttB-superfamily coding gene (DENIP130_03627) as well as its corrinoid co-factor (DENIP130_03626) and an adjacent methylcorrinoid:THF

methyltransferase (DENIP130_03628) were very highly expressed, with the MttB-superfamily coding gene being the 35th most highly expressed gene. Only one other MttB-superfamily coding gene was expressed at low level and all others were not expressed above the inclusion threshold (supplementary table 4). One of the three pyrrolysine cassettes, spanning from DENIP130_00894 to DENIP130_00905 was medium to high expressed. The trimethylamine:corrinoid methyltransferase coding gene located within this operon was likewise highly expressed, as was its adjacent corrinoid protein coding gene. The methylcorrinoid:tetrahydrofolate methyltransferase within this operon was medium expressed. However, the ethanolamine utilization coding gene (DENIP130_00899) and the Fis family transcriptional regulator (DENIP130_00900) were expressed at low and below inclusion threshold, respectively. None of the genes of the two other pyrrolysine cassettes were expressed above inclusion threshold, nor were the methyltransferase genes within the operons coding for the cassettes. Genes coding for proteins involved in sulfate reduction as well as the Wood-Ljungdahl pathway for carbon fixation were all highly expressed, with genes coding for dissimilatory sulfite reduction (*dsrAB*), adenylyl-sulfate reductase (*aprA*), carbon monoxide dehydrogenase/acetyl-CoA synthase (*codh*) and formate—tetrahydrofolate ligase (*fhs*) all within the 100 most highly expressed genes. All expression data can be found in supplementary data S1.

5.3 Discussion

Here, we have demonstrated the utilization of a range of methylated compounds by a new methylotrophic *Desulfosporosinus nitroreducens* strain P130 isolated from Black Sea sediments. Despite the genomic potential for methylotrophy of many marine SRM including *D. nitroreducens* strains 59.4B^{T} and 59.4F, little in-depth inquiry into the genomic and physiological capabilities of anaerobic marine bacterial methylotrophy has been conducted so far, and most research has focused on the interplay between bacteria and methanogenic archaea or the general patterns of the degradation of methylated compounds (198, 245, 326, 327). Both Black Sea sediment enrichments S5 and S29 with methanol as the only ubiquitous electron donor became dominated over time by one organism when methanogenesis was inhibited, as evidenced by reads per basepairs of the metagenome sequencing attributed to these specific MAGs. These MAGs, S29_bin.2 and S5_bin.4 have high genome similarity to the isolated strains P125 and P130, respectively. While S5_bin.5 contained the genes for sulfate reduction, the only putative methylotrophic gene in its genome was an MttB-superfamily gene, without any corrinoid co-factor coding gene. Furthermore, S5_bin.1 and S29_bin.4 are possibly capable of sulfide oxidation, as suggested by the presence of an NADP-dependent sulfide oxidoreductase in their genomes

and the depletion of sulfide when growing on TMAO (data not shown). All other MAGs obtained from the enrichments were determined to be heterotrophic fermenters, based on the absence of respiratory genes in the genomes and characterization of strains with high similarity to the MAGs. For example, S5_bin.1 and S29_bin.4 are classified in the genus *Labilibaculum*, the same genus as the heterotroph *Labilibaculum euxinus*, similarly isolated from the Black Sea (242). This suggests the methylotrophic strains in the enrichment are primarily responsible for the turnover of methylated compounds to biomass, which can then be used by the other organisms in the enrichments. The decrease of diversity and relative abundance of these heterotrophs from the initial community profiling at 12 months compared to the enrichment metagenome at 24 months further corroborates this point, as leftover biomass and more recalcitrant organic matter from the inoculum source became depleted over successive transfers. Using traditional culturing techniques, coupled with genomic analyses, we can provide a potential model on the utilization of methylated compounds in marine sulfate-reducing bacteria isolated from this enrichment 2. At in-situ conditions of 10°C and 1.8% salinity detected in Black Sea sediments, strain P130 is thus able to persist in the environment from which it is isolated, in contrast to strain 59.4B^{T} which was suggested to persist in sporulated form in the Baltic Sea sediments from which it was isolated (198, 328). Copies of the key genes involved in sulfate reduction, dissimilatory sulfite reductase A and B (*dsrAB*) closely related to those of *D. nitroreducens* were identified in a comprehensive study of sulfate reduction in Black Sea sediments (74). As such, it is likely that *D. nitroreducens*, and similar bacteria are active in Black Sea sediments. Whether it is actively using methylated compounds in this environment requires further research.

Due to the more favorable substrate specificity and kinetics of the methanol methyltransferase pathway compared to methanol dehydrogenase, methanogens have traditionally been assumed to outcompete SRM for methylated compounds (46). However, there is a large body of evidence of the presence of methyltransferases in acetogens from environments other than marine sediments(134, 238, 329). Additionally, in the sulfate reducer *Desulfofundulus kuznetzovi* both methyltransferase and dehydrogenase pathways are present in the genome (330). Previously, proteomics of *D. kuznetsovii* grown on methanol showed that the proteins for methanol methyltransferase were present as long as cobalt was present (45). Under conditions of high cobalt concentration, proteins for both dehydrogenases and methyltransferase were present (45). Likewise, in strain P130, both a complete operon for the methanol methyltransferase pathway and several alcohol dehydrogenases are present in its genome. Strain P130 is capable of using methanol as sole carbon and energy source. This

signals methanol serves as a suitable electron donor comparable to other substrates. Whether dehydrogenases or methyltransferases are expressed while growing on methanol is unknown, although it is likely that, like in *D. kuznetzovi*, strain P130 can utilize both pathways due to this previous work.

The majority of trimethylamine-utilizing microorganisms are methanogenic archaea (244, 247). Trimethylamine:corrinoid methyltransferase is one of just three known major genes utilizing the 22nd amino acid pyrrolysine, with the other two genes being dimethylamine and monomethylamine methyltransferases (55). In *Acetohalobium arabaticum*, the expression of the biosynthesis cassette for pyrrolysine is upregulated in the presence of TMA (243). Strain P130 is likewise expected to be able to upregulate the biosynthesis and incorporation of pyrrolysine as a reaction to the presence of TMA. The close localization of pyrrolysine biosynthesis and incorporation genes with the *mtmB* and two *mttB* genes in the genome of strain P130, suggests biosynthesis of pyrrolysine and utilization of trimethylamine are intrinsically linked in strain P130 (figure 2A-B). There is similar close localization of pyrrolysine biosynthesis with monomethylamine methyltransferase (figure 3C). Several organisms related to strain P130 similarly harbor closely localized pyrrolysine biosynthesis and methylated amine methyltransferase genes, for example *Desulfosporosinus fructosivorans*, *Desulfosporosinus lacus*, *D. nitroreducens* 59.4B^{T} as well as the known TMA degrader *A. arabaticum* (198, 202, 243). It is remarkable that of strain P130 is unable to grow on monomethylamine, despite the proximity of all necessary genes in the genome. Both operons with trimethylamine methyltransferase contained genes annotated as transcription regulators, which may have an influence on the incorporation of pyrrolysine in MttB. Additionally, the localization of methylamine methyltransferase is upstream of tRNA-pyl, as opposed to the downstream presence in both trimethylamine methyltransferase operons. Only one operon, spanning from DENIP130_00894 through DENIP130_00905, was expressed at medium or high levels (figure 2A). In this operon, *mttABC* is located directly after the pyrrolysine incorporation gene *pylS*. This could suggest the exact placement of tRNA-pyl and the pyrrolysine incorporation might be of influence for incorporation of pyrrolysine in protein biosynthesis for sequential translation in the correct order to ensure pyrrolysine is incorporated in *mttB*. As such, physiological screening is essential for any organism containing pyrrolysine biosynthesis, as the presence of these genes does not necessarily translate to actual usage of methylated amines such as TMA or MMA.

It was thought that in anoxic environments, larger methylated compounds such as glycine betaine are fermented by bacteria, and that the formed methylated

compounds, such as TMA, are released in the environment (18). These small methylated compounds could then be utilized in the methyltransferase pathway by methanogens (46). This has indeed been observed in some recent enrichment studies on glycine betaine (331–333). However, *Desulfitobacterium hafniense*, isolated from sewage sludge, has been shown to use the methyltransferase pathway to grow on glycine betaine, forming N,N-dimethylglycine (50). Glycine betaine has been described to be utilized by marine methanogens within the genus *Methanococcoides*, the marine sulfate-reducing bacterium *Desulfobacterium* strain PM4 and the acetogenic organism *Eubacterium limosum*, likewise producing dimethylglycine rather than TMA (16, 53, 178). The genomes of all these organisms have been shown to contain close homologs to the gene encoding glycine betaine:corrinoid methyltransferase MtgB of *D. hafniense*, except for *Desulfitobacterium* strain PM4, of which no genome is available. Strain P130 uses glycine betaine as carbon and energy source, producing N,N-dimethylglycine as well as acetate and TMA.

The presence of *mtgB* and *grd*-complex suggests glycine betaine can be used as both a direct substrate for demethylation as well as reduction to acetylphosphate and TMA, after which acetylphosphate is further oxidized to acetate. In *Eubacterium acidaminophilum* and *Sporomusa ovata*, the reducing power for the conversion of glycine betaine to trimethylamine and acetylphosphate was suggested to originate through the oxidation of a methyl group of glycine betaine to CO_2 although the genes involved in this methyl transfer were never identified (17, 323). In *Sporomusa* strain An4, oxidation of a methyl group of TMA to CO_2 was suggested to generate reducing equivalents, which are then used to power glycine betaine reductase (123). However, close inspection of the genome of *Sporomusa* An4 reveals the gene assigned as *mttB* in that study lacks pyrrolysine and, as such, is unlikely to be a trimethylamine methyltransferase. A re-examination of this organism is needed to determine the actual function of the gene annotated as *mttB*, although it seems likely this is a glycine betaine:corrinoid methyltransferase coding gene. In *Acetobacterium woodii*, which lacks *grdHI* and *grdABCD*, glycine betaine is demethylated to dimethylglycine through a protein encoded by a *mtgB* homolog, after which the reducing equivalent obtained is used to reduce CO_2 to CO in the carboxylic branch of the Wood-Ljungdahl pathway (238). Based on the presence of both a GB reductase complex and a GB methyltransferase system, both of which were highly expressed in our transcriptome analysis, we propose that in strain P130, MtgB (DENIP130_03627), and its associated corrinoid co-factor protein catalyze the methyl transfer from glycine betaine to tetrahydrofolate, forming dimethylglycine and methyl-THF. Methyl-THF is then oxidized to CO_2 through a reversal of the methyl-branch of the Wood-Ljungdahl path-

way, generating reducing equivalent to reduce a second molecule of glycine betaine to trimethylamine and acetylphosphate, which is then oxidized to acetate (figure 4). Excess reducing equivalents are coupled to reduction of sulfate or another electron acceptor. This process would yield an ATP through substrate level phosphorylation in both the reverse Wood-Ljungdahl pathway as well as the substrate level phosphorylation driven by the conversion of acetylphosphate to acetate. The coupling of reducing equivalent to sulfate reduction additionally yields ATP through electron transport phosphorylation. The produced TMA can then serve as substrate for a pyrrolysine incorporating methyltransferase, producing DMA. While we did not measure any DMA in our physiological tests, pyrrolysine biosynthesis and TMA methyltransferase were all highly expressed in our transcriptomic study, suggesting that some produced TMA could indeed be converted into DMA. Conversely, while we did see production of acetate in our physiological study, the sole acetate kinase coding gene (DENIP130_00417) was only expessed at low levels. As equimolar amounts of TMA and acetate are formed, incorporation of formed acetyl-P in the central carbon metabolism is not likely. It is possible a low constitute expression is enough for this step. Due to the dependence of pyrrolysine for TMA methyltransferase and the dependence of selenocysteine in glycine betaine reductase, this suggests that *D. nitroreducens* strain P130 when growing on glycine betaine relies on all 22 known amino acids (334).

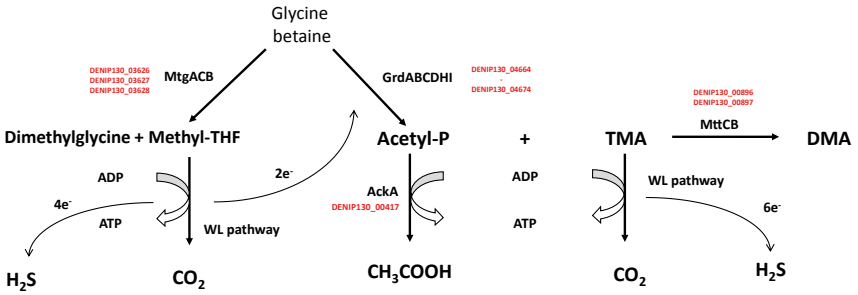

Figure 4: Proposed pathway of glycine betaine utilization by Desulfosporosinus nitroreducens strain P130. Names of genes expressed by strain P130 when growing on glycine betaine are displayed in red. Abbreviations: TMA: trimethylamine; DMA: dimethylamine; Methyl-THF: methylated tetrahydrofolate; Acetyl-P: acetyl-phosphate; MtgACB: glycine betaine:corrinoid methyltransferase pathway; GrdABCDHI: glycine betaine reductase pathway; MttCB: trimethylamine:corrinoid methyltransferase pathway; AckA: acetate kinase; ACS: acetyl-CoA synthase; WL pathway: Wood-Ljungdahl pathway.

5.4 Conclusions

Here, we have isolated a novel strain P130 of *Desulfosporosinus nitroreducens* able to grow on methylated compounds and under Black Sea sediment *in-situ* conditions of salinity and temperature. Genome and transcriptome analysis revealed that this bacterium likely uses the methyltransferase pathway, similar as employed by methanogens. Likewise, this organism contains the pyrrolysine biosynthesis cassette required for incorporation of the 22nd amino acid pyrrolysine, crucial in the TMA:corrinoid methyltransferase catalysis. Strain P130 is capable of growth on the ubiquitous marine methylated compounds methanol and glycine betaine, and its genome codes for MttB-superfamily coding genes and associated corrinoid protein coding genes, suggesting it can metabolize a broader range of methylated amines. These results indicate possible bacterial methylotrophy in marine sediments, although *in-situ* experiments will be required to confirm active methylotrophy of *D. nitroreducens* strain P130 and other similar bacteria in marine sediments.

5.5 Material and methods

5.5.1 Inoculum source and enrichment

Black Sea sediment samples were collected in August 2018 from station 1 (42° 53.8' N 30° 40.7' E, Bulgarian exclusive economic zone) of the 64PE444 research cruise of R/V Pelagia. Sediment cores with a diameter of 10 cm and lengths of 30-45 cm were collected with a multicorer, sliced in 1 cm disks and immediately transferred into a glovebag (Aldrich© AtmosBag) flushed with N_2. The upper sediment, consisting of unstable coccolith ooze was discarded. Sediment disks of 5-6 cm below seafloor level (bsf) and 29-30 cm bsf were transferred to sterile 1L bottles and amended with 500 mL anoxic reduced basal medium as described previously, yielding roughly 600 mL of sediment slurry (240). Medium consisted of (g L^{-1}) NaCl 17.16; KCl 0.3715; NaBr 0.0557; NH_4Cl 0.1548; K_2HPO_4 0.5653; $MgCl_2$ 3.511; $CaCl_2$ 0.091; Na_2S 0.360. Medium was appended with 1 mL L^{-1} of vitamin solution consisting of (mg L^{-1}) biotin 20; nicotinic acid; 200; pyridoxine 300; riboflavin 50; thiamine 200; cyanocobalamin 100; p-aminobenzoic acid 80; pantothenic acid 100; lipoic acid 30 and folic acid 80. Additionally, 1 mL L^{-1} each of acid and base trace element solution was added, consisting of (mg L^{-1}) acid: H_3BO_3 10; $MnCl_2$ 53; $FeCl_2$ 943; $CoCl_2$ 42; $ZnSO_4$ 70; $NiCl_2$ 25; $CuCl_2$ 20; KI 10 $SrCl_2$ 10; $CeCl_3$ 10 and 13 mL L^{-1} HCl 25%; base: Na_2MoO_4 36; Na_2WO_4 8; Na_2SeO_3 6; Na_3VO_4 1; NaOH 400. 1; mL L^{-1} 0.1% w/v Na-resazurin solution was added as redox indicator. Sediment slurry was used immediately to create enrichment cultures by adding 5 mL slurry to 45 mL anoxic reduced basal medium amended with 10 mM magnesium sulfate as electron acceptor, 10 mM methanol as

electron donor, 20 mM 2-bromo ethanosulfonate to inhibit methanogenesis, and 0.1 g · L^{-1} yeast extract from anoxic, separately autoclaved stock solutions in 117 mL serum bottles. Bottles were sealed with butyl rubber stoppers (Rubber BV, Hilversum, Netherlands) and capped with aluminum caps. Sediment enrichments were kept at in-situ temperatures of 10°C with 100% N_2 at 1.5 atm as headspace for 80 days during transport, after which the headspace was replaced with a mixture of 80/20 (v/v) N_2/CO_2 headspace at 1.5 atm and enrichments were incubated at 20°C. Enrichments were monitored frequently for depletion of carbon source (see analytical techniques), and transferred to fresh medium monthly using 10% v/v inoculum or after full depletion of carbon source. Inoculum slurry with no additional carbon source was preserved at 4°C.

5.5.2 Microbial diversity analysis of enrichments based on 16S rRNA gene amplicon sequencing

The microbial community composition of enrichment cultures after 12 months of successive transfers was analyzed by 16S rRNA amplicon sequencing. Technical triplicates of DNA was isolated from 15 mL of enrichment culture. Each sample was centrifuged for 20 minutes at 4500 g. A total of 14.5 mL of the supernatant was discarded, and pellet was resuspended in the remaining 0.5 mL of supernatant. DNA was extracted using the FastDNA Spin Kit for soil (MP Bio, Irvine, CA) using supplier's instructions. Obtained DNA was cleaned and concentrated using the Zymo CleanDNA kit (Zymo Research, Irvine, CA). Fragments of the 16S rRNA gene were amplified from the extracted DNA using polymerase chain reaction (PCR) to obtain 16S rRNA amplicons. As positive control, two defined mock communities were added to the sample set. DNA was diluted to 20 ng/µL in nuclease free water. PCR reactions were performed in triplicate reactions of 50 µL PCR mix, which consisted of 0.2 mM dNTPs, 0.02 U μL^{-1} Phusion Hot Start II DNA polymerase (Thermo Scientific, Waltham, MA) 500 nM of 515F (5'-GTGTGYCAGCMGCCGCGGTAA-'3) and 806R (3'-CCGGACTACNVGGGTWTCTAAT-'5) primer pairs, 10 µL HF PCR buffer and 1 µL diluted DNA. The amplification program consisted of an initial denaturing step at 98°C for 30 seconds, followed by 25 cycles of 10 seconds of denaturation at 98°C, 10 seconds of annealing at 50°C and 10 seconds of elongation at 72°C, after which DNA was allowed a final elongation at 72°C for 7 minutes. PCR product was cleaned and purified using magnetic beads (MagBio Genomics, Gaithersburg, MD) and quantified using Qubit (Thermo Fisher, Waltham, USA). Amplicon fragments were sequenced by Eurofins Genomics (Luxemburg, Luxemburg) using the Illumina HiSeq2500 platform yielding paired-end reads. All samples were sequenced in triplicate.

16S rRNA amplicon sequencing data were analyzed using the CASCABEL pipeline (335). Amplicon fragments were clustered into amplicon sequence variants (ASVs) at >98.5% and assigned taxonomy using the SILVA non-redundant r138 database as reference using qiime2 (336, 337). To eliminate singletons, minimum ASV relative abundance inclusion threshold was set at 0.01%.

5.5.3 Metagenome sequencing

Metagenome sequencing was performed after 24 months of subsequent transfers. DNA was isolated by sampling 20 mL of enrichment culture, which was centrifuged for 20 minutes at 4500 g. 19.5 mL of supernatant was discarded, and pellet was resuspended in the remaining 0.5 mL of supernatant. DNA was extracted using the FastDNA SPIN Kit for Soil (MP Bio, Irvine, CA) using supplier's instructions. Obtained DNA was cleaned and concentrated using the Zymo kit (Zymo Research, Irvine, CA). DNA concentrations were quantified using Qubit (Thermo Fisher, Waltham, MA). Metagenome sequencing and quality assessment was performed by Novogene using the Illumina HiSeq2500 platform (Hong Kong, China). Demultiplexed, quality controlled and trimmed 150 bp reads were obtained. Reads were assembled in contigs using SPAdes v3.15 with function metaSPAdes at default settings (306). Assembly quality was assessed using Quast v5.0.2 (338) at default settings and reads were mapped back to contigs using Bowtie2 v2.4.2 (339) at default settings. Contigs were assigned to metagenome assembled genomes (MAGs) using three binning algorithms: MetaBAT2 v2.12.1, concoct v1.0.0 and maxbin2 v2.0 within the metaWRAP 1.2.1 environment at default settings (272–274, 307). Obtained MAGs were refined and clustered in consensus bins using metaWRAP v1.2.1 at default settings. MAG quality was checked for completeness and contamination throughout using CheckM v1.0.12 with function lineage workflow (277). Bins with a completeness >60% and contamination <10% were included in assessment. Taxonomic classification of obtained MAGs was obtained with gtdb-tk v1.3.0 using function classify_wf (278). Initial genome annotation of all MAGs was performed using PROKKA v1.13 at default settings and function –kingdom Bacteria (291). Additional gene annotation for methyltransferase genes of interest was performed with HMMer suite v3.3 using profile HMMs as described in chapter 3 (281).

5.5.4 Isolation and identification of strains P130 and P125

Single colonies were grown from both enrichments S29 and S5 by using the roll tube technique (340). Basal medium was amended with 10 g L^{-1} agarose (Carl Roth, Karlsruhe, Germany) and 10 mL was dispensed in 50 mL tubes, sealed with butyl stoppers and aluminum caps and flushed with 80:20 v/v N_2/CO_2. After six weeks of incuba-

tion at 20°C small, white colonies with a diameter of 0.2-0.5 mm formed. Single colonies were picked and transferred to fresh liquid medium. A dilution series followed by an additional growth in roll tubes was performed to ensure axenity. Purity was verified by subcultivation in basal medium with 2 g L^{-1} Wilkins-Chalgren broth to promote growth of potential contaminants and Sanger sequencing of full length 16S rRNA gene. To obtain DNA for sequencing, 10 mL liquid culture was centrifuged for 15 minutes at 4500g. From the supernatant, 9.5 mL was discarded. DNA was extracted using the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany) using supplier's instructions. The obtained DNA was used as template material for PCR using 27F and 1492R primers. PCR product was checked with 1% agarose gel electrophoresis and cleaned and concentrated with the Zymo kit (Zymo Research, Irvine, CA). Cleaned PCR product was sequenced using Sanger sequencing at Novogene with 27F and 1492R primers (Novogene, Hong Kong, China).

5.5.5 Physiological characterization of the isolated strain and analytical techniques

Growth tests were performed on strain P130 in 50 mL bicarbonate buffered medium as described previously, using thiosulfate as electron acceptor to optimize growth conditions (341). Medium was supplemented with 1 g L^{-1} yeast extract (YE) and a negative control with only YE was used to correct for the influence of YE in all assays. The following methylated substrates as carbon and energy source were used: methanol, dimethylsulfide (DMS), glycine betaine (GBT), trimethylamine (TMA), dimethylamine (DMA) and methylamine (MMA). Growth was monitored by visual inspection by phase-contrast microscopy (Leica DM2000, Leica Microsystems GmbH, Wetzlar, Germany), and by measuring decrease of electron donor as well as increase in sulfide.

Concentrations of methanol were determined through gas chromatography using a GC2010 system equipped with a HS-20 headspace analyzer (Shimadzu Corporation, Kyoto, Japan). The GC carried a DB-WAX Ultra Inert column, 30m x 0.32mm x 0.50 um (Agilent Technologies, Santa Clara, CA) that was held at 50°C. Carrier gas was nitrogen, with an applied pressure of 100 kPa. A sample of 100 ul liquid was brought into a 10 ml headspace vial using 10 mM propanol as internal standard. Next the vial was transferred to the oven of the autosampler that was held at 60°C. After an equilibrating time of 5 minutes a sample of the headspace wat brought onto the column. Detector was a flame ionization detector. Concentrations of MMA were determined using gas chromatography using the GC2010 system (Shimadzu Corporation, Kyoto, Japan) equipped with a Select CP-Volamine column, 30m x 0.54 mm x 0.5 um (Agilent Technologies, Santa Clara, CA) which was held at 100°C using nitrogen

as carrier gas at 100 kPa and using a flame ionization detector. Dimethylamine and trimethylamine concentrations were determined using ion exchange-chromatography using the ICS2100 system equipped with an IonPac SCS1 column, 250 mm x 2 mm set to 30°C (Thermo Fisher, Waltham, MA). As eluent 3 mM methanesulfonic acid in 10/90 (v/v) acetonitrile and milliQ water was used at a flow rate of 0.25 mL/minute and using a non-suppressed conductivity detector. Sulfate and thiosulfate concentrations were determined using ion exchange-chromatography using the ICS2100 system equipped with an AS17 column, 250 mm x 2 mm set to a temperature of 30°C (Thermo Fisher, Waltham, MA). The eluent consisted of a gradient that was generated with a KOH-cartridge. The hydroxide concentration ranged from 1 mM during the first two minutes to 40 mM after 20 minutes. The flow was set at 0.30 ml/min. Detection was done with a suppressed conductivity detector. Sulfide concentrations were determined photometrically using the methylene blue method after fixation of the sample in 20 mM zinc-chloride (342).

5.5.6 Whole genome sequencing and analysis

Freeze dried *Desulfosporosinus nitroreducens* 59.4BT^{T} (DSM101608^{T}) was obtained from the German collection of of Microorganisms and Cell cultures (DSMZ) and reactivated following their instructions. Both *D. nitroreducens 59.4BT^{T}* and strain P130 were subsequently cultured in anoxic basal medium prepared as described above. DNA of strain P130 and *D. nitroreducens 59.4BT^{T}* was obtained and sequenced as described above. Retrieved reads were assembled using SPAdes v3.15 at default settings. Short contigs with aberrant coverage of a factor two or above were removed from the assembly. Assembly quality was assessed using Quast v5.0.2 at default settings and reads were mapped back to contigs using Bowtie2 v2.4.2 at default settings to obtain genome coverage. Genome completeness and contamination was assessed with CheckM v1.0.12 with function lineage workflow. Initial genome annotation of was performed using PROKKA v1.13 (settings: --kingdom Bacteria). Additional gene annotation for methyltransferase genes of interest was performed with HMMer suite v3.3 using profile HMMs as described in chapter 3. Alcohol dehydrogenase annotations were performed by building a custom HMM using function hmmbuild of HMMer suite v3.3 using the alcohol dehydrogenase coding genes within the family *Peptococcacea* as described by Friedeheim et al. (manuscript in preparation). Average nucleotide identity and average amino acid identity between strain P130 and *D. nitroreducens 59.4BT^{T}* was calculated using the ani.rb and aai.rb scripts from the enveomics collection, respectively (343). To further assess strain purity, metagenome binning as described in enrichment metagenome sequencing was performed on the retrieved reads, which yielded a single genome for each sample.

5.5.7 RNA extraction and transcriptome analysis

Strain P130 was grown in triplicate in 250 mL bicarbonate buffered medium in 500 mL bottles with glycine betaine as carbon and energy source. Growth was monitored through measurements of N,N-dimethylamine and trimethylamine as described above and biomass was harvested anaerobically when accumulation of products equated half of added substrate. To quench translational activity, 200 mL of ice cold buffered medium was added to each bottle and all movements were conducted with the bottles on ice. Biomass was obtained by centrifuging medium at 10.000 x g for 20 minutes at 4°C in a Sorvall LYNX 4000 centrifuge (Thermo Fisher Scientific, Waltham, MA). Supernatant was discarded and the pellet was washed with ice cold PBS buffer and centrifuged again using a Sorvall Legend XTR centrifuge at 10.000 x g for 10 minutes at 4°C. Supernatant was removed and the obtained biomass was snap-frozen using liquid nitrogen and stored at -70°C until further use. Cells were thawed and DNA and RNA was extracted using the Masterpure Gram Positive DNA Purification Kit according to manufacturer's instructions (Biosearch Technologies,Hoddesdon, United Kingdom). To inhibit RNase, β-mercaptoethanol was added to the lysis solution. RNA was then purified and extracted with a Maxwell RSC simpyRNA cells kit, following manufacturer's instructions, utilizing the Maxwell RSC instrument (Promega, Madison, WI). Samples were sent for rRNA depletion and sequencing at Novogene (Hong Kong, China). Sequencing was performed utilizing a NovaSeq 6000 platform which yielded paired-end reads of 150 bp.

Quality of the obtained reads was assessed using FastQC v0.11.9 (308). Adapters, PhiX contamination and low quality sequences were trimmed using bbduk.sh from the bbtools v38.62 suite with functions ktrim=r, k=23, mink=7, hdist=1, qtrim=rl, trimq=30, ftm=5, maq=20, minlen=50 (305). In total, between 10.06 and 14.41% of the reads were discarded. Reads were mapped to the full *D. nitroreducens* strain P130 genome to assess mapping rate, then to all protein coding sequences of the genome to infer gene expression using function bbsplit.sh from the bbtools v38.62 suite at default settings (305). Counts per gene were normalized by calculating transcripts-per-million (TPM) by dividing counts by the corresponding gene length in kilobasepairs (kbp). Normalized counts in kbp were then divided by the total sample sum and multiplied by one million.

5.6 Data availability

The demultiplexed Illumina Hiseq reads of the 16S rRNA gene amplicon sequencing were deposited at the European Nucleotide Archive (ENA) under study PRJEB48164 in fastq format with accession numbers ERS7696150-ERS7696161.

Metagenome assembled genomes are deposited at the National Centre for Biotechnology Information (NCBI) under Bioproject PRJNA769205 with accession numbers SAMN22312441- SAMN22312451. The genome of *Desulfosporosinus nitroreducens* strain P130 is deposited at the NCBI with accession number SAMN28464218. The genome of *Desulfosporosinus nitroreducens 59.4F*^{T} is deposited at the NCBI under accession number SAMN28463191. Supplementary material can be downloaded from doi.org/10.6084/m9.figshare.c.6564901.v1

Simultaneous consumption of glycine betaine and methanol by a co-culture of a sulfate-reducing microorganism and a methanogen

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Abstract

Methylated compounds are commonly produced by marine organisms as osmolytes. In marine sediments, these methylated compounds are considered non-competitive substrates for methanogens, allowing them to co-exist with sulfate-reducing microorganisms. However, there is an increasing body of evidence suggesting that sulfate-reducing microorganisms play an important role in metabolizing methylotrophic substrates and might in fact compete for these substrates. This suggests the fate of methylated compounds in marine sediments may be more complex than previously assumed. To further investigate the interactions between methanogens and sulfate-reducing organisms, we performed co-cultivation studies between *Desulfosporosinus nitroreducens* strain P130 and a methylotrophic methanogenic enrichment dominated by the methanogen *Candidatus* Methanococcoides chernomoris, obtained from Black Sea sediments. In these experiments, both methylotrophic microorganisms co-existed when growing on methanol. When growing on glycine betaine, simultaneous oxidative and reductive cleavage occurs, indicated by the simultaneous production of TMA, acetate and N,N-dimethylglycine. Through a transcriptomic approach we compared the co-culture of strain P130 and methanogenic enrichment with their individual cultures. We have identified a strong upregulation of the genes involved in methyltransferase-dependent cleavage of trimethylamine, dimethylamine and monomethylamine in the methylotrophic methanogen, whereas glycine betaine specific methyltransferases were downregulated compared to growth without the sulfate reducer present. In the sulfate-reducing microorganism, genes coding for both the oxidative methyltransferase and reductive cleavage of glycine betaine as well as the methyltransferase-dependent cleavage of trimethylamine were more highly expressed in the co-culture compared to the monoculture. Our results further our understanding of the increasingly complex interactions surrounding methylated compounds in marine sediments.

6.1 Introduction

Marine sediments are some of the most diverse habitats known in the world. In these sediments, organic material accumulates through deposition from the water column as well as through microbial turnover, mineralization and remineralization (2). While the uppermost layers of sediment is oxic, oxygen is rapidly depleted, causing all underlying sediments to be anoxic. In the absence of oxygen, the most common electron acceptor for microbial respiration is sulfate, which is present in millimolar amounts in most marine systems (39). Another important marine microbial metabolism is methanogenesis. As sulfate reduction is more kinetically and thermodynamically favorable over methanogenesis for their common substrates such as hydrogen and acetate, sulfate reduction and methanogenesis are considered mutually exclusive metabolisms. In deeper sediments where no sulfate is present, significant methanogenic activity is observed (344). Despite this, some methanogenic activity in sediments rich in sulfate has been observed. This is considered to be facilitated by the utilization of methanogenic substrates where there is little to no competition with sulfate reducers, allowing them to co-exist (40). These non-competitive substrates are commonly recognized as small methylated compounds such as methanol or trimethylamine (TMA). Methylated compounds are ubiquitous in marine environments as fermentation products of osmolytes that are produced in bulk by most marine organisms (19, 321).

Methanogens utilize these compounds through the transfer of methyl groups from the methylated substrate into methanogenesis through the methyltransferase pathway (35). The methyltransferase pathway generally consists of two proteins, MTI and MTII. MTI is a substrate specific, two-domain protein, which cleaves off the methyl group from the donor and transfers it to a corrinoid cofactor. Then, MTII facilitates the transfer of the methyl-corrinoid complex to a tetrahydromethanopterin (H_4MPT) acceptor, integrating it in the methanogenesis pathway. In the methylotrophic methanogenesis pathway, one in four methyl-H_4MPT is oxidized to CO_2 in a reversal of the hydrogenotrophic methanogenesis pathway to obtain reducing equivalent to reduce the remaining three molecules of methyl-H_4MPT to methane (226, 230, 345). While sulfate reduction with methylated compounds has a higher theoretical energy yield than methanogenesis (for example, ΔG^0 -364 kJ/reaction versus -311 kJ/reaction under standard conditions for methanol oxidization with sulfate and methanol derived methanogenesis, respectively), the more favorable kinetics of the methyltransferase pathway in comparison with the dehydrogenase proteins utilized by sulfate-reducing microorganisms are thought to facilitate co-existence of these metabolisms (57, 121).

Despite the long-held assumption that methylated compounds are indeed "non-competitive", there is an increasing body of evidence that suggests this is not the case. Methyltransferase pathways have been found to be present in the genomes a wide range of non-methanogenic marine microorganisms, including sulfate-reducing organisms and acetogens (45, 243, 245). Methanol methyltransferases were found to be present in high numbers in the proteome of the sulfate reducer *Desulfofundulus kuznetsovii* when growing on methanol (45). Furthermore, methylated quaternary amine compounds have been found to serve as a direct substrate for methyl transfer for both methanogens and bacteria (50, 54, 236, 238). Homologs for the genes coding for these quaternary amine methyltransferases are widely present in the genomes of many anoxic marine microorganisms. Likewise, some methanogens have been demonstrated to utilize quaternary amines as a direct substrate for methanogenesis (52, 53, 178, 317). Thus, the fate of methylated compounds in marine sediments may be different than previously assumed. However, the relationship between sulfate reducers and methanogens when growing on common methylated substrates remain largely unexplored and little is known about their potential interactions when growing on these substrates.

To further elucidate the intricate relationship between sulfate reducers and methylotrophic methanogens potentially taking place in marine sediments, we performed co-cultivation studies between the recently described sulfate-reducing *Desulfosporosinus nitroreducens* strain P130 (chapter 5) and a methylotrophic methanogenic enrichment, M32, dominated by the methanogen *Ca*. Methanococcoides chernomoris, which also contains the saccharolytic microorganisms *Ca*. Izemoplasma apoleimma and *Ca*. Pulmentibacterium quisquilia (chapter 4). Both *D. nitroreducens* strain P130 and *Ca*. Mc. chernomoris were obtained from Black Sea sediments and are capable of growing at *in situ* salinity and temperatures of 1.8‰ and 10°C, respectively. *D. nitroreducens* strain P130 is capable of growth on the methylated compounds methanol, TMA and glycine betaine (GB) and codes for their respective methyltransferase genes *mtaB*, *mttB* and *mtgB*. A transcriptomic investigation in the utilization of glycine betaine of *D. nitroreducens* strain P130 revealed the simultaneous expression of a glycine betaine:corrinoid methyltransferase and a glycine betaine reductase pathway. The methanogenic enrichment dominated by *Ca*. Mc. chernomoris is likewise capable of growth on the methylated compounds methanol and GB, as well as tetramethylamine (QMA), trimethylamine, dimethylamine (DMA) and monomethylamine (MMA). Growth rates of both organisms in co-culture on methanol and glycine betaine were compared. Additionally, a transcriptomic approach was employed to elucidate the simultaneous expression of genes involved in the consumption of glycine betaine by both organisms.

6.2 Material and methods

6.2.1 Source of the microorganisms and construction of the co-culture

Desulfosporosinus nitroreducens Strain P130 and the methylotrophic methanogenic enrichment M32 were obtained in previous studies. Individual cultures were grown under the same conditions at 25°C in saline bicarbonate buffered growth medium, consisting of (g L^{-1}) NaCl 17.16; KCl 0.3715; NaBr 0.0557; NH_4Cl 0.1548; K_2HPO_4 0.5653; $MgCl_2$ 3.511; $CaCl_2$ 0.091; sulfide 0.360. Medium was amended with 1 mL L^{-1} of vitamin solution consisting of (mg L^{-1}) biotin 20; nicotinic acid; 200; pyridoxine 300; riboflavin 50; thiamine 200; cyanocobalamin 100; p-aminobenzoic acid 80; pantothenic acid 100; lipoic acid 30 and folic acid 80. Additionally, 1 mL L^{-1} each of acid and base trace element solution was added, consisting of (mg L^{-1}): H_3BO_3 10; $MnCl_2$ 53; $FeCl_2$ 943; $CoCl_2$ 42; $ZnSO_4$ 70; $NiCl_2$ 25; $CuCl_2$ 20; KI 10 $SrCl_2$ 10; $CeCl_3$ 10 and 13 mL L^{-1} HCl 25% for the acid trace mix and Na_2MoO_4 36; Na_2WO_4 8; Na_2SeO_3 6; Na_3VO_4 1; NaOH 400 for the base trace mix. Additionally, 1 mL L^{-1} 0.1% w/v Na-resazurin solution was added as redox indicator. Finally, 0.1 ml L^{-1} of yeast extract was added to enhance growth. All growth tests were conducted in 250 mL anoxic serum bottles, sealed with butyl rubber stoppers (Rubber BV, Hilversum, the Netherlands) and capped with aluminum caps. As headspace, 80/20 (v/v) N_2:CO_2 was used.

Unless stated otherwise, all growth tests were performed in the described basal anoxic medium with 80:20 (v/v) N_2/CO_2 as headspace at 20°C and pH 7. Growth tests were performed in triplicate for co-culture with 10 mM methanol and 10 mM glycine betaine as carbon and electron donor. Additionally, growth tests were performed on methanogenic enrichment culture with 10 mM of sulfate and 10 mM of glycine betaine as carbon and electron donor.

Both *Desulfosporosinus* strain P130 and methanogenic enrichment cultures were pregrown individually with 10 mM sulfate and either 10 mM methanol or 10 mM glycine betaine at 25°C. Initial co-cultures were established by adding 10% (v/v) methanogenic enrichment to serum bottles containing strain P130 at the start of its exponential phase to account for the longer lag phase of strain P130. Co-cultures were grown until all substrate was depleted, after which 10% (v/v) was transferred to fresh medium. After two subsequent transfers, growth tests were initiated.

6.2.2 Analytical techniques

Methane production was tested using gas chromatography utilizing a GC2010 system

(Shimadzu corporation, Kyoto, Japan) equipped with a Porabond Q column, 25m x 0.53mm x 10 um (Agilent Technologies, Santa Clara, CA). As carrier gas nitrogen was used at 100 kPa, with a column temperature of 40°C and using a flame ionization detector. Methanol consumption was tested using gas chromatography utilizing a GC2010 system with a HS20 gas phase autosampler (Shimadzu corporation, Kyoto, Japan) equipped with a DB-WAX Ultra Inert column, 30m x 0.32mm x 0.5 um (Agilent Technologies, Santa Clara, CA). As carrier gas nitrogen was used at a column oven temperature of 50°C with a flame ionization detector. Dimethylglycine, trimethylamine and dimethylamine were measured using ion exchange chromatography utilizing a DIONEX 2100 system (Thermo Fisher, Waltham, MA) equipped with a IonPac SCS1 column, 250mm x 2 mm, set to 30°C (Thermo Fisher, Waltham, MA). As eluent, 3 mM methanesulfonic acid in 10/90 (v/v) acetonitrile/milliQ water was used at a flow rate of 0.25 mL/minute using a non-suppressed conductivity detector. Due to overlapping peaks with other ions, no glycine betaine could be measured. Sulfate was measured using ion exchange chromatography utilizing a DIONEX 2100 system equipped with an AS17 column, 250 mm x 2 mm set to 30°C (Thermo Fisher, Waltham, MA). The eluent consisted of a gradient that was generated with a KOH-cartridge. The hydroxide concentration ranged from 1 mM during the first two minutes to 40 mM after 20 minutes. The flow was set at 0.30 ml/min. Detection was done with a suppressed conductivity detector. Acetate was measured using high pressure liquid chromatography, utilizing an LC2030-plus system, equipped with a Shodex SH1821 column, 300mm x 8mm at 40°C (Shodex, Japan) and using refractive index detector. As eluent, 0.5 mM sulfuric acid in milliQ water was used. Sulfide was measured colorimetrically, using the methylene blue method after fixation of the sample in 20 mM zinc-chloride (342).

6.2.3 RNA extraction and metatranscriptome sequencing

Co-cultures and methylotrophic methanogen enrichment were grown in triplicate in 250 mL bicarbonate-buffered medium as described above in 500 mL serum bottles with glycine betaine as carbon and energy source. Biomass was harvested under anoxic conditions when dimethylglycine concentrations were half of the total added glycine betaine. To each bottle, 200 mL ice cold reduced medium was added to arrest transcriptional activity. Cultures were centrifuged at 10.000 x g at 4°C for 20 minutes in a Sorvall Lynx 4000 centrifuge (Therma Fisher Scientific, Waltham, MA). From this, supernatant was discarded and biomass pellet was resuspended in ice cold PBS buffer, then centrifuged again in a Sorvall Legend XTR centrifuge (Thermo Fisher Scientific, Waltham, MA) at 10.000 x g for 10 minutes at 4°C. Obtained supernatant was discarded and biomass pellet was snap-frozen using liquid nitrogen and stored

at -70°C until RNA extraction. RNA and DNA were extracted using the Masterpure Gram Positive DNA Purification Kit (Biosearch Technologies, Hoddesdon, UK) using manufacturer's instructions, with an additional step of β-mercaptoethanol addition to inhibit RNases. RNA was purified using the Maxwell RSC simplyRNA platform with the Maxwell RSC instrument (Promega, Madison, WI) following manufacturer's instructions. RNA extracts were sequenced at Novogene (Hong Kong, China), utilizing the NovaSeq 6000 platform, yielding paired-end reads of 150 bp.

6.2.4 Transcriptomic analysis

Quality of the obtained reads was analyzed with FastQC v0.11.9 (308). Low quality sequences, adapter sequences and PhiX contamination were trimmed using bbduk.sh from the bbtools v38.62 suite using functions ktrim=r, k=23, mink=7, hdist=1, qtrim=rl, trimq=30, ftm=5, maq=20, minlen=50 (305). Trimmed reads were mapped back to the draft enrichment genomes using function bbsplit.sh from the bbtools v38.62 suite at default settings. Mapping quality statistics and counts per gene were compiled using function samtools view from the samtools v1.10 suite using the genomes of *D. nitroreducens* strain P130 and the 4 constituent strains of the methanogenic enrichments (271). For differential expression analysis, *D. nitroreducens* strain P130 expression data of chapter 5 was used. Expression data was normalized and analyzed for differential expression and statistical significance using DESeq2 version 1.34.0 (309). Briefly, differential expression is calculated by fitting normalized count data per gene to a generalized linear model using gamma-Poisson distribution, followed by a Wald significance test between conditions, yielding log2fold changes and adjusted p-values between conditions. Count data per gene were normalized to transcripts-per-million (TPM) by dividing normalized counts by the corresponding gene length in kilobasepairs (kbp). Normalized counts per kbp were then divided by the total sample sum and multiplied by $1{\cdot}10^6$ to obtain the TPM. Visualization of results was performed in R version 4.2.2 using package pheatmap with additional editing in Adobe InDesign (211).

6.3 Results

6.3.1 Physiology of the *D. nitroreducens* strain P130 and methanogenic enrichment co-cultures

A co-culture consisting of *Desulfosporosinus nitroreducens* strain P130 and methanogenic enrichment M32 dominated by *Ca*. Methanococcoides chernomoris was constructed. Incubation of co-cultures with both methanol and glycine betaine resulted in the production of both methane and sulfide, indicating activity of both

the SRM and methanogen in the co-culture. Growth on methanol yielded reproducible results in all replicates, with immediate consumption of methanol and production of methane and sulfide, despite the lag phase in both the monoculture of *D. nitrodeducens* strain P130 and the methanogenic enrichment when growing on methanol (Figure 1a). In contrast, growth of the triplicate cultures on glycine betaine was less reproducible and therefore the results in this text are shown for all the triplicates individually (Figure 1b). Intermediates and final products in each triplicate were the same, yet the ratio between intermediates and final products differed. Each replicate produced methane, sulfide, N,N-dimethylglycine trimethylamine, dimethylamine and acetate. Either a large amount of N,N-dimethylglycine and a low amount of TMA, DMA and acetate was produced (95% to 5% for N,N-dimethylglycine and TMA, respectively), a low amount of N,N-dimethylglycine and a large amount of TMA (26% to 74%, for N,N-dimethylglycine and TMA, respectively), or an intermediary between these two results (66% to 34% for N,N-dimethylglycine and TMA, respectively) (figure 1b). In the replicate with the highest observed amount of N,N-dimethylglycine, a larger amount of methane and a lower amount of sulfide was produced than in the replicate with the lowest observed N,N-dimethylglycine. In all replicates, production of acetate was observed, following the same trend as TMA production. DMA production was observed after the initial peak of TMA in all replicates. For all replicates, a lag phase of 7 days was observed and DMA production started only after TMA was produced.

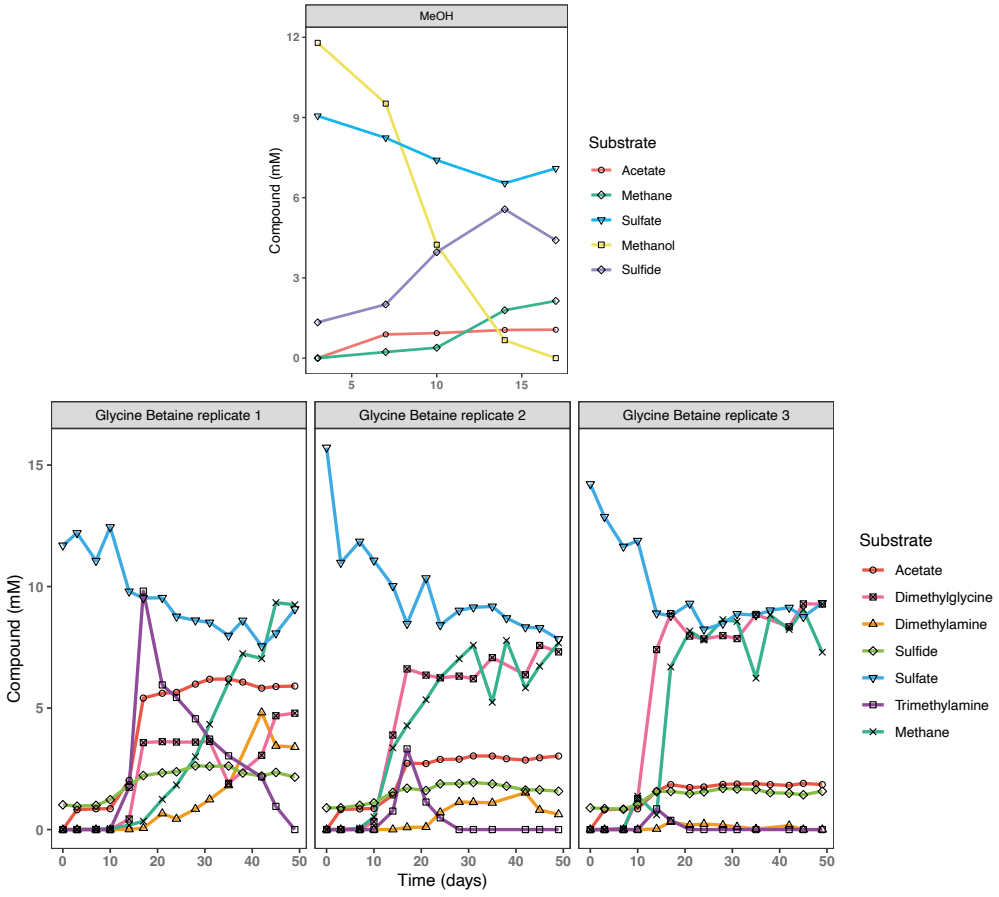

Figure 1: Growth rates of A) co-culture of Desulfosporosinus nitroreducens strain P130 with methanogenic enrichment M32 growing on methanol (triplicate data, error bars are standard deviation) B) Individual replicates of the co-culture of Desulfosporosinus nitroreducens strain P130 with methanogenic enrichment M32 growing on glycine betaine

6.3.2 Gene expression analysis of co-culture growing on glycine betaine

Expression data for a monoculture of *D. nitroreducens* strain P130, methanogenic enrichment community and co-culture of strain P130 with methanogenic community, all growing on glycine betaine, was obtained. For each replicate for each condition, the overall mapping rate was over 97% of all trimmed reads mapped back to the genomes of the organisms present. One exception was one triplicate of the methanogenic enrichment, where the mapping rate was 92%. This indicates the majority of obtained reads are retraceable and the data is of sufficient quality. Each triplicate of the methanogenic enrichment displayed similar mapping statistics between the three

members of the enrichment as in chapter 4, with an average mapping rate of 90.7% to *Ca*. Mc. chernomoris, 5% to *Ca*. Pulmentibacterium quisquilia and 4.3% to Ca. Izemoplasma apoleimma.

In the co-culture mapping rates between the constituents differed. In two replicates, the co-culture was dominated by *D. nitroreducens* strain P130 with 89.3% and 93.1% of all mapped reads mapped to this organism and 9.2% and 6.3% to *Ca*. Mc. chernomoris, 1.2% and 0.5% to *Ca*. P. quisquilia and less than 0.1% in each to *Ca*. I. apoleimma, respectively. For clarity, the two co-cultures dominated by *D. nitroreducens* strain P130 will be referred to as *co-culture replicate 1* and *co-culture replicate 2*. In the third replicate, however, 38.6%, 53.9%, 4.9% and 2.1% of reads mapped to *D. nitroreducens* strain P130, *Ca*. Mc. chernomoris, *Ca*. P. quisquilia and *Ca*. I. apoleimma, respectively and will be referred to as *co-culture replicate 3*. In the expression profiles of many genes of interest involved in methylotrophy in both *D. nitroreducens* strain P130 and *Ca*. Mc. chernomoris, co-culture replicate 3 displayed different transcripts per million than the two comparable replicates. For example, the gene annotated as glycine betaine methyltransferase (*mtgB*) in *D. nitroreducens* strain P130, DENIP130_03627 and its corrinoid co-factor coding gene (*mtgC*) DENIP130_03628 were expressed at TPM of 4398 and 6852 respectively in co-culture replicate 3. In co-culture replicates 1 and 2, this was 1874/2575 for *mtgB* and 2959/2617 for *mtgC*. Due to this incongruity, no meaningful statistics can be performed on the differential expression between the co-culture and *D. nitroreducens* strain P130 monoculture or between the co-culture and methanogenic enrichment. Despite the lack of replicates, general trends were observed in expression profiles of co-culture replicates 1 and 2 and results will be discussed as such. Based on the total expression profile, all genes with a TPM of 25 or more were included in the analysis. The expression was defined as low with TPM between 25 and 100, medium with TPM between 101 and 500, high with TPM between 501 and 1000 and very high with TPM higher than 1000. All expression data of all organisms can be found in supplementary table S1. The expression profiles of *Ca*. P. quisquilia and *Ca*. I. apoleimma displayed similar trends as observed in chapter 4, with little influence on methylotrophy, and shall not be discussed further. Total expression profiles of *Ca*. P. quisquilia and *Ca*. I. apoleimma can be found in supplementary tables S1-2.

6.3.4 *Desulfosporosinus nitroreducens* strain P130 gene expression in monoculture and co-culture with methanogenic enrichment M32

For *D. nitroreducens* strain P130 growth under all conditions resulted in the high expression of genes coding for the glycine betaine reductase complex

(DENIP130_4664-4676) as well as one out of the 12 MttB-superfamily coding genes, its corresponding corrinoid protein and the adjacently located methylcorrinoid:THF methyltransferase (DENIP130_03626-3628). For all replicates for both monoculture and co-culture, genes of the glycine betaine reductase pathway were in the top 10 most expressed genes. In co-culture replicate 3 and all replicates of the monoculture, both the glycine betaine:corrinoid methyltransferase as well as the adjacent methylcorrinoid:THF methyltransferase genes were in the 50 most expressed genes. Additionally, the corrinoid protein coding gene was in the top 100 most expressed genes. In co-culture replicates 1 and 2, expression of these glycine betaine:corrinoid methyltransferase coding genes was lower than in co-culture replicate 3, although these genes still were in the 100 most expressed genes for these co-cultures. Of the remaining putative MttB-superfamily coding genes, all were expressed under or slightly above the inclusion threshold of 25 transcripts per million in co-culture 3 and all monoculture triplicates. In co-culture replicates 1 and 2, two MttB-family coding genes (DENIP130_04785 and DENIP130_04946) were expressed at medium level, and all others were expressed below or slightly above inclusion threshold.

In co-culture replicates 1 and 2, one of the two trimethylamine:corrinoid methyltransferase and its corrinoid protein coding gene (DENIP130_00896-00897) were highly expressed. In co-culture replicate 1, trimethylamine:corrinoid methyltransferase was the second most highly expressed gene while it was the 18th most highly expressed in co-culture replicate 2. In co-culture replicate 3 and all triplicates of the monoculture, these genes were expressed as well, albeit to a much lesser degree and outside of the 200 most expressed genes. The pyrrolysine biosynthesis cassette on the operon surrounding this *mttBC* was medium expressed in all replicates of both the co-culture and monoculture. The second *mttBC* and its pyrrolysine biosynthesis cassette (DENIP130_04562-04570) were expressed at low levels in co-culture replicates 1 and 2 and below inclusion threshold in co-culture replicate 3 and all triplicates of the monoculture. All genes on the operon coding for the third pyrrolysine biosynthesis cassette and the monomethylamine:corrinoid methyltransferase (DENIP130_00873-00881) were not expressed above inclusion threshold in any culture under any condition. Figure 2 gives an overview of the discussed genes with their transcripts per million. All expression profiles can be found in supplementary table S3

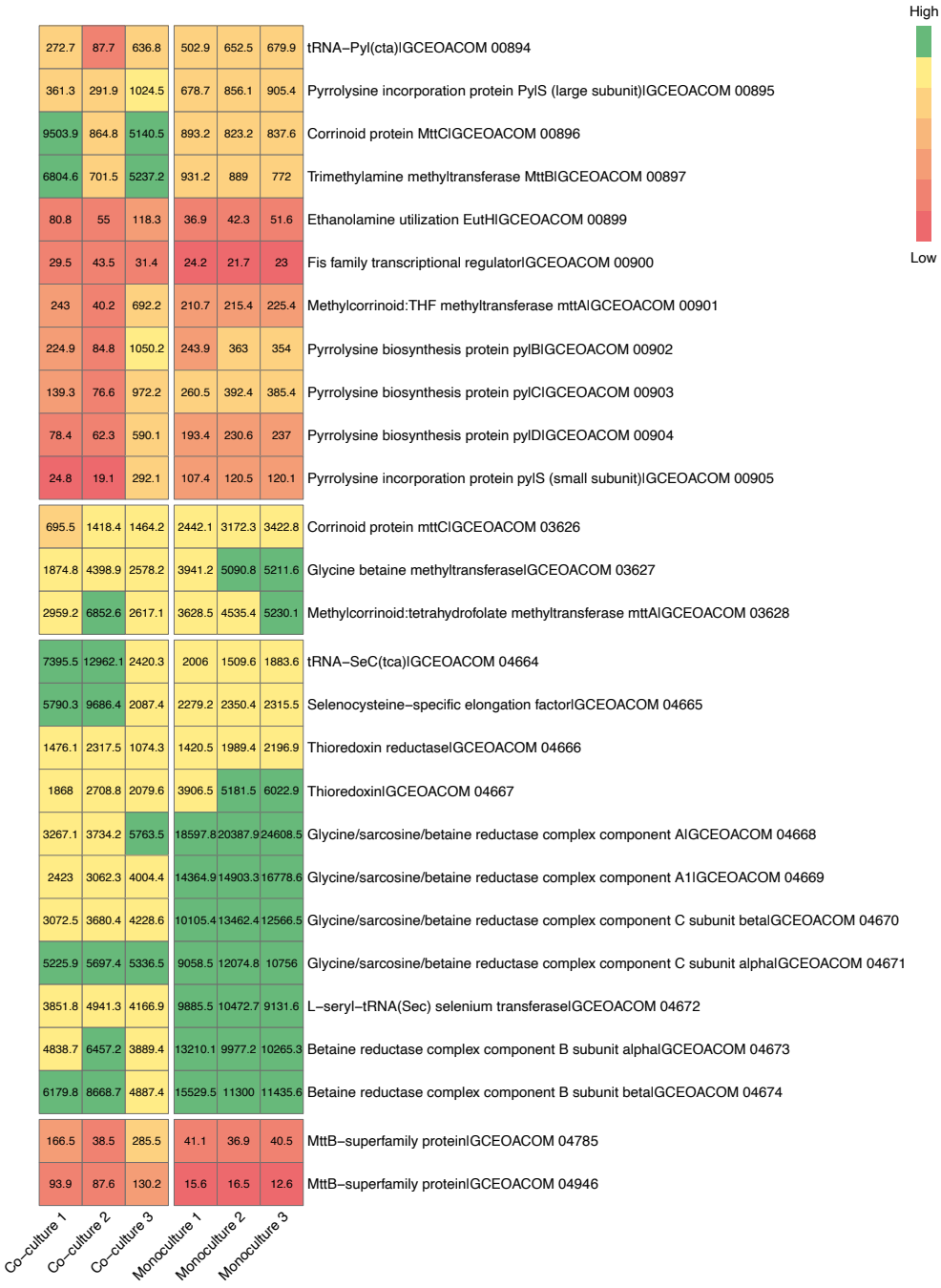

Figure 2: Heatmap of the expression profiles of selected genes from Desulfosporosinus nitroreducens strain P130 growing on glycine betaine in monoculture (left) and in co-culture with methanogenic enrichment M32 (right)

6.3.5 *Ca*. Methanococcoides chernomoris gene expression in enrichment M32 and co-culture with *Desulfosporosinus nitroreducens* strain P130

In the gene expression profile of Mc. chernomoris, less obvious trends between co-culture replicate 3 and co-culture replicates 1 and 2 were observed. Under all conditions, the genes coding for the final step of methanogenesis, methyl co-enzyme M reductase (Mcr) were all among the 10 most highly expressed genes. Of the two non-pyrrolysine MttB-superfamily coding genes present in the genome, one (METCH_00354) was expressed slightly above inclusion threshold in all conditions. The second MttB-superfamily coding gene (METCH_02409) was amongst the 25 most expressed genes while its associated corrinoid protein (METCH_02408) was expressed amongst the 75 most expressed genes when growing in the enrichment. In all three co-culture replicates, these genes were still expressed, albeit much less so, with the *mttB* gene outside of the 250 most expressed genes and *mttC* outside of the 800 most expressed genes. In contrast, the non-pyrrolysine MtmB-family gene and its associated corrinoid protein coding gene (METCH_00361-00362) were expressed below or slightly above the inclusion threshold in the enrichment M32, yet these genes were very highly expressed in the co-culture. In co-culture replicate 3, these genes were expressed amongst the 100 most expressed genes, while they were expressed in the 250 most expressed genes in co-culture replicates 1 and 2.

The sole pyrrolysine biosynthesis cassette was expressed at medium levels in all conditions. Of the two pyrrolysine encoding methylamine:corrinoid methyltransferases, one (METCH _01175) was expressed slightly above inclusion threshold in all conditions, as was its associated corrinoid protein coding gene (METCH_01176). The second methylamine:corrinoid methyltransferase (METCH_01166) was very highly expressed in all co-culture replicates, where this gene was in the 10 most expressed genes. The associated corrinoid protein coding gene (METCH_01165) was likewise very highly expressed. In the enrichment M32, these genes were likewise very highly expressed, yet outside the 50 most expressed genes. Similarly, one dimethylamine:corrinoid methyltransferase coding gene (METCH_02312) and its associated corrinoid protein coding gene (METCH_02314) were expressed within the 50 most expressed genes in the co-cultures, and outside the 300 most expressed genes in the enrichment M32. The second dimethylamine:corrinoid methyltransferase coding gene and its associated corrinoid protein coding gene (METCH_1405-1406) were expressed in all co-culture replicates outside of the 250 most expressed genes and outside of the 1000 most expressed genes in the enrichment M32. The trimethylamine:corrinoid methyltransferase coding gene (METCH_01314) and its associated

corrinoid protein coding gene (METC_01316) were in the 25 most expressed genes in all co-cultures replicates, while they were outside the 250 most expressed genes in the enrichment M32. Two methylcorrinoid:CoM methyltransferase coding proteins were very highly expressed under all conditions: METCH_00028 and METCH_00689. All of the other methylcorrinoid:CoM methyltransferase coding genes were expressed at medium levels or below. Figure 3 gives an overview of the discussed genes. All expression profiles can be found in supplementary table S4.

Figure 3: Heatmap of the expression profiles of selected genes from Ca. *Methanococcoides chernomoris growing on glycine betaine in the enrichment M32 (left) and in co-culture with* Desulfosporosinus nitroreducens *strain P130 (right)*

6.4 Discussion

In this work, we studied the utilization of the methylated compounds methanol and glycine betaine by a co-culture containing a sulfate-reducing microorganism and methanogen, both able to use these compounds for growth. The aim was to observe the different relations between these two organisms when growing on these substrates utilized by both. Both the sulfate-reducing microorganism *Desulfosporosinus nitroreducens* strain P130 and the methanogenic enrichment M32 containing the methanogen *Ca*. Mc. chernomoris have been shown to readily grow on these methylated compounds. Both methanol and GB are widespread in marine sediments where these organisms originated, and as such have been identified to be a source for microbial metabolism (46, 218, 238, 321). Our results demonstrate that under the conditions of our experiment, both methanogenesis and sulfate reduction can occur when growing on methanol (figure 1a). Based on the relative production of sulfide and methane, the growth rates of *Desulfosporosinus nitroreducens* strain P130 and *Ca*. Methanococcoides chernomoris were 1.41 and 0.91 day $^{-1}$, respectively when growing on methanol. The genome of *D. nitroreducens* strain P130 codes for both a complete methanol methyltransferase pathway as well as several alcohol dehydrogenase coding genes. Earlier work on similar organisms with both pathways demonstrated simultaneous protein presence under optimal conditions (45). In *Desulfovibrio carbinolicus* and other gram-negative SRM, the methyltransferase pathway is not present and these organisms grew poorly on methanol in comparison to other alcohols, suggesting a limited role of bacterial methanol oxidation if only dehydrogenases are present (346). Thus, it seems likely that in environments where methanol is present in suitable amounts and SRM coding for methyltransferases are present, methanogens and SRM can co-exist and thrive on methylated substrates. If these conditions are not met, other outcomes, where either methanogens or SRM dominate can be observed, leading to the seemingly contradictory results observed in environmental studies regarding methanol utilization (46, 57, 259). However, more thorough investigations in co-cultures and in situ measurements are needed to confirm this hypothesis.

The reductive glycine betaine cleavage pathway, yielding TMA and acetate, is well described. Furthermore, it has long been known that glycine betaine) could be utilized as a substrate for oxidative anaerobic methylotrophic metabolism as well, yielding N,N-dimethylglycine (16, 17, 323). However, thorough investigation into the specific pathways involved in the oxidative metabolism of GB is relatively recent. The oxidative anaerobic pathway for glycine betaine, using glycine betaine:corrinoid methyltransferase was described only in 2014 (50). As a result of this, glycine

betaine metabolism in marine sediments has mostly been regarded as bacterial reduction of GB to acetate and TMA, after which TMA could be utilized as a non-competitive substrate for methanogens (18, 327). More recent investigations from axenic cultures have demonstrated that the reality may be more complex. Direct methanogenic growth on glycine betaine has been demonstrated in *Methanococcoides vulcani*, *Methanolobus vulcani* and now in *Ca*. Methanococcoides and non-pyrrolysine coding homologs of *mttB* are present in many genomes of methylotrophic methanogens, suggesting the potential utilization of this compound or other quaternary amines is widespread (53, 178, 235). Likewise, a wide range of bacterial strains from marine sediments and elsewhere are described to harbor non-pyrrolysine coding *mttB* homologs, suggesting that quaternary amine methylotrophy might play an important role as substrates in many environments (54). This complexity is reflected in the results observed in this study. Despite using the same inoculum and starting conditions, no reliably reproducible results were observed over several replicates, with differing production rates of N,N-dimethylglycine and TMA/acetate throughout, suggesting no clear preference of the oxidative or reductive metabolism of GB. In contrast, for both the monoculture of *D. nitroreducens* strain P130 and the methanogenic enrichment M32, no such irregular results were observed. As all scenarios had similar lag phases, it is possible that one of the two strains, due to stochastic effects, has an earlier start in some cultures, causing the other to have to catch up. Depending on which strain this was, more N,N-dimethylglycine would be produced if *Ca*. Mc. chernomoris is first, as this is the only substrate available for it to grow on. Alternatively, if *D. nitroreducens* strain P130 is first, GB is mostly utilized reductively, with some oxidative demethylation to obtain reducing equivalent to power reductive cleavage. Both strain P130 and *Ca*. Mc. chernomoris can then subsequently utilize the produced TMA and *Ca*. Mc. chernomoris can further utilize the produced DMA unavailable to *D. nitroreducens* strain P130, which can oxidate acetate.This succession can be seen in the quick production of methane in the culture with high N,N-dimethylamine and much later production of methane in the culture with high TMA/acetate, indicating either early or later growth of *Ca*. Mc. chernomoris compared to *D. nitroreducens* strain p130. In the culture with a more mixed ratio between N,N-dimethylamine and TMA/acetate, there was likely a shorter time gap between the start of the two strains than in the cultures with a more extreme ratio.

This hypothesis is further strengthened by the growth of these organisms for our transcriptomic tests, where two out of three replicates were dominated by strain P130, whereas in the third a more mixed culture between strain P130 and Ca. Mc. chernomoris was observed despite simultaneous inoculation from the same source.

Different expression levels between these replicates were observed, further suggesting the specific composition of the co-culture at time of RNA isolation influences expression rates of the constituent microorganisms. While the ratio of genes coding for the glycine betaine reductase pathway to genes coding for glycine betaine:corrinoid methyltransferase is similar in all triplicates of the co-culture as well as in the monoculture of *D. nitroreducens* strain P130, the relative expression of trimethylamine:corrinoid methyltransferase was much higher in co-culture replicates 1 and 2 than in co-culture replicate 3. This likely indicates that glycine betaine was actively being consumed in all co-cultures, utilizing the same simultaneous reduction and oxidation as described in the monoculture in chapter 5. However, in co-culture replicates 1 and 2, dominated by *D. nitroreducens* strain P130, the produced trimethylamine from glycine betaine reduction is then more readily consumed by strain P130 as a result of the higher expression of trimethylamine:corrinoid methyltransferase than in the mixed co-culture replicate 3. Whether this higher expression of TMA:corrinoid methyltransferase is a cause or a consequence of the dominance of strain P130 remains to be investigated.

Conversely, in the transcripts of *Ca.* Mc. chernomoris, the relative expression of trimethylamine:corrinoid, dimethylamine:corrinoid and monomethylamine:corrinoid methyltransferase coding genes was much higher in the co-culture replicate 3 than in co-culture replicates 1 and 2. Regardless, the relative expression of these methylated amine:corrinoid methyltranferase coding genes was much higher in all co-cultures than in the cultures without strain P130, although all these genes were still expressed. The likely gene carrying out glycine betaine:corrinoid methyltransferase in *Ca.* Mc. chernomoris, METCH_02409, was highly expressed when growing without strain P130. In the presence of strain P130, this gene was also expressed, yet on similar levels as in the enrichments grown on TMA or QMA in chapter 4. Similarly, the genes coding for trimethylamine:methylcorrinoid, dimethylamine:corrinoid and especially monomethylamine:corrinoid methyltransferases were expressed in the enrichment cultures without strain P130, even though none of these substrates were measured at any point during our growth tests. While the genomes of neither bacterial strains (*Ca.* I. apoleimma and *Ca.* Pulmentibacterium quisquilia) in the enrichment M32 code for any known protein related to oxidative or reductive metabolism of glycine betaine, it is possible that small amounts of TMA can be produced by these organisms using undescribed pathways, which is then directly cycled by *Ca.* Mc. chernomoris. Alternatively, these may be genes that are activated in the presence of glycine betaine, as in most environments, TMA will become available through bacterial degradation of glycine betaine. Finally, it has been suggested that in some meth-

anogens, key metabolic genes for preferred substrates are constitutively expressed, which may be the case for the expression of these methyltransferase coding genes in the absence of TMA (235).

Our results demonstrate that the utilization of the methylated compounds methanol and glycine betaine by marine microorganisms is likely much less straightforward than commonly assumed. While the methylotrophic archaeon in our experiments was capable of growing on the provided methylated compounds, it did not readily outcompete the sulfate reducer as was suggested since neither methanol nor glycine betaine were non-competitive compounds. Based on our transcriptomic results, the produced trimethylamine by reductive cleavage of glycine betaine was likewise used by both the methanogen and sulfate reducer, indicating this compound is not non-competitive either. These results are in line with previous reports in microcosm experiments and *in-situ* metatranscriptome analysis where bacterial utilization of methylated compounds was suggested in marine sediments and microbial mats, further indicating the more complex interplay between methanogens and sulfate-reducing bacteria for common substrates (44, 245, 258).

6.5 Conclusions

In this work, we investigated the consumption of common methylated substrates by a co-culture of a methylotrophic methanogen and a sulfate-reducing microorganism. While methylated compounds are commonly considered non-competitive substrates for methylotrophic methanogens, here we demonstrate the simultaneous consumption of methanol by both methanogen and sulfate reducer. Furthermore, we demonstrate that as a response to currently unknown factors, different ratios of oxidative or reductive consumption of glycine betaine are observed, producing differing ratios of N,N-dimethylamine and TMA/acetate. Subsequently, the gene expression analysis of this study observed different expression ratios of genes coding for substrate specific methyltransferases for glycine betaine, TMA, DMA and MMA and glycine betaine reductase, depending on the relative abundance of the constituent organisms in the co-culture. Regardless, our results demonstrate that the utilization of methylated compounds by marine microorganisms is much more complex than previously thought.

6.6 Supplementary data

All supplementary data can be downloaded from 10.6084/m9.figshare.c.6386033

6.7 Acknowledgements

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General discussion

7.1 Canonical view on anaerobic marine methylotrophy

At the beginning of this research, the explicit goal was stated to determine which microorganisms are capable of utilizing methylated compounds in Black Sea sediments. Utilizing a combination of traditional cultivation techniques and more modern omics-based techniques, we have achieved a deeper understanding of different microorganisms capable of metabolizing methylated compounds. We have isolated a methylotrophic sulfate-reducing microorganism and have given insights in the wide diversity of microorganisms with genes linked with methylotrophy in their genomes. At the same time, our results have opened up novel research questions that will hopefully fuel future research. Regardless, to start this general discussion on future perspectives, we need to travel back 40 years in time.

Methylated compounds have been called "non-competitive" substrates ever since studies in the 1980s identified methylotrophic methanogenesis in sulfate rich environments. Interestingly, these studies did recognize that while methanogenesis from methylated compounds does occur in sulfate rich sediments, it is not the only process occurring on these substrates. Indeed, the abstract of one of the most cited scientific articles on methylotrophic methanogenesis reads as follows:

"Ratios of $^{14}CH_4/^{14}CO_2$ from [^{14}C]trimethylamine and methylamine in uninhibited sediments indicated that a significant fraction of these substrates were catabolized via a non-methanogenic process. (...) [^{14}C]methanol was oxidized relatively slowly compared with the other substrates and was catabolized mainly to $^{14}CO_2$. Results from experiments with molybdate and 2-bromoethanesulfonic acid suggested that methanol was oxidized primarily through sulfate reduction. In Lowes Cove sediments, trimethylamine accounted for 35.1 to 61.1% of total methane production."

(King, Klug & Lovley, 1983)

Despite these clear observations and statements in this quote, the article in reference is usually cited as claiming compounds like methanol or trimethylamine (TMA) are *non-competitive*, without any further attention to the conclusions introduced by the original authors. A 1983 overview of methanogenesis in marine sediments, sarcastically uses the word "non-competitive" in its title, in quotes, to emphasize that they are not non-competitive whatsoever, and that the reality is more nuanced (40). However, subsequent research apparently failed to read the word "non-competitive" the way it was intended and started to claim the opposite. Perhaps as a result, research on the fate of methylated compounds in marine sediments has been stagnant

for many decades due to the false assumption that all methylated compounds were shuttled to methanogenesis. It is therefore crucial for any scientist to recognize the impact of using specific words in scientific literature can have on subsequent publications, as well as the necessity of carefully reading through any literature a scientific work cites to determine whether this actually supports any claims made.

Due to these blind spots, after almost 40 years of research and technological improvements, the question then remains the same: outside of methanogenesis, what is the fate of methylated compounds in marine sediments? This question is not so straightforward. Different methylated compounds have different theoretical energy yields and different methyltransferases have different affinity and specificity constants for their substrate. Furthermore, the specific amount of these different compounds available in the environment has an impact on their eventual fate due to substrate preference between different species. As such, a holistic approach is needed to understand what is going on in an environment, which starts with understanding the physiology of individual strains, expands with the knowledge of which other microorganisms are present in its environment and ends with having to understand the environment itself. This is a task not suited for a singular scientist – a mineralogist will have a different view of an environment than a biogeochemist. Through collaboration between different scientific fields, and through the utilization of the different frameworks obtained by the scientists working in different fields, such a holistic view can be obtained. The work presented here attempts to be a part of this holistic approach, and we used key findings from biogeochemistry, microbiology, bioinformatics and beyond to reach the conclusions discussed here. However, much more research is needed to fully answer the research question this thesis started out with.

7.2 Methanogens, sulfate reducers, and methyltransferases: a love story?

At the core of this thesis are the methyltransferase genes and the enzymes coded by these genes. The biochemical intricacies of these enzymes are well-studied and well-known, especially for the traditional substrates methanol and trimethylamine (315, 316). The presence of catabolism-linked methyltransferases in bacteria has been known since the 1990s (347, 348). However, most of the research on these enzymes has been conducted on archaea, specifically methanogens (126, 182). A range of marine methylotrophic sulfate-reducing bacteria have been described, as outlined in **chapter 2**, and many more from other environments such as freshwater systems, anaerobic digesters and wastewater treatment plants. Nevertheless, microbial diversity of anaerobic methylotrophs is surely underestimated, even within currently

isolated microbes, as methanol and other methylated compounds are not standardly tested during physiological characterization of novel microbes. As a result, there are still unknowns regarding the pathways of methanol utilization in sulfate-reducing microorganisms and other metabolisms outside of methanogenic archaea. This is despite the widespread use of methanol in wastewater treatment facilities as a supplemental electron donor to facilitate biological sulfate reduction, where methanogens are also present and could therefore compete (33, 48, 295). A possible explanation for this could be that, especially in human engineered systems like wastewater treatment plants, the scientific focus has been more on performance of the system as a whole rather than the individual microbial players contributing to this. The conversion of other methylated compounds such as trimethylamine or glycine betaine is even less studied. The anaerobic bacterial oxidation of TMA through methyltransferases has previously only been described in *Acetohalobium arabaticum* (243). TMA dehydrogenases have been described and do not need oxygen to function, yet their environmental impact in anoxic systems is not known, especially as these dehydrogenases can also function in the general nitrogen metabolism of bacteria and thus the involvement of these proteins in TMA catabolism is uncertain (349, 350).

It is likewise not clear how methyltransferases have evolved and diverged over time. There are indications that methylotrophic methanogenesis may be the oldest form of methanogenesis and linkage between methanogenesis and the Wood-Ljungdahl (WL) pathway occurred only later in its evolution (351). Evidence for this is the widespread presence of methyltransferases in archaea that appear to be vertically evolved from an early Archaean origin (351). Furthermore, WL-independent methanogenesis occurs in some archaea, which can use hydrogen oxidation to obtain reducing equivalent (33, 295). This hypothesis does not take into account the diversity of the different substrate specific methyltransferase coding genes *mtxB* and rather treats them as a whole. This is interesting, as there is little commonality on amino acid level between the different known methyltransferases, even between the pyrrolysine encoding mono- di- and trimethylamine methyltransferases that share this unique amino acid in their active site. However, as all known methyltransferases appear to function in the same way, as well as the substrate independence of MTII, it is possible the methylotrophic methanogen ancestor only utilized a single methylated compound, and all other substrates suitable for methyltransferase activity early on evolved from there, leading to the diversity in substrates observed today. To further investigate this, we will look at the presence of the best described methylated compound:corrinoid methyltransferase systems, those acting on methanol and TMA, in a range of microorganisms to discuss their evolutionary origins and divergence.

7.3.1 Methanol methyltransferases

Using the methodology to search for methyltransferases described in **chapter 3,** we assessed all 965 publicly available *Desulfobacterota* and *Thermodesulfobacterota* genomes, which are the bacterial phyla classically associated with sulfate reduction. Only six of these genomes (all from different families) showed the presence of methanol:corrinoid methyltransferase coding genes. In **chapter 3**, we identified four methanol:corrinoid methyltransferase coding genes. Two of these genes belonged to the same genus within the phylum *Desulfobacterota*, suggesting currently unsequenced or uncultivated marine representatives within the phylum *Desulfobacterota* may reveal more methanol:corrinoid methyltransferases. In contrast, homologs to alcohol dehydrogenases are present in 634 *Desulfobacterota* and *Thermodesulfobacterota* genomes. However, as explored in **chapter 2**, alcohol dehydrogenases are not substrate specific and do not necessarily code proteins involved in the oxidation of methanol as other alcohols can be the substrate as well. Formaldehyde dehydrogenase, active on the product of methanol dehydrogenase, is present in 57 genomes. While this formaldehyde can also be derived from other compounds like TMA, this does suggest that alcohol dehydrogenase is the preferred pathway in these phyla and methyltransferase occurs only in a small subset of these SRM.

In contrast, methanol:corrinoid methyltransferase coding genes are much more widespread in sulfate-reducing microorganisms in the phylum *Firmicutes*. In the genus *Desulfosporosinus*, to which strain P130 isolated in **chapter 5** belongs, all available genomes contain a methanol:corrinoid methyltransferase coding gene, as do most genomes of the genus *Desulfitobacterium* and other genera formerly classified as *Desulfotomaculum*. The phylum *Firmicutes* and especially the order *Eubacteriales* within this phylum, to which all mentioned genera belong, consists of many acetogenic bacteria as well. These bacteria are well associated with methanol utilization through methyltransferases and do not appear to involve dehydrogenases (225, 352). Additionally, methanol:corrinoid methyltransferases can be found in some *Chloroflexota*, including in two MAGs uncovered in **chapter 3**, the bacterial phyla *Spirochaetes* and *Planctomycetota*. However, in none of these phyla the presence of methanol:corrinoid methyltransferases appears to be widespread, occurring only in a handful out of hundreds of publicly available genomes for each phylum. In *Archaea*, genomes from the phyla *Brockarchaeota Thaumarchaeota,* and *Thermoproteota* all contained methanol:corrinoid methyltransferases. As with bacteria, only few genomes for these phyla contain this methyltransferase. However, there are much fewer available genomes for these phyla making it more difficult to assess how widespread methanol:corrinoid methyltransferases are within these lineages.

A phylogenetic analysis of the recovered methanol:corrinoid methyltransferase coding genes reveals all genes from methanogenic genomes cluster closely together, in a single branch (Figure 1). All other archaeal genes cluster together in a separate branch. Between the methanogenic and other archaeal clusters is a cluster consisting of two subclusters of bacterial *mtaB* genes, one cluster from all *Desulfobacterota* species described to contain an *mtaB* gene, including those described in **chapter 3**. The second subcluster consists of *mtaB* genes from *Firmicutes*. It is possible this branching within Archaea is due to an early horizontal gene transfer event (HGT), where an archaeal host transferred an *mtaB* gene to an ancestral *Desulfobacterota* acceptor. A secondary HGT event then transferred this gene to an ancestral *Firmicutes* genome. Outside of this cluster between archaeal clusters, the other bacterial methanol:corrinoid methyltransferase clusters adhere little to taxonomy. Two separate clusters of *mtaB* genes originating from mainly *Firmicutes* exist. One of these two clusters harbors a cluster of *mtaB g*enes originating from *Spirochaetes* genomes, likely due to a HGT event as all four *Spirochaete mtaB* genes cluster closely together and originate from organisms within the same genus, obtained from a single metagenome dataset. A third branch consists of *mtaB* genes from three related *Firmicutes* genomes, two *Chloroflexota* genomes and a *Planctomycetota* genome. Furthermore, several *mtaB* genes from *Planctomycetota* genomes are branch singularly. As most genomes from *Planctomyetota* and *Chloroflexota* do not contain *mtaB* genes and the genomes that do are closely related, it is unlikely the ancestral *Chloroflexota* and *Planctomycetota* coded for *mtaB* and gene loss events amongst most lineages led to the currently observed dispersal of *mtaB* within these lineages. More likely, this indicates several instances of HGT between bacterial species. However, due to the limited amount of genes it is currently unknown whether *mtaB* originated within *Bacteria*, *Archaea* or before the archaeal-bacterial split. As more genomes, especially from archaeal lineages, become available this question may be answered. However, these observations do suggest that the emergence of bacterial methylotrophy occurred early in evolution, and is likely as old as methylotrophic methanogenesis. Most likely the presence of methanol methyltransferases in bacterial groups is not the result of a HGT event later in the evolutionary timeline (figure 1).

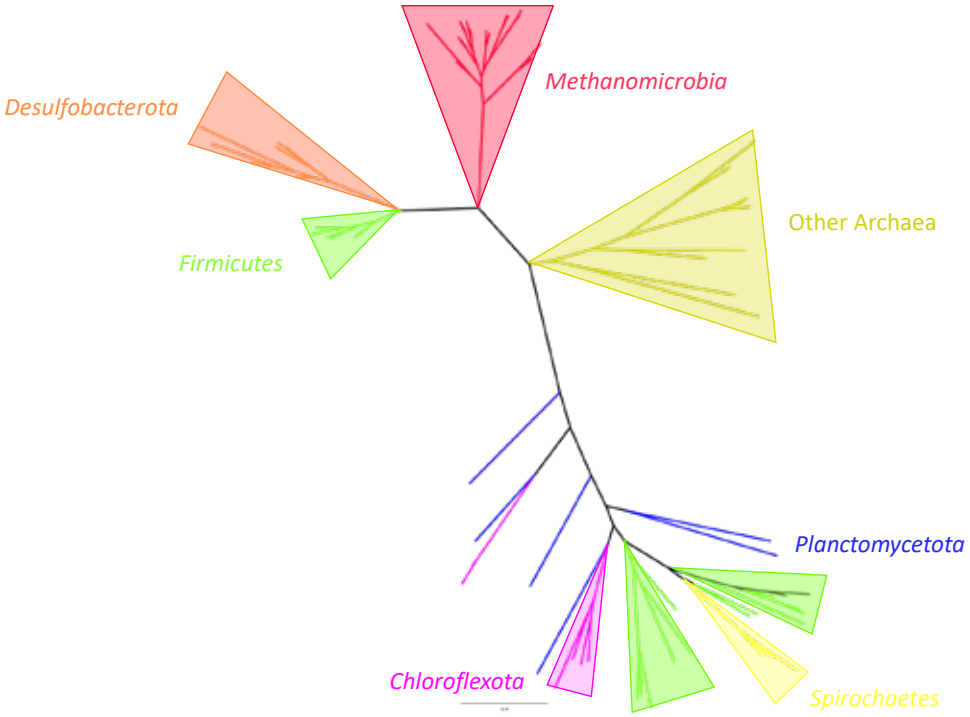

Figure 1: Unrooted phylogenetic tree of recovered mtaB sequences. Color codes represent the phylum of origin. Green: Firmicutes; orange: Desulfobacterota; blue: Planctomycetota; pink: Chloroflexota; yellow: Spirochaetes; red: Methanomicrobia; brown: all other Archaea. The tree was reconstructed by using all known genomes of the named phyla (downloaded from the integrated microbial genomes & microbiomes (IMG) database). Genomes were mined for the presence of mtaB homologs following the methodology described in chapter 3. Obtained mtaB homologs were aligned using mafft-linsi and the tree was built with IQ-tree using LG+F+R+C60 models and visualized in FigTree. All included nodes have bootstrap values higher than 0.9.

7.3.2 Trimethylamine methyltransferases

The divergence of pyrrolysine-incorporating MttB coding genes display a similar pattern as with MtaB coding genes described above, with a range of archaeal and bacterial genomes containing MttB coding genes. The majority of the bona fide bacterial MttB coding genes belong to the phylum *Firmicutes*, as is the case for methanol:corrinoid methyltransferases. However, this restrictive occurrence within *Firmicutes* is even more strict than with methanol:corrinoid methyltransferases. Even though the pyrrolysine biosynthesis cassette can be found in a range of *Thermodesulfobacterota* and *Desulfobacterota*, much fewer publicly available genomes contain an *mttB* gene coding for an in-frame pyrrolysine (245). Likewise, while pyrrolysine biosynthesis genes can be found in some *Spirochaetes* and *Planctomycetes* genomes,

none contained any *mttB* gene with the in-frame pyrrolysine incorporation amber codon. In *Chloroflexota*, multiple genomes contain *mttB* genes; six from the dataset in **chapter 3** and all others from a single marine metagenome dataset. Similarly, we were unable to find any archaeal MttB-coding genes outside the known methanogenic lineages. However, pyrrolysine biosynthesis is described in archaea outside of known methanogens, for example in *Brockarchaeota* and *Asgardarchaeota* (59, 60). This may mean there are pyrrolysine-coding proteins of unknown function to be discovered – or lost in the evolution of these lineages. While trimethylamine:corrinoid methyltransferases have been described in some archaea, for example in *Brockarchaeota* (59), our investigations revealed the genes annotated as trimethylamine:corrinoid methyltransferase did not contain any pyrrolysine and as such, are not bona fide coding genes for this exact protein, but rather putative quaternary amine methyltransferases. This highlights the need for careful annotation and curation of metagenome assembled genomes (MAGs). The chance of mis-annotating is high when dealing with large datasets, which makes subsequent ecological claims about the utilization of substrates of the organisms these MAGs code for difficult to interpret. This also further highlights the need to corroborate genomic predictions with laboratory studies, before claims on the physiology of microorganisms can be substantiated.

The phylogeny of all *mttB* genes show similarities to the phylogeny of *mtaB* genes. All *mttB* genes from archaeal genomes cluster together, mostly following archaeal phylogeny. The majority of bacterial trimethylamine methyltransferases cluster together as well, although with less adherence to bacterial phylogeny. The genomes of several organisms contain multiple copies of *mttB* yet these genes from within the same organism do not cluster together. All *mttB* genes of *Desulfobacterota* origin cluster within branches of *Firmicutes*. All *mttB* genes obtained from **chapter 3** cluster together within a branch of *Firmicutes*. The other *mttB* genes of *Chloroflexota* origin cluster together in a separate branch. This suggests that the presence of trimethylamine:corrinoid methyltransferase in both *Deltaproteobacteria* and *Chloroflexota* could be due to HGT events from Firmicutes (figure 2).

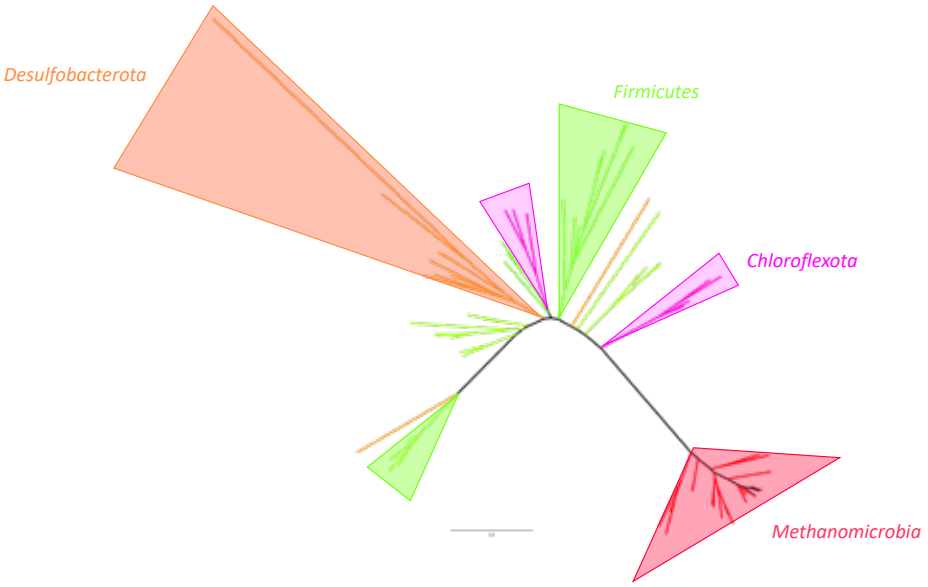

Figure 2: unrooted phylogenetic tree of recovered mttB *sequences. Color codes represent the phylum of origin. Green:* Firmicutes*; orange: (Thermo)*Desulfobacterota*; pink:* Chloroflexota*; red:* Methanomicrobia*. The tree was constructed by downloading all known genomes of the named phyla as well as* Spirochaetes*,* Planctomycetota*,* Brockarchaeota*,* Asgardarchaeota *and* Sifarchaeota *from the integrated microbial genomes & microbiomes (IMG) database. Genomes were mined for the presence of* mttB *homologs following the methodology described in chapter 3 and manually checked for in-frame pyrrolysine coding. Obtained* mttB *homologs were aligned using mafft-linsi and the tree was built using IQ-tree using LG+F+R+C60 models. All included nodes have bootstrap values higher than 0.9*

7.3.3 Divergence of methyl:corrinoid methyltransferases

Thus, it seems likely that utilization of methanol and trimethylamine through methyltransferases is widespread a wide range of microorganisms, but mainly concentrated in bacteria from the phylum *Firmicutes* and methanogenic archaea. In other lineages, this may be gained through horizontal gene transfer from either *Firmicutes* or methanogens. *Firmicutes* are not as abundant in marine sediments as *Desulfobacterota* and *Thermodesulfobacterota*, with datasets commonly measuring less than 1% of obtained reads in metagenome or amplicon datasets belonging to *Firmicutes*, compared to abundances upwards of 15% for *Desulfobacterota* and *Thermodesulfobacterota*. This is also reflected in the diversity of MAGs observed in **chapter 3.** However, methanogenic archaea have similar observed abundances in these marine sediments as Firmicutes. Furthermore, our analyses here and in **chapter 3** revealed potential methylotrophy in the phyla *Chloroflexota* and *Planctomycetota*,

both of which are more abundant in marine sediment datasets than *Desulfobacterota* and *Thermodesulfobacterota* (267, 353–355). As such, it appears that bacterial methylotrophy could, based on genomic information, compare with the presence of archaeal methanogenic methylotrophy in marine sediments.

In **chapter 3**, we demonstrate the presence of methyltransferase genes in a wide range of bacterial phyla. In the dataset described in this chapter, we recovered a wide range of micro-organisms not previously linked with methylotrophy, for example lineages within *Acidobacteriota*, *Zixibacteri* and *Actinobacteriota*. An increasing amount of bacterial and archaeal genomes phyla are described to contain genes coding for methyltransferases (60, 245, 247, 324). We have expanded this list extensively. The vast majority of the recovered methyltransferase coding genes in **chapter 3** are non-pyrrolysine containing homologs of the trimethylamine methyltransferase, which appear to occupy a much more important role in marine methylotrophy than the much better described methanol or trimethylamine metabolisms. In **chapters 4** and **5** we demonstrate the presence and expression of these non-pyrrolysine containing genes in microorganisms obtained from Black Sea sediments. This all suggests that methylotrophy utilizing genes of the MttB-family is likely a much more widespread strategy to obtain energy than previously assumed. Before these pyrrolyisine lacking methyltransferases can be discussed, it is important to look at pyrrolysine itself, and the central role it plays in methylated amine metabolism, to fully understand the fundamental meaning of the lack of it in this wide range of methyltransferases.

7.4 Pyrrolysine presence and absence in methyltransferases

Pyrrolysine exhibits a peculiar place in biology. It is one of just two non-canonical amino acids that are directly encoded by a codon in the genome. The other non-canonical amino acid, selenocysteine, is present in a relatively wide range of proteins throughout all domains of life (356). In contrast, pyrrolysine is mainly incorporated in the methyltransferase proteins involved in demethylating TMA, DMA and MMA. Additionally, pyrrolysine occupies a minor role in certain transposases and in $tRNA^{His}$-guanylyltransferase Thg1 in some strains of *Methanosarcinales* (357, 358). The evolutionary origins of pyrrolysine are not clear. Some evidence suggests that it emerged post last universal ancestor (LUCA) (251, 359). This hypothesis suggests it arose after the bacterial-archaeal split in methylotrophic methanogens of the family *Methanosarcinaea* and horizontal gene transfer led to its wider dispersion. Another hypothesis posits it existed in a hypothetical fourth kingdom that existed pre-LUCA, which has since become extinct (360). The presence of pyrrolysine biosynthesis in extant lineages is inferred through horizontal gene transfer to bacteria and archaea

after the bacterial-archaeal split. An origin through HGT, however, does not fully agree with the observations of the dispersal of pyrrolysine in **chapter 3** as well as its expression as inferred in **chapter 5**. While the list of bacterial phyla containing a complete pyrrolysine synthesis cassette is still small, the taxonomic dispersal of it covers a range of bacteria that have diverged early in bacterial evolution (361). Thus, multiple HGT events to different bacterial clades, either originating from *Methanosarcinaea* or a hypothetical fourth kingdom, is unlikely. A thorough phylogenetic assessment of the three distinct types of pyrrolysine tRNA synthetase (*pylRS*), which exists in genomes either as a single gene, two distinct genes or a shortened single gene lacking the n-terminus, revealed a strict taxonomic divide between the three pylRS variants (362). These results posited the hypothesis that pylRS arose pre-LUCA, likely inferring pyrrolysine biosynthesis and incorporation simultaneously arose due to the close association of pylRS with all other pyl genes in most known genomes. Similarly, a phylogenetic assessment of the conserved pyrrolysine biosynthesis coding gene *pylB* showed a clear distinction between *pylB* from *Archaea*, *Firmicutes* and *Deltaproteobacteria*, further supporting an early divergence of pyrrolysine biosynthesis between bacterial and archaeal genomes (245).

Nearly all research on the evolution of pyrrolysine has neglected to incorporate the evolution of the three pyrrolysine-incorporating methyltransferases. Due to the strong link between pyrrolysine and these methyltransferases, this seems like an oversight. If these methyltransferases and pyrrolysine biosynthesis display a similar evolutionary path, this could mean pyrrolysine and methyltransferases evolved together as a response to the availability of methylated amines as a potential substrate. Alternatively, if there are differing evolutionary paths, pyrrolysine may have had a role in proteins other than methyltransferase when it originated and only later did an adaptation to methylamines occur within the established framework of pyrrolysine biosynthesis and adaptation. Pyrrolysine including homologs of *mttB* are much less common than pyrrolysine biosynthesis in bacteria. Similarly, no pyrrolysine including homologs of *mtbB* genes, coding for dimethylamine:corrinoid methyltransferase, are observed in any genome of *Spirochaetes* and *Planctomycetota*. In *Chloroflexota* and *Desulfobacterota*, this gene was only found in some of the genomes where trimethylamine:corrinoid methyltransferase was also present. Homologs of pyrrolysine including *mtmB*, coding for Monomethylamine:corrinoid methyltransferase was not observed in genomes of *Planctomycetota* and *Desulfobacterota*. However, this gene was present in one *Spirochaete* genome and four more genomes of *Chloroflexota* than *mttB* or *mtdB*. As such, it is difficult to assess the relative evolutionary path of the known pyrrolysine containing proteins in comparison to the evolution of pyrro-

lysine biosynthesis. The fact that genes coding for the incorporation of pyrrolysine are less common than its biosynthesis could infer there may be as of yet unknown proteins incorporating this amino acid. Alternatively, this may indicate the process of gene loss of methyltransferases where pyrrolysine biosynthesis still remains.

7.5 Methylated quaternary amines: an overlooked source of methylotrophy

In contrast to the methyltransferases described above, the phylogeny of non-pyrrolysine coding homologs of *mttB* is much less defined, revealing no obvious clusters based on taxonomy, as observed in **chapter 3** and elsewhere (54). However, all MttB-family coding genes without an in-frame pyrrolysine that have been described thus far have been demonstrated to be active on quaternary amines. The lack of pyrrolysine on these compounds is unsurprising; the function of pyrrolysine is to form a covalent bond between the pyrrole ring with the nitrogen group of the target methylated amine, changing the electron configuration of the amine to a quaternary state, allowing for nucleophilic attack and subsequent demethylation (55). Thus, if the target substrate is by itself a quaternary amine, there is no need for pyrrolysine.

The widespread presence of non-pyrrolysine MttB-superfamily homologs and to a lesser degree MtmB-family homologs in marine organisms is not unexpected. As demonstrated in **chapter 3**, there is a high correlation between the presence of genes coding for enzymes in the Wood-Ljungdahl pathway and the presence of such genes. Methyl-THF is an intermediary of this pathway, in which a corrinoid methyltransferase carries the methyl group of methyl-THF to acetyl-coA synthetase. This corrinoid methyltransferase requires the ATP-dependent activation of the catalytic cobalt to switch between the I and III states which is carried out by homologs to the methanogenic RamA observed to perform this cobalt activation in methylotrophic methanogens (224, 363). As such, several major components for methylotrophy are present in organisms utilizing the WL pathway as a source of carbon. Reversible WL reactions have been described in a range of sulfate-reducing bacteria, but this may not be intrinsically reversible (364). For example, *Desulfobacterium autotrophicum* is capable of the reductive, but not oxidative acetyl-CoA pathway (365). However, most bottlenecks in switching between oxidative and reductive Wood-Ljungdahl appear to be adaptations in carbon monoxide dehydrogenase/acetyl-CoA synthase, which is circumvented in oxidative methylated compound metabolism, where oxidation starts from methyl-THF. Almost all research on the oxidative Wood-Ljungdahl pathway from methylated compounds has been performed on acetogens, where the reductive equivalents obtained from methyl-THF oxidation to CO_2 are used to power CO_2 reduction to

CO and its subsequent incorporation in acetyl-CoA synthesis (134, 225, 238). In **chapter 5** we have demonstrated the genetic framework for a partial oxidative pathway, where the carboxylic branch of the WL pathway is likely not used. In theory, *Desulfosporosinus nitroreducens* strain P130 isolated in **chapter 5** could be able to grow on glycine betaine as sole carbon and energy source; from methyl-THF, both oxidation to CO_2 and reduction to acetyl-CoA is possible based on its genetics. In contrast to acetogenic microorganisms, where CO_2 is captured as the only available electron acceptor, strain P130 is capable of transferring excess electrons to reduce sulfate as well as additional glycine betaine.

Thus, with an HGT event of an MttB-superfamily coding gene and its associated corrinoid protein coding gene, the utilization of a new substrate becomes possible that requires little to no additional genomic adaptation as long as the WL pathway is present. As the corrinoid proteins coding genes are usually located in close proximity to the methyltransferase coding genes, a single HGT event would be sufficient for an organism to expand its metabolism to include methylated quaternary amines. Especially in marine sediments, where there are plentiful methylated quaternary amines present and there is a higher density of microorganisms than in seawater and consequentially, close microbe-microbe contact, there is a high likelihood of such HGT events occurring (2, 218).

We have demonstrated the utilization of the methylated quaternary amines glycine betaine and tetramethylamine in **chapters 4, 5** and **6.** However, the genome of *Desulfosporosinus nitroreducens* strain P130 as well as many other bacterial genomes contain a multitude of MttB-superfamily and MtmB-family genes, many of which with an adjacent gene coding for an associated corrinoid protein, as demonstrated in **chapter 3**. The best studied organism in regards to methylated quaternary amine metabolism, *Eubacterium limosum*, contains over 40 non-pyrrolysine homologs of *mttB*. Three of these homologs have been described to be coding for substrate specific proline betaine, carnitine and γ-butyrobetaine:corrinoid methyltransferases (49, 51, 227). While no glycine betaine specific methyltransferase has been described in this organism, production of N,N-dimethylglycine when growing on glycine betaine has been described (329), making it likely one of the homologs of *mttB* codes for glycine betaine:corrinoid methyltransferase. The fact that homologs of *mttB* are widespread and present in high numbers in many genomes makes the hypothesis that each individual homolog codes for a protein specific for a single methylated quaternary amine appealing, especially in the context of a single HGT event being sufficient for full adaptation to this new substrate.

The commonality of glycine betaine in marine contexts has been discussed throughout this thesis. The quaternary amine discussed in **chapter 4**, tetramethylamine, is found as a toxin in marine snails of the family Neptunea, yet further research on the natural distribution is unknown (366). Thus, the ecological relevance of methanogenic degradation in natural habitats is difficult to gauge. It is, however, common in industrial use as an etchant in thin-film and semiconductor production (367). As a pollutant with detrimental effects to human health, proper management of tetramethylammonium waste streams is paramount. Methanogenic degradation of tetramethylammonium in bioreactors has been shown to be a possible avenue to mitigate these waste streams (368).

Other methylated quaternary amines are less well described in environmental contexts, but play important roles in intracellular processes of both prokaryotes and eukaryotes. Many secondary metabolites are N-methylated amino acids. N-methylation provides higher stability against proteolytic degradation or increase the permeability through membranes compared to unmethylated amino acids (369, 370). For example, glycine betaine itself is a methylated form of the amino acid glycine, and carnitine is a methylated derivative of lysine (371). Due to the intrinsic zwitterionic nature of quaternary amine amino acid derivates, they can act as osmolytes in the "high salt-in" strategy (13, 14). While not much research has been conducted on the prevalence of such compounds outside of glycine betaine, trimethylated serine has been identified as the major compatible solute in the marine cyanobacterium *Trichodesmium erythraeum* (372). Furthermore, chemical assays on trimethyltaurine identified it as a suitable zwitterionic osmolyte throughout the physiological pH range (373). Many of such amino acids and its derivatives have been detected to be freely available in the cytosol of marine organisms such as red algae and sponges (374, 375). As such, an interesting avenue of future research is to identify marine methylated quaternary amines that are common as osmolytes or have other major biological functions. Once identified and purified, these compounds can be tested for potential methylotrophic metabolism with organisms that can serve as model organisms such as *Desulfosporosinus nitroreducens* strain P130 or *Methanococcoides vulcani*. Additionally, it is important to obtain a broader view of the potential of microorganisms from marine sediments.. This can be done through isolation and cultivation of microorganisms under laboratory conditions and through meta-'omic approaches. Both of these have major benefits and drawbacks, which shall be discussed next.

7.6 Laboratory cultivation of environmental microorganisms

The majority of this thesis has been founded on samples obtained during a single sampling campaign, undertaken over the course of four days - two weeks if travel to and from the Black Sea station is included. From the obtained samples, we have performed a combination of modern sequencing technology, most notably in **chapter 3** and classical isolation techniques, most notably in **chapter 4** and **chapter 5.** Both these classical and modern techniques have their benefits and drawbacks.

The major shortcoming in classical cultivation techniques is the cultivability of certain microorganisms over others. Four major phyla – *Actinobacteria, Bacteroidetes*, *Firmicutes* and *Proteobacteria* – represent the majority of cultured microorganisms (376). This discrepancy is apparent in the diversity of cultured methylotrophic microorganisms as outlined in **chapter 2**, a very short list representing all anoxic marine systems worldwide, and all these bacterial strains reside in one of these four phyla. In 2022, 17470 validly described and named bacterial and archaeal species were on record (377). With estimates of at least 400.000 microbial species and possibly orders of magnitude more existing, this is only a small fraction of the actual diversity (378–380). It is therefore very likely that due to this disparity between diversity in nature and in the lab, unknown metabolisms and microbial functions remain as of yet undescribed.

An oft-repeated axiom in microbiology is that only 1% of all bacteria are culturable in the laboratory, the so-called "great plate count anomaly" (381). While there is some merit in this claim, the reality is there is a large variance in the percentage of organisms amenable to culturing depending on the ecosystem, and the methodologies used to measure/estimate cultured versus uncultured. A meta-analysis showed only 0.5% of detected 16S rRNA gene amplicons worldwide could be grown in standard culture, but the interquartile ranged between 0.025 and 4.3% (382, 383). However, individual studies were able to culture up to 70% of all cell counts from the human gut system and other human engineered environments had similar culturable ratios (384). The ratio of cultured organisms in metagenomic or metatranscriptomic assays was much lower, with an average of 65% of bacterial and 71% of archaeal of sequences belonging to currently uncultured phyla, highlighting the black box on microbial diversity outside of the laboratory (382, 383). Reasons for the absence of cultured representatives are diverse. Traditionally, culturing is performed on agar plates, rich in nutrients. This favors organisms with fast turnover times, capable of quickly becoming dominant. Additionally, the sterilization process of agar can produce radicals, further inhibiting growth of less opportunistic organisms (385).

Other physicochemical factors such as the correct hydrostatic pressure, presence of rare trace elements such as lanthanides and presence of a surface material on which to grow all contribute to the ability of a microorganism to grow in the laboratory (145, 241, 242, 386). Furthermore, an often overlooked aspect in culturing is time. Turnover of organic material in marine sediments is measured in weeks in the most upper layers to eons in the deep subsurface (266, 387). While it is as of yet impossible to perform laboratory experiments over geological timescales, patience in cultivation is still required. *Candidatus* Promethoarchaeum syntrophicum, an important microorganism in understanding the bridge between prokaryotic and eukaryotic life, took over a decade to obtain in a highly enriched culture (388). With PhD contracts commonly lasting much shorter than this, it is therefore important for laboratories to invest in permanent talent that can provide a continuous effort in culturing difficult microorganisms over timescales longer than a single PhD project.

With this in mind, many of the standard hurdles can be mitigated by more targeted approaches towards cultivating microorganisms. Rather than using traditional culturing media, the medium utilized for the cultivation of the target microbes in **chapter 4** and **chapter 5** closely resembles the mineral makeup of the Black Sea (66). As such, once inoculated with sediments from the Black Sea, conditions favored all organisms, rather than opportunistic fast growing microorganisms. Regardless, even the relatively fast growing *Desulfosporosinus nitroreducens* strain P130 required several years of consequent transferring under conditions optimal for its growth before isolation attempts were successful. Other opportunistic organisms from the enrichment culture, such as heterotrophic strains from the genera *Labilibaculum* and *Sedimentibacter* were more readily isolated from the enrichment culture, despite conditions of the enrichment culture not favoring these organisms. Acquiring the genome of all constituents of the enrichment cultures allowed us to make more accurate predictions on the physiology of the target organisms, which yielded an isolate in **chapter 5** and a more highly enriched culture in **chapter 4.** The availability of cheap, reliable sequencing technologies such as Illumina Novaseq and Oxford Nanopore has made the routine incorporation of genomic information in cultivation efforts possible. This has also yielded novel successful cultivation techniques, such as reverse genomics, where fluorescently labeled antibodies against specific membrane-bound enzymes of the target organism are created. Cells to which these antibodies are then bound can be sorted utilizing fluorescent-activated cell sorting. However, such techniques come with many practical drawbacks and while the cell-sorting technique was tried to isolate microorganisms in this research, this resulted in failure. Despite this, the cell sorting technique has been demonstrated to isolate a member of the previously

uncultivated phylum *Saccharibacteria* (389). The utilization of modern techniques such as genomics, but also live staining, microchips or single droplet cultivation to obtain axenic cultures has been recently reviewed thoroughly and as such, shall not be discussed in further detail (390).

7.7 Meta-'omics techniques

The advent of techniques to gain insights in the molecular blueprint of an organism through sequencing DNA has opened a multitude of new research possibilities. The advent of *affordable* DNA sequencing has multiplied these possibilities even further. When the human genome was partially sequenced in 2003, it took over a decade and $3.000.000.000 to cover the 3 billion bases of the human genome, a dollar per sequenced base (391). Since the unveiling of this monumental watershed moment in biological research, costs have dramatically decreased. In 2022, the sequencing of a human genome has become routine and costs as little as $400 (392). In the microbiological world, these advancements have had similar effects. The sequencing of environmental samples to obtain insights in its microbiological constituents is a routine effort. **Chapter 3** is an example of such a routine effort. Together with the ability to obtain high resolution metagenomic insights in the genomes present in an environment, the expression of genes in these genomes through metatranscriptomics and presence of the proteins coded by the genes through metaproteomics has become available. Combined, this has allowed analyses of various environments to a degree of resolution that were unthinkable a decade ago. Many reviews have been written on the benefits of these meta-'omics to gain insights in environments as diverse as cattle rumen (393), zooplankton (394), soils (395) or marine waters (396).

The proliferation of these techniques has led to the mass abundance of datasets. Thanks to good scientific practice of allowing these datasets to be publicly available, many scientists can further build on these results. As an example, the availability of these datasets have led to our analyses in **chapter 2**, which allowed us to draw conclusions on the presence of genes of interest in a wide range of anoxic marine metagenome datasets. This may or may not have been the intention of the original authors. However, the proliferation of these techniques have also led to an overreliance on this type of data, and incomplete assumptions on what the data represents. In this thesis, the differences between MttB-superfamily coding genes which do or do not code for an in-frame amber codon have been discussed in detail. This difference of three nucleotides drastically alters the function of a homolog. As this is not necessarily commonly known, this means that many non-pyrrolysine MttB-superfamily genes are wrongly annotated as a trimethylamine:corrinoid methyltransferase. As a

result, organisms with MttB-superfamily genes yet lacking in pyrrolysine biosynthesis have likely been misinterpreted as growing on TMA. This highlights the need for careful validation of meta-'omics results with physiological experiments and a thorough examination of the claims made in any paper surrounding potential utilization of substrates or pathways by both authors and reviewers of such works. The current onslaught of data generation and the need to publish quickly to avoid being scooped is often at odds with such careful examination and validation. However, in order to deduce the intricate networks of ecosystems such as marine sediments, it is crucial to take a patient approach.

7.8 Global significance

One of the stated goals of this thesis was to revisit commonly held assumptions on methylotrophic methanogens in marine sediments. The end product of methanogens is methane, a gas with global repercussions on the climate. After CO_2, methane is the strongest contributor to the climate change, with an estimated 30% of the rise in global temperatures since 1900 attributed to increased anthropogenic methane emissions (397). An estimated 70% of these methane emissions were due to biotic production by methanogens (29, 398). Methanogens occur in virtually any habitat where oxygen is absent and especially methanogens in the gastrointestinal tract of ruminant cattle, landfills and rice paddies contribute strongly to the increased anthropogenic emissions of methane (29). In contrast, marine ecosystems contribute an estimated 3% to total methane emissions, mostly from shallow coastal areas (399, 400). While relatively modest in comparison to other major emission sources, this still represents 6-12 Tg of emitted methane on a yearly basis, equivalent to 150-300 million tons of CO_2 – roughly as much as the total greenhouse gas emissions in the Netherlands in 2020 on the low end of this scale, or France on the high end. The majority of these marine methane emissions are thought to originate from methylotrophic methanogenesis. Additionally, novel lineages of methylotrophic methanogens are suggested to occupy important niches in other environments, such as manmade ponds or wetlands (33, 34).

In the light of this, understanding each detail of the marine methane cycle is crucial to further refine our models on methane emissions from methylated substrates. Once identified which methylated compounds can contribute to a higher rate of methanogenesis and which compounds are more readily oxidized by sulfate-reducing bacteria, acetogens or any of the lineages suggested in **chapter 3**, better models can be created to assess the impact of anoxic marine systems in the global methane budget. This is especially relevant in the context of expanding anoxic marine zones. Glob-

ally, less oxygen is becoming available in oceans and coastal waters (401). Higher temperatures decreases the solubility of oxygen in water. Due to the dependence of methanogens for strictly anoxic conditions, a positive feedback loop is created between oceanic anoxic zones causing sediments to become anoxic, which then produce more methane, exacerbating emissions, causing higher temperatures, which in turn creates more anoxic zones. Such feedback loops have been suggested to have been a major part of the most significant extinction event in Earth's history, the Permian-Triassic extinction (402). Furthermore, due to intensified agricultural runoff and waste-water discharges, nominally rate limiting compounds such as nitrogen and phosphorous are increasingly available in marine systems. As a result, massive algal blooms occur. As these blooms die, an excessive amount of organic carbon becomes available, the degradation of which consumes all available oxygen (403, 404). As a result, over 400 of such marine "dead zones" have been identified (401, 405). As demonstrated throughout this thesis, the combination of anoxia and organic material yields the anaerobic metabolism of methylated compounds. Thus, to understand to what extent methanogenesis occurs in these new anoxic areas, it is important to understand the fate of all methylated compounds and which metabolisms are involved in its degradation.

7.9 Conclusions

The work presented in this thesis pertains to the utilization of methylated compounds in anoxic marine sediments in general, using Black Sea sediments as a model site. We have expanded the knowledge on the potential of methylotrophy in several lineages of bacteria previously unknown to utilize these compounds through metagenomic analyses, including the widely proliferated sulfate-reducing bacteria. We have also demonstrated the presence of key methyltransferase genes in a wide array of publicly available metagenome datasets. These results all demonstrate that methylated compounds are very likely to be utilized as competitive rather than "non-competitive" substrates by methanogens in marine sediment, in contrast to what has been widely claimed in scientific literature. Especially methylated quaternary amines, which are widely available in marine systems as ubiquitous osmolytes in many marine organisms, are suggested in this work to form a major source of methylotrophy for anaerobic bacteria and archaea. Genes coding for the family of proteins suspected to be involved in methyl cleavage of quaternary methylated amines were present in the majority of metagenome assembled genomes in the Black Sea. This strongly suggests a major catalytic role of the proteins coded by these genes for the incorporation of methylated quaternary amines in the metabolism of micro-organisms in the Black Sea.

Through classical cultivation efforts, an isolated methylotrophic sulfate-reducing microorganism and a highly enriched methylotrophic methanogenic consortium were obtained. Both the isolate and the enrichment were capable of growth on the methylated compounds methanol, trimethylamine and glycine betaine. Through a combination of genomic and transcriptomic efforts, we have demonstrated the possibility of simultaneous degradation of glycine betaine through an oxidative and reductive pathway by the sulfate-reducing microorganism *Desulfosporosinus nitroreducens* strain P130. Furthermore, we have provided strong evidence that a non-pyrrolysine coding homolog of monomethyl:corrinoid methyltransferase is involved in demethylating the simplest methylated quaternary amine tetramethylammonium in the methanogen *Candidatus* Methanococcoides chernomoris. When grown together on common methylated substrates, both sulfide and methane were produced, indicating co-subsistence rather than methylated compounds serving as non-competitive substrates for methanogens. These results demonstrate a closer look at the cycle of methylated compounds in anaerobic sediments is needed to fully understand the role they play in the global carbon, nitrogen and sulfate cycles. In this work, we have given strong evidence that the reality of methylated compound utilization in anoxic marine sediments is much more nuanced than previously assumed.

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Summary

Methylated compounds, which we define in this thesis as any small organic compound with one or more methyl (CH_3) groups attached to a non-carbon atom, such as sulfur, nitrogen or oxygen play important roles in the global nutrient cycles. Marine organisms produce small organic methylated compounds such as glycine betaine or choline and retain them in their cells to counter the stress from to the large amounts of salt in the sea. As these organisms die, they sink to the seafloor. Once at the bottom, release the methylated compounds to the environment. In the sediment beneath the seafloor, these compounds are either directly used by some of the present microorganisms, or fermented to smaller methylated compounds such as methanol or trimethylamine by other microorganisms. As such, anoxic marine sediments are a hotbed of microbial activity. In the absence of oxygen, microorganisms can use sulfate as an alternative to "breathe," using a wide range of organic and inorganic compounds as fuel. These organisms that utilize sulfate are called sulfate-reducing microorganisms, or SRM. Deeper in the sediment, where little to no sulfate is present, methanogenic archaea exist, capable of producing the potent greenhouse gas methane from organic and inorganic substrates. SRM and methanogenic archaea are seen as mutually exclusive organisms, as they use the same substrates as fuel. Yet, for a long time, it was assumed methanogenic archaea were able to co-exist in sediments rich in sulfate by using methylated compounds, which SRM were thought not to use. However, recent research has demonstrated this may not be the case. To gain a deeper understanding of the fate of different methylated compounds in anoxic marine sediments, we performed the work presented in this thesis.

In chapter 2, we set out to compile what is already known on the marine methanol cycle. Methanol is seen as the most important methylated compound in marine sediments. Through a literature research, we identified the different concentrations of methanol in different marine sediments worldwide. We provide an overview of the different ways in which methanol becomes available in marine sediments and list the known anoxic marine microorganisms that have been studied in laboratories throughout the world capable of growing on methanol. The different biochemical methods these organisms use to obtain energy from methanol are described in detail, and we hypothesize on the interactions between these different microorganisms when growing on methanol. Finally, to gain insights on the worldwide distribution of methanol use, we mined publicly available datasets of genetic information of marine sediments for the presence of genes involved in methanol utilization.

To be able to gain specific insights of our model ecosystem, we set sail to the Black Sea and collected sediments from the top 30 centimeters below the sea floor. In chapter 3, we set out to obtain the DNA sequences of as many microorganisms from these sediments as possible. In total, we obtained 394 unique genomes of acceptable quality as a representative of the microbial diversity of this ecosystem. In the majority of these genomes, we found genes likely coding for enzymes involved in the utilization of a specific group of methylated compounds called quaternary methylated amines. We identified the presence of these genes in groups of bacteria not previously associated with the use of methylated compounds, including groups such as *Desulfobacterota* and *Chloroflexota*, which are found in great numbers throughout the world's oceans.

As genomic information by itself does not paint a complete picture, we wanted to obtain organisms capable of growing on methylated compounds in the laboratory as well. From the obtained Black Sea sediments, enrichments were set up to obtain these organisms. In chapter 4, we describe the enrichment of Black Sea sediments to obtain a consortium of three microorganisms capable of producing methane from the methylated compounds methanol, glycine betaine, monomethylamine, dimethylamine, trimethylamine and tetramethylamine. From these three organisms, only one, the novel methanogenic archaeon *Candidatus* Methanococcoides chernomoris codes for the genes involved in the degradation of these compounds. We provide strong evidence that a gene from the so-called MtmB-family of genes codes for a protein likely to be involved in the degradation of tetramethylamine. In chapter 5 we describe the isolation of a sulfate-reducing microorganism, *Desulfosporosinus nitroreducens* strain P130. This organism is capable of growth on the methylated compounds methanol, trimethylamine and glycine betaine. We have provided evidence this organism codes for the same genes as methanogenic archaea to code to use these compounds. We further demonstrate that this organism is capable of growing on glycine betaine using two degradation pathways simultaneously.

In our final experimental chapter, chapter 6, we grow the enrichment obtained in chapter 4 and the sulfate-reducing microorganism obtained in chapter 5 in a co-culture growing on their shared substrates methanol and glycine betaine. Under batch conditions, both *Desulfosporosinus nitroreducens* strain P130 and *Ca.* Methanococoides chernomoris were able to grow at similar growth rates, indicating that as long as substrate is available, neither SRM or methanogenic archaea outcompete the other. Using data on the expression of all present genes, we were able to demonstrate a cascading network of glycine betaine conversion by the sulfate-reducing microorganism and subsequent use of products by both SRM and methanogenic archaea.

Finally, in chapter 7, we discuss the findings of this thesis in a broader scientific context. We hypothesize on the evolutionary origins of genes involved in the conversion of methylated compounds. Additionally, we discuss the presence of quaternary methylated amines as a likely major substrate in anoxic marine sediments. We discuss the benefits of drawbacks of traditional laboratory culture of microorganisms and modern genome-based technologies. To close, the environmental impacts on the global methane cycle of our results are discussed.

Samenvatting

Gemethyleerde stoffen in deze thesis worden gedefinieerd als kleine organische verbindingen met één of meerdere methylgroepen (CH_3) verbonden aan een atoom dat géén koolstof is, zoals stikstof, zwavel of zuurstof. Deze stoffen spelen een belangrijke rol in de wereldwijde kringlopen van koolstof, stikstof en zwavel. Vrijwel alle organismen die in de zee leven produceren gemethyleerde stoffen zoals glycine-betaine of choline in hun cellen als verdedigingsmiddel tegen te hoge zoutgehaltes in de zee. Wanneer deze organismen sterven, zinken ze naar beneden en komen de gemethyleerde stoffen vrij in de zeebodem. In het sediment van de zeebodem kunnen deze stoffen vervolgens gebruikt worden door micro-organismen die hier leven, zowel door directe verbranding als door fermentatie tot kleinere gemethyleerde stoffen zoals methanol of trimethylamine. In de zeebodem raakt zuurstof snel op en al na enkele centimeters diepte is er geen zuurstof meer aanwezig. In de afwezigheid van zuurstof kunnen bepaalde micro-organismen gebruikmaken van sulfaat voor de 'verbranding,' waarbij een ruime hoeveelheid aan organische en anorganische stoffen kunnen dienen als brandstof. Organismen die sulfaat kunnen gebruiken heten sulfaatreducerende micro-organismen, of SRM. Dieper in het sediment, waar weinig tot geen sulfaat aanwezig is, leven bepaalde microorganismen, die methanogene archaea worden genoemd. Methanogene archaea produceren het sterke broeikasgas methaan door organische en anorganische stoffen te gebruiken. SRM en methanogene archaea sluiten elkaars aanwezigheid uit vanwege het gebruik van gemeenschappelijke brandstoffen waarbij verbranding met sulfaat veel meer energie oplevert. Toch wordt sinds lange tijd gedacht dat in de bovenste laag van de zeebodem, waar veel sulfaat aanwezig is, deze organismen konden samenleven omdat methanogene archaea als enige gemethyleerde stoffen konden gebruiken. Echter heeft recent onderzoek reden gegeven dat dit niet per sé waar is. Om een beter begrip te ontwikkelen over het lot van gemethyleerde stoffen in zuurstofloze zeebodems is het werk in deze thesis uitgevoerd.

In hoofdstuk 2 hebben we ons ten doel gesteld om informatie te verzamelen over wat al bekend was over de mariene methanolcyclus. Methanol wordt gezien als één van de belangrijkste gemethyleerde stoffen in zeeën. Door middel van een literatuuronderzoek hebben we de kennis over de concentraties van methanol in verschillende zeebodems wereldwijd verzameld. Ook geven we een overzicht over de verschillende manieren hoe deze stof beschikbaar wordt in zeebodems en beschrijven we welke mariene micro-organismen al geïsoleerd zijn die kunnen groeien op methanol. De

verschillende biochemische routes die deze micro-organismen gebruiken om energie op te wekken uit methanol worden in detail beschreven en we beschrijven hypotheses over de mogelijke interacties tussen deze organismen. Om inzicht te krijgen in hoe wijdverspreid het gebruik van methanol in zuurstofloze bodems is, hebben we alle publiekelijk beschikbare gegevens onderzocht op de aanwezigheid van genen die betrokken zijn bij deze biochemische routes.

Om specifieke inzichten te krijgen in ons modelsysteem, zijn we uitgevaren naar de Zwarte Zee en hebben daar sediment van de bovenste 30 centimeter zeebodem opgevist. In hoofdstuk 3 beschrijven we hoe we het DNA in kaart hebben gebracht van zo veel mogelijk micro-organismen uit deze zeebodem. In totaal beschrijven we het DNA van 394 unieke micro-organismen die de diversiteit van de Zwarte Zeebodem als geheel weergeven. In de meerderheid van deze organismen hebben we aanwijzingen gevonden dat ze mogelijk een specifieke groep gemethyleerde stoffen kunnen gebruiken: *quaternaire gemethyleerde amines*. We hebben aangetoond dat dit voorkomt in groepen bacteriën die niet eerder in verband zijn gebracht met het gebruik van gemethyleerde stoffen, zoals *Desulfobacteriota* en *Chloroflexota*, groepen die veel voorkomen in vrijwel alle zuurstofloze mariene systemen over de hele wereld.

Aangezien informatie uit DNA niet voldoende is om een totaalbeeld te schetsen, hebben we geprobeerd voorheen onbekende micro-organismen uit de Zwarte Zeebodem die in staat zijn gemethyleerde stoffen te gebruiken in ons laboratorium te kweken. In hoofdstuk 4 beschrijven we hoe we vanuit ruwe Zwarte Zeemodder een consortium van drie micro-organismen hebben verkregen die in staat is methaan te produceren uit de gemethyleerde stoffen methanol, glycine-betaine, monomethylamine, dimethylamine, trimethylamine en tetramethylamine. Van deze drie organismen is er slechts één die de benodigde genen heeft om de afbraak van deze stoffen te faciliteren: *Candidatus* Methanococcoides chernomoris, een niet eerder beschreven methanogeen archaeon. Tot dusver waren er geen genen bekend die betrokken zijn bij de afbraak van tetramethylamine. We geven sterk bewijs dat een gen uit de MtmB-familie van genen verantwoordelijk is voor de afbraak van tetramethylamine in *Candidatus* Methanococcoides chernomoris. In hoofdstuk 5 beschrijven we de isolatie van een sulfaatreducerend micro-organisme: *Desulfosporosinus nitroreducens* P130. Dit organisme is in staat te groeien door het gebruiken van de gemethyleerde stoffen methanol, glycine-betaine en trimethylamine. We hebben bewijs geleverd dat dit organisme voor dezelfde genen codeert als methanogene archaea voor het gebruik van deze gemethyleerde stoffen. Verder tonen we aan dat dit organisme tegelijkertijd twee verschillende biochemische routes gebruikt om glycine-betaine af te breken.

In ons laatste experimentele hoofdstuk, hoofdstuk 6, voegen we het methanogene consortium en het sulfaatreducerende micro-organisme samen om hun interacties te volgen wanneer er methanol of glycine-betaine wordt toegevoegd, waar beiden op kunnen groeien. We tonen aan dat bij voldoende aanwezigheid van het substraat beiden in staat zijn op gelijke voet te groeien. De uitdrukking van genen die betrokken zijn bij het gebruik van glycine-betaine toont aan dat een trapsgewijze afbraak plaatsvindt van deze stof door het sulfaatreducerende organisme, waarna de afbraakproducten worden geconsumeerd door zowel de SRM als het methanogene archaeon.

Tot slot, in hoofdstuk 7, bespreken we de behaalde resultaten in een bredere wetenschappelijke context. We voeren hypotheses op over de evolutionaire ontwikkeling van de genen die betrokken zijn bij de afbraak van gemethyleerde stoffen. Verder wordt het gebruik van quaternaire gemethyleerde amines besproken als een onderbelicht substraat voor vele mariene micro-organismen. We bespreken ook de positieve en negatieve aspecten van traditioneel groeien in een laboratorium en meer moderne DNA-technieken. Tot slot wordt de impact van onze resultaten op het begrip van de globale methaancyclus besproken.

Oettreksel

Gemethyleerde sfoff'n wördt in dizze thesis omschrev'n as kleene organische verbinding'n met één of meer methylgroep'n (CH_3) verbeund'n an 'n atoom, dat gen koolstof is, zo as stikstof, zwaofel of zuurstof. Dizze stoff'n speult ne veurname rol in de kringloop'n van koolstof, stikstof en zwaofel van oawer de gaanze weerld. Host alle organism'n die in de zee leaft, produceert gemethyleerde stoff'n as glycine-betaine of choline in hear cell'n as ofwearmiddel teg'n te hoog zoaltinhoald in de zee. As dizze stearft, zakt ze naor onnerd'n en komt gemethyleerde stoff'n vriej in 'n boad'm van de zee. In 't slip van 'n zeeboad'm koomp dizze stoff'n vriej, die doornoa wördt broekt duur micro-organism'n, die nig alleen leaft duur directe verbraanding, mer ok duur fermentatie tot klenere gemethyleerde stoff'n as methanol of trimethylamine. In 'n zeeboad'm raakt 'n zuurstof rap op en noa 'n paar centimeter in de deepte is d'r gen zuurstof mear te beken'n. As d'r gen zurstof mear is, könt bepoalde micro-organism'n gebroek maak'n van sulfaat vuur 't verbraan'n, woarbiej ne roeme moat an organische en anorganische stoff'n könt deen'n as braandstof. Organism'n, die sulfaat könt broek'n, die sulfaatreducerende micro-organism'n of SRM wordt neumd. Deeper in 't slip, woar gen of wenig sulfaat in zit, leaf't bepaolde micro-organism'n, die methanogene archaea wördt neumd. Methanogene archaea maakt 't staerke breuikasgas methaan duur organische en anorganische stoff'n zo, dat het kan won broek't. SRM en methanogene archaea sloet meka oet duur 't gebroek van braandstoff'n die ze tehoop broekt, woarbiej verbraanding völ meer energie opleavert. Toch wördt al lange tied dacht, dat in de boavenste loag van 'n zeeboad'm, woar völ sofaat wördt antröff'n, dizze organism'n te hoop kont leav'n, omdat methanogene archaea as enige gemethyleerde stoff'n kon'n broek'n. Mer de leste tied hef onnerzeuk oetwez'n, dat dit nig per se waor is. Um het lot van gemethyleerde stoff'n in 'n zeeboad'm better te konn'n begriep'n, is het onnerzeuk in dizze thesis oetvoerd.

In Kepitel 2 bint wie an de gang goan um informatie te verzamel'n oawer wat bekeand was oawer de mariene methanolcyclus. Methanol wördt zeen as één van de veurnaamste gemethyleerde stoff'n in zeeën. Duur 'n literatuuronnerzeuk hebt wiej kennis biej meka gadderd oawer de concentraties van methanol in verschill'nde zeeboadems oawer de godganske weerld. Ok geft wiej 'n oawerzicht oawer de verschiedene manèèrn hoe dizze stoff'n in zeeboad'm vuur haand'n is en wat vunne micro-organism'n a isoleard bint, die op methanol kunt greui'n. De verschill'nde bio-chemische weag'n die dizze micro-organism'n broekt um energie oet methanol op te wekk'n wördt tot in de klenste kleanighed'n verkloard en wiej duudt hypotheses

over de mögeluke interacties tuske dizze organism'n oet, um inzicht te krieg'n in hoe wied en zied het gebroek van methanol in boadems zonner zuurstof is, hebt wiej aln's wat in 't openbaar opscheav'n steet, onnerzocht op het bestoan van geen'n, die biej dizze biochemische weag'n betrökk'n bint.

Um specifieke inzicht'n in oons modelsysteem te kreig'n, bint wiej noar de Zwarte Zee trökk'n en heb wiej daor drek van de boavenste loag vean dettig centimeter van 'n zeeboad'm ofhaald. In Kepitel 3 duudt wiej het DNA oet van zo völ micro-organism van dizze zeeboad'm. Al met meka beschrieft wiej het DNA van 394 unieke micro-organism'n um de diversiteit van 'n Zwarte Zeeboad'm in zien grote geheel wier te geff'n. In de meeste gevall'n van dizze organism'n hebt wiej aanwiezing'n vuer'n, dat ze mögeluke specifieke gemethyleerde stoff'n könt broek'n: quaternaire gemethyleerde amines. Wiej hebt loat'n zeen, dat dit veurkoamp in groep'n bacteriën, die nig earder in verbaand bint bracht met 't gebroek van gemethyleerde stoff'n, zo as *Desulfobacteriota* en *Chloroflexota,* groep'n die vuurkomt in host alle marine systeem'n zonner zuurstof van oawer de godganske weerld.

Umdat informatie oet DNA nich genog is um 'n totaalbeeld oet te teek'n, hebt wiej prebeerd vrogger onbekeande micro-organism'n oet 'n Zwarte Zeeboad'm, die in stoat bint gemethyleerde stoff'n te broek'n, in 'n laboratorium te kweek'n. In Kepitel 4 beschrieft wiej op welke wieze wiej roew'n drek van oet de Zwarte Zee ne groep van gelieksoartige stoff'n van dree micro-organism'n hebt kreg'n, die methaan könt maak'n oet de gemethyleerde stoff'n methanol, glycine-betaine, monomethylamine, dimethylamine, trimethylamine en tetramethylamine. Van dizze dree organism'n is d'r mer één, dat oawer de neudige geen'n geschikt um het ofbrekk'n van dizze stoff'n mögeluk te maak'n: *Candidatus* Methanococcoides chernomoris, nig earder bescrhreff'n methanogeen archaeon. Tot noe too wan'n d'r gen geen'n bekeand, die betrökk'n bint biej het ofbrekk'n van tetramethylamine. Wiej leaver't 'n staerk bewies, dat 'n gen oet de MtmB-familie verantwoardelijk is vuur het ofbrekk'n van tetramethylamine in *Ca.* Methanococcoides chernomoris.

In Kepitel 5 duudt wiej het isoleer'n van 'n sulfaatreducerend micro-organisme oet: *Desulfosporosinus nitroreducens* stam P130. Dit organisme is in stoat te greuien duur 't gebroek van gemethyleerde stoff'n methanol, glycine-betaine en trimethylamine. Wiej hebt bewez'n, dat dit organisme vuur dezölfde geen'n codeert as methanogene archaea vuur 't gebroek van dizze gemethyleerde stoff'n. Wieder duud't wiej oet, dat dir organisme tegliekertied twee verschill'nde biochemische weag'n broekt um glycine-betaine of te brekk'n.

In oons leste experimentele kepitel, Kepitel 6, doot wiej ’t methanogene consortium en ’t sulfaatreducerende micro-organisme bie meka um hear interacties te volg’n, as methanol of glycine-betaine too wördt voogd, op meka wearkt, waorop ze op geliek’n voot kunt wasn’t en greui’n. Wie loat zeen dat, as der genog substraat is, albear op geliek’n voor könt greui’n. De wieze woarop de geen’n zich oetdrukt biej het gebroek van glycine-betaine lat zeen, dat ne trapsgewieze ofbraak van dizze stof an de geng geet duur ’t sulfaatreducerend organisme, waarnoa de ofbraakproduct’n wördt opget’n, duur ni gallenig de SRM maar ok duur ’t methanogene archaeon.

Op ’t allerlest, in Kepitel 7, duudt wie oet hoe de verkreg’n feit’n in ’n grötter wetenschappelijk verbaand met meka stoat. Wie voert hypotheses op en loat ’t bestoan van evolutionaire ontwikkeling’n van geen’n zeen, die biej het ofbrekk’n van gemethyleerde stoff’n betrökk’n bint. Wieder wördt het gebroek van quaternaire gemethyleerde amines besprökk’n as ’n onnerbelecht substraat vuur marine micro-organism’n. Wiej besprekt de goeie en de slechte kaant’n van de gebroekelijke wieze van greui’n in ’n laboratorium en meer DNA-techniek’n van dizze tied.

Um ’t af te sloet’n besprekt wiej de umgevingsoetwerking van oonze resultaot’n op ’t begrip van ’n globaal’n methaancyclus.

List of publications

Peter Q. Fischer, Irene Sánchez-Andrea, Alfons J. M. Stams, Laura Villanueva, Diana Z. Sousa (2021). Anaerobic microbial methanol conversion in marine sediments. *Environmental microbiology* 23(3):1348-1362. DOI: 10.1111/1462-2920.15434

Sara Cantera, Peter Q. Fischer, Irene Sánchez-Andrea, David Marín, Diana Z. Sousa, Raúl Muñoz (2021). Impact of the algal-bacterial community structure, physio-types and biological and environmental interactions on the performance of a high rate algal pond treating biogas and wastewater. *Fuel* 302(15):121148. DOI: 10.1016/j.fuel.2021.121148

Karin Stultiens, Maartje A.H.J. van Kessel, Jeroen Frank, Peter Q. Fischer, Chris Pelzer, Theo A. van Alen, Boran Kartal, Huub J.M. Op den Camp, Mike S.M. Jetten (2020). Diversity, enrichment, and genomic potential of anaerobic methane-and ammonium-oxidizing microorganisms from a brewery wastewater treatment plant. *Applied Microbiology and Biotechnology* 104:7201-7212. DOI: 10.1007/s00253-020-10748-z

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Peter Q. Fischer, Alfons J.M. Stams, Laura Villanueva, Diana Z. Sousa. A novel Desulfosporosinus nitroreducens strain P130 capable of anaerobic methylotrophy isolated from Black Sea sediments

About the author

Peter "Q." Fischer was born on the 19th of May, 1989 in Almelo, the Netherlands. He received his secondary education at CSG Het Noordik Almelo. Afterwards, he studied Biology at the University of Groningen, with a specialization in Evolutionary Biology and Ecology. In 2015 he continued with a Master's program in Biology at Radboud University Nijmegen, with a specialization in Microbiology. This included a thesis internship at the department of microbiology at Radboud University, where he studied the metagenomic composition of an enrichment of an anaerobic methane- and ammonium-oxidizing microbial community. This was followed up with an internship at Bioclear Earth B.V. in Groningen, where he developed models to imply agricultural soil quality based on their microbial composition.

After graduating in 2017, he worked as a project officer on the topic of sustainable soil management at Van Hall Larenstein University of Applied Sciences. In 2018 he started his PhD project at Wageningen University & Research, joining the Microbial Physiology Group at the Laboratory of Microbiology. The results of this PhD project can be read in this thesis. Currently he is employed at AquaLab Zuid B.V. as advisor drinking water quality.

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- HPC advanced course, Wageningen University (2018)

Management and Didactic Skills Training

- Supervising MSc student with thesis entitled 'Researching the metabolic flexibility of sulfate reducing bacteria' (2020)
- Supervising BSc student with thesis entitled 'Interactions between a sulphate reducer and a methanogen: constructing a co-culture' (2019)
- Teaching in the MSc course 'Research Methods in Microbiology' (2018-2020)
- Member of the SENSE PhD board (2019-2022)
- Initiator and member of the Microbiology PhD board (2019-2021)
- PhD trip organization for MIB (2019-2022)

Oral Presentations

- *Methylotrophy in the deep: metagenomics and physiology of microbial enrichments from Black Sea sediments*. Extremophiles 2022, 18-22 September 2022, Loutraki, Greece
- *Expanded diversity of anoxic marine methylotrophy: physiological capabilities of a non-canonical methylotroph on a range of methylated compounds*. KNVM, 5-6 April, 2022, Papendal, The Netherlands
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