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Minireview

Methyl/alkyl-coenzyme M reductase-based anaerobic alkane oxidation in archaea

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Summary

Methyl-coenzyme M reductase (MCR) has been originally identified to catalyse the final step of the methanogenesis pathway. About 20 years ago *an*aerobic *m*ethane-oxidizing archaea (ANME) were discovered that use MCR enzymes to activate methane. ANME thrive at the thermodynamic limit of life, are slow-growing, and in most cases form syntrophic consortia with sulfate-reducing bacteria. Recently, archaea that have the ability to anaerobically oxidize non-methane multi-carbon alkanes such as ethane and *n*-butane were described in both enrichment cultures and environmental samples. These *an*aerobic multi-carbon al*k*ane-oxidizing archaea (ANKA) use enzymes homologous to MCR named alkyl-coenzyme M reductase (ACR). Here we review the recent progresses on the diversity, distribution and functioning of both ANME and ANKA by presenting a detailed MCR/ACR-based phylogeny, compare their metabolic pathways and discuss the gaps in our knowledge of physiology of these organisms. To improve our understanding of alkane oxidation in archaea, we identified three directions for future research: (i) expanding cultivation attempts to validate omics-based metabolic models of yet-uncultured organisms, (ii) performing biochemical and structural analyses of key enzymes to understand thermodynamic and steric constraints and (iii) investigating the evolution of anaerobic alkane metabolisms and their impact on biogeochemical cycles.

Introduction

Methane (CH_4) is the simplest and most abundant hydrocarbon on Earth (Wahlen, 1993) and a greenhouse gas that substantially influences the Earth's climate (Conrad, 2009; Yvon-Durocher *et al*., 2014). Most of the methane in natural environments, such as marine sediments, cold seeps, wetlands, freshwater lakes and hot springs, is produced by methanogenic archaea (Reeburgh, 2007; Thauer, 2019). Methane is also a component of subsurface-derived natural gas originating from deep marine and terrestrial reservoirs. These natural gases are a diverse mix of methane and short-chain alkanes including ethane, propane, butane, pentane and hexane produced by thermocatalytic decomposition of organic matters in geothermally heated sediments (Stolper *et al*., 2014).

Both methane and other alkanes can be utilized by microorganisms as energy and carbon sources. Under oxic conditions, microorganisms activate alkanes using methane or alkane monooxygenases that produce methanol or other alcohols as primary intermediates (Wang *et al*., 2017). Such monooxygenases are key enzymes present in numerous bacterial lineages, and strains of almost 30 genera have been cultured that oxidize methane or other alkanes (Kallistova *et al*., 2017; Dedysh and

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Knief, 2018). These aerobic alkane oxidizers are the dominant alkanotrophs in oxic and hypoxic soils, surface sediments and aquatic environments (Kallistova *et al*., 2017). However, alkanes are also consumed in anoxic habitats such as marine or freshwater sediments, cold seeps and subsurface ecosystems (reviewed in Evans *et al*., 2019; Bhattarai *et al*., 2019). For instance, it was estimated that the anaerobic oxidation of methane (AOM) removes around 80% of the methane in marine sediments (Reeburgh, 2007; Knittel and Boetius, 2009; Boetius and Wenzhöfer, 2013). To our current knowledge, the anaerobic oxidation of methane and ethane is performed exclusively by archaea (Chen *et al*., 2019; reviewed by Thauer, 2019), while the anaerobic oxidation of propane and butane can be performed by both archaea (Laso-Pérez *et al*., 2016; Wang *et al*., 2019a) and bacteria (Kniemeyer *et al*., 2007; Jaekel *et al*., 2013). The anaerobic oxidation of longer chain alkanes has only been described for bacteria (Aeckersberg *et al*., 1991; Coates *et al*., 1997), yet metagenomic surveys indicate a large unexplored potential for alkane degradation in the archaeal domain (Borrel *et al*., 2019; Laso-Pérez *et al*., 2019; Wang *et al*., 2019b). In this review we mainly present an updated diversity of anaerobic alkane-oxidizing archaea, illustrate their global distribution using public metagenomic data sets from natural environments and summarize the biochemistry of anaerobic methane and multi-carbon alkane-oxidizing pathways.

Diversity and distribution of anaerobic alkane-oxidizing archaea

All so far cultured anaerobic methane, ethane, propane and *n*-butane-oxidizing archaea belong to the phylum Euryarchaeota (Table 1, Nauhaus *et al*., 2007; Holler *et al*., 2011; Laso-Pérez *et al*., 2016; Chen *et al*., 2019; Hahn *et al*., 2020). They are closely related to methanogens and hence contain most genes of the methanogenesis pathway. Yet, anaerobic alkane-oxidizing archaea use the methanogenesis pathway in a reverse direction, thereby consuming alkanes instead of producing them. The key enzyme of the reverse methanogenesis pathway is a methyl-coenzyme M reductase (MCR) or alkyl-coenzyme M reductase (ACR) that activates the methane or other alkanes as methyl-CoM or alkyl-CoM respectively (Thauer, 2019). Global surveys based on 16S rRNA gene sequences showed that *an*aerobic *me*thane-oxidizing archaea (ANME) are widely distributed in marine cold seep environments (Knittel *et al*., 2005; Ruff *et al*., 2015). In most ANME and *an*aerobic multi-carbon al*ka*ne-oxidizing archaea (here abbreviated as ANKA), the MCR or ACR-encoding genes are well conserved and can be used as phylogenetic markers (Friedrich, 2005; Evans *et al*., 2019). We screened public metagenomic data sets for MCR and ACR sequences and found that both ANME and ANKA are widely distributed across the globe, being particularly abundant at methane- and alkane-rich ecosystems (Fig. 1). We show that different types of anaerobic alkane-oxidizing archaea appear in distinct ecosystems or distinct niches in the same ecosystem.

Diversity of anaerobic methane-oxidizing archaea

ANME belong to two orders in the phylum Euryarchaeota, i.e., *Ca*. Methanophagales (ANME-1) and Methanosarcinales (including ANME-2a/b/c/d, ANME-3; Fig.2). ANME-1 were originally described at cold seeps (Hinrichs *et al*., 1999; Michaelis *et al*., 2002), but they thrive in a wide range of environments particularly in marine and lacustrine sediments (Fig.1). In marine sediments, ANME-1 often dominate deeper parts of sulfate–methane transition zones (SMTZ). Additionally, thermophilic ANME-1 subtypes occur in hydrothermally heated sediments of the Guaymas Basin (Teske *et al*., 2002; Holler *et al*., 2011; Wang *et al*., 2019a). In most cases, ANME-1 form consortia with the sulfate-reducing *Deltaproteobacteria* of the SEEP-SRB1/2 clade or with *Ca*. Desulfofervidus, the only isolated partner bacterium involved in AOM to date (Holler *et al*., 2011; Krukenberg *et al*., 2016). Occasionally, ANME-1 occur without obvious partners (Orphan *et al*., 2002; Ruff *et al*., 2016), and in sulfate-depleted horizons below SMTZ. These observations indicate that ANME are capable to use alternative electron acceptors or thrive as methanogens (Lloyd *et al*., 2011; Bertram *et al*., 2013; Niu *et al*., 2017; Beulig *et al*., 2019). Yet, to date ANME-1 with alternative lifestyles have not been cultured.

Archaea of the ANME-2a/b clade dominate most marine cold seeps (Fig. 1; Orphan *et al*., 2002; Mills *et al*., 2003; Wegener *et al*., 2008; Ruff *et al*., 2015). ANME-2a/b preferentially occur in cold or moderately temperate environments and in enrichment cultures, they frequently outcompete other ANME clades (Zhang *et al*., 2011; Wegener *et al*., 2016). The MCR from ANME-2c is closely related to that of ANME-2a/b (Fig. 2), and the two ANME clades occupy similar ecological niches (Felden *et al*., 2014; Krukenberg *et al*., 2018). They also form dense consortia with sulfate-reducing SEEP-SRB1 or SEEP-SRB2 partner bacteria (Schreiber *et al*., 2010; Kleindienst *et al*., 2012).

Ca. Methanoperedenaceae (ANME-2d) are closely related to ANME-2a/b on the McrA phylogenetic tree (Fig. 2), yet are capable of thriving without partner bacteria (Haroon *et al*., 2013). All cultivated members of this group and most environmental sequences derive from freshwater settings (Fig. 1; Cui *et al*., 2015; Welte *et al*., 2016). *Ca*. Methanoperedens nitroreducens encodes

Table 1 MCR/ACR-based anaerobic alkane-oxidizing archaea.

Organism[a]	MCR/ACR function	Electron acceptor	References
ANME-1[b]	Methane activation	SO_4^{2-}	Hinrichs *et al*., 1999; Boetius *et al*., 2000
ANME-2a/b		SO_4^{2-}	Orphan *et al*., 2002; Wang *et al*., 2014
ANME-2c		SO_4^{2-}	Krukenberg *et al*., 2018; Wang *et al*., 2019a
ANME-2d[c]		NO_3^-, Fe^{3+}, Mn^{4+}	Haroon *et al*., 2013; Arshad *et al*., 2015; Cai *et al*., 2018; Leu *et al*., 2020
ANME-3		SO_4^{2-}	Niemann *et al*., 2006; Omoregie *et al*., 2008
Ca. Syntrophoarchaeum	*n*-Butane/propane activation	SO_4^{2-}	Laso-Pérez *et al*., 2016; Wang *et al*., 2019a
Ca. Argoarchaeum	Ethane activation	SO_4^{2-}	Chen *et al*., 2019
Ca. Ethanoperedens			Hahn *et al*., 2020
Ca. Verstraetearchaeota[d]	Unknown, potentially in methane metabolism	Unknown	Vanwonterghem *et al*., 2016
Ca. Nezharchaeota			Wang *et al*., 2019b; Hua *et al*., 2019
Ca. Korarchaeota			Wang *et al*., 2019b; McKay *et al*., 2019
Thaumarchaeota			Hua *et al*., 2019
Archaeoglobi[e]			Wang *et al*., 2019b; Colman *et al*., 2019
Ca. Bathyarchaeota[d]	Unknown, potentially in long-chain alkane metabolism	Unknown	Evans *et al*., 2015
Ca. Hadesarchaeota			Wang *et al*., 2019b; Hua *et al*., 2019
Ca. Helarchaeota			Seitz *et al*., 2019
Archaeoglobi[e]			Wang *et al*., 2019b; Boyd *et al*., 2019
Ca. Methanoliparia			Borrel *et al*., 2019; Laso-Pérez *et al*., 2019

[a]Reported at different phylogenetic levels.
[b]ANME-1 is now classified as the novel order *Candidatus* (*Ca*.) Methanophagales.
[c]ANME-2d is now classified as the family *Ca*. Methanoperedenaceae.
[d]*Ca*. Verstraetearchaeota and *Ca*. Bathyarchaeota were the first discovered organisms with these MCR-encoding genes outside the Euryarchaeota phylum (Evans *et al*., 2015; Vanwonterghem *et al*., 2016). Due to unknown function, we refer to the Verstraetearchaeota-type and Bathyarchaeota-type MCRs (see Fig. 2).
[e]Different MAGs of Archaeoglobi contain either Verstraetearchaeota-type MCR or Bathyarchaeota-type MCR-encoding genes.

nitrate reductases (*nar*) and couples methane oxidation to the reduction of nitrate to nitrite. Hence, it does not require a partner bacterium, but it benefits from partnerships with nitrite-reducing partners (Haroon *et al*., 2013; Arshad *et al*., 2015). Recently it has been discovered that other *Ca*. Methanoperedens strains coupled AOM to the reduction of iron oxides and other metal oxides (Cai *et al*., 2018; Liang et al. 2019; Leu *et al*., 2020).

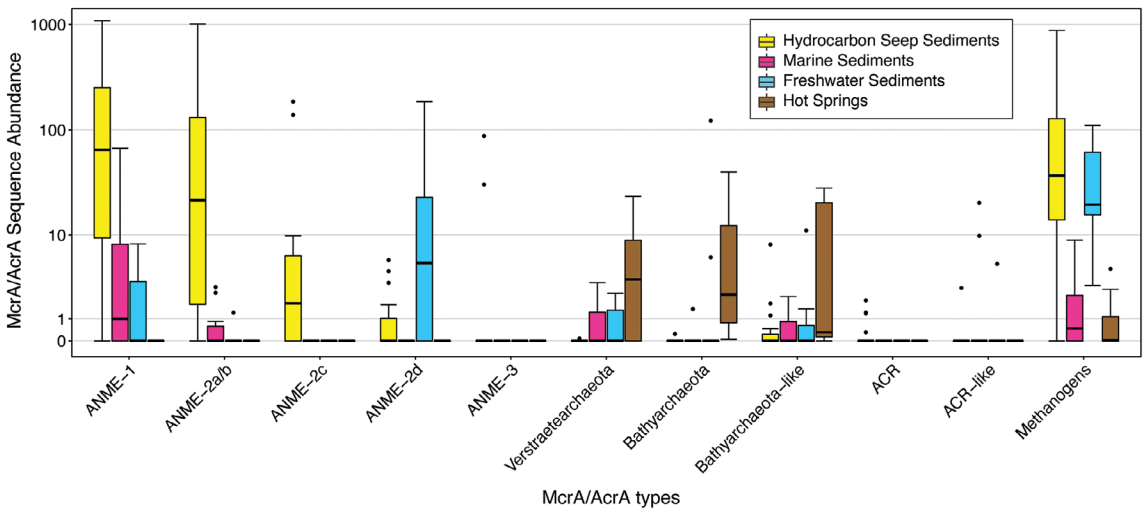

Fig 1 Abundances of gene sequences affiliating with eleven types of McrA/AcrA encoding genes.
The sequences derived from public metagenomes in the NCBI SRA database. They originated from hydrocarbon seeps (n = 17), marine sediments (n = 10), freshwater sediments (n = 10) and hot springs (n = 8). For comparison, all abundance values are normalized, i.e., the abundance of each McrA/AcrA type in a metagenome was divided by the arithmetical mean of this type across all studied metagenomes. Sequence abundance is shown on a pseudo-log scale to include zeros, i.e., data sets/ecosystems in which certain McrA/AcrA gene sequences were not detected. Many potential McrA sequences have best hits with AcrA or Bathyarchaeota-type McrA sequences, but with <60% amino acid identities. Hence, they are classified as Bathyarchaeota-like or AcrA-like sequences respectively.

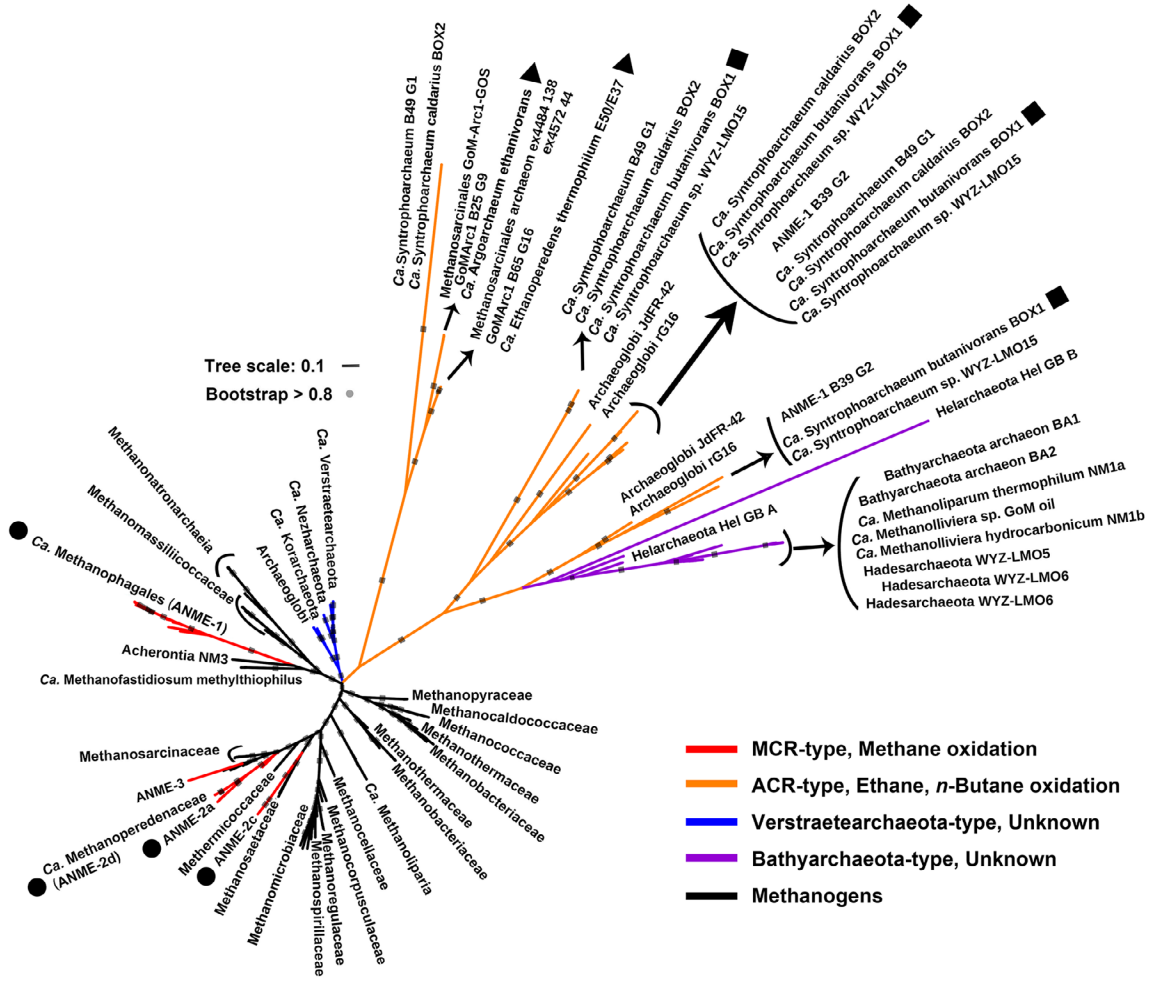

Fig 2 McrA/AcrA phylogenetic tree.
The tree was constructed based on alignments of McrA/AcrA sequences generated using MAFFT and then filtered with trimAl. The tree was built by the IQ-Tree method with model LG + C60 + F + G using 1000 bootstrap iterations. Black cycles, triangles or squares show experimentally confirmed anaerobic methane, ethane and *n*-butane-oxidizing archaea respectively. Note that the two cultured *Ca*. Syntrophoarchaeum strains encode four different ACRs.

Members of the ANME-3 clade dominate methane-rich arctic mud volcanoes (Niemann *et al*., 2006; Lee *et al*., 2019; Ruff *et al*., 2019), cold seep sediments in the Eastern Mediterranean (Omoregie *et al*., 2008) as well as sediments of the Marine Lake Grevelingen (Cassarini *et al*., 2019). ANME-3 usually forms consortia with bacteria related to the genus *Desulfobulbus*. ANME-3 has not been cultured, and genomic information for this clade is lacking. The clade is closely related to methanogens of the genus *Methanococcoides* (Fig. 2) indicating that the capability to reverse methanogenesis may have evolved independently in several archaeal lineages.

Diversity of anaerobic multi-carbon alkane-oxidizing archaea

More than 15years after the discovery of ANME, a thermophilic enrichment culture was established that contained consortia of syntrophic archaea and bacteria mediating the anaerobic oxidation of *n*-butane (Laso-Pérez *et al*., 2016). The archaea were identified as *Ca*. Syntrophoarchaeum (formerly GoM-Arc87 clade) in the new order *Ca*. Syntropharchaeales (Adam *et al*., 2017). Their partner bacterium is *Ca*. Desulfofervidus, which also form consortia with thermophilic ANME-1. *Ca*. Syntrophoarchaeum contains four *mcr*-like gene operons that are highly divergent from those of methanogens and methanotrophs based on amino acid identity. The encoded enzyme enables organisms from this genus and the later discovered *Ca*. Argoarchaeum (Chen *et al*., 2019) to activate non-methane alkanes such as *n*-butane, propane and ethane. Hence these enzymes were called alkyl-coenzyme M reductases (ACR, Chen *et al*., 2019). ACR sequences are mostly found in marine hydrocarbon seep environments, oil reservoirs or hydrothermal environments that are rich in multi-carbon

alkanes (Fig. 2; Wang *et al*., 2019a). *Ca*. Syntrophoarchaeum express four highly divergent *acr* gene operons, yet it is not known which of the encoded enzymes is required to activate specific alkanes such as *n*-butane (Laso-Pérez *et al*., 2016). A study on hydrothermal sediments in Guaymas Basin showed that these archaea were also active in natural environments (Wang *et al*., 2019a). *Ca.* Argoarchaeum and its recently discovered related genus *Ca*. Ethanoperedens contain one *acr* gene operon that encode enzymes optimized for the activation of ethane (Fig. 2;Chen *et al*., 2019; Hahn *et al*., 2020). These archaea belong to the order Methanosarcinales and are closely related to ANME-2d. Physiological experiments confirmed that these archaea oxidize ethane coupled to sulfate reduction (Chen *et al*., 2019; Hahn *et al*., 2020). Biochemical and structural characterization of these enzymes is needed to resolve the functional details and specificity of this alkane-oxidizing ACR family.

Diversity of MCR/ACR-containing archaea with unknown function

Until recently, *mcr* genes were only found in members of the phylum Euryarchaeota with methane as single substrate or reaction product. However, Evans *et al*. (2015) described two metagenome-assembled genomes (MAGs) affiliating with the candidate phylum Bathyarchaeota that contained *mcr* genes. The catalytic (alpha) subunits of MCRs from Bathyarchaeota have low amino acid similarity (i.e., < 60%) to alpha subunits of MCRs of the known ANMEs and methanogens. To date, members of the phylum Bathyarchaeota have defied cultivation and the exact function of the Bathyarchaeota-type MCR is still unknown. The Bathyarchaeota-type MCR sequences were detected in many marine and terrestrial environments (Fig. 1). This MCR type appears in MAGs from a variety of different lineages including *Ca*. Hadesarchaeota (Hua *et al*., 2019; Wang *et al*., 2019b), the candidate phylum Helarchaeota from the Asgard superphylum (Seitz *et al*., 2019), *Ca*. Methanoliparia (Borrel *et al*., 2019; Laso-Pérez *et al*., 2019) and Archaeoglobi in the Euryarchaeota phylum (Boyd *et al*., 2019; Wang *et al*., 2019b), suggesting horizontal gene transfers. In MAGs of *Ca*. Methanoliparia, the Bathyarchaeota-type *mcr* gene operon appears together with a canonical *mcr* gene operon. Based on the co-occurrence of these apparently functionally different MCR types, *Ca*. Methanoliparia may couple alkane degradation to the formation of methane (Borrel *et al*., 2019; Laso-Pérez *et al*., 2019).

A different type of *mcr* gene was discovered in MAGs of the candidate phylum Verstraetearchaeota in the TACK superphylum (Fig.2; Vanwonterghem *et al*., 2016; Berghuis *et al*., 2019). The Verstraetearchaeota-type *mcr* genes additionally appear in MAGs of the candidate phylum Korarchaeota (Borrel *et al*., 2019; McKay *et al*., 2019; Wang *et al*., 2019b), *Ca*. Nezharchaeota (Hua *et al*., 2019; Wang *et al*., 2019b), Thaumarchaeota (Hua *et al*., 2019) and Archaeoglobi (Colman *et al*., 2019; Hua *et al*., 2019; Wang *et al*., 2019b). The Verstraetearchaeota-type MCR were mostly found in hot springs (Fig.1). Interestingly, some archaeal lineages belonging to Archaeoglobi and *Ca*. Korarchaeota possess both MCR and dissimilatory sulfate reductase–encoding genes, suggesting that these organisms might be able to couple methane oxidation and sulfate reduction without involving syntrophic partners (Colman *et al*., 2019; McKay *et al*., 2019; Wang *et al*., 2019b). Organisms with Verstraetearchaeota-type MCR have not yet been cultured; hence the function of this MCR family is unknown. The large diversity of archaeal MAGs with *mcr* gene operons but unknown functioning underlines the great demand for cultivation in this field of research.

Metabolism of anaerobic alkane-oxidizing archaea

The AOM process is catalysed by enzymes similar to those of the methanogenesis pathway. They allow the activation of methane and the complete oxidation of methyl groups to CO_2. For the anaerobic multi-carbon alkane oxidation, ANKA activate alkanes via ACR, analogous to the activity of MCR in ANME, yet they also combine a fatty acid degradation and the Wood–Ljungdahl pathway. Additionally, ANKA require a machinery for the transformation of alkyl-CoM to acyl-CoA, yet so far, enzymes catalysing these reactions have not been identified. Electrons released in anaerobic alkane oxidation are subsequently transferred via electron carriers to partner bacteria or directly to terminal electron acceptors.

Activation of methane and other alkanes via MCR/ACR enzymes

In all so far described anaerobic alkane-oxidizing archaea, the first step for alkane oxidation is the transformation of methane or multi-carbon alkanes to methyl- or alkyl-CoM via MCR or ACR respectively (Fig. 3A, Krüger *et al*., 2003; Shima *et al*., 2012; Laso-Pérez *et al*., 2016; Chen *et al*., 2019). The MCR sequences of methane oxidizers are highly similar to those of methanogens (Fig. 2), and their phylogeny suggests that methane-activating MCRs may have evolved from multiple methanogenic MCR lineages. The three-dimensional structure and functioning of methanogenic and methane-activating MCRs were resolved by protein crystallization and biochemical approaches (Ermler *et al*., 1997; Shima *et al*., 2012; Wongnate *et al*., 2016; also reviewed in Thauer, 2019).

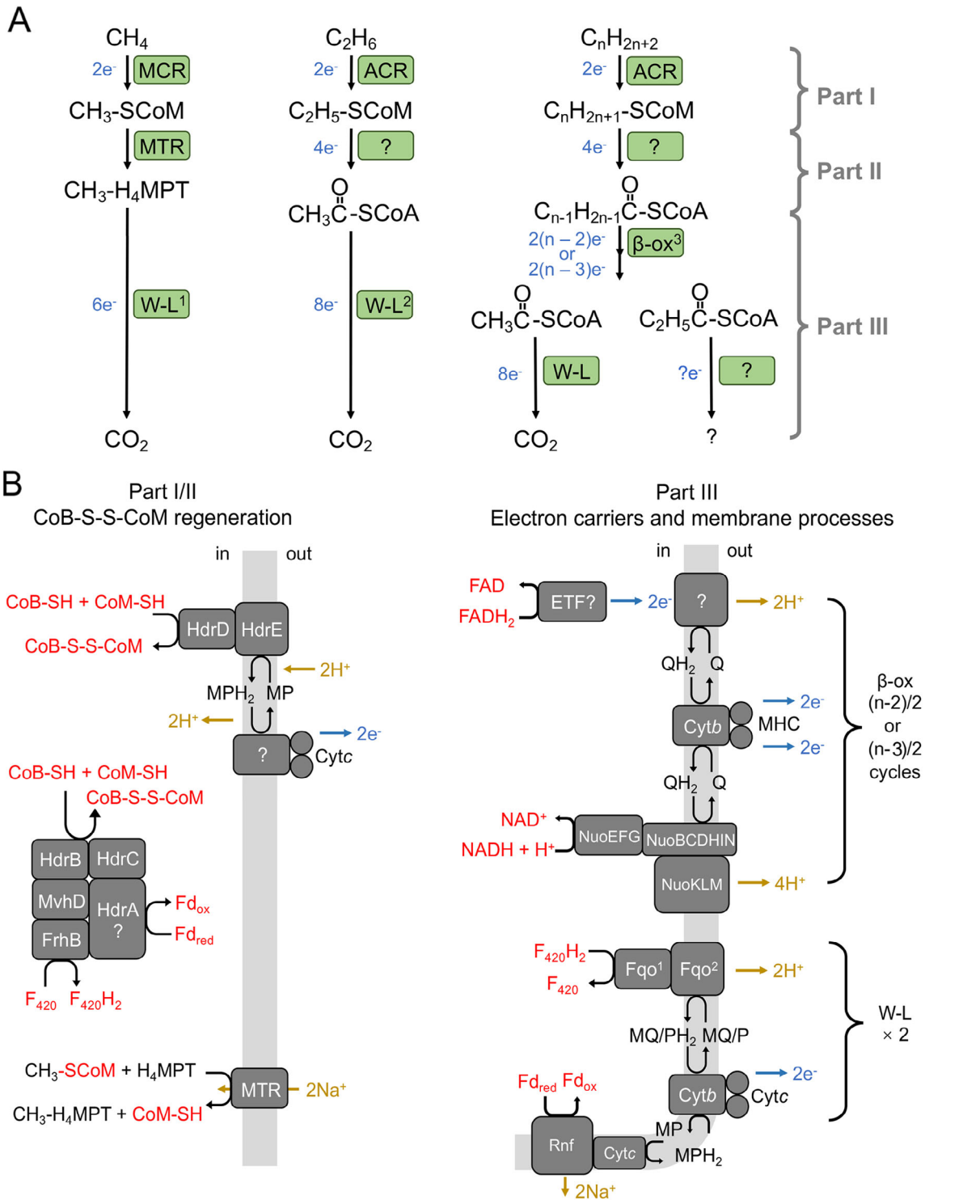

Fig 3 Pathways of alkanes oxidation and electron flows in anaerobic alkane-oxidizing archaea.
A. Schematic of the pathways for anaerobic methane, ethane and multi-carbon alkanes oxidation, including the activation of alkanes to alkyl-CoM (Part I); the transfer of methyl- or alkyl-CoM to methyl-H_4MPT or acyl-CoA, respectively (Part II); the beta-oxidation and Wood–Ljungdahl pathway for complete oxidation of methyl-H_4MPT or acyl-CoA, respectively (Part III); in C_nH_{2n+2}, $n > 3$. W-L: Wood-Ljungdahl pathway; β-ox: beta-oxidation pathway. Remarks: [1]The genomes of ANME-1 and *Ca*. Syntrophoarchaeum do not encode a methylenetetrahydromethanopterin (H_4MPT) reductase (Mer). Mer might be replaced by methylenetetrahydrofolate (H_4MF) reductase (MetF). [2]Some of the published MAGs of *Ca.* Argoarchaeum do not code for a formylmethanofuran tetrahydromethanopterin formyltransferase (Ftr); it could be replaced by methylenetetrahydrofolate dehydrogenase (FolD). [3]The even- and odd-carbon numbered alkane degradation may finally yield an acetyl-CoA or a propionyl-CoA, respectively, and the metabolism of propionyl-CoA in ANKA is still unknown.

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These MCRs are highly similar, and their active centres are identical. Yet the MCR of ANME-1 contains cysteine-rich patches near the active centre and several post-translational amino acid modifications that are not found in the MCR of methanogens. Furthermore, in ANME-1, the cofactor F430 contains an additional methylthiol that is not observed in ANME-2 and ANME-3 or methanogens (Mayr *et al*., 2008). Based on simulated three-dimensional structures inferred from amino acid sequences, ACRs largely resemble typical MCRs. Proposed modifications of ACR include a replacement of aromatic by aliphatic amino acids near or at the active pocket (Borrel *et al*., 2019; Chen *et al*., 2019), which might be needed to sterically accommodate multi-carbon substrates. However, only enzyme crystallization will allow resolving the true structure of these enzymes.

Transfer of methyl- or alkyl-CoM to methyl-H_4MPT or acyl-CoA respectively

ANMEs encode a tetrahydromethanopterin S-methyltransferase (MTR) to transfer the methyl group from CoM to tetrahydromethanopterin to allow further reactions that are required for complete oxidation (Fig.3A, Hallam*et al.,2004;*Timmers *et al*., 2017). Ethane oxidizers *Ca*. Argoarchaeum and *Ca*. Ethanoperedens also encode for MTR (Chen *et al*., 2019; Hahn *et al*., 2020). Chen *et al*.(2019) proposed that the MTR of *Ca*. Argoarchaeum catalyses the ethyl transfer to a yet unknown intermediate (Fig.3A, Chen *et al*., 2019). However, MTR is not known to catalyse multi-carbon compound transformations. Alternatively, MTR may recycle small quantities of methyl-CoM that form as a by-product in ACR (Hahn *et al*., 2020). Some of the required transformation steps of ethyl-CoM to acetyl-CoA might be catalysed by aldehyde ferredoxin oxidoreductases (Hahn *et al*., 2020). The propane and *n*-butane-oxidizing archaea *Ca*. Syntrophoarchaeum do not have MTR, indicating that this enzyme is not essential for multi-carbon alkane metabolism in archaea. Laso-Pérez *et al*.(2016) and Wang *et al*. (2019a) suggested that the transfer between butyl-CoM to butyryl-CoA might be catalysed by homologues of methylcobamide:CoM methyltransferase that are found in all *Ca*. Syntrophoarchaeum genomes (Laso-Pérez *et al*., 2016; Wang *et al*., 2019a). Yet this reaction cannot explain the alkyl oxidation and transfer to CoA. Extensive metabolite analyses of the ANKA cultures are required to clarify the role of MTR in ethane oxidizers and to resolve how CoM-bound alkyl units are transferred to CoA-bound fatty acids. Further insights into the role of the above-mentioned enzymes could be retrieved by heterologous gene expressions followed by *in vitro* enzyme experiments.

Beta-oxidation and Wood–Ljungdahl pathway for complete oxidation of alkanes

In ANMEs, methyl-H_4MPT can be subsequently oxidized to CO_2 by the C1 branch of Wood–Ljungdahl pathway. Ethane-oxidizing *Ca*. Argoarchaeum and *Ca*. Ethanoperedens also use the complete Wood–Ljungdahl pathway for acetyl-CoA degradation. In *Ca*. Syntrophoarchaeum and potential other long-chain alkane degraders, the formed acyl-CoA is first subjected to beta-oxidation, then the produced acetyl-CoA is oxidized via the Wood–Ljungdahl pathway (Fig. 3A, Laso-Pérez *et al*., 2016, 2019; Wang *et al*., 2019a). The beta-oxidation pathway is widespread in all domains of life, and it can also be used in the generation of fatty acid (Dibrova *et al*., 2014). The pathway involves at least four enzymes: acyl-CoA dehydrogenase (FadE), crotonase (FadB1), 3-hydroxyacyl-CoA dehydrogenase (FadB2) and acetyl-CoA acetyltransferase (AtoB). *Ca*. Syntrophoarchaeum possess and express several of these genes (Laso-Pérez *et al*., 2016; Wang *et al*., 2019a). Interestingly, ANME-1 and ANME-2d also contain genes encoding beta-oxidation (Wang *et al*., 2019a). However ANME show only low expression of these genes (Krukenberg *et al*., 2018); hence, the encoded enzymes should not have catabolic functions. Instead they may be involved in the production or degradation of rare long-chain fatty acids (Jagersma *et al*., 2012).

In ANMEs, the complete Wood–Ljungdahl pathway with the acetyl-CoA synthase:CO dehydrogenase complex has a central anabolic role as it produces acetyl-CoA for biomass production. Interestingly, isotope-labelling studies revealed that many ANME strains assimilate inorganic carbon (DIC) and only small amounts of methane when forming lipids and total biomass, and hence they should be considered as autotrophs (Kellermann *et al*., 2012; Wegener *et al*., 2016). This is puzzling because the Wood–Ljungdahl pathway is able to catalyse both DIC assimilation and methane oxidation. To regulate the extent of carbon fixation, both processes might be temporally separated. Alternatively, ANMEs may have a yet unidentified additional enzymatic route for carbon fixation or methyl group oxidation. For ANKA, *Ca*. Argoarchaeum and *Ca*. Syntrophoarchaeum produce acetyl-CoA as an

B. Electron transfer and coenzymes cycling with emphasis on CoB-S-S-CoM regeneration, electron carriers (coloured in red), electron transfer (coloured in blue) and proton or sodium ion translocation (coloured in yellow). Particularly, the oxidation of CoB-SH and CoM-SH deliver two electrons; the oxidation of $FADH_2$ and NADH deliver four electrons; the oxidation of $F_{420}H_2$ deliver two electrons. Fqo^1 (Fqo/FpoAHJKLMN), Fqo^2 (Fqo/FpoBCDFIO).

intermediate in the degradation of their substrate (Fig. 3A, Laso-Pérez *et al*., 2016; Chen *et al*., 2019). Hence, these organisms should be heterotrophs. To confirm this hypothesis, isotope-labelling experiments need to be performed.

Electron transfer and coenzyme cycling

The oxidation of methane and other alkanes according to the general catabolic reaction (1)

$$C_nH_{2n+2} + 2nH_2O \rightarrow nCO_2 + (6n+2)H^+ + (6n+2)e^- \quad (1)$$

releases 8 (methane), 14 (ethane) or 26 (butane) electrons. These electrons are transferred to terminal electron acceptors via a number of electron-shuttling coenzymes generating transmembrane proton or sodium gradients for ATP synthesis. The first step of anaerobic alkane-oxidizing archaea is the oxidation of alkanes by MCR/ACR. The electron acceptor of this reaction is CoM-S-S-CoB, which is reduced to alkyl-S-CoM, now carrying the covalently bound alkane chain, and HS-CoB. The subsequent regeneration of CoM-S-S-CoB releases two electrons that are transferred to cytoplasmic electron carriers or directly to the membrane. For CoM-S-S-CoB regeneration, ANME-2a/b/c/d and *Ca*. Argoarchaeum have a cytoplasmic (HdrABC) and a membrane-bound (HdrDE) heterodisulfide reductase. HdrDE catalyses CoM-S-S-CoB regeneration but requires the inflow of two protons (Wang *et al*., 2014; Arshad *et al*., 2015; Krukenberg *et al*., 2018; Chen *et al*., 2019). In contrast, ANME-1 and *Ca*. Syntrophoarchaeum possess only the cytoplasmic HdrABC (Stokke *et al*., 2012; Laso-Pérez *et al*., 2016; Wang *et al*., 2019a). Unlike HdrDE, HdrABC alone cannot catalyse the regeneration of CoM-S-S-CoB (Wagner *et al*., 2017). Arshad *et al*. (2015) suggested that an enzyme complex consisting of HdrABC and a coenzyme F_{420} hydrogenase (FrhB) couples CoM-S-S-CoB regeneration and ferredoxin oxidation to the reduction of two molecules of F_{420} (Fig. 3B, Arshad *et al*., 2015).

In ANME, the transfer of the methyl group from CoM to H_4MPT is catalysed by MTR with the inflow of two sodium ions. The further oxidation of CH_3-H_4MPT via the Wood–Ljungdahl pathway involves the reduction of F_{420}/$F_{420}H_2$. In total, four electrons are transferred from CH_3-H_4MPT to two molecules of F_{420} by forming two $F_{420}H_2$ and CH-H_4MPT in two reactions. ANME-2a/b/c/d and *Ca*. Argoarchaeum can reoxidize $F_{420}H_2$ with membrane-bound $F_{420}H_2$:phenazine oxidoreductase (Fpo, Wang *et al*., 2014; Arshad *et al*., 2015; Krukenberg *et al*., 2018; Chen *et al*., 2019). In contrast, ANME-1 and *Ca*. Syntrophoarchaeum use $F_{420}H_2$:quinone oxidoreductases (Fqo) for this function (Fig. 3B, Meyerdierks *et al*., 2010; Krukenberg *et al*., 2018; Laso-Pérez *et al*., 2016; Wang *et al*., 2019a). Through methanophenazine (MP) or menaquinone (MQ), electrons are transferred to cytochromes and external sinks, a process that is coupled to proton translocations (Fig. 3B). During the last step of the Wood–Ljungdahl pathway, two electrons from formyl group oxidation are transferred to ferredoxin, which can be replenished by the previously described reaction catalysed by HdrABC in all ANMEs. ANME-2a/b and ANME-2c additionally encode a membrane-integral, sodium-motive ferredoxin:NAD^+ oxidoreductase complex (Rnf). The reaction catalysed by Rnf involves the translocation of two sodium ions (Fig. 3B, Wang *et al*., 2014; Krukenberg *et al*., 2018; Wang *et al*., 2019a).

In ANKA, the transformation of alkyl-CoM to acyl-CoA should release four electrons to yet unknown electron carriers. Anaerobic ethane oxidizers, similar to ANMEs, use the Wood–Ljungdahl pathway to degrade acetyl-CoA, which releases another eight electrons if completely oxidized to CO_2 (Fig. 3A). *Ca*. Syntrophoarchaeum uses the beta-oxidation pathway for acyl-CoA degradation. For instance, the degradation of butyryl-CoA into two units of acetyl-CoA, releases eight electrons that are transferred to two units of FAD and NAD^+ (Fig. 3B). These electron carriers are reoxidized by enzymes of the respiratory chain translocating around six protons per acetyl-CoA released (Villanueva *et al*., 2017).

Most ANME and ANKA transfer the electrons from alkane oxidation to partner bacteria, while specific strains of ANME-2d deliver electrons to nitrate or metal oxides. MAGs of ANME and ANKA contain many genes encoding cytochromes and archaellum-like proteins. These genes are highly expressed, and microscopic analyses revealed high abundance of extracellular cytochromes partly coupled to the S-layer of archaea (McGlynn *et al*., 2015; Wegener *et al*., 2015; Laso-Pérez *et al*., 2016; Krukenberg *et al*., 2018). Moreover, the partner bacteria produce cytochromes and pili-based nanowires. Together these cell structures may allow direct interspecies electron transfer from the archaea to their partner bacteria. Heterologous expression experiments in model organisms as shown for pili-based nanowires (Walker *et al*., 2018) will help to assess unresolved pathways in anaerobic alkane oxidation and the coupling to electron acceptors.

In AOM, the combined membrane-coupled reactions yield a surplus of about two protons in the periplasm per molecule methane oxidized depending on the different types of ANME. Based on the required inflow of five protons per ATP (Thauer, 2011), or the free energy released from the methane oxidation ($\Delta G'^m = -35$ kJ mol^{-1}, McGlynn, 2017), this translates to a yield of only 0.3–0.4 mol ATP per molecule methane oxidized. In ANKA some catabolic reaction steps and their energy

requirements are unknown; hence, their proton translocations cannot be budgeted. However, thermodynamic considerations for the net reaction of multi-carbon alkane oxidation suggest more proton translocations per mole substrate oxidized, which would explain the higher growth yields of these organisms compared with ANME (Hahn *et al*., 2020).

Conclusion and perspectives

The physiology and ecology of ANME have been studied intensively for 20years. In contrast, the first ANKA have been identified about 4 years ago. Hence, to date, only three genera of non-methane alkane oxidizers have been cultured, and all belong to the class Methanomicrobia in the Euyrarchaeota phylum. Based on metagenomic studies, an enormous diversity of ACR-containing archaea awaits their cultivation. A larger collection of cultures will help to understand the physiology, biochemistry, evolution and ecological roles of the fascinating alkane-metabolizing archaea. Structural and functional features of MCR/ACR enzymes that determine the preference for methane or multi-carbon alkane substrates needs to be resolved, ideally by crystallization of their native forms. Required biochemical characterizations include substrate spectra and kinetic analyses. Also, the transformation of CoM-bound alkyl units to CoA-bound fatty acids is not understood. As these reactions lack analogues in other organisms, extended metabolomics analyses are required. To resolve the role of alkane-metabolizing archaea in the global carbon cycle, metagenomic information needs to be combined with environmental information including alkane, sulfate/sulfide, nitrate/nitrite and metal ions fluxes. This environmental research should include understudied environments including the terrestrial subsurface, deep-sea ecosystems and hydrocarbon reservoirs.

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