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# Methane oxidation by anaerobic archaea for conversion to liquid fuels

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**Abstract** Given the recent increases in natural gas reserves and associated drawbacks of current gas-to-liquids technologies, the development of a bioconversion process to directly convert methane to liquid fuels would generate considerable industrial interest. Several clades of anaerobic methanotrophic archaea (ANME) are capable of performing anaerobic oxidation of methane (AOM). AOM carried out by ANME offers carbon efficiency advantages over aerobic oxidation by conserving the entire carbon flux without losing one out of three carbon atoms to carbon dioxide. This review highlights the recent advances in understanding the key enzymes involved in AOM (i.e., methyl-coenzyme M reductase), the ecological niches of a number of ANME, the putative metabolic pathways for AOM, and the syntrophic consortia that they typically form.

**Keywords** Archaea · Anaerobic oxidation of methane · Anaerobic methanotrophic archaea · ANME

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## Introduction

Methane is not only an important modulator of global climate as a potent greenhouse gas [33, 71] but also by far the largest constituent of natural gas deposits. Global proven natural gas resources have been estimated at 6,800 trillion cubic feet (tcf), which when converted to barrels of oil equivalent is approximately 69 % of the global proved crude oil reserves [37]. China, Argentina, Algeria, the United States, and Canada have the largest technically recoverable shale gas reserves [85]. The increased development of shale gas resources is expected to be an important contributor to the predicted 56 % increase in natural gas reserves in the United States from 2012 to 2040 [5]. Over the last ten reported years (ending in 2012) the United States has seen increases in proved reserves of dry natural gas (i.e. after the removal of nonhydrocarbon gases and liquefiable hydrocarbons) [61].

The low energy density and lack of infrastructure for the use of compressed natural gas [33] are spearheading the use of gas-to-liquid (GTL) technologies. The Fischer–Tropsch process is the current industrial standard used to generate liquid fuels from synthesis gas (i.e., syngas, a mixture of hydrogen, carbon monoxide, and carbon dioxide). Syngas is produced through steam reforming of natural gas and then fed to the Fischer–Tropsch process, where it is converted to hydrocarbons that can be further refined to produce high-value fuels, including diesel and gasoline [33]. Approximately

25–45 % of the carbon is recovered as hydrocarbon products [21]. GTL-Fischer–Tropsch (GTL-FT) technologies require large-scale plants with multi-billion dollar capital expenditures (CapEx). These plants must produce upwards of 10,000 barrels of oil equivalent per day (BPD) before their CapEx/BPD become economically attractive. Direct bioconversion processes have the potential to avoid the need for very high CapEx through bypassing syngas formation, avoiding large temperature and pressure changes, and directly converting methane to liquid fuels [33]. For example, corn-ethanol plants with similar CapEx/BPD to GTL-FT plants typically have capacities between 500 and 5,000 BPD [33]. Nevertheless, significant challenges exist in terms of elucidation of the relevant organisms and pathways, enzyme optimization, and scale-up.

Methane oxidation is performed both aerobically and anaerobically within the ocean biome. Aerobic methanotrophs utilize methane monooxygenases (MMOs) that are classified as either soluble (sMMO) or membrane-bound particulate (pMMO) [59]. Aerobic methane oxidation converts methane to formaldehyde through methanol, which connects with the rest of metabolism through either the ribulose monophosphate pathway as seen in type 1 methanotrophs, or through the serine pathway as seen in type 2 methanotrophs [33]. An existing bioconversion process for the anaerobic oxidation of methane (AOM) has already been uncovered in the oceans. Oceans contribute only approximately 2 % of the global methane budget [71] despite the multiple sources and rates of contribution. This surprisingly low net total is due in large part to the offset provided by the AOM performed within the marine sediments, estimated at 70–300 teragrams (Tg) of methane per year [93]. Therefore, significant changes to the global climate are plausible if oceanic methane oxidation processes become saturated [39]. Methane enters the oceans via a number of different sources including coastal runoff and rivers [17, 79], diffusion from organic-rich anoxic sediments, seeps, vents, and mud volcanoes [71]. These seeps supply methane to a number of regions including the Black Sea, which is the world's largest surface water reservoir of dissolved methane [78] contributing between 0.03 and 0.15 Tg of methane per year [20]. The oceans are also a large reservoir of methane, most of which is contained within methane clathrate hydrates. Methane clathrates are solid nonstoichiometric compounds [35] formed from methane and water under low temperatures and high pressures, typically found along continental margins at depths of 600–3000 m [71]. Clathrates are dynamic structures undergoing breakdown at the top end and formation at the bottom end of the zone of stability [19]. Previous estimates have placed the total methane contained in clathrates as high as 10,000 gigatonnes (Gt) of carbon [71]. More recent estimates approximate this total as 3000 Gt of carbon [11], which is 5,900 and 7,300 times greater than estimates of yearly carbon from methane sources according to process-based and atmospheric inversion models, respectively [40].

Microorganisms in anoxic environments have been estimated to oxidize more than 80 % of the methane produced in the world's oceans [66]. Anaerobic methanotrophs predominantly exist within the sulfate-methane transition zone (SMTZ), a region in the sediments where the methane rising from below and the sulfate sinking from above form a region suitable for anaerobic methanotrophy. Sulfate is necessary to support the sulfate reducing bacteria (SRBs) that commonly form consortia with anaerobic methanotrophic archaea (ANME). The SMTZ can vary in both size and location depending on a number of factors, including the depth of organic matter and methane production rates [41, 71, 82]. Several different clades of ANME have been discovered that are capable of anaerobically oxidizing methane. AOM does not require two external electrons to activate methane, unlike aerobic methanotrophs that utilize MMOs by consuming NADH or NADPH [33]. As is discussed in more detail in the pathway section below, while anaerobic methane oxidation is thermodynamically infeasible unless coupled with an electron sink, its higher carbon efficiency makes it appealing if paired with the correct electron acceptor. ANME perform AOM in a number of different environments with a number of different electron acceptors [6, 65]. While many of these organisms form consortia with a variety of SRBs, there is still debate as to the exact mechanism by which the syntrophy is facilitated [53, 62]. The pathway by which these organisms oxidize methane is commonly agreed upon to be the reversal of the methanogenesis pathway found in archaeal methanogens [31].

## Environmental conditions and species associated with ANME

ANME have been categorized using 16 s rRNA sequences into three clades, ANME-1, ANME-2, and ANME-3. ANME-1 and ANME-2 have been found in a wide variety of locations, while ANME-3 has been found most commonly near mud volcanoes [43, 49]. ANME-1 is distantly related to the orders *Methanosarcinales* and *Methanomicrobiales* [53] while both ANME-2 and ANME-3 belong to the *Methanosarcinales* order [65]. Two of these clades are further divided into subgroups, giving rise to ANME-1a and ANME-1b, along with ANME-2a–d [55]. Despite their shared capability to perform AOM, it is expected that the members of the three clades belong to different families and orders, as even the subgroups of ANME-2 have low intergroup similarity [43].

ANME populations are typically heterogeneous with a mixture of the clades present. In these populations one clade is frequently dominant, typically ANME-2 or

ANME-3 [41] with a few exceptions. For example, ANME-1b was the only ANME subgroup found in the samples taken from shallow sediments near a mud volcano in the Gulf of Mexico. The authors suggest that geochemical factors such as high salinity and the consistent lack of oxygen, as the sediments are permanently anoxic, contribute to the dominance of ANME-1b [48]. Knittel et al. suggested that ANME-1 may be more sensitive to oxygen than ANME-2 [43]. This difference could also account for the dominance of ANME-1 in the Black Sea mats where the ANME-1 cells account for between 40 and 50 % of the total number of cells as they are exposed exclusively to anoxic bottom waters [43]. The community composition at a site such as Hydrate Ridge at the Cascadia Margin in the Pacific Ocean is composed predominantly of ANME-2 [43], which may be due in part to the fact that the surface sediments are occasionally flushed with oxygen [92].

The clades have also adapted to a wide variety of temperatures and pH values. Typically the optimal conditions for AOM are 5–10 °C above the in situ temperature and a pH value between 7.7 and 7.9 [8]. However some ANME populations have adapted to extreme environments, from the Haakon Mosby mud volcano where the bottom water is −1.5 °C [46] to populations in the hydrothermal sediments of the Guaymas Basin at temperatures up to 95 °C [76]. Hydrate Ridge, the Gulf of Mexico, and the Black Sea are three of the more commonly sampled areas and have in situ temperatures of 4, 6, and 8 °C, respectively [48, 63]. Similarly a large range of pH values are tolerated by certain populations ranging from a pH as low as four in Yonaguni Knoll to 9–11 in the Lost City hydrothermal field [10, 36].

The ANME clades can also be distinguished based on the type of consortia they form and the organisms with which they cooperate. ANME organisms most commonly form consortia with SRBs. Organisms of the ANME-1 and ANME-2 clades have been shown to form consortia with SRBs of the *Desulfosarcina/Desulfococcus* (DSS) branch of *Deltaproteobacteria* [43, 54]. Some ANME-2 along with ANME-3 methanotrophs associate with *Desulfobulbus*-related (DBB) SRB [49, 69]. SEEP-SRB1 is a subset of the DSS clade and the SEEP-SRB1a subcluster has been reported to be the most commonly associated partner for between 75 and 95 % of the consortia with ANME-2a and ANME-2c [42, 77]. ANME-2c has also been reported to be in consortia with a subgroup of *Desulfobulbaceae* [69]. Some of these consortia have a preference for nitrate, and the nitrogen sources in sediments could define the niches that allow for the coexistence with ANME/DSS consortia [29].

The three clades vary in both their individual shape and the type of aggregate that they typically form. ANME-1 cells are commonly rectangular whereas ANME-2 and ANME-3 cells are coccoid [49, 67]. Of the three clades ANME-1 is most frequently found as single cells [67]; however, when it forms a consortium ANME-1 assumes a mat-type association as observed in samples from the Black Sea [90]. ANME-2a/DSS aggregates have been reported as mixed- and shell-type while ANME-3 has been found to form shell-type aggregates [41]. Figure 1 shows a representation of the microbial reef structures that have been found in the Black Sea to illustrate both the aggregate shapes as well as the presence of the SMTZ. The Black Sea is one of the most common sampling sites for ANME populations and was the source of the samples used to find the structure of ANME-1 Mcr [80].

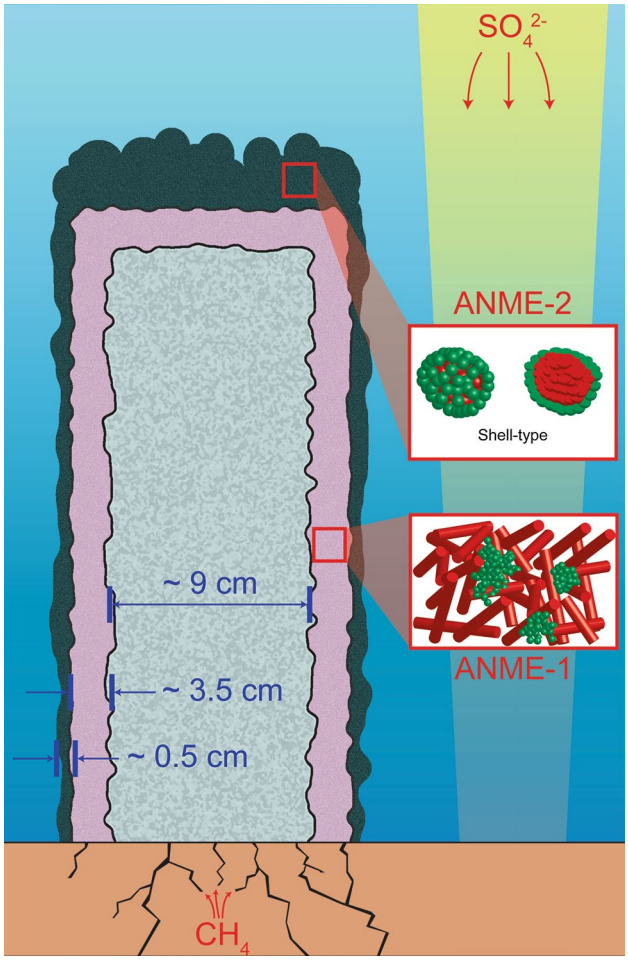


**Fig. 1** Representation of the microbial reefs found in the Black Sea. Microbial reef structures form over methane seeps in the SMTZ. The inner structure is a porous carbonate precipitate (*grey*) [90]. The carbonate is covered by a layer of ANME-1 (*pink*) in a mat-type consortium. The outer layer (*black*) is composed of ANME-2 in shell-type consortia. This layer is described as nodular and is thicker at the top of the reef. In the insets, the ANME cells (*red*) and SRB cells (*green*) have colors matching those visualized by FISH [41]. The sizes of microbial reefs vary but estimates were provided by Treude et al. [91]. Descriptions of color and photos of the structures can be found in several works [41, 43, 73] (color figure online)

Despite the potential of ANME to revolutionize methane bio-activation, a number of significant challenges have so far prevented the development of industrially viable bioconversion processes. The most pressing obstacle is that no pure culture of an ANME organism has been achieved due in large part to their exceptionally slow native growth rates and the presence of a syntrophic partner for many ANME [8]. This slow growth rate also limits the ability to quickly cultivate the ANME populations necessary for large-scale processes. Further investigations into genetic elements underpinning growth are necessary with some research already underway [8]. In addition, despite the bioconversion of enormous amounts of methane into biomass by oceanic AOM, the pathways involved, regulatory structures, and possible interacting partners are still poorly characterized.

## Pathway for AOM

The metabolic pathway(s) by which ANME, alone or in syntrophy, catalyze the oxidation of methane in anoxic environments are still not fully understood. Using $^{13}C$ labeled methane it was shown that pure cultures of methanogenic organisms exhibit trace methane oxidation [58]. This combined with the lack of methane oxidation in organisms that do not contain methyl-coenzyme M reductase (Mcr) provides support for the reversal of methanogenesis as the main pathway used for AOM [58]. While it must be paired with the reduction of another compound, reverse methanogenesis has higher carbon efficiency than aerobic methane oxidation.

Genomics analysis [31] and later the generation of a draft genome for ANME-1 [53] confirmed that ANME-1 contains all genes of the methanogenesis pathway except for methylene-tetrahydromethanopterin reductase (Mer) (Fig. 2). The authors suggested that the methyl group is converted into a methylated compound that is redirected into the reverse methanogenesis pathway (Fig. 2, dashed pathway). ANME-2 has been shown to contain and express all of the genes for methanogenesis [95]. The presence of these genes along with the demonstrated ability of the pathway to operate in the reverse direction [58] lend credence to the hypothesis that reverse methanogenesis is the main pathway for AOM in ANME-2. Several of these enzymes have been the focus of further investigation. A homolog to Mcr was purified and characterized from anoxic sediments and shown to bind coenzyme M and coenzyme B [80] and initiate the first step of reverse methanogenesis in the presence of sulfate [45, 52]. Homologs from ANME-1 for three other methanogenesis enzymes, formyl-MFR:$H_4$MPT formyltransferase (Ftr), methenyl-$H_4$MPT cyclohydrolase (Mch), and $F_{420}$-dependent methylene-$H_4$MPT dehydrogenase (Mtd) were synthesized and expressed in *Escherichia coli* [44]. In the presence of the corresponding coenzymes, the purified enzymes showed activity for their native substrates [44]. The presence of these methanogenesis pathway genes in ANME organisms along with the ability of Mcr to catalyze the first step in the reverse methanogenic pathway [45, 52] provides support for the activity of reverse methanogenesis in ANME. Reverse methanogenesis is not the only putative pathway for AOM. The addition of methane to fumarate was also suggested as a possible methane activation mechanism [7, 12, 89]. In this proposed mechanism a glycyl radical enzyme extracts a hydrogen atom from methane, forming a methyl radical that then reacts with fumarate to form a methylsuccinyl radical, which then reacts with the enzyme to reform the glycyl radical and 2-methylsuccinate [7, 12, 89]. This mechanism was

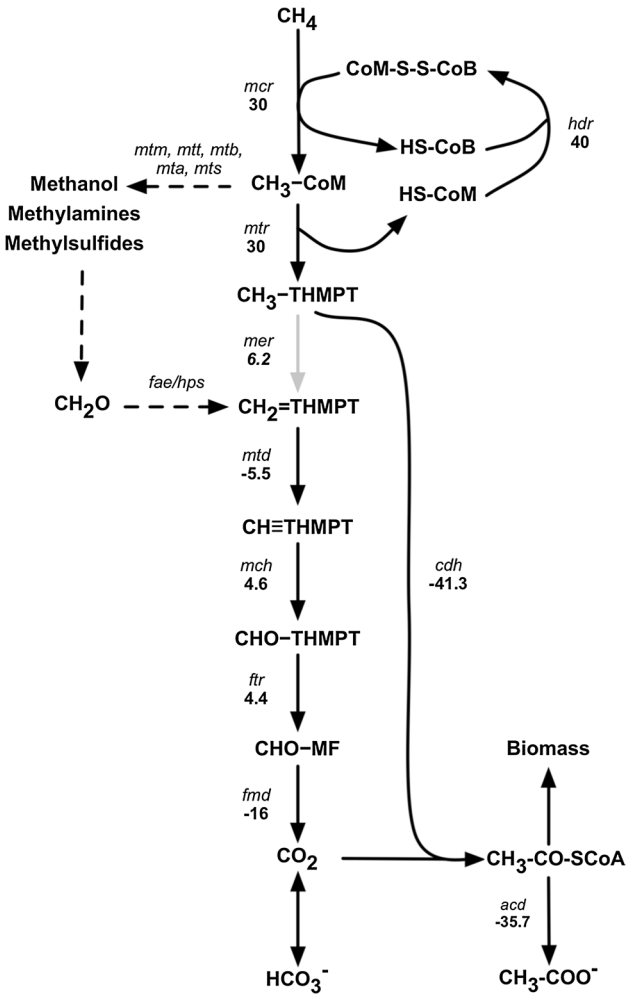


**Fig. 2** Reverse methanogenesis pathway. The Mer enzyme (*grey*) is found in ANME-2 but not ANME-1. The proposed alternative pathway for ANME-1 by Meyerdierks et al. [53] is shown in *dotted arrows*. Gene names are italicized and $\Delta G$ values (in *bold* below gene names) are from Thauer [86] with updated values for Mcr [81] and Fmd [87] (color figure online)

**Table 1** Comparison of $\Delta G$ and carbon efficiency for anaerobic and aerobic methane oxidation

| Product | $\Delta G^{\circ\prime}$ per methane oxidized, (kJ mol$^{-1}$ $CH_4$) | | | Overall efficiency | | | |
|---|---|---|---|---|---|---|---|
| | | | | Reverse methanogenesis | | Aerobic methane oxidation (MMO) | |
| | $SO_4^{2-} \rightarrow HS^-$ | $NO_3^- \rightarrow N_2$ | $O_2 \rightarrow H_2O$ | Carbon efficiency (%) | Energy efficiency (%) | Carbon efficiency (%) | Energy efficiency (%) |
| Methanol | 58.97 | −124.68 | −385.73 | 100 | 79.6 | 66.7 | 53.0 |
| Ethanol | 24.87 | −158.78 | −408.46 | 100 | 76.9 | 66.7 | 51.3 |
| Butanol | 13.43 | −170.22 | −416.09 | 100 | 76.5 | 66.7 | 51.0 |

$\Delta G$ calculations were performed for three different electron acceptors (sulfate, nitrate, and oxygen) and three different products. Values were calculated assuming all compounds were in the aqueous phase at pH 7, 25 °C, and an $I$ value of 0.25 M using the formula supplied by Alberty [1]. Carbon efficiency was calculated as the ratio of the number of carbons in the product to the carbons fed to the pathway. Energy efficiency is calculated from the lower heating value (LHV) of the product divided by the LHV of methane supplied to the pathway

initially proposed in analogy to the anaerobic alkane activation mechanism under sulfate or nitrate reducing conditions on non-methane alkanes [12].

Unlike the aerobic oxidation of methane, the reverse methanogenesis pathway is not by itself thermodynamically feasible; however, it has the potential for 100 % carbon efficiency as compared to the 66.7 % achieved by aerobic methane oxidation (see Table 1). Reverse methanogenesis of the aceticlastic pathway produces acetyl-CoA whereas aerobic methanotrophs typically fix methane using the ribose monophosphate cycle to produce glyceraldehyde-3-phosphate. While the production of acetyl-CoA from glyceraldehyde-3-phosphate produces more energy than reverse methanogenesis, it does so at the expense of one carbon lost as $CO_2$. Therefore, reverse methanogenesis can retain all carbon from methane promising better product yields. Aerobic methanotrophs transfer all of their electrons to oxygen, either directly via the methane monooxygenase reaction, or indirectly through the electron transport system. For example with the production of acetate the pathway produces 10 electrons worth of reducing equivalents as compared to the eight reducing equivalents produced in reverse methanogenesis from the carbon dioxide reduction pathway. If reverse methanogenesis is paired with an electron acceptor (such as nitrate) that makes the production of alcohols thermodynamically feasible, then the increased carbon efficiency of the pathway makes it an attractive alternative to aerobic methanotrophs.

## Potential electron acceptors and syntrophic interactions

A number of different compounds can be reduced in conjunction with AOM. Table 2 shows the overall ΔG values of AOM paired with the reduction of these acceptors, and Fig. 3 illustrates several proposed syntrophic interactions between ANME and bacterial partners. The most prominent of these is sulfate, which is reduced by SRBs that form consortia with ANMEs. A number of different syntrophic

**Table 2** $\Delta G^{\circ\prime}$ values for overall reactions using different electron acceptors

| Reaction | $\Delta G^{\circ\prime}$ (kJ mol$^{-1}$ $CH_4$) |
|---|---|
| $CH_4 + SO_4^{2-} \rightarrow HCO_3^- + HS^- + H_2O$ | −31.4 |
| $CH_4 + 4NO_3^- \rightarrow CO_2 + 4NO_2^- + 2H_2O$ | −519.9 |
| $CH_4 + 4MnO_2 + 7H^+ \rightarrow HCO_3^- + 4Mn^{2+} + 5H_2O$ | −511.6 |
| $CH_4 + 8Fe(OH)_3 + 15H^+ \rightarrow HCO_3^- + 8Fe^{2+} + 21H_2O$ | −1691.7 |

Overall reactions from Knittel et al. [41], Haroon et al. [32], and Beal et al. [6]. $\Delta G^{\circ\prime}$ values were calculated at pH 7, 25 °C, I value of 0.25 M using the formula supplied by Alberty [1]. The $\Delta G^{\circ\prime}$ value for the iron reduction reaction was calculated assuming that ferric hydroxide dissociates into its respective ions

interactions have been proposed for ANME and SRBs (Fig. 3a–d) [57, 94, 96]. SRBs play an important role in the sulfur cycle as they reduce sulfate to hydrogen sulfide [60]. ANME organisms have been shown to grow independently [66] suggesting that physically associated SRBs are not obligatory for AOM. A possible mechanism for ANME cells to circumvent an associated SRB organism involves the formation of a zero-valent intracellular or deposited sulfur as an intermediate [56]. ANME-2 oxidizes methane with a concomitant reduction of sulfate to disulfide through an unknown enzymatic mechanism while deltaproteobacteria disproportionates disulfide to sulfide and sulfate. This reformed sulfate is then reused by the archaea (Fig. 3d) [56].

Several different mechanisms have been proposed to facilitate the syntrophic interactions between ANME and their bacteria partners. Intermediates from reverse methanogenesis, including hydrogen, formate, methanol, methanethiol, and acetate, have all been proposed as possible electron carriers [4, 53, 57]. Arguments against these compounds acting as intermediates are summarized in the work by Nauhaus et al. in which the authors supplied hydrogen,

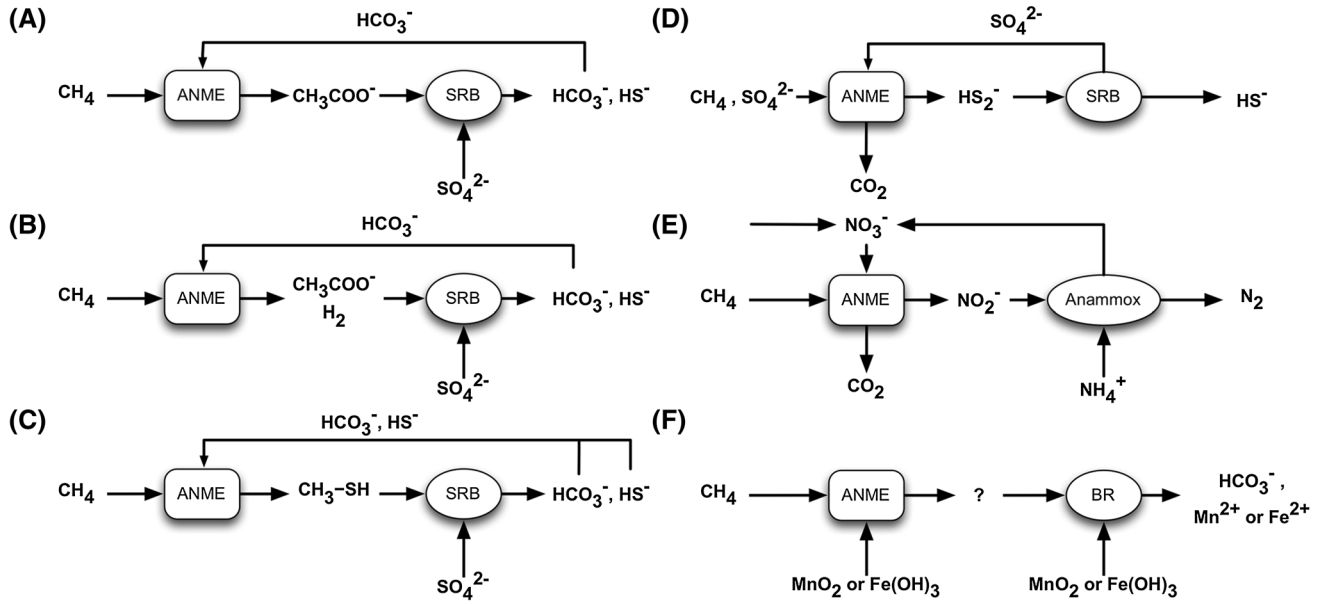


**Fig. 3** Proposed syntrophy interactions for various electron acceptors. **a** AOM by ANME-SRB syntrophy based on Zehnder and Brock [96] and Hoehler et al. [34]. **b** AOM by ANME-SRB syntrophy based on Valentine and Reeburgh [94]. **c** AOM by ANME-SRB syntrophy based on Moran et al. [57]. **d** AOM by ANME with disulfide disproportionation by SRB based on Milucka et al. [56]. **e** AOM by ANME-Anammox syntrophy based on Haroon et al. [32]. **f** AOM by ANME-BR syntrophy or BR alone based on Beal et al. [6]. Anammox is an anaerobic ammonium oxidizer. BR is a bacterial reducer

formate, acetate, or methanol to consortia with and without methane and found either slower rates of sulfate reduction or no change in sulfate reduction, respectively [62]. Moran et al. suggested methyl sulfides as possible syntrophic intermediates as they would require a specialized SRB, which may account for the limited number of SRBs that associate with ANME (Fig. 3c) [57]. In the analysis of the ANME-1 draft genome and mRNA expression analyses a set of clustered genes was annotated as secreted multiheme c-type cytochromes. These were proposed as a possible system for the direct electron transport to the SRBs [53]. Similarly nanowires have been proposed as a possible mechanism for the transport of electrons [72, 83]. While nanowires are capable of facilitating AOM at reported rates [3, 64], they do not account for the number of both ANME-1 and ANME-2 cells that are found outside of consortia [66].

ANME consortia have also been found to use a number of other electron acceptors in addition to sulfate such as $NO_3^-$, $Fe^{3+}$, and $Mn^{4+}$ all with more negative $\Delta G$ values than sulfate-dependent AOM (Table 2) [93]. In fact even SRBs have even been shown to use electron acceptors other than sulfate [16, 84]. Raghoebarsing et al. proposed the coupling of denitrification with AOM. The system required 0.37 and 0.62 mol of methane per mole of nitrite and nitrate reduced, respectively, effectively requiring 1 mol of methane per mole of nitrogen gas produced. Experimental studies revealed that the denitrification rate was almost double that of methane oxidation, indicating that other organic compounds were also oxidized [70]. It was shown that archaea were not essential for AOM with nitrite as an electron acceptor, and one bacterium, *Methylomirabilis oxyfera*, has a complete aerobic methanotrophic pathway. This pathway could use the oxygen produced from the reaction of nitric oxide to dinitrogen and oxygen as a metabolic intermediate [23, 24]. Recent work by Haroon et al. [32] has shown that ANME-2d can be the dominant population in a bioreactor fed with nitrate, nitrite, and methane. *M. oxyfera* and known SRBs were not detected in the reactor. The ANME-2d genome was recently sequenced and a complete methanogenesis pathway along with the nitrate reductase complex that had been laterally acquired from a bacterial donor was found and highly expressed in the bioreactor compared to housekeeping genes [32]. Given the comparable expression levels of nitrate reductases between ANME-2d and the flanking populations it appears that ANME-2d also performs the majority of nitrate reduction within the bioreactor. The nitrite generated from this pairing of AOM with nitrate reduction is then consumed by anaerobic ammonium oxidizing bacteria that outcompetes *M. Oxyfera* for the available nitrite (Fig. 3e) [32]. The addition of methane to fumarate discussed by Thauer and Shima was initially proposed given the higher (i.e., 1000-fold) catalytic efficiency ($k\text{cat}/K_m$) for AOM with nitrate [70] compared to AOM with sulfate using reverse methanogenesis [89]. Continual methane consumption even though archaea cell density is reduced over time [24, 70] suggests that another consortium member such as *M. oxyfera* could be performing

methane addition to fumarate. The discovery of the complete methanogenesis pathway and nitrate reductase complex in the ANME-2D genome and their high levels of expression [32] supports the hypothesis that ANME are capable of pairing reverse methanogenesis with the reduction of nitrate.

Beal et al. showed that both manganese and iron (in the forms of birnessite and ferrihydrate, respectively) could be used as electron acceptors. The authors suggest that manganese-dependent AOM could be carried out by ANME-1, ANME-3 with a bacterial partner, or solely by bacteria (Fig. 3f). They also note that if the global flux of manganese and iron were used to oxidize methane it would account for roughly 25 % of current AOM [6]. While manganese and iron have the favorable $\Delta G$ values for pairing with AOM, their applicability for large-scale applications is limited by the fact that manganese and iron oxides are largely insoluble solids, and therefore less accessible than sulfate. Beal et al. showed that AOM paired with either of these compounds occurred at slower rates than those paired with sulfate [6]. The confirmation of archaeal AOM paired with nitrate reduction is much more recent than its sulfate counterpart [32]. As seen in Table 1 the overall $\Delta G$ is negative for growth for ANME-SRB and ANME-Anammox, however, Table 2 shows that the production of alcohols appears to be thermodynamically feasible only for the ANME-Anammox association. This implies that an alternative electron acceptor such as nitrate may need to be considered for an alcohol-based biofuel processes.

## Methyl-coenzyme M reductase

The central enzyme to all ANME consortia is a homolog to the methanogenic Mcr. ANME Mcr (the Mcr homolog found in ANME) catalyzes the redox reaction that couples the oxidation of methane to methyl-coenzyme M ($CH_3$-S-CoM) with the reduction of the coenzyme M-coenzyme B heterodisulfide (CoM-S–S-CoB) to coenzyme B (CoB). ANME Mcr is critical for the bioconversion of methane as it is asked to selectively activate methane by overcoming the 438.9 kJ $mol^{-1}$ of free energy required to break the first carbon-hydrogen bond without proceeding with breaking the remaining carbon-hydrogen bonds even though the free energy barrier is lower. The essentiality of this enzyme is demonstrated by the fact that Mcr can be used to assess phylogeny for both methanogens and ANME [30, 50]. The *mcrA* a–b monophyletic groups are related to the ANME-1 clade, while the *mcrA* c–d groups are associated with the ANME-2 clade [30]. The presence of *mcrA* in both methanogens and methanotrophs along with the fact that AOM is inhibited by bromoethane-sulfonate, a known inhibitor of Mcr, lends support to the reverse methanogenesis hypothesis [26, 31, 63].

Mcr structural information for three methanogens has been available for several years [22, 28], but the structure of ANME-1 Mcr in complex with its prosthetic group and coenzymes (PDB: 3SQG) was only recently elucidated from samples taken from the Black Sea [80]. The Mcr active site is shielded from solvent contact after a presumed conformational change upon substrate binding [27, 75, 81]. Methanogenic Mcrs and the ANME-1 Mcr are largely identical. Key differences include a different pattern of post-translationally modified residues and a cysteine-rich patch in ANME-1 Mcr which may be involved in a redox-relay system [80]. All methanogenic and ANME Mcrs contain an essential nickel tetrahydrocorphin prosthetic group, which is either coenzyme $F_{430}$ or a variant of coenzyme $F_{430}$ [2, 38, 52].

The mechanism by which methanotrophy occurs in ANME Mcr has yet to be determined. The reverse methanogenesis hypothesis suggests that ANME Mcr and methanogenic Mcr make use of the same mechanism but are driven in opposite directions. Thus, originally there have been two proposed reaction mechanisms for ANME Mcr. Mechanism I involves formation of a methyl-Ni(III) intermediate [22, 27] while Mechanism II includes a methyl radical intermediate [14, 68]. An in-depth discussion providing supporting evidence for each of the two proposed mechanisms can be found in Thauer et al. [88]. The ANME Mcr reaction mechanism remains unresolved as recent evidence supports the function of both Mechanism I [13, 18] and Mechanism II [15, 75]. This led to Mechanism III, a hybrid reaction mechanism that explains the simultaneous presence of key intermediates for both Mechanism I and Mechanism II [47]. Mechanisms I–III are depicted in Fig. 4. The $\Delta G°$ for ANME Mcr is 30 ± 10 kJ/mol, which is revised from the previous value of 45 kJ/mol [81]. A mechanism that couples the endergonic step at one active site with an exergonic step at the second active site was proposed to overcome the unfavorable thermodynamics [25].

The catalytic activity of ANME Mcr has not yet been determined because the purified enzyme was isolated in an inactive form [80]. Despite this, a methanogenic Mcr capable of AOM showed a specific activity of approximately 11.4 nmol/min/mg Mcr at 60 °C and 1 bar methane [74]. However, ANME-1 Mcr was maintained in artificial seawater medium at 8 °C [80]. The different growth conditions for ANME Mcr and methanogenic Mcr could explain the discrepancies in their structures. The specific activity of methane monooxygenases is 5090 nmol/min/mg enzyme for sMMO [9] and 160 nmol/min/mg enzyme for pMMO [51]. The observed specific activity for AOM by methanogenic Mcr is thus one to two orders of magnitude lower than that of methane monooxygenases. Since ANME Mcr was isolated in an inactive form, its true activity is unknown. Given that the only measured activity was for an enzyme evolved to carry out the reverse activity (i.e.,

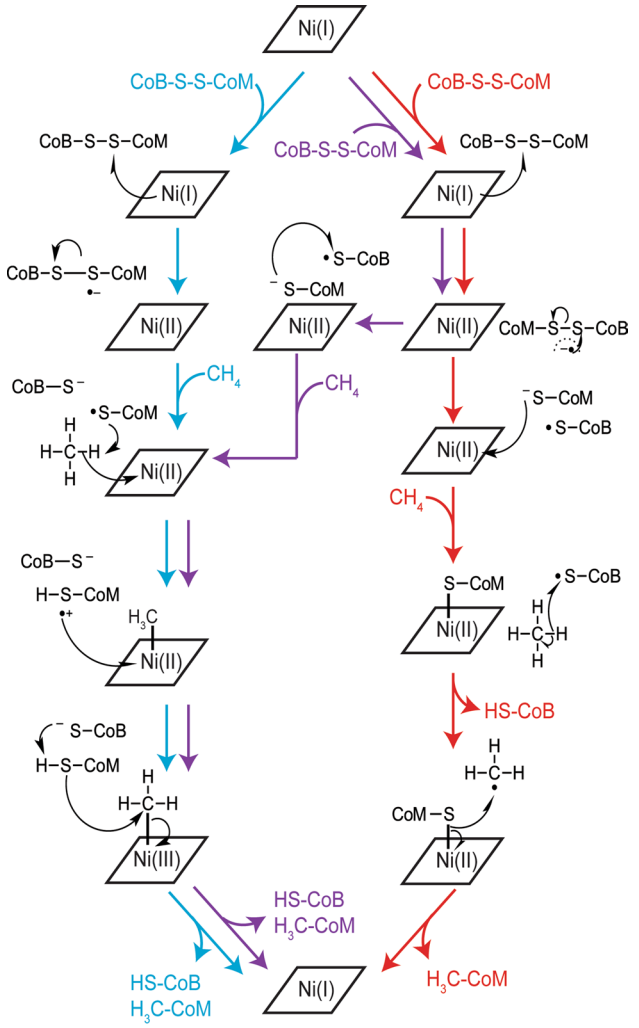


**Fig. 4** Proposed reaction mechanisms for ANME Mcr. Mechanism I (*blue*) involves formation of a methyl-Ni(III) intermediate [22, 27], and Mechanism II (*red*) includes generation of coenzyme B and methyl radical intermediates [14, 68]. Mechanism III (*purple*) was developed after key intermediates were found from both Mechanism I and II [47]. The nickel atom in the figure is part of coenzyme $F_{430}$, and Mcr is only active if nickel is in the +1 oxidation state, as depicted in each mechanism (color figure online)

methanogenesis), ANME Mcr may ultimately reach a substantially higher activity than the one measured for methanogenic Mcr. ANME Mcr catalysis is slow due to scission of the strong C–H bond of methane. Protein engineering efforts could pave the way for improving the catalytic activity of this enzyme into the range of 300 nmol/mg/min to enable an industrially viable bioprocess.

## Summary

The ANME clades, which are able to survive across a wide variety of environmental conditions, provide a number of interesting possibilities in the development of bioconversion alternatives to current GTL processes. These organisms are thought to reverse the methanogenesis pathway in order to anaerobically oxidize methane. When compared with aerobic methane oxidation this pathway is more effective at conserving carbon for the production of compounds derived from acetyl-CoA. Methane diffusion into the liquid phase is the limiting factor in the size of the bioreactor. Anaerobic fermentation offers bioprocess advantages over aerobic fermentation by obviating the need for diffusing oxygen and monitoring flammability limits. Steps towards an industrially viable bioconversion process implementing ANME will require advancements in a number of areas. Isolation and purification of ANME organisms, research into the ANME-Anammox consortia, or identification of other electron acceptors would pave the way for the design of engineered systems that enable the thermodynamically feasible anaerobic conversion of methane into liquid fuels and biorenewables. Furthermore, investigation into the regulation of reverse methanogenesis and improvements of the activity of the ANME Mcr enzyme to at least 300 nmol/mg/min (which would satisfy a rate of methane activation of 1 $g_{CH4}$/L/hr, assuming that Mcr comprises at least 20 % of cellular protein, which is 55 % of cell dry weight, and that the average cell density is 32 g DW/L) could usher viable bioprocess designs.

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**Conflict of interest** The authors declare that they have no conflict of interest.

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