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MINIREVIEW

Model Organisms To Study Methanogenesis, a Uniquely Archaeal Metabolism

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ABSTRACT Methanogenic archaea are the only organisms that produce CH_4 as part of their energy-generating metabolism. They are ubiquitous in oxidant-depleted, anoxic environments such as aquatic sediments, anaerobic digesters, inundated agricultural fields, the rumen of cattle, and the hindgut of termites, where they catalyze the terminal reactions in the degradation of organic matter. Methanogenesis is the only metabolism that is restricted to members of the domain *Archaea*. Here, we discuss the importance of model organisms in the history of methanogen research, including their role in the discovery of the archaea and in the biochemical and genetic characterization of methanogenesis. We also discuss outstanding questions in the field and newly emerging model systems that will expand our understanding of this uniquely archaeal metabolism.

KEYWORDS archaea, methanogenesis, model organisms

METHANOGENESIS

Methanogenic archaea are the only organisms on Earth that produce CH_4 as part of their energy-generating metabolism and are essential for the complete remineralization of organic matter in oxidant-depleted, anoxic environments, such as marine and freshwater sediments, the intestinal tracts of some insects and other animals, wastewater treatment plants, and agricultural plots that rely on inundation. It is estimated that methanogens account for the annual production of ~750 Tg of CH_4 or 560 Tg of C (1). Given that during methanogenesis from biomass, one CO_2 is produced per CH_4, methanogenesis is responsible for the annual remineralization of about 1,100 Tg of C or 1.1% of all the photosynthetically produced organic carbon on Earth.

Cultivated methanogens fall into two metabolic subtypes. The hydrogenotrophs, or methanogens without cytochromes, generally grow by the reduction of CO_2 to CH_4 using either H_2 or formate as the electron donor, although some species can also oxidize primary and secondary alcohols and carbon monoxide or reduce methyl compounds with H_2 (2). Methanogens with cytochromes, which include carboxydotrophic, most methylotrophic, and all aceticlastic methanogens, can often reduce CO_2 as well. However, most species grow primarily by the reduction of methyl groups (e.g., methanol, methylamines, and methylsulfides) or the methyl carbon of acetate to CH_4 (2). In the case of aceticlastic methanogenesis, the reduction of the methyl carbon of acetate is coupled to the oxidation of the carboxylate carbon (3). In the case of methanol, methylamines, or methylsulfides, three methyl groups are reduced using electrons from the complete oxidation of a fourth methyl group to CO_2. Hydrogenotrophic methanogenesis is thought to be an evolutionarily ancient process, possibly arising at or near the origin of life (4), although some have argued for a methylotrophic origin of methanogenesis (5). In contrast, aceticlastic methanogenesis likely evolved within the last ~500 million years (6).

Methanogens represent multiple extremes in biodiversity that have made their study challenging. They are strict anaerobes and require specialized cultivation techniques. Many are lithotrophs, and growth with H_2 requires specialized gas handling and safety

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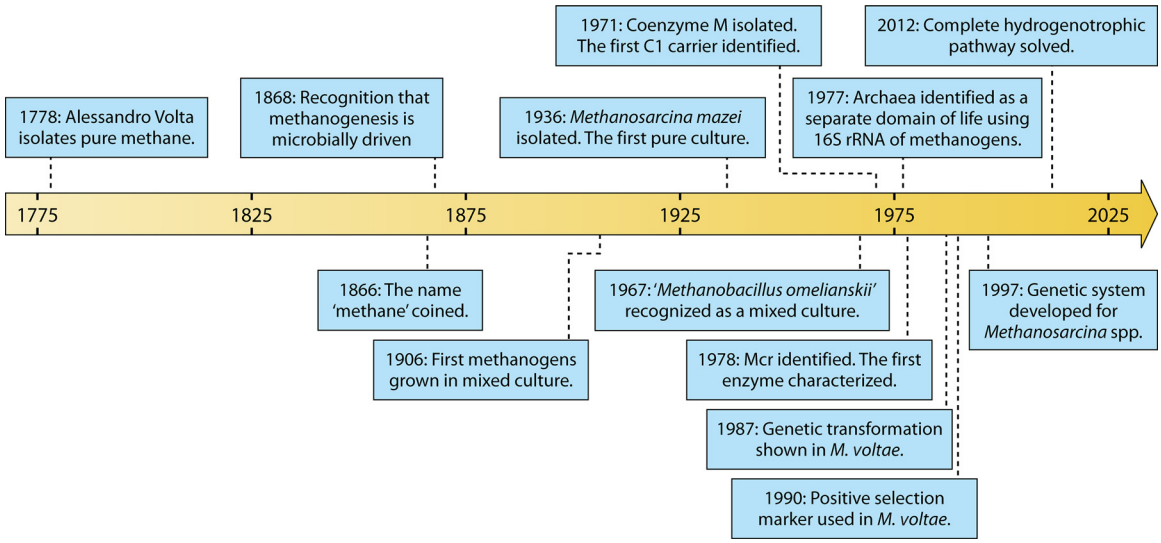

FIG 1 A timeline of key discoveries involving methanogens.

equipment. Many are also extremophiles that grow only at high temperatures or concentrations of salt and require additional specialized cultivation techniques and equipment. Finally, they are archaea, and many of the common molecular biological tools used to study bacteria are ineffective.

The development of model organisms enabled research in this field to rapidly progress. Although model organisms often fail to represent the full diversity of a group, they offer some important advantages. First, model organisms are often selected because they possess properties that make them easy to study, such as a relatively simple lifestyle or rapid growth. Second, for fastidious organisms like methanogens, the development of efficient cultivation techniques is a major initial challenge requiring substantial investments in time and equipment. Often, the methodology that is developed fails to generalize to more than a few species. Therefore, once this obstacle has been overcome, the benefits are harvested by continuing work on the particular group. Finally, knowledge is cumulative, and the discovery of the properties of a model organism enables more detailed investigations. Each of these points is illustrated below.

While methanogens all belong to the archaeal domain and rely on the formation of CH_4 for energy, the diversity of the organisms is very large, and it was necessary to select different model organisms at different stages in their study. In this review, we give an overview of the history of methanogen research, highlight significant scientific discoveries resulting from the study of model methanogens, describe how and why certain species were used as models to study the two physiologically distinct subgroups, and discuss future directions. A timeline of major discoveries is highlighted in Fig. 1. Related organisms catalyzing reactions such as the anaerobic oxidation of methane (reverse methanogenesis) and the reduction of short-chain alkanes are not discussed here due to our focus on model organisms that form CH_4 as a product of their primary metabolism.

ISOLATION OF CH_4 AND THE FIRST CULTIVATED METHANOGENS

The phenomenon of "flammable air" was first investigated by Alessandro Volta working in Lake Maggiore, Italy, in 1776. Volta's initial experiments focused on the isolation and identification of this gas as CH_4. He characterized CH_4 by igniting gas collected from lake sediments or by using simple combustion chambers to propel a projectile (7); similar experiments were also being performed in the Americas (8). Even at this time, it was apparent that CH_4 production was a by-product of the biologically catalyzed degradation of organic matter, but it was not until nearly a century later that scientists began to appreciate that methanogenesis was microbially catalyzed (9–15). Moreover, many of the early studies were limited due to a lack of pure cultures for characterization. It was not until the 1930s

that H. A. Barker (16) and M. Stephenson and L. H. Stickland (17) reported the first "pure" cultures of methanogenic organisms growing with substrates such as H_2 and formate to reduce CO_2 to CH_4 or with acetate via the aceticlastic reaction. Based on their descriptions and 20-20 hindsight, it is unlikely that these cultures were, in fact, axenic. They were isolated by the dilution-to-extinction technique in broth, and many of their properties were inconsistent with those of pure cultures that were subsequently isolated as better methods became available. Nevertheless, since they have been lost, their purity cannot be reexamined.

In 1931, Stephenson and Stickland described a culture of a hydrogenotroph that was isolated by dilution under conditions where the disproportionation of formic acid to CH_4 and CO_2 supported growth (17). Using suspensions of this organism, they found that it was also capable of the oxidation of H_2 coupled with the reductions of CO_2, CO, formaldehyde, methanol, and sulfate. Today, methanogens are not known to use sulfate as an electron acceptor in respiratory metabolism, so it is likely that these cultures contained sulfate-reducing bacteria in addition to methanogens. Several years later, Barker (16) described additional cultures: *Methanococcus mazei* (isolated on acetate-containing medium, later reisolated, and now known as *Methanosarcina mazei* [18]), *Methanobacterium söhngenii* (capable of acetate and butyrate fermentation), and "*Methanobacillus omelianskii*" (isolated on alcohols). All of these cultures had properties suggesting that they were mixed cultures containing a methanogen as well as bacteria. While these early studies demonstrated the existence of these unique microbial metabolisms, they also illustrated the difficulties in obtaining pure cultures of these fastidious microorganisms with the techniques available at the time.

The greatest challenge with the growth of methanogens was a strict requirement to maintain anoxia. Early studies used excess reductants, medium sparged with oxygen-free gas, and standard microbiological culture tubes capped with rubber stoppers to maintain these conditions (for example, see reference 19). While often effective, these techniques did not allow the reliable and consistent growth of axenic cultures. It was not until R. E. Hungate (19) modified methods and designed glassware designed specifically for the growth of anaerobic microorganisms and A. Aranki and R. Frêter (20) and D. Coy (https://coylab.com) designed a reliable, inexpensive anaerobic chamber that rigorous study of the physiology of methanogens became possible. The techniques developed by Hungate were further optimized by T. L. Miller and M. J. Wolin (21) and W. E. Balch and R. S. Wolfe (22) to allow growth in pressurized glassware and incubation vessels. Techniques for large-scale cultivation were also established (23). Reproducible and reliable growth led to breakthroughs in the biochemistry and genetics of methanogenesis.

THE DISCOVERY OF SYNTROPHY AND THE REALIZATION THAT "*METHANOBACILLUS OMELIANSKII*" WAS A MIXED CULTURE

While additional cultures continued to be isolated, much of the work performed in the early years focused on defining the substrate range of Barker's cultures, with a particular focus on "*M. omelianskii*." This culture was attractive as a model system because it grew much faster than the other cultures available at the time and was technically easier with which to work (16). Moreover, unlike *M. mazei*, which had a complicated life cycle with multiple morphological forms, its simple lifestyle allowed the study of energy metabolism in the absence of complicating factors (16).

"*M. omelianskii*" was initially cultivated on medium using ethanol as a reductant for CO_2 reduction to CH_4 (16) and was later found to use other reductants, importantly H_2. A key observation was that "*M. omelianskii*" failed to ferment ethanol after cultivation in medium with H_2 supplied as the reductant (24). The careful isolation of single colonies on different media led to the identification of two organisms in the culture, a methanogen that could grow only via the H_2-dependent reduction of CO_2 and a bacterium that could ferment ethanol to acetate and H_2. The bacterium became known as the "S organism," but this culture has since been lost; the methanogen was later named *Methanobacterium bryantii* M.o.H. to recognize the contributions of M. P. Bryant in its isolation (25). The combined activities of two organisms to catabolize a substrate that neither one can use in axenic culture are known as syntrophy (from the Greek *syn*, meaning together, and *trophe*, meaning nourishment) (26).

The discovery of syntrophy between the S organism and *M. bryantii* was a key observation in the recognition of interspecies H_2 transfer, which drives the complete remineralization of organic matter in anoxic environments. The overall reaction is thermodynamically favorable under standard conditions (equation 1). The S organism grows by the fermentation of ethanol according to equation 2, and *M. bryantii* grows by methanogenesis according to equation 3. The S organism relies on a methanogen to maintain a low partial pressure of H_2, which creates conditions where ethanol oxidation is favorable, and *M. bryantii* relies on the S organism for the production of H_2.

$$2CH_3CH_2OH + CO_2 \rightarrow 2CH_3COO^- + 2H^+ + CH_4 \left(\Delta G^{0'} = -112\,\text{kJ mol}^{-1}\right) \quad (1)$$

$$CH_3CH_2OH + H_2O \rightarrow CH_3COO^- + H^+ + 2H_2 \left(\Delta G^{0'} = +9.6\,\text{kJ mol}^{-1}\right) \quad (2)$$

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \left(\Delta G^{0'} = -131\,\text{kJ mol}^{-1}\right) \quad (3)$$

Other substrates can support the syntrophic growth of bacteria (e.g., propanol, propionate, butyrate, and benzoate) and methanogens (e.g., H_2, formate, or direct interspecies electron transfer [DIET]). Today, many studies on syntrophic nutrient exchange have focused on pairing *Methanospirillum hungatei*, *Methanobacterium* spp., or *Methanosarcina* spp. with syntrophic bacteria (e.g., see references 27–29). In fact, these methanogens can be used as "bait" when enriching or isolating bacterial syntrophs. While the initial cultivation of *M. bryantii* and the S organism suggested the importance of interspecies H_2 exchange for maintaining these interactions, recent evidence suggests that alternate intermediates such as formate may be essential for maintaining a stable syntrophic association (30–32). These small organic acids are highly soluble and easily diffuse between partners in aqueous medium. New evidence also suggests that in *Methanosarcina*-dominated syntrophy, bacterial and archaeal outer membrane multiheme cytochromes and cytochrome-based "nanowire" filaments may facilitate DIET (28, 33, 34). DIET removes the need for a soluble intermediate between partner organisms and may be more efficient for electron transfer. (For comprehensive reviews on the topic of syntrophy, see references 29 and 35. For a more detailed account of the laboratory experiments surrounding the discovery of syntrophy, see reference 36.)

M. BRYANTII M.o.H. AS A MODEL ORGANISM TO IDENTIFY THE C_1 CARRIERS AND REDOX-ACTIVE COFACTORS OF METHANOGENESIS

The isolation of *M. bryantii* from the "*M. omelianskii*" mixed culture led to the development of a model system based on a pure culture of a methanogen, and the characterization of the C_1 intermediates, cofactors, vitamins, and enzymes for methanogenesis began in earnest. Much of this work was performed in the laboratory of R. S. Wolfe (see his autobiographical article in *Annual Reviews of Microbiology* for more details [36]). B. C. McBride and R. S. Wolfe (37) and C. D. Taylor and R. S. Wolfe (38) isolated the first carbon carrier from methanogens and identified it as 2-mercaptoethanesulfonate, which carries a methyl group that is reduced to CH_4; therefore, it was named HS-CoM (coenzyme that carries a methyl carbon). This was one of the first clues that methanogens were a unique biological group as this "vitamin" had not been previously observed in another biological process (although today, HS-CoM is also known to be present in bacteria [e.g., see reference 39]). At around this time, Wolfe's and Bryant's laboratories, in collaboration with G. D. Vogel's laboratory, also solved the structure of F_{420} (40–42), a previously unknown electron carrier, providing more evidence of a unique metabolism. Additional electron and C_1 carriers were subsequently isolated from *M. bryantii* and other methanogens, and a partial list of some of these molecules can be found in Fig. 2 (reviewed in reference 43).

ARCHAEA AS THE THIRD DOMAIN OF LIFE AND A SWITCH TO *METHANOTHERMOBACTER* SPECIES AS MODEL METHANOGENS

While many of the vitamins and cofactors of methanogenesis have subsequently been identified in bacteria, the unique (at the time) nature of the biochemistry of

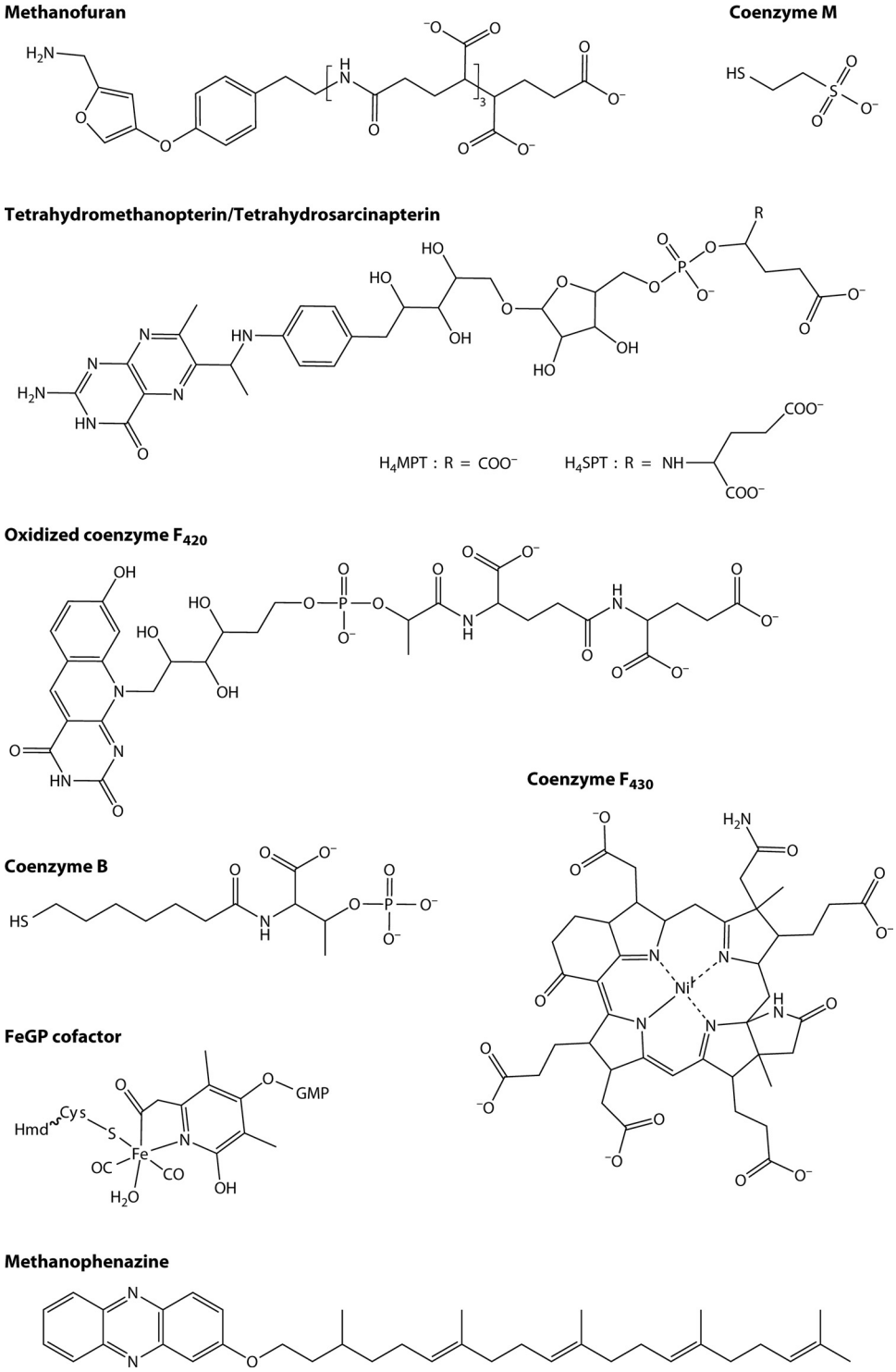

FIG 2 Coenzymes and C_1 and electron carriers used in methanogenesis. H_4MPT, tetrahydromethanopterin; H_4SPT, tetrahydrosarcinapterin.

methanogens was one of the first clues that they were distinct from bacteria. In around 1976, C. R. Woese began his groundbreaking work to create a universal tree of life using the small-subunit rRNA (16S for prokaryotes) of the ribosome as an evolutionary marker. The initial findings of Woese's work were published in 1977 (44), which led to the formal proposal of the name "*Archaea*" in 1990 (45). Woese was performing his work at the University of Illinois and collecting ^{32}P-labeled rRNA from a variety of organisms; naturally, Wolfe's methanogens were

included in these analyses because of their unique physiology. ^{32}P labeling was particularly difficult for slow-growing methanogens as there were issues with this radioactive element killing the cultures before sufficient incorporation could occur. At around this time, J. G. Zeikus in the Wolfe laboratory isolated *Methanothermobacter thermautotrophicus* (originally *Methanobacterium thermoautotrophicus*) (46). This organism has a short generation time, which is ideal for ^{32}P incorporation studies, and could be grown easily in large fermenters. These attributes also made it an excellent candidate for biochemical studies that would drive the field for the next 20 years (see the next section).

Woese's analysis of the 16S rRNA of *M. thermautotrophicus* lead to the realization that the ribosomes of methanogens were as different from those of bacteria as those of bacteria were from those of eukaryotes and the conclusion that methanogens were a different domain of life (44). This was also true when other methanogens and other archaeal 16S rRNAs were analyzed. In addition to the unique biochemistry of methanogenesis and the molecular evidence from 16S rRNA, O. Kandler et al. soon found that the cell walls of archaea were comprised of different polymeric substances than the cell walls of bacteria (47–49). In 1978, it was found that *M. thermautotrophicus* contained lipids composed of phytanyl-glycerol ethers and squalenes, also distinct from bacterial lipids (50). Additional lines of evidence that archaea were a distinct domain continued to accumulate (see reference 25 for an argument that was put forward at the time, and see reference 51 for a more recent treatment of the argument that archaea are a domain distinct from bacteria). Over the ensuing decades, additional evidence accumulated. Of note is that the first archaeal genome sequence, that of *Methanocaldococcus jannaschii*, was completed in 1996 (52). In addition to providing additional genetic support for this argument, it widely popularized the concept among biologists outside microbiology.

DEVELOPMENT OF *METHANOTHERMOBACTER* AND OTHER MODEL SYSTEMS FOR THE ISOLATION OF ENZYMES

The isolation of unique cofactors from *M. bryantii* provided the impetus for studies on the enzymology of methanogenesis. During the 1970s, there was a rapid increase in the isolation of pure cultures of methanogens and the availability of potential model systems. Improvements to its large-scale cultivation, its rapid growth, and the stability of thermophilic enzymes at room temperature made *M. thermautotrophicus* an attractive model for the biochemistry of methanogenesis. A closely related species, *Methanothermobacter marburgensis*, was used as a model organism in the R. K. Thauer laboratory in Marburg, Germany, for similar reasons (53). Particularly important for the study of the reactions at the intermediate levels of CO_2 reduction as well as methylotrophic methanogenesis were *Methanosarcina* strain Gö1, used by the G. Gottschalk laboratory in Göttingen, Germany, and *Methanosarcina barkeri* strain MS, used by the G. D. Vogel laboratory in Nijmegen, The Netherlands. Although their growth was slower than that of *Methanothermobacter*, the *Methanosarcina* spp. were particularly useful model systems because they could be grown on methanol in the absence of H_2, which was technically simpler and much safer than H_2 growth. They were also ideal for studies of methyl group oxidation, which was believed at the time to be the reverse of CO_2 reduction.

The first enzyme to receive significant attention was methyl-CoM reductase (Mcr), the enzyme that catalyzes the CH_4-forming step of methanogenesis (54, 55). Mcr is biochemically interesting because it is one of the most oxygen-sensitive enzymes known. It relies on a low-potential nickel ($E^{0'}$ of less than −600 mV versus a standard hydrogen electrode [56, 57]) coordinated by the tetrapyrrole coenzyme F_{430} to reduce methyl-CoM to CH_4 using electrons from the thiol coenzyme B (HS-CoB), also generating a heterodisulfide of HS-CoM and HS-CoB (CoM-S-S-CoB).

After the isolation and characterization of Mcr, the remainder of the pathway for the reduction of CO_2 to CH_4 was elucidated, the details of which have been well described in recent reviews (1, 2, 58–60). Two additional C_1 carriers, methanofuran (MFR) and tetrahydromethanopterin (H_4MPT) (61–66), are needed for methanogenesis. The initial CO_2-reducing reaction is carried out by formyl-methanofuran (formyl-MFR)

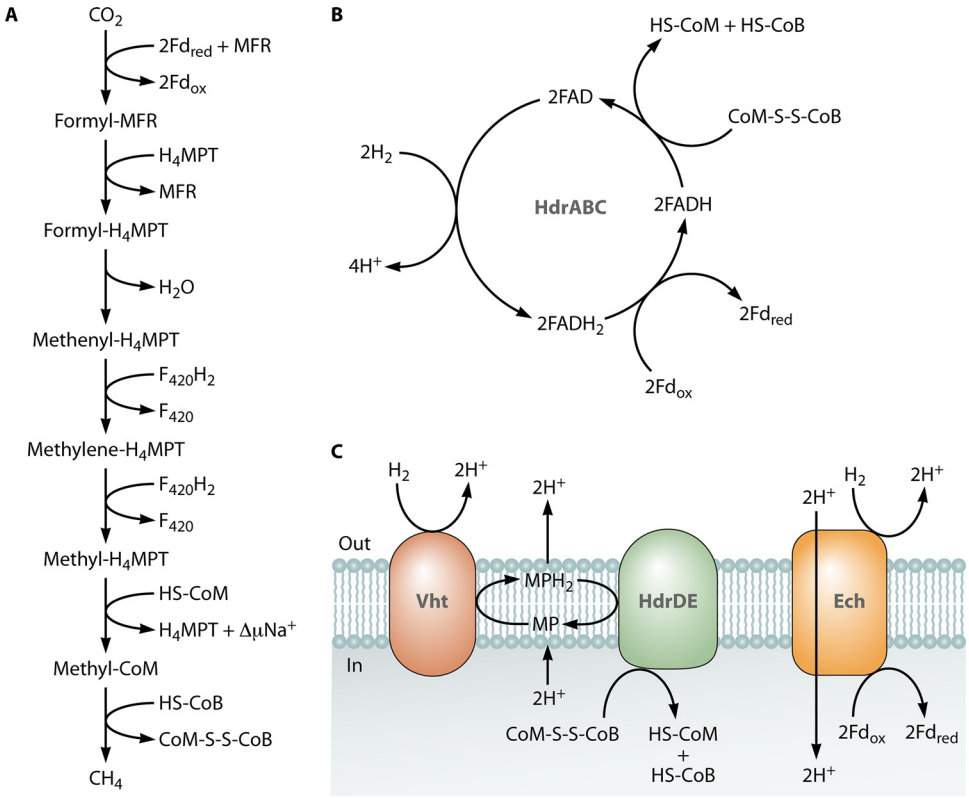

FIG 3 The CO_2-reducing pathway of methanogenesis. (A) The pathway used by all methanogens (with and without cytochromes) that reduce CO_2. In *Methanosarcinales* methanogens, tetrahydrosarcinapterin (H_4SPT) may replace tetrahydromethanopterin (H_4MPT). (B) Reactions catalyzed by the cytoplasmic, flavin-based, electron-bifurcating heterodisulfide reductase (Hdr) of hydrogenotrophs. The ferredoxin (Fd) reduced in these reactions is used in the initial CO_2-reducing step of methanogenesis, rendering methanogenesis a cycle in these organisms. The hydrogenotrophic pathway is known as the Wolfe cycle (195). (C) An example of a membrane-bound electron transport chain found in *Methanosarcina* spp. MFR, methanofuran; HS-CoM, coenzyme M; HS-CoB, coenzyme B; F_{420}, coenzyme F_{420}; MP, methanophenazine; Vht, methanophenazine-reducing hydrogenase; Ech, energy-converting hydrogenase; FAD, flavin adenine dinucleotide; FADH2, fully reduced form of FAD; FADH, partially reduced flavosemiquinone.

dehydrogenase (Fmd), which fixes CO_2 to a formyl group covalently attached to MFR (67). Next, a formyl-MFR:formyl-H_4MPT formyltransferase (68) and a formyl-H_4MPT cyclohydrolase generate methenyl-H_4MPT (69), which is further reduced to methyl-H_4MPT. Finally, methyl transfer from H_4MPT to HS-CoM is catalyzed by a membrane-bound, Na^+-translocating methyltransferase (70). This methyltransferase comprises one of the key steps in the generation of energy for the cell. Mcr catalyzes the final, CH_4-producing step of methanogenesis. The series of reactions for the reduction of CO_2 to CH_4 can be found in Fig. 3A.

A side product of CH_4 formation is CoM-S-S-CoB, which must be reduced to provide the thiol forms of HS-CoM and HS-CoB for subsequent rounds of methanogenesis. This is accomplished by heterodisulfide reductase (Hdr). In hydrogenotrophic methanogens, Hdr is a cytoplasmic enzyme that uses H_2 as an electron donor via an associated hydrogenase (71). Several decades later, it was discovered that some methanogens can use formate as an electron donor for this reaction via an associated formate dehydrogenase (72–74). Interestingly, early observations by R. P. Gunsalus and R. S. Wolfe (54) and T. A. Bobik and R. S. Wolfe (75) found that CoM-S-S-CoB or methyl-CoM, which is converted to CH_4 and CoM-S-S-CoB in the cell, also stimulated CO_2 reduction to CH_4 in cell extracts. The reason for this became clear in 2011 when A.-K. Kaster et al. showed that Hdr is capable of generating reduced ferredoxin (an endergonic reaction with H_2 as an electron donor) coupled with the reduction of CoM-S-S-CoB (an exergonic reaction) via flavin-based electron bifurcation (76) (Fig. 3B). Since this discovery, Hdr from methanogenic archaea has been used as a model enzyme to study electron-bifurcating reactions. In methanogens from the order *Methanosarcinales*, both cytoplasmic

Hdr and a second membrane-bound isoform of Hdr (Fig. 3C), which is discussed below, are essential for growth (77).

In addition to the unique biochemistry of methanogenesis, hydrogenotrophic methanogens were models for studies of hydrogenases. *M. marburgensis* was an early model for characterizing nickel-containing hydrogenases that are found in bacteria, archaea, and eukaryotes. In methanogens, there are cytoplasmic F_{420}-reducing ($F_{420}H_2$ donates electrons for the reduction of methenyl-H_4MPT to methylene-H_4MPT) and F_{420}-nonreducing (for donating electrons to Hdr) [NiFe]-hydrogenases and membrane-bound [NiFe]-hydrogenases (for assimilatory reactions) (58). Similarly, *Methanococcus voltae* was a model of selenium metabolism and [NiFeSe]-hydrogenases (78). Additionally, some methanogens contain an unusual [Fe]-hydrogenase (Hmd) that is found only in archaea and catalyzes an alternate methenyl-H_4MPT-reducing reaction (79, 80). Hmd contains no nickel and instead possesses a novel iron-guanylylpyridinol (FeGP) (Fig. 2) cofactor. The biosynthesis of the FeGP cofactor is only now being elucidated (58, 81, 82).

THE FIRST GENETIC SYSTEMS IN METHANOGENS

Recombinant DNA technology was invented in the 1970s, and by the late 1970s, it became obvious that genetics could offer tremendous insights into methanogenesis. In the Wolfe laboratory, W. E. Balch maintained a collection of methanogens for his studies on their taxonomy (25). Among this collection, one of us (W. B. Whitman) selected *Methanococcus voltae* as a candidate for genetic studies because it possessed many properties considered ideal. Although its nutrition was not yet characterized, it grew rapidly in complex media and possessed a single-cell morphology. Therefore, it was believed to be a good candidate for plating. The absence of a pseudomurein cell wall was also a key factor because, at the time, it was difficult to isolate intact DNA from many methanogens. Early studies with *M. voltae* defined its nutrient requirements and developed protocols for efficient plating on agar medium (83, 84). Related strains were isolated to search for plasmids and phages, work that would later lead to the development of a shuttle vector (85, 86). This organism is also competent for natural transformation (87, 88). P. Gernhardt et al. (89) demonstrated the first use of antibiotic selection in a methanogen by introducing a puromycin resistance cassette from *Streptomyces alboniger* (90) via natural transformation into *M. voltae* albeit with a low efficiency of ~8 transformants μg^{-1} of DNA (89). Because of its greater sensitivity to puromycin and the availability of cryptic plasmids in recent isolates, *Methanococcus maripaludis* was also explored as a model system. The major advance that enabled reproducible and efficient transformation was the generation of protoplasts (87, 91), which increased the transformation efficiencies ~10^2- to 10^5-fold.

While the natural transformation of *M. maripaludis* could be used to generate mutations, the efficiency of transformation remained relatively modest, with a maximal efficiency of ~10^3 transformants μg^{-1} DNA (92). A polyethylene glycol (PEG)-based protocol was established, which allowed transformation efficiencies of ~10^6 transformants μg^{-1} DNA (91, 92). The PEG protocol greatly expanded the utility of a genetic system for *M. maripaludis* and has been adapted for the introduction of both shuttle and suicide vectors and DNA-protein complexes (85, 86, 93–96). This has resulted in protocols for markerless mutagenesis (94, 97), heterologous gene expression and the expression of epitope-tagged proteins (97–99), transposon mutagenesis (96, 100, 101), and the use of fluorescent reporters (102, 103). Both CRISPR-Cas12a-based and CRISPR-Cas9-based systems have also been recently reported for mutagenesis (104, 105).

Since the development of genetic tools, *Methanococcus* spp. have served as models for several aspects of archaeal biology and methanogenesis, including studies focused on the genetics of methanogens (95, 106), the substrate range of hydrogenotrophic methanogens (73, 97, 100, 107–110), archaeal nitrogen fixation (98, 111–113), selenocysteine biosynthesis and selenoprotein biochemistry (114–117), archaeal sulfur metabolism (118–120), pilus and archaellar assembly and function (121–128), the synthesis of the FeGP

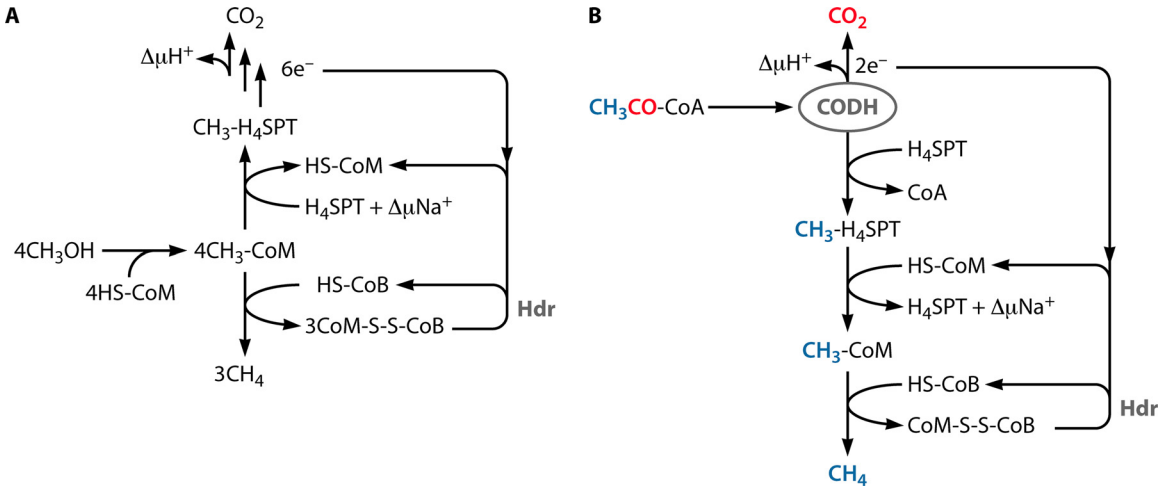

FIG 4 Additional reactions of methanogenesis catalyzed by *Methanosarcina* spp. (A) Disproportionation of methanol to CH_4 and CO_2. (B) The aceticlastic pathway of methanogenesis. H_4SPT, tetrahydrosarcinapterin; HS-CoM, coenzyme M; HS-CoB, coenzyme B; CoA, coenzyme A; CODH, carbon monoxide dehydrogenase/acetyl-CoA synthase.

cofactor of the [Fe]-hydrogenase (81, 82, 129, 130), and methanogen transcriptomics and proteomics (131, 132).

While *M. maripaludis* is an excellent model for the study of the CO_2-reducing pathway and general archaeal cell biology, an expanded understanding of methylotrophic and aceticlastic methanogenesis required the development of additional model organisms. From a biochemical perspective, the core CO_2-reducing pathway in *Methanosarcina* spp. is largely the same as that in hydrogenotrophic methanogens except that membrane-bound Hdr and methanophenazine, which plays a quinone-like role in electron transport, are used for energy conservation (1, 2) (Fig. 3C). The membrane-bound form of Hdr has profound implications for energy conservation that have been discussed (1), but this also means that the first and last steps of methanogenesis are not obligately coupled by flavin-based electron bifurcation. This expands the substrate range of *Methanosarcina* spp. as C_1 can theoretically enter the pathway at any oxidation state. For example, the methyl group of acetate enters the pathway as methyl-tetrahydrosarcinapterin (H_4SPT), a derivative of H_4MPT used in *Methanosarcina* spp. (133), and methanol enters as methyl-CoM (Fig. 4). In addition to membrane-bound Hdr, additional enzymes are needed to use methanol, methylamines, and methylsulfides. Thus, *Methanosarcina* spp. are ideal models for understanding methanogens with a diverse substrate range. For example, B. A. Blaylock and T. C. Stadtman performed many of the pioneering experiments with this organism, showing that methylcobalamin could be reduced to CH_4 (134). Pyrrolysine, the "22nd amino acid," was also first characterized in methanogens from the *Methanosarcinales* where it is essential for the activity of enzymes that catalyze methyl transfer reactions from methylamines and methylsulfides (135–137; for a recent review of methylotrophic methanogenesis, see reference 60).

Like *Methanococcus*, the development of a genetic system for *Methanosarcina* spp. was successful only after detailed studies of its nutrition and biology were performed. In the original media used, *Methanosarcina* spp. typically grew in large granules or clumps bound together by an amorphous heteropolysaccharide. However, upon the isolation and characterization of marine species, it was discovered that growth in high-salt media suppressed the production of heteropolysaccharide, allowing growth as single cells (138). Under these conditions, it was possible to lyse the cells with detergents, isolate high-molecular-weight DNA, and screen for cryptic plasmids (139). These technical developments enabled the construction of the first methanosarcinal shuttle vector in 1997 using a liposome-based DNA delivery system (140), and recently, CRISPR-Cas9-based tools have been established (141). These advances resulted in protocols for markerless mutagenesis, heterologous gene expression, and the expression of epitope-tagged proteins (106, 140, 142);

transposon mutagenesis (143); the use of fluorescent reporters (103); and inducible gene expression (144). Due to the broad substrate range of *Methanosarcina* spp., mutagenesis has been successful in the isolation of strains unable to grow on one substrate while supplying another (e.g., mutagenesis of acetate utilization in cultures grown on methanol and H_2) (145, 146). Thus, the effects of eliminating certain enzymes in the core CO_2-reducing pathway have been studied only in members of the *Methanosarcinales* (145, 147). In particular, the biochemistry of membrane-bound hydrogenases has been most extensively studied in these organisms (146, 148). Due to their metabolic versatility, *Methanosarcina* spp. have also been targeted for engineering new metabolic capabilities into methanogens (149–153).

In addition to being excellent models to understand the substrate range of methanogens, *Methanosarcina* spp. have been instrumental for our understanding of archaeal surface layer structure and chemistry (154), nitrogen fixation (155, 156), genome-scale metabolic modeling of methanogenesis (157–159), carbon monoxide metabolism (160, 161), dehalogenation reactions (162, 163), γ carbonic anhydrases (164, 165), the use of compatible solutes for salt adaptation (166), extracellular electron transfer (167, 168), and unique biochemical reactions catalyzed by iron-sulfur proteins (169–172).

EMERGING MODEL ORGANISMS: GENETIC TOOLS IN *METHANOCALDOCOCCUS*, *METHANOTHERMOBACTER*, AND *METHANOCULLEUS* SPECIES

A major criticism of the use of model organisms is that they fail to capture the full diversity of a group of microorganisms and lead to the perilous assumption that a model organism is typical of the entire group. With methanogens, we know from comparative genomics that they possess substantial diversity and combinations of genes unexpected from those observed in the model organisms. Therefore, the development of additional model organisms will prove extremely valuable.

As the first sequenced archaeal genome and due to its hyperthermophilic growth temperatures, *M. jannaschii* has been a model organism for structural studies on archaeal proteins (52). However, until recently, this organism lacked a genetic system, so all structural work required the purification of native proteins or their recombinant expression. Two groups recently reported the development of positive selection, negative selection, and genetic complementation in this methanogen (173, 174). Using these tools, it should be possible to epitope tag proteins for targeted purification, allowing higher-throughput structural studies. Additionally, *Methanocaldococcus* spp. are capable of nitrogen fixation at the highest temperatures known (175), and genetic tools will lead to a more complete understanding of this metabolism.

Methanogens from the orders *Methanobacteriales* and *Methanomicrobiales* are ubiquitous and often numerically dominant in industrial and municipal anaerobic reactors (e.g., see references 176 and 177). Despite their importance, only in the last few years have genetic systems been developed in these organisms. While the tools available for genetic manipulations are still in their infancy, we want to highlight these emerging model systems that will expand our understanding of these understudied methanogen groups.

Methanothermobacter spp. have been some of the most important models for understanding the biochemistry of methanogenesis, but only in the last few years have tools for robust and repeatable genetic manipulations been described (178, 179). While early attempts to perform genetic manipulations in this organism aimed to leverage its natural competence (180), they had a low efficiency and were difficult to reproduce. The new genetic system leverages conjugal DNA transfer from *Escherichia coli* (178) similarly to a system that has been used in other methanogens (181). Genetic manipulations of *Methanothermobacter* spp. will allow the testing of the roles of various methanogenesis enzymes *in vivo* and enable the exploration of unique physiological structures, such as the role of fimbriae in cellular attachment (182).

Methanogens from the *Methanomicrobiales* grow more slowly and are more difficult to culture than many of the hydrogenotrophs previously chosen as model organisms. However, one exception is *Methanoculleus thermophilus*, which has a doubling time of

~4 h and can be plated with high efficiency (72, 183). Some strains of *M. thermophilus* had been used to study F_{420}-dependent alcohol dehydrogenases to understand how the oxidation of secondary alcohols to ketones could feed electrons into methanogenesis (184). It was recently discovered that *M. thermophilus* is naturally competent, providing the basis for a genetic system (92). Using this genetic system, the epitope-tagged purification of Hdr led to the finding that formate dehydrogenase is the only electron-donating enzyme for the reduction of CoM-S-S-CoB (Fig. 3) (72). This was also shown with Hdr purified from cells of *M. hungatei* (74), which, along with *Methanobacterium formicicum*, was an early model organism for the enzymatic characterization of F_{420}-dependent formate dehydrogenases (185–187). While more work needs to be done with organisms from this group, these results suggest that, unlike other hydrogenotrophic methanogens, organisms from the *Methanomicrobiales* may be formate specialists. A functional genetic system for *M. thermophilus* will enable future studies focused on formate and alcohol oxidation in hydrogenotrophic methanogens.

FUTURE DIRECTIONS

While advances in the study of methanogenesis have historically adapted tools used for the manipulation of model bacteria, innovations leading to improved anaerobic manipulation and cultivation are usually required before these tools are useful for strict anaerobes. To leverage state-of-the-art approaches in genetic engineering and structural biology (e.g., large-scale screening of mutant libraries, high-throughput screening of protein structures, microfluidic manipulations of cultures, and direct monitoring of live cells using microscopy), researchers will need to adapt these tools for inexpensive and efficient use under anoxic conditions. The greatest advance could come from the use of robotics designed to work in environments with high levels of CO_2 and sulfide, which are required for the growth of most methanogens. Additionally, many databases and algorithms that predict metabolic networks and protein structures have been developed with bacterial and eukaryotic systems in mind, with a bias toward easily cultured aerobic organisms. Anaerobes, especially methanogens that use electron carriers and cofactors not commonly found in other model organisms, are poorly represented in the data sets used to construct these tools. Thus, concerted effort is needed before large-scale predictive data sets can be applied for the optimization of methanogenic metabolism.

The use of model organisms to study methanogenesis has been vital for an understanding of the metabolic versatility of this diverse group. However, many additional questions remain to be answered. Due to their importance in the complete degradation of organic matter in anoxic environments, a detailed understanding of the interactions between methanogens and their syntrophic partners, whether they require DIET or the exchange of small molecules like H_2 or formate, is vital for the optimization of anaerobic remediation processes. In addition to archaeon-bacterium interactions through syntrophy, many methanogens can grow as intracellular symbionts with single-celled eukaryotes (188, 189) or as members of the microbiota of humans and animals, where they are correlated with altered health outcomes (190, 191). In particular, *Methanobrevibacter* spp. have been useful models for understanding host-methanogen and methanogen-bacterium interactions in the human oral cavity and gut (192–194); the establishment of genetic tools in these organisms will be vital for an in-depth understanding of these interactions. Finally, leveraging the growing number of model methanogenic organisms will empower efforts to understand the biochemistry of the Hmd hydrogenase (the uniquely archaeal hydrogenase), Mcr, and flavin-based electron bifurcations catalyzed by Hdr. While the number of model organisms used to study methanogenesis has expanded in recent years, efforts to isolate methanogens from other phyla and relatives of methanogens that catalyze the anaerobic oxidation of methane will be essential to harness the full potential of this unique metabolic pathway.

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