



# Energy Conservation and Hydrogenase Function in Methanogenic Archaea, in Particular the Genus *Methanosarcina*

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**SUMMARY** The biological production of methane is vital to the global carbon cycle and accounts for ca. 74% of total methane emissions. The organisms that facilitate this process, methanogenic archaea, belong to a large and phylogenetically diverse group that thrives in a wide range of anaerobic environments. Two main subgroups exist within methanogenic archaea: those with and those without cytochromes. Although a variety of metabolisms exist within this group, the reduction of growth substrates to methane using electrons from molecular hydrogen is, in a phylogenetic sense, the most widespread methanogenic pathway. Methanogens without cytochromes typically generate methane by the reduction of CO<sub>2</sub> with electrons derived from H<sub>2</sub>, formate, or secondary alcohols, generating a transmembrane ion gradient for ATP production via an Na<sup>+</sup>-translocating methyltransferase (Mtr). These organisms also conserve energy with a novel flavin-based electron bifurcation mechanism, wherein the endergonic reduction of ferredoxin is facilitated by the exergonic reduction of a disulfide terminal electron acceptor coupled to either H<sub>2</sub> or formate oxidation. Methanogens that utilize cytochromes have a broader substrate range, and can convert acetate and methylated compounds to methane, in addition to the ability to reduce CO<sub>2</sub>. Cytochrome-containing methanogens are able to supplement the ion motive force generated by Mtr with an H<sup>+</sup>-translocating electron transport system. In both groups, enzymes known as hydrogenases, which reversibly interconvert protons and electrons to molecular hydrogen, play a central role in the methanogenic process. This review discusses recent insight into methanogen metabolism and energy conservation mechanisms with a particular focus on the genus *Methanosarcina*.

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## INTRODUCTION

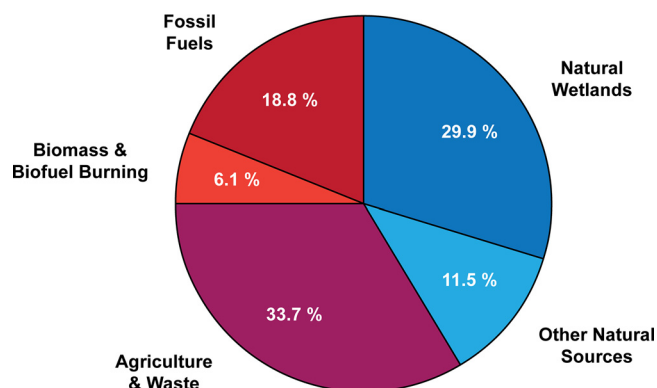
Methanogenic archaea (methanogens) play a critical role in the global carbon cycle by virtue of their ability to facilitate the decomposition of organic matter to methane and carbon dioxide (CO<sub>2</sub>) in anoxic environments that lack superior electron acceptors such as Fe(III) and sulfate (1). It is estimated that approximately 2% of CO<sub>2</sub> that is fixed into biomass by photosynthetic organisms is eventually converted into methane, a process that produces around 1 billion tons of methane annually (2, 3). Approximately 60% of this methane is oxidized to CO<sub>2</sub> by methane-consuming organisms, while ca. 40% is released into the atmosphere (2, 3). Although geological sources of methane are significant, biological production accounts for approximately 72 to 74% of global emissions (Fig. 1) (4, 5). Moreover, human activities, especially agriculture and the petrochemical industry, have significantly increased the inputs of methane into the atmosphere. As a result, atmospheric methane concentrations have more than doubled since the onset of the industrial revolution (late 1700s), and levels continue to rise (5, 6). Evidence from carbon isotope data suggests that this increase can be primarily attributed to the enhancement of biologically produced methane by human activities (6). Rising atmospheric methane concentrations are of particular concern, as methane has a global warming potential that is 28 to 34 times higher than that of CO<sub>2</sub> over a 100-year period (5). On the other hand, biogenic methane is a clean-burning, carbon-neutral, renewable energy source that has the potential to mitigate human-induced climate change. In either case, a more in-depth understanding of the metabolism and physiology of the organisms responsible for methane production is required to effectively manage the process.

Methanogens are nearly ubiquitous in anaerobic environments and have been found to thrive in environments with a wide range of temperatures, salinities, and pHs. These include marine and freshwater sediments, wetlands, geothermal systems, permafrost soils, anaerobic sewage digesters, landfills, and the intestinal tracts of ruminants, humans, and termites (4). Wetlands are by far the largest natural source of biological methane production; however, anthropogenic production of methane by methanogens remains the largest overall source (Fig. 1). This includes emissions related to agriculture (ruminant-associated methanogenesis [18% of total methane emissions] and rice paddy soils [5%]) and landfill and waste decomposition (10%) (5).

Due to their prolific production of methane and the diverse environments in which they thrive, methanogens are ideally suited for the production of biogas, a renewable alternative to fossil natural gas that is composed primarily of methane. A variety of engineered systems have been established for the conversion of organic matter into methane by methanogens (7). More than 2,000 biogas production facilities currently operate in the United States, and it is estimated that full implementation of all potential sites could provide enough energy to power more than 3.5 million homes (8). Improving the rate and efficiency of this process could increase this number even further (7). Because biogas must be purified and methane enriched to be compatible with existing gas distribution networks, a careful consideration of the physiology of methanogens will enable both an enhancement of methane production in biogas reactors and a reduction in overall methane emissions. In this review, we discuss recent insight into the metabolism and energy conservation mechanisms utilized by methanogens, with a particular focus on how H<sub>2</sub> is used both as a substrate for methanogenesis and as an electron carrier by cytochrome-containing methanogens.

## DIVERSITY OF METHANOGENS

Methanogenic archaea belong to a large and phylogenetically diverse group with various metabolic capabilities and requirements. Until recently, known methanogens were found solely within the phylum *Euryarchaeota*, assigned to 8 different orders based on 16S rRNA sequence similarity. These include *Methanobacteriales*, *Methanococcales*, *Methanomicrobiales*, *Methanosarcinales*, *Methanopyrales*, *Methanocellales*, *Methanomassiliicoccales*, and *Methanonatronarchaeales* (4, 9–11). Recently, three novel taxa of methanogenic archaea have been proposed based on metagenomic data



**FIG 1** Sources of atmospheric methane. The proportion of atmospheric methane emissions caused by anthropogenic activities (red), produced by biogenic processes (blue), and the overlap between the two categories (purple) is shown. Percentages of each methane source were determined from atmospheric observation-based calculations reported by Dean et al. (5). The category “other natural sources” is primarily composed of biologically produced methane (8% of total) in freshwater and marine sediments and geologically produced methane (3% of total).

of uncultured organisms, including “*Candidatus Methanofastidiosa*” and members of the “*Candidatus Bathyarchaeota*” and “*Candidatus Verstraetearchaeota*” phyla (12–14), with the latter two falling outside of the *Euryarchaeota* in the TACK superphylum (12, 14). The discovery of methanogen lineages outside of *Euryarchaeota* has led to the hypothesis that the last archaeal common ancestor (LACA) was potentially a methanogen (15); however, this conclusion is not supported by recent phylogenetic analyses (16). In addition to their genetic diversity, each order of methanogenic archaea has unique characteristics, including their metabolic capabilities and the environments from which they can be isolated.

Members of the *Methanobacteriales* order have been isolated from a wide variety of environments, including marine and freshwater sediments, permafrost, hot springs, alkaline lakes, and the gastrointestinal tracts of various animals. Accordingly, the requirements for growth in this order are quite varied. Most species are limited to utilizing  $\text{CO}_2$  and  $\text{H}_2$  for methanogenesis, but some members of this order are capable of using formate,  $\text{CO}$ , or secondary alcohols (e.g., 2-propanol and 2-butanol) as electron donors for reduction of  $\text{CO}_2$  to methane (17). While some strains are autotrophs, many are heterotrophs that require exogenous acetate, amino acids, or vitamins for growth. Lastly, one genus, *Methanosphaera*, cannot reduce  $\text{CO}_2$  to methane and is limited to utilizing methanol with  $\text{H}_2$  for methanogenesis (18, 19).

All members of the *Methanococcales* order were isolated from marine environments and are capable of utilizing  $\text{CO}_2$  and  $\text{H}_2$  as substrates for methanogenesis. Some species are also able to use formate as an electron donor. Many members of this order are thermophilic or hyperthermophilic and were isolated from deep-sea hydrothermal vents or other marine sediments (17). As a corollary, *Methanococcales* species have some of the highest growth rates of all methanogens, with a rate as high as  $2.4 \text{ h}^{-1}$  found in *Methanocaldococcus jannaschii* (20, 21).

Most members of the *Methanomicrobiales* order are mesophilic and require near-neutral pH conditions for ideal growth. Like members of *Methanococcales*, all *Methanomicrobiales* species can reduce  $\text{CO}_2$  to methane with electrons derived from  $\text{H}_2$ , and most can also use formate as an electron source. Additionally, some species are able to oxidize secondary alcohols for the reduction of  $\text{CO}_2$ . Many species also require supplementation with acetate or other organic substrates, such as yeast extract or rumen fluid (17). This order includes two organisms, *Methanofollis ethanolicus* and *Methanogenium organophilum*, with the unique ability to utilize the primary alcohol, ethanol, for methanogenesis (22, 23).

The orders *Methanopyrales*, *Methanomassiliococcales*, and *Methanocellales* contain few cultivated organisms, with a single species each for the first two and a single genus

with three species for the last (17). Aside from that commonality, organisms from each of these orders are drastically different. *Methanopyrus kandleri*, isolated from hydrothermal marine sediments, grows by reduction of CO<sub>2</sub> to methane using electrons from H<sub>2</sub> and is the only known species capable of methanogenesis above 100°C (24, 25). The *Methanomassiliicoccales* order consists of one isolated species, *Methanomassiliicoccus luminyensis*, and at least three other “*Candidatus*” species identified within enrichment cultures (10, 26–28). *M. luminyensis* grows solely by reduction of C<sub>1</sub> compounds with H<sub>2</sub> and was isolated from human fecal matter (26, 29). Lastly, all three species of the *Methanocellales* order were isolated from the soil of rice paddies, and all utilize H<sub>2</sub> and CO<sub>2</sub> for methanogenesis. Two of the species can also use formate as an electron donor, and all three species require supplementation with acetate for growth (17). Sequencing of the genome of *Methanocella paludicola* revealed a lack of genes encoding carbon monoxide dehydrogenase/acetyl coenzyme A (acetyl-CoA) synthase (CODH/ACS); thus, this organism is unable to assimilate CO<sub>2</sub> and requires acetate for biosynthesis (30).

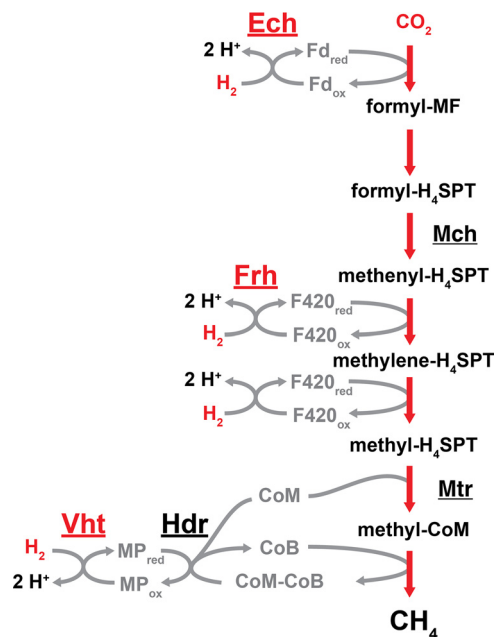
*Methanonatronarchaeales* is the most recently recognized order of methanogens and consists of organisms isolated from sediments of hypersaline chloride-sulfate and soda lakes (11). Most organisms from this order are thermophiles and alkaliphiles, with optimal growth conditions of 50°C and a pH of 9.5 to 9.8. Additionally, all are halophiles with an optimum Na<sup>+</sup> concentration of 4.0 M. In a mechanism that is unique among halophilic methanogens, this order overcomes osmotic stress by maintaining high intracellular potassium concentrations. Lastly, all members of this order, like those of the *Methanomassiliicoccales* order, generate methane by reducing methyl compounds with electrons derived from H<sub>2</sub>. However, unlike other taxa that are limited to reducing methyl compounds with H<sub>2</sub>, the *Methanonatronarchaeales* are also able to utilize formate as an electron source (11).

Members of the final order, *Methanosarcinales*, are widely distributed and can be found in environments ranging from marine and freshwater sediments to gastrointestinal tracts and anaerobic sewage digesters. Species from this order have the widest substrate range of all methanogens and can utilize CO<sub>2</sub> plus H<sub>2</sub>, CO, acetate, or methyl compounds (e.g., methanol, methylamines, and methyl sulfides) for methanogenesis (2, 17). In addition to these substrates, a single isolate, *Methermicoccus shengliensis*, is able to use methoxylated aromatic compounds, which can be found in immature coal deposits, for methanogenesis via an enigmatic mechanism (31). Despite this broad substrate range, no member of the *Methanosarcinales* order is able to utilize formate as an electron donor, which is in stark contrast to the majority of other methanogen orders. A final difference between members of *Methanosarcinales* and all other orders of methanogenic archaea is that they alone contain cytochromes and the lipid-soluble electron carrier methanophenazine, the significance of which is discussed in the following sections (2).

Two putatively methanogenic taxa outside of the *Euryarchaeota* phylum, “*Ca. Bathyarchaeota*” and “*Ca. Verstraetearchaeota*,” were recently identified based on the presence of genes required for methanogenesis in metagenome-assembled genomes (MAGs) (12, 14). Apart from this similarity, they were obtained from quite different environments: “*Ca. Bathyarchaeota*” from a coal bed-associated deep aquifer and “*Ca. Verstraetearchaeota*” from a cellulose-degrading bioreactor. Despite the lack of a cultivated isolate from either taxon, it is thought that both reduce methyl compounds to methane based on the presence of methyl transferase genes (12, 14, 16). Additionally, a recent “*Ca. Verstraetearchaeota*” MAG obtained from a Yellowstone National Park hot spring appears to contain all genes required to utilize H<sub>2</sub> and CO<sub>2</sub> (32). While speculative, these findings suggest that the phylogenetic diversity of methanogens is significantly broader than was initially appreciated.

## METABOLIC PATHWAYS FOR METHANOGENESIS

A common characteristic of all known methanogens is that they are obligate methane producers, meaning that their only system for energy conservation comes from the reduction of growth substrates to methane. As described in the previous

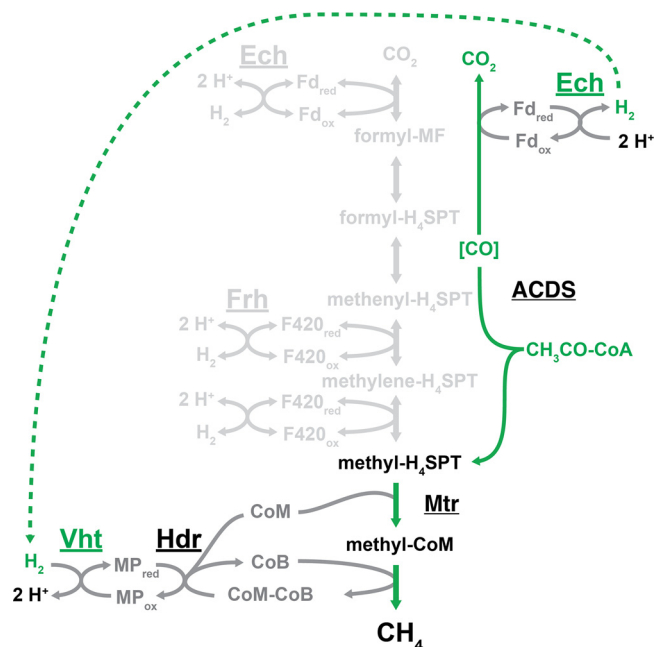


**FIG 2** CO<sub>2</sub> reduction pathway of *Methanosarcina*. In this pathway, CO<sub>2</sub> is reduced to CH<sub>4</sub> in a stepwise manner. For each reduction step, electrons are derived from the oxidation of H<sub>2</sub> by the energy-converting hydrogenase (Ech), the F420-reducing hydrogenase (Frh), and the methanophenazine-reducing hydrogenase (Vht), as indicated. In the final methanogenic step, the coenzyme M (CoM)-bound methyl group is reduced by coenzyme B (CoB), thereby forming CH<sub>4</sub> and a disulfide of the two coenzymes (CoM-CoB). Reduced forms of CoM and CoB are regenerated from the disulfide by the heterodisulfide reductase (Hdr). Methanogens that lack cytochromes do not encode Ech or Vht and instead use flavin-based electron bifurcation to couple H<sub>2</sub> oxidation to the reduction of ferredoxin (Fd) and CoM-CoB via the MvhADG:HdrABC enzyme complex (not shown). Other abbreviations: Mch, methenyl-tetrahydrosarcinapterin (methenyl-H<sub>4</sub>SPT) cyclohydrolase; Mtr, methyl-H<sub>4</sub>SPT:CoM methyl-transferase; MF, methanofuran; “red” and “ox” subscripts, reduced and oxidized states of electron carriers.

section, nearly all methanogens are capable of reducing CO<sub>2</sub> to methane with electrons derived from the oxidation of H<sub>2</sub> via the CO<sub>2</sub> reduction pathway (4, 17). This pathway differs slightly between methanogens with and without cytochromes, as is discussed in the following sections. The pathway that is utilized by cytochrome-containing methanogens is depicted in Fig. 2. In addition to this pathway, members of the *Methanosarcinales* order can utilize three other methanogenic pathways in order to metabolize acetate (acetate pathway [Fig. 3]) or methyl compounds (methyl reduction pathway and methylotrophic pathway [Fig. 4 and 5]) (4, 33). The two pathways involving methyl compounds differ in the source of electrons used for reduction of the methyl group to methane. H<sub>2</sub> oxidation provides electrons in the methyl reduction pathway, but in the methylotrophic pathway methyl compounds serve as both the oxidant and reductant. Whereas only species in the *Methanosarcinales* order are able to utilize the acetate and methylotrophic pathways, three additional classes of methanogens can use the methyl reduction pathway. These include organisms from the *Methanosphaera* genus in the *Methanobacteriales* order and organisms from the *Methanomassiliicoccales* and *Methanonatronarchaeales* orders (11, 17). Based on metagenomic data, “*Ca. Methanofastidiosa*,” “*Ca. Bathyarchaeota*,” and “*Ca. Verstraetearchaeota*” may also rely on this pathway to reduce methyl compounds (12–14). The following sections primarily focus on the metabolic capabilities of *Methanosarcina* species, due to their ability to use all four methanogenic pathways.

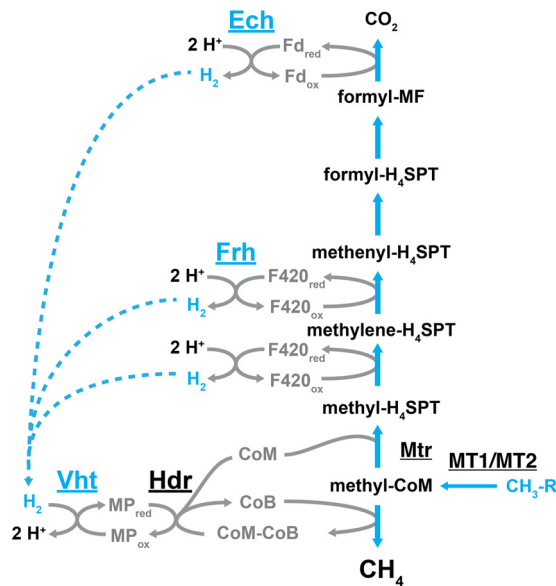
### The CO<sub>2</sub> Reduction Pathway

The CO<sub>2</sub> reduction pathway is typified by the stepwise reduction of CO<sub>2</sub> to methane with electrons derived from the oxidation of H<sub>2</sub>, which is mediated by a group of enzymes known as hydrogenases (Fig. 2). The overall standard free energy change



**FIG 3** The aceticlastic pathway of methanogenesis for *M. barkeri*. Acetate is first converted to acetyl-CoA in an ATP-dependent manner (not shown), which is then split by acetyl-CoA decarbonylase/synthase (ACDS) into an enzyme-bound carbonyl ([CO]) and a methyl group, which gets transferred to the C<sub>1</sub> carrier tetrahydrosarcinapterin. Oxidation of [CO] to CO<sub>2</sub> produces reduced ferredoxin (Fd<sub>red</sub>), which is oxidized by Ech, thereby generating H<sub>2</sub> inside the cell. H<sub>2</sub> diffuses across the cell membrane to the Vht active site, where it is oxidized and electrons are used to reduce methanophenazine (MP<sub>red</sub>). Reduction of the methyl group bound to coenzyme M by coenzyme B produces CH<sub>4</sub> and a disulfide of CoM and CoB, which is regenerated by reduction with electrons from MP<sub>red</sub> via the heterodisulfide reductase enzyme. Portions of the methanogenic pathway that are not required for aceticlastic methanogenesis, including the use of Frh, are shown in light gray.

( $\Delta G^\circ$ ) for this process is  $-131$  kJ per mole. However, when accounting for the low partial pressure of H<sub>2</sub> in natural environments, this value drops to between  $-17$  and  $-40$  kJ per mole. Therefore, less than 1 mole of ATP can be synthesized from ADP and P<sub>i</sub> (within the cell  $\Delta G^\circ = +50$  kJ per mole) for each mole of methane that is produced (2). The initial reduction of CO<sub>2</sub> to a formyl group attached to the C<sub>1</sub> carrier molecule methanofuran (MF) requires energy input in the form of a low-redox-potential ferredoxin (Fd;  $E^\circ \approx -500$  mV) (34–36). Methanogens have several strategies for generating reduced Fd (Fd<sub>red</sub>), which are discussed in the “Energy Conservation Mechanisms of Methanogens” section below. In one example, some species of *Methanosarcina* generate Fd<sub>red</sub> by the membrane-bound, proton-translocating energy-converting hydrogenase (Ech), wherein the energetically unfavorable reduction of Fd by H<sub>2</sub> oxidation is made possible by coupling the reaction to proton translocation from the outside to the inside of the cell (2, 37, 38). The formyl group is subsequently transferred from MF to another C<sub>1</sub> carrier molecule, tetrahydromethanopterin (H<sub>4</sub>MPT; alternatively, tetrahydrosarcinapterin [H<sub>4</sub>SPT] in species of *Methanosarcina*) and then converted to methenyl-H<sub>4</sub>MPT via a condensation reaction catalyzed by the cyclohydrolase enzyme, methenyl-H<sub>4</sub>SPT cyclohydrolase (Mch) (39–41). Two reduction steps follow (methenyl-H<sub>4</sub>MPT to methylene-H<sub>4</sub>MPT and methylene-H<sub>4</sub>MPT to methyl-H<sub>4</sub>MPT), in which the electron donor, reduced coenzyme F420 (F420<sub>red</sub>), is supplied by the F420-reducing hydrogenase (Frh) (42–44). After the exergonic transfer of the methyl group to the thiol of coenzyme M (CoM) by the membrane-bound methyl-H<sub>4</sub>MPT:CoM methyltransferase enzyme (Mtr) (45), the final reduction step occurs with the thiol group of coenzyme B (CoB) serving as the electron donor, resulting in production of methane and a mixed disulfide of CoM and CoB (CoM-CoB) (39, 46, 47). Continued methanogenesis relies on regeneration of CoM and CoB thiols by reducing the CoM-CoB disulfide, which is facilitated by the enzyme heterodisulfide reductase (Hdr). Non-cytochrome-containing

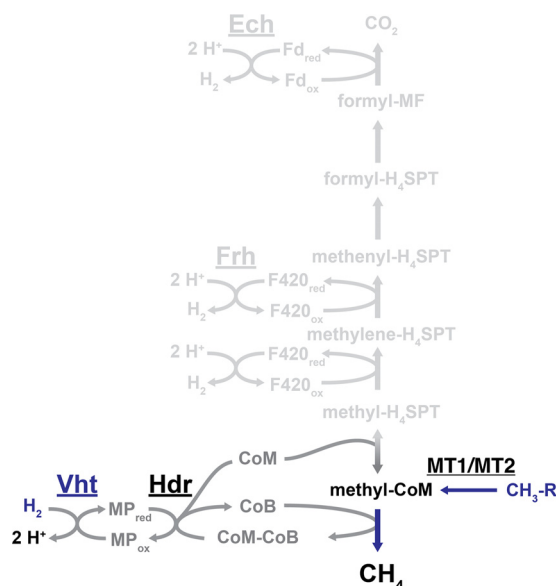


**FIG 4** The methylotrophic pathway of methanogenesis for *M. barkeri*. In this pathway, methyl compounds undergo disproportionation, wherein the oxidation of one methyl group to  $\text{CO}_2$  provides the reducing equivalents for the reduction of three additional methyl groups to  $\text{CH}_4$ . In the oxidation portion of the pathway, methyl groups are first transferred to coenzyme M by the sequential action of methyltransferases MT1 and MT2, and then transferred to tetrahydroscarinapterin by the methyl- $\text{H}_4\text{SPT}$ : CoM methyltransferase. The methyl group is oxidized in two successive steps, first to methylene- $\text{H}_4\text{SPT}$  and then to methenyl- $\text{H}_4\text{SPT}$ , which produces two reducing equivalents in the form of reduced coenzyme F420. The third reducing equivalent comes from the oxidation of formyl-methanofuran, which produces  $\text{CO}_2$  and reduced ferredoxin. In the reduction portion of the pathway, methyl-CoM is reduced by coenzyme B, which forms  $\text{CH}_4$  and a disulfide of CoM and CoB. Regeneration of CoM and CoB requires reduction of the disulfide by the heterodisulfide reductase enzyme with electrons from reduced methanophenazine. The transfer of electrons from the oxidative to reductive portions of the pathway occurs via a hydrogen cycling mechanism in *M. barkeri*. In this mechanism,  $\text{F420}_{\text{red}}$  and  $\text{Fd}_{\text{red}}$  are oxidized by Frh and Ech, respectively, which forms  $\text{H}_2$  on the inside of the cell. The  $\text{H}_2$  then diffuses across the membrane to the Vht active site on the outside of the cell, where it is oxidized and electrons are passed to MP, thereby completing the cycle. The methyl compound R groups include  $-\text{OH}$  (methanol),  $-\text{NH}_2$  (methylamine), and  $-\text{SH}$  (methanethiol). Other examples of methyl compounds include dimethylamine, trimethylamine, and dimethyl sulfide.

methanogens rely on a soluble, electron-bifurcating Hdr enzyme for this reaction, as discussed below. In *Methanosarcina* species, the membrane-bound and cytochrome-containing HdrED catalyzes this reaction with electrons ultimately derived from  $\text{H}_2$  by way of the methanophenazine-reducing hydrogenase (Vht) and the membrane-soluble electron carrier, methanophenazine (MP) (48). Thus, the reduction of  $\text{CO}_2$  to methane with electrons derived from  $\text{H}_2$  in *Methanosarcina* species requires three different types of hydrogenases.

### The Aceticlastic Pathway

It has been estimated that most biologically produced methane (ca. 2/3 globally) comes from the methyl group of acetate by the processes of syntrophic acetate oxidation or aceticlastic methanogenesis (49–51). The former process is facilitated by interaction between an acetate-oxidizing bacterium and an  $\text{H}_2$ -consuming,  $\text{CO}_2$ -reducing methanogen. Oxidation of acetate to  $\text{CO}_2$  and  $\text{H}_2$  is very energetically unfavorable ( $\Delta G^\circ = +95$  kJ per mole), unless the end products are rapidly consumed by the methanogen. Overall, coupled acetate oxidation and  $\text{CO}_2$  reduction is energetically favorable ( $\Delta G^\circ = -36.0$  kJ per mole), although the small amount of available energy is presumably split between the two organisms (51, 52). Syntrophic conversion of acetate to methane was originally identified in a thermophilic coculture containing a rod-shaped, acetate-oxidizing bacterium and a *Methanothermobacter*-like methanogen (51, 53). Subsequently, several other acetate-oxidizing bacteria, such as *Clostridium ultunense* strain BS, *Thermacetogenium phaeum* strain PB, and *Thermotoga lettingae*



**FIG 5** The methyl reductive methanogenic pathway in *Methanosarcina*. In this pathway, methyl groups are transferred to coenzyme M by the sequential action of methyltransferases MT1 and MT2 and then reduced to  $\text{CH}_4$  by coenzyme B, which forms a disulfide of CoM and CoB. Regeneration of CoM and CoB by the reduction of CoM-CoB occurs at the heterodisulfide reductase enzyme with electrons from reduced methanophenazine. The Vht hydrogenase generates  $\text{MP}_{\text{red}}$  with electrons derived from the oxidation of  $\text{H}_2$ . Portions of the core methanogenic pathway that are not used by the methyl reduction pathway, including Ech and Frh, are shown in light gray.

strain TMO, were identified in coculture with  $\text{CO}_2$ -reducing methanogens (*Methanococcus* sp. strain MAB1, *Methanothermobacter thermautotrophicus* strain TM, and *M. thermautotrophicus* strain  $\Delta\text{H}$ , respectively) (54–56).

Aceticlastic methanogenesis, which involves the dismutation of acetate to  $\text{CO}_2$  and methane by a single organism, has been observed only in two genera from the *Methanosarcinales* order, *Methanosarcina* and *Methanothermobacter* (formerly referred to as *Methanosaeta* [57]). Under standard conditions, aceticlastic methanogenesis has the lowest free energy of the known methane-producing pathways ( $\Delta G^\circ = -36$  kJ per mole) (58–60). In the first step of this pathway, acetate is converted into acetyl-CoA in an ATP-dependent manner. The acetyl group is then split into an enzyme-bound carbonyl group and a methyl group by the acetyl-CoA decarbonylase/synthase (ACDS) enzyme complex (Fig. 3) (61, 62). Oxidation of the carbonyl to  $\text{CO}_2$  results in  $\text{Fd}_{\text{red}}$ , which is used to reduce the methyl group to methane. After the initial cleavage of acetate, the methyl group is transferred to  $\text{H}_4\text{SPT}$ , and the same reduction process involving CoM, CoB, Hdr, and  $\text{MP}_{\text{red}}$  as described above for the  $\text{CO}_2$  reduction pathway occurs (60). In *Methanosarcina* species, there are two different mechanisms for transferring electrons from the oxidative to the reductive branches of the pathway. Species with active hydrogenases, such as *Methanosarcina barkeri*, use a hydrogen cycling mechanism, whereas species that are hydrogenase deficient, such as *Methanosarcina acetivorans*, have an  $\text{H}_2$ -independent electron transport system. In the  $\text{H}_2$  cycling mechanism, the Ech hydrogenase proceeds in the direction opposite that described for the  $\text{CO}_2$  reduction pathway by oxidizing  $\text{Fd}_{\text{red}}$ , which generates  $\text{H}_2$  and allows for proton translocation across the membrane, thereby contributing to the proton motive force (63).  $\text{H}_2$  then diffuses across the membrane and is oxidized by the Vht hydrogenase, where the electrons are used to reduce CoM-CoB via  $\text{MP}_{\text{red}}$  and Hdr (Fig. 3) (37, 64, 65). Species that utilize the  $\text{H}_2$ -independent electron transport system do not have Ech and instead use an Rnf complex to catalyze the transfer of electrons from  $\text{Fd}_{\text{red}}$  to MP (66, 67). Like Ech, Rnf translocates ions across the membrane due to the exergonic nature of MP reduction with  $\text{Fd}_{\text{red}}$  ( $\Delta G^\circ = -68$  kJ per mole). However, whereas Ech translocates protons, Rnf conserves energy by translocating sodium ions (68). Despite



these differences, *M. barkeri* and *M. acetivorans* have similar growth rates and yields on acetate, indicating that the two electron transport systems conserve approximately equivalent amounts of energy (69).

### The Methylo-trophic Pathway

Only members of the *Methanosarcinales* order, excluding *Methanotherix* species, are capable of utilizing the methylo-trophic pathway for methanogenesis (17). In this pathway, methyl compounds such as methanol, methylamines, and methyl sulfides serve as the source of carbon and energy. Under standard conditions, substantially more energy is available from methylo-trophic methanogenesis ( $\Delta G^\circ = -95$  and  $-90$  kJ per mole of methane from methanol and methylamine, respectively) than from acetoclastic methanogenesis (52, 59, 70). To serve as both the reductant and oxidant, methyl compounds are disproportionated such that the oxidation of one methyl group to  $\text{CO}_2$  provides the reducing equivalents needed for the reduction of three methyl groups to methane (46). At the outset, methyl groups are transferred to CoM by the sequential action of two methyltransferases (MT1 and MT2). *Methanosarcina* species encode multiple MT1 and MT2 isozymes that are specific for different methyl group-containing substrates (71). From the methyl-CoM level, methyl groups are either oxidized to  $\text{CO}_2$  in a reversal of the  $\text{CO}_2$  reduction pathway or reduced to methane by CoB in a manner that is identical in all four methanogenic pathways (Fig. 4). The oxidative portion of the pathway generates  $\text{F420}_{\text{red}}$  and  $\text{Fd}_{\text{red}}$ , which are used in the reductive branch of the pathway (72). As with acetoclastic methanogenesis, two different mechanisms are used to transfer electrons from the oxidative to the reductive portions of the pathway, depending on hydrogenase availability (33, 73). In hydrogenase-proficient species, such as *M. barkeri*, a hydrogen cycling mechanism is utilized in which  $\text{F420}_{\text{red}}$  and  $\text{Fd}_{\text{red}}$  are oxidized by the Frh and Ech hydrogenases, respectively, thereby generating  $\text{H}_2$  inside the cell. The  $\text{H}_2$  then diffuses across the cell membrane to the Vht hydrogenase active site, where it is oxidized and electrons are passed to MP to be used for the reduction of CoM-CoB (Fig. 4) (65). In hydrogenase-deficient species, such as *M. acetivorans*, two different membrane-bound enzyme complexes are utilized. The first, Rnf, transfers electrons from  $\text{Fd}_{\text{red}}$  to MP and functions as described for the acetoclastic pathway (66–68). The second enzyme complex, F420 dehydrogenase (Fpo), catalyzes the transfer of electrons from  $\text{F420}_{\text{red}}$  to MP (48). Fpo is closely related to NADH dehydrogenases found in bacteria and eukaryotes, and it similarly contributes to proton motive force by moving protons across the cell membrane (74, 75). It should be noted that *M. barkeri* is capable of both  $\text{H}_2$ -dependent and  $\text{H}_2$ -independent electron transport; however, mutant strains that lack Frh, and are thus limited to utilizing Fpo for F420:MP oxidoreductase activity, grow far slower than the wild-type strain (42).

### The Methyl Reduction Pathway

In addition to the *Methanosarcinales* order, only three other classes of methanogens are able to utilize the methyl reduction pathway, which includes a single genus (*Methanosphaera*) of the *Methanobacteriales* order, the *Methanomassiliicoccales* order, and the *Methanonatronarchaeales* order. Based on metagenomic data, newly discovered methanogens "*Ca. Methanofastidiosa*," "*Ca. Bathyarchaeota*," and "*Ca. Verstraetearchaeota*" are also likely to rely on this pathway (12–14). In the methyl reduction pathway, methyl compounds are reduced to methane with electrons derived from  $\text{H}_2$  (76). The amount of energy made available by this reaction is fairly high under standard conditions ( $\Delta G^\circ = -95$  kJ per mole), but is likely to be much lower under the  $\text{H}_2$ -limiting conditions found in natural environments (2). Additionally, a strain of *Methanosphaera* that was isolated from a kangaroo forestomach (sp. strain WGK6) is uniquely able to reduce methanol with electrons derived from ethanol oxidation (77). As many organisms are able to reduce  $\text{CO}_2$  with electrons derived from primary and secondary alcohols, it would not be surprising for future studies to identify organisms that can couple the oxidation of a variety of substrates to methyl reduction.

In *Methanosarcina* species, which are unable to utilize alcohols (other than methanol) as a substrate for methanogenesis, the transfer of a methyl group to CoM, reduction to methane by CoB, reduction of CoM-CoB by Hdr, and H<sub>2</sub>:MP oxidoreductase activity of Vht all occur as described for the CO<sub>2</sub> reduction, acetoclastic, and methylotrophic pathways (Fig. 5). The model for the methyl reduction pathway predicts that Ech and Frh should not be required for methanogenesis, which has been confirmed by mutational studies (37, 42). However, despite the ability to produce methane, the strain lacking Ech was unable to grow via this pathway unless supplemented with acetate or pyruvate, indicating that Ech has a biosynthetic role during growth with methyl compounds and H<sub>2</sub>. It is presumed that Ech is required for the H<sub>2</sub>-dependent production of Fd<sub>red</sub> that is required for acetyl-CoA and pyruvate synthesis (37).

### ENERGY CONSERVATION MECHANISMS OF METHANOGENS

Methanogens, like all living organisms, use energy derived from metabolic processes to drive growth and cellular maintenance. However, they are unable to generate net ATP via substrate-level phosphorylation and instead require ion gradient-dependent ATP generation via an ATP synthase as their principal energy conservation mechanism (70, 76). Due to the limited amount of energy available from methanogenic substrates, very few steps in the four methanogenic pathways are sufficiently exergonic to translocate ions across the cell membrane (2). Further, as mentioned above, there is a significant divide in energy conservation systems between methanogens that contain cytochromes and those that do not (2). Most methanogenic species belong to the second group, but cytochromes are ubiquitous within the order *Methanosarcinales*. While the utilization of cytochromes enables a unique energy conservation system, both groups share energy conservation mechanisms that are common to all methanogens (2, 70).

#### Establishing a Primary Na<sup>+</sup> Ion Gradient with Mtr

A characteristic feature of all characterized methanogens is the dependence on sodium ions (Na<sup>+</sup>) for growth and methanogenesis (2, 78, 79). This requirement is likely due to the energy conservation mechanism employed by the methyl-H<sub>4</sub>MPT:CoM methyltransferase (Mtr) (45, 80, 81). The transfer of a methyl group from H<sub>4</sub>MPT to CoM is exergonic ( $\Delta G^{\circ} = -29$  kJ per mole), thereby allowing for the translocation of approximately 2 Na<sup>+</sup> ions across the membrane and contributing to ion motive force (58). All methanogens that are able to utilize the CO<sub>2</sub> reduction or acetoclastic pathway (Fig. 2 and 3) have this mechanism for energy conservation. During methylotrophic methanogenesis, methyl transfer also occurs in the opposite direction (Fig. 4), such that consumption of the Na<sup>+</sup> ion motive force is required to facilitate this endergonic reaction (70, 82).

#### Electron Bifurcation and Methanogenesis as a Cycle

All methanogenic pathways generate a CoM-CoB disulfide in the terminal methane-generating step. Reduction of CoM-CoB is another energy-conserving reaction common to all methanogens; however, different mechanisms for this process have evolved in methanogens with cytochromes versus those without cytochromes. Those without cytochromes utilize an energy conservation mechanism that was only recently discovered: flavin-based electron bifurcation (FBEB) (2, 83). With this mechanism, the endergonic reduction of ferredoxin (Fd;  $E^{\circ} \approx -500$  mV) by H<sub>2</sub> oxidation ( $E^{\circ} = -414$  mV) is coupled to the exergonic reduction of CoM-CoB ( $E^{\circ} = -140$  mV) by H<sub>2</sub> oxidation. Thus, the overall reaction ( $\text{Fd}_{\text{ox}} + \text{CoM-CoB} + 2\text{H}_2 \rightarrow \text{Fd}_{\text{red}} + \text{CoM} + \text{CoB} + 2\text{H}^+$ ) is energetically favorable ( $\Delta G^{\circ} = -50$  kJ per mole) (83). The FBEB mechanism is facilitated by the MvhADG:HdrABC enzyme complex, wherein MvhADG catalyzes H<sub>2</sub> oxidation and HdrABC catalyzes CoM-CoB and Fd reduction (83–85). The HdrABC enzyme is a class of heterodisulfide reductase that is distinct from the HdrED enzyme found in *Methanosarcina* species and is neither cytochrome containing nor membrane bound (86, 87). Some cytochrome-deficient methanogens are also able to couple Fd and

CoM-CoB reduction to formate oxidation. In these organisms, HdrABC also forms a complex with a formate dehydrogenase (Fdh) (88, 89). The  $Fd_{red}$  produced by FBEB is required for the initial step in the  $CO_2$  reduction pathway, the reduction of  $CO_2$  to formyl-MF (Fig. 2), which has a reduction potential of  $\approx -500$  mV (83). Thus, in cytochrome-deficient methanogens the first and final steps of the methanogenic pathway are energetically linked via FBEB, leaving the  $Na^+$ -dependent Mtr reaction as the only mechanism for generating an ion gradient for ATP synthesis.

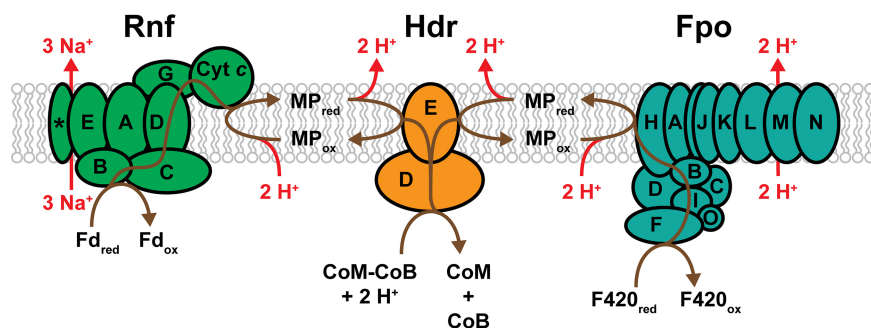
Coupling of the first and last methanogenic steps by FBEB confirms the cyclical nature of methanogenesis, as proposed 30 years ago by Rouvière and Wolfe (90). Recent elucidation of anaplerotic mechanisms required to replenish cycle intermediates that are withdrawn for biosynthesis further supports methanogenesis as a cycle (91). The cyclization of methanogenesis by way of FBEB is now referred to as the Wolfe cycle to honor the influence of Ralph S. Wolfe on the field of methanogenic biochemistry (92).

Some methanogens with cytochromes also encode one or more putative electron bifurcating heterodisulfide reductases that are homologous to HdrABC (93). In *Methanosarcina* species, the HdrA2 homolog is comprised of a domain similar to MvhD (as found in the MvhADG:HdrABC enzyme complex of cytochrome-deficient methanogens) fused to a C-terminal domain with homology to HdrA (85). *In vitro* data suggest that the HdrA2B2C2 complex from *M. acetivorans* catalyzes the endergonic reduction of Fd ( $E^\circ' \approx -500$  mV) by oxidation of  $F420_{red}$  ( $E^\circ' = -360$  mV) coupled to the exergonic reduction of CoM-CoB ( $E^\circ' = -140$  mV) by  $F420_{red}$ . The reaction ( $Fd_{ox} + CoM-CoB + 2 F420_{red} \rightarrow Fd_{red} + CoM + CoB + 2 F420_{ox}$ ) is energetically favorable, with a free energy of  $\approx -30$  kJ per mole (94). Although biochemically characterized, the *in vivo* role for this FBEB mechanism in *Methanosarcina* species has not been fully established. Indeed, a modified strain of *M. acetivorans* that does not produce HdrA2B2C2 is unaffected during growth with most substrates, with the exception of slower growth with acetate (87). As  $F420$  is not required for the acetoclastic pathway, it is not clear how this form of FBEB integrates into the energy conservation network of methanogens with cytochromes.

### Electron Transport in Methanogens with Cytochromes

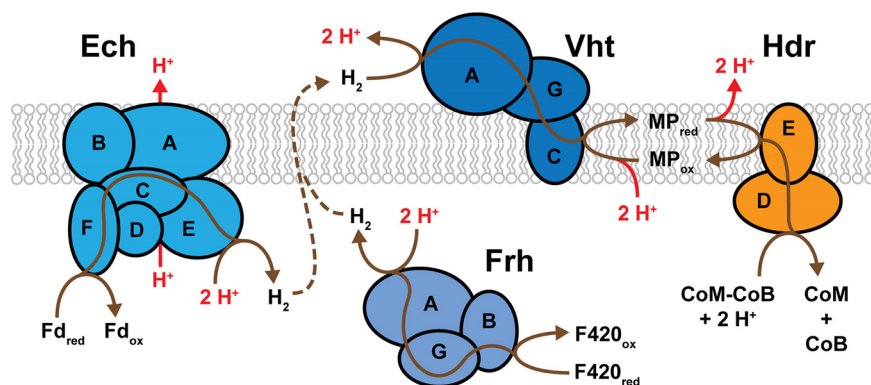
In contrast to methanogens without cytochromes, which can establish a transmembrane  $Na^+$  gradient only via Mtr, methanogens with cytochromes have multiple mechanisms for establishing an ion gradient via membrane-bound electron transport systems (2, 70). In each, the CoM-CoB disulfide serves as the terminal electron acceptor with translocation of  $H^+$  or  $Na^+$  ions across the membrane during electron transfer from a variety of donors (48). The electron transport mechanisms depend on the electron source ( $F420_{red}$ ,  $Fd_{red}$ , or  $H_2$ ) and on whether the organism can utilize  $H_2$  as a substrate (Fig. 6 and 7). Production of simultaneous  $H^+$  and  $Na^+$  electrochemical ion gradients is a unique characteristic of these methanogens, and efficient energy conservation requires that the electrical potential from both ions be used for ATP generation (95). Recent evidence suggests that the  $A_1A_O$  ATP synthase from *M. acetivorans* can translocate both  $H^+$  and  $Na^+$  ions for ATP synthesis, a unique property that is potentially shared by other *Methanosarcina* species (96). Nevertheless, these organisms also encode multiple  $Na^+/H^+$  antiporters, such as Mrp, to convert one ion gradient into the other or to optimize the ion ratio for the ATP synthase (70, 97). Significantly, the additional ion translocation sites within methanogens with cytochromes allow growth yields that are more than double than that of methanogens without cytochromes. Accordingly, the ATP yield per mole of methane generated during growth with  $CO_2$  and  $H_2$  is estimated to be 1.5 in methanogens with cytochromes and 0.5 in methanogens without cytochromes, which enables a growth yield of up to 7 g of cells per mole of methane in the former versus 3 g per mole of methane in the latter (2).

All characterized *Methanosarcina* species have an  $H_2$ -independent  $F420_{red}$ :CoM-CoB electron transport pathway (60, 70). During methylotrophic methanogenesis,  $F420_{red}$  ( $E^\circ' = -360$  mV) is produced during the oxidation of methyl groups to  $CO_2$ , and



**FIG 6** The  $H_2$ -independent electron transport system of *Methanosarcina*. Electrons enter the transport system as either reduced coenzyme F420 or reduced ferredoxin and are used for the reduction of the terminal electron acceptor, CoM-CoB. Reduction of CoM-CoB by the heterodisulfide reductase regenerates CoM and CoB for continued methanogenesis. F420 dehydrogenase (Fpo) catalyzes the exergonic transfer of electrons from F420<sub>red</sub> to MP with concomitant translocation of 2  $H^+$  ions to the outside of the cell. Similarly, the Rnf enzyme complex facilitates the exergonic transfer of electrons from Fd<sub>red</sub> to MP, but instead translocates 3  $Na^+$  ions outside of the cell. In both pathways, an additional 2  $H^+$  ions are consumed from the cytoplasm during MP reduction and released outside of the cell upon MP oxidation. Thus, the transport of 2 electrons results in 4  $H^+$  ions translocated for the F420<sub>red</sub>:CoM-CoB pathway and 3  $Na^+$  plus 2  $H^+$  ions translocated for the Fd<sub>red</sub>:CoM-CoB pathway (indicated in red). Brown lines trace the putative pathways of electron transport. Enzyme subunits are identified by letters, with the exception of the cytochrome c subunit (Cyt c) and a membrane-integral subunit with unknown function (\*) that are cotranscribed with the rest of the *rnf* operon.

CoM-CoB ( $E^{\circ\prime} = -143$  mV) is produced during the reduction of methyl groups to methane (Fig. 4) (98). The F420<sub>red</sub>:CoM-CoB electron transport pathway allows for the transfer of reducing equivalents between these oxidized and reduced electron carriers while also conserving energy in the form of an electrochemical  $H^+$  gradient. The key players in this electron transport chain are the 14-subunit F420 dehydrogenase (FpoABCDHFHIJJKLNMNO) and the membrane-bound, cytochrome-containing heterodisulfide reductase (HdrED). The two enzymes are linked by the membrane-soluble electron carrier methanophenazine ( $E^{\circ\prime} = -165$  mV [Fig. 6]) (98–102). The initial reduction of MP with electrons from F420<sub>red</sub> is exergonic ( $\Delta G^{\circ\prime} = -37.6$  kJ per mole), which allows 2  $H^+$  to be translocated to the outside of the cell by Fpo (75). Analysis of



**FIG 7** The  $H_2$ -dependent electron transport system of *Methanosarcina*. Electrons enter the transport system as either externally provided  $H_2$  or internally generated Fd<sub>red</sub> or F420<sub>red</sub>. All pathways involve the oxidation of  $H_2$  by Vht, and the transport of electrons by MP to the heterodisulfide reductase for reduction of the terminal electron acceptor, CoM-CoB. Regeneration of CoM and CoB enables continued methanogenesis. Frh and Ech generate  $H_2$  with electrons from F420<sub>red</sub> and Fd<sub>red</sub>, respectively. The internally produced  $H_2$  diffuses across the membrane (dashed line) and is oxidized by Vht, thereby transferring 2  $H^+$  ions from the inside to the outside of the cell. The exergonic reaction catalyzed by Ech facilitates additional proton translocation. For each pathway, 2  $H^+$  ions are consumed from the cytoplasm during MP reduction and released outside of the cell upon MP oxidation. Thus, the transport of 2 electrons results in 4  $H^+$  ions translocated for the F420<sub>red</sub>:CoM-CoB pathway and 5  $H^+$  ions translocated for the Fd<sub>red</sub>:CoM-CoB pathway (indicated in red). Brown lines trace the putative pathways of electron transport. Enzyme subunits are identified by letters.

the amino acid sequence of Fpo from *Methanosarcina* species indicates a high level of similarity to the NADH:quinone oxidoreductases (complex I) of bacteria and eukaryotes, which are also H<sup>+</sup>-translocating enzyme complexes (60). Thus, it seems likely that the two enzymes share energy conservation mechanisms (75, 103). A key difference between the two is the module for electron input. Fpo lacks a homolog to the NADH-oxidizing module of complex I (NuoEFG) and instead contains an F420<sub>red</sub>-oxidizing subunit, FpoF (75). An additional 2 H<sup>+</sup> ions are moved across the membrane during the transfer of electrons from MP<sub>red</sub> to CoM-CoB via HdrED, for a total of 4 H<sup>+</sup> ions translocated for every 2 electrons transported by the F420<sub>red</sub>:CoM-CoB system. The HdrE subunit is a membrane-bound, heme *b*-containing cytochrome, and it has been suggested that protons are translocated by a mechanism similar to that of the bacterial quinone loop (the MP loop) (104, 105). Reduction of CoM-CoB occurs at the active site of HdrD with electrons obtained from HdrE, thereby completing the electron transport pathway and regenerating the free CoM and CoB thiols for further rounds of methanogenesis (106).

Some species of *Methanosarcina*, such as *M. acetivorans*, also have an H<sub>2</sub>-independent Fd<sub>red</sub>:CoM-CoB electron transport pathway. With this system, Fd<sub>red</sub> generated by the oxidation of an enzyme-bound carbonyl group to CO<sub>2</sub> in the acetoclastic pathway (Fig. 3) or by the oxidation of formyl-MF to CO<sub>2</sub> in the methylotrophic pathway (Fig. 4) is used to reduce the terminal electron acceptor, CoM-CoB. In a mechanism similar to the F420<sub>red</sub>:CoM-CoB pathway, electrons from the reduced electron carrier (Fd<sub>red</sub>) are transferred to MP, which is used by HdrED to reduce CoM-CoB. The exergonic transport of electrons from Fd<sub>red</sub> ( $E^{\circ} = -500$  mV) to MP<sub>ox</sub> ( $E^{\circ} = -165$  mV;  $\Delta G^{\circ} = -65$  kJ per mole) is facilitated by the membrane-bound, Na<sup>+</sup>-translocating enzyme complex, Rnf (Fig. 6) (66–68, 107). It is estimated that 3 Na<sup>+</sup> ions are translocated for every 2 electrons transported by Rnf (68, 108). Combining this value with protons translocated by the HdrED-mediated MP loop brings the electrochemical total to 3 Na<sup>+</sup> ions 2 H<sup>+</sup> ions moved to the outside of the cell for every 2 electrons that pass through the Fd<sub>red</sub>:CoM-CoB electron transport pathway. The Rnf complex has been identified in a wide array of bacterial species and typically catalyzes Fd<sub>red</sub>:NAD<sup>+</sup> oxidoreductase activity with concomitant Na<sup>+</sup> translocation (109). However, it has been shown that Rnf from *Methanosarcina* does not interact with NADH/NAD<sup>+</sup> (66, 68). Additionally, the gene cluster encoding Rnf (*rnfCDGEAB*) differs from all known bacterial versions by encoding a multiheme *c*-type cytochrome upstream of *rnfC* and a gene downstream of *rnfB* that is predicted to integrate into the membrane. Both genes are cotranscribed with the rest of the *rnf* operon, and it is thought that the *cytochrome c* subunit is responsible for transferring electrons to MP (67).

*Methanosarcina* species that are able to metabolize H<sub>2</sub>, such as *M. barkeri*, have an H<sub>2</sub>-dependent electron transport system, wherein H<sub>2</sub> can serve as the sole source of electrons (H<sub>2</sub>:CoM-CoB pathway) or as an intermediate (Fd<sub>red</sub>:CoM-CoB and F420<sub>red</sub>:CoM-CoB pathways). In all three, the transport of electrons from H<sub>2</sub> to CoM-CoB (i.e., the H<sub>2</sub>:CoM-CoB pathway) occurs by the same mechanism; only the source of H<sub>2</sub> varies between the pathways (Fig. 7). Electron transport is initiated by the membrane-bound Vht, which oxidizes H<sub>2</sub> at an externally located active site. Electrons are then transferred to MP by way of the cytochrome *b*-containing subunit (VhtC) (48, 74, 110). HdrED facilitates the transfer of electrons from MP<sub>red</sub> to CoM-CoB, as described above for H<sub>2</sub>-independent electron transport systems. Oxidation of H<sub>2</sub> by Vht produces 2 external H<sup>+</sup> ions, which, when combined with translocated protons from the MP loop, results in a total proton motive force of 4 H<sup>+</sup> ions per 2 electrons transported (74).

The only electron source for the CO<sub>2</sub> reduction (Fig. 2) and methyl reduction (Fig. 5) methanogenic pathways is external H<sub>2</sub>. During growth via these pathways, *M. barkeri* uses the H<sub>2</sub>:CoM-CoB electron transport pathway for energy conservation (111). However, metabolism of acetate and methyl compounds via the acetoclastic and methylotrophic pathways produces internal electron sources (Fd<sub>red</sub> and F420<sub>red</sub>) as described above. The transport of electrons from Fd<sub>red</sub> and F420<sub>red</sub> to CoM-CoB via the H<sub>2</sub>-dependent electron transport pathways involves a hydrogen cycling mechanism (Fig. 7)

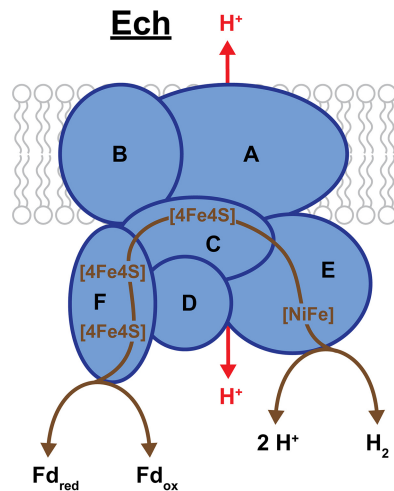
(37, 64, 65). In the first part of this mechanism,  $Fd_{red}$  and  $F420_{red}$  are oxidized by the Ech and Frh hydrogenases, respectively, and electrons are used to generate  $H_2$  on the inside of the cell with concomitant consumption of cytoplasmic protons. The  $H_2$  then diffuses across the membrane to the Vht active site, where it is oxidized releasing extracellular protons and feeding electrons to CoM-CoB via the  $H_2$ :CoM-CoB electron transport pathway. The hydrogen cycling mechanism was confirmed in a series of hydrogenase mutants, wherein removal of Vht caused rapid accumulation of  $H_2$ , diminished methanogenesis, and cell death. However, when both Vht and Frh were removed this effect was abrogated, indicating that Vht is required to capture  $H_2$  produced by Frh in order to maintain redox balance (65). The production of  $H_2$  inside the cell consumes 2 internal  $H^+$  ions, and oxidation by Vht produces 2 external  $H^+$  ions. Combination with protons translocated via the MP loop yields a total of 4  $H^+$  moved across the membrane for 2 electrons that pass through the electron transport chain (42, 65). Additional energy is conserved by the proton-pumping Ech hydrogenase during the generation of  $H_2$  from  $Fd_{red}$ , as this exergonic reaction enables auxiliary proton translocation (63). Thus, a similar amount of energy is conserved as an ion motive force by the  $H_2$ -dependent and  $H_2$ -independent electron transport systems (69). As highlighted by the use of  $H_2$  as an electron source and as an electron carrier, this molecule serves as an important metabolite, and the hydrogenase enzymes that enable its use are an essential component of methanogenic energy conservation systems.

### **HYDROGENASES OF METHANOGENS**

Organisms from all three domains of life rely on the activity of hydrogenases to consume  $H_2$  as a substrate or to produce  $H_2$  from the reduction of protons. Hydrogenases belong to a large and diverse group of enzymes and are classified into groups based on their cognate electron-carrying redox partners ( $NAD^+$ , Fd, F420, MP, etc.), active-site cofactors ([NiFe], [FeFe], or [Fe]), location within the cell (membrane bound or cytoplasm), or ability to conserve energy by ion translocation (112, 113). Methanogens use five different types of hydrogenase. Four of these belong to the [NiFe] group of hydrogenases, as classified by the active site transition-metals used for catalysis (112). The core of all [NiFe] hydrogenases consists of a heterodimer containing a "large" and a "small" subunit. The large subunit contains the [NiFe] active site, and the small subunit typically contains three linearly arranged Fe-S clusters of the cubane [4Fe4S]-type, which facilitate electron transport between the active site and cognate redox partner (113). Despite different mechanisms for energy conservation between methanogens with and without cytochromes, two types of [NiFe] hydrogenases are present in both methanogen classes. These include the membrane-bound energy-converting hydrogenases, such as the Ech hydrogenase found in *M. barkeri*, and a cytoplasmic F420-reducing hydrogenase, such as Frh. A third type of hydrogenase, exemplified by Vht in *M. barkeri*, contains a cytochrome and is found only in species of *Methanosarcina* (3). The fourth type of [NiFe] hydrogenase is Mvh, which forms a complex with the electron-bifurcating HdrABC and which is found solely in noncytochrome methanogens. Finally, a unique hydrogenase that is also found only in cytochrome-deficient methanogens is the nickel-free [Fe]-hydrogenase, which contains a unique single-Fe cofactor that is not found in any other enzyme, including the [Ni/Fe] and [Fe/Fe] hydrogenases (3, 85, 114, 115). Under nickel-limiting conditions, the [Fe]-hydrogenase replaces the F420-reducing [NiFe] hydrogenase activity required for the reduction of methenyl- $H_4$ MPT to methylene- $H_4$ MPT (Fig. 2) (116, 117). Under these growth conditions, the [Fe]-hydrogenase is also responsible for synthesis of reduced F420, which is required for numerous metabolic processes, by coupling the F420-dependent and  $H_2$ -dependent methylene dehydrogenase activities.

#### **The Energy-Converting Hydrogenase of *Methanosarcina barkeri***

The membrane-bound, energy-converting [NiFe] hydrogenases are evolutionarily distinct from other hydrogenases based on amino acid sequence alignments (113, 118). Aside from the conserved residues required to coordinate the [NiFe] active site and Fe-S



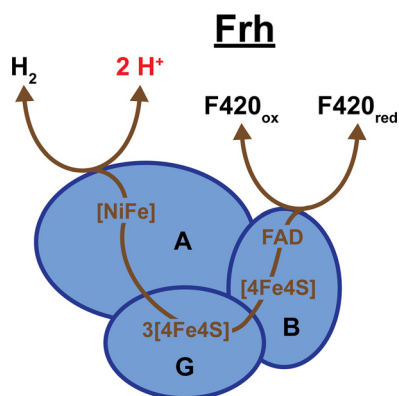
**FIG 8** Ech of *M. barkeri*. Ech consists of 6 subunits (EchABCDEF), of which EchA and EchB are membrane bound and EchA is the likely location for H<sup>+</sup> translocation (shown in red). The remaining 4 subunits are located in the cytoplasm and facilitate the transfer of electrons between Fd and H<sub>2</sub>/H<sup>+</sup> by way of 3 cubane Fe-S clusters ([4Fe4S]). EchE contains the bimetallic [NiFe] active site for H<sub>2</sub> formation/oxidation. Bidirectional arrows indicate Ech is a reversible enzyme, and brown lines trace the putative pathway of electron transport. In one direction, the exothermic oxidation of Fd<sub>red</sub> allows for internal H<sub>2</sub> production and translocation of H<sub>2</sub> to the outside of the cell.

clusters, this type of hydrogenase has very little sequence similarity to other [NiFe] hydrogenases (38). The core of all energy-converting hydrogenases contains at least six subunits; two membrane-bound hydrophobic subunits, two soluble hydrophilic subunits, and the large and small hydrogenase subunits. These six subunits, as typified by Ech from *M. barkeri* (Fig. 8), are highly similar to the subunits that form the catalytic core of the ion-translocating NADH:quinone oxidoreductase (complex I) of mitochondria and bacteria (38, 119). In fact, energy-converting hydrogenases are predicted to be ancestral to complex I (38).

All *Methanosarcina* species that are able to reduce CO<sub>2</sub> with H<sub>2</sub> encode an energy-converting hydrogenase. Initial identification of this enzyme in *M. barkeri* indicated that it was highly similar to *Escherichia coli*-3-type hydrogenase, and it was thereby designated Ech (64, 120). Further elucidation of the coupled hydrogenase and ion-translocating activities of this enzyme led to the more accurate “energy-converting hydrogenase” designation for Ech (118). This 6-subunit enzyme (EchABCDEF) drives the endergonic reduction of Fd ( $E^{\circ'} \approx -500$  mV) with electrons from H<sub>2</sub> oxidation ( $E^{\circ'} = -414$  mV) by utilizing the proton motive force (Fig. 8) (38, 64, 121). Ech is reversible, such that oxidation of Fd<sub>red</sub> leads to both the generation of H<sub>2</sub> and the translocation of H<sup>+</sup> outside of the cell (63, 122, 123). EchA and EchB are the membrane-bound subunits, with EchA being the most probable location for H<sup>+</sup> translocation based on sequence similarity to ion-translocating proteins in other organisms. The large and small hydrogenase subunits, EchE and EchC, are soluble and located within the cytoplasm. EchE contains the [NiFe] active site, and EchC contains only one [4Fe4S] cluster, which distinguishes it from the small subunit of other [NiFe] hydrogenases that typically have three clusters. However, another subunit, EchF, contains two additional [4Fe4S] clusters and is the location of Fd oxidation/reduction (3, 38, 124). Studies of an *M. barkeri* mutant lacking Ech indicate that this hydrogenase is required for production of methane via both the CO<sub>2</sub> reduction and acetoclastic pathways (Fig. 2 and 3) (37, 111). Additionally, this mutant strain requires supplementation with biosynthetic precursors during growth via the methyl reduction pathway (Fig. 5), showing that Ech is required to provide the Fd<sub>red</sub> needed for pyruvate and acetyl-CoA synthesis (37).

### The Coenzyme F420-Reducing Hydrogenase

The F420-reducing hydrogenase (Frh) has a critical role in the CO<sub>2</sub> reduction



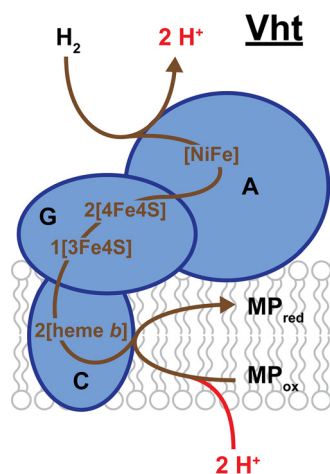
**FIG 9** Frh. Frh is a heterotrimeric enzyme, consisting of a large subunit (FrhA), small subunit (FrhG), and a flavin adenine dinucleotide (FAD)-containing subunit (FrhB). FrhA contains the [NiFe] active site for formation or oxidation of  $H_2$ , and FrhB has an FAD-containing active site for coenzyme F420 oxidation or reduction. Electrons are transmitted between active sites by four cubane Fe-S clusters ([4Fe4S]) located in FrhG and FrhB. Bidirectional arrows indicate Frh is a reversible enzyme, and brown lines trace the putative pathway of electron transport.

pathway of methanogenesis (Fig. 2) by providing  $F420_{red}$  required for two reduction steps. Thus, all methanogens that are able to reduce  $CO_2$  with  $H_2$ -derived electrons require an Frh hydrogenase, and this enzyme has been purified from methanogens both with and without cytochromes (125–129). Frh is a reversible enzyme that catalyzes the reduction of F420 with electron obtained by the oxidation of  $H_2$  (Fig. 9). Under standard conditions this reaction has a free energy change of  $\Delta G^{\circ'} = -11$  kJ per mole. However, under *in vivo* conditions there is very little free energy change, and Frh activity is not directly coupled to energy conservation (3). Indeed, the redox state of F420 has been found to be in rapid equilibrium with the external concentration (partial pressure) of  $H_2$  (65, 130).

Initial purification and characterization of Frh indicated that the core of this hydrogenase consists of three subunits (FrhAGB) that form a membrane-associated heterotrimer (129, 131, 132). However, Frh was later found to be located within the cytoplasm and not membrane bound, as none of the subunits contain transmembrane helices. Frh, as purified from *Methanothermobacter*, forms a complex consisting of at least 8  $\alpha/\beta/\gamma$  heterotrimers (129, 131). Recently, the structure of Frh from *Methanothermobacter marburgensis* was determined by cryo-electron microscopy, which suggested that the enzyme is made up of 12 copies of the  $\alpha/\beta/\gamma$  heterotrimer (133). The structure determination also verified the locations of key components for electron transport within this enzyme: the [NiFe] active site, [4Fe4S] clusters, and flavin adenine dinucleotide (FAD) (Fig. 9). FrhA is the large hydrogenase subunit and contains the bimetallic [NiFe] active site for  $H_2$  oxidation or formation. FrhB facilitates F420 redox reactions with an FAD-containing active site. Transport of electrons between the [NiFe] and FAD active sites occurs via four [4Fe4S] clusters. Three of these clusters are located in the small hydrogenase subunit, FrhG, and the fourth is located within FrhB (3, 133).

Whereas methanogens without cytochromes use Frh solely to produce  $F420_{red}$  from  $H_2$  for two sequential reduction steps in the  $CO_2$  reduction pathway, methanogens with cytochromes are also able to take advantage of the reversibility of Frh during methylotrophic methanogenesis (Fig. 4) (42, 65). In this pathway,  $F420_{red}$  generated by the oxidation of methyl groups to  $CO_2$  is oxidized by Frh to form  $H_2$ . Diffusion of  $H_2$  to the external Vht hydrogenase active site allows electrons from  $F420_{red}$  to enter the electron transport system by way of the hydrogen cycling mechanism discussed above (65). A mutant strain of *M. barkeri* that lacks the Frh hydrogenase is still able to utilize the methylotrophic pathway for methanogenesis; however, the growth rate and final yield are severely diminished, indicating that the Frh-Vht mediated hydrogen cycling mechanism is the preferred method of electron transport (42). Thus, Frh plays an important role in both the  $CO_2$  reduction and methylotrophic pathways.





**FIG 10** Vht of *M. barkeri*. The large hydrogenase subunit (VhtA) is located on the outside face of the cytoplasmic membrane and contains the bimetallic [NiFe] active site where  $H_2$  is oxidized. This reaction generates 2 external  $H^+$  ions that contribute to proton motive force (shown in red) and 2 electrons that are transported via Fe-S clusters ([4Fe4S] and [3Fe4S]) located in the small subunit (VhtG) to heme *b* groups in the membrane-bound cytochrome *b* subunit (VhtC). Reduction of the electron carrier MP by VhtC involves uptake of 2  $H^+$  ions from the cytoplasm, which are released externally upon MP oxidation (not shown). Brown lines trace the putative pathway of electron transport.

### The Methanophenazine-Reducing Hydrogenase

Only methanogens with cytochromes have an MP-reducing hydrogenase, which plays a critical role in all four methanogenic pathways. Vht (as the MP-reducing hydrogenase is designated in *M. barkeri*) catalyzes the exergonic reduction of MP with electrons obtained by the oxidation of  $H_2$  ( $\Delta G^{\circ\prime} = -50$  kJ per mole) (2). Energy is conserved by the production of 2 external  $H^+$  ions from  $H_2$  oxidation at the Vht active site on the outside of the cell, thereby contributing to the transmembrane proton gradient (60). The Vht-generated  $MP_{red}$  is then used for reduction of the terminal electron acceptor common to all methanogenic pathways, CoM-CoB, as described for the  $H_2$ -dependent electron transport system (48, 74). During methanogenesis via the  $CO_2$  reduction and methyl reduction pathways  $H_2$  is provided externally, and for the acetoclastic and methylotrophic pathways  $H_2$  is produced internally by Ech and Frh (37, 64, 65). The inability of an *M. barkeri* mutant strain lacking Vht to grow on any substrate supports the essentiality of Vht in *Methanosarcina* strains that rely on  $H_2$  as a substrate and for electron transport (65).

Vht was first characterized from cell extracts of *Methanosarcina mazei* and *M. barkeri*. Initial purification of Vht from membrane fractions indicated that it was a two-subunit, membrane-bound hydrogenase (134, 135). These subunits consisted of VhtA, the large subunit containing the [NiFe] active site, and VhtG, the small subunit containing three Fe-S clusters (Fig. 10). The third subunit, VhtC, was later identified by experiments searching for genes that encode Vht, as the *vht* operon contains genes for all three subunits (136, 137). VhtC is a membrane-spanning cytochrome *b* protein that contains two heme *b* prosthetic groups as the location for MP reduction. The VhtA and VhtG subunits were determined to be located on the outer face of the cell membrane based on the presence of a twin-arginine-translocation (Tat) signal peptide on the N terminus of VhtG and on the high level of homology to cytochrome *b*-containing hydrogenases from bacterial species that are located in the periplasm (3, 138, 139). Thus, the external active site of Vht allows for the extraction of electrons from  $H_2$  while simultaneously contributing to the proton gradient, which can be used to generate ATP.

### CONCLUSION

Despite wide-ranging environmental and phylogenetic diversity, most methanogens lack cytochromes and are limited to a single process for methane production, the  $CO_2$  reduction pathway. However, overlapping metabolic pathways of cytochrome-

containing methanogens, as exemplified by *Methanosarcina* species, allow this type of methanogen to use a comparatively large number of substrates for growth and methanogenesis. While the methanogenic pathways have been largely characterized, important aspects of energy conservation mechanisms in both types of methanogens, such as electron bifurcation and the involvement of hydrogenases in electron flow, are continually being discovered (2, 65, 83, 111). These concepts have a broad impact, as similar mechanisms have been found in multiple domains of life (65, 94). Additionally, investigation of newly identified methanogen classes, both within and outside of the *Euryarchaeota* phylum, may reveal novel methanogenic and energy conservation mechanisms. Thus, the unique biochemistry of methanogens continues to be a rich source for future studies uncovering fundamental properties of life.

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