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REVIEW

Methyl-Based Methanogenesis: an Ecological and Genomic Review

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SUMMARY Methyl-based methanogenesis is one of three broad categories of archaeal anaerobic methanogenesis, including both the methyl dismutation (methylotrophic) pathway and the methyl-reducing (also known as hydrogen-dependent methylotrophic) pathway. Methyl-based methanogenesis is increasingly recognized as an important source of methane in a variety of environments. Here, we provide an overview of methyl-based methanogenesis research, including the conditions under which methyl-based methanogenesis can be a dominant source of methane emissions, experimental methods for distinguishing different pathways of methane production, molecular details of the biochemical pathways involved, and the genes and organisms involved in these processes. We also identify the current gaps in knowledge and present a genomic and metagenomic survey of methyl-based methanogenesis genes, highlighting the diversity of methyl-based methanogens at multiple taxonomic levels and the widespread distribution of known methyl-based methanogenesis genes and families across different environments.

KEYWORDS *Archaea*, anaerobic catabolic pathways, methane, methanogenesis, methanogens, methylated compounds, methylotrophs

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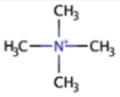
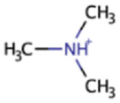
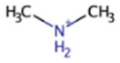
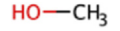
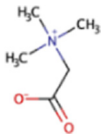
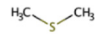
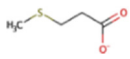
INTRODUCTION

Methanogenesis (the production of methane gas [CH_4] as a result of energy conservation) is an ancient microbial metabolism that likely played an important role in the evolution of life on Earth (1–3). Methanogenesis occurs as the final step in decomposition of organic matter in anaerobic environments and contributes most of the biotically produced methane, which makes up 70 to 90% of the methane produced on Earth today (the remainder is produced abiotically) (4–7). Today, methane (and consequently methanogenesis) is the second largest contributor to the greenhouse gas effect causing global climate change but at the same time is an important source of energy for human societies as the principal ingredient of natural gas (8, 9). Atmospheric methane concentrations have increased by about 2.5-fold from ~729 ppb in 1750 to ~1,857 ppb in 2018, which is the highest level reached in the past 800,000 years (7). The resulting radiative forcing of this increase is linked to an increase in temperature of 0.5°C when comparing 1850 to 1900 levels to 2010 to 2019 levels (8).

We have a keen interest in understanding the methane cycle from both basic and applied perspectives. Studies of the fundamental biology involved in methanogenesis are yielding new insights into anaerobic metabolism, the function of many poorly understood archaeal and extremophilic taxa, and the evolution of life on Earth as well as discoveries of taxa that can be useful for bioremediation efforts or for improving human health (10–12). We also need an improved and mechanistic understanding of the methane cycle to inform modeling efforts to more accurately understand, predict, and mitigate climate change. While great progress has been made and continues to be made on reconciling global methane sources and sinks, there are still many uncertainties (7, 13, 14). In particular, anthropogenic distortion of the natural methane cycle is a major contributor to climate change, yet new science-based technologies, such as animal feed additives (15) and rice paddy management practices (16), have the potential to reduce emissions. Furthermore, many questions remain regarding positive and negative feedbacks in the climate system, including feedbacks involving methane as a central player (17). For example, will Arctic warming and thawing permafrost contribute to increased methane fluxes? How will increased salinization from sea level rise and/or droughts affect methane fluxes from coastal wetlands? The answers to these questions depend on knowledge of the underlying microbiological processes and how different pathways of methane production will be affected. Another application for methanogenesis research is to study sources of natural gas, a key source of energy for human society. Natural gas sources are diverse, including hydrates, shales, deep aquifers, and coalbeds (9), and contain a substantial amount of biogenic methane from methanogens, including methyl-based methanogens in addition to some thermogenic methane (18, 19). Finally, anaerobic digesters of biomass, manure, or sludge from sewage treatment plants to produce methane to burn as energy rely on microorganisms to convert those products to methane (20). Thus, these two forms of energy production rely on methanogens, and a better understanding of methanogens can help us predict energy sources and produce a greater quantity of energy more efficiently.

There are three major types of methanogenesis: acetoclastic, hydrogenotrophic, and methyl-based methanogenesis (including methyl dismutation and methyl reduction). The relative contributions of these three broad categories vary among ecosystems and are influenced by environmental conditions such as temperature, the extent to which organic matter is degraded, and the other fermentation processes at work (21). There is also a recently described alternative form of methanogenesis from methylated compounds known as methoxydotrophic methanogenesis, whereby aromatic compounds are demethoxylated in a process suggested to be performed by both methanogens and nonmethanogens (22, 23). Furthermore, there are several pathways that yield methane that are not performed by methanogens, including aerobic methylphosphonate degradation (24), aerobic aspartate aminotransferase (25), cyanobacterial photosynthetic (26), and nitrogen fixation (27) pathways as well as proposed plant, animal, and fungal pathways (28, 29). These alternative pathways have been only recently

TABLE 1 Methyl-based methanogenic substrates

Name[a]	Abbreviation	Formula	Structure[b]	CH_3-Dism.	CH_3-Red.	Environments[c]	Genes[d]
Tetramethylammonium	QMA	$(CH_3)_4N$		X		Marine sediment, industrial wastewater	*mtqBC*[e], *mtqA*[e]
Trimethylamine	TMA	$(CH_3)_3N$		X	X	Marine sediment, hypersaline sediment, gut	*mttBC*, *mtbA*
Dimethylamine	DMA	$(CH_3)_2NH$		X	X	Marine sediment, hypersaline sediment, gut	*mtbBC*, *mtbA*
Monomethylamine	MMA	CH_3NH_2		X	X	Marine sediment, hypersaline sediment, gut	*mtmBC*, *mtbA*
Methanol	MeOH	CH_3OH		X	X	Marine sediment, freshwater sediment	*mtaBC*, *mtaA*
Glycine betaine	GB	$C_5H_{11}NO_2$		X		Marine sediment, hypersaline sediment	*mtgBC*, *mtgA*
Dimethyl sulfide	DMS	$(CH_3)_2S$		X		Marine sediment, hypersaline sediment	*mtsAB*, *mtpC*, *mtsDEF*
Methanethiol	MT	CH_3SH		X		Marine sediment, hypersaline sediment, freshwater sediment	*mtsAB*, *mtpC*, *mtsF*
Methylthiopropanoate	MMPA	$C_5H_{10}O_2S$		X		Marine sediment, hypersaline sediment	*mtpP*, *mtsA*, *mtpA*, *mtpCAP*

[a]Substrate names, abbreviations, formulas, structures, use in methyl dismutation (CH_3-Dism.), use in methyl reduction (CH_3-Red.), environments in which the substrates are present, and genes involved in the demethylation and methyl transfer to coenzyme M are shown.
[b]Structure diagrams from ModelSEED.
[c]Marine sediments here include ocean sediments as well as coastal and estuarine sediments, which are influenced by seawater.
[d]Pathway-specific genes. All pathways would additionally need *mcrABG*. Some pathways would additionally require the Wood-Ljungdahl methyl branch and hydrogenases and membrane-bound proteins (Fig. 2).
[e]Hypothesized but only demonstrated by one study.

described and are still poorly understood, both in terms of the biochemistry involved and the overall contribution to the global methane budget. Archaeal methanogenesis has been reviewed elsewhere (4, 20, 21, 30–33), but generally, more focus has been given to acetoclastic and hydrogenotrophic methanogenesis. Here, we specifically review methyl-based methanogenesis, as recent work has increasingly pointed to it as an important source of methane in a variety of environments. Methyl-based methanogenesis includes methanogenesis from the following methylated compounds: tetramethylammonium, trimethylamine (TMA), dimethylamine (DMA), monomethylamine (MMA), methanol (MeOH), glycine betaine (GB), dimethylsulfide (DMS), methanethiol (MT), and methylthiopropanoate (MMPA) (Table 1). We synthesize information from the literature on the environments in which methyl-based methanogenesis is prevalent, review methods for determining the source of methane, integrate and summarize biochemical information from the literature and the KEGG (34), BioCyc (35), and ModelSeed (36) databases for each methylated methanogenic substrate, and analyze the distribution and abundances of methyl-based methanogenesis genes and taxa using publicly available genomes and metagenomes in the Joint Genome Institute's Integrated Microbial Genomes and Microbiomes (IMG/M) database (37).

CONDITIONS FAVORABLE FOR METHYL-BASED METHANOGENESIS

To understand when and where methyl-based methanogenesis represents an important microbial metabolic strategy, we must consider both abiotic conditions and biotic

interactions. Important abiotic factors are substrate availability and any other environmental filters on the methanogenic archaea, such as temperature, pH, salinity, carbon availability, and nutrient concentrations (38). Biotic interactions include positive syntrophic associations and negative competitive interactions for the same limited resources. Methyl-based methanogenesis is performed by a variety of archaeal genera and species across multiple phyla, including *Euryarchaeota*, *Halobacteriota*, *Thermoplasmatota*, and potentially *Crenarchaeota* (suggested by genomic data but not confirmed experimentally) (39, 40). Environments that favor these organisms will also favor methyl-based methanogenesis.

As the energy yield of methanogenesis is typically low at only −33 to −131 $\Delta G°$ kJ/mol CH_4 depending on the pathway (but could also reach a maximum of −241 $\Delta G°$ kJ/mol CH_4 with glycine betaine), methanogens can be easily outcompeted by other organisms in most conditions (30). More specifically, the energy yield of the acetoclastic pathway is −33 to −36 $\Delta G°$ kJ/mol CH_4, that of the hydrogenotrophic pathway is −37 to −131 $\Delta G°$ kJ/mol CH_4 (depending on whether the electron donor is hydrogen, ethanol, or isopropanol and assuming abundant electron donors), that of the methyl dismutation pathway is −31 to −241 $\Delta G°$ kJ/mol CH_4 (depending on the substrate), and that of the methyl-reducing pathway with methanol is −100 to −113 $\Delta G°$ kJ/mol CH_4 (depending on whether hydrogen or ethanol is used as the electron donor) (30, 33). In fact, it has been proposed that methanogenesis occurs only when other more energetically favorable electron acceptors, such as nitrate (NO_3^-), iron (Fe^{3+}), manganese (Mn^{4+}), and sulfate (SO_4^{2-}), have been depleted (41). For each of the aforementioned electron acceptors, experimental manipulations have shown decreased methane production when those molecules were added (42, 43). One key line of work on methanogenesis has demonstrated that sulfate reducers can outcompete methanogens when sulfate concentrations are high. Importantly, this only applies to the "competitive substrates" acetate and hydrogen, which are also used by sulfate reducers (42, 44). In key early experiments, hydrogenotrophic and acetoclastic methanogenesis were inhibited by sulfate-reducing bacteria, while methyl-based methanogens avoided this competition and remained active even under high sulfate conditions (44–50). More recent studies have built on this work by pointing to the prevalence of methyl-based methanogens and substrates in the sulfate-reducing zone in the upper layer of marine sediments (51–59). While many studies tend to label all methylated methanogenic substrates as "noncompetitive" with sulfate reducers, it is important to note that methanol is used by some sulfate reducers just like H_2 and acetate (60). Some studies have still shown methanol-driven increases in methanogenesis concurrent with sulfate reduction (55, 58), but it is also possible that there could be competition between sulfate reducers and methanogens for methanol (61).

Methyl-based methanogenesis can be the dominant methanogenesis pathway in anaerobic ocean environments (e.g., sediments and hydrothermal vents), coastal environments (e.g., seagrass meadows and coastal wetlands), other environments with high salt and/or sulfate concentrations (e.g., solar salterns, hypersaline lakes, and microbial mats) (30, 51–59, 61–68), and even insect guts (69). In some cases, the high salt/high sulfate conditions go hand in hand, and the prevalence of the methyl-based pathway is likely due to other methanogens being outcompeted by sulfate reducers. In other cases where hypersaline environments have low sulfate concentrations or low sulfate reducer populations (70), the prevalence of methyl-based methanogenesis is not due to other methanogens being outcompeted by sulfate reducers but to the environmental conditions not being favorable for survival by other methanogens. This is because some methylotrophic methanogens such as *Methanolobus*, *Methanosalsum*, *Methanohalophilus*, *Methanohalobium*, and a potential new *Methanosarcinaceae* genus identified by metagenomics, are halotolerant or halophilic organisms; while they group phylogenetically with other methanogens, they contain the biochemical mechanisms necessary to cope with high salt via production of compatible solutes or by having an acidic proteome (39, 62, 71). Methyl-based methanogens are also favored in saline environments because the compatible solute glycine betaine produced by halophilic

bacteria is fermented to TMA or used directly, which fuels methyl-based methanogens. Another potential reason why these methyl-based methanogenic taxa persist in hypersaline environments while other types of methanogens do not is that the relative energy yield of the methyl-based pathway is higher, which enables them to divert more energy to compatible solute production (72).

Such results have implications for predicting the changes in methane fluxes that will occur as many coastal and inland ecosystems are increasingly impacted by sea level rise and seawater intrusion, decreases in freshwater inputs, or human activities (e.g., irrigation and salting roads) (73, 74) that increase salinity or sulfate or both salinity and sulfate together. Ecosystems influenced by seawater will experience increases in both salinity and sulfate. Crop irrigation with groundwater or river water can also lead to increases in both salinity and sulfate, especially in arid to semiarid regions (75, 76). The salting of roads in winter increases salinity but not sulfate, as typically NaCl rather than sulfate salts are used for this purpose. However, some human activities lead to increases in sulfate and not salinity. Commonly used nitrogen, phosphorus, and potassium fertilizers are a major source of sulfates in ecosystems affected by agricultural runoff (75, 77, 78). Additionally, fossil fuel-fired power plants are associated with increases in sulfate but not salinity due to wet and dry deposition of sulfur emissions to the atmosphere (79). This source of sulfate has decreased in the United States and Europe (80) but still contributes a substantial amount of sulfate in some parts of the world (81).

METHODS FOR ASSESSING METHYL-BASED METHANOGENESIS

There are several different ways in which researchers can shed light on the relative contributions of different pathways, including but not limited to methyl-based pathways, to methanogenesis. These can be broadly grouped into four categories: isotope-based methods, geochemical measurements, nucleic acid sequencing, and substrate addition experiments. Each category has its own set of advantages and disadvantages (Table 2). The most convincing and conclusive claims about dominant methane sources will use a combination of these methods, as has been done recently by several studies on methyl-based methanogenesis (59, 64, 65).

Isotope-Based Methods

Heavy isotopes are a useful tool for tracking the flow of elements through biological systems. In particular, to study methanogenic pathways, heavy isotopes of carbon and/or hydrogen can be used. Due to the activities of enzymes and their preference for certain isotopes, products of enzymatic reactions will have a different isotopic makeup than the background in the environment.

Isotopic profiling to determine methane sources could involve one of three methods: ^{13}C and/or ^{2}H fractionation of methane and substrates, ^{13}C fractionation of the biomass or lipids of the methanogens, or heavy isotope labeling. Isotopic profiling methods can be performed on environmental samples or combined with substrate addition experiments in the laboratory on microcosms or isolates. The most common isotopic method is examination of the natural abundance of ^{13}C and/or ^{2}H in methane from a particular environment; this method has been used since the late 1950s (82, 83) and can be used to distinguish between biotic and abiotic sources of methane and different pathways of biotic and abiotic methanogenesis (5). Early observations of the differences between the $\delta^{13}C$ and $\delta^{2}H$ values of methane in marine and freshwater sediments suggested that different substrates might yield different isotopic profiles and led to subsequent experimental work with isolates to match signatures to substrates (82). Isotopic profiling of methane is recognized as an important method for understanding sources of methane at regional to global scales (13).

Carbon fractionation values are more frequently used than hydrogen fractionation values and have been specifically determined for many methanogenic substrates (84). For example, the aerobic bacterial methylphosphonate degradation pathway that produces methane as a byproduct of phosphorus acquisition yields methane molecules with only minor depletion in ^{13}C (mean = $-1.3‰$) (85), while for anaerobic methanogenesis, ^{13}C depletion values range from $-9‰$ to $-95‰$ (Table 3). Within the anaerobic

TABLE 2 Summary of the advantages and disadvantages of different methods to determine the importance of different methanogenesis pathways

Method	Advantages	Disadvantages
Isotope-based methods	Can clearly distinguish some pathways based on natural ^{13}C abundance	Can be ambiguous, as some substrates have overlapping signatures
	Can clearly track carbon from substrate to product with ^{14}C label	Can be influenced by other biochemical processes/pathways such as methanotrophy that affect natural ^{13}C abundances
		Can be ambiguous if different CH_4 pools in the environment mix physically before measurement
Substrate quantification	Identifies which substrates are present	Can be difficult for some substrates and require specialized analytical methods
	Quantifies concentration of substrate to help interpret its relevance	Measures substrate pools rather than production and consumption rates
Nucleic acid sequencing	Enables high-throughput processing of many samples	Can be affected by organisms and genes that are present but not active or expressed (DNA)
	Provides a deeper understanding of the ecology and taxonomy	Can be affected by relic DNA
	Facilitates comparisons to databases and other studies	Can be difficult for RNA, which is easily degraded
	Can assess which genes are actively expressed (RNA)	
	Provides a clear relationship between substrate concentration and CH_4 produced	
Substrate addition experiments	Enable manipulating other environmental variables of interest	Are performed under laboratory conditions that cannot completely mimic field conditions
	Can be performed on microcosms or isolates	Test potential rates of methanogenesis, not the actual field rates
	Can utilize inhibitors to help indicate importance of a pathway	
	Can be combined with isotope-based methods to clearly link substrate to methane	
	Can be combined with microscopy-based methods to show the spatial arrangement of microorganisms	

methanogenesis pathways, there is also substantial variation in fractionation values, distinguishing hydrogenotrophic, acetoclastic, and methyl-based methanogenesis (84). Notably, there are still many methylated substrates that have not been analyzed for ^{13}C depletion, which is an area for future work (Table 3). ^{2}H fractionation studies are less common than those on ^{13}C, but there is some evidence of differences in ^{2}H fractionation between CO_2-derived methane and acetate-derived methane (86, 87). Although isotopic methods can be clear and definitive in some cases (i.e., when there is no overlap in values), or at least enable researchers to eliminate potential pathways (i.e., when values are out of the range of at least one substrate), there are also some drawbacks, including nondefinitive cases in the overlapping ranges of substrates. The carbon isotope fractionation also depends on other variables, most notably temperature but also growth phase, hydrogen supply, substrate concentration, methanogen species, effects of other enzymes involved in carbon acquisition, and methanotrophy, as methanotrophs preferentially consume ^{12}C-CH_4 over ^{13}C-CH_4 (86, 88–90). Environmental measurements of isotope fractionation in methane can also be impacted by the mixing of multiple gas sources (91).

A related but less frequently used stable isotope method involves looking at the carbon fractionation not of the methane itself, but of the biomass or lipids of the microorganisms hypothesized to be performing the methanogenesis. Archaeal lipids are composed of an isoprenoid chain, an ether linkage, and a glycerol-1-phosphate backbone, while bacterial phospholipids consist of a fatty acid chain, an ester linkage, and a glycerol-3-phosphate backbone. The archaeal domain contains substantial variation in the length, composition, and configuration of the isoprenoid chain and modifications to the polar head groups (92). Differences in lipid and biomass ^{13}C fractionation are expected among different methanogens due to differences in their carbon assimilation pathways (88). This method was developed in the versatile *Methanosarcina barkeri* species but was found to not be as conclusive as studying ^{13}C fractionation of the

TABLE 3 Summary of isotope delta and fractionation value ranges from the literature

Pathway	Substrate	$\delta^{13}C_{substrate}$[a]	$\delta^{2}H_{CH4}$[a]	$\delta^{13}C_{CH4}$[a]	Exptl $\varepsilon_{CH4\text{-}substrate}$[a]	Refs
Bacterial	MPn	−100.7 to −95.86[b]		−39	−5.11 to 2.91	85, 104
Methyl based	QMA					
	TMA	−36.9 to −29.5[b]		−97 to −83	−83 to −39	84, 86, 88, 93, 225
	DMA					
	MMA					
	MeOH	−46.2 to −37.7[b]		−129.6 to −46.4	−94 to −68	84–86, 88
	GB					
	DMS	−24.4 to −18.6[c]			−54 to −44	86, 225
	MT					
	MMPA					
Hydrogenotrophic	CO_2	−49.7[c] to 16.7[b]	−266 to −153	−108 to −60	−95 to −23	82, 84–87, 94, 226
Acetoclastic	Acetate	−36.4 to −22.1[b]	−396 to −266	−70 to −27	−35 to −6	84, 85, 87, 227

[a]The $\delta^{13}C$ of the substrates (from the environment or experimental reagents), the $\delta^{2}H$ of methane, the $\delta^{13}C$ of methane, and the ^{13}C fractionation factor (methane-substrate) ε, calculated from experiments as described elsewhere (84) and is commonly reported in the literature, are shown. All numbers reflect per mille (‰) values. $\delta^{2}H$ is calculated using the Vienna Standard Mean Ocean Water standard, while $\delta^{13}C$ is calculated using the Vienna Pee Dee Belemnite standard. Blank cells indicate no known reports in the literature as of October 2022.
[b]Reagent used in experiments.
[c]From natural environments.

methane molecule itself, as the fractionation of methane was more pronounced (88). Even so, it can be used in concert with other methods, and several studies have reported the $\delta^{13}C$ of biomass or archaeal lipids (59, 93, 94), although this likely includes nonmethanogens and does not distinguish among organisms using similar carbon assimilation pathways but different energy conservation pathways. Lipids were enriched in ^{13}C relative to the substrate and bulk biomass only when methanogens were grown with added acetate and not H_2/CO_2, methanol, or trimethylamine under conditions of limited substrate availability (88).

A third isotopic method is to use a ^{14}C tracer to track carbon from a labeled substrate to the methane molecule; in such experiments, a suspected methane-producing environmental sample (e.g., sediment) is brought back to the lab, known quantities of ^{14}C-labeled substrates are added, and ^{14}C-CH_4 is quantified. An advantage of this method is that it enables quantification of the contribution of different pathways to the overall methane flux. The contributions of acetate, CO_2, trimethylamine, monomethylamine, methanol, and glycine betaine have been studied by this method while, to our knowledge, to date, quaternary methylammonium or tetramethylammonium, dimethylamine, dimethylsulfide, methanethiol, and methylthiopropanoate have not. Such experiments have been done using a variety of sediments, including those from freshwater lakes, the deep ocean, coastal salt marshes, and rice fields as well as rumen fluid and feed (59, 61, 63, 65, 95–100).

Finally, stable isotope probing (SIP) can be used to assess microbial function in environmental samples by using isotopic probes to link identity and function. A wide variety of SIP techniques, including DNA-SIP, RNA-SIP, protein-SIP, phospholipid fatty acid analysis-SIP (PLFA-SIP), and metabolite-SIP have been developed to focus on different aspects of biology and biochemistry (101), and new methods are still actively being developed (e.g., flow-SIP) to minimize uncertainties arising from microbial cross-feeding (102). SIP is often combined with secondary ion mass spectrometry (SIMS) for isotopic and elemental analysis and with *in situ* hybridization methods, such as fluorescence *in situ* hybridization (FISH), to link microbial identity to isotopic enrichment.

Substrate Quantification

A seemingly simple way to test if a pathway is relevant in an environment is to directly measure the concentration of the substrate. However, this can be challenging in terms of both measurement and interpretation, as such measurements only quantify pools and not active production or uptake. Many of the methylated methanogenic substrates as well as methylated methane precursors in bacterial methane production pathways are notoriously hard to identify and detect, can be strongly sorbed to sediments, and require specific

extraction and spectrometry methods (63, 103). For example, clearly identifying a pool of methylphosphonate required ^{31}P nuclear magnetic resonance spectroscopy (104). In addition to the substrate concentrations themselves informing likely methanogenesis pathways, a second step is to take those concentrations and make thermodynamic calculations that can be used in concert with the substrate concentration data and other environmental parameters to assess the likelihood of the occurrence of a pathway. Assuming that -15 kJ mol substrate^{-1} energy must be available to be usefully harnessed and support basic biochemical integrity and function (105, 106), if certain pathways fall below this cutoff, it is likely that they do not occur (59). Such conclusions, however, depend on accurately measuring the substrate concentrations, which can be challenging (see above).

Nucleic Acid Sequencing

DNA and RNA sequencing methods can be used to identify relevant pathways via the presence, abundance, or expression of key genes and taxa. This works because many of the pathways have genes, enzymes, and proteins isolated, purified, and described, and many taxa are known methanogens with available cultures and experimental evidence. Thus, metagenomic or genomic data (or better metatranscriptomic or transcriptomic data) can show which genes are present or expressed, while 16S rRNA or *mcrA* marker gene surveys can identify potentially methanogenic archaeal taxa, many of which have been cultured and have known substrates that they can or cannot metabolize. The 16S or *mcrA* gene is used to identify organisms in the sample; assessment of methanogenesis capabilities would be based on prior experiments done with the identified taxa. The *mcrA* gene is also present in anaerobic methanotrophic (ANME) archaea so this gene in itself is not necessarily a marker of methanogens, although it is commonly used as such due to its presence in all methanogens (i.e., all methanogens have *mcrA* but not all *mcrA*-containing organisms are methanogens). Sequencing data can be an effective method for broad surveys and to answer other biological, ecological, and functional questions at the same time, and there are extensive databases from many environments that can be queried for genes or taxa of interest.

But sequencing data also have disadvantages. For example, metagenomic data could provide support for a pathway but not conclusive evidence, as it would not show if the genes were actively expressed. This can become especially problematic considering that some taxa are versatile methanogens and may harbor genes for multiple methanogenesis pathways but only be metabolizing a specific substrate under the conditions of the particular sample. Likewise, using taxonomy could also be inconclusive depending on the taxa involved. For example, members of the family *Methanosarcinaceae* can perform all four major archaeal methanogenic pathways, so an operational taxonomic unit (OTU) or amplicon sequence variant (ASV) assigned only to that family would be inconclusive in terms of identifying the methanogenic pathway, while identifying a particular species that has been verified in culture to use only specific substrates would constitute much stronger evidence. Of the sequence-based methods, generating metatranscriptomic or transcriptomic data under methanogenic conditions would be the most conclusive as it would show the actively expressed genes. Yet, even the conclusions from metatranscriptomic or transcriptomic data can be limited when protein or activity quantification are lacking.

Substrate or Inhibitor Addition Experiments

A final method for distinguishing methane pathways is to perform substrate addition experiments, in which known concentrations of substrates are added to microcosms or cultures, and methane production is measured over time and compared to controls. The benefits of this method are that the production of methane can be validated, the rate of production can be quantified, and the methane flux from different potential substrates can be compared. This is commonly done for pure methanogen cultures to characterize the capabilities of specific isolates (107) but is also done at the microcosm level (70).

In addition to testing methane production from different substrates, chemical inhibitors can also be added to specifically inhibit certain pathways and thereby strengthen conclusions about other pathways. 2-Bromoethanesulfonic acid or chloroform is used

to inhibit methanogens and confirm biogenic methane origin (108–110). Sodium molybdate inhibits sulfate reduction, and fluoroacetate inhibits acetate metabolism (100). Methyl fluoride specifically inhibits acetoclastic methanogenesis (84, 99).

Lastly, the substrate addition method can be combined with stable and radioactive isotope methods by performing an isotopically labeled substrate addition (see above section). This would add additional clarity to the interpretation of the results, as potential contributions of nonadditive substrates to net methane production in unlabeled enrichments could confound those results. Substrate addition experiments could also be combined with recently developed microscopy-based methods such as the combination of FISH and biorthogonal noncanonical amino acid tagging (BONCAT), which has been demonstrated to be a powerful tool for assessing microbial function as it enables visualization of newly made proteins (111, 112). BONCAT-FISH has been used to study methane-oxidizing microbial consortia (113) and could be used to identify different methanogenesis pathways by linking microbial taxonomy and translational activity in environmental samples with different substrate additions. The downside of substrate addition experiments is that laboratory conditions do not mimic field conditions, and, as such, the experiment provides information only on the capacity of the microorganisms in a sample to produce methane from a substrate and not the field production rates or even that they actively produce methane in the field.

In summary, there are four main types of methods for determining methane sources (Table 2), and the strongest cases about the relative importance of different pathways can be made by combining multiple lines of evidence, as has been done by several studies on methylotrophic methanogenesis (59, 64, 65).

SUBSTRATE SOURCES AND BIOCHEMISTRY

As the methyl dismutation and methyl-reducing pathways of methanogenesis both involve the demethylation of methylated compounds, a variety of compounds can serve as the primary substrates and carbon sources for methanogenesis. Known methylated substrates include QMA, TMA, DMA, MMA, methanol, methanethiol, MMPA, DMS, dimethylsulfoniopropionate (DMSP), glycine betaine (GB), choline, methionine, and dimethylethanolamine (DMEA). An overview of the biochemical reactions and genes involved in these pathways is presented in Fig. 1, with the exception of choline, methionine, and DMEA, which have demonstrated methane-producing potential but may only function as precursors, as the mechanisms have not been fully characterized (114–116).

Generally, methanogenesis from methylated compounds using the methyl dismutation pathway follows a two-step process in which the substrate is demethylated, with the methyl group first transferred to a substrate-specific corrinoid protein and then to coenzyme M (CoM) to produce methyl-CoM (35, 117). Methyl-CoM then reacts with coenzyme B (CoB) to yield methane in the reaction catalyzed by methyl-CoM reductase (encoded by *mcrABG*). This is the same ultimate methane-forming reaction that occurs in the other methanogenesis pathways. Methanogens performing methyl dismutation reduce 75% of methyl groups using electrons obtained by oxidizing 25% of the methyl groups to CO_2 with the methyl branch of the Wood-Ljungdahl pathway (33). Energy conservation happens during membrane-bound electron transport (Fig. 2).

Even though methyl-based methanogenesis differs significantly from hydrogenotrophic methanogenesis, the methyl reduction pathway is dependent on hydrogen (or formate or ethanol) for reducing electrons (33, 39, 118, 119). Genes for the methyl reduction pathway have been found in the *Korarchaeota* phylum (120), the *Methanonatronarchaeia* class (121), the *Methanomassiliicoccales* order (39, 122), the *Methanosphaera* genus (*Methanobacteriaceae*), and the *Methanosarcinales* order (69), the latter of which also contains members performing methyl dismutation methanogenesis without hydrogen as well as acetoclastic and hydrogenotrophic methanogenesis. Taxa performing methyl-reducing methanogenesis lack the methyl branch of the Wood-Ljungdahl pathway and instead use H_2 (or formate or ethanol) as the electron donor. Genomes of organisms that perform the methyl-reducing pathway may completely or partially lack the genes of the methyl branch

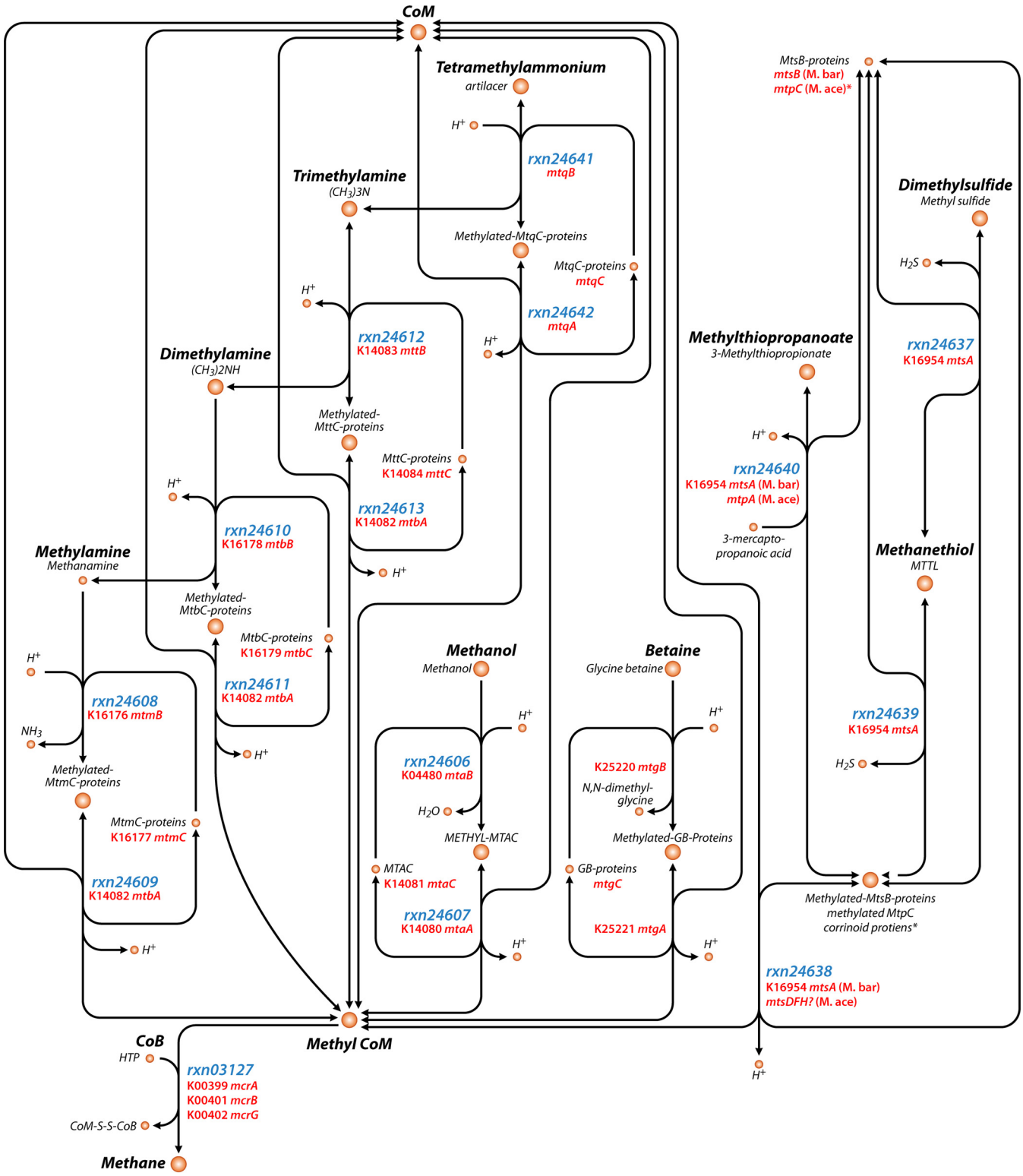


FIG 1 Simplified map of methyl dismutation methanogenesis pathways showing ModelSeed compound names and reaction IDs as well as KEGG Orthology (KO) identifiers (when available) for the genes involved in the reactions. This map focuses on the methyl dismutation steps and does not show all of the proteins and pathways for energy conservation. The map was made with Escher based on MetaCyc, KEGG, and ModelSeed annotations and only includes those pathways in at least one of those databases as of the time of writing. Note that some gene names are described but do not yet have KO assignments. Also note that some reactions are not in ModelSeed (e.g., for betaine). The asterisk (*) indicates that methanogenesis from methylthiopropanoate differs in *Ms. barkeri* ("*M. bar*") and *Ms. acetivorans* ("*M. ace*"), with *Ms. acetivorans* using MtsC proteins instead of MtsB proteins. A question mark (?) denotes hypothesized genes in need of further confirmation.

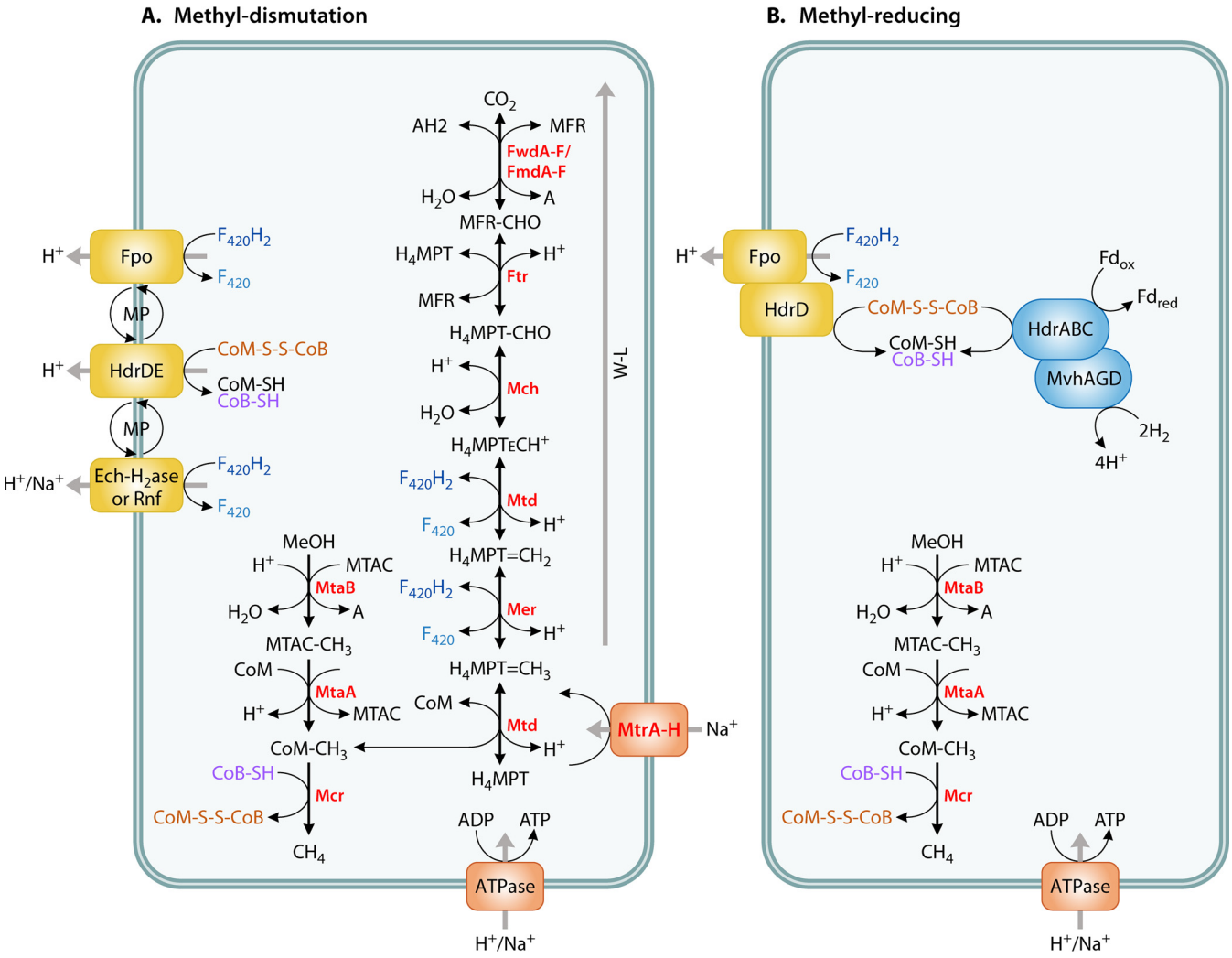


FIG 2 (A and B) Diagram of methyl dismutation (A) versus methyl-reducing (B) pathways from methanol (adapted from Kurth et al. [33]). The pathway in *Methanomassiliicoccus luminyensis* is shown in B. Note that methyl-reducing taxa may or may not contain the genes for the methyl branch of the Wood-Ljungdahl pathway, and activity/growth experiments are recommended to confirm the methyl-reducing pathway; WL, Wood-Ljungdahl; MFR, methanofuran; H_4MPT, tetrahydromethanopterin; Fwd/Fmd, formylmethanofuran dehydrogenase; Ftr, formylmethanofurantetrahydromethanopterin formyltransferase; Mch, methenyltetrahydromethanopterin cyclohydrolase; Mtd, methylenetetrahydromethanopterin dehydrogenase; Mer, 5,10-methylenetetrahydromethanopterin; Mtr, tetrahydromethanopterin *S*-methyltransferase; Mta = methyl-coenzyme M methyltransferase (methanol/glycine betaine-specific corrinoid protein); Mcr, methyl-coenzyme M reductase; Fpo, $F_{420}H_2$ dehydrogenase; Hdr, membrane-bound heterodisulfide reductase; Ech-H_2ase, energy-conserving hydrogenase; Rnf, Na^+-translocating ferredoxin:NAD^+ oxidoreductase complex; MP, methanophenazine; AH2, hydrogen donor; A, hydrogen acceptor; H_4MPT, tetrahydromethanopterin; MTAC, Col-corrinoid-Fe-S-proteins; CoM, coenzyme M; CoB, coenzyme B; CoM-S-S-Cob, coenzyme B-coenzyme M heterodisulfide; F_{420}, coenzyme F_{420}; Fd, ferredoxin, a two electron carrier; red, reduced; ox, oxidized. Na^+/H^+ translation stoichiometry is not represented in the figure.

of the Wood-Ljungdahl pathway (123, 124). This is a key difference between the methyl dismutation and methyl-reducing pathways (Fig. 2). The methyl-reducing pathway may involve one of several different systems of membrane-bound electron transport depending on the species (Fig. S1 in the supplemental material) (33). The methyl-reducing pathway can be confirmed by activity and growth experiments that demonstrate a lack of methane production in the absence of H_2 or other electron donors.

There are key differences in how energy is conserved among the hydrogenotrophic, acetoclastic, methyl dismutation, and methyl reduction methanogenesis pathways. In hydrogenotrophic methanogenesis, energy conservation happens exclusively during a methyl transfer reaction involving the membrane-bound Mtr methyltransferase, which transports sodium ions across the membrane, building up a sodium motive force that can be used by ATP synthase (125). In acetoclastic methanogenesis, energy conservation happens at the Mtr step as well as in a membrane-bound ferredoxin-heterodisulfide

electron transport chain (33, 126). In contrast to hydrogenotrophic and acetoclastic methanogenesis, energy conservation in methyl-based methanogenesis does not occur at the Mtr step because it operates in reverse (Fig. 2). In methanogenesis via methyl dismutation, energy conservation occurs during membrane-bound electron transport involving ferredoxin, heterodisulfide, and methanophenazine, although there are differences among taxa, such as the model organisms *Methanosarcina acetivorans* and *Methanosarcina barkeri*. The hydrogenase-proficient *Ms. barkeri* uses the Frh, Ech, and Vht hydrogenases, while the hydrogenase-deficient *Ms. acetivorans* uses the Rnf enzyme complex and the dehydrogenase Fpo (125). In methanogenesis via methyl reduction, energy conservation occurs during membrane-bound electron transport involving ferredoxin and heterodisulfide; whether methanophenazine is used or not and which protein is used varies among taxa (Fig. 2; Fig. S1) (33, 126). Furthermore, in certain instances of methyl reduction, energy conservation may also occur with a sodium motive force, such as that generated by EhbA-Q in *Methanosphaera stadtmanae* (33, 126, 127).

In the following sections, we will provide an overview of the sources and sinks of each substrate, including biosynthesis and nonmethanogenic degradation pathways that both affect the available pools of the substrates and then describe the methanogenesis pathway from each substrate (Fig. 1).

Tetramethylammonium

Tetramethylammonium or quaternary methylammonium (QMA) is commonly present in a variety of marine animals, including the phyla *Cnidaria*, *Mollusca*, and *Bryozoa* (128). QMA often forms salts of chloride or hydroxide, the latter of which is toxic and is a constituent of industrial wastewater (129). QMA hydroxide is produced for several manufacturing industries for uses that include electronic chips, semiconductors, liquid crystal displays, and light-emitting diodes. QMA is also abundant in wastewater from these industries, which has sparked interest in QMA-degrading microbes (12). The hypothesized methanogenesis pathway from QMA begins when it reacts with H^+ and a Co(I) QMA-specific corrinoid protein (MtqC) to form TMA and a methyl-Co(III) QMA-specific corrinoid protein in a reaction catalyzed by a methyltransferase enzyme encoded by *mtqB*. Next, a second methyltransferase enzyme encoded by *mtqA* catalyzes the reaction to methylate coenzyme M with the methyl-Co(III) QMA-specific corrinoid protein, yielding methyl-CoM, H^+, and the Co(I) QMA-specific corrinoid protein to be recycled back into the first reaction (130) (Fig. 1).

Trimethylamine

Trimethylamine (TMA) is produced via the degradation of several precursors, including QMA (see above), choline, glycine betaine, trimethylamine-*N*-oxide (TMAO), carnitine, and diacylglyceryl hydroxymethyl *N*,*N*,*N*-trimethyl-β-alanine (DGTA) (131). TMA appears to be released by the cordgrass *Spartina alterniflora* in salt marshes, is present in benthic animals and phytoplankton in marine ecosystems where it can contribute to methanogenesis (132), and is also present in many plant and fungal species in marine and terrestrial ecosystems (133). TMA is also present in ruminants as a product of betaine degradation (96). In humans, TMA is produced from L-carnitine and choline by gut microflora, is excreted in urine, and has been associated with disease, effects on the circulatory system, and other negative effects (134–136). Because of this, there is interest in the role of methanogenic archaea in the gut to potentially remove TMA (and DMA and MMA) locally (11).

TMA has gained a lot of attention as an important methylated methanogenic substrate due to the ubiquity and abundance of the aforementioned precursors. Choline, glycine betaine, and TMAO are produced abundantly and ubiquitously by both prokaryotic and eukaryotic organisms, particularly in marine environments (93, 137), and genes encoding proteins involved in their degradation to TMA are similarly ubiquitous and abundant (138, 139). TMA dominated the exchangeable pool of amines in a salt marsh (132). Open water and sediment porewater concentrations of TMA range from 0.05 nM to 50 μM (131). In hypersaline environments, TMA can be formed primarily by

breaking down glycine betaine, which is an abundant compatible solute in those environments (137). TMA itself has also been suggested to be a compatible solute, and its concentrations vary seasonally and with salinity as a function of benthic invertebrate concentrations (140). TMA has been found to be more abundant in the solid phase than in the dissolved pool, suggesting strong adsorption to sediments, a factor that must be taken into account when considering TMA sinks (103). In shallow marine sediments, TMA and DMA were both detected while MMA was not, suggesting some differences in the cycling of these three methylated amines. In the same study, TMA and DMA concentrations increased as organic matter content increased (141).

TMA can be degraded aerobically and anaerobically via several nonmethanogenic pathways, which thereby decrease pools available for methanogenesis (131, 142–144). Aerobic marine bacteria, particularly those in the *Roseobacteria* clade, use TMA monooxygenase (encoded by the *tmm* gene) to use TMA as a carbon and nitrogen source, with some specialized methylotrophs capable of growing on TMA as their sole source of carbon and energy (145). Anaerobic denitrifying bacteria can use TMA as a carbon source for growth with nitrate (142, 146). A second anaerobic pathway is the TMA dehydrogenase pathway, which produces DMA, MMA, formaldehyde, and ammonia (131). TMA can also be used to form acetate by *Acetohalobium*, a versatile halophilic bacterium (147). In methanogenesis from TMA, TMA is first degraded to DMA in a two-step reaction where a methyl group, catalyzed by the MttB methyltransferase enzyme, is first transferred to the MttC corrinoid protein to generate H^+ and methylated MttC (148). The methyl group is then transferred from methyl-MttC to CoM to form methyl-CoM in a reaction catalyzed by a second methyltransferase enzyme (encoded by *mtbA*), which is the same enzyme and gene used for methyl-CoM production from DMA and MMA (Fig. 1, see below) (144).

An important aspect of methyl group transfer reactions is that they depend on the redox state of the corrinoid protein (e.g., highly reducing Co[I], inactive Co[II], and methylated Co[III]). The iron-sulfur RamA protein, which is often encoded near the methyltransferases in methanogen genomes, is necessary to activate the methyltransferase reactions in the TMA, DMA, and MMA pathways (149, 150). Without RamA, adventitious oxidation of the corrinoid protein to the Co(II) state would inactivate the methyltransferase reactions. RamA returns the corrinoid protein to the Co(I) state via ATP-dependent reduction (150). Recent work on this protein has demonstrated its dependence on ions such as potassium and ammonium and described the steady state kinetics of ATP dependence (151).

Dimethylamine

Environmental dimethylamine (DMA) is likely primarily produced as a product of trimethylamine degradation (see above) and degradation of trimethylamine *N*-oxide (143). It follows that DMA is ubiquitous in marine waters and sediments just as these two precursors and their precursors are. DMA is also found in human and rat guts, where it is produced from choline, lecithin, methylamine, and methionine and is excreted in urine (134). DMA can be found in plants and fungi; while it was found in only 2 of 28 marine plant species, which is much fewer than TMA (23 of 28 species), DMA was similarly widespread in many species of *Basidiomycete* fungi (133). DMA (and also MMA) increased seasonally in a salt marsh as a result of new organic matter inputs from senescing marsh grasses (103).

Besides serving as a substrate for methanogenesis, DMA can be broken down photochemically to form *N*-nitrosodimethylamine (152) as well as by aerobic marine bacteria that use DMA monooxygenase (encoded by *dmmABC* genes) to form MMA (143). Anaerobic bacteria can also use DMA dehydrogenase to produce MMA and formaldehyde (131). While denitrification involving TMA and MMA has been demonstrated, it is unclear whether denitrification involving DMA can occur or if DMA is only an intermediate in TMA-dependent denitrification (142, 146, 153). Methanogenesis from DMA follows a similar pathway as the pathway for TMA, but MtbB and MtbC proteins are involved instead of MttB and MttC proteins (Fig. 1) (144).

Monomethylamine

Monomethylamine (MMA) is produced in marine environments from microbial degradation of DMA and glycine betaine (154) (see above and below), in animal guts from sarcosine, glycine, creatine, and epinephrine (155, 156), and in terrestrial ecosystems by flowering plants and fungi (133). MMA is present in ruminant guts, where it makes up a substantial portion of the nitrogen content along with ammonia (157), and in human and rat guts, where it is produced primarily from sarcosine and oxidized to CO_2 and NH_3 (156). Human adults can excrete several milligrams of MMA per day in their urine (158). MMA is a smaller component of animal tissue than TMA, but worms may excrete MMA and contribute to MMA pools in salt marshes where methanogenesis is known to occur in sediments (132, 159).

Like TMA and DMA, aerobic marine bacteria can use MMA as a source of carbon, nitrogen, and energy, in some instances as the sole source (145). MMA can also be used as an electron donor in anaerobic denitrification (153). In the last step of the dehydrogenase pathway originating with TMA, MMA dehydrogenase is used by anaerobic and aerobic bacteria to form ammonia and formaldehyde from MMA (131). Methanogenesis from MMA follows the same general pathway as TMA and DMA except that MtmB and MtmC proteins are used (Fig. 1) (144).

Methanol

Globally, the primary source of methanol (MeOH) is production from plants and subsequent release as a volatile organic compound (160). Dissolved methanol can also be present in coastal and freshwater wetlands as a product of pectin, xylan, lignin, or aromatic acid degradation (98, 161–164). Although these precursors constitute a large fraction of plant-derived organic matter, methanol typically does not contribute as much methane flux as acetate or H_2/CO_2, perhaps only 1 to 10% (46, 165). Even in saline environments where methanol might be predicted to play a larger role, TMA appears to contribute more to methane flux (46). Methanol is also released by chemical and enzymatic methylation of methoxy groups. In marine environments, *in situ* production and external deposition from terrestrial ecosystems and the atmosphere contribute to methanol concentrations in ocean sediments ranging from 0.3 μM to 111.7 μM depending on the depth (60).

Methanol can be oxidized aerobically to CO_2 by bacteria and fungi that use it as a carbon and energy source (166). Anaerobically, methanol serves as a carbon source and electron donor for denitrification, acetogenesis, and sulfate reduction, all of which consume methanol and could lower the available methanol for methanogenesis (60, 167, 168). Methanogenesis from methanol follows a similar path as TMA, DMA, and MMA but with MtaB and MtaC proteins in the initial step (169) and MtaA instead of MtbA to transfer the methyl group from the corrinoid protein to CoM (170). Similar to RamA in the TMA, DMA, and MMA methyltransferase reactions, the RamM protein is required to reduce the corrinoid-binding proteins in the methanol pathway (149).

Glycine Betaine

Glycine betaine (GB) is an important osmolyte that can be found in high concentrations in saline to hypersaline environments, both inside and outside cells (137, 171). A wide variety of organisms, including plants, archaea, cyanobacteria, and mammals, have been reported to accumulate GB as a compatible solute (137). As it is present in plants, GB consequently is present in the diets of ruminants and other herbivores (96). Five pathways of glycine betaine biosynthesis have been described, encompassing those from choline performed by Gram-positive bacteria, Gram-negative bacteria, and plants and two pathways from glycine (via sarcosine) performed by archaea and bacteria (35). Genes for GB biosynthesis and transport have been found in hypothesized halotolerant methanogenic archaea and methylphosphonate-degrading bacteria (71, 172).

In addition to serving as a substrate for methanogenesis, GB can be degraded via three other pathways, which would consume GB and decrease pools available for methanogenesis. GB degradation forms many other metabolites, including *N*,*N*-dimethylglycine,

sarcosine, L-serine, pyruvate, and acetate (35, 173). In the ruminant gut, trimethylamine, dimethylglycine, and methionine can be produced from GB (96). Members of the sulfur-reducing archaeal genus *Halalkaliarchaeum* are able to use GB as a carbon source and electron donor for sulfur reduction (174). The pathway for methanogenesis from GB has been recently described and involves a methyltransferase enzyme (encoded by the *mtgB* gene) in a reaction between GB and H^+ to produce *N*,*N*-dimethylglycine and a methyl-Co (III) glycine betaine-specific corrinoid protein MtgC. Then, a methyltransferase enzyme encoded by *mtgA*, which is homologous to the one involved in methyl-CoM formation from methanol, is used to methylate CoM to produce methyl-CoM and H^+ and MtgC, which can be recycled back into the first reaction (175, 176). Additionally, as was the case for methanol, the RamM protein is required to reduce the bound corrinoid of MtgC (176).

Dimethylsulfide

Dimethylsulfide (DMS) is a volatile organic sulfur compound estimated to constitute up to 90% of reduced sulfur in surface seawater. DMS concentrations in seawater follow a clear seasonal pattern of low concentrations during winter and high concentrations during summer, suggesting a biological origin (177). The most likely primary source of DMS in marine ecosystems is the multifunctional compound dimethylsulfoniopropionate (DMSP), which is produced in large quantities by marine plants, algae, phytoplankton, and other organisms and may account for up to 10% of the carbon fixed by marine phytoplankton (178–180). DMSP has been described as an osmoprotectant, cryoprotectant, predator deterrent, and antioxidant (181–183). DMSP can be degraded via several different pathways that occur in both marine and freshwater environments involving bacterial and algal lyase enzymes (184). Two such cleavage pathways directly form DMS as a product, while a third forms methylthiopropanoate (MMPA; see below) (180, 185, 186). Secondary sources of DMS include methionine, methanethiol methylation, dimethyl sulfoxide reduction, and sulfonium salts other than DMSP, such as *S*-methylmethionine or trimethylsulfonium (35, 181, 187). DMS levels may increase with salinity, as a positive relationship between salinity and DMS levels was reported in algal cultures (188).

DMS can be degraded with DMS monooxygenase (encoded by *dmoAB*) to form methanethiol (MT) and formaldehyde (189), oxidized to dimethyl sulfoxide with DMS oxygenase (encoded by *dsoBCDEF*) (190), or oxidized by sulfate- and nitrate-reducing bacteria (191). Methane production from DMS, MT, and MMPA all follow similar pathways (192). A key difference between methanogenesis from DMS, MT, and MMPA and the other six substrates (described above and shown in Fig. 1) is that only one methyltransferase enzyme is present, and it catalyzes both the methylation and demethylation of the corrinoid protein. There are also some differences that have been described between *Methanosarcina barkeri* and *Methanosarcina acetivorans* (117, 192). In methanogenesis from DMS in *Ms. barkeri*, DMS, H^+, and MtsB proteins react to form methylated MtsB proteins, a reaction catalyzed by a methyltransferase encoded by *mtsA*. Then, MtsA combines CoM and the methylated MtsB proteins to form methyl-CoM, H^+, and free MtsB proteins to be recycled back into the first reaction (Fig. 1) (192, 193). It has been suggested that in *Ms. acetivorans*, MtsDFH may be used as the methyltransferase instead of MtsA, and MtsB is not needed (117, 194).

Methanethiol

MT is a volatile sulfur compound with a single methyl group (CH_3) and an active thiol (-SH) group that appears as a gas or as a labile acid. As with DMS, MT is produced via degradation of DMSP and methionine, perhaps in even greater levels than DMS (179, 187). In this pathway, which differs from the two DMS-producing cleavage pathways, MMPA is produced as an intermediate, which is transformed through three subsequent reactions to MT (180). Bacteria were shown to take up 15 to 40% of [^{35}S]DMSP sulfur. *Alphaproteobacteria* degraded DMSP to MT and rapidly incorporated it into macromolecules (195). In addition to these bacteria, algae and plants, particularly in marine environments, can also generate MT. For example, one survey found that MT was produced by

87 of the 118 herbaceous plants studied (196). MT is also produced from DMS with DMS monooxygenase or methanogenesis (see above), from H_2S via thiol transmethylation, and from MMPA (179, 181, 193). Substantial rates of MT formation have been observed in *Sphagnum* peat bogs, salt marsh sediments, and freshwater habitats. MT in the atmosphere is oxidized to dimethyl disulfide (DMDS), while in marine environments, MT could be converted to carbonyl sulfide (181). While some MT is taken up by microorganisms, some MT is also likely sorbed to dissolved organic matter (179).

The methanogenesis pathway from MT is similar to DMS. In *Ms. barkeri*, MtsB proteins and MT react to produce H_2S and methylated MtsB proteins in a reaction catalyzed by a methyltransferase enzyme encoded by *mtsA*. Subsequently, MtsA catalyzes the reaction to form methyl-CoM as above (Fig. 1) (192). In *Methanosarcina acetivorans*, MtsF may be used instead of MtsA, with no need for MtsB (117).

Methylthiopropanoate

Methylthiopropanoate (MMPA) is the third of the methylated sulfur compounds that can be produced from DMSP. MMPA can also be produced from methionine or via oxidation of 1,2-dihydroxy-3-keto-5-methylthiopentene in bacteria and plants (35, 197, 198). Besides methanogenesis, it can be demethylated into 3-mercaptopropanoic acid or cleaved into MT, acetaldehyde, and CO_2 (184).

Methanogenesis from MMPA was first shown to follow a similar path as DMS and MT in *Ms. barkeri* (192, 193). In the first reaction, MtsA methylates the MtsB proteins with MMPA and H^+, forming 3-mercaptopropanoic acid. In the second reaction, MtsA demethylates the MtsB proteins to methylate CoM. Subsequent work suggested additional mechanisms involving other MMPA-specific genes in *Ms. acetivorans* (117). In particular, the *mtpCAP* suite of genes was implicated in growth specifically with MMPA but not with DMS or MT. While not necessary under laboratory conditions, *mtpP* was suggested as a transporter for MMPA in nature. MtpC proteins were suggested to take the place of MtbB proteins, and MtpA was suggested to be the methyltransferase of the two reactions (117). It was later shown that MtpA only catalyzes the first reaction (methylation of MtpC proteins) but not the second reaction (methylation of CoM). MtsDFH were suggested to play a role, but the second methyltransferase remains unidentified (199). Also, of note for methanogenesis from MMPA in *Ms. acetivorans* is that the *msrH* gene is required for transcription of *mtpCAP* (117).

GENE AND TAXA ENVIRONMENTAL ABUNDANCES

We used the Joint Genome Institute's Integrated Microbial Genomes and Microbiomes Database (IMG/M) (37) to conduct several different analyses of the distribution of methanogenesis genes and taxa using publicly available genomes (isolate genomes and high-quality metagenome-assembled genomes [MAGs]) and metagenomes. The goal of the analysis was to assess the diversity of organisms containing some of the methyl-based methanogenesis genes, compare the abundance of certain genes involved in methyl-based, hydrogenotrophic, and acetoclastic methanogenesis across a variety of methanogenic environments, and compare the abundance of methanogen families across those same environments. The environments include landfills, sewage treatment plants, wetlands (freshwater and coastal), rice fields, ocean (sediment), human guts, ruminant (cow and sheep) guts, termite guts, hydrothermal vents (plume, sediment, seawater, microbial mat, and host-associated), and hypersaline environments. These environments are hypothesized to have different dominant methanogenic substrates (21, 30, 62). Human and livestock guts, termite guts, and hydrothermal vents are rich in H_2, while ocean, coastal wetland, and hypersaline sediments are thought to be rich in methylated compounds. Landfills, sewage treatment, freshwater wetlands, and rice fields are rich in both H_2 and acetate, although for simplicity, we will classify these to be hypothesized acetoclastic dominated because the majority of methane is produced by acetoclastic methanogenesis where acetate is abundant (21).

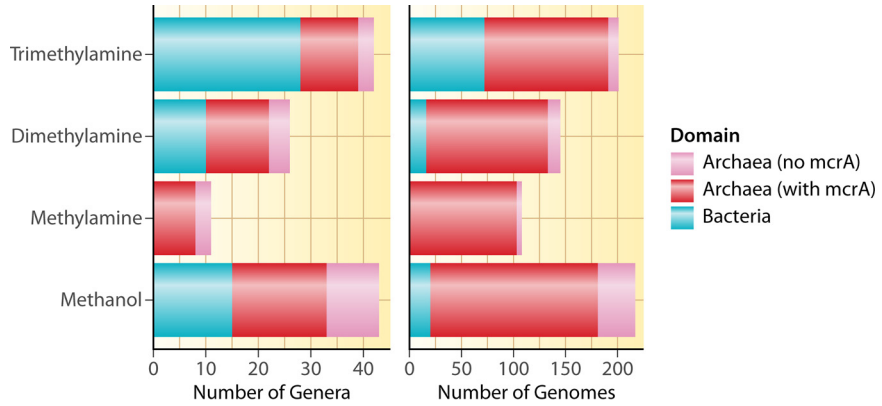


FIG 3 Number of genera and genomes of archaea and bacteria containing genes for the first step of demethylation for each of the four methylated compounds with complete KO annotations. Archaeal genomes are separated into those containing *mcrA* and those without *mcrA*; trimethylamine = *mttB* and *mttC*; dimethylamine = *mtbB* and *mtbC*; methylamine = *mtmB* and *mtmC*; methanol = *mtaB* and *mtaC*. Only four bacterial genomes contained *mtbA* encoding the second enzyme to produce methyl-CoM from TMA/DMA/MMA, but none of these four contained *mttBC*, *mtbBC*, or *mtmBC*. Twenty-six bacterial genomes contained *mtaA*, encoding the second enzyme to produce methyl-CoM from methanol, and 13 of these contained *mtaBC*. However, the vast majority (>90%) of archaeal genomes shown here also contained *mtbA* (TMA/DMA/MMA) or *mtaA* (methanol). Note that only archaea perform the subsequent step of reducing methyl-CoM to methane (with McrABG) and that this is just for demethylation for methanogenesis; other pathways also degrade these compounds. Also note that the *x* axis scale is different on each graph. We searched for *mttB*, *mttC*, *mtbB*, *mtbC*, *mtmB*, *mtmC*, *mtaB*, and *mtaC* genes on IMG/M on May 11, 2022. The table of genomes containing each gene was downloaded and filtered to include only isolate genomes or high-quality metagenome assembled genomes (224). Only genomes containing both of the genes in each pair were counted.

Genome Survey

The isolate and metagenome-assembled genome (MAG) genome survey demonstrated that while the pairs of genes for initial demethylation of TMA, DMA, MMA, and MeOH are present in more archaeal genomes than bacterial genomes, they are present in many bacteria too, with the exception of MMA demethylation genes (Fig. 3). This highlights that both of these domains may perform initial degradation of methylated carbon compounds in the same manner in addition to other degradation pathways that are known to be performed by bacteria (131, 166). While fermentation and acetogenesis performed by bacteria have long been implicated as generating important precursors to archaeal methanogenesis (200), it remains to be seen how bacteria with these particular demethylation genes might interact with or otherwise affect (positively or negatively) methanogenic archaea. The analysis also shows that more genomes contain methanol degradation genes than genes for other pathways, while at the genus level, TMA degradation genes are the most prevalent among methanogenic archaeal genera, followed by genes for degradation of DMA and then MMA. This is generally in line with the ecology of these substrates, with TMA and methanol being produced from more sources than DMA and MMA.

There was a total of 117 unique genomes with the complete suite of genes for at least one of the methylated amines (TMA, DMA, or MMA) pathways (i.e., containing the pair of genes for the initial demethylation, *mtbA* for methyl-CoM production, and *mcrA* for CH_4 production). Most of the genomes that contained TMA-processing genes (n = 115) also contained genes to process DMA (n = 113) or MMA (n = 103), with 97 genomes containing the complete suite for all three methylated amines. No single genome examined here contained only TMA-, only DMA-, or only MMA-degrading genes. Overall, this supports the hypothesis that most methanogens that produce methane from TMA can also produce methane from DMA and MMA, which would enable them to generate additional energy from the products of the reactions (i.e., DMA from TMA and MMA from DMA). This is partially supported by isolate culture experiments that test methane production from a suite of substrates and often show methane production for TMA, DMA, and MMA (201–207), although more substrate specificity has also been shown (208).

Metagenome Gene Survey

We analyzed the metagenomes for certain genes that are present in different methanogenesis pathways, which provides information on the potential of different environments to harbor taxa containing those genes. Certain environments were previously hypothesized to have relatively greater contributions of certain methanogenic pathways based on substrate availability and previous work on methanogenic community composition (30). Gene counts were separated into those harbored by archaeal taxa, bacterial taxa, and all other taxa/unknown taxa based on the taxonomic assignment of metagenomic scaffolds by the IMG/M annotation pipeline, since only archaeal genes are expected to participate in methanogenesis pathways. The gene abundance survey of metagenomes demonstrated significant differences in all eight of the genes analyzed, and this held true for the total gene count across all taxa, the gene counts from just the archaeal assigned scaffolds, and the gene counts of scaffolds not assigned to bacteria or archaea (Kruskal-Wallis, $P < 0.05$; Fig. 4; Table S2 in the supplemental material). For gene counts from bacterial-assigned scaffolds, six of the eight gene abundances were significantly different among the ecosystems, while *mcrA* (not present in bacteria) and *mtsA* (only present in very few bacterial scaffolds) were not (Fig. 4; Table S2). This is not surprising due to the known differences in environmental conditions and known taxonomic preferences across these habitats.

The total abundance of the *cdhD* gene involved in acetoclastic methanogenesis as well as other pathways found in bacteria was significantly greater than hydrogenotrophic and methyl-based methanogenesis genes in five of the six habitats where those two pathways are hypothesized to be dominant and was also greater than *mcrA*. Indeed, many of the *cdhD* counts were derived from bacteria, especially in freshwater wetlands, termite guts, and ocean sediments (Fig. 4). When analyzing just the archaeal-assigned scaffolds, *cdhD* abundance was greatest in hydrothermal vents, followed by freshwater wetland and ocean sediments (Fig. 4). In two instances (ocean sediments and freshwater wetlands), archaeal *cdhD* abundance was greater than *mcrA* abundance, suggesting multiple copies per genome and/or presence of *cdhD* in nonmethanogenic archaea. The total abundance and archaeal abundance of the *frhA* gene involved in hydrogenotrophic methanogenesis was greatest in hydrothermal vents and was least abundant in ocean and hypersaline sediments (Fig. 4).

In terms of the methyl-based methanogenesis genes, their total abundances generally followed a pattern of genes for utilization of methanol or TMA being the most abundant, followed by DMA, MMA, and DMS (Fig. 4). This pattern was also true for abundance in the archaeal-assigned scaffolds, except that *mtbC* (for DMA utilization) was the most abundant methyl-based gene in termite guts (Fig. 4). Abundance in the three ecosystems hypothesized to be dominated by methyl-based methanogenesis was not significantly greater than in the other ecosystems except for *mtaA* (methanol) and *mttC* (TMA) in ocean sediments.

These metagenomic gene abundances can be confounded by several factors, which could obscure the true patterns of the activity levels of each methanogenic pathway. Most importantly, some of the genes are not strictly specific to each pathway, and most of the genes are not specific even to archaea (Fig. 4). To aid in the interpretation of abundances, genes from archaeal and bacterial scaffolds were counted separately; however, this step still has limitations, as there are many scaffolds that are not taxonomically assigned. In particular, the *cdhD* gene, while required for acetoclastic methanogenesis and thus present in all acetoclastic methanogens, is not an exclusive marker of acetoclastic methanogenesis, as it is also present in some hydrogenotrophs and other bacterial taxa (acetogens, sulfate-reducers) that use the Wood-Ljungdahl pathway, and this inflated the *cdhD* counts. However, a low abundance of *cdhD*, and in particular of archaeal *cdhD*, does mean a low abundance of acetoclastic taxa (and other taxa with *cdhD*). This interpretation applies to all of the genes, in fact, as most of them (except *mcrA* and *mtsA*) are found in bacteria as well (Fig. 4). Similarly, the TMA methyltransferases have homologs in bacteria that are involved in glycine betaine metabolism (209).

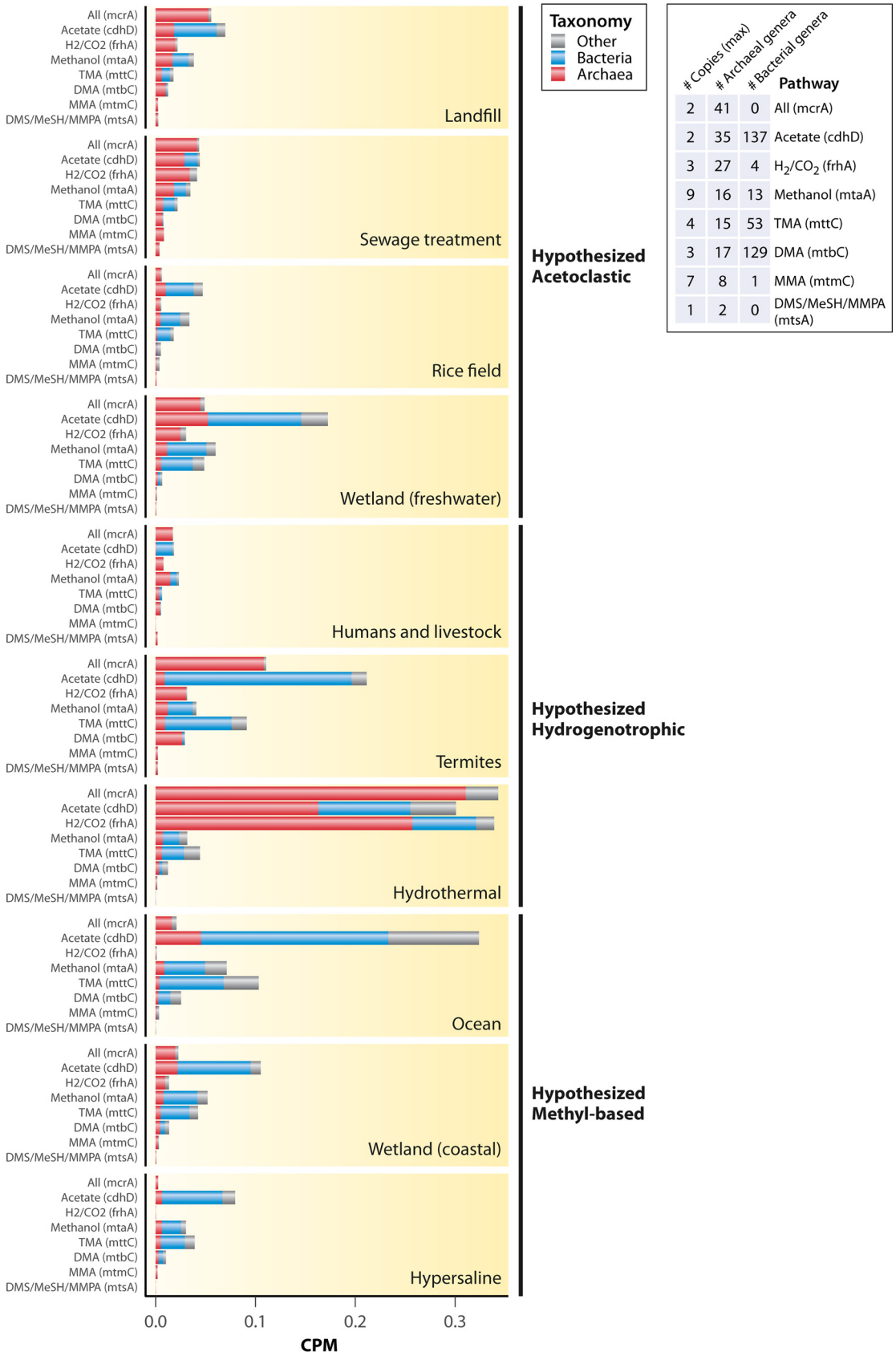


FIG 4 Counts per million (CPM) assembled reads normalized abundance of genes for all core methanogenesis pathways (methyl-CoM reduction, *mcrA*), acetoclastic methanogenesis (*cdhD*), hydrogenotrophic methanogenesis (*frhA*), and methyl-based methanogenesis from methanol (*mtaA*), trimethylamine

(Continued on next page)

The estimates are further complicated by the fact that some taxa contain genes for multiple pathways, and metagenomic data do not distinguish which of them are actively expressed. This is a well-known issue with the versatile methanogens in the *Methanosarcinales* order, as discussed elsewhere (70). In the case of distinguishing between methyl dismutation and methyl reduction, a complete or partial absence of the methyl branch of the Wood-Ljungdahl pathway would suggest the methyl reduction pathway, but it is also possible that methyl-reducing taxa have those genes but do not express them (33). Still, we suggest that researchers working with genomic data assess the genomes or MAGs for multiple suites of genes to evaluate the presence of pathways more effectively. For metagenomic data, this is more difficult, as multiple organisms are represented in the metagenome. Furthermore, some genes can be present in multiple copies (Fig. 4), which can affect their overall abundances in metagenomes. More metatranscriptomic surveys are necessary to complement this analysis and more effectively assess the dominant active pathways. Another confounding factor for ocean sediments is depth, which was not available in the IMG/M metadata. Methyl-based methanogenesis is expected to be more abundant in the top layer of sediment, while hydrogenotrophic and acetoclastic methanogenesis are expected to increase further down the sediment profile below the sulfate reduction zone (55).

Metagenome Taxonomic Survey

The metagenome-based taxonomic survey showed major differences at the family level of methanogens across the different habitat types, with all families exhibiting significant differences in abundance among the different environments (Fig. 5; Table S2). Hydrothermal vents had the highest overall abundance of methanogens in terms of counts per million assembled metagenomic reads and were dominated by *Methanocaldococcaceae*, *Methanococcaceae*, and, to a lesser extent, *Methanosarcinaceae*. *Methanococcaceae* and *Methanocaldococcaceae* were dominant methanogenic families only in hydrothermal vents, likely due to the extreme thermal adaptations present in some members of these families. *Methanosarcinaceae* was the most abundant family across the whole data set, with mean counts per million (CPM) >1 in all ecosystems but human and livestock gastrointestinal tracts. These ecosystems were dominated by hydrogenotrophic *Methanobacteriaceae*, although 14 other methanogen families were present at very low abundances. *Methanoregulaceae* was the second most abundant

FIG 4 Legend (Continued)
(*mttC*), dimethylamine (*mtbC*), methylamine (*mtmC*), or the methylated sulfides (*mtsA*), which include dimethylsulfide (DMS), methanethiol (MeSH), and methylthiopropanoate (MMPA). Gene counts are split taxonomically between archaea, bacteria, and other (eukaryotes and unassigned) according to the scaffold taxonomic assignments from the IMG/M annotation pipeline. Also shown are the number of copies of the genes in genomes containing the *mcrA* gene (methanogens), the number of archaeal genera containing the genes, and the number of bacterial genera containing the genes based on isolate genomes and high-quality MAGs in the IMG/M database. Note that "Humans and livestock" and "Termites" refer to gut samples. The following words were searched in IMG/M for each habitat type (the search was performed on February 5, 2022): landfill = "landfill", sewage treatment = "sewage", wetlands = "wetland", rice fields = "rice", ocean = "ocean", humans and livestock = "human gut" or "ruminant" or "cow", termites = "termite", hydrothermal vents = "hydrothermal", and hypersaline = "hypersaline." The results were first filtered to only metagenomes (i.e., metatranscriptomes and isolate genomes were removed). Results were then further filtered based on the information in the study name or genome name to ensure that the metagenomes were actually from the targeted habitats, and only metagenomes containing the *mcrA* gene were retained. Coastal wetlands were separated from freshwater wetlands using "grep" to extract metagenomes containing the word "coastal" or based on our knowledge of the metagenomic study. Furthermore, only metagenomes with either unrestricted public use status or explicit permission from the principal investigators in the case of restricted use status for metagenomes sequenced at Joint Genome Institute (JGI) (JGI Data Utilization Status = "Restricted" in IMG/M) were used (Table S1 in the supplemental material). The KEGG Orthology (KO) gene counts were downloaded using the "Statistical Analysis" tool on IMG/M, which uses lastal 983 and KEGG Genes v77.1 to assign KO terms. To acquire KO gene counts of just the archaeal portion of the metagenomes, the KO profiles were filtered to include only those found on scaffolds assigned to the domain *Archaea*. The same was performed for the domain *Bacteria*. Archaeal and bacterial counts were subtracted from the total counts to yield the counts for all eukaryotic taxa as well as scaffolds with no taxonomic assignment. This was performed using a custom Python script to process three of the output files generated by the IMG/M annotation pipeline, (i) the KO terms of the genes, (ii) the taxonomic assignments of the scaffolds, and (iii) the gene to scaffold mapping. Metagenomes that contained fewer than 1,000 reads with family-level taxonomic information were removed from the data set. The final sample size was 465, including samples from all over the world (Fig. S2; landfill, $n = 12$; sewage treatment, $n = 27$; rice field, $n = 11$; wetlands (freshwater), $n = 90$; humans and livestock, $n = 50$; termites, $n = 62$; hydrothermal vent, $n = 130$; wetlands (coastal), $n = 52$; ocean, $n = 13$; hypersaline, $n = 18$). Correlations between the selected gene and the other genes in each pathway are shown in Fig. S3. Tables of all genomes containing each of these genes were downloaded from IMG/M to acquire counts of genera per domain that contain each gene. To aid in interpretation of abundances, we also downloaded a KO profile of all IMG/M isolate genomes and high-quality metagenome assembled genomes containing the *mcrA* gene (the search was performed May 11, 2022; $n = 282$ genomes), selected the genes of interest by their KOs, and examined the maximum and minimum number of copies across all of the genomes.

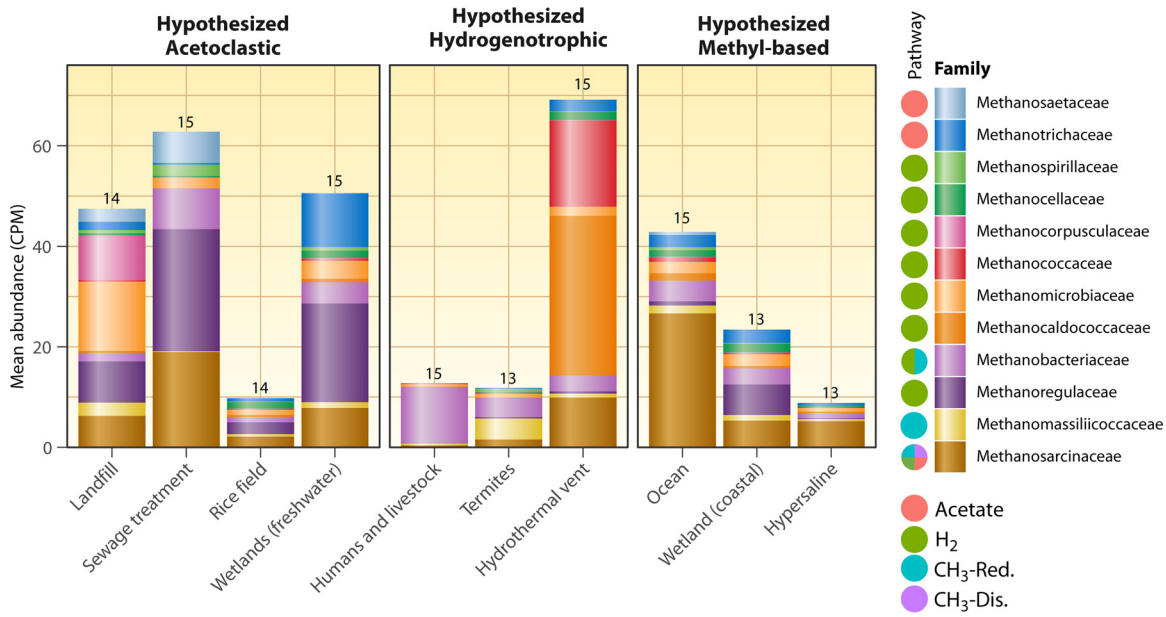


FIG 5 Counts per million normalized abundance of different methanogen families across different methanogenesis habitats. Numbers above the columns are the number of methanogenic families present (the total number of methanogen families in the whole data set was 18). Also shown are the known substrates/pathways that can be used as published previously (39) but with the methyl-reducing pathway added to *Methanosarcinaceae* (69). Note that only the top 12 families in the data set are shown here (these families had greater than 1.6 CPM in at least one habitat type). Families are sorted by pathway and then by overall abundance. The data set was assembled as in Fig. 3, except a family-level taxonomic profile was downloaded with the "Statistical Analysis" tool on IMG/M, which uses lastal 983 and the IMG-NR reference database to assign the taxonomy. Methanogenic families were selected as those containing the string "Methano," followed by filtering out any methanotrophs based on the literature. Known methanogenic pathways performed by the families were taken from the literature (39, 69).

family overall; this was driven by their abundance in sewage treatment samples, wetlands, landfills, and rice fields (Fig. 5).

These taxonomic data provide mixed support for some of the proposed dominant pathways, although again it is difficult to know where members of the versatile *Methanosarcinaceae* family are performing more of the acetoclastic, hydrogenotrophic, methyl dismutation, or methyl-reducing pathways with these data. However, we can use the other two families that perform exclusively acetoclastic methanogenesis (*Methanosaetaceae* and *Methanotrichaceae*) as markers; they are indeed more abundant in the hypothesized acetoclastic-dominant habitats with perhaps the exception of rice fields, which had an even distribution of family abundances. Note that *Methanosaetaceae* was renamed *Methanotrichaceae*, and these families represent the same group of organisms, but older taxonomic databases and older annotations by the IMG/M pipeline contain both families. *Methanosaetaceae*/*Methanotrichaceae* were most abundant in landfills, sewage treatment plants, and freshwater wetlands, but were also abundant in ocean sediments, hydrothermal vents, and coastal wetlands. Human and livestock guts and hydrothermal vents were dominated by hydrogenotrophic taxa, in support of the hypothesized dominance of the hydrogenotrophic pathway there (Fig. S4) even though the hydrogenotrophic gene *frhA* was not as abundant as *cdhD* in either of those habitats or in termite guts. Termite guts had about equal mean abundances of hydrogenotrophic (most *Methanobacteriaceae*) and methyl-reducing methanogens (*Methanomassiliicoccaceae*). Ocean sediments, which are predicted to be dominated by methylotrophs, were dominated by *Methanosarcinaceae*, which could indeed be performing methyl-based methanogenesis. Hydrogenotrophic and acetoclastic taxa were present in ocean sediments as well, although to a lesser extent (Fig. 5).

CONCLUSIONS AND FUTURE DIRECTIONS

Methanogens were first cultured in the 1920s. Since then, hundreds of methanogens have been isolated and sequenced, and many new methanogenic substrates

have been identified. We now know that archaeal methanogens are more diverse taxonomically and functionally than previously predicted and that biological methane production is not limited to the archaeal domain. Methanogenesis as a mode of growth and energy conservation, however, is still limited to the archaeal domain. Many new studies have come out in recent years demonstrating that methyl-based methanogenesis is the dominant pathway in some environments. These studies use multiple methods, including isotopic profiling, molecular sequencing, substrate quantification, and microcosm incubation methods to draw robust conclusions (59, 63). Genes involved in methyl-based methanogenesis are also widely distributed across taxa and ecosystems, suggesting that it may contribute to methane production in a broader range of environments than previously thought (Fig. 1, 4, and 5) (39).

In the context of two key ongoing global change processes (drought and sea level rise), it is possible that the relative contribution of methyl-based methanogenesis to methane production may increase due to the increasingly saline conditions in ecosystems affected by these processes (210), even if overall methane emissions might decrease (211, 212). Salinity is expected not only to increase but also to become more variable (74). As noted earlier, climate change has already and will continue to increase salinity, especially in estuarine and coastal wetlands, as has been shown by many studies in many places (74, 213, 214). This is due to a combination of sea level rise and declining freshwater inputs as well as other human activities, such as salting roads in winter, irrigation, and vegetation clearing (74). These factors combined with decreasing precipitation in some areas mean that methyl-based methanogenesis could also become more relevant in inland freshwater wetlands.

There is still much work to be done to fully characterize some pathways as well as their isotope fractionations (Fig. 1; Table 3) and to quantify the relative contributions of each substrate to the overall methane production in different environments. We also call for improved computational methods for metabolic modeling of methyl-based methanogenesis as well as methanogenesis in general. Automated genome-scale metabolic modeling methods are continuously making excellent and important methodological improvements. However, it still takes a high degree of manual curation to build accurate metabolic models for methanogens (215–222). This is an active area of research that we expect will soon enable model development for a greater number and broader diversity of methanogens. Research on methyl-based methanogenesis and methanogenesis in general is an exciting and challenging field of research that will continue to capitalize on improving molecular and computational methods, enabling discoveries with significant implications for climate change mitigation and natural gas energy production.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, XLSX file, 0.1 MB.

SUPPLEMENTAL FILE 2, PDF file, 0.6 MB.

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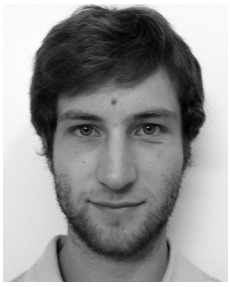

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