Fundamentals of methanogenic pathways that are key to the biomethanation of complex biomass
James G Ferry

The conversion of biomass to CH₄ (biomethanation) involves an anaerobic microbial food chain composed of at least three metabolic groups of which the first two decompose the complex biomass primarily to acetate, formate, and H₂. The thermodynamics of these conversions are unfavorable requiring a symbiosis with the CH₄-producing group (methanogens) that metabolize the decomposition products to favorable concentrations. The methanogens produce CH₄ by two major pathways, conversion of the methyl group of acetate and reduction of CO₂ coupled to the oxidation of formate or H₂. This review covers recent advances in the fundamental understanding of both methanogenic pathways with the view of stimulating research towards improving the rate and reliability of the overall biomethanation process.

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Introduction
Earth’s biosphere contains diverse oxygen-free (anaerobic) environments where complex organic matter is decomposed to CH₄ in a process called biomethanation that is an essential link in the global carbon cycle (Figure 1). In the cycle, atmospheric CO₂ is fixed into plant biomass primarily via oxygenic photosynthesis (step 1). Microbes in aerobic environments digest the biomass consuming O₂ and returning CO₂ to the atmosphere (step 2). A portion of the biomass also enters diverse O₂-free environments (step 3) such as the lower intestinal tract of humans, wetlands, rice paddy soils, and the rumen of livestock where anaerobic microbes digest the organic matter producing CO₂ and CH₄ (steps 4–6) in a ratio of approximately 1:1 (biogas). In freshwater environments, biomethanation involves a minimum of three metabolic groups from the domains Bacteria and Archaea. The fermentative group decomposes biomass primarily to butyrate, propionate, acetate, formate, and H₂ plus CO₂ (step 4). The obligate proton-reducing acetogenic group (acetogens) converts the butyrate and propionate to acetate, CO₂, and H₂ or formate (step 5). The thermodynamics of these conversions are unfavorable under standard conditions of equimolar reactants and products (Table 1) requiring a symbiosis with the methane-producing group (methanogens) that metabolizes the products to thermodynamically favorable levels (step 6). The methanogens produce CH₄ by two major pathways (Table 1). In the CO₂-reduction pathway, formate or H₂ is oxidized and CO₂ is reduced to CH₄. In the aceticlastic pathway, acetate is cleaved with the carbonyl group oxidized to CO₂ and the methyl group reduced to CH₄. In most freshwater environments, the aceticlastic group is responsible for approximately two-thirds with most of the remaining one-third produced by CO₂-reducing methanogens. Smaller, yet significant amounts of CH₄ are produced from the methyl groups of methanol, methylamines, and dimethylsulfide. Some of the CH₄ is oxidized to CO₂ (step 7) by a consortia of anaerobes that reduce either sulfate, nitrate, manganese, or iron [1]. The CH₄ also diffuses into aerobic environments (step 8) where O₂-requiring methanotrophic microbes oxidize it to CO₂ (step 9).

The biomethanation of organic matter is exploited for the disposal of organic waste and production of biogas from renewable plant material as an alternative to fossil fuels. Domestic and industrial wastes are disposed of in large-scale municipal sewage treatment plants, reducing the volume of volatile solids as much as 75% and producing electricity with biogas-powered generators that serve the facility and contribute to the power grid. In rural areas of developing countries, biomethanation is utilized for the small-scale disposal of domestic waste and use of the biogas for home heating and cooking. Large-scale production of biogas from sustainable plant biomass has the potential for contributing to the replacement of fossil fuels. On a weight for weight basis, the energy content of CH₄ is approximately 3-fold greater than H₂, and CH₄ is stored and transported in a more efficient and safer manner than H₂. Through catalytic reformation, CH₄ is converted into methanol as a chemical feed stock that further reduces the dependence on petroleum. This review of the recent literature introduces the physiology of CH₄-producing microbes, key players in the production of biogas from organic matter, providing the reader with a background suitable for stimulating research.
aimed towards improving the process that will ensure biogas among the competitive alternatives to fossil fuels.

Reactions common to aceticlastic and CO₂-reducing pathways

Figure 2 shows a composite of CO₂-reducing and aceticlastic pathways, and the overall reactions are shown in Table 1. Both pathways have in common reactions 10–12 (Figure 2) but differ in reactions generating either methyl-tetrahydrosarcinapterin (CH₃-H₄MPT) in the aceticlastic pathway (reactions 1–4) or methyl-tetrahydromethanopterin (CH₃-H₄MPT) in the CO₂-reduction pathway (reactions 5–9). The H₄SPT and H₄MPT cofactors are functionally equivalent analogs of tetrahydrofolate. In reaction 10, the methyl group of CH₃-H₄M(S)PT is transferred to HS-CoM followed by reductive demethylation of CH₃-S-CoM to CH₃ with electrons donated by HS-CoB (reaction 11). Reaction 10 is catalyzed by CH₃-H₄M(S)PT:coenzyme M (HS-CoM) methyltransferase (Mtr), a membrane-bound eight-subunit complex that couples the exergonic methyl transfer to the generation of a sodium ion gradient (high outside).

Methyl-coenzyme M reductase (Mcr) catalyzes reaction 11 wherein the methyl group of CH₃-S-CoM is reduced to CH₃ with HS-CoB the electron donor and the heterodisulfide CoM-S-S-CoB is an added product. The enzyme is also proposed to activate CH₃ for anaerobic oxidation to CO₂ [2]. The crystal structure of Mcr from *Methanothermobacter marburgensis* (f. *Methanobacterium thermoautotrophicum* strain Marburg) shows two active sites, each with a Ni-containing cofactor called F₄₃₀ [3]. In dispute are two proposed mechanisms, investigated at late, that diverge in how the C–S bond of CH₃-S-CoM is cleaved. Mechanism A (also referred to as mechanism II) predicts the nickel of Ni₄F₄₃₀ attacking the sulfur atom of CH₃-S-CoM producing a CH₃ radical and CoM-S-Ni₄F₄₃₀ [4]. The methyl radical then abstracts a hydrogen from HS-CoB producing

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**Table 1**

<table>
<thead>
<tr>
<th>Reactions in the syntrophic metabolism of obligate proton-reducing acetogens and methanogens.</th>
<th>ΔG°′ (kJ/mol)</th>
</tr>
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<tbody>
<tr>
<td>Propionate + 3H₂O → acetate + HCO₃⁻ + H⁺ + 3H₂</td>
<td>+76.1</td>
</tr>
<tr>
<td>Butyrate + 2H₂O → 2acetate + H⁺ + 2H₂</td>
<td>+48.6</td>
</tr>
<tr>
<td>4H₂ + HCO₃⁻ + H⁺ → CH₄ + 3H₂O</td>
<td>−135.6</td>
</tr>
<tr>
<td>4Formate + H⁺ + H₂O → 3HCO₃⁻ + CH₄</td>
<td>−130.4</td>
</tr>
<tr>
<td>Acetate → HCO₃⁻ + H⁺ + CH₄</td>
<td>−36.0</td>
</tr>
</tbody>
</table>
\(^*\)S-CoB and CH\(_4\). The \(^*\)S-CoB radical reacts with CoM-S-Ni\(^{11}\)F\(_{430}\) producing the anion radical CoM-S-S\(^*\)-CoB\(^-\) that donates the extra electron to Ni\(^{11}\)F\(_{430}\) regenerating the active Ni\(^{11}\)F\(_{430}\) species and yielding CoM-S-S-CoB. Mechanism B (also called mechanism 1) proposes a nucleophilic attack of Ni\(^{3+}\) on the methyl of CH\(_2\)-S-CoM generating CH\(_2\)-Ni\(^{11}\)F\(_{430}\) and \(^*\)S-CoM. Transfer of an electron from \(\text{S-CoM} \rightarrow \text{CH}_2\text{-Ni}^{11}\text{F}_{430}\) produces the \(^*\)S-CoM radical and CH\(_2\)-Ni\(^{11}\)F\(_{430}\), the protonolysis of which produces CH\(_4\). The Ni\(^{11}\)F\(_{430}\) is reduced to the active Ni\(^{11}\)F\(_{430}\) form and CoM-S-S-CoB is produced the same as in mechanism A. Unfortunately a crystal structure of the reduced active enzyme reacted with the natural substrate CH\(_2\)-S-CoM has not been obtained and the proposed intermediates have not been trapped. Nonetheless, deuterium exchange studies are the basis for a proposal considering both \(\sigma\)-alkane-Ni\(^{11}\)F\(_{430}\) and alkane-Ni\(^{11}\)F\(_{430}\) complexes as intermediates [5]. X-ray absorption spectroscopy of Mcr reacted with methyl iodide, together with density functional theory, indicates a stable CH\(_2\)-Ni\(^{11}\)F\(_{430}\) species consistent with mechanism B [6]. Stable synthetic CH\(_2\)-Ni\(^{11}\) complexes are reported [7] that contain Ni-C bond lengths equivalent to the lengths reported for the enzyme-containing CH\(_2\)-Ni\(^{11}\)F\(_{430}\) species [6]. Further support for mechanism B was obtained by reacting Mcr with the natural substrate CH\(_2\)-S-CoM and an analog of HS-CoB at reduced rates which allowed transient kinetics to detect an alkyl-Ni\(^{11}\) intermediate and an unidentified organic radical that decays at a catalytically competent rate [8]. Finally, it has been suggested that binding of HS-CoB subsequent to binding of CH\(_2\)-S-CoM induces a conformational change that moves CH\(_2\)-S-CoM to within a distance of F\(_{430}\) that initiates the reaction. Recent crystal structures of inactive Mcr (Ni\(^{11}\)F\(_{430}\)) failed to show significant conformational changes when complexed with HS-CoB analogs [9]. On the other hand, crystallographic and spectroscopic approaches investigating Mcr(Ni\(^3\)) complexed with an HS-CoB analog show that binding of HS-CoB induces a major conformational change in the active site with implications for predicting Ni coordination geometries [10]. One approach that has not been employed effectively to resolve the catalytic mechanism is the use of site-directed mutagenesis of the over-expressed enzyme in conjunction with substrate analogs and crystallography to trap and identify reaction intermediates. However, over-expression of an active form of an iron-containing carbonic anhydrase (Cam) from Methanosarcina thermophila in Methanosarcina acetivorans provides precedent for over-expression of other metalloenzymes such as Mcr in an active form [11].

The CoM-S-S-CoB produced by Mcr is reduced to HS-CoB and HS-CoM (reaction 12, Figure 2) catalyzed by heterodisulfide reductase (Hdr) for which there are two types. A two-subunit (HdRE) enzyme functions in the acetaticlastic pathway of Methanosarcina species [12], and a three-subunit (HdRBC) enzyme functions in obligate CO\(_2\)-reducers [13**].

**Electron transport and ATP synthesis in the acetaticlastic and CO\(_2\)-reducing pathways**

All acetotrophic methanogens obtain energy for growth by coupling electron transfer from ferredoxin to CoM-S-CoB with generation of a proton gradient that drives ATP synthesis via the proton-translocating “archaeal” A\(_3\)F\(_{2}\)-type ATP synthase [14**,15]. In freshwater Methanosarcina species (Figure 3), ferredoxin donates electrons to a membrane-bound hydrogenase (Ech) that evolves H\(_2\) and generates a proton gradient driving ATP synthesis [16,17]. It is proposed that a membrane-bound F\(_{430}\)-nonreducing hydrogenase (Vho) reoxidizes H\(_2\) and donates electrons to a quinone-like electron carrier methanophenazine that mediates electron transfer to the heterodisulfide reductase while translocating protons that contribute to the proton gradient. On the other hand, the acetotrophic marine isolate M. acetivorans does not encode an Ech hydrogenase, and proteomic analyses indicate that acetate-grown cells synthesize subunits with sequence identity to an Rnf complex first described in Rhodobacter capsulatus from the domain Bacteria [18]. Gene knockouts confirm that the complex is essential for growth.
for growth on acetate [12]. Thus, it is anticipated that the Rnf complex replaces Ech as an acceptor of electrons from ferredoxin and donor to methanophenazine accompanied by translocation of ions contributing to the gradient driving ATP synthesis.

Electron transfer from H₂ to CoM-S-S-CoB (reaction 12, Figure 2) in the CO₂-reducing pathways of freshwater Methanosarcina and obligate CO₂-reducing species differs significantly. In freshwater Methanosarcina, the H₂:CoM-S-S-CoB oxidoreductase system is identical to the proton pumping segment of electron transport in the aceticlastic pathway involving the F₄₂₀-nonreducing hydrogenase, methanophenazine, and the HdrDE heterodisulfide reductase. However, the membrane-bound electron transport chain of Methanosarcina species is absent in obligate CO₂-reducing species with no apparent mechanism for generating an ion gradient. Instead, the H₂:CoM-S-S-CoB oxidoreductase system is composed of the cytoplasmic F₄₂₀-nonreducing hydrogenase (MvhAGD) tightly bound to HdrABC with no experimentally determined mechanism for generating an ion gradient. One possibility for ATP synthesis is the sodium gradient generated by the membrane-bound methyl-H₄SPT:coenzyme M methyltransferase complex (Figure 2, reaction 10) driving a sodium translocating ATP synthase [13**].

Electron transport coupled to ATP synthesis in the CO₂-reduction pathway of the marine isolate M. acicorans is distinct from H₂-metabolizing Methanosarcina and obligate CO₂-reducing methanogens [19*,20]. M. acicorans does not metabolize H₂, and the only electron donor for reduction of CO₂ to CH₄ is CO. Coenzyme F₄₂₀ is the electron carrier that donates electrons to a membrane-bound electron transport chain terminating with the heterodisulfide reductase [19*]. Uprogulation of the F₄₂₀H₂ dehydrogenase complex (Fpo) in response to growth with acetate suggests it is the entry point to the electron transport chain and that methanophenazine mediates electron transfer from the dehydrogenase to the heterodisulfide reductase generating the proton gradient that drives ATP synthesis.

**Synthesis of methyl-H₄SPT in the aceticlastic pathway**

Methyl-H₄SPT is synthesized by reactions 1–4 (Figure 2) in the aceticlastic pathway of Methanosarcina species. Unlike the CO₂-reduction pathway, homologs of the enzymes catalyzing these reactions are wide-spread in diverse anaerobes from the domain Bacteria. Homologs of acetate kinase and phosphotransacetylase, catalyzing the reverse of reactions 1 and 2 (Figure 1), are the primary energy-conserving enzymes of the fermentative and obligate proton-reducing acetogenic groups (Figure 1) converting acetyl-CoA to ATP and acetate [21**]. Reaction 3 is central to the aceticlastic pathway catalyzed by the CO dehydrogenase/acetyl-CoA synthase (Cdh) complex that cleaves the C–C and C–S bonds of acetyl-CoA transferring the methyl group to H₄SPT and oxidizing the carbonyl group to CO₂ with transfer of electrons to ferredoxin. Acetogens from the domains Bacteria and Archaean contain Cdh homologs that function in acetate-producing energy-conserving pathways, and also pathways for generating acetyl-CoA from a methyl group and CO₂ for cell biosynthesis [22]. The five-subunit CdhABCDE complex from Methanosarcina species is resolvable into three components. The CdhC component cleaves the C–S bond of acetyl-CoA with transfer of the acetyl group to the “A” cluster composed of a 4Fe–4S center bridged by a cysteine thiolate to a binuclear Ni–Ni site [23] similar to the homolog from Moorella thermoacetica, an acetate-producing species from the domain Bacteria [24]. Cleavage of the acetyl group yields enzyme-bound CO derived from the carbonyl group and a methyl group that is transferred to the corrinoid cofactor of the CdhDE component that in turn donates the methyl group to H₄SPT. The CdhAE component accepts CO from CdhC oxidizing it to CO₂ and reducing ferredoxin. The crystal structure of CdhAE from Methanosarcina barkeri reveals 4Fe–4S clusters proposed to transfer electrons to ferredoxin from the active site “C” center, a pseudocubane NiFe₂S₄ cluster bridged to an exogenous iron atom [25*]. A gas channel in the crystal structure extends from the “C” center to the protein surface with potential to interact with the CdhC component. A mechanism is proposed in which transfer of an electron from the “C” cluster of CdhAE to cluster “A” of CdhC maintains the reduced catalytically active Ni(I) redox state [26].

The conversion of acetate to CH₄ and CO₂ provides only a marginal amount of energy available for growth (ΔG°′ = −36 kJ/CH₄). Thus, it is postulated that a carbonic anhydrase (Cam) is located outside the cell membrane where it converts CO₂ to membrane-impermeable HCO₃⁻ (Figure 2, reaction 4) facilitating removal of CO₂ from the cytoplasm that optimizes the thermodynamic efficiency of growth. Cam from M. thermophila is the archetype of the γ class of carbonic anhydrases for which homologs are widely distributed in the domains Bacteria and Archaea [27]. Contrary to all prokaryotic carbonic anhydrases that contain zinc, Cam, and the Cam homolog CamH contain Fe²⁺ in the active site [11*,28].

Although of lesser importance for the biomethanation of renewable plant material to biogas, Methanosarcina species also utilize the methylotrophic substrates methanol, trimethylamines, and methylsulfide for growth and methanogenesis. Compared to the aceticlastic pathway, more energy is available via the methylotrophic pathway for which the encoding genes are upregulated when cells are exposed to both acetate and methylotrophic substrates. A significant understanding of global regulation and genes specific to methylotrophic pathways has been
advanced recently for *Methanosarina* species [29,30]. However, with one exception [31], nothing is known of the regulatory proteins and mechanisms specific for genes of the aceticlastic pathway for which research is urgently needed.

**Synthesis of methyl-H₄MPT in the CO₂-reduction pathway**

The CO₂-reducing methanogens can be further divided according to the diversity of substrates used for growth and CH₄ production. Obligate CO₂-reducing species only reduce CO₂ to CH₄ with either H₂ or formate whereas CO₂-reducing *Methanosarina* species of the genus also grow and produce CH₄ by converting the methyl groups of acetate, methanol, methylamines, and dimethylsulfide to CH₄. Both types reduce CO₂ via reactions 5–9 (Figure 2) to a methyl group bound either to tetrahydro-methanopterin (H₄MPT) in obligate CO₂ reducers or to tetrahydroscarcinapterin (H₄SPT) in *Methanosarina* species. The three electron pairs required for reactions 5, 8, and 9 originate from oxidation of H₂, CO, or formate and reduction of ferredoxin or coenzyme F₄₂₀ serving as electron carriers. In freshwater *Methanosarina* species CO is first oxidized to H₂ and CO₂ whereas in the marine isolate *M. acetivorans* the oxidation of CO is coupled to reduction of ferredoxin and F₄₂₀ avoiding H₂ as an intermediate [19*]. The utilization of formate is limited to obligate CO₂-reducing species. Although formic hydrogenase systems have been described that convert formate to H₂ and CO₂, the role of H₂ as an intermediate during growth with formate is still in question [32,33].

Reaction 5 (Figure 2) catalyzed by formyl-methanofuran (MF) dehydrogenase is endergonic in the environment where partial pressures of H₂ are held in the range of 1–10 Pa and therefore requires energy [13**]. The Ech hydrogenase of freshwater *Methanosarina* species reduces ferredoxin driven by a proton gradient (high outside) generated by the membrane-bound electron transport chain originating with oxidation of H₂ and ending with reduction of CoM-S-S-CoB (reaction 12, Figure 2) [14*]. Less understood is the mechanism that drives reaction 5 in obligate CO₂-reducers that do not synthesize a membrane-bound electron transport chain. It is hypothesized that the exergonic H₂-dependent reduction of CoM-S-S-CoB is mechanistically coupled to the endergonic reduction of ferredoxin driving reaction 5 [13**]. Supporting this hypothesis is the recent report of a protein complex from *Methanothrix maripaludis* that contains heterodisulfide reductase, formyl-MF dehydrogenase, and a hydrogenase [34**].

In the next steps (reactions 6–9) the formyl group of formyl-MF is transferred to H₂M(S)PT followed by two reduction steps culminating with CH₃-H₂M(S)PT. The two-electron donor F₄₂₀ is reduced with a nickel-containing hydrogenase [35]. Under Ni-limiting conditions, involvement of the F₄₂₀-reducing hydrogenase is bypassed by a novel iron-only hydrogenase that directly oxidizes H₂ and reduces CH≡H₄MPT⁺⁺ (reaction 8b) [35].

**Biotechnological applications**

The biomethanation of easily degradable organic waste and renewable plant material has been abundantly applied in past decades. More recently there has been interest in understanding the biomethanation of more recalcitrant substrates such as peat and higher rank coals to improve yields of coal bed methane with some success [36–39]. Finally, an engineered methanogenic pathway has been described derived from the domains *Bacteria* and *Archaea* that utilizes the methyl esters of acetate and propionate for growth and methanogenesis [40**]. The fragile interactions of multispecies food chains converting complex biomass to methane are easily disrupted, a major impediment to efficient and reliable conversion of renewable biomass as an alternative to fossil fuels. The engineered pathway expands the exceptionally narrow range of substrates utilized by methanogens, exemplifying the simplification of food chains leading to the more-efficient conversion of complex biomass to methane.

**Conclusions and perspectives**

Methanogens are key players in the biomethanation of complex biomass serving as terminal organisms of the food chain and maintaining H₂, formate, and acetate concentrations at levels that are thermodynamically favorable for fermentative and acetogenic members at the front of the food chain. Thus, a fundamental understanding of aceticlastic and CO₂-reducing methanogenic pathways is necessary to identify factors that optimize the rate and reliability for the biomethanation process to be economically competitive with fossil fuels. Although considerable progress has been made in understanding pathway enzymes, additional research is necessary to understand other factors such as the stress response and regulatory mechanisms, particularly of aceticlastic methanogens. Finally, a fundamental understanding of pathways combined with recent advances in genetics provides a platform for a synthetic genomic approach to engineer methanogens with properties superior to native species that further enhance the biomethanation process.

**Acknowledgements**

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**References and recommended reading**

Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest


Describes the first experimental evidence for the hypothesized coupling of heterodisulfide reduction with the first reductive step in the CO₂ pathway of obligate CO₂-reducing methanogens.


Describes the first engineered methanogenic pathway expanding the substrate range of methanogens.