This is an electronic reprint of the original article.
This reprint may differ from the original in pagination and typographic detail.

Scheller, Silvan; Ermler, Ulrich; Shima, Seigo

Catabolic Pathways and Enzymes Involved in Anaerobic Methane Oxidation

Published in:
Anaerobic Utilization of Hydrocarbons, Oils, and Lipids

Published: 01/01/2017

Document Version
Peer reviewed version

Published under the following license:
Unspecified

Please cite the original version:
Scheller, S., Ermler, U., & Shima, S. (2017). Catabolic Pathways and Enzymes Involved in Anaerobic Methane Oxidation. In M. Boll (Ed.), *Anaerobic Utilization of Hydrocarbons, Oils, and Lipids* (Handbook of Hydrocarbon and Lipid Microbiology). SPRINGER. https://doi.org/10.1007/978-3-319-33598-8_3-1

This material is protected by copyright and other intellectual property rights, and duplication or sale of all or part of any of the repository collections is not permitted, except that material may be duplicated by you for your research use or educational purposes in electronic or print form. You must obtain permission for any other use. Electronic or print copies may not be offered, whether for sale or otherwise to anyone who is not an authorised user.

Catabolic Pathways and Enzymes Involved in the Anaerobic Oxidation of Methane (revised: Jan. 31st 2017)

Silvan Scheller, Ulrich Ermler and Seigo Shima

Prof. Dr. Silvan Scheller; Aalto University, Kemistintie 1; 02150 Espoo; Finland.
silvan.scheller@aalto.fi

PD Dr. Ulrich Ermler; Max-Planck-Institut für Biophysik, Max-von-Laue-Straße 3; 60438 Frankfurt am Main; Deutschland. ulrich.ermler@biophys.mpg.de

Dr. Seigo Shima; Max Planck Institute for Terrestrial Microbiology, Karl-von-Frisch-Strasse 10; 35043 Marburg; Deutschland. shima@mpi-marburg.mpg.de

Content:

- 1. Abstract**
- 2. Introduction**
- 3. Catabolic pathways involved in methane oxidation**
 - 3.1 Geobiochemical pathways of methane oxidation
 - 3.2 Anaerobic oxidation of methane (AOM): the reverse of methanogenesis
 - 3.3 AOM coupled to nitrate reduction
 - 3.4 AOM coupled to sulfate reduction
 - 3.5 AOM with release of single electrons
 - 3.6 AOM coupled to metal oxide reduction
- 4 Enzymes involved in AOM**
 - 4.1 General aspects of AOM biochemistry
 - 4.2 Methyl-Coenzyme M Reductase (Mcr)
 - 4.2.1 Primary structure and isolation of ANME Mcr
 - 4.2.2 Structure of ANME Mcr
 - 4.2.3 Mechanistic basis of methane oxidation by Mcr
 - 4.2.4 Optimization of Mcr
 - 4.3 Heterodisulfide reductase (Hdr)
 - 4.4 Methyl-tetrahydromethanopterin:coenzyme M methyltransferase (Mtr)
 - 4.5 Methylene-tetrahydromethanopterin reductase (Mer)
 - 4.6 Methylene-H₄MPT dehydrogenase (Mtd), cyclohydrolase (Mch) and formyltransferase (Ftr)
 - 4.7 Formylmethanofuran dehydrogenase (Fmd and Fwd)
- 5 Research needs**
- 6 Literature**
- 7 Acknowledgements**

1 Abstract

Microbes use two distinct catabolic pathways for life with the fuel methane: aerobic methane oxidation carried out by bacteria, and anaerobic methane oxidation carried out by archaea. The archaea capable of anaerobic oxidation of methane, anaerobic methanotrophs (ANME), are phylogenetically related to methanogens. While the carbon metabolism in ANME follows the pathway of reverse methanogenesis, the mode of electron transfer from methane oxidation to the terminal oxidant is remarkably versatile.

This chapter discusses the catabolic pathways of methane oxidation coupled to the reduction of nitrate, sulfate and metal oxides. Methane oxidation with sulfate and metal oxides are hypothesized to involve direct interspecies electron transfer and extracellular electron transfer. Cultivation of ANME, their mechanisms of energy conservation, and details about the electron transfer pathways to the ultimate oxidants are rather new and quickly developing research fields, which may reveal novel metabolisms and redox reactions. The second section focuses on the carbon catabolism from methane to CO₂ and the biochemistry in ANME with its unique enzymes containing Fe, Ni, Co, Mo and W, that are compared with their homologues found in methanogens.

2 Introduction

Life can be sustained with energy from only slightly exergonic chemical reactions. The **anaerobic oxidation of methane (AOM)** with sulfate is an extreme example, as the overall free energy yield allows for the production of less than one **ATP** per methane oxidized. In addition to this small thermodynamic driving force, the activation energy for the non-catalyzed reaction is particularly high, because methane and sulfate are inert compounds. Nevertheless, AOM with sulfate is the globally most important anaerobic sink of the potent **greenhouse gas** methane and thus relevant in the context of global climate-changes.

AOM with sulfate as the oxidant is carried out syntrophically (**syntrophy** = Greek, “eating together”), which means that this catabolic process is split into two distinct organisms. Anaerobic methanotrophs (**ANME archaea**) carry out the oxidative (anodic) half-reaction from methane to CO₂, and sulfate-reducing bacteria carry out the reductive (cathodic) half-reaction from sulfate to hydrogen sulfide. As a consequence, the two partners are strictly dependent on each other and need to share the small energy yield derived from their overall catabolism. This chapter summarizes current knowledge about the syntrophy of AOM with sulfate and discusses methane oxidation pathways with the oxidants nitrate and metal oxides.

3. Catabolic pathways involved in methane oxidation

3.1 Geobiochemical pathways of methane oxidation

Around 1.5 Gt per year of methane are produced via microbial and geochemical processes. Microbes consume most of it via oxidation with various electron acceptors in the subsurface, which limit input into the atmosphere. **Figure 1** summarizes the global methane cycle with its most important sources and sinks, indicating different electron acceptors used for methane oxidation.

Figure 1: Summary of global methane cycle. In oxic environments, methane is oxidized with molecular oxygen, a very exergonic reaction that provides ample energy for bacterial growth. In the anaerobic zones, the alternative oxidants nitrite, nitrate, metal oxides and sulfate are respired for microbial methane catabolism (modified from Thauer 2011).

In the presence of molecular oxygen, Alphaproteobacteria, Gammaproteobacteria (Semarau et al. 2010) and Verrucomicrobia (Op den Camp et al. 2009) can thrive from methane oxidation using methane monooxygenases that utilize oxygen-derived radicals for the activation of methane. Methanol is obtained as the first intermediate that is further catabolized via formaldehyde and formate to CO₂ (Chistoserdova et al. 2005). The anaerobic organism *Methylomirabilis oxyfera* developed the ability to produce its own oxygen for the conversion of methane to methanol. It uses nitrite as the oxidant and converts it via nitric oxide to N₂ and O₂ that is utilized for radical methane activation (Ettwig et al. 2010). *M. oxyfera* may thus be classified as an obligate anaerobe (Luesken et al. 2012) that carries out an aerobic pathway of methane activation. Here we describe pathways and enzymes related to methane oxidation devoid of molecular oxygen.

3.2 Anaerobic oxidation of methane (AOM): the reverse of methanogenesis

A special class of archaea termed anaerobic methanotrophs (ANME archaea) that are phylogenetically related to methanogens carry out AOM. It has originally been speculated that they carry out the reverse of hydrogenotrophic methanogenesis according to eq. 1 (Hoehler et al. 1994):

Metagenomic analysis (Hallam et al. 2004) and enzymatic studies (Scheller et al. 2010) support a catabolic pathway of CH_4 oxidation to CO_2 via the reverse of methanogenesis. The reducing equivalents from AOM, however, are not released via hydrogen formation, because hydrogen concentrations would need to be kept below 1 nM to conserve energy from eq. 1 ($\Delta G' < 0$, eq. 1'). At such low hydrogen concentrations, the possible maximal diffusion rates would be distinctly smaller than the diffusion rates calculated for the observed methane oxidation rates (Orcutt and Meile 2008).

The pathway of reverse methanogenesis is initiated by the enzyme methyl-coenzyme M reductase (Mcr) that cleaves the C-H bond in methane using its nickel cofactor F430 (Jaun and Thauer 2007) and transfers the resulting methyl group to the thiolate moiety of coenzyme M. The sulfur-bound methyl-group in methyl-S-CoM is subsequently transferred to N5 of the C_1 -carrier tetrahydromethanopterin (H_4MPT) and further oxidized to CO_2 as depicted in figure 2 A.

Figure 2: Catabolic pathway of AOM and methanogenesis. Mcr: methyl-S-CoM reductase. Hdr: heterodisulfide reductase. C_1 -carriers and enzymes are discussed in the second part of this chapter; methylotrophic methanogenic archaea contain tetrahydrosarcinapterin (H_4SPT) instead of the H_4MPT . **A)** Complete reverse methanogenesis pathway from CH_4 to CO_2 as present in ANME archaea. **B)** Metabolism in methylotrophic methanogens that disproportionate the substrates methanol, methylamines or methanethiol ($\text{CH}_3\text{-X}$) to CH_4 and CO_2 for comparison.

The pathway from **methyl-S-CoM** to CO₂ is identical to the oxidative branch in methylotrophic methanogens, where methyl-S-CoM derived from methanol, methylamines or methanethiol (X = O, N, or S), is disproportionated to CH₄ and CO₂ in a 3:1 stoichiometry (**figure 2 B**). Therefore, only the reversal of the enzyme Mcr and the reversal of the **heterodisulfide reductase (Hdr)** are unique to AOM and unprecedented in methanogens.

For methylotrophs, the 6 electrons generated from the oxidation of the methyl group are internally re-cycled to reduce 3 heterodisulfides (CoM-S-S-CoB), which allows disposing these electrons via methane production (**figure 2 B**). In the case of full methane oxidation, all 8 electrons obtained need to be disposed externally (**figure 2 A**) via different transfer pathways to the ultimate oxidants as described below.

3.3 AOM coupled to **nitrate reduction**

Anaerobic oxidation of methane with nitrate (Raghoebarsing et al. 2006) is currently the best-understood mode of anaerobic methane oxidation, because highly enriched **co-cultures** are available that yielded comprehensive genomic and transcriptomic data (Haroon et al. 2013). AOM is carried out by *Candidatus 'Methanoperedens nitroreducens'*, also known as ANME-2d, which uses a **nitrate reductase** to dispose methane-derived electrons via the formation of nitrite (eq. 2):

Reported enrichments of ANME-2d require co-cultures with partner-bacteria that remove the toxic product nitrite efficiently. Incubations with methane and nitrate as the sole energy substrates resulted in a co-culture dominated by ANME-2d and *M. oxyfera*, whereby both organisms carry out methane oxidation (Ettwig et al. 2008). The nitrogen metabolism is partitioned, ANME-2d reduces nitrate to nitrite (eq. 2), and the *M. oxyfera* partner reduces nitrite to molecular nitrogen (**figure 3 A**).

Figure 3: Co-cultures of ANME-2d with partner-bacteria that remove the toxic product nitrite efficiently. **A)** Combination of anaerobic and aerobic methanotrophy involving ANME-2d and *M. oxyfera* (Raghoebarsing et al. 2006). **B)** Co-culture of ANME-2d with *Kuenenia* for nitrite removal (Haroon et al. 2013).

By feeding equimolar amounts of ammonium and nitrate to the methanotrophic culture, ANME-2d are enriched with *Kuenia* partner bacteria instead of *M. oxyfera* (Haroon et al. 2013). In this case, ANME-2d are the sole species with methanotrophic capabilities. The *Kuenia* bacteria remove the toxic product nitrite via the anammox pathway, the **comproportionation** of nitrite and ammonium to molecular nitrogen

(figure 3 B). Evaluation of the substrate and product fluxes in the bioreactors experimentally verified the expected stoichiometry between the overall substrates methane, nitrate and ammonium and the product nitrogen (eq 3) (Haroon et al. 2013):

ANME-2d may serve as the model ANME for physiological studies and for isolation of active enzymes, since highly enriched co-cultures are available. Genome sequencing confirmed the presence of all genes required for the pathway of reverse methanogenesis; most of them, in particular the key enzyme Mcr, are highly expressed (Haroon et al. 2013).

Metagenomic data of related organisms suggests that nitrate used as the oxidant for AOM might be fully reduced to ammonium by the ANME-2d alone (Arshad et al. 2015), but physiological details were not reported yet. Such studies highlight that we have just begun to gain control over the cultivation and physiology of some ANME members, and that the microbial diversity is a treasure box filled with metabolic surprises.

3.4 AOM coupled to sulfate reduction

Geochemists reported long before the discovery of ANME archaea that a pathway of methane oxidation coupled to sulfate reduction (eq. 4) must be present at benthic methane seeps, based on inverse sulfate-methane concentration gradients measured (Iversen and Jorgensen 1985).

Fluorescence-in-situ-hybridization with oligonucleotide probes targeting the 16S rRNA allowed the visualization of microorganisms apparently mediating AOM coupled to sulfate reduction (figure 4 A) (Boetius et al. 2000).

The high diversity of ANME **phylotypes** involved in the AOM with sulfate can be dissected into two main groups with distinct morphology. ANME-1, which are related to methanogens of the order Methanomicrobiales, form loose associations with sulfate-reducing bacteria (SRB) and are often found as single cells (Orphan et al. 2002; Lösekann et al. 2007). ANME-2a-c and ANME-3, which are related to Methanosarcinales (Knittel and Boetius 2009), form tight **aggregates** with SRB as exemplified in figure 4 A.

Figure 4: A) Microscopy image of ANME-SRB consortium apparently mediating AOM: red (dark color) = ANME, green (light color) = SRB; scale bar = 5 μm (transferred from Boetius et al. 2000). B) Cartoon of the metabolism of AOM with sulfate and its compartmentalization into ANME archaea and SRB.

The correlation of the phylogenetic identity with stable isotope analyses indicated that those archaea are carrying out methane oxidation (Orphan et al. 2001). AOM with sulfate is thus a syntrophic reaction requiring both, ANME and SRB. This process can be considered as a single overall metabolism that is split into two distinct organisms, which requires transfer of reducing equivalents (**figure 4 B**). ANME archaea are responsible for the carbon metabolism and SRB are responsible for the sulfur metabolism. A study involving sulfur stable isotope probing suggested that ANME may carry out both methane oxidation and sulfate reduction (Milucka et al. 2012), but the responsible archaeal genes for sulfate-reduction have not been assigned to date.

The mechanism of electron transfer from ANME to their SRB partners has been a mystery for a long time. Investigation of AOM communities is hampered by high genetic diversity, yielding only incomplete genomic data, and by the slow grow rates, with doubling times range between 2 and 9 months under laboratory conditions (Girguis et al. 2005; Nauhaus et al. 2007; Meulepas et al. 2009; Holler et al. 2011).

A major goal in the field is the decoupling of the syntrophy between ANME and SRB that would open the door to grow the individual organisms in pure cultures. Supplying the specific substrate (**intermediate**) for one partner only, or adding a compound that removes the specific product (**intermediate**) efficiently, should induce selective stimulation of only one type of organisms. Putative intermediates, such as hydrogen, formate and acetate, have been extensively tested in laboratory studies (Nauhaus et al. 2005; Meulepas et al. 2010). For a thermophilic enrichment of ANME-1 and SRB, the bacterial partner of the ANME-1 could be stimulated by hydrogen addition (Wegener et al. 2015; Wegener et al. 2016), which allowed to highly enrich (> 95%) these sulfate-reducing bacteria (Krukenberg et al. 2016). Hydrogen, however, appears to be an alternative electron donor for these particular SRB and not to be produced by the corresponding ANME during sulfate-driven AOM (Wegener et al. 2015; Wegener et al. 2016). All attempts failed to detect diffusive intermediates consumed or produced by the ANME.

Single cell activity mapping via **secondary-ion-mass-spectrometry** at the nanometer scale of active AOM-SRB consortia in combination with metabolic modeling excluded diffusive intermediates as electron carriers and suggested that the syntrophy is based on direct interspecies electron transfer from the ANME to their bacterial partners (McGlynn et al. 2015).

3.5 AOM with release of single electrons

Multi-heme c-type cytochromes possibly involved in single electron transfer were discovered in metagenome sequence analyses (Meyerdierks et al. 2010; Kletzin et al. 2015). Later, the hypothesis of direct **interspecies electron transfer (DIET)** as the coupling mechanism between ANME and SRB could be derived from two independent studies using ANME-1 and ANME-2, respectively. In the study with ANME-1, evidence for methane oxidation via the release of single electrons was derived from putative **nanowires** visualized by electron-microscopy (**figure 5A**) and transcriptional studies (Wegener et al. 2015). In the study with ANME-2, the putative multi-heme c-type cytochromes were stained *in situ* and localized by electron-microscopy (McGlynn et al. 2015).

Figure 5: Mechanism of electron transfer from ANME to SRB. **A)** Putative microbial nano wires (arrows) between ANME-1 (A) and HotSeep-1 SRB (H) visualized via electron microscopy (scale bar = 300 nm; transferred from Wegener et al. 2015). **B)** Simplified scheme for the electron transfer from ANME-2a-c to their SRB partners. Reducing equivalents from methane oxidation generate reduced methanophenazine (MPH₂) via different membrane-bound enzymes (putatively Hdr, Fpo and Rnf). Multi-heme *c*-type cytochromes (orange) transport the electrons from MPH₂ through the membrane and the S-layer domain to the outside of the cell to be taken up by the syntrophic partner (modified from McGlynn et al. 2015).

Mechanistically, methane-derived electrons travel via **electrical conductance** to the bacterial partners of the ANME. The basis to achieve such an electron transfer is an “**electron-hopping**” mechanism following the array of aligned iron centers in the multi-heme *c*-type cytochromes (Pirbadian and El-Naggar 2012). Catabolic pathways involving release of single electrons and DIET are known from the laboratory organisms *Shewanella* and *Geobacter* (Gorby et al. 2006; Gralnick and Newman 2007; Lovely 2012). According to this hypothesis of DIET in ANME-SRB consortia, electrons derived from reverse methanogenesis reduce the methanophenazine, which is present in the membrane of ANME, from which single electrons are transferred via multi-heme *c*-type cytochromes to the surface of the cell, through the S-layer domain towards the bacterial partner that is tightly aggregated to the ANME (**figure 5 B**). Apparently both ANME-1 and ANME-2a-c simply carry out the half reaction of methane oxidation (eq. 5), which suggests that the overall metabolism of methane oxidation with sulfate is equally split into the carbon and sulfur **half reactions** without transfer of molecular primary metabolites.

Following this proposal, it should be possible to decouple AOM from sulfate reduction via an auxiliary cathodic half-reaction that removes single electrons. Experiments with single electron acceptors, such as **anthraquinone-disulfonate (AQDS)**, demonstrated methane oxidation by ANME-2a+c in the absence of sulfate (Scheller et al. 2016) according to eq. 6.

With AQDS, the catabolic rate of AOM and biomass synthesis in ANME-2a+c cells was similar as with the native oxidant sulfate, but the bacterial partners remained completely inactive (Scheller et al. 2016). Similar results were obtained with a range of different single electron acceptors, such as soluble iron(III)-complexes or **humic acids**, which is expected according to the hypothesis of DIET as the syntrophic mechanism between ANME and their sulfate-reducing partners.

This metabolic separation of archaeal methane oxidation from their partner bacteria lays the foundation to grow ANME in pure cultures, because the bacterial partners may be outcompeted by the auxiliary electron acceptors.

3.6 AOM coupled to metal oxide reduction

Iron and manganese oxides have been reported to serve as oxidants for methane oxidation (Beal et al. 2009), but no specific organism could be directly attributed to carry out this catabolism. The apparent ability of multiple members of ANME to carry out AOM with release of single electrons may explain these findings on the basis of extracellular electron transfer from ANME to the metal oxide particles (**figure 6**), a proposal that currently lacks direct experimental support.

Figure 6: Extracellular electron transfer from ANME to the environment. **A)** Electron transfer to soluble iron(III)-complexes reported for ANME-2a+c (Scheller et al. 2016) **B)** Putative respiratory metabolism allowing for AOM coupled to metal oxide reduction (Rotaru and Thamdrup 2016).

While highly specialized in their carbon metabolism, ANME seem to be **promiscuous** for the terminal oxidant, implying that one specific type of organism may use sulfate-reducing bacteria or metal oxides as the sink for methane-derived electrons. Aggregates of ANME archaea belonging to different clades have been found to be active in the absence of a bacterial partner (Orphan et al. 2002), which could be explained by a metabolism of electron transfer from these ANME to redox active components present in the corresponding environment, such as metal oxide particles.

Extracellular electron transfer to various oxidants may be an **evolutionary** transient solution of a methanogen on its way to a **syntrophic partnership** with sulfate-reducing bacteria. Perhaps methylotrophs evolved coupling of AOM to metal oxide reduction as a first, and syntrophy with sulfate-reducing bacteria as a second evolutionary step (Scheller et al. 2016). However, further research is needed to substantiate such a speculation and to shed light on the process of AOM coupled to metal oxide reduction.

4 Enzymes involved in AOM

4.1 General aspects of AOM biochemistry

Homologues of methanogenic enzymes catalyze the oxidation of methane to CO_2 . This bi-directionality of the methanogenesis pathway is enabled via the relatively flat energy-profile of all intermediates involved (Thauer 1998; Hedderich and Whiteman 2006; Thauer et al. 2008; Ferry 2010; Thauer 2011; Welte and Deppenmeyer 2014; Costa and Leigh 2014). The utilization of the same catabolic pathway in both directions highlights the role of **enzymes** to simply lower transition state energies between intermediates. The equilibria between the intermediates and thus the directionality of the overall pathway are determined by the substrate and product concentrations, coupling to **redox-cofactors**, and by ion-gradient-consuming or producing enzymes. The exact mechanism of **energy conservation** in ANME is still speculative and may vary between different phylotypes. **Figure 7** presents the catabolic pathway of reverse methanogenesis with its corresponding enzymes and redox-cofactors.

Figure 7: Predicted reverse methanogenesis pathway operating in ANME-1 archaea, modified from (Shima 2014). The biochemistry for ANME-2 is discussed elsewhere (McGlynn 2017; Timmers et al. 2017). Genes encoding for the Mer enzyme (in gray) have not been found in the ANME-1 metagenome sequence but they are present in ANME-2a and ANME-2d (see later in this document). Na^+/H^+ antiporters appear to be required for this pathway, but the genes are not annotated in the ANME-1 metagenome.

Mcr: methyl-S-CoM reductase. Mtr: integral membrane methyltransferase. Mer: F_{420} dependent methyl-H₄MPT reductase. Mtd: F_{420} dependent methylene-H₄MPT dehydrogenase. Mch: cyclohydrolase. Ftr: formyltransferase. Fwd: tungsten-containing formylmethanofuran dehydrogenase. Hdr/MvhD/FrhB: hypothetical Hdr/ F_{420} oxidoreductase complex.

Reverse methanogenesis implicates a different chemistry for the challenging step of alkane activation in comparison to the pathway present in most organisms carrying out **anaerobic oxidation of long-chain alkanes**. For long-chain alkanes, enzyme-bound radicals abstract a H atom of the alkane, which is then transferred to fumarate (Widdel and Grundmann 2010). The only exception currently known is the anaerobic

oxidation of butane by archaea, which has just recently been discovered (Laso-Pérez et al. 2016). For methane, the particularly strong C-H bond is cleaved via **organometallic chemistry** rather than via a conventional radical abstraction. This reaction is carried out by methyl-coenzyme M reductase (Mcr) (Gunsalus and Wolfe, 1980) that uses CoM-S-S-CoB as the oxidant. For the enzymatic part of this contribution, we discuss this unique reaction in detail. The Mcr-catalyzed reaction of **methane activation** and methyl-S-CoM formation is associated with a Gibbs free energy difference ($\Delta G^{\circ'}$) of + 30 kJ mol⁻¹ at standard conditions. With 8 bars of methane and 1 mM intracellular CoM-S-S-CoB, for example, net methane oxidation occurs if the intermediates methyl-S-CoM and HS-CoB are kept at concentrations below 0.2 mM each ($\Delta G' = 0$). The enzymes Mtr and Hdr (**figure 7**) probably keep the concentrations of methyl-S-CoM and HS-CoB low in order to drive methane oxidation. Hdr catalyzes formation of CoM-S-S-CoB from HS-CoM, HS-CoB and reduced ferredoxin; the electron acceptor is postulated to be two molecules of F₄₂₀, but not experimentally confirmed.

The endergonic methyl group transfer from methyl-S-CoM to methyl-H₄MPT ($\Delta G^{\circ'} = +30$ kJ/mol) is driven by coupling to the exergonic **sodium ion influx** through the cytoplasmic membrane ($\Delta G' = -30$ kJ/mol) (Gottschalk and Thauer 2001; Thauer et al. 2008). Methyl-H₄MPT is dehydrogenated to methylene-H₄MPT ($\Delta G^{\circ'} = +6.2$ kJ/mol). Methylene-H₄MPT is dehydrogenated to methenyl-H₄MPT⁺ by F₄₂₀ dependent methylene-H₄MPT reductase reaction ($\Delta G^{\circ'} = -5.5$ kJ/mol). Methenyl-H₄MPT⁺ is hydrolyzed to formyl-H₄MPT and then the formyl group is transferred to methanofuran. The last reaction in AOM is catalyzed by formylmethanofuran dehydrogenase (Fmd or Fwd), by which formylmethanofuran (formyl-MFR) is oxidized to CO₂ and methanofuran (MFR). This reaction is almost in equilibrium at standard conditions ($\Delta G^{\circ'} = 0$ kJ/mol) because the redox potential of **ferredoxins** ($E_0' = -500$ mV) and CO₂ + MFR/formyl-MFR couple ($E_0' = -530$ mV) are similar (Kaster et al. 2011).

The predicted metabolic pathway and the primary structure of the enzymes involved in reverse methanogenesis was deduced from metagenomic data of sulfate-dependent ANME-1 and ANME-2b, and nitrate-dependent ANME-2d (Hallam et al. 2004; Meyerdierks et al. 2010; Stokke et al. 2012; Haroon et al. 2013; Arshad et al. 2015). Some ANME-1 enzymes have been heterologously produced and characterized (Kojima et al. 2014), and ANME Mcr could even be crystallized from extracted ANME-1 biomass derived from microbial mats (Krüger et al. 2003) as described below.

4.2 Methyl-Coenzyme M Reductase (Mcr)

4.2.1 Primary structure and isolation of ANME Mcr

Metagenome sequencing of microbial mats performing AOM always revealed genes encoding for ANME-1, 2 and 3 Mcr according to the three branches found (Caldwell et al. 2008; Meyerdierks et al. 2010; Reeburgh 2007). Sequence identities between ANME and methanogenic, ANME-1 and ANME-2 as well as ANME-2 and ANME-3 Mcr are around 50%, 45% and 70%, respectively. Methane metabolism on the basis of the methanogenic pathways has been suggested for various Bathyarchaeota (Evans et al. 2015), with sequence identities between Bathyarchaeota and methanogenic/ANME Mcr of around 45%/40%. Profound biochemical studies on isolated Mcr to prove its function are difficult because Bathyarchaeota and methanotropic Euryarchaea are currently not available in pure cultures. Fortunately,

microbial mats collected from the northwestern Black sea shelf, provided sufficient biomass of multispecies ANME consortia containing abundant but heterogeneous ANME-1 and ANME-2 Mcr. Chromatographic purification of these mat-extracts yielded a higher Ni-protein I population hosting methylthio-F430 (**figure 8 B**) and a lower Ni-protein II population with F430 bound (Krüger et al. 2003). The Ni-protein I elution peak is composed of at least six different ANME-1 Mcr populations (Shima et al. 2012). SDS-PAGE analysis revealed the three Mcr subunits α , β and γ as expected (Krüger et al. 2003). The catalytic activity and the substrate specificity are not measurable because ANME Mcr could only be isolated in an inactive form, which could not be reactivated so far. The capability of the phylogenetically related methanogenic Mcr to oxidize methane to methyl-S-CoM (**figure 8 A**) at specific rates sufficient to account for those observed *in vivo* for AOM (Scheller et al. 2010) supports the postulated function of ANME Mcr as a methane oxidase. Structural data would allow more profound information about the **active site** and the applied substrates.

4.2.2 Structure of AMNE-I Mcr

The proportion of the Ni-protein I in the mat extracts was calculated to be ca. 7% of the total protein extracted which opened a door for structural analysis (Krüger et al. 2003). Despite the highly heterogeneous sample solution after purification, crystals grew. The resulting **crystal structure** (**Figure 8 C**) at 2.1 Å resolution revealed ANME-1 Mcr of a single organism (D1JBK2-4) confirmed by a mass-spectrometric analysis of the dissolved crystal used for structure determination (Shima et al. 2012).

AMNE-1 Mcr was found as a compact heterohexameric ($\alpha\beta\gamma$)₂ complex of 300 kDa (**Figure 8 C**) which is virtually identical to that of methanogenic Mcr (Ermler et al. 1997; Grabarse et al. 2001). The protein complex harbors two active sites built up of subunits α , α' , β and γ that are ca. 50 Å apart from each other. Each of them is positioned at the bottom of a 30 Å long funnel-shaped channel. Methylthio-F430 sits at its base and is non-covalently fixed to the polypeptide scaffold by multiple mostly polar interactions to its propionate, acetate and acetamide side-chains (**Figure 8 D**). In addition to the four equatorial nitrogens of the **tetrapyrrole** ring, the nickel centers are axially coordinated to the side chain amide oxygen of Gln α' 155 (**Figure 8 D**).

The X-ray structure of AMNE-1 Mcr contains nickel of methylthio-F430 in an inactive Ni(II) oxidation state and HS-CoM and HS-CoB in the same positions as found in the methanogenic Mcr_{ox1-silent} structure (Ermler et al. 1997). HS-CoM sits in front of its hydrocorphin plane of methylthio-F430. Its thiol group is coordinated to the sixth ligation site of the nickel and interacts with the hydroxyl groups of Tyr α 347 and Tyr β 363. HS-CoB sits above HS-CoM in the narrowest segment of the channel (**Figure 8 D**). Two hydrogen donors (the amide and peptide nitrogens of Asn α 500 and Val α 501) contact the sulfur of HS-CoB and thus facilitate the release of the proton. The space in the active site between nickel and the thiol group of HS-CoB is completely inaccessible for bulk solvent after binding of HS-CoB and lined up by an annular arrangement of hydrophobic aromatic residues (Phe α 344, Tyr α 347, Phe α 462, Phe β 357 and Tyr β 363) (**Figure 8 D**). No specific **gas channel** for methane could be detected in the ANME-1 structure, suggesting that methane enters via the **substrate channel**, but other possible routes via cavities adjacent to the active site cannot be excluded.

AMNE-I Mcr in complex with the substrates CoM-S-S-CoB and/or methane could not be structurally characterized, but the corresponding structure for the methanogenic

(Mcr_{silent} state) is available that contains CoM-S-S-CoB (Ermler et al. 1997), albeit not in a catalytically competent position. The S-CoM moiety is shifted more than 4 Å apart from its binding site in the AMNE-1 Mcr structure such that one oxygen atom of the sulfonate group becomes axially coordinated to Ni(II). Different enzyme states can be characterized via EPR spectroscopy, which depend on the redox state of the nickel center and on the nature of substrates, products, intermediates, artificial **substrate analogues** or **inhibitors** bound. ¹⁹F-ENDOR data on active methanogenic Mcr in the presence of HS-CoM and CF₃-S-CoB indicated a shift of the 7-thioheptanoyl chain towards nickel by more than 2 Å relative to its position in the structurally determined inactive form (Ebner et al. 2010), which reflects the potential of Mcr to undergo a major **conformational change** of the protein during catalysis. In the active conformation, the sulfonate tail of HS-CoM and the threonine phosphate tail of HS-CoB presumably remain fixed in their binding sites. The structural data for ANME and methanogenic Mcr reveal virtually identical overall and active site structures, as well as the same substrates and products, which implicates the same catalytic mechanism.

Figure 8: **A)** Mcr-catalyzed reaction of methane and CoM-S-S-CoB to methyl-S-CoM and coenzyme B, requiring anaerobic conditions under participation of the unique nickel hydrocorphin cofactor F430. **B)** Structure of cofactor **F430**. ANME-1 Mcr contains 17''-methylthio-F430 (Mayr et al. 2008) with R=S-CH₃ and ANME-2+3 contain F430 as found in methanogens with R=H. **C)** Overall architecture of AMNE-I Mcr. The heterohexameric Mcr complex (α in lightblue, α' in azurblue, β in orangered, β' in brightred, γ in brightgreen and γ' in forestgreen) has a size of about 120 Å by 85 Å by 80 Å. Minor deviations in the overall structures between methanogenic and methylotrophic Mcr are found in a few loop regions and at the N- and C-terminal ends of the subunits only. Coenzyme F430 (C in black) sits at the channel bottom and HS-CoB (C in black) in its most narrow segment with the

elongated thioheptanoyl pointing towards methylthio-F430 and the threoninephosphate group pointing towards its entrance. HS-CoM (C in black) sits in between methylthio-F430 and HS-CoB. **D)** Active site. The active site state of AMNE-1 Mcr is nearly congruent to that of the methanogenic Mcr_{ox1-silent} state. The main difference between AMNE-1 and methanogenic Mcr are five pronounced cysteine residues between methylthio-F430 and the protein surface. This striking cysteine-rich patch might be used as a redox-relay system for the reduction of ANME-1 Mcr from the inactive Ni(II) to the active Ni(I) oxidation state (Prakash et al. 2014). Another source of structural diversity are several post-translational modifications that differ between the structurally known AMNE-1 and methanogenic Mcr, but also within methanogenic Mcr variants (Wagner et al. 2016a).

4.2.3 Mechanistic basis of methane oxidation by Mcr

Methane oxidation under mild conditions, in aqueous solution and, in particular, in the absence of O₂ is a considerable chemical challenge due to the strong C-H bond in methane with a dissociation energy of 439 kJ/mol. Mcr with its Nickel cofactor F430 (**Figure 8 B**) is able to catalyze such a reaction by converting methane and a disulfide (CoM-S-S-CoB) into a methylthioether (methyl-S-CoM) and a thiol (HS-CoB). The catalytic mechanism is still disputed because no intermediates with the native substrates could be characterized and no precedence from laboratory chemistry is available for such a reaction.

To become enzymatically active, F430 Ni(II), or 17''-methylthio-F430 Ni(II), of the resting state has to be reduced to the reactive Ni(I) oxidation state (Goubeaud et al. 1997). *In vivo*, Ni(II) to Ni(I) reduction is achieved by a protein complex using iron/sulfur cluster containing proteins as electron donor whose redox potential is decreased via ATP hydrolysis (Prakash et al. 2014). Interestingly, nickel has suitable redox and ligation properties for catalysis only after integration into a special hydrocorphin ring system created by nature exclusively for Mcr that allows metal-centered reduction and oxidation to Ni(I) and Ni(III) oxidation states, respectively (Jaun and Pfaltz; 1986; Jaun 1990). Mcr is quite specific for the substrates methane and methyl-S-CoM. For Mcr from *M. marburgensis*, ethane and ethyl-S-CoM serve as alternative substrates but overall turnovers are slow; propyl-S-CoM and allyl-S-CoM do not react (Scheller et al. 2013a) although they bind to the active site (Goenrich et al. 2004). A new family of enzymes related to Mcr has been described to be responsible for the conversion of butane to butyl-S-CoM (Laso-Pérez et al. 2016), thereby enlarging the substrate repertoire of this type of reaction.

According to the principle of microscopic reversibility, the reaction mechanism is identical for both directions of the catalyzed reaction. Substantial net turnover *in vitro* can only be performed in the methanogenic direction, because the equilibrium for the Mcr-catalyzed reaction is on the methane side under standard conditions ($\Delta G^{\circ} = -30$ kJ mol⁻¹ for methane formation). Methane oxidation *in vitro* could be monitored under equilibrium conditions with the use of ¹²C and ¹³C isotopes (Scheller et al. 2010).

Figure 9 provides an overview of reaction pathways with their corresponding intermediates that are derived from EPR studies, DFT calculations and analogies to organometallic laboratory chemistry. A central question is whether H abstraction from methane proceeds radically or in a non-radical fashion, which leads to two distinct types of mechanisms that are discussed here.

Intermediates on the upper half (labeled I-a to I-d) correspond to radical mechanisms and involve interaction of the nickel center with the sulfur atom of coenzyme M as the first step in both directions. Intermediates drawn on the lower half (labeled II-a to II-f)

correspond to organometallic 2-electron mechanisms and involve interaction of the nickel center with carbon, hydrogen or methane as the first step.

Figure 9: Intermediates for conceivable reaction pathways from the ternary enzyme + methane + CoM-S-S-CoB complex to the ternary enzyme + methyl-S-CoM + HS-CoB complex. The large ^{13}C -kinetic isotope effect shows that C-S bond formation must be the rate-determining step (Scheller et al. 2013b), indicated “RDS”. **Upper half:** Sequence involving radical intermediates and Ni(II), where the nickel center is interacting with the sulfur atom of methyl-S-CoM or CoM-S-S-CoB as a first step in both directions (Pelmenschikov and Siegbahn, 2003). **Lower half:** Sequences involving non-radical intermediates highlighting alternative reaction pathways. In these sequences, the nickel center is interacting with methane, hydrogen or carbon as a first step of catalysis. Methane activation may also be initiated via the Ni(III) complex I-d (reaction I-d to II-a). The bent in F430 symbolizes states where one tetrapyrrol ring is out of plane allowing for two adjacent coordination sites available for catalysis as inferred from EPR data (Harmer et al. 2008). Intermediates from the lower part may also be able to form species with nickel in the +2 oxidation state and sulfur-based radicals on the coenzymes.

Radical mechanisms

Based on hybrid density functional theory calculations (B3LYP method), a radical mechanism via intermediates I-a and I-b and transition state I-c has been proposed (Chen et al. 2012; Pelmenchikov and Siegbahn 2003), involving a highly endergonic hydrogen atom abstraction by a thiyl radical (Int I-b) from methane to form a methyl-radical (Intermediate I-c, or transition state I-c), which then immediately combines with a nickel(II)-thiolate to form methyl-S-CoM and F430 in the Ni(I) oxidation state (Figure 9, upper half). This mechanism has been calculated using the following components: F430 without methyl-, acetamide-, or carboxylate substituents, *p*-cresols

to mimic Tyr333 and Tyr367, acetamide to mimic Gln147, thioacetone to mimic HS-CoM and ethane thiol to mimic HS-CoB. The methyl radical has originally been calculated to be an intermediate (int. I-c) (Pelmenschikov et al. 2002), which is not compatible with the experimental finding of net configurational inversion of the reaction (Ahn et al. 1991) because alkyl-radicals are expected to undergo rapid racemisation. Therefore, the calculation was adjusted to a “transient methyl-radial” as a transition state (transition state I-c) that does not undergo racemisation (Pelmenschikov and Siegbahn, 2003). In this mechanistic proposal, homolytic cleavage of the C-H bond in methane and C-S bond formation occur in one step, which requires a linear array of 5 atoms (Ni, S, C, H, S) in the transition state (TS I-c), in which 2 bonds are broken and two bonds are formed simultaneously.

Using the substrate analog of HS-CoB that is one methylene-group shorter denoted “HS-CoB₆”, the putative intermediate I-b has been characterized via **MCD spectroscopy** (Wongnate et al. 2016). Further, secondary deuterium isotope effects are in line with formation of a methyl radical (Scheller et al. 2013b). However, the **thiyl radical** in I-b could not be clearly identified via EPR spectroscopy (Wongnate et al. 2016).

Hydrogen atom abstraction from methane by a thiyl radical (int. I-b) is highly endergonic and has never been shown experimentally. It is unusual that the alkane is not interacting with the transition metal center directly, as this is the key feature to achieve alkane C-H bond activation in organometallic chemistry.

Organometallic mechanisms

EPR spectroscopic data demonstrate that cofactor F430 is able to form Ni-C, Ni-H and Ni-S bonds (Harmer et al. 2005; Harmer et al. 2008; Hinderberger et al. 2006; Sarangi et al. 2009; Yang et al. 2007). We compiled conceivable intermediates that involve two-electron reactions (**Figure 9, lower half**) as alternative pathways to the radical mechanism discussed above.

Non-radical C-H bond activation is initiated by a coordination of the methane to the nickel center of F430 to allow for an organometallic intermediate II-a or II-d, which is in analogy to a sigma-methane complex characterized for rhodium(I) (Bernskoetter et al. 2009). Such complexes are usually the first intermediates in alkane C-H bond cleavage reactions known from **oxidative addition** or Shilov reactions in laboratory chemistry.

In the methanogenic direction, S_N2 reaction via nucleophilic attack of the nickel center on the methyl group of CH₃-S-CoM yielding intermediate II-c appears to be a reasonable mechanism analogous to the chemistry of Co(I) in cobalamine-dependent enzymes. Such a mechanism is supported by the methyl-Ni(III) formation observed via CH₃-I addition to active enzyme as a replacement for methyl-S-CoM (Sarangi et al. 2009; Yang et al. 2007), but secondary deuterium isotope effects (1.19 per deuterium in the methyl-group) exclude an S_N2 reaction as the **rate-determining step** (Scheller et al. 2013b). In addition, the putative intermediate characterized recently with the alternative substrate HS-CoB₆ contains nickel in the +2 oxidation state and not in the expected +3 state (Wongnate et al. 2016).

Involvement of nickel-hydrides (intermediates II-e or II-f) is conceivable based on Ni(III) hydrides characterized via EPR spectroscopy (Harmer et al. 2008). Furthermore, nickel-hydride formation before the rate-limiting step in the methanogenic direction would account for the large deuterium equilibrium isotope effect observed when enzymatic methane formation is carried out in deuterated

medium; an equimolar mixture of H₂O and D₂O in the reaction buffer yields 21% CH₃D and 79% CH₄ without a significant kinetic isotope effect (Mayr 2009).

The existence of a rhombic nickel hydride in Mcr (Harmer et al. 2008) implies the availability of 2 adjacent coordination sites at F430 during catalysis that would enable formation of the methyl-hydrido-Ni(III) complex II-e that is prerequisite for a mechanism of reductive elimination/oxidative addition. However, the exact reaction pathway, in particular the reaction from intermediate II-e to II-f is speculative and would possibly involves additional steps.

Mechanistic conclusions

While the calculated radical mechanism (Pelmenschikov and Siegbahn 2003) is supported by recent spectroscopic evidence (Wongnate et al. 2016) and matches measured kinetic isotope effects (Scheller et al. 2013a; Scheller et al. 2013b), many experimental findings remain unexplained. The substrate analogue allyl-S-CoM binds to the active site (Goenrich et al. 2004) but does not react at all (Scheller et al. 2013a), even though allyl-radical formation is expected to proceed much more readily than formation of methyl-radicals. The ability of Mcr to provide two adjacent coordination sites on F430 for catalysis (Harmer et al. 2008) and to undergo major **conformational changes** in the protein (Ebner et al. 2010) are likely relevant for enzyme performance, but are not accounted for in the calculated radical mechanism as currently formulated. In summary, the catalytic mechanism of Mcr is still nebulous and further experimental data is required to unravel the exact chemistry behind this amazing transformation designed by nature that lacks precedent to man-made catalysts.

4.2.4 Optimization of Mcr oxidation

The Mcr reaction is slow, $v_{\max} = 100 \mu\text{mol min}^{-1} \text{mg}^{-1}$ measured for the *M. marburgensis* enzyme (Goubeaud et al. 1997), and considered rate-limiting in the methanogenesis and AOM catabolism. Therefore, it is conceivable that nature created solutions to optimize catalysis on the level of the enzyme, cell and organism, probably without directly changing the above-discussed enzymatic mechanism.

Because the active site is already highly optimized as reflected in its strict conservation among different Mcr variants, we speculate that **post-translational modifications** fine-tune the active site geometry/electrostatics in order to lower the activation barrier of the enzymatic process, if improvements by ordinary substitutions of amino acids are not possible anymore. Mcr from methanotrophic and methanogenic archaea contain several not strictly conserved post-translational modifications, which include 1-N-methylhistidine, S-methylcysteine, (S)-5-methylarginine, (S)-2-methylglutamine, 2,3-didehydroaspartate, 7-hydroxytryptophan, S-oxymethionine and thiopeptide-bound **thioglycine** (Grabarse et al. 2000; Kahnt et al. 2007; Wagner et al. 2016a). All of them are close to the active site but do not appear to be directly involved in the catalytic cycle, perhaps except for the thioglycine.

Coupling of two active sites – 50 Å apart in Mcr (**Figure 8 C**) – in a manner that the energy produced in one active site is used to reduce the transition energy of the rate-limiting step in the second active site may further increase the **catalytic efficiency**. Structural studies suggest a connection between the two active sites via the tight associated subunits α and α' allowing the transfer of local conformational changes over long distances. Spectroscopic studies on Mcr of *M. marburgensis* substantiated this proposal as only 50% of the enzyme can be transformed between two active states (Mcr_{red1} into Mcr_{red2}) after addition of HS-CoM and HS-CoB (Goenrich et al.

2005). Conformational rearrangement of a glycine-rich loop in the active site and an increased flexibility over a wide protein area was found in the structure of the Mcr_{red-silent} state devoid of substrates (Grabarse et al. 2001) and a movement of HS-CoB towards the nickel during the reaction cycle presumably accompanied by conformational changes was inferred from ¹⁹F ENDOR studies (Ebner et al. 2010) and transient kinetic studies (Dey et al. 2010).

The rate of methane oxidation can also be increased by expressing larger amounts of ANME Mcr in the cell, which is realized by the ANME consortia in the Black sea where 7% and 3% of the total protein content is ANME 1 and 2 Mcr (Krüger et al. 2003). Many hydrogenotrophic methanogens synthesize two Mcr isoenzymes with a sequence identity of 60–70%, which is not found for methanotrophic archaea. Their differential expression in *M. marburgensis* are known to be regulated by the growth conditions – temperature and pH (Bonacker et al. 1992) – suggesting an adaptation of their respective active sites to specific environmental conditions. The microbial diversity of ANME consortia with slightly modified Mcr might serve the same purpose.

4.3 Heterodisulfide reductase (Hdr)

The metagenome analysis indicated the presence of five copies of HdrABC in the genomes of ANME-1 archaea from the Black sea microbial mat (Meyerdierks et al. 2010). However, the genes encoding MvhABG, which is CoM-S-S-CoB reductase-associating hydrogenase found in methanogens, was not found in the genome of any ANME. The absence of genes encoding for MvhABG suggests that in ANME, the Hdr does not donate electrons to H⁺ forming H₂, although H₂ is the electron donor of soluble Hdr system in methanogenic archaea. Interestingly, one of the *hdr* gene clusters contains genes encoding MvhD and FdhB. MvhD is proposed to be an electronic connector of the HdrABC-MvhAGD complex. FdhB belongs to an F₄₂₀ binding protein family, which are associated with F₄₂₀-reducing enzymes, such as F₄₂₀-reducing hydrogenase (FrhAGB), which suggests that electrons from ferredoxin, HS-CoM and HS-CoB may be transferred to F₄₂₀ in the methanotrophic direction (Meyerdierks et al. 2010).

ANME-2a and ANME-2d archaea contain an integral membrane Hdr (HdrDE), which catalyzes the reduction of methanophenazin ($E_0' = -165\text{mV}$ (Tietze et al. 2003)) with the oxidation of HS-CoM and HS-CoB ($E_0' = -143\text{ mV}$ (Tietze et al. 2003)) to CoB-S-S-CoB. This endergonic reaction ($\Delta G^{\circ'} = +4.2\text{ kJ/mol}$) (Bäumer et al. 1998) could be driven by a proton ion influx. HdrABC type enzyme was also found in the genome of ANME-2a and ANME-2d. In the genome of ANME-2d, the encoding genes for MvhD- and FrhB-homologues are found near the genes encoding HdrABC, suggesting that FrhB might be complexed with HdrABC and serves as an enzyme complex catalyzing electron confurcation from reduced ferredoxin, HS-CoM and HS-CoB to two molecules of F₄₂₀ (Arshad et al. 2015). In the genome analysis of ANME-2a, presence of MvhD and the F₄₂₀-oxidoreductase subunit was not mentioned (Wang et al. 2014).

4.4 Methyl-tetrahydromethanopterin:coenzyme M methyltransferase (Mtr)

In ANME archaea, the MtrA–H complex catalyzes methyl transfer from methyl-S-CoM to H₄MPT. This endergonic reaction is driven by a coupling to sodium-ion influx. There are two homologues *mtrA* genes encoded in methanogenic archaea from Methanomicrobiales and Methanococcales, one of which is fused with the *mtrG* and

mtrF genes, respectively (Wang et al. 2015). In the *mtr* operon of ANME-1 archaea, only one *mtrA* gene is coded and fused with *mtrF* gene in an *mtrA-H* gene cluster (CBH38255). In ANME-2b archaea, two sets of *mtrA-H* genes are detected. One of the gene sets is expressed more strongly, suggesting that only this set is functional (Wang et al. 2014).

4.5 Methylene-tetrahydromethanopterin reductase (Mer)

In ANME-2, Mer catalyzes the reversible dehydrogenation of methyl-H₄MPT to methylene-H₄MPT with F₄₂₀H₂ as the electron acceptor. F₄₂₀ is a deazaflavin derivative with a structure similar to flavin but with a redox potential of $E_0' = -360$ mV (Thauer 1998), like NAD(P)H ($E_0' = -320$ mV). The crystal structure of Mer from the methanogen *M. barkeri* complexed with F₄₂₀ is available (Aufhammer et al. 2005). The Mer-encoding gene was found in the genomes of ANME-2a and ANME-2d but not in those of ANME-1 (Meyerdierks et al. 2010; Stokke et al. 2012). It has been proposed that a protein homologous to MetF, which is a methylene-tetrahydrofolate reductase structurally similar to Mer, could catalyze the Mer-reaction in ANME-1, but this hypothesis has not been tested yet (Stokke et al. 2012). We tried to heterologously overexpress the *metF* gene homolog from ANME-1, but the protein formed inclusion bodies that disabled functional studies (Shima et al. unpublished result).

4.6 Methylene-H₄MPT dehydrogenase (Mtd), cyclohydrolase (Mch) and formyltransferase (Ftr)

The genes from the metagenom sequences of ANME-1 were heterologously overexpressed and the enzymes Mtd, Mch and Ftr were purified to homogeneity by column chromatography without tags (Kojima et al. 2014). The three enzymes revealed high activity at 10 °C, which is near the physiological temperature of the ANME-1 archaea harvested. The specific activity substantiated their predicted functions in the AOM pathway. Mtd is quite unstable in buffer solutions with low salt concentrations. Interestingly, the stability also decreases by lowering the temperature; the half-life of the enzymatic activity at 20 °C, 10 °C (near the *in situ* temperature of the Black Sea mat) and on ice were 10 h, 3 h and 0.5 h, respectively. The Mtd enzyme from ANME-1 was stabilized by addition of 0.5 M potassium phosphate. The stability of Mch from ANME-1 is not dependent on the presence of salt, but the activity was found to be dependent on the presence of potassium phosphate, the maximum activity was observed at 0.6-0.8 M of potassium phosphate; at low salt concentrations, no activity could be detected.

It is known that the cytoplasm of the hyperthermophile *Methanopyrus kandleri* contains very high concentrations of salt, which is mainly composed of ~1 M cyclic 2,3-diphosphoglycerate, probably with potassium as the main cation. The activity and stability of some of the methanogenic enzymes are increased on the presence of lyotropic salts, i.e. with potassium phosphate, ammonium sulfate and potassium cyclic 2,3-diphosphoglycerate (Shima et al. 1998). Salt-dependent stabilization of Mtd and salt-dependent activation of Mch from ANME-1 archaea suggested that ANME-1 archaea contains high concentrations of **lyotropic salts** in the cells.

4.7 Formylmethanofuran dehydrogenase (Fmd and Fwd)

The formyl group in formyl-methanofuran is oxidized and hydrolyzed to CO₂ by two enzyme variants, Fmd or Fwd. Ferredoxin serves as the natural electron acceptor. The

two **isoenzymes** Fmd (ANME-2b and ANME-2d) and Fwd (ANME-1) contain **molybdenum** and **tungsten** in the pterin cofactor, respectively. The biochemically characterized Fwd complex from methanogenic archaea is composed of six subunits (FwdABCDGF) (Wagner et al. 2016b). FwdB contains the sequence motif for binding of the pterin cofactor. FwdA, an amidohydrolase, catalyzes the hydrolysis of formylmethanofuran, FwdF is a polyferredoxin and carries eight **4Fe-4S clusters**. The genes encoding Fwd proteins are found in ANME-1 archaea but those for FwdG and FwdH iron-sulfur cluster proteins are not detected in this genome (Meyerdierks et al. 2010). However, FmdG and FmdH are annotated in the ANME-2b and ANME-2d genomes.

5 Research needs

Pathways

Knowledge about the physiologies of different ANME clades has substantially increased over the last decade due to metagenomic sequencing, the enrichment of ANME-2d co-cultures, and via the recently proposed hypothesis of direct interspecies electron transfer as a syntrophic coupling mechanism for some members of ANME. Now, the focus on AOM needs to transition from –omics-driven investigations towards the isolation of ANME in pure cultures, and activity studies that include detection and quantification of the metabolic reactions, biochemical characterization of active ANME enzymes. Hypotheses of energy conservation in distinct ANME clades need to be probed experimentally, the minimal energy requirements for growth tested, and the exact stoichiometries between methane molecules oxidized, ion-gradients formed and ATP-yields quantified. Once elucidated, the distinct physiologies of ANME and methanogens can then be compared to gain a deeper understanding of the putative evolution of ANME from methanogens.

Niche differentiation among various ANME clades apparently doing the same overall reaction is poorly understood. ANME-1, for example, apparently lack the *mer*-gene that seems required for the primary metabolism of AOM. The Mer-step may be replaced by an alternative enzyme that needs to be characterized to understand why ANME-1 are not utilizing Mer. The metagenomics-derived proposal that some members of the ANME-2d clade carry out full denitrification from nitrate to ammonium requires clear experimental verification and isolation of the corresponding species in pure cultures to study their physiology in depth. The presence of multi-heme *c*-type cytochromes in ANME-2d may also allow for extracellular electron transfer to oxidants other than nitrate.

The hypothesis of direct interspecies electron transfer from ANME-1 and ANME-2a-c to their partner-bacteria needs profound analysis to enable growth of ANME in pure cultures by substituting the electron-“disposing” partner-bacteria with suitable laboratory settings. Cultivation of ANME on **electrodes** with defined redox potential may serve as a platform to probe their physiology. The electrode can mimic the metal oxide particles and thus simulate specific environmental conditions.

Pure cultures of ANME open the door for genetic engineering enabling ultimate control over the AOM-process, which may be relevant to apply the mechanism of methane oxidation with release of single electrons for **electric power** generation from **natural gas** at ambient temperatures.

Enzymes

As all enzymes in reverse methanogenesis operate close to the thermodynamic equilibrium, they may reveal catalytic strategies of how enzymes are optimized to carry out difficult chemical transformations in an energy-efficient manner that may be useful for **bio-catalytic** conversions in an industrial setting. Heterologous expression of all enzymes involved in reverse methanogenesis is advantageous, but some of them require elucidation of the genes required to biosynthesize special cofactors and install the posttranslational modifications present, in particular Mcr with its F430 cofactor and the 6 posttranslational modifications.

The mechanism of the Mcr-catalyzed reaction remains mysterious despite progress made via DFT calculations, characterization of intermediates with substrate analogs and measurements of isotope effects. The reason for the ability of Mcr to provide two adjacent sites for catalysis on F430 concomitant with a major conformational change in the protein, as well as a chemical involvement of the thioglycine residue during catalysis needs to be elucidated. Understanding the nickel-chemistry of F430-driven methane activation may lead to an integration of the Mcr-reaction into the toolbox of organometallic chemistry to selectively functionalize alkanes with the cheap transition metal nickel. The recent discovery of nickel-catalyzed conversion of butane to butyl-S-CoM broadens the scope for this type of catalysis.

Finally, the study of multi-heme *c*-type cytochromes as a tool in biology to conduct electrical current between microbes is in its infancy, but may find industrial utility as a tool to convert **fuel**-derived electrons directly to **electricity**. These processes require substantial empirical and theoretical research for a thorough understanding.

6 Literature

- [1] Ahn YH, Krzycki JA, Floss HG (1991) Steric course of the reduction of ethyl coenzyme m to ethane catalyzed by methyl coenzyme m reductase from *methanosarcina-barkeri*. Journal of the American Chemical Society 113: 4700-4701.
- [2] Arshad A, Speth DR, de Graaf RM, Op den Camp HJ, Jetten MS, Welte CU (2015) A metagenomics-based metabolic model of nitrate-dependent anaerobic oxidation of methane by *Methanoperedens*-like archaea. Front. Microbiol. 6: 1423.
- [3] Aufhammer SW, Warkentin E, Ermler U, Hagemeyer CH, Thauer RK, Shima S (2005) Crystal structure of methylenetetrahydromethanopterin reductase (Mer) in complex with coenzyme F₄₂₀: Architecture of the F₄₂₀/FMN binding site of enzymes within the nonprolyl cis-peptide containing bacterial luciferase family. Protein Sci. 14: 1840-1849.
- [4] Bäumer S, Murakami E, Brodersen J, Gottschalk G, Ragsdale SW, Deppenmeier U (1998) The F₄₂₀H₂:heterodisulfide oxidoreductase system from *Methanosarcina* species. 2-Hydroxyphenazine mediates electron transfer from F₄₂₀H₂ dehydrogenase to heterodisulfide reductase. FEBS Lett. 428: 295-298.
- [5] Beal EJ, House CH, Orphan VJ (2009) Manganese- and Iron-Dependent Marine Methane Oxidation. Science 325: 184-187.
- [6] Bernskoetter WH, Schauer CK, Goldberg KI, Brookhart M (2009)

Characterization of a Rhodium(I) sigma-Methane Complex in Solution. *Science* 326: 553-556.

- [7] Boetius A, Ravensschlag K, Schubert CJ, Rickert D, Widdel F, Gieseke A, Amann R, Jorgensen BB, Witte U, Pfannkuche O (2000) A marine microbial consortium apparently mediating anaerobic oxidation of methane. *Nature* 407: 623-626.
- [8] Bonacker LG, Baudner S, Thauer RK (1992) Differential expression of the two methyl-coenzyme M reductases in *Methanobacterium thermoautotrophicum* as determined immunochemically via isoenzyme-specific antisera. *Eur. J. Biochem.* 206: 87-92.
- [9] Caldwell SL, Laidler JR, Brewer EA, Eberly JO, Sandborgh SC, Colwell FS (2008) Anaerobic oxidation of methane: mechanisms, bioenergetics, and the ecology of associated microorganisms. *Environ Sci Technol* 42: 6791-6799.
- [10] Chen SL, Blomberg MRA, Siegbahn PEM (2012) How is methane formed and oxidized reversibly when catalyzed by Ni-containing methyl-coenzyme M reductase?, *Chem. Eur. J.* 18: 6309-6315.
- [11] Chistoserdova L, Vorholt Julia A, Lidstrom Mary E (2005) A genomic view of methane oxidation by aerobic bacteria and anaerobic archaea. *Genome biology* 6: 208.
- [12] Costa KC, Leigh JA (2014) Metabolic versatility in methanogens. *Current Opinion in Biotechnology* 29: 70-75.
- [13] Dey M, Li XH, Kunz RC, Ragsdale SW (2010) Detection of Organometallic and Radical Intermediates in the Catalytic Mechanism of Methyl-Coenzyme M Reductase Using the Natural Substrate Methyl-Coenzyme M and a Coenzyme B Substrate Analogue. *Biochemistry* 49: 10902-10911.
- [14] Ebner S, Jaun B, Goenrich M, Thauer RK, Harmer J (2010) Binding of Coenzyme B Induces a Major Conformational Change in the Active Site of Methyl-Coenzyme M Reductase. *Journal of the American Chemical Society* 132: 567-575.
- [15] Ermler U, Grabarse W, Shima S, Goubeaud M, Thauer RK (1997) Crystal structure of methyl-coenzyme M reductase: the key enzyme of biological methane formation. *Science* 278: 1457-1462.
- [16] Ettwig KF, Butler MK, Paslier DL, Pelletier E, Mangenot S, Kuypers MMM, Schreiber F, Dutilh BE, Zedelius J, Dirk de Beer JG, Wessels HJCT, Theo van Alen FL, Wu ML, Pas-Schoonen KTvd, Camp HJMod, Janssen-Megens EM, Francoijs K-J, Stunnenberg H, Weissenbach J, Jetten MSM, Strous M (2010) Nitrite-driven anaerobic methane oxidation by oxygenic bacteria. *Nature* 464: 543.
- [17] Ettwig KF, Shima S, Van De Pas-Schoonen KT, Kahnt J, Medema MH, Op Den Camp HJM, Jetten MSM, Strous M (2008) Denitrifying bacteria anaerobically oxidize methane in the absence of Archaea. *Environmental Microbiology* 10: 3164-3173.
- [18] Evans PN, Parks DH, Chadwick GL, Robbins SJ, Orphan VJ, Golding SD, Tyson GW (2015) Methane metabolism in the archaeal phylum Bathyarchaeota revealed by genome-centric metagenomics. *Science* 350: 434-

- [19] Ferry JG (2010) The chemical biology of methanogenesis. *Planetary and Space Science* 58: 1775-1783.
- [20] Girguis PR, Cozen AE, DeLong EF (2005) Growth and population dynamics of anaerobic methane-oxidizing archaea and sulfate-reducing bacteria in a continuous-flow bioreactor. *Applied and Environmental Microbiology* 71: 3725-3733.
- [21] Goenrich M, Duin EC, Mahlert F, Thauer RK (2005) Temperature dependence of methyl-coenzyme M reductase activity and of the formation of the methyl-coenzyme M reductase red2 state induced by coenzyme B. *Journal of Biological Inorganic Chemistry* 10: 333-342.
- [22] Goenrich M, Mahlert F, Duin EC, Bauer C, Jaun B, Thauer RK (2004) Probing the reactivity of Ni in the active site of methyl-coenzyme M reductase with substrate analogues. *Journal of Biological Inorganic Chemistry* 9: 691-705.
- [23] Gorby YA, Yanina S, McLean JS, Rosso KM, Moyles D, Dohnalkova A, Beveridge TJ, Chang IS, Kim BH, Kim KS (2006) Electrically conductive bacterial nanowires produced by *Shewanella oneidensis* strain MR-1 and other microorganisms. *Proceedings of the National Academy of Sciences* 103: 11358-11363.
- [24] Gottschalk G, Thauer RK (2001) The Na⁺-translocating methyltransferase complex from methanogenic archaea. *Biochim. Biophys. Acta* 1505: 28-36.
- [25] Goubeau M, Schreiner G, Thauer RK (1997) Purified methyl-coenzyme-M reductase is activated when the enzyme-bound coenzyme F430 is reduced to the nickel(I) oxidation state by titanium(III) citrate. *Eur. J. Biochem.* 243: 110-114.
- [26] Grabarse W, Mahlert F, Duin EC, Goubeaud M, Shima S, Thauer RK, Lamzin V, Ermler U (2001) On the mechanism of biological methane formation: Structural evidence for conformational changes in methyl-coenzyme M reductase upon substrate binding. *J. Mol. Biol.* 309: 315-330.
- [27] Grabarse WG, Mahlert F, Shima S, Thauer RK, Ermler U (2000) Comparison of three methyl-coenzyme M reductases from phylogenetically distant organisms: Unusual amino acid modification, conservation and adaptation. *J. Mol. Biol.* 303: 329-344.
- [28] Gralnick JA, Newman DK (2007) Extracellular respiration. *Molecular microbiology* 65: 1-11.
- [29] Gunsalus RP, Wolfe RS (1980) Methyl coenzyme-M reductase from *methanobacterium-thermoautotrophicum* - Resolution and properties of the components. *Journal of Biological Chemistry* 255: 1891-1895.
- [30] Hallam SJ, Putnam N, Preston CM, Detter JC, Rokhsar D, Richardson PM, DeLong EF (2004) Reverse methanogenesis: Testing the hypothesis with environmental genomics. *Science* 305: 1457-1462.
- [31] Harmer J, Finazzo C, Piskorski R, Bauer C, Jaun B, Duin EC, Goenrich M, Thauer RK, Van Doorslaer S, Schweiger A (2005) Spin Density and

Coenzyme M Coordination Geometry of the ox1 Form of Methyl-Coenzyme M Reductase: A Pulse EPR Study. *Journal of the American Chemical Society* 127: 17744-17755.

- [32] Harmer J, Finazzo C, Piskorski R, Ebner S, Duin EC, Goenrich M, Thauer RK, Reiher M, Schweiger A, Hinderberger D, Jaun B (2008) A nickel hydride complex in the active site of methyl-coenzyme M reductase: Implications for the catalytic cycle. *Journal of the American Chemical Society* 130: 10907-10920.
- [33] Haroon MF, Hu SH, Shi Y, Imelfort M, Keller J, Hugenholtz P, Yuan ZG, Tyson GW (2013) Anaerobic oxidation of methane coupled to nitrate reduction in a novel archaeal lineage. *Nature* 501: 567-570.
- [34] Hedderich R, Whitman WB, in *The Prokaryotes: Volume 2: Ecophysiology and Biochemistry* (Eds.: M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer, E. Stackebrandt), Springer New York, New York, NY, **2006**, pp. 1050-1079.
- [35] Hinderberger D, Piskorski RP, Goenrich M, Thauer RK, Schweiger A, Harmer J, Jaun B (2006) A nickel-alkyl bond in an inactivated state of the enzyme catalyzing methane formation. *Angewandte Chemie, International Edition* 45: 3602-3607.
- [36] Hoehler TM, Alperin MJ, Albert DB, Martens CS (1994) Field and laboratory studies of methane oxidation in an anoxic marine sediment - evidence for a methanogen-sulfate reducer consortium. *Global Biogeochemical Cycles* 8: 451-463.
- [37] Holler T, Widdel F, Knittel K, Amann R, Kellermann MY, Hinrichs KU, Teske A, Boetius A, Wegener G (2011) Thermophilic anaerobic oxidation of methane by marine microbial consortia. *Isme Journal* 5: 1946-1956.
- [38] Iversen N, Jorgensen BB (1985) Anaerobic methane oxidation rates at the sulfate-methane transition in marine sediments from Kattegat and Skagerrak (Denmark). *Limnology and Oceanography* 30: 944-955.
- [39] Jaun B (1990) Coenzyme F430 from methanogenic bacteria: oxidation of F430 pentamethyl ester to the nickel(III) form. *Helvetica Chimica Acta* 73: 2209-2217.
- [40] Jaun B, Pfaltz A (1986) Coenzyme F430 from methanogenic bacteria: reversible one-electron reduction of F430 pentamethyl ester to the nickel(I) form. *Journal of the Chemical Society, Chemical Communications*: 1327-1329.
- [41] Jaun B, Thauer RK (2007) Methyl-Coenzyme M reductase and its nickel corphin coenzyme F430 in methanogenic Archaea. *Metal Ions in Life Sciences* 2: 323-356.
- [42] Kahnt J, Buchenau B, Mahlert F, Kruger M, Shima S, Thauer RK (2007) Post-translational modifications in the active site region of methyl-coenzyme M reductase from methanogenic and methanotrophic archaea. *FEBS J.* 274: 4913-4921.
- [43] Kaster AK, Moll J, Parey K, Thauer RK (2011) Coupling of ferredoxin and heterodisulfide reduction via electron bifurcation in hydrogenotrophic

- methanogenic archaea. *Proc. Natl. Acad. Sci. USA* 108: 2981-2986.
- [44] Kletzin A, Heimerl T, Flechsler J, van Niftrik L, Rachel R, Klingl A (2015) Cytochromes c in Archaea: distribution, maturation, cell architecture, and the special case of *Ignicoccus hospitalis*. *Frontiers in microbiology* 6: 439.
 - [45] Knittel K, Boetius A (2009) Anaerobic oxidation of methane: progress with an unknown process. *Annu. Rev. Microbiol.* 63: 311-334.
 - [46] Kojima H, Moll J, Kahnt J, Fukui M, Shima S (2014) A reversed genetic approach reveals the coenzyme specificity and other catalytic properties of three enzymes putatively involved in anaerobic oxidation of methane with sulfate. *Environmental Microbiology* 16: 3431-3442.
 - [47] Krüger M, Meyerdierks A, Glockner FO, Amann R, Widdel F, Kube M, Reinhardt R, Kahnt J, Böcher R, Thauer RK, Shima S (2003) A conspicuous nickel protein in microbial mats that oxidize methane anaerobically. *Nature* 426: 878-881.
 - [48] Krukenberg V, Harding K, Richter M, Glöckner FO, Gruber-Vodicka HR, Adam B, Berg JS, Knittel K, Tegetmeyer HE, Boetius A, Wegener G (2016) *Candidatus Desulfofervidus auxilii*, a hydrogenotrophic sulfate-reducing bacterium involved in the thermophilic anaerobic oxidation of methane. *Environmental Microbiolog* 18: 3073-3091.
 - [49] Laso-Pérez R, Wegener G, Knittel K, Widdel F, Harding KJ, Krukenberg V, Meier DV, Richter M, Tegetmeyer HE, Riedel D, Richnow H, Adrian L, Reemtsma T, Lechtenfeld OJ, Musat F (2016) Thermophilic archaea activate butane via alkyl-coenzyme M formation. *Nature* 539: 396-401.
 - [50] Losekann T, Knittel K, Nadalig T, Fuchs B, Niemann H, Boetius A, Amann R (2007) Diversity and abundance of aerobic and anaerobic methane oxidizers at the Haakon Mosby mud volcano, Barents Sea. *Applied and Environmental Microbiology* 73: 3348-3362.
 - [51] Lovley DR (2012) Electromicrobiology. *Annual review of microbiology* 66: 391-409.
 - [52] Luesken FA, Wu ML, Op den Camp HJM, Keltjens JT, Stunnenberg H, Francoijs K-J, Strous M, Jetten MSM (2012) Effect of oxygen on the anaerobic methanotroph ‘*Candidatus Methyloirabilis oxyfera*’: kinetic and transcriptional analysis. *Environmental Microbiology* 14: 1024-1034.
 - [53] Mayr S, PhD Thesis from Eidgenössische Technische Hochschule, Diss ETH Nr. 18549, p. 175 (Zurich), **2009**.
 - [54] Mayr S, Latkoczy C, Krüger M, Gunther D, Shima S, Thauer RK, Widdel F, Jaun B (2008) Structure of an F₄₃₀ variant from archaea associated with anaerobic oxidation of methane. *J Am Chem Soc* 130: 10758-10767.
 - [55] McGlynn SE, Chadwick GL, Kempes CP, Orphan VJ (2015) Single cell activity reveals direct electron transfer in methanotrophic consortia. *Nature* 526: 531-535.
 - [56] McGlynn SE (2017) Energy metabolism during anaerobic methane oxidation in ANME archaea. *Microbes and Environments*. doi:10.1264/jsme2.ME16166, in press.

- [57] Meulepas RJW, Jagersma CG, Gieteling J, Buisman CJN, Stams AJM, Lens PNL (2009) Enrichment of Anaerobic Methanotrophs in Sulfate-Reducing Membrane Bioreactors. *Biotechnology and Bioengineering* 104: 458-470.
- [58] Meulepas RJW, Jagersma CG, Khadem AF, Stams AJM, Lens PNL (2010) Effect of methanogenic substrates on anaerobic oxidation of methane and sulfate reduction by an anaerobic methanotrophic enrichment. *Applied Microbiology and Biotechnology* 87: 1499-1506.
- [59] Meyerdierks A, Kube M, Kostadinov I, Teeling H, Glockner FO, Reinhardt R, Amann R (2010) Metagenome and mRNA expression analyses of anaerobic methanotrophic archaea of the ANME-1 group. *Environmental Microbiology* 12: 422-439.
- [60] Milucka J, Ferdelman TG, Polerecky L, Franzke D, Wegener G, Schmid M, Lieberwirth I, Wagner M, Widdel F, Kuypers MMM (2012) Zero-valent sulphur is a key intermediate in marine methane oxidation. *Nature* 491: 541-546.
- [61] Nauhaus K, Albrecht M, Elvert M, Boetius A, Widdel F (2007) In vitro cell growth of marine archaeal-bacterial consortia during anaerobic oxidation of methane with sulfate. *Environmental Microbiology* 9: 187-196.
- [62] Nauhaus K, Treude T, Boetius A, Krueger M (2005) Environmental regulation of the anaerobic oxidation of methane: A comparison of ANME-I and ANME-II communities. *Environmental Microbiology* 7: 98-106.
- [63] Op den Camp HJM, Islam T, Stott MB, Harhangi HR, Hynes A, Schouten S, Jetten MSM, Birkeland N-K, Pol A, Dunfield PF (2009) Environmental, genomic and taxonomic perspectives on methanotrophic Verrucomicrobia. *Environmental Microbiology Reports* 1: 293-306.
- [64] Orcutt B, Meile C (2008) Constraints on mechanisms and rates of anaerobic oxidation of methane by microbial consortia: process-based modeling of ANME-2 archaea and sulfate reducing bacteria interactions. *Biogeosciences* 5: 1587-1599.
- [65] Orphan VJ, House CH, Hinrichs K-U, McKeegan KD, DeLong EF (2002) Multiple archaeal groups mediate methane oxidation in anoxic cold seep sediments. *Proceedings of the National Academy of Sciences of the United States of America* 99: 7663-7668.
- [66] Orphan VJ, House CH, Hinrichs KU, McKeegan KD, DeLong EF (2001) Methane-consuming archaea revealed by directly coupled isotopic and phylogenetic analysis. *Science* 293: 484-487.
- [67] Pelmeshnikov V, Blomberg MRA, Siegbahn PEM, Crabtree RH (2002) A mechanism from quantum chemical studies for methane formation in methanogenesis. *J. Am. Chem. Soc.* 124: 4039-4049.
- [68] Pelmeshnikov V, Siegbahn PEM (2003) Catalysis by methyl-coenzyme M reductase: a theoretical study for heterodisulfide product formation. *J. Biol. Inorg. Chem.* 8: 653-662.
- [69] Pirbadian S, El-Naggar MY (2012) Multistep hopping and extracellular charge transfer in microbial redox chains. *Physical Chemistry Chemical Physics* 14: 13802-13808.

- [70] Prakash D, Wu YN, Suh SJ, Duin EC (2014) Elucidating the process of activation of methyl-coenzyme M reductase. *J. Bacteriol.* 196: 2491-2498.
- [71] Raghoebarsing AA, Pol A, van de Pas-Schoonen KT, Smolders AJP, Ettwig KF, Rijpstra WIC, Schouten S, Damste JSS, Op den Camp HJM, Jetten MSM, Strous M (2006) A microbial consortium couples anaerobic methane oxidation to denitrification. *Nature* 440: 918-921.
- [72] Reeburgh WS (2007) Oceanic methane biogeochemistry. *Chem. Rev.* 107: 486-513.
- [73] Rotaru A-E, Thamdrup B (2016) A new diet for methane oxidizers. *Science* 351: 658-658.
- [74] Sarangi R, Dey M, Ragsdale SW (2009) Geometric and Electronic Structures of the Ni-I and Methyl-Ni-III Intermediates of Methyl-Coenzyme M Reductase. *Biochemistry* 48: 3146-3156.
- [75] Scheller S, Goenrich M, Boecher R, Thauer RK, Jaun B (2010) The key nickel enzyme of methanogenesis catalyses the anaerobic oxidation of methane. *Nature* 465: 606-608.
- [76] Scheller S, Goenrich M, Thauer RK, Jaun B (2013b) Methyl-coenzyme M reductase from methanogenic archaea: isotope effects on the formation and anaerobic oxidation of methane. *J. Am. Chem. Soc.* 135: 14975-14984.
- [77] Scheller S, Goenrich M, Thauer RK, Jaun B (2013a) Methyl-coenzyme M reductase from methanogenic archaea: isotope effects on label exchange and ethane formation with the homologous substrate ethyl-coenzyme M. *J. Am. Chem. Soc.* 135: 14985-14995.
- [78] Scheller S, Yu H, Chadwick GL, McGlynn SE, Orphan VJ (2016) Artificial electron acceptors decouple archaeal methane oxidation from sulfate reduction. *Science* 351: 703-707.
- [79] Semrau JD, DiSpirito AA, Yoon S (2010) Methanotrophs and copper. *FEMS Microbiology Reviews* 34: 496-531.
- [80] Shima S (2014) Enzyme chemistry of methanogenesis and anaerobic oxidation of methane. *Kagakutoseibutsu* 52: 307-312.
- [81] Shima S, Krueger M, Weinert T, Demmer U, Kahnt J, Thauer RK, Ermler U (2012) Structure of a methyl-coenzyme M reductase from Black Sea mats that oxidize methane anaerobically. *Nature* 481: 98-101.
- [82] Shima S, Tziatzios C, Schubert D, Fukada H, Takahashi K, Ermler U, Thauer RK (1998) Lyotropic-salt-induced changes in monomer/dimer/tetramer association equilibrium of formyltransferase from the hyperthermophilic *Methanopyrus kandleri* in relation to the activity and thermostability of the enzyme. *Eur. J. Biochem.* 258: 85-92.
- [83] Stokke R, Roalkvam I, Lanzen A, Haflidason H, Steen IH (2012) Integrated metagenomic and metaproteomic analyses of an ANME-1-dominated community in marine cold seep sediments. *Environ. Microbiol.* 14: 1333-1346.
- [84] Thauer RK (1998) Biochemistry of methanogenesis: a tribute to Marjory Stephenson. *Microbiology* 144: 2377-2406.

- [85] Thauer RK (2011) Anaerobic oxidation of methane with sulfate: on the reversibility of the reactions that are catalyzed by enzymes also involved in methanogenesis from CO₂. *Curr. Opin. Microbiol.* 14: 292-299.
- [86] Thauer RK, Kaster AK, Seedorf H, Buckel W, Hedderich R (2008) Methanogenic archaea: ecologically relevant differences in energy conservation. *Nat. Rev. Microbiol.* 6: 579-591.
- [87] Tietze M, Beuchle A, Lamla I, Orth N, Dehler M, Greiner G, Beifuss U (2003) Redox Potentials of Methanophenazine and CoB-S-S-CoM, Factors Involved in Electron Transport in Methanogenic Archaea. *ChemBioChem* 4: 333-335.
- [88] Timmers PHA, Welte CU, Koehorst JJ, Plugge CM, Jetten MSM, Stams AJM (2017) Reverse Methanogenesis and Respiration in Methanotrophic Archaea. *Archaea*. 2017: 1–22.
- [89] Wagner T, Ermler U, Shima S (2016b) The methanogenic CO₂ reducing-and-fixing enzyme is bifunctional and contains 46 [4Fe-4S] clusters. *Science* 354: 114-117.
- [90] Wagner T, Kahnt J, Ermler U, Shima S (2016a) Didehydroaspartate Modification in Methyl-Coenzyme M Reductase Catalyzing Methane Formation. *Angewandte Chemie International Edition* 55: 10630-10633.
- [91] Wang FP, Zhang Y, Chen Y, He Y, Qi J, Hinrichs KU, Zhang XX, Xiao X, Boon N (2014) Methanotrophic archaea possessing diverging methane-oxidizing and electron-transporting pathways. *Isme Journal* 8: 1069-1078.
- [92] Wang S, Chen Y, Cao Q, Lou H (2015) Long-lasting gene conversion shapes the convergent evolution of the critical methanogenesis genes. *G3 (Bethesda)* 5: 2475-2486.
- [93] Wegener G, Krukenberg V, Riedel D, Tegetmeyer HE, Boetius A (2015) Intercellular wiring enables electron transfer between methanotrophic archaea and bacteria. *Nature* 526: 587-590.
- [94] Wegener G, Krukenberg V, Ruff SE, Kellermann MY, Knittel K (2016) Metabolic Capabilities of Microorganisms Involved in and Associated with the Anaerobic Oxidation of Methane. *Frontiers in microbiology* 7.
- [95] Welte C, Deppenmeier U (2014) Bioenergetics and anaerobic respiratory chains of acetoclastic methanogens. *Biochimica et Biophysica Acta (BBA) - Bioenergetics* 1837: 1130-1147.
- [96] Widdel F, Grundmann O, in *Handbook of Hydrocarbon and Lipid Microbiology* (Ed.: K. N. Timmis), Springer Berlin Heidelberg, Berlin, Heidelberg, **2010**, pp. 909-924.
- [97] Wongnate T, Sliwa D, Ginovska B, Smith D, Wolf MW, Lehnert N, Raugei S, Ragsdale SW (2016) The radical mechanism of biological methane synthesis by methyl-coenzyme M reductase. *Science* 352: 953-958.
- [98] Yang N, Reiher M, Wang M, Harmer J, Duin EC (2007) Formation of a nickel-methyl species in methyl-coenzyme M reductase, an enzyme catalyzing methane formation. *Journal of the American Chemical Society* 129: 11028-11029.