Electron Bifurcation and Confurcation in Methanogenesis and Reverse Methanogenesis

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Reduction of the disulfide of coenzyme M and coenzyme B (CoMS–SCoB) by heterodisulfide reductases (HdrED and HdrABC) is the final step in all methanogenic pathways. Flavin-based electron bifurcation (FBEB) by soluble HdrABC homologs play additional roles in driving essential endergonic reactions at the expense of the exergonic reduction of CoMS–SCoM. In the first step of the CO₂ reduction pathway, HdrABC complexed with hydrogenase or formate dehydrogenase generates reduced ferredoxin (Fdx₂⁻) for the exergonic reduction of CO₂ coupled to the exergonic reduction of CoMS–SCoB dependent on FBEB of electrons from H₂ or formate. Roles for HdrABC:hydrogenase complexes are also proposed for pathways wherein the methyl group of methanol is reduced to methane with electrons from H₂. The HdrABC complexes catalyze FBEB-dependent oxidation of H₂ for the endergonic reduction of Fdx driven by the exergonic reduction of CoMS–SCoB. The Fdx₂⁻ supplies electrons for reduction of the methyl group to methane. In H₂⁻ independent pathways, three-fourths of the methyl groups are oxidized producing Fdx₂⁻ and reduced coenzyme F₄₂₀ (F₄₂₀H₂). The F₄₂₀H₂ donates electrons for reduction of the remaining methyl groups to methane requiring transfer of electrons from Fdx₂⁻ to F₄₂₀. HdrA1B1C1 is proposed to catalyze FBEB-dependent oxidation of Fdx₂⁻ for the endergonic reduction of F₄₂₀ driven by the exergonic reduction of CoMS–SCoB. In H₂⁻ independent acetotrophic pathways, the methyl group of acetate is reduced to methane with electrons derived from oxidation of the carbonyl group mediated by Fdx. Electron transport involves a membrane-bound complex (Rnf) that oxidizes Fdx₂⁻ and generates a Na⁺ gradient driving ATP synthesis. It is postulated that F₄₂₀ is reduced by Rnf requiring HdrA2B2C2 catalyzing FBEB-dependent oxidation of F₄₂₀H₂ for the endergonic reduction of F₄₂₀ driven by the exergonic reduction of CoMS–SCoB. The Fdx₂⁻ is recycled by Rnf and HdrA2B2C2 thereby conserving energy. The HdrA2B2C2 is also proposed to play a role in Fe(III)-dependent reverse methanogenesis. A flavin-based electron confurcating (FBE) HdrABC complex is proposed for nitrate-dependent reverse methanogenesis in which the oxidation of CoM-SH/CoB-SH and Fdx₂⁻ is coupled to reduction of F₄₂₀. The F₄₂₀H₂ donates electrons to a membrane complex that generates a proton gradient driving ATP synthesis.

Keywords: archaea, heterodisulfide reductase, methane, ferredoxin, hydrogen, acetate, formate
INTRODUCTION

Methane-producing archaea (methanogens) are terminal organisms of anaerobic microbial food chains decomposing complex organic matter in Earth’s anaerobic biosphere which includes the lower intestinal tract of humans, the hind gut of termites, the rumen of animals, natural wetlands and rice paddies. As such, methanogens are an essential link in the global carbon cycle (Figure 1). In step 1, CO₂ is incorporated into biomass by photosynthetic plants and microbes. In oxygenated environments, O₂-respiring microbes oxidize the biomass producing CO₂ that re-enters the carbon cycle (step 2).

A significant fraction of the biomass enters anaerobic biospheres where it is converted to CO₂ and CH₄ by microbial food chains comprised of at least four metabolic groups (steps 3–6). The fermentative group digests the complex biomass producing acetate, H₂, and CO₂ along with other volatile fatty acids (step 3) that are oxidized to acetate plus either formate or H₂ (step 4) by syntrophic acetogens. The CO₂-reducing methanogen group reduces CO₂ to CH₄ with electrons derived from oxidation of H₂ or formate (step 5). This group forms symbioses with the acetogens that supply H₂ or formate the methanogens metabolize to concentrations thermodynamically favorable for the acetogens in a process termed interspecies electron transfer (ISET) (Sieber et al., 2009). The acetate-utilizing (acetoclastic) methanogen group converts the methyl group to CH₄ and the carbonyl group to CO₂ (step 6). A portion of the CH₄ is oxidized to CO₂ (step 7) by the anaerobic oxidation of methane (AOM) proposed to involve the reversal of methanogenic pathways. The CO₂ and remaining CH₄ escapes into oxygenated zones where O₂-respiring methanotrophic microbes oxidize CH₄ to CO₂ (step 8), closing the carbon cycle. As a greenhouse gas, methane is nearly 20-fold more potent than CO₂; thus, the aerobic and anaerobic oxidation of CH₄ plays an important role in controlling Earth's climate (Valentine, 2002; Rhee et al., 2009).

Electron transport is much less understood than the comprehensive biochemical understanding of carbon transformations in methanogenic and reverse methanogenic pathways. Herein is reviewed the current understanding of electron transport with a focus on the role of flavin-based electron bifurcation (FBEB) and confurcation (FBEC).

OBLIGATE CO₂ REDUCING METHANOGENS

As the name implies, this group only produces CH₄ by reducing CO₂, primarily with electrons from oxidation of H₂ or formate. The pathway (Figure 2) is the subject of reviews (Liu and Whitman, 2008; Thauer et al., 2008; Ferry, 2010). The first step is reduction of CO₂ to formyl-methanofuran (CHO-MF) catalyzed by formylmethanofuran dehydrogenase (Fwd or Fmd) (Wagner et al., 2016). The reaction is endergonic and dependent on reduced ferredoxin (Fdx₂⁻). The formyl group of CHO-MF is transferred to tetrahydromethanopterin (H₂MPT) and reduced to yield CH₃ – H₄MPT. Most methanogens contain H₄MPT, whereas Methanosarcina species contain the functionally equivalent tetrahydrosarcinapterin (H₄SPT). The electron donor is reduced coenzyme F₄₂₀ (F₄₂₀H₂) generated from H₂ or formate by F₄₂₀-dependent hydrogenases (Fru, Frc, Frh) or formate dehydrogenase (Fdh) (Thauer et al., 2010). Coenzyme F₄₂₀ is an obligate two-electron carrier donating or accepting a hydride. The methyl group of CH₃ – H₄MPT is transferred to coenzyme M (HS-CoM) catalyzed by methyltransferase (Mtr) to generate CH₃ – SCoM. This exergonic reaction is linked to translocation of Na⁺ outside the membrane generating a gradient (high outside). Without cytochromes in obligate CO₂ reducing methanogens, this is the only mechanism generating an ion gradient that drives ATP synthesis (Thauer et al., 2008). Methyl-SCoM methylreductase (Mcr) catalyzes the reductive demethylation of CH₃ – SCoM to CH₄ involving coenzyme B (HS-CoB) accompanied by formation of CoMS-SCoB. Heterodisulfide reductase (HdrABC) reduces the disulfide bond with electrons supplied from the oxidation of 2H₂ or 2HCO₂H (E°′ ≈ –420 mV) catalyzed by F₄₂₀-independent hydrogenase or Fdh. The exergonic reduction of CoMS-SCoB (E°′ = –140 mV) drives the endergonic reduction of CO₂ (E°′ = –500 mV) in the first step (Figure 2) via FBEB by HdrABC (Buckel and Thauer, 2013).

Two variations are proposed for FBEB of obligate CO₂-reducing methanogens (Figure 2). FBEB in strictly hydrogenotrophic Methanothermobacter marburgensis involves the soluble MvhAGD:HdrABC for which the crystal structure of the heterododecameric complex from Methanothermococcus thermolithotrophicus supports a proposed mechanism (Figure 3; Thauer et al., 2008; Kaster et al., 2011; Buckel and Thauer, 2013; Wagner et al., 2017). H₂ is oxidized at the catalytic [NiFe] center of MvhA with transfer of electrons to the [2Fe-2S] cluster of MvhD mediated by the [4Fe-4S] clusters of MvhA and MvhG. The bifurcating FAD of HdrA sequentially accepts two electrons from the [2Fe-2S] cluster that contrasts with other
characterized FBEB enzymes for which a hydride is donated to FAD (Lubner et al., 2017; Peters and Lubner, 2017). Any of three conformational changes are proposed to overcome the >30 Å distance observed in the crystal structure that otherwise would prohibit electron transfer between the [2Fe-2S] cluster of MvhD and FAD of HdrA. At this juncture the electrons from reduced FAD (FADH$^-$) bifurcate into a high-potential and a low-potential electron. The high-potential electron from FADH$^-$ is transported via [4Fe-4S] clusters of HdrA and HdrC to the active-site non-cubane [4Fe-4S] clusters of HdrB where CoMS-SCoB is reduced. The low-potential electron of the resulting semiquinone radical (FADH) is transported via [4Fe-4S] clusters (HA3, HA5, and HA6) of HdrA to Fdx. It is proposed that a conformational change overcomes the 21.5 Å distance in the crystal structure that would otherwise prohibit electron transfer between HA3 and HA5 (Figure 3). The structure reveals residues adjacent to the isoalloxazine ring of FAD proposed to achieve the low-potential neutral FADH radical and a postulated anionic semiquinone (FAD$^-$) intermediate during reduction of FAD. The process occurs twice yielding HSCoM, HSCoB, and Fdx$^{2-}$ that donates electrons to Fwd reducing CO$_2$ to CHO-MF (Figure 2). Generation of Fdx$^{2-}$ by the membrane-bound energy-converting hydrogenase Eha (Figure 4) of obligate CO$_2$ reducing methanogens serves an anaplerotic role and validates the essentiality of FBEB (Lie et al., 2012).

In contrast to strictly hydrogenotrophic *M. marburgensis, Methanococcus maripaludis* utilizes either H$_2$ or formate as electron donors for reduction of CO$_2$ to CH$_4$ requiring FBEB mechanisms for each substrate (Figure 2). A protein complex isolated from formate-grown cells contains HdrABC, F$_{120}$-non-reducing selenocysteine-containing hydrogenase (Vhu), Fdh, and the tungsten-containing Fwd (Costa et al., 2010). This result lead to the conclusion that Fdh oxidizes formate with direct transfer of electrons to HdrABC without first producing H$_2$ by a F$_{120}$-dependent formic hydrogenlyase system and then oxidation of the H$_2$ by the F$_{120}$-independent MvhAGD hydrogenase as in FBEB by *M. marburgensis*. Direct transfer without participation of H$_2$ as an intermediate is supported by robust growth with formate, although not H$_2$, for a mutant deleted of genes encoding subunits of the selenocysteine-containing (vhu) and cysteine-containing (vhc) F$_{120}$-independent hydrogenases associated with HdrABC. However, the mutant retained the vhuD and vhcD genes homologous to mvhD of *M. marburgensis* obscuring potential

**FIGURE 2** | Roles for flavin-based electron bifurcation (FBEB) by heterodisulfide reductases in the formate- and H$_2$-dependent CO$_2$ reduction pathway of methanogenesis in *Methanothermococcus thermolithotrophicus* and *Methanococcus maripaludis*. The red and green arrows represent the FBEB-dependent generation of electrons by HdrABC originating from oxidation of H$_2$ and HCO$_2$H, respectively, for reduction of CO$_2$ in the first step of the pathway.

**FIGURE 3** | Proposed electron-transfer pathway in the heterodisulfide reductase [NiFe]-hydrogenase complex (HdrABC-MvhAGD) from *Methanococcus maripaludis*. The red and green arrows indicate the electron-transfer pathway composed of iron-sulfur clusters at distances less than 13.5 Å. The dashed arrows correspond to a hypothetical electron-transfer pathway with distances longer than 15 Å between the redox centers. Reproduced by permission (Wagner et al., 2017).

**FIGURE 4** | Schematic representation of the structure and function of the energy-converting [NiFe]-hydrogenases EchA-F, EhaA-T, EhbA-Q, and MbhA-N found in methanogenic archaea. The energy-converting hydrogenase EchA-F is composed only of the six conserved core subunits, which are shown in shades of gray. The energy-converting hydrogenases EhaA-T, EhbA-Q, and MbhA-N also contain several hydrophobic and hydrophilic subunits of unknown function. These subunits are symbolized by areas with dashed boundaries. Abbreviations: Fd$_{ox}$, oxidized ferredoxin (Fdx); Fd$_{red}^{2-}$, two-electron-reduced ferredoxin (Fdx$^{2-}$). Adapted from (Thauer et al., 2010).
roles for VhuD and VhcD. When grown under conditions where both Fdh and Vhu are expressed, the enzymes compete for binding to VhuD, and are fully functional and bound to VhuD (Costa et al., 2013). Further, Fdh co-purifies with VhuD in the absence of other hydrogenase subunits. It was concluded that VhuD, also containing a [2Fe-2S] cluster, functions analogous to MvhD by mediating direct electron transfer from Vhu or Fdh to HdrABC (Figure 2; Costa et al., 2013). The mechanism for transfer of electrons from HdrABC to Fdh is unknown although likely mediated by Fdh as for M. marburgensis (Costa et al., 2010). Not reported is biochemical validation of electron bifurcation by the proposed complex as was shown for the MvhAGD:HdrABC and HdrA2B2C2 complexes of M. marburgensis and Methanosarcina acetivorans (Kaster et al., 2011; Yan et al., 2017). Nonetheless, an in silico genome-scale metabolic reconstruction of M. maripaludis indicates the organism is unable to grow without the energy-conserving complex (Richards et al., 2016).

In addition to supplying Fdx$^{2-}$ for the first step in the CO$_2$-reduction pathway of methanogenesis, it is proposed that energy-conserving FBE by the heterodisulfide reductase in obligate CO$_2$-reducing methanogens of the order Methanomicrobiales is missing genes encoding MvhA and MvhG but encode MvhD and HdrABC (Brown et al., 2016). These methanogens could form an MvhD/HdrABC complex associated with energy-converting hydrogenases EchA–F, EhaA–T, or MbhA–N dependent on ion gradients to supply Fdx$^{2-}$ for reduction of CO$_2$ to CHO-MF, although reduction of CoMS-SCoB would be energy consuming. Thus, it is proposed that these methanogens substitute MvhA and MvhG with FrhA and FrhG of the F$_{420}$-reducing hydrogenase (FrhABG) contained in all methanogens without cytochromes (Thauer et al., 2010; Gilmore et al., 2017). In this way, FrhAG would be present in an FrhAG/MvhD/HdrABC complex with the potential for FBE by HdrABC of H$_2$ that generates the Fdx$^{2-}$ required for reduction of CO$_2$ to CHO-MF (Figure 5) with the added advantage of conserving energy. In this scenario, the energy-converting hydrogenases play a role in only providing Fdx$^{2-}$ for biosynthesis (Major et al., 2010). However, the possibility of an FrhABG/MvhD/HdrABC complex (Figure 5) cannot be ruled out at this juncture. Inclusion of the F$_{420}$-binding FrhB subunit invokes electron transport dependent on FrhABG producing F$_{420}$H$_2$ for which the electron pair is bifurcated by MvhD/HdrABC reducing Fdx and CoMS-SCoB analogous to the HdrA2B2C2 of M. acetivorans (Vitt et al., 2014; Yan et al., 2017). However, it is unknown which FBE pathway is physiologically relevant.

METHYLOTROPHIC METHANOGENS

Methanogens from the order Methanosarcinales grow and produce CH$_4$ with methyl-containing substrates (methanol, methylamines, and methyl sulfides) (Liu and Whitman, 2008). A few also grow by reducing CO$_2$ with H$_2$. Unlike obligate CO$_2$ reducers, these methanogens contain cytochromes and generate a proton gradient dependent on electron transport involving hydrogenases (Thauer et al., 2008). Like obligate CO$_2$ reducers, Fdx$^{2-}$ is required to supply electrons to Fwd/Fmd catalyzing reduction of CO$_2$ to CHO-MF; however, Fdx$^{2-}$ is generated independent of FBE by the membrane-bound energy-converting Ech hydrogenase driven by the proton gradient (Figure 4).

The methylopathway of the order Methanosarcinales involves transfer of substrate methyl groups to HSCoM forming a CH$_3$-SCoM pool of which one-fourth of the methyl groups are oxidized to CO$_2$ via reversal of the CO$_2$-reduction pathway to supply F$_{420}$H$_2$ and Fdx$^{2-}$ required for reductive demethylation of the remaining three-fourths CH$_3$-SCoM to CH$_4$ (Figure 6). The F$_{420}$H$_2$ is oxidized by a membrane-bound complex (FpO) that donates electrons to a quinone-like electron carrier (methanophenazine, MP) coupled to generation of a proton gradient. It is proposed that Fdx$^{2-}$ is re-oxidized by reducing F$_{420}$ although the mechanism is unknown. The production of CH$_4$ from CH$_3$-SCoM is similar to obligate CO$_2$ reducers involving HSCoM and Mcr with the exception of the membrane-bound heterodisulfide reductase (HdrDE) that reduces CoMS-SCoB to the sulfhydryl forms of the cofactors. Electrons are supplied to HdrDE by MPH$_2$ with the scalar translocation of protons contributing to the proton gradient that drives ATP synthesis. However, the genomes of all sequenced Methanosarcinales also contain genes encoding the HdrABC homologs HdrA1B1C1 and HdrA2B2C2 (Buan and Metcalf, 2010). HdrA1B1C1 is elevated during methylopathy growth of M. acetivorans for which the ΔhdrA1B1C1 mutant strain is growth impaired. Thus, a role in methylopathy growth is proposed wherein FBE by HdrA1B1C1 reduces F$_{420}$ and CoMS-SCoB with electron pairs donated by two Fdx$^{2-}$ generated in...
the oxidation of CHO-MF (Figure 6), thereby allowing energy conservation via Fpo (Buan and Metcalf, 2010).

*Methanosphaera stadtmanae*, isolated from the human gut, is also a methylotrophic methanogen reducing the methyl group of methanol to CH₄ although belonging to the order *Methanobacteriales* that do not contain cytochromes necessary for electron-transport coupled proton translocation that drives ATP synthesis. The genome also lacks a complete gene set necessary for reversal of the CO₂ reduction pathway and therefore requires the oxidation of H₂ to supply electrons for reductive demethylation of CH₃-SCOm to CH₄ (Fricke et al., 2006). A scheme is proposed that includes FBEB of H₂ by an MvhADG:HdrABC complex to explain the finding that ATP synthesis is driven by an ion gradient (Figure 7; Sparling et al., 1993; Thauer et al., 2008). The Fdx²⁻ produced donates electrons to the membrane-bound energy-converting Ehb complex that generates a Na⁺ gradient driving ATP synthesis and regenerates H₂ recycled for FBEB by the MvhADG:HdrABC complex. A similar FBEB/H₂ cycling scheme is proposed for a sixth class of methanogens, ‘*Candidatus Methanofastidiosa*’, based on metagenome-derived draft genomes that also lack cytochromes and genes encoding enzymes for reversal of the CO₂ reducing pathway (Nobu et al., 2016). However, this class is restricted to reducing methylthiols with H₂. A seventh order, the *Methanomassiliicoccales*, also grows by reducing the methyl groups of methylotrophic substrates with H₂. Analyses of several genomes show this class also lacks cytochromes and genes required for reversal of the CO₂ reducing pathway (Borrel et al., 2014; Kroninger et al., 2015; Lang et al., 2015). Unlike *M. stadtmanae* and the ‘*Candidatus Methanofastidiosa*’ class, genes encoding the membrane-bound energy-converting complexes are absent and genes encoding an Fpo-like complex and the HdrD subunit of HdrDE are present. Figure 8 shows the pathway proposed for *Methanomassiliicoccus luminyensis*. The Fpo complex oxidizes Fdx²⁻ generated via FBEB of H₂ by the MvhADG:HdrABC complex. HdrD accepts electrons from Fpo and reduces CoMS-SCOβ coupled to generation of a H⁺ gradient. Roles for involvement of the Ech₁ and Ech₂ hydrogenases are ruled out based on low abundance of transcripts and low membrane-bound hydrogenase activity (Kroninger et al., 2015). Thus, CoMS-SCOβ is essential for both FBEB and the terminal electron acceptor which is distinct from that proposed for *M. stadtmanae* and the ‘*Candidatus Methanofastidiosa*’ class which involves H₂ cycling (Figure 7).

**ACETOTROPHIC METHANOGENS**

*Methanosarcina* and *Methanosaeta* are the only described genera of acetotrophic methanogens that are the subject of recent reviews (Ferry, 2013; Schlegel and Muller, 2013; Welte and Deppenmeier, 2014; Ferry, 2015). Most biochemical investigations have involved *Methanosarcina* species. *M. acetivorans* is a model for species that do not metabolize H₂ which constitute the majority of *Methanosarcina* species (Figure 9A). Acetate is converted to acetyl-CoA at the expense of one ATP followed by cleavage of the C-C and C-S bonds yielding a methyl group that is transferred to H₄SPT and a carbonyl group that is oxidized to CO₂ with transfer of electrons to Fdx. The methyl group of CH₃-H₄SPT is transferred to HS-CoM followed by reductive demethylation of CH₃S-CoM to methane involving reactions common to all methanogenic pathways. The Mtr complex catalyzes the exergonic methyl transfer coupled to generation of a Na⁺ gradient. The reduced Fdx²⁻ is electron donor to the Na⁺-pumping Rnf complex that donates electrons to cytochrome c that is the electron donor to MP (Wang et al., 2011). As in the methylotrophic pathway, HdrDE oxidizes MPH₂ and reduces CoMS-SCOβ with scalar translocation of H⁺ that generates a gradient. The multisubunit Na⁺/H⁺ antipporter Mrp
FIGURE 8 | Proposed role for FBEB by heterodisulfide reductase in energy-conservation by Methanosalbacillicoccus luminyensis. Ech1 and Ech2 are covered with crosses to indicate non-involvement in generating the proton gradient driving ATP synthesis (Kroninger et al., 2015). The question mark indicates a reaction not experimentally verified, although includes 2H\(^+\) pumped by the Fpo complex and another 2H\(^+\) translocated scalar via reduction and re-oxidation of methanophenazine. Fdx\(^{\text{ox}}\), oxidized ferredoxin (Fdx); Fdx\(^{\text{red}}\), two-electron-reduced ferredoxin (Fdx\(^{2-}\)). Reproduced by permission (Kroninger et al., 2015).

adjusts the Na\(^+\)/H\(^+\) ratio optimal for the ATP synthase which is dependent on both Na\(^+\) and H\(^+\) gradients (Schlegel et al., 2012a; Jasso-Chavez et al., 2013, 2016).

When switched from growth with methanol to growth with acetate, \textit{M. acetivorans} up regulates an electron bifurcating heterodisulfide reductase (HdrA2B2C2) that oxidizes F\(_{420}\)H\(_2\) (\(E_m = -380\) mV) and reduces Fdx (\(E_m = -520\) mV) driven by reduction of CoMS-SCoB (\(E_m = -140\) mV) (Yan et al., 2017). A role has been proposed for HdrA2B2C2 dependent on reduction of NAD-like coenzyme F\(_{420}\) (F\(_{420}\)) by the Rnf complex analogous to Fdx-dependent reduction of NAD\(^+\) by homologous Rnf complexes from the domain \textit{Bacteria} (Figure 9B; Buckel and Thauer, 2018a,b). In this way, Fdx reduced by HdrA2B2C2 is re-oxidized by Rnf thereby supplementing the translocation of Na\(^+\). The Na\(^+\) gradient formed by Rnf and Mtr could be exchanged with H\(^+\) by Mrp to adjust the Na\(^+\)/H\(^+\) ratio optimal for ATP synthesis. The process generates more ATP than electron transport involving MP and HdrDE (Figure 9). However, it is reported that HdrDE is essential for acetotrophic growth suggesting the possibility of both electron transport pathways oxidizing Fdx\(^{2-}\) and reducing CoMS-SCoB (Buan and Metcalf, 2010). Having alternate electron transport pathways with different thermodynamic efficiencies could provide the cell with options for responding to fluctuations in available free energy proportional to levels of acetate in the environment. Indeed, the conversion of acetate to CH\(_4\) and CO\(_2\) provides only a marginal amount of energy available for growth (\(\Delta G^{\circ'} = -36\) kJ/CH\(_4\)) that requires cells to maximize the thermodynamic efficiency.

A genome-wide analysis of \textit{Methanosoeta thermophila} revealed genes encoding enzymes catalyzing carbon transformation reactions in the pathway of acetate to CH\(_4\) similar to \textit{Methanosarcina} species (Smith and Ingram-Smith, 2007). However, genes encoding the Rnf complex are absent in the genome of \textit{Methanosoeta} suggesting an unknown alternative electron transport pathway and mechanism for energy conservation.

**REVERSE METHANOGENESIS**

It is postulated that AOM is accomplished by a reversal of methanogenic pathways based on environmental metagenomic and metatranscriptomic analyses of sediments (Hallam et al., 2004; McGlynn, 2017; Timmers et al., 2017). Although discovered nearly four decades ago, the unavailability of pure cultures...
prevented biochemical investigations of AOM. However, *M. acetivorans* is capable of "trace methane oxidation" (TMO) defined as reverse methanogenesis during net CH₄ production from growth substrates (Moran et al., 2005, 2007; Timmers et al., 2017). More recently, methanotrophic growth dependent on reduction of Fe(III) was documented for *M. acetivorans* (Soo et al., 2016). **Figure 10** illustrates the reverse methanogenesis pathway proposed for *M. acetivorans* based on a biochemical understanding of Fe(III)-dependent mechanisms driving endergonic reactions and energy conservation essential for methanotrophic growth (Yan et al., 2018). It is remarkably similar to the pathway proposed for anaerobic methanotrophic archaea (ANME) based on metagenomic and transcriptomic analyses of uncultured *Methanosarcinales* sp. ANME-2a (Wang et al., 2014).

The *M. acetivorans* pathway is a reversal of established acetate-utilizing and CO₂-reducing methanogenic pathways (Li et al., 2005, 2006, 2007; Lessner et al., 2006; Ferry, 2008; Wang et al., 2011; Schlegel et al., 2012b; Welte and Deppenmeier, 2014). Methane is oxidized by Mcr (Rxn. 1) with the methyl group of CH₃-CoM transferred to H₂-SPT by Mtr (Rxn. 2) representing the reversal of reactions common to all methanogenic pathways. The HdrDE oxidizes HSCOm and HSCOβ coupled to reduction of Fe(III) that regenerates CoMS-CoB (Rxn. 4). Removal of HSCOm, HSCOβ, and CH₃-CoM products by HdrDE and Mtr drives the endergonic oxidation of CH₄ by Mcr. The endergonic methyl transfer producing CH₃-H₂-SPT is driven with the Na⁺ gradient generated by the Nrf/cytochrome c complex catalyzing the highly exergonic oxidation of Fdx²⁻ and reduction of Fe(III) (Rxn. 3). Fdx²⁻ is also utilized in reduction of CO₂ that supplies the carboxyl group for condensation with the methyl group of CH₃-H₂-SPT producing acetate (Rxn. 5 and 6). Fdx²⁻ and F₂H₂ are generated in reversal of the CO₂ reduction pathway (Rxn. 7 and 8). F₂H₂ is oxidized by the Fpo complex (Rxn. 9) with transfer of electrons to MP and Fe(III) coupled to generation of a H⁺ gradient. The H⁺ gradient, together with the Na⁺ gradient, drives ATP synthesis assisted by the Mrp antiporter that optimizes the H⁺/Na⁺ ratio optimal for the ATP synthase dependent on both H⁺ and Na⁺ (Rxn. 14 and 15) (Schlegel et al., 2012a; Jasso-Chavez et al., 2013). The reverse methanogenesis pathway is remarkably similar to that proposed for uncultivated *Methanosarcinales* sp. ANME-2a present in marine sediments that perform AOM. It is also proposed that Fdx²⁻ is generated by HdrA2B2C2 previously shown to oxidize F₂H₂ and reduce Fdx coupled to reduction of CoMS-CoB via energy-conserving FEBE (Rxn. 10–12) (Yan et al., 2017). The HSCOm and HSCOβ produced are oxidized by HdrDE coupled to the reduction of Fe(III) regenerating CoMS-CoB (Rxn. 13). This proposed role would be essential in the environment where low availability of Fe(III) limits the generation of Na⁺ and H⁺ gradients by the Rnf and Fpo complexes. In this scenario, the Fdx²⁻ produced by HdrA2B2C2 is used to reduce CO₂ for the synthesis of acetate and ATP by substrate level phosphorylation (Rxn. 5 and 6). Notably, the *Methanosarcinales* sp. ANME-2a metagencode encodes HdrA2, HdrB2, and HdrC2 homologs with 59, 72, and 59% identities (Supplementary Figure S1) consistent with a role in reverse methanogenesis by ANME.

Unlike the HdrA1B1C1 of *M. acetivorans* and the HdrABC of obligate CO₂-reducing methanogens, the C-terminal domain of HdrA2 extends with sequences homologous to MvhD (Yan et al., 2017). Although the function of this fused MvhD is unknown, HdrA2 homologs are ubiquitous in acetotrophic and methylotrophic species of the order *Methanosarcinales* suggesting important functions. Remarkably, HdrA2 and HdrBC homologs are present in non-methanogenic species of the domain *Bacteria* signaling diverse functions.

A metagenomics-based metabolic model of electron transport is proposed for the nitrate-dependent reverse methanogenesis by *Methanoperedens*-like ANME (Figure 11). Apart from the F₂H₂, HSCOm/HSCOβ, and Fdx²⁻ generated by reverse methanogenesis, the model contrasts with the Fe(III)-dependent pathway of *M. acetivorans* (Arshad et al., 2015). Foremost, the genome encodes a Rieske-type protein, cytochromes c and b, and a nitrate reductase that reduces nitrate to nitrite with reduced menaquinone (MQ₂) generated by a F₂H₂ dehydrogenase (Fpo) that combine to generate a proton gradient driving ATP synthesis. Reduction of MQ is also accomplished by oxidation of HSCOm/HSCOβ with HdrDE. An energy-converting hydrogenase homolog (Ech) is proposed to oxidize Fdx²⁻ and contribute to the proton gradient although the fate of produced H₂ is unknown. Alternatively, Fdx²⁻ is the electron donor to a flavin-based electron confurcating complex comprised of an HdrABC homolog oxidizing HSCOm/HSCOβ and donating electrons to the F₂H₂-dependent hydrogenase subunit (FrhB) proposed to oxidize Fdx²⁻ and reduce F₂H₂. Energy is conserved in the confurcation reaction rather than lost as heat should FrhB alone oxidize Fdx²⁻ and reduce F₂H₂ (ΔE°′ = 120 mV). The genome of "*Candidatus Methanoperedens nitroreducens*" encodes a homolog of *M. acetivorans* HdrA2 which presents the possibility of an HdrA2B2C2 homolog catalyzing the confurcation reaction (Berger et al., 2017; Yan et al., 2017).

**CONCLUSION**

Methanogenic and reverse methanogenic pathways are proposed to involve FEBE or FEBC in electron transport that also serve as mechanisms of energy conservation. However, there is a significant lack of understanding requiring further investigation.

1. Biochemical confirmation of FEBE is needed for the several proposed complexes other than that shown for the purified MvhADG:HdrABC of *M. marburgensis* and HdrA2B2C2 of *M. acetivorans*.

2. A more detailed understanding of the FEBE mechanism of HdrABC is needed. The crystal structure of MvhADG:HdrABC from *M. thermolithotrophicus* has provided a guide for experiments to address questions of electron gating and stabilization of reduced flavin intermediates. The ability to produce the catalytically active recombinant HdrA2B2C2 of *M. acetivorans*, combined with the crystal structure of MvhADG:HdrABC, provides a foundation for genetic approaches generating enzyme variants that will facilitate a detailed understanding of FEBE.
(3) Validation is needed for the proposed role of HdrA1B1C1 in the methylotrophic pathway of \textit{M. acetivorans} and related methylotrophic methanogens; in particular, the proposal that HdrA1B1C1 of \textit{M. acetivorans} oxidizes F$_{420}$H$_2$ in analogy to that shown for HdrA2B2C2. Also worthy of investigation are the uncharacterized HdrA2B2C2 homologs in the order \textit{Methanosarcinales} and the domain \textit{Bacteria}.

(4) Investigations are in order to determine the mechanism by which H$_2$ is oxidized and electrons are delivered to the proposed MvhD/HdrABC and MvhD/HdrABC/Fwd complexes of methanogens in the orders \textit{Methanocellales} and \textit{Methanomicrobiales}.

(5) The proposed roles for FBEB and FBEC in reverse methanogenesis pathways require validation via analyses of deletion mutants.
AUTHOR CONTRIBUTIONS
ZY performed the research. JF wrote the manuscript.

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SUPPLEMENTARY MATERIAL
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