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

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Abstract

The conversion of biomass to CH_4 (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 H_2. The thermodynamics of these conversions are unfavorable requiring a symbiosis with the CH_4-producing group (methanogens) that metabolize the decomposition products to favorable concentrations. The methanogens produce CH_4 by two major pathways, conversion of the methyl group of acetate and reduction of CO_2 coupled to the oxidation of formate or H_2. 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.

Introduction

Earth's biosphere contains diverse oxygen-free (anaerobic) environments where complex organic matter is decomposed to CH_4 in a process called biomethanation that is an essential link in the global carbon cycle (Figure 1). In the cycle, atmospheric CO_2 is fixed into plant biomass primarily *via* oxygenic photosynthesis (step 1). Microbes in aerobic environments digest the biomass consuming O_2 and returning CO_2 to the atmosphere (step 2). A portion of the biomass also enters diverse O_2-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 CO_2 and CH_4 (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 H_2 plus CO_2 (step 4). The obligate proton-reducing acetogenic group (acetogens) converts the butyrate and propionate to acetate, CO_2, and H_2 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 CH_4 by two major pathways (Table 1). In the CO_2-reduction pathway, formate or H_2 is oxidized and CO_2 is reduced to CH_4. In the aceticlastic pathway, acetate is cleaved with the carbonyl group oxidized to CO_2 and the methyl group reduced to CH_4. In most freshwater environments, the aceticlastic group is responsible for approximately two-thirds with most of the remaining one-third produced by CO_2-reducing methanogens. Smaller, yet significant amounts of CH_4, are produced from the methyl groups of methanol, methylamines, and dimethylsulfide. Some of the CH_4 is oxidized to CO_2 (step 7) by a consortia of anaerobes that reduce either sulfate, nitrate,

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manganese, or iron [1]. The CH_4 also diffuses into aerobic environments (step 8) where O_2-requiring methanotrophic microbes oxidize it to CO_2 (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 CH_4 is approximately 3-fold greater than H_2, and CH_4 is stored and transported in a more efficient and safer manner than H_2. Through catalytic reformation, CH_4 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 CH_4-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 CO_2-reducing pathways

Figure 2 shows a composite of CO_2-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 (CH_3-H_4SPT) in the aceticlastic pathway (reactions 1–4) or methyl-tetrahydromethanopterin (CH_3-H_4MPT) in the CO_2-reduction pathway (reactions 5–9). The H_4SPT and H_4MPT cofactors are functionally equivalent analogs of tetrahydrofolate. In reaction 10, the methyl group of CH_3-H_4M(S)PT is transferred to HS-CoM followed by reductive demethylation of CH_3-S-CoM to CH_4 with electrons donated by HS-CoB (reaction 11). Reaction 10 is catalyzed by CH_3-H_4M(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 CH_3-S-CoM is reduced to CH_4 with HS-CoB the electron donor and the heterodisulfide CoM-S-S-CoB is an added product. The enzyme is also proposed to activate CH_4 for anaerobic oxidation to CO_2 [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 F_{430} [3]. In dispute are two proposed mechanisms, investigated of late, that diverge in how the C–S bond of CH_3-S-CoM is cleaved. Mechanism A (also referred to as mechanism II) predicts the nickel of $Ni^I F_{430}$ attacking the sulfur atom of CH_3-S-CoM producing a $^\bullet CH_3$ radical and CoM-S-$Ni^{II} F_{430}$ [4]. The methyl radical then abstracts a hydrogen from HS-CoB producing $^\bullet$S-CoB and CH_4. The $^\bullet$S-CoB radical reacts with CoM-S-$Ni^{II} F_{430}$ producing the anion radical CoM-S-S$^{\bullet-}$-CoB$^-$ that donates the extra electron to $Ni^{II} F_{430}$ regenerating the active $Ni^I F_{430}$ species and yielding CoM-S-S-CoB. Mechanism B (also called mechanism I) proposes a nucleophilic attack of Ni^I on the methyl of CH_3-S-CoM generating CH_3-$Ni^{III} F_{430}$ and $^-$S-CoM. Transfer of an electron from $^-$S-CoM to CH_3-$Ni^{III} F_{430}$ produces the $^\bullet$S-CoM radical and CH_3-$Ni^{II} F_{430}$, the protonolysis of which produces CH_4. The $Ni^{II} F_{430}$ is reduced to the active $Ni^I F_{430}$ 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_3-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 σ-alkane-$Ni^{III}F_{430}$ and alkane-$Ni^I F_{430}$ complexes as intermediates [5]. X-ray absorption spectroscopy of Mcr reacted with methyl iodide, together with density functional theory, indicates a stable CH_3-$Ni^{III}F_{430}$ species consistent with mechanism B [6]. Stable synthetic CH_3-Ni^{III} complexes are reported [7] that contain Ni–C bond lengths equivalent to the lengths reported for the enzyme-containing CH_3-$Ni^{III}F_{430}$ species [6]. Further support for mechanism B was obtained by reacting Mcr with the natural substrate CH_3-S-CoM and an analog of HS-CoB at reduced rates which allowed transient kinetics to detect an alkyl-Ni^{III} 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_3-S-CoM induces a conformational change that moves CH_3-S-CoM to within a distance of F_{430} that initiates the reaction. Recent crystal structures of inactive Mcr ($Ni^{II}F_{430}$) failed to show significant conformational changes when complexed with HS-CoB analogs [9]. On the other hand, crystallographic and spectroscopic approaches investigating Mcr(Ni^I) 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 (HdrDE) enzyme functions in the aceticlastic pathway of *Methanosarcina* species [12], and a three-subunit (HdrABC) enzyme functions in obligate CO_2-reducers [13••].

Electron transport and ATP synthesis in the aceticlastic and CO_2-reducing pathways

All acetotrophic methanogens obtain energy for growth by coupling electron transfer from ferredoxin to CoM-S-S-CoB with generation of a proton gradient that drives ATP synthesis *via* the proton-translocating "archaeal" A_1A_0-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_{420}-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 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 H_2 to CoM-S-S-CoB (reaction 12, Figure 2) in the CO_2-reducing pathways of freshwater *Methanosarcina* and obligate CO_2-reducing species differs significantly. In freshwater *Methanosarcina*, the H_2:CoM-S-S-CoB oxidoreductase system is identical to the proton pumping segment of electron transport in the aceticlastic pathway involving the F_{420}-nonreducing hydrogenase, methanophenazine, and the HdrDE

heterodisulfide reductase. However, the membrane-bound electron transport chain of *Methanosarcina* species is absent in obligate CO_2-reducing species with no apparent mechanism for generating an ion gradient. Instead, the H_2:CoM-S-S-CoB oxidoreductase system is composed of the cytoplasmic F_{420}-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-H_4MPT:coenzyme M methyltransferase complex (Figure 2, reaction 10) driving a sodium translocating ATP synthase [13••].

Electron transport coupled to ATP synthesis in the CO_2-reduction pathway of the marine isolate *M. acetivorans* is distinct from H_2-metabolizing *Methanosarcina* and obligate CO_2-reducing methanogens [19•,20]. *M. acetivorans* does not metabolize H_2, and the only electron donor for reduction of CO_2 to CH_4 is CO. Coenzyme F_{420} is the electron carrier that donates electrons to a membrane-bound electron transport chain terminating with the heterodisulfide reductase [19•]. Upregulation of the $F_{420}H_2$ 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-H_4SPT in the aceticlastic pathway

Methyl-H_4SPT is synthesized by reactions 1–4 (Figure 2) in the aceticlastic pathway of *Methanosarcina* species. Unlike the CO_2-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 H_4SPT and oxidizing the carbonyl group to CO_2 with transfer of electrons to ferredoxin. Acetogens from the domains *Bacteria* and *Archaea* contain Cdh homologs that function in acetate-producing energy-conserving pathways, and also pathways for generating acetyl-CoA from a methyl group and CO_2 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 H_4SPT. The CdhAE component accepts CO from CdhC oxidizing it to CO_2 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 $NiFe_3S_4$ cluster bridged to an exogeneous iron atom [25•]. A gas channel in the crystal structure extends from the "C" cluster 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 CH_4 and CO_2 provides only a marginal amount of energy available for growth ($\Delta G^{\circ\prime} = -36$ kJ/CH_4). Thus, it is postulated that a carbonic anhydrase (Cam) is located outside the cell membrane where it converts CO_2 to membrane-impermeable HCO_3^- (Figure 2, reaction 4) facilitating removal of CO_2 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 Fe^{2+} 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 *Methanosarcina* 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-H$_4$MPT in the CO$_2$-reduction pathway

The CO$_2$-reducing methanogens can be further divided according to the diversity of substrates used for growth and CH$_4$ production. Obligate CO$_2$-reducing species only reduce CO$_2$ to CH$_4$ with either H$_2$ or formate whereas CO$_2$-reducing *Methanosarcina* species of the genus also grow and produce CH$_4$ by converting the methyl groups of acetate, methanol, methylamines, and dimethylsulfide to CH$_4$. Both types reduce CO$_2$ *via* reactions 5–9 (Figure 2) to a methyl group bound either to tetrahydromethanopterin (H$_4$MPT) in obligate CO$_2$ reducers or to tetrahydrosarcinapterin (H$_4$SPT) in *Methanosarcina* species. The three electron pairs required for reactions 5, 8, and 9 originate from oxidation of H$_2$, CO, or formate and reduction of ferredoxin or coenzyme F$_{420}$ serving as electron carriers. In freshwater *Methanosarcina* species CO is first oxidized to H$_2$ and CO$_2$ whereas in the marine isolate *M. acetivorans* the oxidation of CO is coupled to reduction of ferredoxin and F$_{420}$ avoiding H$_2$ as an intermediate [19•]. The utilization of formate is limited to obligate CO$_2$-reducing species. Although formic hydrogenlyase systems have been described that convert formate to H$_2$ and CO$_2$, the role of H$_2$ 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 H$_2$ are held in the range of 1–10 Pa and therefore requires energy [13••]. The Ech hydrogenase of freshwater *Methanosarcina* species reduces ferredoxin driven by a proton gradient (high outside) generated by the membrane-bound electron transport chain originating with oxidation of H$_2$ 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 CO$_2$-reducers that do not synthesize a membrane-bound electron transport chain. It is hypothesized that the exergonic H$_2$-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 *Methanococcus 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 H$_4$M(S)PT followed by two reduction steps culminating with CH$_3$-H$_4$M(S)PT. The two-electron donor F$_{420}$ is reduced with a nickel-containing hydrogenase [35]. Under Ni-limiting conditions, involvement of the F$_{420}$-reducing hydrogenase is bypassed by a novel iron-only hydrogenase that directly oxidizes H$_2$ and reduces CH≡H$_4$MPT$^+$ (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 H_2, 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 CO_2-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.

Acknowledgments

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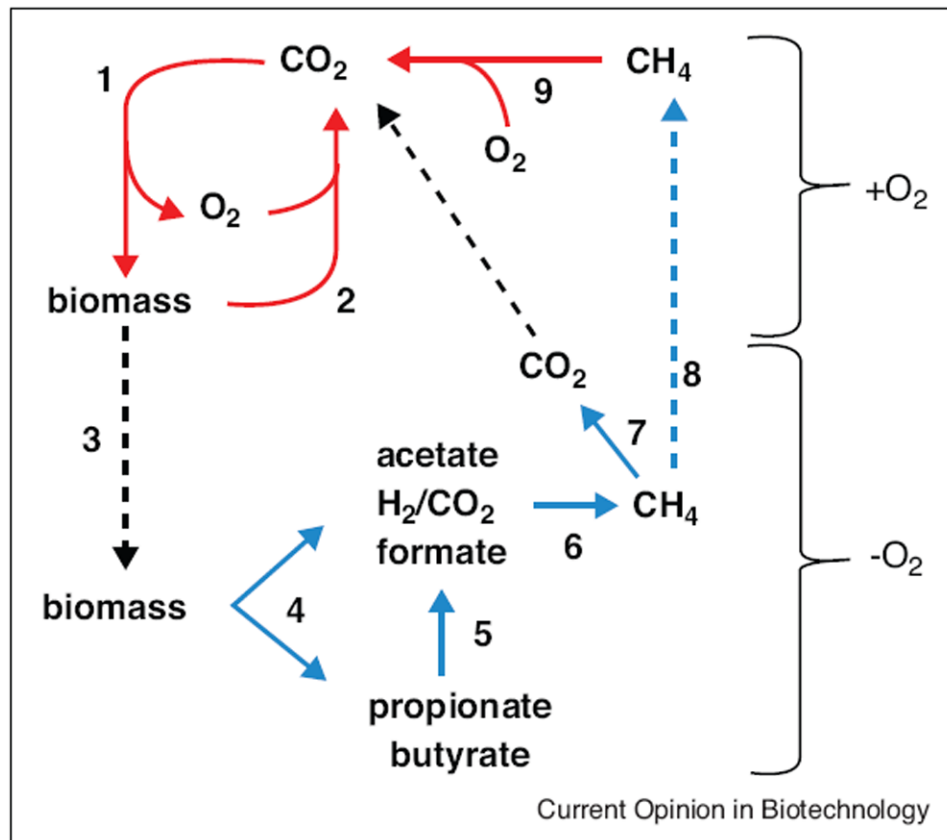
Figure 1.
The global carbon cycle. Aerobic O_2-requiring conversions are shown in solid red arrows and anaerobic conversions in solid blue arrows. The brackets denote aerobic ($+O_2$) and anaerobic ($-O_2$) habitats. Black dotted arrows symbolize diffusion of substrates and products across the interface of zones.
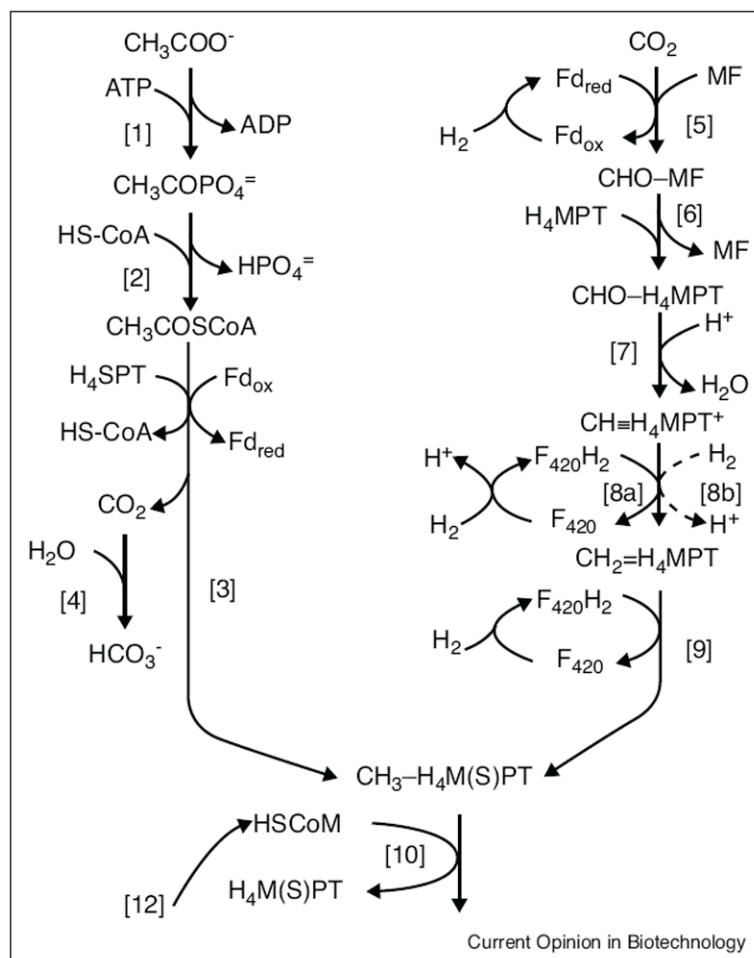
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Figure 2.
Composite of CO_2-reduction and aceticlastic methane-producing pathways. The left arm leading to CH_3-$H_4M(S)PT$ shows reactions (1–4) unique to the aceticlastic pathway, and the right arm leading to CH_3-$H_4M(S)PT$ shows reactions (5–9) unique to the CO_2-reduction pathway. Both pathways have in common reactions (10–12) leading to the formation of CH_4 from the methyl groups of CH_3-$H_4M(S)PT$.

Abbreviations: ATP, adenosine triphosphate; H_4SPT, tetrahydrosarcinapterin; H_4MPT, tetrahydromethanopterin, Fd, ferredoxin; CoA, coenzyme A; CoM, coenzyme M; CoB, coenzyme B; MF, methanofuran; F_{420}, coenzyme F_{420}.
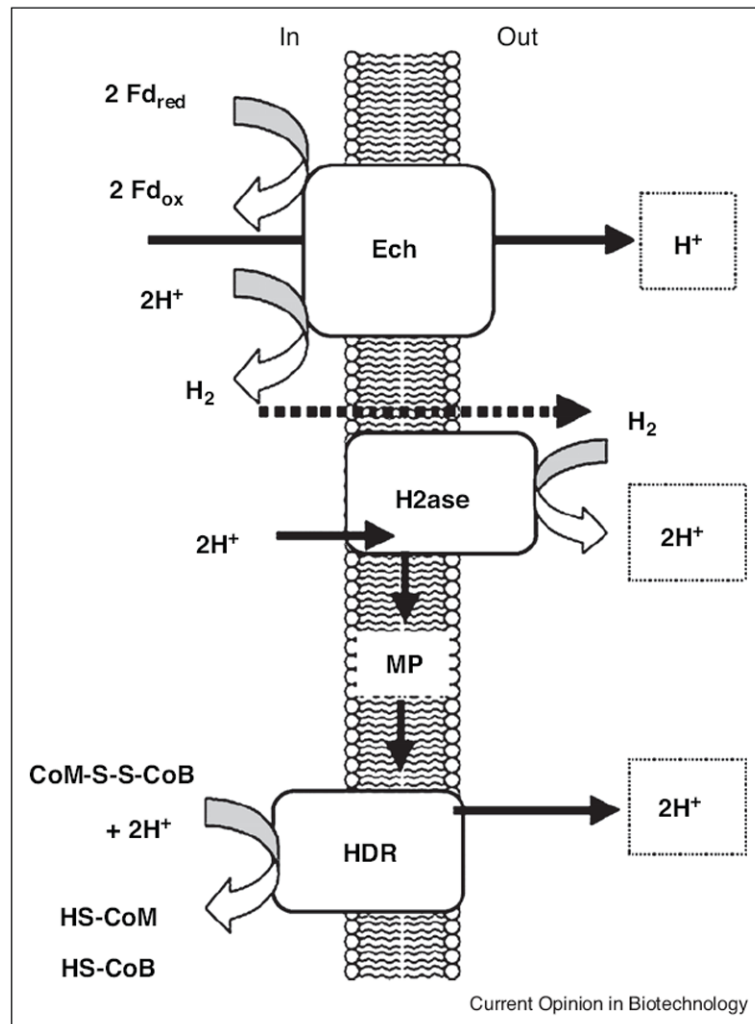
Figure 3.
Proposed model of ferredoxin-dependent electron transport chain in the freshwater isolate *Methanosarcina mazei*. H$_2$ase, F$_{420}$-nonreducing hydrogenase (Vho); HDR, heterodisulfide reductase; MP, methanophenazine. By permission from Ref. [16].

Table 1

Reactions involved in the syntrophic metabolism of obligate proton-reducing acetogens and methanogens.

Reactions	$\Delta G^{\circ\prime}$ (kJ/mol)
Propionate$^-$ + 3H$_2$O → acetate$^-$ + HCO$_3^-$ + H$^+$ + 3H$_2$	+76.1
Butyrate$^-$ + 2H$_2$O → 2acetate$^-$ + H$^+$ + 2H$_2$	+48.6
4H$_2$ + HCO$_3^-$ + H$^+$ → CH$_4$ + 3H$_2$O	−135.6
4Formate$^-$ + H$^+$ + H$_2$O → 3HCO$_3^-$ + CH$_4$	−130.4
Acetate$^-$ → HCO$_3^-$ + H$^+$ + CH$_4$	−36.0