Handbook of
Hydrocarbon and Lipid Microbiology

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Alfons J. M. Stams · Diana Z. Sousa *Editors*

Biogenesis of Hydrocarbons

Springer

Handbook of Hydrocarbon and Lipid Microbiology

This handbook is the unique and definitive resource of current knowledge on the diverse and multifaceted aspects of microbial interactions with hydrocarbons and lipids, the microbial players, the physiological mechanisms and adaptive strategies underlying microbial life and activities at hydrophobic material:aqueous liquid interfaces, and the multitude of health, environmental and biotechnological consequences of these activities.

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Editors

Biogenesis of Hydrocarbons

With 66 Figures and 33 Tables

Springer

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Preface

Microorganisms are able to produce a wide range of hydrocarbons, from the simplest hydrocarbon, methane, to longer chain molecules, including aliphatic hydrocarbons and oils. This book compiles information on hydrocarbon biogenesis, covering aspects from biochemistry, microbial diversity and taxonomy, and application of hydrocarbon-producing microbes. An introductory section on the bioenergetics of microbial hydrocarbon production is given in Part 1. Further on, a main segment of the book is dedicated to methanogens (Parts 2–4), while the biogenesis of longer hydrocarbons is covered in Part 5. Methanogens are microorganisms belonging to the archaeal domain, which can produce methane from various substrates (hydrogen plus CO_2, carbon monoxide, acetate, and other methylated compounds). These microorganisms may strive in extreme environments (very hot, very cold environments) but are commonly abundant in, for example, wetlands and agricultural lands (e.g., rice paddy fields) and in the intestinal tract of animals. Their high activity in these natural environments is responsible for the production and release of substantial amounts of methane to the atmosphere, where it exerts a greenhouse effect stronger than CO_2 (per molecule). On the other hand, methane can be used as biofuel or to produce electricity, when produced in confined and controlled anaerobic digesters. Conversion of wastes and biomass to methane has been exploited in the frame of circular economy. Part 2 includes several chapters on the fundamentals of methanogenic metabolism. A chapter dedicated to the importance of methanogens in syntrophic metabolism, indispensable for the anaerobic conversion of molecules such as fatty acids, is also included. Part 3 gives further insight into the methanogenic diversity on different natural and man-made environments, including recent advances provided by (functional) genomics and metagenomics analyses. As production of methane by methanogens is commonly coupled to its consumption by methanotrophic communities, two chapters on methane oxidation and methane cycling are included in Part 4 of this book. The final part of the book (Part 5) contains one chapter covering the diversity and taxonomy of aliphatic hydrocarbon producers and two chapters on the metabolism of alkane and oil biosynthesis by bacteria.

As a final note, we would like to thank all the authors who wrote the chapters and allowed the concretization of this book. We also acknowledge Prof. Kenneth Timmis for the initiative on this series of books and colleagues at Springer for their always prompt support.

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Alfons J. M. Stams
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Kenneth Timmis studied microbiology and obtained his Ph.D. at Bristol University. He undertook postdoctoral training at the Ruhr-University Bochum, Yale and Stanford, at the latter two as a Fellow of the Helen Hay Whitney Foundation. He was then appointed Head of an independent research group at the Max Planck Institute for Molecular Genetics in Berlin and subsequently Professor of Biochemistry in the University of Geneva, Faculty of Medicine. Thereafter, for almost 20 years, he was Director of the Division of Microbiology at the National Research Centre for Biotechnology (GBF)/now the Helmholtz Centre for Infection Research (HZI), and concomitantly Professor of Microbiology in the Institute of Microbiology of the Technical University Braunschweig. He is currently Emeritus Professor in this institute.

The Editor-in-Chief has worked for more than 30 years in the area of environmental microbiology and biotechnology, has published over 400 papers in international journals, and is an ISI Highly Cited Microbiology-100 researcher. His group has worked for many years, inter alia, on the biodegradation of oil hydrocarbons, especially the genetics and regulation of toluene degradation, and on the ecology of hydrocarbon-degrading microbial communities, discovered the new group of marine oil-degrading hydrocarbonoclastic bacteria, initiated genome sequencing projects on bacteria that are paradigms of microbes

that degrade organic compounds (*Pseudomonas putida* and *Alcanivorax borkumensis*), and pioneered the topic of experimental evolution of novel catabolic activities.

He is Fellow of the Royal Society, Member of the European Molecular Biology Organisation, Fellow of the American Academy of Microbiology, Member of the European Academy of Microbiology, and Recipient of the Erwin Schrödinger Prize. He is the founder and Editor-in-Chief of the journals *Environmental Microbiology*, *Environmental Microbiology Reports*, and *Microbial Biotechnology.*

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Introduction to Microbial Hydrocarbon Production: Bioenergetics

1

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Abstract

Microorganisms play an essential role in the global carbon budget with methanogenesis being a significant global source of methane. The ability to produce hydrocarbons other than methane is widespread among microorganisms, and the diversity of hydrocarbon structures that are made is remarkable. However, other than microbial methane production, we know very little about the biochemical processes involved in microbial hydrocarbon formation. Methane production from natural polymers involves a consortium of interacting microbial species. Gibbs free energy yields associated with methanogenesis depend significantly on environmental conditions, especially temperature, activities (concentrations) of substrates and products, and pH, and are typically substantially smaller in natural systems than in growth-optimized cultures. The Gibbs free energy changes involved in the conversion of hydrocarbons, fatty and aromatic acids, alcohols, and hydrogen to methane are close to thermodynamic equilibrium. The low Gibbs free energy changes by which methanogenic consortia operate imply the existence of a minimum free energy change needed to sustain microbial growth, e.g., a biological energy quantum (BEQ), which is supported both by theoretical considerations and experimental data. Methanogenic consortia provide excellent models to study interspecies interactions and highly efficient energy economies.

1 Introduction: Scope of Microbial Hydrocarbon Production

Microorganisms play a significant role in the production of the simplest hydrocarbon, methane (CH_4). Methane is an important fuel and a potent greenhouse gas, and its atmospheric concentration has nearly tripled since preindustrial times (Lelieveld et al. 1998). Estimates for the annual global methane budget range from 500 to 600 Teragram (Tg) (1 Tg equals 10^{12} g) with about 70% (350 – 400 Tg) due to microbial activity (Ehhalt et al. 2001). Important sources of microbially produced methane are wetlands including tundra, bogs and swamps, ocean sediments, rice paddies, ruminant animals, oceans, termites, landfills, and waste treatment facilities. Some years ago, Keppler et al. (2006) showed that also plants may emit methane, which may account for about 10–30% of the total methane entering the atmosphere. Because most of the microbially produced methane comes from the decomposition of biomass, methanogenesis is an integral component of the global carbon cycle. Microbial methane production is an ancient process dating to the early Archaean era, 3.5 Gyr ago (Ueno et al. 2006).

Microorganisms make a variety of hydrocarbons other than CH_4 (Table 1) (Ladygina et al. 2006; Tornabene 1980, 1982; Wackett 2008). Soil microorganisms are significant producers of ethylene (Ilag and Curtis 1968; Lynch 1972) and volatile alkanes and alkenes with two to four carbons (Ladygina et al. 2006). Geochemical evidence implicates microorganisms in the formation of ethane and propane in deep marine sediments (Hinrichs et al. 2006). Long-chain alkane production by marine algae is well documented (Table 1) although the amounts made by most algae

Table 1 Types of hydrocarbons produced by microorganisms[a]

Hydrocarbon type	Microorganisms
CH_4	Methanogens
C-2 to C-4 alkanes and alkenes	Many different soil microorganisms
Long-chain alkanes	
n-Pentadecane (C-15) and n-heptadecane (C-17)	Brown and red algae
C-17 to C-36 alkanes	*Botryococcus braunii*
Saturated and unsaturated C-17 straight chain hydrocarbons and 6-methyl hexadecane and 4-methyl octadecane	*Dunaliella salina* (green alga)
Pristane, (C-19), phytane (C-20),	Phototrophic bacteria
C-15 to C-31 alkanes	Various bacteria and fungi
C-17 alkanes; 7, 9-dimethyl hexadecane; and 7- and 8-methyl-heptadecane	Cyanobacteria
Alkenes	
n-Heneicosahexaene (C-21:6); up to 1% of dry weight	Freshwater and marine algae
Olefins and polyunsaturated alkenes	Marine algae
Di-unsaturated hydrocarbons; botryococcane	Chlorophytes
C-21 to C-29 alkenes	*Micrococcus* and *Kocuria*
Terpenes (Isoprenoids)	
Isoprene (2-methyl-1,3-butadiene)	Actinomyces *Bacillus subtilis*
Carotenes	Fungi, yeasts, algae, bacteria
Squalene (C-30) and isoprenoids, hydroisoprenoids and isopranoids of different chain lengths	Fungi, yeasts, algae, bacteria, and archaea
Lipids	
Long-chain fatty acids (C-12 to C-18)	Bacteria
Mycolic acids (C-60 to C-90)	Mycobacteria, *Rhodococcus*
Isoprenoids (C-20 to C-40)	Archaea

[a]Data from Koga and Mori (2007), Ladygina et al. (2006), Tornabene (1980, 1982), and Wackett (2008) and references therein

are low (Ladygina et al. 2006; Tornabene 1980, 1982). Brown algae contain n-pentadecane, red algae contain n-heptadecane, and green algae contain C-17-cyclopropylalkane (Youngblood and Blumer 1973). *Dunaliella salina* produces 6-methyl-hexadecane and 4-methyl-octadecane (Tornabene 1980). Cyanobacteria contain C-17-alkanes and methylated alkanes. The green microalga, *Botryococcus braunii*, is unusual in that it accumulates hydrocarbons up to 75% of its dry mass and may be a promising source for biofuels in the future (Kalacheva et al. 2002). A cobalt-porphyrin enzyme was purified from microsomes of *B. braunii* that decarbonylated octadecanal to heptadecane, CO, and some CO_2 (Dennis and Kolattukudy 1992). These data indicate that the pathway for alkane synthesize involves the reduction of fatty acids to aldehydes, which are then decarbonylated

to alkanes. A number of bacteria are also known to make long-chain alkanes as well as fatty acids (Table 1). Some members of the genera *Micrococcus* and *Kocuria* produce a range of alkenes with 12 to 29 carbons with subterminal branching. The unsaturated bond is in the middle of the molecule suggesting an interesting biosynthetic reaction possibly involving decarbonylation or decarboxylation and head-to-head condensation of two fatty acids (Tornabene 1980, 1982). Park (2005) found that membrane fractions of *Vibrio furnissii* made pentadecane and hexadecane from hexadecanoic acid and detected labeled hexadecanal and hexadecanol from labeled hexadecanoic acid. Pentadecane formation can be explained by the decarbonylation pathway above, but the formation of hexadecane must involve some as yet undescribed mechanism as no loss of carbon occurred. However, there is some uncertainty about the ability of *V. furnissii* to produce large amounts of alkanes (Wackett 2008).

Another important class of hydrocarbons made by microorganisms is terpenes. *Actinomyces* and *Bacillus* species are major sources of isoprene (2-methyl-1,3-butadiene) (Ladygina et al. 2006). Mutational analysis shows that isoprene synthesis occurs by the methylerythritol phosphate pathway in *Bacillus subtilis* (Julsing et al. 2007). Once the intermediates, isopentenyl-diphosphate and dimethylallyl diphosphate, are formed, terpenes of 10 to 110 carbons can be made by a series of condensation reactions. Again, details of the pathways are sketchy particularly the mechanism(s) by which carboxylic acid intermediates are converted to hydrocarbons. Finally, all archaea synthesize significant amounts of isoprenoid lipids of C-20 to C-40 chain length where considerable variation exists regarding the degree of molecule saturation, cyclization, and methylation (Koga and Morii 2007). Archaeal isoprenoid biosynthesis proceeds by the mevalonic acid pathway or a modified version of this pathway involving isopentenyl-phosphate rather than diphosphomevalonic acid as an intermediate. Details of these interesting biochemical reactions as well as the microbes involved will be discussed in the chapters subsequent to this section of the handbook.

2 Methanogenesis

The conversion of natural polymers such as polysaccharides, proteins, nucleic acids, and lipids to CO_2 and CH_4 is called methanogenesis and involves a number of diverse, interacting microbial species. First, numerous fermentative bacteria hydrolyze the polymers and ferment the hydrolysis products to acetate and longer-chain fatty acids, CO_2, formate, and H_2 (McInerney et al. 2008; Schink 1997). Acetogenic bacteria use methanol (from methyl groups of pectin), methyl groups of methoxylated aromatic compounds, some hydroxylated aromatic compounds, and H_2 and CO_2 to produce acetate (Drake 1994). A second group of microorganisms works cooperatively with methanogenic archaea to syntrophically metabolize the products of fermentative metabolism (e.g., propionate and longer-chain fatty acids, alcohols, and aromatic acids) to the methanogenic substrates, H_2, formate, and acetate. In syntrophic metabolism, the degradation of the parent compound, e.g., the fatty acid, is thermodynamically unfavorable unless the hydrogen, formate, and

Table 2 Reactions involved in syntrophic metabolism

Reaction	$\Delta G^{o,a}$	pH_2 (atm) for $-\Delta G'^{b}$
Ethanol + H_2O ➔ acetate$^-$ + H^+ + 2 H_2	+9.6	$<10^{-1}$
Propionate$^-$ + 3H_2O ➔ acetate$^-$ + HCO_3^- + H^+ + 3 H_2	+76.1	$<10^{-4}$
Butyrate$^-$ + 2 H_2O ➔ 2 acetate$^-$ + H^+ + 2 H_2	+48.3	$<10^{-4}$
Toluene + 9 H_2O ➔ 3 acetate$^-$ + HCO_3^- + 4 H^+ + 6 H_2	+166.1	$<10^{-5}$

[a]Gibbs free energy changes are from Thauer et al. (1977) except for toluene which is from Heider et al. (1999)

[b]The partial pressure of hydrogen needed for the reaction to be thermodynamically favorable ($-\Delta G'$), which was calculated for hydrogen in the gaseous state rather than the liquid state as in Figs. 1 and 2, substrate and acetate concentrations of 0.1 mM, and a bicarbonate concentration of 100 mM

acetate produced by the fatty acid degrader are kept low by the partner methanogens (Table 2). Lastly, two different groups of methanogens, the hydrogenotrophic methanogens and the acetotrophic methanogens, complete the process by converting the acetate, formate, and hydrogen made by other microorganisms to methane and carbon dioxide (Deppenmeier 2002; Schaefer et al. 1999; Hedderich and Whitman 2006; Zinder 1993). The biochemical details of microbial methane production will be discussed in a subsequent chapter of the handbook.

In the gastrointestinal tract of animals, mainly fermentative bacteria and hydrogenotrophic methanogens are active (Mackie and White 1997; Hedderich and Whitman 2006). Acetotrophic methanogens and organisms capable of syntrophic metabolism grow too slowly to be effectively maintained in significant numbers in these ecosystems. Thus, organic matter is degraded to acetate and longer-chain fatty acids (mainly propionate and butyrate), which accumulate and are absorbed and used by the host animal as energy sources. The amount of energy released per unit of biomass degraded during methanogenesis is very low as most of the energy is retained in methane. For this reason, methanogenesis is the treatment of choice for wastes. Aiyuk et al. (2006) recently reviewed new methanogenic treatment technologies.

The methanogenic degradation of hydrocarbons is an important process that affects the recovery and economic value of crude oil. Biodegradation of crude oil decreases the saturated hydrocarbon content and increases the oil density, acidity, viscosity, and sulfur and metal content, making refining more costly and recovery more difficult (Head et al. 2003). It was long thought that biological alterations to crude oil were the result of aerobic metabolism. However, we now know that many anaerobic microorganisms can degrade hydrocarbons (Heider et al. 1999; Widdel et al. 2006; Zedelius et al. 2011; Laso-Perez et al. 2016) and several studies implicate methanogenic degradation as the mechanism for crude oil bioalterations in many oil reservoirs (Aitken et al. 2004; Head et al. 2003; Jones et al. 2008; Wilhelms et al. 2001). On the positive side, it may be possible to use in situ methanogenic hydrocarbon biodegradation activity for economic gain. In many reservoirs, only about one-third of the crude oil can be recovered by current technologies; the rest remains entrapped in the formation. The conversion of entrapped crude oil to methane would

be a mechanism to recover the energy content of the oil. In situ methanogenic crude oil degradation is a slow process, but a recent study showed that rapid methane production from a variety of petroliferous rocks is possible with an oil-degrading enrichment as the inoculum (Gieg et al. 2008).

3 Thermodynamics of Microbial Growth

A thermodynamic approach has been developed to estimate biomass yields based on the Gibbs free energy that must be dissipated to produce 1 C-mol of biomass from a given carbon source (D_s^0/r_x; kJ • C-mol^{-1} of biomass) (Eq. 1) (Heijnen 1999):

$$Y_{sx} = \left[\gamma_D\left(\Delta G_{eD}{}^0 - \Delta G_{eA}{}^0\right)/\left(D_s{}^0/r_x\right) + \gamma_x\left(\Delta G_{eD}{}^0 - \Delta G_{eA}{}^0\right)\right] \quad (1)$$

where Y_{sx} is the yield of biomass of a substrate or electron donor (C-mol biomass • C-mol^{-1} of the donor); γ is the degree of reduction of the chemical compound (donor (D), acceptor (A), or biomass (X)); $\Delta G_{eD}{}^0$ and $\Delta G_{eA}{}^0$ are the Gibbs energies of formation per electron donor (kJ mol^{-1} of electron) and acceptor (kJ mol electron of donor^{-1}), respectively; $D_s{}^0$ is the Gibbs energy dissipation (kJ m^{-3} h^{-1}); and r_X is the net growth rate (mol m^{-3} h^{-1}) (Heijnen 1994, 1999). Rather than using Gibbs free energy of formation values to calculate the thermodynamics, a reference system is used where each chemical compound is characterized by its electron content, which is equal to its degree of reduction, γ, (electrons • C-mol^{-1} of the compound), and the Gibbs free energy of formation per electron ($\Delta G_e{}^o$; kJ mol^{-1} of electron) (see Table 3 in Heijnen (1999) for a listing of these values). The reference system simplifies the thermodynamic calculations in that only the γ and $\Delta G_e{}^o$ of the electron donor (γ_D and $\Delta G_{eD}{}^0$) and of the electron acceptor (γ_A and $\Delta G_{eA}{}^0$) are needed. The numerator on the right side of Eq. 1 is the Gibbs free energy change of the reaction (e.g., $\Delta G_r = \gamma_D\,(\Delta G_{eD}{}^0 - \Delta G_{eA}{}^0)$). Two correlations (Eqs. 2 and 3 in Heijnen (1999)) are used to calculate $D_s{}^0/r_x$ if the growth rate is known. The approach is able to predict biomass yields (Y_{sx}, C-mol biomass • C-mol^{-1} of substrate) from the stoichiometry and the thermodynamics of the catabolic reaction so long as a growth rate is known. The approach has an accuracy of about 10–20% over a range of 0.01–0.8 mol biomass • C-mol^{-1} of substrate for many carbon substrates, but the authors stress that the Y_{sx} values should only be considered a preliminary estimate because there is often more than one biochemical pathway to degrade a compound.

$D_s{}^{01}/r_x$ can be considered to be a measure of the amount of biochemical work needed to convert the carbon source into biomass (Heijnen and vanDijken 1992). Values for $D_s{}^{01}/r_x$ range from 150 to 3500 kJ (mol biomass C)$^{-1}$. Chemolithotrophic bacteria that use CO_2 as a carbon source and use reverse electron transport such as nitrifiers and thiobacilli have high $D_s{}^{01}/r_x$ values. Interestingly, *Syntrophobacter fumaroxidans*, which needs reverse electron transport for hydrogen production during syntrophic propionate metabolism, also has a very large $D_s{}^{01}/r_x$ value, about 3,500 kJ (mol biomass C)$^{-1}$ (Scholten and Conrad, 2000).

Table 3 Free energy changes for toluene degradation with different electron acceptors[a]

Electron acceptor	Reaction	$\Delta G^{o'}$ (kJe-mol^{-1})
Oxygen	$C_7H_8 + 9\ O_2 + 3\ H_2O \rightarrow\ 7\ HCO_3^- + 7\ H^+$	−105.3
Nitrate	$C_7H_8 + 7.2\ NO_3^- + 0.2\ H^+ \rightarrow 7\ HCO_3^- + 3.6\ N_2 + 0.6\ H_2O$	−98.7
Iron	$C_7H_8 + 94\ Fe(OH)_3 + 3\ H_2O \rightarrow\ 7\ FeCO_3 + 29\ Fe_3O_4 + 145\ H_2O$	−94.4
Sulfate	$C_7H_8 + 4.5\ SO_4^- + 3\ H_2O \rightarrow 7\ HCO_3^- + 4.5\ HS^- + 2.5\ H^+$	−5.7
CO_2	$C_7H_8 + 7.5\ H_2O \rightarrow 4.5\ CH_4 + 2.5\ HCO_3^- + 2.5\ H^+$	−3.6

[a]Stoichiometries and free energy changes from Heider et al. (1999)

The thermodynamic approach predicts that catabolic reactions with more favorable Gibbs free energy changes should result in higher growth yields of the organism, and this is generally true. For example, toluene oxidation coupled to aerobic, nitrate, and iron respirations (Table 3) releases large amounts of Gibbs free energy per electron compared to toluene oxidation coupled to sulfate reduction or methanogenesis (Table 3). Aerobes, denitrifiers, and iron reducers that use toluene have higher yields than sulfate reducers or methanogenic consortia (Zwolinski et al. 2000). An interesting question is why, for methane formation, more than one organism is needed to degrade the parent substrate (toluene in this case) to CO_2 and CH_4, but a single species is able to do so with other electron acceptors. McInerney and Beaty (1988) noted that the Gibbs free energy released per electron for glucose degradation to CO_2 and CH_4 or to CO_2 only with sulfate as electron acceptor was much lower than that for mineralization of glucose with other electron acceptors or by various glucose fermentations. McCarty (1971) proposed that the free energy released per electron is a major factor determining whether an organism will be maintained in anaerobic digestors. A low-energy yield per electron should result in low biomass yields according to Eq. 1. Organisms with low cell yields will have difficulties to maintain a sufficient population size if substrate concentrations are low or if there is competition for the substrate. While thermodynamic analysis seemingly predicts the appropriate scenario for methane formation, e.g., an association is needed for toluene degradation (Ficker et al. 1999; Meckenstock 1999), it is not useful in predicting the scenario for sulfate reduction. Several sulfate reducers completely mineralize toluene in pure culture (Widdel et al. 2006), and interspecies hydrogen transfer was not needed for toluene degradation by aquifer microorganisms with sulfate as electron acceptor (Elshahed and McInerney 2001). Most likely, considering only the Gibbs free energy released per electron only and without kinetic analyses is too simplistic. Ecological theory predicts that the rate of ATP formation is important. When resources (e. g., substrate) are limiting, organisms that produce ATP at high rates but low yields are favored over those that produce ATP at high yields but at low rates (Pfeiffer et al. 2001). Similarly, kinetic theory for optimal pathway design implies that the optimal pathway length is one that maximizes the rate of ATP production (Costa et al. 2006). As a consequence, we may conclude that the overall ATP yield of biomass conversion to methane and CO_2 may be too small

to sustain a long reaction chain from sugar degradation through glycolysis and the entire pathway of methane formation via acetate, hydrogen, and/or formate and that therefore a separation of the overall process into two to three subsections may appear feasible.

4 Impact of Environmental Conditions on the Thermodynamics of Methanogenesis

The Gibbs free energy yields associated with methanogenesis depend significantly on environmental conditions and are typically substantially lower in natural systems than in growth-optimized cultures or than is suggested by standard free energy changes. Among factors that influence free energy yields are temperature, activities (concentrations) of substrates and products, pH, and pressure when considering their variation in naturally occurring biological systems. The first two have the largest potential effect on energy yield and are considered in some detail below; pH and pressure which have a more modest effect are addressed only briefly.

4.1 Substrate and Product Concentrations

The free energy change of any chemical reaction is affected by variations in the activities of products and reactants, as follows (Eq. 2):

$$\Delta G_{\mathrm{r}} = \Delta G^{\circ}(T) + RT \cdot \ln\left(\frac{\prod P^{y}}{\prod R^{z}}\right) \tag{2}$$

where ΔG_{r} is the free energy available under in situ conditions; ΔG° is the temperature-adjusted free energy change under standard conditions; T is temperature in Kelvin; R is the universal gas constant; and ΠP^{y} and ΠR^{x} are the mathematical products of the activities of reaction products and reactants, respectively, with each raised to its stoichiometric power (e.g., a reactant having a stoichiometric coefficient of 3 would have its activity raised to the third power in the calculation of ΔG). For reactions in which environmental activities of substrates and products differ markedly from standard state (defined as 1 M for all species) – *especially* for species exhibiting high reaction stoichiometry – in situ free energy yields may thus differ dramatically from standard reaction free energy changes.

Typical environmental substrate and product concentrations result in free energy yields for hydrogenotrophic methanogenesis that are smaller – in some cases by more than one order of magnitude (Hoehler, 2004) – than the standard free energy change (-131 kJ·(mol CH_4)$^{-1}$ for the reaction written as CO_2 (g) + 4 H_2 (g) → CH_4 (g) + 2 H_2O (liq). While the activities of each of the substrates and products in this reaction under environmental conditions are subject to significant natural variation, hydrogen exerts the greatest influence on the energetics of methanogenesis in most systems. This is due to both its high stoichiometric

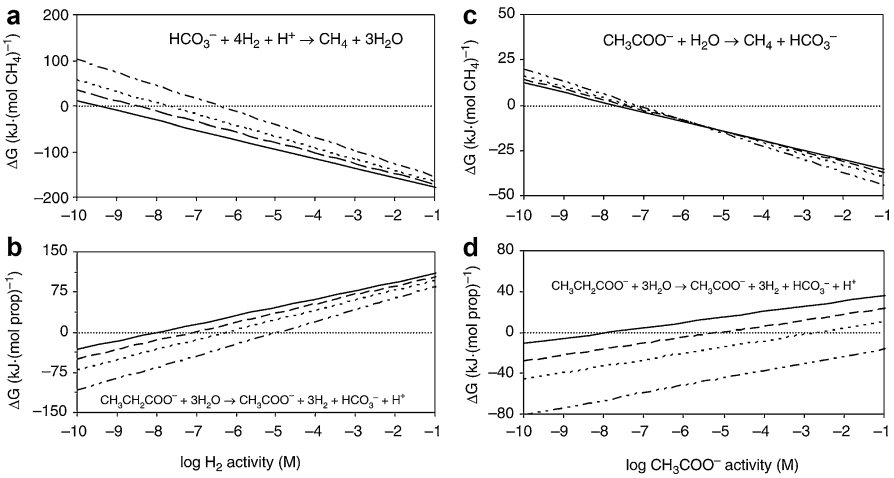

Fig. 1 The effect of activity and temperature on the Gibbs free energy change for (**a**) hydrogenotrophic methanogenesis versus aqueous H_2 activity; (**b**) syntrophic propionate metabolism versus aqueous H_2 activity; (**c**) acetoclastic methanogenesis versus aqueous acetate activity; and (**d**) syntrophic propionate metabolism versus aqueous acetate activity. Values are plotted for four temperatures: 0 °C (solid line), 25 °C (long dashes), 50 °C (short dashes), and 100 °C (alternating short/long dashes). Reaction free energy changes were calculated using Eq. 2. Standard free energies of reaction were calculated using thermodynamic data from Shock and Helgeson (1988, 1990), with temperature dependence calculated via the Gibbs-Helmholtz equation (for the latter calculation, the small temperature dependence of ΔH over the range considered was neglected). Calculations are for the reactions shown, with all species considered in the aqueous form. The standard free energy change for hydrogenotrophic methanogenesis (written as HCO_3^- (aq) + 4 H_2 (aq) + H^+ (aq) → CH_4 (aq) + 3H_2O (liq)) is −229 kJ·(mol CH_4)$^{-1}$. Calculations assume the following concentrations (M), unless otherwise indicated by a variable axis: propionate and acetate = 10^{-5}; HCO_3^- = $2{\cdot}10^{-2}$; CH_4 = 10^{-3}; H^+ = 10^{-7}; H_2 = 10^{-7}

coefficient (and a corresponding fourfold greater effect than any other species in the reaction) and its typically short half-life (and corresponding potential for rapid change) in many ecosystems (Hoehler et al. 2002). Across a realistic range of hydrogen activities for anaerobic systems in nature, the free energy change of H_2-based methanogenesis varies by more than 200 kJ·(mol CH_4)$^{-1}$ (Fig. 1a). In contrast, changes in acetate concentrations yield a much smaller variation in the free energy yield of acetoclastic methanogenesis ($CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$), by virtue of a unit stoichiometric coefficient (Fig. 1c).

Hydrogen can be delivered to methanogenic communities by either abiotic reactions (e.g., water-rock reactions in hydrothermal systems) or by biological production (e.g., by fermentation of organic matter). The former case may yield H_2 activities up to tens to hundreds of millimolar, depending on the fluid source. H_2 production by anaerobic fermentation reactions, however, typically encounters thermodynamic inhibition at much lower H_2 activities. As with methanogenesis, the high stoichiometric coefficient of H_2 in most fermentation reactions (e.g., fermentation of propionate: $CH_3CH_3COO^- + 3H_2O \rightarrow CH_3COO^- + HCO_3^- + H^+ + 3H_2$)

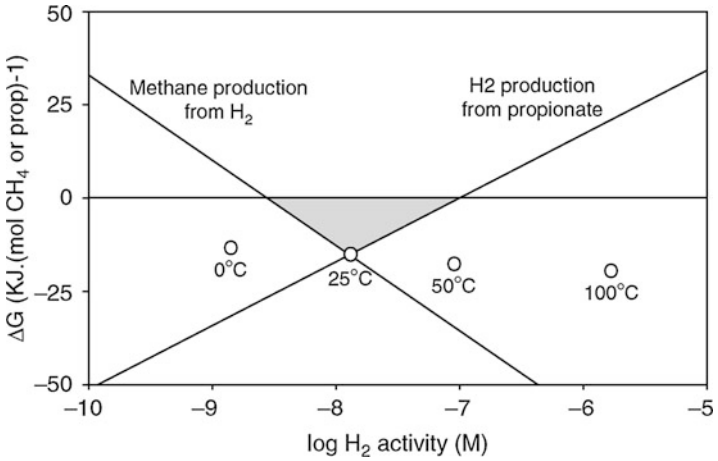

Fig. 2 The zone where the production and consumption of H_2 is both thermodynamically favorable. The shaded region represents the range of H_2 activities at 25 °C in which hydrogenotrophic methanogenesis and syntrophic propionate fermentation are thermodynamically favorable (note that this range may be smaller when considering biological minimum free energy thresholds). Syntrophic conversion of propionate to methane is not possible outside of this range. The open symbol labeled "25 °C" represents the H_2 activity at which the free energy yields for the methanogenic and syntrophic reactions are equalized, along with the energy yield associated with each reaction. Equivalent values are also plotted as open symbols for 0, 50, and 100 °C. Note that the midpoint H_2 activity increases by more than three orders of magnitude over this range in temperature, while the free energy yield available to each reaction increases slightly

renders their energetics highly sensitive to variations in H_2 activity (Fig. 1b). For this reason, H_2 concentrations in systems driven by anaerobic decomposition of organic matter are constrained to a range in which both production and consumption of H_2 are thermodynamically and biologically favorable (Fig. 2). The permissive range depends on the specific nature of the fermentation reactions, as well as substrate and product activities and temperature (see below), but this phenomenon generally results in methanogenic energy yields much lower than are typical of growth-optimized cultures. A comparable effect can also be inferred for acetate production/consumption (Fig. 1c and d). Notably, however, the free energy changes of both fermentative production and methanogenic consumption of acetate are fourfold less sensitive to acetate concentrations than in the corresponding case of H_2. Free energy yields for acetoclastic methanogenesis are therefore constrained within a narrower range across the spectrum of environmentally realistic acetate concentrations, and differences between natural systems and cultures are less exaggerated than for H_2-based methanogenesis. An elegant alternative to syntrophic propionate oxidation is its conversion to acetate plus H_2 by *Smithella propionica* which is energetically more favorable and channels the electrons to a major part into the acetate pool (Dolfing 2013). The matter has been reviewed recently in detail (Leng et al. 2018).

4.2 Temperature

Methanogens are represented across much of the biologically tolerated range of temperature, and variation over this range can significantly impact the free energy yield of methanogenesis. Temperature has two main effects on reaction energetics, as indicated by Eq. 2. First, the entropic contribution to free energy increases linearly with temperature, $\Delta G = \Delta H - T\Delta S$, so that the standard free energy change, ΔG°, is temperature-sensitive. For reactions having large entropy changes, this effect can be quite pronounced across the biologically tolerated range of temperatures, especially when considering processes that occur with small in situ free energy yields. This is the case for H_2-consuming methanogenesis and is generally true for most fermentation reactions that involve high stoichiometries of H_2 production. Second, the impact of substrate and product activities that deviate from standard (unit activity) conditions becomes more pronounced with increasing temperature. This effect is most important for reactions in which one or more species have in situ activities that lie far from unit activity, particularly if those species have high stoichiometric coefficients, e.g., syntrophic acetate metabolism where $4H_2$ are made per acetate. Again, this is generally the case for naturally occurring H_2-consuming methanogenesis and fermentation reactions that produce H_2 with high stoichiometry, while the effect is less pronounced for acetoclastic methanogenesis. The effects of temperature on free energy yields of methanogenesis and propionate fermentation are shown in Fig. 1. Noting that this magnitude is (by virtue of the second temperature effect) specific to the choice of substrate and product activities, it is nonetheless clear that for environmentally plausible conditions, temperature shifts across the biologically tolerated range can cause free energy yields to increase or decrease by several times the typical in situ yield. Such changes (particularly in the positive direction) may significantly shift the range of substrate or product concentrations over which a given reaction is favorable. As shown in Fig. 2, for example, the range of H_2 activities over which both methanogenesis and propionate fermentation are favorable (and the midpoint H_2 activity, at which the energy yields of the two reactions are equal) shifts by more than three orders of magnitude in going from 0 °C to 100 °C.

4.3 pH

The effects of environmental pH on free energy yield, though potentially substantial if calculated over the full range of pH in natural aquatic systems, are less straightforward to interpret. These effects can be direct, for reactions that involve "H^+" or "OH^-" as substrates or products, or indirect, by affecting the speciation of pH-sensitive products or reactants (e.g., carbonates, organic acids, etc.). However, given extremes of pH, organisms can employ a variety of mechanisms to regulate the pH of the intracellular medium (where biochemical reaction energetics must be calculated) at more clement levels than the surrounding environment (Krulwich 1995, 2000). The specific mechanism employed, and extent to which pH is

regulated, may thus variably mitigate and modify impacts on free energy change that are predicted based on environmental pH values.

4.4 Pressure

Pressure exhibits a natural range of three to four orders of magnitude across biological systems, from atmospheric (approx. 1 bar) to >1000 bar in ocean trench or deep subsurface ecosystems. However, the direct effect of this range on the thermodynamics of aqueous reactions (including the metabolic reactions of methanogens) is modest. For example, holding all other factors constant, a pressure change from 1 to 1000 bar changes the free energy change associated with hydrogenotrophic methanogenesis changes by only about -3 kJ·(mol CH_4)$^{-1}$. The secondary, and potentially more important, effect of pressure in biological systems is on the solubility of gaseous substrates or end products where H_2 and CH_4 can (and do) reach dissolved concentrations hundreds of times higher at the ocean floor than in surface ecosystems (Boetius et al. 2000). Because it is the dissolved concentration of substrate or product that affects the free energy changes of aqueous biochemical reactions, this effect can significantly alter the energetics of methanogenesis. The impact of changing substrate and product concentrations is considered below.

In summary, the impacts of temperature, pH, pressure, and nonstandard activities of substrates and products, collectively, can shift in situ free energy yields dramatically away from values suggested by standard free energies of reaction or from those typically experienced by organisms grown in culture. This effect is particularly pronounced for processes that produce or consume H_2 with high stoichiometry, such as hydrogenotrophic methanogenesis and many fermentation reactions.

5 Thresholds and Minimum Free Energy Change

As discussed above (Eq. 2), there is a strong effect of in situ concentration on the Gibbs free energy of the catabolic reaction for some anaerobic processes. The activity of methanogens is essential to maintain hydrogen and formate levels low enough for syntrophic metabolism to be energetically favorable (Table 2; Figs. 1 and 2) (Schink 1997; Montag and Schink 2015; Schink et al. 2017). Consistent with thermodynamic predictions, thresholds for substrate metabolism, defined as the concentration below which further substrate decay is not observed, have been observed for syntrophic metabolism and methanogenesis (Cord-Ruwisch et al. 1988; Dwyer et al. 1988; Hoehler 2004; Jackson and McInerney 2002; Lovley 1985; Schöcke and Schink 1997; Seitz et al. 1990; Warikoo et al. 1996).

The free energy changes for syntrophic metabolism and methanogenesis approach a minimum free energy value when substrate thresholds are reached (Hoehler 2004; Schink 1997). This minimum free energy, also referred to as the biological energy quantum (BEQ) , predicts the favorability of continuous biological activity. BEQ values of 12–15 kJ per mol of transformation reaction have been

estimated based on the free energy change needed for ATP synthesis under physiological conditions (about −60 to −70 kJ mol^{-1}) and the number of protons needed to make an ATP molecule by ATP synthase (three to five protons per ATP) (Hoehler 2004; Schink 1997; Schink and Stams 2002). Experimental evidence shows that several syntrophic metabolisms operate at free energy changes in the range of 15–20 kJ mol^{-1}, close to the theoretically predicted BEQ (Schink 1997; Scholten and Conrad, 2000). Recent studies have shown that the homoacetogenic bacterium *Acetobacterium woodii* can produce ATP at lower energy expenditure (Spahn et al. 2015) than the values measured before with, e. g., well energy-supplied *Escherichia coli* cells (Tran and Unden 1998). These lower phosphorylation potentials and ATPase stoichiometries of up to five protons per ATP may decrease the minimum energy requirement for maintenance of life to a range similar to those values calculated from substrate concentrations that were measured in marine sediments (−10 kJ per mol reaction; Hoehler et al. 2001; Lever et al. 2015). One may speculate that the recently developed concept of electron bifurcation (Li et al. 2008; Buckel and Thauer 2013) may allow even smaller energy spans to be translated into membrane potentials that can be coupled to ATP synthesis, especially for microbes surviving in extremely energy-deprived deep sediments.

The small amounts of free energy released during syntrophic metabolism must be shared among the partners (Schink 1997). Thus, it is appropriate to describe syntrophy as an extreme existence, a lifestyle that involves a marginal or near-equilibrium energy economy, where the direction of metabolism depends on the prevailing environmental conditions. The existence of a near-equilibrium energy economy is illustrated quite impressively by the metabolism of *Thermacetogenium phaeum* (Hattori et al. 2005). This bacterium is a fascinating microorganism that – in a similar manner as the formerly nicknamed “Reversibacterium” (Zinder and Koch 1984) – syntrophically oxidizes acetate to CO_2 and H_2 in coculture with a methanogen that keeps the hydrogen partial pressure low. It can also make acetate from CO_2 and H_2 in pure culture if the hydrogen partial pressure is high. The enzymes of the homoacetogenic Wood-Ljungdahl pathway are present both during acetate utilization and acetate formation (Hattori et al. 2005). This bacterium exemplifies how close to thermodynamic equilibrium an anaerobic metabolism can operate, depending on the concentrations of substrates and products.

6 Research Needs

The global importance of microbial methane production is well documented; however, we do not fully understand the importance or function of the microbial production of hydrocarbons other than methane. Hydrocarbons are produced by diverse microorganisms. In many cases, the amount of hydrocarbons produced is low, and the biochemical mechanisms for their formation are poorly understood. In contrast, a diverse range of fatty acids and isoprenoid lipids are synthesized for incorporation into bacterial and archaeal cell membranes. However, many details of their pathways and/or metabolic control are poorly understood. Clearly, much work

is needed if we are to exploit non-methane microbial hydrocarbon metabolism for biofuel production. Microbial hydrocarbon metabolism may provide a means for the continued use of hydrocarbons in a carbon-neutral fashion.

Methanogenesis and in particular syntrophic metabolism operate at very small free energy changes, suggesting that a minimum free energy change is needed to sustain biological activity. However, the biochemical mechanisms of energy conservation and its regulation in bacteria capable of syntrophic metabolism are poorly understood. The stoichiometry of ions translocated per mole substrate consumed by the syntrophic metabolizer and the stoichiometry of ions consumed in support of ATP synthesis are critical issues that remain unresolved.

Bacteria and archaea reside at the interface between the inhabited and uninhabited realms of our planet. They represent the ultimate biological arbiters of chemical exchange between those spheres. In some environments such as the deep subsurface, the energy flux and growth rates are orders of magnitude below anything we have observed in the laboratory. How is it possible to maintain complex microbial communities and critical cell functions at energy economies that barely allow cell growth? Do these organisms have properties beyond our current understanding of microbial biochemistry, or are energy sources available there that we have not yet identified?

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Diversity and Taxonomy of Methanogens

2

Zhe Lyu and Yuchen Liu

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Abstract

Methanogens are strictly anaerobic, methane-producing archaea. All characterized members belong to the phylum *Euryarchaeota*, but methanogenesis pathway is also predicted to be present in the newly proposed phyla *Bathyarchaeota* and *Verstraetearchaeota*. This indicates that the diversity of methanogens may be larger than previously excepted. Although methanogens share a set of physiological characteristics, they are phylogenetically very diverse. The current taxonomy classifies methanogens into seven well established orders: *Methanobacteriales*, *Methanococcales*, *Methanomicrobiales*, *Methanosarcinales*, *Methanopyrales*, *Methanocellales*, and *Methanomassiliicoccales*. This taxonomy is supported by 16S rRNA gene sequences as well as a number of physiological properties, e.g. substrates for methanogenesis, nutritional requirements, morphologies, and structures of cell envelopes. Methanogens are abundant in a wide variety of anaerobic environments where they catalyze the terminal step in the anaerobic food chain by converting methanogenic substrates to methane. The complexity of methanogenesis pathways suggests an ancient monophyletic origin of methanogens, a hypothesis that is supported by phylogenetic analyses based upon DNA sequences.

1 Introduction

Methanogens are microorganisms that produce methane as the end-product of their anaerobic respiration. All methanogens share three common features. (i) They are obligate methane producers, obtaining all or most of their energy for growth from producing large quantities of methane. (ii) They are archaea, belonging to the phylum *Euryarchaeota* and possibly other archaeal phyla too. (iii) They are obligate anaerobes, limiting their growth to anaerobic environments.

Then known methanogens can only utilize a restricted number of substrates for methane production or methanogenesis. The substrates are limited to three major types: $CO_2 + H_2$ or a few other electron donors such as formate, methyl-group containing compounds, and acetate. Methanogens using these three types of substrates are classified as hydrogenotrophs, methylotrophs, and acetotrophs, respectively. Most organic substances, for instance, carbohydrates, proteins, and long-chain fatty acids and alcohols, are not substrates for methanogenesis. Exceptions are that some hydrogenotrophs can also use secondary alcohols, such as 2-propanol, 2-butanol, and cyclopentanol, as electron donors. A small number can use ethanol (Widdel 1986; Widdel et al. 1988; Bleicher et al. 1989; Frimmer and Widdel 1989). Athough these organic compounds can obviously be assimilated, they are only incompletely oxidized to ketones (secondary alcohols) and acetate (ethanol), and methane is derived from CO_2 reduction.

Methanogenesis is a complex process that requires a number of unique enzyme complexes and unusual coenzymes (reviewed in Hedderich and Whitman (2006)). Although the methanogenesis pathways of the three nutritional groups start differently, the final steps leading to methane are common in virtually all methanogens. The

bioenergetics of methanogenesis employs both proton and sodium gradients generated by primary pumps for ATP synthesis. Due to the complexity of methanogenesis, all modern methanogens perhaps originate from a common ancient ancestor.

2 Taxonomy and Phylogeny of Methanogens

Although methanogens are united by a few common features, they are phylogenetically diverse. The taxonomy of methanogens that has been developed in the last three decades has aimed to reflect the phylogenetic diversity of methanogens and be consistent with the taxonomy of other prokaryotes (Balch et al. 1979; Boone et al. 1993b; Whitman et al. 2001b). An overview of the current taxonomy of methanogens is given in Table 1. Organisms from different orders have less than 82% 16S rRNA sequence similarity. Organisms with less than 88–93% and less than 93–95% 16S rRNA sequence similarity are separated into different families and genera, respectively. Organisms are distinguished as separate species if their DNA reassociation is less than 70%, the change in the melting temperature of their hybrid DNA is greater than 5 °C, and substantial phenotypic differences exist (Wayne et al. 1987; Stackebrandt et al. 2002). When 16S rRNA data are available, organisms with a similarity of less than 98% are considered as separate species. However, sequence similarity of greater than 98% is not considered as a sufficient evidence that two organisms belong to the same species.

All modern methanogens share the same set of homologous enzymes and cofactors required for methanogenesis, suggesting an ancient monophyletic origin of methanogens. In the phylogenetic tree based on 16S rRNA gene sequences, methanogens are separated into seven orders (Fig. 1). Non-methanogenic lineages such as *Archaeoglobales* and *Thermoplasmatales*, are interspersed in the tree. Phylogenomic studies using more gene markers including ribosomal proteins and/or methanogenesis proteins further classified methanogens collectively into three classes (Bapteste et al. 2005; Anderson et al. 2009). The Class I methanogens include *Methanobacteriales*, *Methanococcales*, and *Methanopyrales*, the Class II methanogens include *Methanomicrobiales*, and the Class III methanogens include *Methanosarcinales*. However, when *Methanocellales* was included in phylogenomic analyses, the boundaries between the Classes II and III could not be fully resolved, suggesting that they could also belong to a single class (Lyu and Lu 2017). Although the seventh order *Methanomassiliicoccales* is distantly related to all three methanogen classes, its close affiliation to the Class Thermoplasmata could not warrant an immediate establishment of a fourth methanogen class.

Four hypotheses are proposed to explain the branching of methanogens. (1) Methanogens and these non-methanogen lineages shared a common ancestor, and genes required for methanogenesis were lost in these non-methanogens. This hypothesis is supported by the presence of a few genes encoding methanogenesis enzymes in the genome of *Archaeoglobus fulgidus* but is challenged by aerobic growth in both the *Halobacteriales* and *Thermoplasmatales*. This hypothesis also suggests that the common ancestor of *Euryarchaeota* was a methanogen (Gribaldo and Brochier-Armanet 2006). However, this view is now challenged by the possible

presence of methanogens outside *Euryarchaeota* as shown by metagenomic surveys (Evans et al. 2015; Vanwonterghem et al. 2016). (2) Methanogenesis in various branches was acquired by horizontal gene transfer (HGT). However, the core genes required for methanogenesis are not linked on the genomes of methanogens, thus the

Table 1 Taxonomy of methanogens (Modified from Liu (2010e))

Order	Family	Genus	Species[b]
Methanobacteriales	*Methanobacteriaceae*	*Methanobacterium*	*M. aarhusense*, *M. alcaliphilum*, *M. beijingense*, *M. bryantii*, *M. congolense*, *M. espanolae*, ***M. formicicum***, *M. ivanovii*, *M. oryzae*, *M. palustre*, *M. subterraneum*, *M. uliginosum*, *M. aggregans*, *M. arcticum*, *M. ferruginis*, *M. flexile*, *M. kanagiense*, *M. lacus*, *M. movens*, *M. movilense*, *M. paludis*, *M. petrolearium*, *M. veterum*
		Methanobrevibacter	*M. acididurans*, *M. arboriphilus*, *M. curvatus*, *M. cuticularis*, *M. filiformis*, *M. gottschalkii*, *M. millerae*, *M. olleyae*, *M. oralis*, ***M. ruminantium***, *M. smithii*, *M. thaueri*, *M. woesei*, *M. wolinii*, *M. boviskoreani*
		Methanosphaera	*M. cuniculi*, ***M. stadtmanae***
		Methanothermobacter	*M. defluvii*, *M. marburgensis*, ***M. thermoautotrophicus***, *M. thermoflexus*, *M. thermophilus*, *M. wolfeii*, *M. crinale*, *M. tenebrarum*
	Methanothermaceae	*Methanothermus*	***M. fervidus***, *M. sociabilis*
Methanococcales	*Methanococcaceae*	*Methanococcus*	*M. aeolicus*, *M. maripaludis*, ***M. vannielii***, *M. voltae*
		Methanothermococcus	*M. okinawensis*, ***M. thermolithotrophicus***
	Methanocaldococcaceae	*Methanocaldococcus*	*M. fervens*, *M. indicus*, *M. infernus*, ***M. jannaschii***, *M. vulcanius*, *M. villosus*, *M. bathoardescens*
		Methanotorris	*M. formicicus*, ***M. igneus***

(*continued*)

Table 1 (continued)

Order	Family	Genus	Species[b]
Methanomicrobiales	*Methanomicrobiaceae*	*Methanoculleus*	***M. bourgensis***, *M. chikugoensis*, *M. marisnigri*, *M. palmolei*, *M. submarinus*, *M. thermophiles*, *M. horonobensis*, *M. hydrogenitrophicus*, *M. receptaculi*, *M. sediminis*, *M. taiwanensis*
		Methanofollis	*M. aquaemaris*, *M. formosanus*, *M. liminatans*, ***M. tationis***, *M. ethanolicus*
		Methanogenium	***M. cariaci***, *M. frigidum*, *M. marinum*, *M. organophilum*
		Methanolacinia	***M. paynteri***, *M. petrolearius*
		Methanomicrobium	***M. mobile***
		Methanoplanus	*M. endosymbiosus*, ***M. limicola***
	Methanospirillaceae	*Methanospirillum*	***M. hungatei***, *M. lacunae*, *M. psychrodurum*, *M. stamsii*
	Methanocorpusculaceae	*Methanocorpusculum*	*M. bavaricum*, *M. labreanum*, ***M. parvum***, *M. sinense*
	Methanoregulaceae	*Methanolinea*	*M. tarda*, *M. mesophila*
		Methanoregula	*M. boonei*
		Methanosphaerula	*M. palustris*
	Unassigned	*Methanocalculus*[a]	*M. chunghsingensis*, ***M. halotolerans***, *M. pumilus*, *M. taiwanensis*, *M. natronophilus*, *M. alkaliphilus*
Methanosarcinales	*Methanosarcinaceae*	*Methanosarcina*	*M. acetivorans*, *M. baltica*, ***M. barkeri***, *M. lacustris*, *M. mazei*, *M. semesiae*, *M. siciliae*, *M. thermophila*, *M. vacuolata*, *M. horonobensis*, *M. soligelidi*, *M. splelaei*, *M. subterranea*
		Methanococcoides	*M. alaskense*, *M. burtonii*, ***M. methylutens***, *M. vulcani*
		Methanohalobium	***M. evestigatum***
		Methanohalophilus	*M. halophilus*, ***M. mahii***, *M. portucalensis*, *M. levihalophilus*

(*continued*)

Table 1 (continued)

Order	Family	Genus	Species[b]
		Methanolobus	*M. bombayensis*, *M. oregonensis*, *M. taylorii*, ***M. tindarius***, *M. vulcani*, *M. chelungpuianus*, *M. profundi*, *M. zinderi*
		Methanomethylovorans	***M. hollandica***, *M. thermophile*, *M. uponensis*
		Methanimicrococcus[a]	***M. blatticola***
		Methanosalsum	***M. zhilinae***, *M. natronophilum*
	Methanosaetaceae	*Methanosaeta*	***M. concilii***, *M. harundinacea*, *M. thermophila*
	Methermicoccaceae	*Methermicoccus*	***M. shengliensis***
Methanopyrales	*Methanopyraceae*	*Methanopyrus*	***M. kandleri***
Methanocellales	*Methanocellaceae*	*Methanocella*	***M. paludicola***, *M. avoryzae*, *M. conradii*
Methanomassiliicoccales	*Methanomassiliicoccaceae*	*Methanomassiliicoccus*	***M. luminyensis***

[a]Placement in higher taxon is tentative
[b]Type species of the genera are in bold

simultaneous acquisition via lateral transfer is unlikely, and the transfer of single genes would not confer a selective advantage (Gribaldo and Brochier-Armanet 2006). (3) The phylogeny based on 16S rRNA gene is misleading, and methanogens and *Archaeoglobus* shared a common ancestor exclusive of all other archaea. This hypothesis is supported by phylogenomics analyses showing that 10 proteins are exclusively shared in methanogens and *A. fulgidus* (Gao and Gupta 2007), while no proteins are exclusively shared in methanogens and any of the *Halobacteriales* or *Thermoplasmatales* (Gao and Gupta 2007). Therefore, methanogens and *Archaeoglobus* appear to have a closer relationship within the *Euryarchaeota*. However, the presence of methanogens in the *Thermoplasmata* suggests otherwise. (4) The last archaeal common ancestor was a methanogen, and the methanogenesis pathway was inherited, modified or lost in various lineages throughout evolution. This view is supported by (i) recent metagenomics surveys that indicate possible presence of methanogens in at least two other archaeal phyla besides the *Euryarchaeota* (Evans et al. 2015; Vanwonterghem et al. 2016), and (ii) the root of the archaeal tree based on phylogenomic analyses was placed between *Euryarchaeota* and the rest of archaeal phyla (Petitjean et al. 2015).

Methanogens are currently classified into seven orders: *Methanobacteriales*, *Methanococcales*, *Methanomicrobiales*, *Methanosarcinales*, *Methanomassiliicoccales*, *Methanocellales* and *Methanopyrales* (Whitman et al. 2001b, 2006; Sakai et al. 2008; Iino et al. 2013). This taxonomy is supported by comparative 16S rRNA gene sequence and phylogenomic analyses as well as distinctive phenotypic properties, such as different cell envelope structures, lipid compositions, and substrate ranges. Some representative characteristics are listed in Table 2 and further described in following subsections.

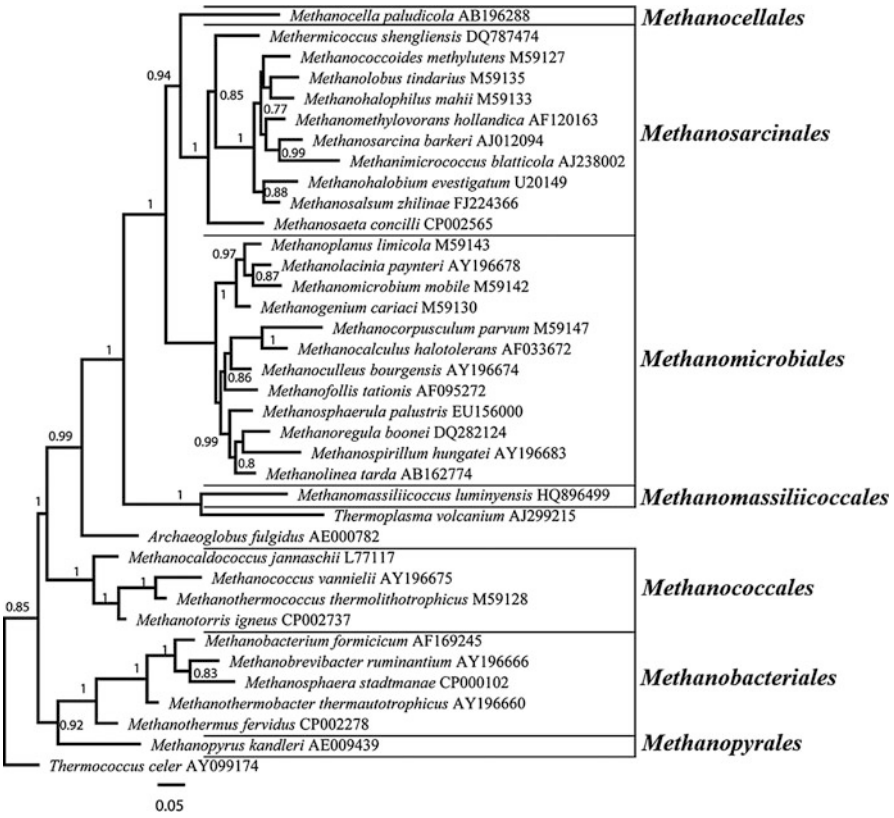

Fig. 1 Maximum-likelihood tree based on nearly full length 16S rRNA gene sequences from type species of 34 methanogen genera. The tree was built by FastTree 2.1.5 using *Thermococcus celer* as an outgroup. Bootstrap values >0.77 are indicated at nodes and were based on 1000 replicates (Price 2010). There were a total of 1555 positions in the final dataset, which were aligned in the RDP 11 database. The scale bar represents substitutions per position. The GenBank accesion numbers are indicated following the species name

2.1 Methanobacteriales

Methanobacteriales are currently classified into two families and five genera based upon 16S rRNA sequences, DNA reassociation levels, and phenotypic characteristics. The two families *Methanobacteriaceae* and *Methanothermaceae* are distinguished by 16S rRNA sequence similarities below 89% and differences in cell wall structure and growth temperatures. The family *Methanobacteriaceae* contains three mesophilic genera – *Methanobacterium*, *Methanobrevibacter*, and *Methanosphaera* – and one thermophilic genus *Methanothermobacter*. Members of the *Methanobacteriaceae* possess pseudomurein as a major component of the cellular envelope. The family *Methanothermaceae* is represented by one hyperthermophilic genus, *Methanothermus*. Members of the *Methanothermaceae* possess a protein surface layer in addition to the pseudomurein layer.

Table 2 Some characteristics of the methanogen orders (Modified from Liu (2010e))

Order	Shape	Methanogenesis substrates[a]	Motility	Cell wall	Cellular lipids[b]	
					Core lipids	Polar lipids
Methanobacteriales	Rods, cocci	$H_2 + CO_2$, (formate, CO, methanol, secondary alcohols)	−[c]	Pseudomurein, protein	Caldarchaeol, archaeol, hydroxyarchaeol	Glucose, *N*-acetylglucosamine, *myo*-inositol, ethanolamine, serine
Methanococcales	Cocci	$H_2 + CO_2$, formate	+	Protein	Archaeol, caldarchaeol, hydroxyarchaeol, macrocyclic archaeol	Glucose, *N*-acetylglucosamine, serine, ethanolamine
Methanomicrobiales	Cocci, rods, spirals, sheathed rods	$H_2 + CO_2$, formate, (secondary alcohols)	+/−	Protein, glycoprotein	Archaeol, caldarchaeol	Glucose, galactose, aminopentanetetrol, glycerol
Methanosarcinales	Pseudosarcina, cocci, sheathed rods	Methanol, methylamine, acetate, ($H_2 + CO_2$, methoxylated aromatic compounds)	−	Protein, glycoprotein	Archaeol, hydroxyarchaeol, caldarchaeol	Glucose, galactose, mannose, *myo*-inositol, ethanolamine, serine, glycerol
Methanopyrales	Rods	$H_2 + CO_2$	+	Pseudomurein	Archaeol	nd
Methanocellales	Rods	$H_2 + CO_2$, formate	−	nd	nd	nd
Methanomassiliicoccales	Cocci	H_2 + methanol, methylamine	−	nd	nd	nd

Abbreviation: *nd* not determined

[a]Major substrates utilized for methanogenesis. Parentheses means utilized sometimes

[b]Compounds can be contained in cellular lipids, depending on the species

[c]Except the genus *Methanothermus*

The placement of the hyperthermophilic *Methanothermus* into a separate family from other *Methanobacteriales* genera is justified by the deep branching of the phylogeny of its 16S rRNA gene (Schuchmann and Muller 2014). The 16S rRNA gene sequence similarities within the *Methanothermus* species are much higher (98%) than the similarities between *Methanothermus* and other members of the *Methanobacteriales* (83–89%). This classification is further confirmed by DNA reassociation. For instance, the DNA relatedness between *Methanothermus* isolates and *Methanothermobacter thermoautotrophicus* strain IM is 2–8% (Lauerer et al. 1986). Phenotypically, the genus *Methanothermus* is distinguished from other *Methanobacteriales* by their high temperature optima (80–88 °C), double-layered cell wall, and motility by bipolar polytrichous flagellation.

Methanobacteriaceae is a diverse family, including mesophilic and thermophilic species. The phylogeny of the 16S rRNA gene indicates that the thermophilic species are divergent from mesophilic members at the genus level. The 16S rRNA sequence similarities within the thermophilic genus *Methanothermobacter* are above 98%, while the similarities between thermophilic and mesophilic members of *Methanobacteriaceae* are generally below 93% (Wasserfallen et al. 2000). The DNA relatedness between *Methanothermobacter* species are 22–47%, confirming that they are genetically distant and should be assigned to separate species (Boone et al. 2001a).

The separation of mesophilic members of *Methanobacteriales* into three genera is supported by both genetic and phenotypic analyses. Species of *Methanobacterium* are usually autotrophs, while species of *Methanobrevibacter* and *Methanosphaera* are commonly mixotrophic or heterotrophic. Species of *Methanosphaera* use only H_2 and methanol as substrates for methanogenesis, while all species of *Methanobrevibacter* and *Methanobacterium* can use H_2 and CO_2.

Members of the order *Methanobacteriales* use a limited range of substrates for methanogenesis. Most of them reduce CO_2 to CH_4 with H_2. Some *Methanobacterium* species can also reduce methanol with H_2, which are the exclusive substrates for the genus *Methanosphaera*. There is one *Methanobacterium* species that can also reduce methylamine with H_2. Some *Methanobacteriales* members can also use formate, CO, or secondary alcohols as electron donors. Some species can grow autotrophically using CO_2 as the sole carbon source, and some species are mixotrophs or heterotrophs, which may require acetate, amino acids, peptones, yeast extract, vitamins, and/or rumen fluid for growth. Ammonium is a major nitrogen source. Sulfide can serve as the sole sulfur source, and some species can reduce elemental sulfur to sulfide. Cells are generally rod-shaped with a length of 0.6–25 μm, often forming chains or filaments up to 40 μm in length. Cells typically stain Gram positive, but the wall does not contain muramic acid. Pesudomurein is the predominant polymer in the cell wall. Members of the genus *Methanothermus* have double-layered cell wall, consisting of an inner pseudomurein layer and an outer S-layer composed of protein. The cellular lipids contain caldarchaeol, archaeol, and, in some species, hydoxyarchaeol as core lipids. The polar lipids can contain glucose, *N*-acetylglucosamine, *myo*-inositol, ethanolamine, and serine, depending on the species. Most species are nonmotile. However, *Methanobacterium movens* and members of the genus *Methanothermus* are motile via one or two polar flagella

and peritrichous flagella, respectively. The optimum growth temperatures of members of the *Methanobacteriales* vary from 20 °C to 88 °C. The genus *Methanothermus* can grow at temperatures up to 97 °C, while multiple *Methanobacterium* species can grow at as low as 10 °C and one species can even grow at 0 °C. The pH optima of *Methanobacteriales* members vary from 5.5 to 9.

Descriptive properties of the *Methanobacteriales* are summarized in Tables 3, 4, 5, 6, and 7. Further information can be found in Bonin and Boone (2006) and Boone et al. (2001a). Our current knowledge on the diversity of the *Methanobacteriales* is largely incomplete. As an example, investigations of 16S rRNA gene from clone libraries recognized a large number of uncultured *Methanobrevibacter*, especially from the rumen and termite gut (Dighe et al. 2004; Wright et al. 2004). Moreover, the cloned sequences from termite gut formed separate lineages from cultured *Methanobrevibacter* (Dighe et al. 2004). The correlation between ecological habitat and 16S rRNA based phylogeny need more ecological surveys to unravel.

2.2 Methanococcales

The order *Methanococcales* is composed of two families, *Methanocaldococcaceae* and *Methanococcaceae*, which are distinguished by 16S rRNA sequence similarities below 93% and differences in growth temperatures. The *Methanocaldococcaceae* are all hyperthermophilic, while the *Methanococcaceae* are extremely thermophilic and mesophilic. Members of this order are all capable of forming methane by CO_2 reduction with H_2. Many species can use formate as an alternative electron donor. Most species can grow autotrophically.

Phylogenetic analyses with DNA sequences reveal a high diversity of the *Methanococcales*. The sequence similarities of the 16S rRNA genes between hyperthermophilic and mesophilic methanococci are generally below 90%. For instance, the 16S rRNA gene sequence similarity between the mesophile *Methanococcus voltae* and the hyperthermophile *Methanocaldococcus infernus* is about 85%, which is comparable to the similarity between *Escherichia* and *Pseudomonas*. In addition, the mesophilic methanococci possess 91–96% (average 94%) 16S rRNA gene sequence similarities and 5–30% DNA reassociation values, suggesting that they are related only at the genus level (Keswani et al. 1996).

The *Methanococcales* are currently divided into two families and four genera, according to their growth temperatures. The family *Methanocaldococcaceae* includes two hyperthermophilic genera, *Methanocaldococcus* and *Methanotorris*. The family *Methanococcaceae* includes the mesophilic genus *Methanococcus* and the extremely thermophilic genus *Methanothermococcus*. This taxonomy generally agrees with the phylogeny of the 16S rRNA genes (Liu 2010b), in which the lineages formed by the deepest bifurcation represent the two methanococcal families. However, some ambiguity remains. For instance, 16S rRNA gene sequences indicate that *Methanococcus aeolicus* forms a deep branch of the mesophilic methanococci and is more closely related to the thermophile *Methanothermococcus okinawensis* (95% sequence similarity) than to the other *Methanococcus* (91–93% sequence similarity). In addition, *Methanothermococcus okinawensis* also has low sequence similarity to

Table 3 Descriptive characteristics of the species of the genus *Methanobacterium* (Modified from Liu (2010a))

Species	Type strain	Source[a]	Cell width (μm)	Cell length (μm)	Methanogenesis substrates[b]	Required organic compounds	Temperature range (optimum) (°C)	pH range (optimum)	NaCl range (%, w/v)	Doubling time[c] (h)	GC content (mol%)	References
aarhusense	H2-LR	Marine sediment	0.7	5–18	$H_2 + CO_2$	None	>5–<48 (45)	5–9 (7.5–8)	0.6–5.4	nd	34.9 (LC)	(Shlimon et al. 2004)
aggregans	E09F.3	Anaerobic digester	0.2–0.5	2–2.5	$H_2 + CO_2$, formate	None	25–45 (40)	nd (6.5–7.0)	0–0.3	5–6	39.1 (LC)	(Kern et al. 2015)
alcaliphilum	WeN4	Alkaline lake	0.5–0.6	2–25	$H_2 + CO_2$	TP or YE	25–45 (37)	7.0–9.9 (8.1–9.1)	nd	nd	57 (BD)	(Worakit et al. 1986)
arcticum	M2	Permafrost sediments	0.45–0.5	3–6	$H_2 + CO_2$, formate	None	15–45 (37)	5.5–8.5 (6.8–7.2)	0–1.8	nd	38.1 (T_m)	(Shcherbakova et al. 2011)
beijingense	8-2	Anaerobic digestor	0.4–0.5	3–5	$H_2 + CO_2$, formate	YE	25–50 (37)	6.5–8.0 (7.2)	0–3	14	38.9 (T_m)	(Ma et al. 2005)
bryantii	M.o.H.	Anaerobic digestor	0.5–1.0	10–15	$H_2 + CO_2$, (2-propanol, 2-butanol, cyclopentanol)	None	nd (37–39)	nd (6.9–7.2)	nd	nd	33–38 (Bd)	(Boone 1987)
congolense	C	Anaerobic digestor	0.4–0.5	2–10	$H_2 + CO_2$, (2-propanol, 2-butanol, cyclopentanol)	None	25–50 (37–42)	5.9–8.2 (7.2)	nd	7.5	39.5 (LC)	(Cuzin et al. 2001)
espanolae	GP9	Sludge of a bleach-craft mill	0.8	3–22	$H_2 + CO_2$, (2-propanol, 2-butanol)	nd[d]	15–50 (35)	4.6–7.0 (5.6–6.2)	nd	10	34 (T_m)	(Patel et al. 1990)
ferruginis	Mic6c05	Corroded pipe sediment	nd	nd	$H_2 + CO_2$, (2-propanol), (isobutanol), (cyclopentanol)	None	20–45 (40)	5.5–9.0 (6.0–7.5)	0–7	18.5	37.6 (LC)	(Mori and Harayama 2011)
flexile	GH	Lake sediments	0.3–0.5	2–5	$H_2 + CO_2$, formate	YT	10–50 (35–38)	6.5–9.5 (7.0–7.5)	0–0.6	21.7	36.4 (T_m)-	(Zhu et al. 2011)
formicicum	MF	Sewage sludge	0.4–0.8	2–15	$H_2 + CO_2$, formate	None	25–50 (37–45)	6.6–7.8 (7–7.5)	nd	13	41–42 (Bd)	(Boone 1987)

(*continued*)

Table 3 (continued)

Species	Type strain	Source[a]	Cell width (μm)	Cell length (μm)	Methanogenesis substrates[b]	Required organic compounds	Temperature range (optimum) (°C)	pH range (optimum)	NaCl range (%, w/v)	Doubling time[c] (h)	GC content (mol%)	References
ivanovii	Ivanov	Rock core	0.5–0.8	1–15	$H_2 + CO_2$	None	>10–<55 (37–45)	6.5–8.2 (7.0–7.4)	0–1	16–18	36.6 (T_m)	(Belyaev et al. 1983)
kanagiense	169	Rice field	0.35–0.5	1.6–5.0	$H_2 + CO_2$	None	15–45 (40)	6.5–9.6 (7.5–8.5)	0–7	21	39.3 (LC)	(Kitamura et al. 2011)
lacus	17A1	Lake sediments	0.2–0.4	2–15	$H_2 + CO_2$, methanol + H_2	None	14–41 (30)	5.0–8.5 (6.5)	0–2.3	22	37.0 (LC)	(Borrel et al. 2012b)
movens	TS − 2	Lake sediments	0.3–0.5	2–5	$H_2 + CO_2$	YT	10–50 (35–38)	6.0–9.0 (7.2–7.5)	0–1.8	25.7	39.1 (T_m)	(Zhu et al. 2011)
movilense	MC-20	Lake sediments	0.6–0.7	3.5–4.0	$H_2 + CO_2$, formate, 2-propanol, 2-butanol	None	0–44 (33)	6.2–9.9 (7.4)	0.1–3.5	79.2	33.0	(Schirmack et al. 2014)
oryzae	FPi	Rice field	0.3–0.4	3–10	$H_2 + CO_2$, formate	None	20–42 (40)	6.0–8.5 (7.0)	0–2.5	nd	31 (LC)	(Joulian et al. 2000)
paludis	SWAN1	Minerotrophic fen	0.6	1.5–2.8	$H_2 + CO_2$	None	16–40 (32–37)	4.8–6.6 (5.4–5.7)	0–1.5	35	35.7 (G_s)	(Cadillo-Quiroz et al. 2014)

palustre	F	Peat bog	0.5	2.5–5	$H_2 + CO_2$, formate, 2-propanol, (2-butanol)	None	20–45 (33–37)	nd (7.0)	0–1.8	18	34 (T_m)	(Zellner et al. 1988)
	FG694aF	Fault gouge	0.5–0.7	1.7–0.24	$H_2 + CO_2$, formate	None	20–45 (37)	5.7–8.3 (5.7–6.8)	0–3.2	8.6	nd	(Wu and Lai 2011)
petrolearium	Mic5c12	Crude oil sludge	nd	nd	$H_2 + CO_2$	YE, ac	20–40 (35)	5.5–9.0 (6.5)	0–7	39.5	38.3 (LC)	(Mori and Harayama 2011)
subterraneum	A8p	Deep granitic groundwater	0.1–0.15	0.6–1.2	$H_2 + CO_2$, formate	None	3.6–45 (20–40)	6.5–9.2 (7.8–8.8)	0–8	2.5	54.5 (T_m)	(Kotelnikova et al. 1998)
uliginosum	P2St	Marshy soil	0.2–0.6	2–4	$H_2 + CO_2$	None	15–45 (37–40)	6.0–8.5 (6.0–7.5)	nd	11	29.4 (T_m)	(Koenig 1984)
veterum	MK4	Permafrost	0.4–0.45	2.0–8.0	$H_2 + CO_2$, methanol + H_2, methylamine + H_2	None	10–46 (28)	5.2–9.4 (7.2–7.4)	0–1.8	26.7	33.8 (T_m)	(Krivushin et al. 2010)

Abbreviations: *nd* not determined, *TP* trypticase peptones, *YE* yeast extract, *ac* acetate, *LC* liquid chromatography, *BD* buoyant density method, T_m melting point method, G_s genome sequencing method

[a]Environment from which the type strain was isolated

[b]Substrates in parentheses are oxidized, but do not result in growth

[c]Doubling time of the type strain under optimal growth conditions of temperature, pH, and NaCl

[d]Cells grew in vitamin-free medium containing acetate

Table 4 Descriptive characteristics of the species of the genus *Methanobrevibacter* (Modified from Liu (2010a))

Species	Type strain	Source[a]	Cell width (μm)	Cell length (μm)	Methanogenesis substrates	Required organic compounds	Temperature range (optimum) (°C)	pH range (optimum)	NaCl range (%, w/v)	Doubling time[b] (h)	GC content (mol%)	References
acididurans	ATM	Acidogenic digestor	0.3–0.5	0.3–0.5	$H_2 + CO_2$	RF, ac, AAs	25–37 (35)	5.0–7.5 (6.0)	nd	~16	nd	(Savant et al. 2002)
arboriphilus	DH1	Decaying cottonwood tissue	0.5	1.2–1.4	$H_2 + CO_2$, (formate)[c]	B-vit	25–45 (30–37)	6.0–8.6 (7.5–8)	0–0.6	13	25.5–31.6 (Bd or T_m)	(Zeikus and Henning 1975)
boviskoreani	JH1	Bovine rumen	0.6	1.5–1.8	$H_2 + CO_2$, formate	YE, CoM, FA	35–45 (37–40)	5.5–8.0 (6.5–7.0)	0.6–3.0	nd	28 (LC)	(Lee et al. 2013)
curvatus	RFM-2	Termite hindgut	0.34	1.6	$H_2 + CO_2$	RF	10–<37 (30)	6.5–8.5 (7.1–7.2)	nd	40	nd	(Leadbetter and Breznak 1996)
cuticularis	RFM-1	Termite hindgut	0.4	1.2	$H_2 + CO_2$, (formate)[d]	None	10–<42 (37)	6.5–8.5 (7.7)	nd	35	nd	(Leadbetter and Breznak 1996)
filiformis	RFM-3	Termite hindgut	0.23–0.28	4	$H_2 + CO_2$	YE	10–33.5 (30)	6.0–7.5 (7.0–7.2)	nd	37	nd	(Leadbetter et al. 1998)
gottschalkii	HO	Horse faeces	0.7	0.9	$H_2 + CO_2$	ac or YE or TP	27–41 (37)	5.0–10.0 (7)	nd	nd	29 (T_m)	(Miller and Lin 2002)
millerae	ZA-10	Bovine rumen	nd	nd	$H_2 + CO_2$, formate	Ac, YE or TP	33–43 (36–42)	5.5–10.0 (7.0–8.0)	up to 2.6	nd	31–32 (T_m)	(Rea et al. 2007)
olleyae	KM1H5-1P	Ovine rumen	nd	nd	$H_2 + CO_2$, formate	ac	28–42 (36–40)	6.0–10.0 (7.5)	up to 2.6	nd	27–29 (T_m)	(Rea et al. 2007)

oralis	ZR	Human subgingival plaque	0.4–0.5	0.7–1.2	$H_2 + CO_2$	Fecal extract	25–39 (35–38)	6.2–8.0 (6.9–7.4)	0.06–0.6	15	28 (T_m)	(Ferrari et al. 1994)
ruminantium	M1	Bovine rumen	0.7	0.8–1.7	$H_2 + CO_2$, (formate)[d]	ac, B-vit, CoM, 2-MBA, AAs	33–42 (37–39)	5.5–7.7 (6–7)	nd	nd	30.6 (Bd)	(Smith and Hungate 1958)
smithii	PS	Sewage sludge	0.6–0.7	~1	$H_2 + CO_2$, (formate)[d]	ac, B-vit	26–46 (34–46)	5.0–8.5 (5.5–7.0)	nd	nd	30–31 (T_m or Bd)	(Balch et al. 1979)
thaueri	CW	Cow faeces	0.5	0.6–1.2	$H_2 + CO_2$	ac or YE or TP	nd (37)	nd (7)	nd	nd	38 (T_m)	(Miller and Lin 2002)
woesei	GS	Goose faeces	0.6	1	$H_2 + CO_2$, (formate)[d]	ac or YE or TP	nd (37)	nd (7)	nd	nd	31 (T_m)	(Miller and Lin 2002)
wolinii	SH	Sheep faeces	0.6	1.0–1.4	$H_2 + CO_2$	ac or YE or TP	nd (37)	nd (7)	nd	nd	33 (T_m)	(Miller and Lin 2002)

Abbreviations: *nd* not determined, *RF* rumen fluid, *ac* acetate, *AAs* amino acids, *B-vit* B vitamins, *TP* trypticase peptones, *YE* yeast extract, *CoM* 2-mercaptoethanesulfonic acid (conenzyme M), *FA* fatty acids, *2-MBA* 2-methylbutyric acid, *BD* buoyant density method, T_m melting point method

[a]Environment from which the type strain was isolated

[b]Doubling time of the type strain under optimal growth conditions of temperature, pH, and NaCl

[c]Formate is used by some, but not all strains

[d]Growth on formate is poor

Table 5 "Descriptive characteristics of the species of the genus *Methanosphaera*" (Originally published in Liu (2010a), published with kind permission of)

Species	Type strain	Source[a]	Cell width (μm)	Cell length (μm)	Methanogenesis substrates	Required organic compounds	Temperature range (optimum) (°C)	pH range (optimum)	NaCl range (%, w/v)	GC content (mol%)	References
cuniculi	1R7	Rabbit rectum	0.6–1.2	0.6–1.2	H_2 + methanol	ac	>25–<45 (35–40)	nd (6.8)	nd	23 (T_m)	(Biavati et al. 1988)
stadtmanae	MCB3	Human feces	~1	~1	H_2 + methanol	Thiamine, ac, Ile, Leu	30–40 (36–40)	nd (6.5–6.9)	nd	25.8 (T_m)	(Miller and Wolin 1985)

Abbreviations: *nd* not determined, *ac* acetate, *Ile* isoleucine, *Leu* leucine, T_m melting point method
[a]Environment from which the type strain was isolated

Table 6 Descriptive characteristics of the species of the genus *Methanothermobacter* (Modified from Liu (2010a))

Species	Type strain	Source[a]	Cell width (μm)	Cell length (μm)	Methanogenesis substrates	Required organic compounds	Temperature range (optimum) (°C)	pH range (optimum)	NaCl range (%, w/v)	Doubling time[b] (h)	GC content (mol%)	References
crinale	Tm2	Oil sands	0.3	2.2–5.9	$H_2 + CO_2$	ac	45–80 (65)	6.9–8.0 (6.9)	0–4	nd	41.1 (LC)	(Cheng et al. 2011)
defluvii	ADZ	Anaerobic digestor	0.4	3–6	$H_2 + CO_2$, formate	CoM	45–65 (60)	6.0–7.5 (7.0)	0.08–2	1.5	62.2 (T_m)	(Kotelnikova et al. 1993)
marburgenesis	Marburg	Sewage sludge	0.4–0.6	3–6	$H_2 + CO_2$	None	45–70 (65)	5.0–8.0 (6.8–7.4)	0.01–3.5	1.6–2.5	47.6 (T_m)	(Wasserfallen et al. 2000)
tenebrarum	RMAS	Natural gas field	0.5	3.5–10.5	$H_2 + CO_2$	CA, TP, YE, vit	45–80 (70)	5.8–8.7 (6.9–7.7)	0.01–2	12	41.5 (LC)	(Nakamura et al. 2013)
thermoautotrophicus	ΔH	Sewage sludge	0.35–0.6	3–7	$H_2 + CO_2$, (formate)[c]	None	40–75 (65–70)	6.0–8.8 (7.2–7.6)	0.01–3.5	3	49 (T_m)	(Zeikus and Wolee 1972, Schönheit et al. 1980)
thermoplexus	IDZ	Anaerobic digestor	0.4	7–20	$H_2 + CO_2$, formate	CoM	45–70 (55)	7.5–8.5 (7.9–8.2)	0.1–3	3.5	55 (T_m)	(Kotelnikova et al. 1993)
thermophilus	M	Sludge of methane tank	0.36	1.4–6.5	$H_2 + CO_2$	CoM	47–75 (57)	6.5–8.5 (7.5)	0–0.6	2–3	44.7 (T_m)	(Laurinavichyus et al. 1988)
wolfeii	DSM2970	Sewage sludge and river sediment	0.4–0.6	2.5–6	$H_2 + CO_2$, formate	None	37–74 (55–65)	6.0–8.2 (7.0–7.5)	nd (up to 1)	3.5–4	61 (T_m)	(Winter et al. 1984)

Abbreviations: *nd* not determined, *CoM* 2-mercaptoethanesulfonic acid (conenzyme M), *ac* acetate, *CA* casamino acids, *TP* tryptone, *YE* yeast extract, *vit* vitamins, T_m melting point method

[a]Environment from which the type strain was isolated

[b]Doubling time of the type strain under optimal growth conditions of temperature, pH, and NaCl

[c]Formate is used by some, but not all strains

Table 7 "Descriptive characteristics of the species of the genus *Methanothermus*" (Originally published in Liu (2010a), published with kind permission of)

Species	Type strain	Source[a]	Cell width (μm)	Cell length (μm)	Methanogenesis substrates	Required organic compounds	Temperature range (optimum) (°C)	pH range (optimum)	NaCl range (%, w/v)	Doubling time[b] (h)	GC content (mol%)	References
fervidus	V24S	Icelandic hot spring	0.3–0.4	1–3	$H_2 + CO_2$	None	67–97 (80–85)	nd (6.5)	nd	3	33 (T_m)	(Stetter et al. 1981)
sociabilis	Kf1-F1	Icelandic hot spring	0.3–0.4	1–3	$H_2 + CO_2$	None	55–97 (88)	5.5–7.5 (6.5)	nd	3	33 (T_m)	(Lauerer et al. 1986)

Abbreviations: *nd* not determined, T_m melting point method

[a]Environment from which the type strain was isolated

[b]Doubling time of the type strain under optimal growth conditions of temperature, pH, and NaCl

the other thermophile *Methanothermococcus thermolithotrophicus* (95% sequence similarity). Therefore, the phylogenetic analysis implies that *Methanococcus aeolicus* and *Methanothermococcus okinawensis* could be classified into two novel genera. Nevertheless, phylogeny of additional genes and phenotypic differences other than growth temperature should be examined to justify reclassification.

DNA relatedness and cellular protein patterns are often determined for the phylogenetic and taxonomic analyses of methanococci. They are especially useful to distinguish relationships at the species and subspecies levels, at which levels the 16S rRNA gene sequence analysis is frequently incongruent. For instance, two heterotrophic *Methanococcus voltae* strains A2 and A3 exhibit 37% DNA relatedness to the type train PS (Keswani et al. 1996). Similarly, four autotrophic *Methanococcus maripaludis* strains C5, C6, C7, and C8 exhibit 54–69% DNA relatedness to the type strain JJ (Keswani et al. 1996). Moreover, differences in cellular protein patterns between these strains are also readily recognized. Therefore, classification of these strains into separate species is suggested based on their genetic diversities. However, because distinguishable phenotypic properties are few, these strains are not currently considered as novel species.

Autotrophy and thermophily are represented in both methanococcal families, suggesting that the mesophilic methanococci may have evolved from an autotrophic thermophile (Keswani et al. 1996). The heterotrophy of *Methanococcus voltae* is possibly a recently acquired characteristic. This hypothesis is consistent with the presence of enzymes required for autotrophic CO_2 fixation in *M. voltae* (Shieh et al. 1988).

Members of the *Methanococcales* or the methanococci are coccoid methanogens isolated from marine environments. They share a set of phenotypic characteristics. They all use H_2 or formate to reduce CO_2 for methanogenesis. Acetate, methyl-containing compounds, and alcohols are not used as substrates for methanogenesis. Most of them can grow autotrophically with CO_2 as the sole carbon source. Sulfide is a sufficient sulfur source for all methanococci, and elemental sulfur is reduced to sulfide with slight inhibition of growth in most strains. Ammonium is a sufficient nitrogen source for all methanococci, and nitrogen gas, nitrate, and alanine are used as a nitrogen source by some species. They all require sea salts for optimal growth. Cells are irregular cocci, 1–3 μm in diameter during balanced growth. Most of them are motile by means of polar tuft(s) of flagella. Cells strain Gram negative. They are susceptible to lysis by 0.01% (w/v) SDS and hypotonic solutions. Cell envelopes are composed of a protein cell wall or S-layer. Glycoproteins and cell wall carbohydrates are not abundant. The cellular lipids contain archaeol, caldarchaeol, hydroxyarchaeol, and macrocyclic archaeol, depending upon the species. The polar lipids can contain glucose, *N*-acetylglucosamine, serine, and ethanolamine. The optimal growth temperatures of methanococci are diverse, ranging from 35 °C to 88 °C. They are among the fastest growing methanogens at either mesophilic or thermophilic temperatures, with generation times of about 2 h at 37 °C and less than 30 min at 85 °C.

Descriptive properties of the methanococci are summarized in Tables 8 and 9. Further information can be found in Whitman et al. (2001a), and Whitman and Jeanthon (2006). Creation of new families and genera may be necessary with addition of new isolates and identification of new phenotypic and genetic markers. The *Methanotorris* may represent a new family because they have only 92–93% 16S

Table 8 Descriptive characteristics of the species of the genera *Methanocaldococcus* and *Methanotorris* (Modified from Liu (2010b))

			Methanocaldococcus					*Methanotorris*	
Character	*jannaschii*	*infernus*	*fervens*	*indicus*	*villosus*	*bathoardescens*	*vulcanius*	*igneus*	*formicicus*
Type strain	JAL-1	ME	AG86	SL 43	KIN24-T80	JH146	M7	Kol 5	Mc-S-70
Cell diameter (μm)	1.5	1–3	1–2	1–3	1–2	1–2	1–3	1–2	0.8–1.5
Flagella[a]	2 tufts	3 tufts	nd	1 tuft	1 tuft[d]	1 tuft	3 tufts	±	±
Substrates for methanogenesis	$H_2 + CO_2$	$H_2 + CO_2$	$H_2 + CO_2$	$H_2 + CO_2$	$H_2 + CO_2$	$H_2 + CO_2$	$H_2 + CO_2$	$H_2 + CO_2$	$H_2 + CO_2$, formate
Autotrophy	+	+	+	+	+	+	+	+	+
Yeast extract stimulates growth	−	+	+	+	+	−	+	−	−
Selenium simulates growth	+	+	+	+	+	nd	+	−	−
Nitrogen source	NH_3	NH_3, NO_3^-	NH_3, NO_3^-	NH_3, NO_3^-	NH_3, nd	NH_3	NH_3, NO_3^-	NH_3	NH_3, N_2, NO_3^-
Sulfur source	S^{2-}, S^0	S^{2-}, S^0	S^{2-}, S^0	S^{2-}, S^0	S^{2-}, nd	nd	S^{2-}, S^0	S^{2-}, S^0	S^{2-}
Temperature range (°C)	50–91	55–91	48–92	50–86	55–90	58–90	49–89	45–91	55–83
Temperature optimum (°C)	85	85	85	85	80	82	80	88	75
pH range	5.2–7.0	5.25–7.0	5.5–7.6	5.5–6.7	5.5–7.0	4.5–9.0	5.2–7.0	5.0–7.5	6.0–8.5
pH optimum	6.0	6.5	6.5	6.5	6.5	7.0	6.5	5.7	6.7

NaCl range (%, w/v)	1.0–5.0	0.8–3.5	0.5–5.0	1.5–5.0	0.5–5.5	1.6–7.4	0.6–5.6	0.9–5.4	0.4–6.0
NaCl optimum (%, w/v)	3.0	2.0	3.0	3.0	2.5	2.9	2.5	1.8	2.4
GC content (mol%)	31 (Bd)	33 (T_m)	33 (T_m)	31 (LC)	30 (*Gs*)	30.8 (*Gs*)	31 (T_m)	31 (T_m)	33 (LC)
Doubling time (min)[b]	26	35–40	20–30	25–30	45	20	45	30	30
Source[c]	Deep sea hydrothermal vent	Deep sea hydrothermal vent	Deep sea hydrothermal vent	Deep sea hydrothermal vent	Shallow submarine hydrothermal system	Deep sea hydrothermal fluid	Deep sea hydrothermal vent	Shallow marine hydrothermal vent	Deep sea black smoker chimney
References	(Jones et al. 1983a)	(Jeanthon et al. 1998)	(Jeanthon et al. 1999; Zhao et al. 1988)	(L'Haridon et al. 2003)	(Bellack et al. 2011)	(Ver Eecke et al. 2013; Stewart et al. 2015)	(Jeanthon et al. 1999)	(Burggraf et al. 1990)	(Takai et al. 2004)

Abbreviations: *nd* not determined, *Bd* buoyant density method, T_m melting point method, *LC* liquid chromatography, G_s genome sequencing

[a]Number of flagellar tufts. ±, non-motile, but flagella-like structures are observed by electron microscopy

[b]Doubling time of the type strain under optimal growth conditions of temperature, pH, and NaCl

[c]Environment from which the type strain was isolated

[d]tuft is only formed in some cases, which may mediate cell-cell contact. Otherwise, 50 polarly inserted flagella are observed

Table 9 "Descriptive characteristics of the species of the genera *Methanococcus* and *Methanothermococcus*" (Originally published in Liu (2010b), published with kind permission of © Springer Science+Business Media New York, 2003. All rights reserved)

	Methanococcus				*Methanothermococcus*	
Character	*vannielii*	*voltae*	*maripaludis*	*aeolicus*	*thermolithotrophicus*	*okinawensis*
Type strain	SB	PS	JJ	Nankai-3	SN1	IH1
Cell diameter (μm)	1.3	1.3–1.7	0.9–1.3	1.5–2.0	1.5	1.0–1.5
Flagella[a]	2 tufts	Multiple tufts	1 tuft	nd	1 tuft	1 tuft
Substrates for methanogenesis	$H_2 + CO_2$, formate	$H_2 + CO_2$, formate	$H_2 + CO_2$, formate	$H_2 + CO_2$, formate	$H_2 + CO_2$, formate	$H_2 + CO_2$, formate
Autotrophy	+	–[d]	+	+	+	+
Acetate stimulates growth	–	+	+	–	–	–
Amino acids stimulate growth	–	+	+	–	–	–
Selenium simulate growth	+	+	+	+	nd	+
Nitrogen source	NH_3, purines	NH_3,	NH_3, N_2, alanine	NH_3, N_2	NH_3, N_2, NO_3^-	NH_3
Sulfur source	S^{2-}, S^0	S^{2-}, S^0	S^{2-}, S^0, $(S_2O_3^{2-})$[e]	S^{2-}, S^0	S^{2-}, S^0, $S_2O_3^{2-}$, SO_3^{2-}, SO_4^{2-}	S^{2-}
Temperature range (°C)	<20–45	<20–45	<20–45	<20–55	17–70	40–75

Temperature optimum (°C)	35–40	35–40	35–40	46	60–65	60–65
pH range	6.5–8.0	6.5–8.0	6.5–8.0	5.5–7.5	4.9–9.8	4.5–8.5
pH optimum	7–8	6.0–7.0	6.8–7.2	7.0	5.1–7.5	6–7
NaCl range (%, w/v)	0.3–5	0.6–6	0.3–5	0.3–6	0.6–9.4	1.2–9.6
NaCl optimum (%, w/v)	0.6–2	1–2	0.6–2	1–2	2–4	2.5–5.0[f]
GC content (mol%)[a]	33	30	33	32	34	33.5
Doubling time (h)[b]	8	3	2	1.3	~1	0.5
Source[c]	Marine sediments	Marine sediments	Salt marsh sediments	Marine sediments	Coastal geothermally heated sea sediments	Deep sea hydrothermal vent
References	(Stadtman and Barker 1951)	(Balch et al. 1979; Whitman et al. 1982)	(Jones et al. 1983b)	(Kendall et al. 2006)	(Huber et al. 1982)	(Takai et al. 2002)

Abbreviations: *nd* not determined
[a]The G+C content of the DNA determined by liquid chromatography
[b]Doubling time of the type strain under optimal growth conditions of temperature, pH, and NaCl
[c]Environment from which the type strain was isolated
[d]Acetate and the amino acids leucine and isoleucine are required for growth
[e]Thiosulfate is used by some strains

rRNA similarities with the *Methanocaldococcus*. These two groups are also distinguished by the presence of hydroxyarchaeol and the absence of caldarchaeol in the *Methanotorris*. *Methanococcus aeolicus* and *Methanothermococcus okinawensis* may represent two new genera because they form a lineage separate from other *Methanococcaceae* in the 16S rRNA phylogenetic tree.

2.3 Methanomicrobiales

The order *Methanomicrobiales* is composed of four families, *Methanomicrobiaceae*, *Methanocorpusculaceae*, *Methanospirillaceae*, and *Methanoregulaceae*, which are distinguished by 16S rRNA sequence similarities below 89%. The *Methanospirillaceae* is further distinguished from the other two families by its unique morphology of curved rod-shape and exterior sheath. All members of this order are capable to produce methane by CO_2 reduction with H_2. Formate and secondary alcohols are used as alternative electron donors in many species.

Because the members of *Methanomicrobiales* share many phenotypic characteristics, it is difficult to divide them based solely on their physiological properties. Both of the families *Methanomicrobiaceae* and *Methanocorpusculaceae* contain coccoid organisms, and nearly all members require organic carbon sources for growth (except *Methanofollis aquaemaris*). Therefore, they are difficult to distinguish except by molecular phylogenetic analyses. The family *Methanospirillaceae* is distinguished from the other three families by its unique morphology of curved rod-shape and capability of autotrophic growth. The family *Methanoregulaceae* is unique by having members that grow in acidic conditions.

The family *Methanomicrobiaceae* is divided into six genera. The 16S rRNA gene sequence similarities between different genera are 87–95%, suggesting that they are sufficiently distinctive at genus level. The 16S rRNA gene sequence similarities between different species within a genus are above 95.4%. Both *Methanomicrobium* and *Methanolacinia* are represented by a single species. Cells of both genera are rod-shaped, but they can be differentiated by some other physiological characters. In addition to H_2, *Methanolacinia paynteri* can use secondary alcohols to reduce CO_2. In contrast, *Methanomicrobium mobile* can only use H_2 or formate as electron donors for methanogenesis. *Methanolacinia paynteri* is a marine organism, while *Methanomicrobium mobile* was isolated from bovine rumen. Cells of *Methanoculleus*, *Methanofollis*, and *Methanogenium* are irregular cocci. These three genera are difficult to differentiate by phenotypic characteristics. *Methanoplanus* differs from the other genera by its plate or disc cell shape.

The family *Methanospirillaceae* is represented by a single species, *Methanospirillum hungatei.* Cells have a unique spiral shape that is not found in other methanogens. Cell walls consist of an inner protein S-layer and a rigid paracrystalline outer sheath conferring the α-helical spiral shape of the cells (Sprott and McKellar 1980; Sprott et al. 1983). Cells usually grow as single cells or short filaments within their sheath. The cellular lipid of *M. hungatei* contains two unusual phosphoglycolipids, which are derivatives of the dibiphytanyl diglycerol tetraether. One of the free hydroxyls of this tetraether is esterified with glycerophosphoric acid, and the other is linked to a disaccharide (Kushwaha et al. 1981).

The family *Methanocorpusculaceae* is represented by the genus *Methanocorpusculum*. Cells are irregular cocci with diameters generally <1 μm. All species can use formate in addition to H_2 as electron donor for methanogenesis. For some species, secondary alcohols are alternative electron donors. Acetate and either yeast extract, peptones, or rumen fluid are required as carbon sources. The habitats of *Methanocorpusculum* are usually anaerobic digesters or freshwater sediments. They have not been found in marine environments.

The family *Methanoregulaceae* is divided into three genera (Sakai et al. 2012). The 16S rRNA gene sequence similarities between different genera are 93–96%, suggesting that they are sufficiently distinctive at genus level. Both *Methanolinea* (Imachi et al. 2008; Sakai et al. 2012) and *Methanoregula* (Brauer et al. 2006; Wang et al. 2009) are represented by two species, while *Methanosphaerula* is represented by one (Cadillo-Quiroz et al. 2009). *Methanolinea* is morphologically distinct from other *Methanomicrobiales* by forming rod-shaped, multicellular filaments within a sheath-like structure. *Methanoregula* and *Methanosphaerula* are distinguished from others by their acidophilic growth.

The assignment of *Methanocalculus* into a novel family is tentative. The 16S rRNA sequence similarities between all known *Methanocalculus* species are >98%, but those between *Methanocalculus* and other methanogens are <91%. Different species of *Methanocalculus* exhibited <10–51% DNA relatedness. The closest neighbor of *Methanocalculus* in the phylogenic tree based on 16S rRNA gene is *Methanocorpusculum*. All members of *Methanocalculus* are irregular cocci, can only use H_2 and CO_2 or formate for methanogenesis, and require acetate for growth.

All members of the order *Methanomicrobiales* produce methane using CO_2 as the electron acceptor and H_2 as the electron donor. Most species use formate and many species also use secondary alcohols as alternative electron donors, while two unique species can also grow on primary alcohols. They cannot use acetate and methyl-group containing compounds for methanogenesis. Most species are mixotrophic and require acetate as a carbon source; some species also require additional organic growth factors. Their morphologies are diverse, including cocci, rods, and sheathed rods. Most cells have single-layered protein cell walls, but cells of *Methanospirillum hungatei* are surrounded by an external sheath. Peptidoglycan and pseudomurein are absent. The cellular lipids contain archaeol and caldarchaeol as core lipids. Hydroxyarchaeol is absent. Glucose, galactose, aminopentanetetrols, and glycerol are common polar lipids; and aminopentanetetrols are unique to this order of organisms. Motility varies between species. Most species are mesophilic, with the exceptions of two psychrophilic species (*Methanogenium marinum* and *Methanogenium frigidum*) and one thermophilic species (*Methanoculleus thermophilicus*). Most species grow best near neutral pH. Exceptions are *Methanoregula boonei* and *Methanosphaerula palustris*, which have an optimal pH of 5.1~5.7 and were isolated from acidic peat bog; and *Methanocalculus alkaliphilus* and *Methanocalculus natronophilus*, which grow best at pH of 9.5 and were isolated from soda lake sediments. Many species are marine organisms and grow optimally with 0.1–1 M of NaCl. Descriptive properties of the *Methanomicrobiales* are summarized in Table 10. Further information can be found in Boone et al. (2001b) and Garcia et al. (2006).

Table 10 Descriptive characteristics of the species of the order *Methanomicrobiales* (Modified from Liu (2010c))

Organism	Type strain	Source[a]	Dimensions (μm)	Flagella	Methano-genesis substrates[b]
Methanoculleus					
bourgensis	MS2	Anaerobic digestor	Ø 1–2	None	$H_2 + CO_2$, formate, (2-propanol, 2-butanol)
chikugoensis	MG62	Paddy field soil	Ø 1–2	Flagellated[d]	$H_2 + CO_2$, formate, 2-propanol, 2-butanol, cyclopentanol
horonobensis	T10	Deep subsurface groundwater	Ø 0.7–1.6	Flagellated[d]	$H_2 + CO_2$, formate
hydrogenitrophicus	HC	Wetland soil	Ø 0.8–2	None	$H_2 + CO_2$
marisnigri	JR1	Black sea sediments	Ø <1.3	Peritrichous[d]	$H_2 + CO_2$, formate, 2-propanol, 2-butanol
palmolei	INSLUZ	Anaerobic digestor	Ø1.25–2	Flagellated[d]	$H_2 + CO_2$, formate, 2-propanol, 2-butanol, cyclopentanol
receptaculi	ZC-2	Oil field	Ø 0.8–1.7	None	$H_2 + CO_2$, formate
sediminis	S3Fa	Deep marine sediments	Ø 0.5–1.0	None	$H_2 + CO_2$, formate
submarinus	Nankai-1	Deep marine sediments	Ø 0.8–2.0	Flagellated[d]	$H_2 + CO_2$, formate
taiwanensis	CYW4	Deep marine sediments	Ø 0.6–1.5	None	$H_2 + CO_2$, formate
thermophilus	CR-1	Nuclear power plant sediment	Ø 0.6–1.8	Single[e]	$H_2 + CO_2$, formate
Methanofollis					
aquaemaris	N2F9704	Marine–water fish pond	Ø 1.2–2.0	None	$H_2 + CO_2$, formate
ethanolicus	HASU	Lotus field	Ø 2.0–3.0	nd	$H_2 + CO_2$, formate, ethanol, 1-propanol, 1-butanol
formosanus	ML15	Marine–water fish pond	Ø 1.5–2.0	None	$H_2 + CO_2$, formate
liminatans	GKZPZ	Wastewater reactor	Ø 1.25–2.0	Flagellated[f]	$H_2 + CO_2$, formate, 2-propanol, 2-butanol, cyclopentanol

Required organic compounds	Temperature range (optimum) (°C)	pH range (optimum)	NaCl optimum (%, w/v)	Doubling time[c] (h)	GC content (mol%)	References
ac	37–45 (35–40)	5.5–8.0 (6.7)	0.2–1	18	59 (Bd)	(Ollivier et al. 1986)
ac, YE/TP	15–40 (25–30)	6.7–8.0 (6.7–7.2)	0.6	46	62.2 (LC)	(Dianou et al. 2001)
None	25–45 (37–42)	5.8–8.2 (6.7–6.8)	0.6–1.2	6.3–6.9	62.9 (LC)	(Shimizu et al. 2013)
None	18–45 (37)	5.0–8.5 (6.6)	1.2	22.4	60.2 (T_m)	(Tian et al. 2010)
TP	10–45 (20–25)	5.8–7.6 (6.2–6.6)	0.6–1.1	10	61 (Bd)	(Romesser et al. 1979)
ac	22–50 (40)	6.5–8.0 (6.9–7.5)	nd	13.5	59.5 (LC)	(Zellner et al. 1998)
ac	<30–65 (50–55)	6.5–8.5 (7.5–7.8)	1.2	8.3	55.2 (T_m)	(Cheng et al. 2008)
ac	20–50 (37)	5.6–7.5 (7.1)	1.0	15.1	62.3 (G_s)	(Chen et al. 2015)
ac	>10–<55 (45)	5.0–8.7 (6.0–7.5)	0.6–2.3	~6.8	nd	(Mikucki et al. 2003)
None	20–42 (37)	6.5–8.1 (8.1)	0.5	6.7	61.0 (LC)	(Weng et al. 2015)
ac, TP, vit	37–65 (55–60)	6.2–7.8 (6.5–7.2)	1.2	2.5	55–60 (T_m)	(Rivard and Smith 1982)
None	20–43 (37)	6.3–8.0 (6.5)	0.5	13	59.1 (T_m)	(Lai and Chen 2001)
ac[g]	15–40 (37)	6.5–7.5 (7.0)	0	72[i]	60.9 (LC)	(Imachi et al. 2009)
YE, TP	20–42 (40)	5.6–7.3 (6.6–7.0)	3	36	58.4 (T_m)	(Wu et al. 2005)
ac	≥15–44 (40)	nd (7)	0–3.5	7.5	60 (T_m)	(Zellner et al. 1990)

(*continued*)

Table 10 (continued)

Organism	Type strain	Source[a]	Dimensions (μm)	Flagella	Methanogenesis substrates[b]
tationis	Chile 9	Solfataric pool mud	Ø 1.5–3	Peritrichous[f]	$H_2 + CO_2$, formate
Methanogenium					
cariaci	JR1	Marine sediments	Ø <2.6	Pertrichous	$H_2 + CO_2$, formate
frigidum	Ace-2	Anoxic Ace Lake water	Ø 1.5–2.5	None	$H_2 + CO_2$, formate
marinum	AK-1	Marine sediments	Ø 1–1.2	Flagellated[d]	$H_2 + CO_2$, formate
organophilum	CV	Marine mud	Ø 0.5–1.5	None	$H_2 + CO_2$, formate, ethanol, 1-propanol, [1-butanool], 2-propanol, 2-butanol,
Methanolacinia					
paynteri	G2000	Marine sediment	0.6 × 1.5–2.5	Flagellated[d]	$H_2 + CO_2$, 2-propanol, 2-butanol
petrolearius	SEBR4847	Offshore oil field	Ø 1–3	None	$H_2 + CO_2$, formate, 2-propanol
Methanomicrobium					
mobile	BP	Bovine rumen	0.7 × 1.5–2.0	Single	$H_2 + CO_2$, formate
Methanoplanus					
endosymbiosus	MC1	Marine ciliate	0.5–1 × 1.6–3.4	peritrichous	$H_2 + CO_2$, formate
limicola	M3	Swamp	0.1–0.3 × 1.5–2.8	Polar tuft	$H_2 + CO_2$, formate
Methanospirillum					
hungatei	JF-1	Sewage sludge	0.4–0.5 × 7.4–10 (often 15– > 100)	Polar tufts	$H_2 + CO_2$, formate
lacunae	Ki8-1	Puddly soil	0.5–0.6 × 11–25 (often 8–26)	Single or tufted	$H_2 + CO_2$, formate
psychrodurum	X-18	Wetland soil	0.4–0.5 × 11–62	None	$H_2 + CO_2$, formate
stamsii	Pt1	Anaerobic digestor	04–0.5 × 7–25 (sometimes 15– > 100)	tufted5	$H_2 + CO_2$, [formate]
Methanocorpusculum					
bavaricum	SZSXXZ	Sediment of wastewater treatment pond	Ø <1	Flagellated	$H_2 + CO_2$, formate, 2-propanol, 2-butanol

Required organic compounds	Temperature range (optimum) (°C)	pH range (optimum)	NaCl optimum (%, w/v)	Doubling time[c] (h)	GC content (mol%)	References
ac, YE, TP, tung	≥15–44 (40–44)	6.3–8.8 (7)	0.8–1.2	12	54 (T_m)	(Zabel et al. 1984)
ac, YE	10–32 (20–25)	nd (6.8–7.3)	2.7	11	52 (Bd)	(Romesser et al. 1979)
ac	−12[h]–18 (15)	6.5–7.9 (7.5–7.9)	2–3.5	69.6	nd	(Franzmann et al. 1997)
ac	5–25 (25)	5.5–7.5 (6.0)	1.5–7.3	42	nd	(Chong et al. 2002)
ac, PABA, biotin, tung, vit-B_{12}	nd-39 (30–35)	nd (6.4–7.3)	2.0	6	46.7 (T_m)	(Widdel et al. 1988)
ac	20–45 (40)	6.6–7.3 (7.0)	0.88	4.8	44.9 (Bd)	(Rivard et al. 1983)
ac	28–43 (35–40)	5.3–8.2 (7.0)	1–3	10	50 (LC)	(Ollivier et al. 1997, Göker et al. 2014)
Complex	35–45 (40)	5.9–7.7 (6.1–6.9)	nd	nd	48.8 (Bd)	(Paynter and Hungate 1968)
p-Cresol, tung	16–36 (32)	6.1–8.0 (6.8–7.3)	1.5	7	38.7 (T_m)	(Bruggen et al. 1986)
ac	17–41 (40)	nd (6.5–7.5)	1	7	47.5 (T_m)	(Wildgruber et al. 1982)
(ac)	45 (15–50)	6.5–10.0 (7.5–8.5)	0	20.7	45 (Bd)	(Ferry et al. 1974, Iino et al. 2010)
ac/YE	15–37 (30)	6.0–9.5 (7.2–7.5)	0	32.3	45.3 (LC)	(Iino et al. 2010)
YE	15–35 (30)	6.5–8.0 (7.0)	0–0.6	10.7	44.4 (LC)	(Iino et al. 2010)
None	5–37 (20–30)	6.0–10 (7.0–7.5)	0	39.8	40.0 (T_m)	(Parshina et al. 2014)
RF	15–45 (37)	nd (7.0)	nd	~5	51 (LC)	(Zellner et al. 1989)

(*continued*)

Table 10 (continued)

Organism	Type strain	Source[a]	Dimensions (μm)	Flagella	Methanogenesis substrates[b]
labreanum	Z	Lake sediments	Ø 0.4–2.0	None	$H_2 + CO_2$, formate
parvum	XII	Anaerobic digestor	Ø <1	Single	$H_2 + CO_2$, formate, 2-propanol, 2-butanol
sinense	China Z		Ø <1	Flagellated	$H_2 + CO_2$, formate
Methanocalculus					
alkaliphilus	AMF2	Hypersaline soda lake sediments	Ø 1.5–2.5	Peritrichous	$H_2 + CO_2$, formate
chunghsingensis	K1F9705b	Marine water fishpond	Ø 0.7–1.8	Flagellated[e]	$H_2 + CO_2$, formate
halotolerans	SEBR 4845	Oilfield	Ø 0.8–1.0	Peritrichous	$H_2 + CO_2$, formate
natronophilus	Z-7105	Soda lake sediments	Ø 0.2–1.2	Peritrichous	$H_2 + CO_2$, formate
pumilus	MHT-1	Waste disposal site	Ø 0.8–1.0	None	$H_2 + CO_2$, formate
taiwanensis	P2F9704a	Estuary	Ø 0.9–1.4	None	$H_2 + CO_2$, formate
Methanolinea					
mesophila	TNR	Rice field soil	0.3 × 2.0–6.5	nd	$H_2 + CO_2$, formate
tarda	NOBI-1	Sewage sludge	0.7–1.0 × 2.0	None	$H_2 + CO_2$, formate
Methanoregula					
boonei	6A8	Acid peat bog	0.2–0.3 × 0.8–3.0	Flagella-like filaments	$H_2 + CO_2$
Methanosphaerula					
palustris	E1-9c	Minerotrophic fen peatland	Ø 0.5–0.8	Multiple	$H_2 + CO_2$, formate

Abbreviations: *nd* not determined, *RF* rumen fluid, *ac* acetate, *(ac)* acetate required or stimulatory depending on the strain, *PABA* *p*-aminobenzoate, *vit* vitamins, *tung* tungsten, *TP* trypticase peptones, *YE* yeast extract, *CoM* 2-mercaptoethanesulfonic acid (conenzyme M), *Bd* buoyant density method, T_m melting point method, *LC* liquid chromatography, G_s genome sequencing

[a]Environment from which the type strain was isolated

[b]Parentheses mean utilized by some strains, but not all strains; brackets indicate very poor growth and methane production

[c]Doubling time of the type strain under optimal growth conditions of temperature, pH, and NaCl

[d]Nonmotile, although flagella are detected by electron microscopy

[e]Present in some strains

[f]Some strains are non-motile

[g]Acetate is not required for growth on ethanol

[h]The minimum growth temperature is predicted by applying the Ratkowsky model to temperature growth data

[i]Calculated from cultures that grow on ethanol

Required organic compounds	Temperature range (optimum) (°C)	pH range (optimum)	NaCl optimum (%, w/v)	Doubling time[c] (h)	GC content (mol%)	References
YE/TP	<45 (37)	6.5–7.5 (7.0)	0–1.5	~10	50 (Bd)	(Zhao et al. 1989)
ac, YE, tung	15–45 (37)	nd (6.8–7.5)	0–4.7	8	48.5 (T_m)	(Zellner et al. 1987, 1989)
RF	15–45 (30)	nd (7.0)	0	~20	50.0 (LC)	(Zellner et al. 1989)
ac	nd–41 (35)	8–10.2 (9.5)	3.5	nd	51.1 (T_m)	(Sorokin et al. 2015)
ac	20–45 (37)	5.8–7.7 (7.2)	0.5–1.0	7	50.3–50.8 (T_m)	(Lai et al. 2004)
ac	25–45 (38)	7.0–8.4 (7.6)	5	12	55 (LC)	(Ollivier et al. 1998)
ac	15–45 (35)	8.0–10.2 (9.0–9.5)	8.0–11.1	nd	50.2 (T_m)	(Zhilina et al. 2013)
ac	24–45 (35)	5.5–9.0 (6.5–7.5)	1	12	51.9 (LC)	(Mori et al. 2000)
ac	25–42 (37)	5.6–8.3 (6.7)	0.5	7.1	nd	(Lai et al. 2002)
ac	20–40 (37)	6.5–7.4 (7.0)	0	28.8	56.4 (LC)	(Sakai et al. 2012)
ac, YE	35–55 (50)	6.7–8.0 (7.0)	0	98	nd	(Imachi et al. 2008)
ac, YE, coM, vit	10–40 (35–37)	4.5–5.5 (5.1)	<0.1	40.8	54.5 (G_s)	(Brauer et al. 2006, 2011)
ac, CoM, vit	14–35 (30)	4.8–6.4 (5.7)	<0.2	30	58.9 (G_s)	(Cadillo-Quiroz et al. 2008, 2009)

2.4 Methanosarcinales

The order *Methanosarcinales* is divided into three families, *Methanosarcinaceae*, *Methanosaetaceae* and *Methermicoccus* based on phenotypic properties and 16S rRNA gene sequence analysis (Cheng et al. 2007). The three families are distinguished by 16S rRNA sequence similarities below 91% and differences in substrates for methanogenesis, lipid components, and cell wall structures. The *Methanosarcinaceae* are all capable of producing methane from methyl group containing compounds, and some can use acetate or H_2/CO_2. The cells can form aggregates within an outer layer composed of heteropolysaccharide. The *Methanosaetaceae* can only produce methane by splitting acetate. The cells can form chains within a proteinaceous sheath. The family *Methermicoccus* is represented by only one species, which is a thermophilic, methylotrophic methanogen isolated from an oilfield (Cheng et al. 2007).

The family *Methanosarcinaceae* currently comprises eight genera, *Methanococcoides*, *Methanohalobium*, *Methanohalophilus*, *Methanolobus*, *Methanomethylovorans*, *Methanosalsum*, *Methanimicrococcus* and *Methanosarcina*. The genus *Methanosarcina* can be differentiated from other genera by the unique morphology of pseudosarcinae or large cysts, which are formed by aggregation of cells within a common outer layer. The outer layer is composed of heteropolysaccharide, consisting mainly of galactosamine, glucose, mannose, and galacturonic acid. Some *Methanosarcina* species can also be distinguished from other genera of *Methanosarcinaceae* by their ability to split acetate for methanogenesis. The genus *Methanohalobium* is represented by a single species, *M. evestigatum*, which is an extreme halophile that requires 4 M of NaCl for optimal growth. The genus *Methanosalsum* is represented by *M. zhilinae* and *M. natronophilum*, which are moderate halophiles and alkaliphiles. The genus *Methanohalophilus* comprises moderate halophilic and halotolerant species, which grow best with 1–2 M of NaCl. The genera *Methanococcoides* and *Methanolobus* are difficult to differentiate by phenotypic properties, as they all use methylated compounds for methanogenesis; they require phylogenetic analysis for taxonomy. The genus *Methanimicrococcus* is represented by a single spcies *Methanimicrococcus blatticola*, which is a dominant methylotrophic methanogen in the cockroach hindgut (Sprenger et al. 2000). It has 83.4–89.8% 16S rRNA gene sequence similarities with other species of *Methanosarcinales*, suggesting that it could potentially represent a new family. This is further supported by the fact that it cannot disproportionate methyl-group containing compounds, a feature shared by all other *Methanosarcinaceae* spp. Instead, methanol and methylated amines must be reduced with H_2 for methanogenesis. This obligately hydrogenotrophic and methylotrophic mode of growth is shared with *Methanosphaera* and *Methanomassiliicoccus*, which belongs to the *Methanobacteriales* and *Methanomassiliicoccales*, respectively.

Members of the family *Methanosaetaceae* use acetate as the sole energy source. Acetate and CO_2 serve as carbon sources. Cells form filament-like structures within the sheath, which is composed predominantly with proteins and contains carbohydrates.

Methanogens from only two genera, *Methanosarcina* and *Methanosaeta*, can use acetate as a substrate for methanogenesis. However, they metabolize acetate differently. *Methanosarcina* is a relative generalist that prefers methanol and methylamine to acetate, and many species also utilize H_2. *Methanosaeta* is a specialist that uses only acetate. *Methanosaeta* is a superior acetate utilizer in that it can use acetate at concentrations as low as 5–20 μM, while *Methanosarcina* requires a minimum concentration of about 1 mM (Jetten et al. 1992). The difference of acetate affinity is probably due to different systems for acetate activation. Moreover, based upon their genome sequences, these two genera probably have different modes of electron transfer and energy conservation, even though the methanogenesis pathways are likely to be similar (Smith and Ingram-Smith 2007).

The family *Methermicoccus* is represented by *Methermicoccus shengliensis*. Its closest neighbor in the 16S rRNA phylogenetic tree is *Methanosaeta* (< 90.7% sequence similarities). It is morphologically differentiated from *Methanosaeta* by its coccoid-shape and formation of large cysts. Moreover, *M. shengliensis* uses methanol and methylated amines, but not acetate, for methanogenesis.

Members of the order *Methanosarcinales* have the widest substrate range among methanogens. All members can produce methane by disproportionating methyl-group containing compounds (methanol, methylamines, methylethanolamines, betaine, or methyl sulfides) or by splitting acetate. Some mesophilic *Methanosarcia* species can reduce CO_2 with H_2, but formate, secondary alcohols, and ethanol are not used as electron donors. Recently, it has been shown that *Methermicoccus* spp. are surprisingly capable of growth and methane production using methoxylated aromatic compounds (MACs) such as methoxy-benzoate (Mayumi et al. 2016). Ammonium and sulfide serve as the major nitrogen and sulfur sources, respectively. Their cellular morphologies are diverse, including cocci, pseudosarcinae, and sheathed rods. Most cells have protein cell walls, and some cells are surrounded by a sheath or acidic heteropolysaccharide. Most strains are nonmotile. The cellular lipids contain archaeol, hydroxyarchaeol, and caldarchaeol. Polar lipids can contain glucose, galactose, mannose, *myo*-inositol, ethanolamine, serine, and glycerol, depending upon the species. Most species of *Methanosarcinales* are mesophilic. Four species are moderately thermophilic (*Methanosarcina thermophila*, *Methanomethylovorans thermophila*, *Methanosaeta thermophila*, and *Methermicoccus shengliensis*), and six species are psychrotolerant (*Methanococcoides alaskense*, *Methanococcoides burtonii*, *Methanosarcina lacustris*, *Methanosarcina soligelidi*, *Methanosarcina splelaei*, and *Methanosarcina baltica*). Most species grow best at near neutral pH, except for three species that are alkaliphilic (*Methanolobus oregonensis*, *Methanolobus taylorii*, *Methanosalsum natronophilum*, and *Methanosalsum zhilinae*). Many species were isolated from marine environments and require a salinity near that of seawater for optimal growth. Some species are halophilic or halotolerant. Descriptive properties of members of the *Methanosarcinales* are summarized in Table 11. Further information can be found in Boone et al. (2001c) and Kendall and Boone (2006).

Table 11 Descriptive characteristics of the species of the order *Methanosarcinales* (Modified from Liu (2010d))

Organism	Type strain	Source[a]	Dimensions (μm)	Flagella	Methano-genesis substrates[b]
Methanococcoides					
alaskense	AK-5	Marine sediments	1.5–2.0	Flagellated[d]	(Methanol), TMA
burtonii	DSM 6242	Hypolimnion of ice lake	0.8–1.8	Monotrichous	Methanol, $MeNH_2$
methylutens	TMA-10	Submarine canyon sediments	1.0	None	Methanol, $MeNH_2$
vulcani	SLH33	Marine sediments	0.6–1.7	Single to four	Methanol, $MeNH_2$, TMA, DMA, betaine, choline, DMEA
Methanohalobium					
evestigatum	Z-7303	Saline lagoon sediments	0.2–2	None	$MeNH_2$
Methanohalophilus					
euhalobius	283	Mineralized stratal waters of oil deposits	1.0–2.5	None	Methanol, $MeNH_2$
halophilus	Z-7982	Salinarium sediments	0.5–2.0	None	(Methanol), $MeNH_2$
levihalophilus	GTA13	Palaeo-seawater	0.7–1.0	None	TMA, DMA
mahii	SLP	Salt lake sediments	1.0	None	Methanol, $MeNH_2$
portucalensis	FDF-1	Salinarium sediments	0.6–2.0	None	Methanol, $MeNH_2$
Methanolobus					
bombayensis	B-1	Marine sediments	1.0–1.5	None	Methanol, $MeNH_2$, DMS
chelungpuianus	St54 5 Mb	Deep fault sandstone	0.5–0.7	None	Methanol, TMA
oregonensis	WAL1	Alkaline, saline aquifer	1.0–1.5	None	Methanol, $MeNH_2$, DMS
profundi	MobM	Deep sediments of a natural gas field	0.9–1.2	Multiple	methanol, $MeNH_2$, DMA, TMA
taylorii	GS-16	Estuarine sediments	0.5–1.0	None	Methanol, $MeNH_2$, DMS
tindarius	Tindari3	Marine sediments	0.8–1.25	Monotrichous	Methanol, $MeNH_2$
vulcani	PL-12/M	Marine sediments	0.8–1.25	None	Methanol, $MeNH_2$
zinderi	SD1	Saline coal seam	1.0–2.0	None	Methanol, $MeNH_2$, DMA, TMA

Organic growth factors	Temperature range (optimum) (°C)	pH range (optimum)	NaCl range (optimum) (M)	Doubling time[c] (h)	GC content (mol%)	References
None	−2.3–30.6 (23.6)	6.3–7.5 (7.5)	0.1–0.8 (0.3–0.4)	~85	39.5–41.9 (T_m)	(Singh et al. 2005)
None	−2.54[f]–29.5 (23.4)	6.8–8.2 (7.7)	0.2–0.5 (0.2)	24	39.6 (T_m)	(Franzmann et al. 1992)
Biotin	15–35 (30–35)	6.0–8.0 (7.0–7.5)	0.1–1.0 (0.4)	5.2	42 (T_m)	(Sowers and Ferry 1983)
None	nd–35 (30)	6–7.8 (7.0)	0.08–1.02 (0.5)	21	43.4 (LC)	(L'Haridon et al. 2014)
Vit	25–60 (50)	6.0–8.3 (7.0–7.5)	1.7–5.1 (4.3)	nd	37 (T_m)	(Zhilina and Zavarzin 1987a)
Biotin	15–50 (28–37)	5.8–8.0 (6.8–7.3)	0.16–2.3 (1.0)	nd	43.0	(Davidova et al. 1997)
None	18–42 (26–36)	6.3–7.4 (6.5–7.4)	0.3–2.6 (1.2–1.5)	nd	41–44 (T_m)	(Wilharm et al. 1991)
Vit	20–40 (35)	6.2–8.3 (7.0–7.5)	0.2–1.3 (0.35–0.4)	18	43.7	(Katayama et al. 2014)
Biotin, thiamine	10–45 (35)	6.8–8.2 (7.5)	0.4–3.5 (2.0)	nd	48.5 (Bd)	(Paterek and Smith 1988)
Biotin	>25–45 (40)	6.2–8.2 (7.2)	0.5–3.5 (2)	~7	43–44 (Bd)	(Boone et al. 1993a)
None	20–42 (37)	6.2–8.2 (7.2)	0.3–2 (0.5)	4.4	39.2 (LC)	(Kadam et al. 1994)
None	24–45 (37)	6.8–7.4 (7.0)	0–0.678 (0–0.08)	7.6	48.3 (LC)	(Wu and Lai 2011)
Biotin, thiamine	25–42 (35)	8.2–9.2 (8.6)	0.1–1.6 (0.35)	7	40.9 (LC)	(Liu et al. 1990)
None	9–37 (30)	6.1–7.8 (6.5)	0.1–1.0 (0.35)	5	42.4 (LC)	(Wu and Lai 2011)
Biotin	5–42 (37)	5.5–9.2 (8)	0.2–1.2 (0.5)	nd	40.8 (LC)	(Oremland and Boone 1994)
None	10–45 (25)	5.5–8.0 (6.5)	0.06–1.27 (0.5)	nd	40 (T_m)	(Konig and Stetter 1982)
Biotin	13–45 (40)	6.0–7.5 (7.2)	0.1–1.2 (0.5)	5.3	39 (Bd)	(Kadam and Boone 1995)
None	25–50 (45–50)	6.0–9.0 (7.0–8.0)	0.05–1.8 (0.2–0.6)	~9.9	42 (T_m)	(Doerfert et al. 2009)

(*continued*)

Table 11 (continued)

Organism	Type strain	Source[a]	Dimensions (μm)	Flagella	Methanogenesis substrates[b]
Methanomethylovorans					
hollandica	DMS1	Freshwater sediments	1–1.5	None	Methanol, $MeNH_2$, MT, DMS
thermophila	L2FAW	UASB reactor	0.7–1.5	None	Methanol, $MeNH_2$
uponensis	EK1	Wetland sediment	0.9–1.1	nd	Methanol, $MeNH_2$, DMA, TMA, DMS, MT
Methanosalsum					
natronophilum	AME2	Hypersaline soda lake sediments	0.7–2	None	Methanol, TMA, DMS
zhilinae	WeN5	Alkaline, saline lake sediments	0.75–1.5	Mono/ ditrichous	Methanol, $MeNH_2$, DMS
Methanosarcina					
acetivorans	C2A	Marine sediments	1.7–2.1	None	ac, methanol, $MeNH_2$, CO
baltica	GS1-A	Marine sediments	1.5–3.0	Monotrichous	ac, methanol, $MeNH_2$
barkeri	MS	Sewage sludge	1.5–2.0	None	$H_2 + CO_2$, ac, methanol, $MeNH_2$, CO
horonobensis	HB-1	Deep subsurface groundwater	1.4–2.9	None	Methanol, DMA, TMA, DMS, ac
lacustris	ZS	Lake sediments	1.5–3.5	None	$H_2 + CO_2$, methanol, $MeNH_2$
mazei	S-6	Sewage sludge	1.0–3.0	None	($H_2 + CO_2$), (ac), methanol, $MeNH_2$
semesiae	MD1	Mangrove sediment	0.8–2.1	nd	Methanol, $MeNH_2$, MT, DMS
siciliae	T4/M	Marine canyon sedimetns	3.4	nd	Methanol, $MeNH_2$, DMS
soligelidi	SMA-21	Permafrost-affected soil	1.3–2.5	nd	$H_2 + CO_2$, methanol, ac
splelaei	MC-15	Sulphurous subsurface lake	2.0–4.0	nd	$H_2 + CO_2$, methanol, ac, methanol, $MeNH_2$, DMA, TMA
subterranea	HC-2	Subsurface groundwater	0.9–1.4	None	Methanol, $MeNH_2$, DMA, TMA, DMS
thermophila	TM-1	Anaerobic digestor	100[e]	None	ac, methanol, $MeNH_2$, CO

Organic growth factors	Temperature range (optimum) (°C)	pH range (optimum)	NaCl range (optimum) (M)	Doubling time[c] (h)	GC content (mol%)	References
Vit	12–40 (34–37)	6.0–8.0 (6.5–7.0)	0–0.3 (0–0.04)	11.6	34.4 (T_m)	(Lomans et al. 1999)
None	42–58 (50)	5–7.5 (6.5)	<0.3 (0–0.1)	14	37.6 (T_m)	(Jiang et al. 2005)
None	25–40 (37)	5.5–7.5 (6.0–6.5)	0–0.1 (0)	11.6	39.2 (T_m)	(Cha et al. 2013)
None	nd–43 (37)	8.2–10.2 (9.5)	0.5–3.5 (1.5)	nd	44.8 (T_m)	(Sorokin et al. 2015)
None	20–50 (45)	8.0–10 (9.2)	0.2–2.1 (0.4–0.7)	6	39.5 (T_m)	(Mathrani et al. 1988)
None	15–48 (35–40)	5.4–8.5 (6.5–7.0)	0.1–1.0 (0.2)	5.2	41 (T_m)	(Sowers et al. 1984)
None	4–27 (25)	4–8.5 (6.5–7.5)	0.1–0.7 (0.3–0.4)	84	nd	(von Klein et al. 2002)
None	25–50 (30–40)	5.5–7.5 (7.0)	0.1–0.7 (<0.2)	nd	39–44 (Bd)	(Bryant and Boone 1987)
None	20–42 (37)	6.0–7.75 (7.0–7.25)	0–0.35 (0.1)	5.0	41.4 (LC)	(Shimizu et al. 2011)
YE	1–35 (25)	4.5–8.5 (7.0)	nd (nd)	49	43.4 (T_m)	(Simankova et al. 2001)
None	25–45 (35–42)	5.5–8.0 (6.8–7.2)	0.1–0.7 (0.2–0.4)	7	42 (Bd)	(Mah and Kuhn 1984)
nd	18–39 (30–35)	6.2–8.3 (6.5–7.5)	>0– < 1.5 (0.2–0.6)	3.9	nd	(Lyimo et al. 2000)
None	15–42 (40)	5.0–7.8 (6.5–6.8)	0.2–0.8 (0.4–0.6)	7	41–43	(Elberson and Sowers 1997)
None	0–54 (28)	4.8–9.9 (7.8)	0.02–0.6 (0.02)	122.4	40.9 (LC)	(Wagner et al. 2013)
None	0–54 (33)	4.0–10.0 (6.5)	0.02–0.6 (0.05)	122.4	39.0 (LC)	(Ganzert et al. 2014)
None	10–40 (35)	5.9–7.4 (6.6–6.8)	0–0.6 (0.1–0.2)	8.9	41.5 (LC)	(Shimizu et al. 2015)
PABA	<35–55 (50)	5.5–8.0 (6.0–7.0)	0–1.2 (0.6)	5.3	42 (Bd)	(Zinder et al. 1985)

(*continued*)

Table 11 (continued)

Organism	Type strain	Source[a]	Dimensions (μm)	Flagella	Methanogenesis substrates[b]
vacuolata	Z-761	Methanogenic digestor	1.0–2.0	None	$H_2 + CO_2$, ac, methanol, $MeNH_2$
Methanosaeta					
concilii	GP6	Sewage sludge	0.8–1.3 × 2.0–7.0	None	ac
harundinacea	8Ac	UASB reactor	0.8–1.0 × 3.0–5.0	None	ac
thermophila	P_T	Thermophilic anaerobic digestor	0.8–1.3 × 2.0–6.0	None	ac
Methanimicrococcus					
blatticola	PA	Cockroach hindgut	0.8	nd	Methanol, $MeNH_2$, H_2
Methermicoccus					
shengliensis	ZC-1	Oilfield	0.7–1.0	Flagellated	Methanol, $MeNH_2$, MACs

Abbreviations: *nd* not determined, *ac* acetate, *$MeNH_2$* methylamines, *DMS* dimethylsulfide, *MT* methanethiol, *TMA* trimethylamine, *DMA* dimethylamine, *DMEA N,N*-dimethylethanolamine, *MACs* methoxylated aromatic compounds, *vit* vitamins, *TP* trypticase peptone, *YE* yeast extract, *CoM* 2-mercaptoethanesulfonic acid (conenzyme M), *PAPA p*-aminobenzoate, *Bd* buoyant density method, T_m melting point method, *LC* liquid chromatography

[a]Environment from which the type strain was isolated

[b]Parentheses means utilized by some strains, but not all strains

[c]Doubling time of the type strain under optimal growth conditions of temperature, pH, and NaCl

[d]Flagellated in some strains, but not all strains

[e]Irregular aggregates composed of coccoid cells

[f]The minimum growth temperature is predicted by applying the Ratkowsky model to temperature growth data

Organic growth factors	Temperature range (optimum) (°C)	pH range (optimum)	NaCl range (optimum) (M)	Doubling time[c] (h)	GC content (mol%)	References
None	18–42 (37–40)	6.0–8.0 (7.5)	0.1–0.5 (0.1)	nd	36.3 (T_m)	(Zhilina and Zavarzin 1987b)
Vit	>10– ≤ 45 (35–40)	≥6.6– < 7.8 (7.1–7.5)	nd (nd)	65	49.0 (T_m)	(Patel and Sprott 1990)
YE/TP	25–45 (34–37)	6.5–9.0 (7.2–7.6)	nd (nd)	28	55.7 (T_m)	(Ma et al. 2006)
None	>30– ≤ 70 (55–60)	>5.5– ≤ 8.4 (6.5–6.7)	nd (nd)	35.8	52.7–54.3 (LC)	(Kamagata and Mikami 1991)
ac, CoM, YE, tryptic soy broth, vit	20–40 (39)	6.8–8.2 (7.2–7.7)	0–0.3 (<0.1)	3.1	nd	(Sprenger et al. 2000)
YE/TP	50–70 (65)	5.5–8.0 (6.0–6.5)	0.2–1.1 (0.3–0.5)	5	56 (T_m)	(Cheng et al. 2007; Mayumi et al. 2016)

2.5 Methanopyrales

The order of *Methanopyrales* is represented by only one species, *Methanopyrus kandleri*. It is hyperthermophilic and produces methane by CO_2 reduction with H_2. Genomic sequence analysis of *M. kandleri* suggests that it is closely related to *Methanobacteriales* and *Methanococcales* but possesses unusual features.

The phylogenetic position of *M. kandleri* is ambiguous. The phylogenic analyses based on 16S rRNA gene (Burggraf et al. 1991), elongation factor 1α (Rivera and Lake 1996), and transcription factors (Brochier et al. 2004) suggested that *M. kandleri* is distantly related to other methanogens and represent a separate lineage emerging at the base of the euryarchaeal phylum. On the other hand, phylogenetic analyses based on methyl coenzyme M reductase (MCR) operons (Nolling et al. 1996), translation factors (Brochier et al. 2004), and whole genome sequences (Slesarev et al. 2002; Gao and Gupta 2007) suggested that *M. kandleri* is more closely related to other methanogens and grouped with *Methanobacteriales* and *Methanococcales*. Indeed, *M. kandleri* encodes the core of proteins shared uniquely by methanogens such as proteins evolved in the methanogenesis pathway, and it closely resembles other methanogens in terms of local gene order. Therefore, *M. kandleri* very likely belongs to the monophyletic methanogen group and not a deep-branch close to the root of archaea. The deep branching in 16S rRNA phylogenetic tree is probably due to a very high GC content of *M. kandleri*, a characteristic shared by hyperthermophiles outside the methanogen group.

The genome of *M. kandleri* displays several unusual features (Slesarev et al. 2002; Brochier et al. 2004). The RNA polymerase subunit H is replaced by a homologous protein from a distantly related archael lineage. The transcription factor S (TFS) is missing. The diversity of predicted signal transduction systems and DNA-binding proteins are underrepresented. The histone protein is formed by a fusion of two monomers into a single peptide with two tandemly repeated histone folds. *M. kandleri* possesses a unique topoisomerase, Topo V, which is related to eukaryotic topoisomerase I (Slesarev et al. 1994). These unusual features suggest a high level of gene loss, gene capture, and gene fusion in this archaeon.

Methanopyrus kandleri is the only methanogen known so far that catalyzes methanogenesis at temperatures higher than 100 °C. It reduces CO_2 with H_2 for methanogenesis. It is an obligate chemolithoautotroph that uses CO_2 as the sole carbon source. Ammonium and sulfide are the nitrogen and sulfur sources, respectively. The cells are rod-shaped and stain Gram positive. The cell wall is double layered. The inner layer is composed of a new type of pseudomurein, containing ornithine and lysine. The outer layer is detergent-sensitive, indicating a protein composition. The core lipid is composed of an unsaturated terpenoid lipid, which is considered the most primitive lipid in the evolution of membranes (Hafenbradl et al. 1993). The cells are motile via flagella arranged as polar tufts. They grow at temperatures ranging from 84 °C to 110 °C, with an optimum of 98 °C. The range of pH for growth is 5.5–7, with an optimum of 6.5. The optimal NaCl concentration for growth is 2.0% (w/v). The GC content of its DNA is 60 mol%. *M. kandleri* was

isolated from hydrothermally heated deep-sea sediments and from a shallow marine hydrothermal system (Kurr et al. 1991).

2.6 Methanocellales

The order *Methanocellales* is represented by one family and genus, *Methanocellaceae* and *Methanocella*, respectively. Three species have been described, and they are distinguished by 16S rRNA sequence similarities below 92% and differences in growth temperatures, substrates for methanogenesis, possession of a flagellum, doubling time and NaCl range. The low 16S rRNA sequence similarities suggest potential separation into more genera, which is supported by comparative genomic studies (Sakai et al. 2011; Lyu and Lu 2015). The *Methanocella* are all capable of producing methane from H_2/CO_2, but acetate is required for growth. Formate can also be used as an alternative substrate by two species.

Members of *Methanocellales* are isolated from rice soils. They do not appear to grow autotrophically due to the requirement of acetate for growth. Sulfide and ammonium is a sufficient sulfur and nitrogen source, respectively. Cells are typically rods, but coccoid cells are also seen during late stage of growth. Cells can form a unique lens-shaped colony. Cell envelopes are composed of an S-layer as determined in *Methanocella avoryzae*. Cell envelopes have not been determined in *Methanocella paludicola* and *Methanocella conradii*, but they are resistant to lysis by 2.0% and 0.1% of SDS, respectively. A flagellum is also present in both *M. avoryzae* and *M. conradii*, but not in *M. paludicola*. Cellular lipids have not been determined. They all grow optimally in the absence of NaCl and at neutral pH. The optimal growth temperatures range from 37 °C to 55 °C. Descriptive properties of the *Methanocellales* are summarized in Table 12. Further information can be found in Sakai et al. (2008, 2010), and Lü and Lu (2012b).

2.7 Methanomassiliicoccales

The order *Methanomassiliicoccales* is represented by one family and genus, *Methanomassiliicoccaceae* and *Methanomassiliicoccus*, respectively (Dridi et al. 2012; Iino et al. 2013). Although a few enrichment cultures are available, only one species *Methanomassiliicoccus luminyensis* has been described (Borrel et al. 2012a, 2013; Dridi et al. 2012; Iino et al. 2013). This species was isolated from human faeces, and it reduces methanol with H_2 to produce methane. However, genomic, transcriptomic and *in vivo* studies suggest that members of *Methanomassiliicoccales* also reduce tri-, di- and monomethylamine with H_2 (Poulsen et al. 2013; Borrel et al. 2014; Brugere et al. 2014). Cells are non-motile cocci and lysed in 0.1% (w/v) SDS. It grows optimally at 1% of NaCl, 37 °C and at pH 7.6. Descriptive properties of the *Methanomassiliicoccales* are summarized in Table 13. Further information can be found in Dridi et al. (2012) and Brugere et al. (2014).

Table 12 Descriptive characteristics of the species of the genus *Methanocella*

	Methanocella		
Character	*paludicola*	*avoryzae*	*conradii*
Type strain	SANAE	MRE50	HZ254
Cell width (μm)	0.3–0.6	0.4–0.7	0.2–0.3
Cell length (μm)	1.8–2.4	1.3–2.8	1.4–2.8
Flagellum	None	Single	Single
Substrates for methanogenesis	$H_2 + CO_2$, formate	$H_2 + CO_2$, formate	$H_2 + CO_2$
Acetate requirement	+	+	+
Yeast extract stimulates growth	+	+	+
Nitrogen source	NH_3	NH_3[a]	NH_3[a]
Sulfur source	S^{2-}[b]	S^{2-}	S^{2-}[b]
Temperature range (°C)	25–40	37–55	37–60
Temperature optimum (°C)	35–37	45	55
pH range	6.5–7.8	6.0–7.8	6.4–7.2
pH optimum	7.0	7.0	6.8
NaCl range (%, w/v)	0–0.1	0–2	0–0.5
NaCl optimum (%, w/v)	0	0–0.2	0–0.1
GC content (mol%)[c]	54.9 (G_s)	54.6 (G_s)	52.7 (G_s)
Doubling time (h)	100.8	8.0	6.4–7.2
Source	Rice soil	Rice soil	Rice soil
References	(Sakai et al. 2008)	(Sakai et al. 2010)	(Lü and Lu 2012b)

[a]May use N_2 according to genomic predictions (Lyu and Lu 2015)
[b]May use SO_4^{2-} according to genomic predictions (Erkel et al. 2006; Sakai et al. 2011)
[c]G_s genome sequencing

2.8 Potential Novel Taxa

Through metagenomics guided discovery, a few potential novel taxa of methanogens have been proposed recently. That includes a euryarchaeon, *Candidatus* 'Methanofastidiosa', and members of the archaeal phyla Bathyarchaeota (previously known as the Miscellaneous Crenarchaeota Group) and Verstraetearchaeota previously represented by the Terrestrial Miscellaneous Crenarchaeota Group or TMCG) (Evans et al. 2015; Nobu et al. 2016; Vanwonterghem et al. 2016). They are all predicted to reduce different methylated compounds with H_2 for methanogenesis, but members of Bathyarchaeota and Verstraetearchaeota may also use complex substrates such as lactate. Pure cultures are still needed to further confirm these findings, which would likely not only lead to proposals of novel methanogen classes but establishment of methanogen taxa outside the Euryarchaeota.

Table 13 Descriptive characteristics of *Methanomassiliicoccus luminyensis*

Character	*M. luminyensis*
Type strain	B10
Cell diameter (μm)	0.7–1.0
Flagellum	None
Substrates for methanogenesis	H_2 + methanol/TMA[a]/DMA[b]/$MeNH_2$[c]
Acetate requirement	−
Yeast extract requirement	+
Temperature range (°C)	25–45
Temperature optimum (°C)	37
pH range	7.2–8.4
pH optimum	7.6
NaCl range (%, w/v)	0.1–1.5
NaCl optimum (%, w/v)	1
GC content (mol%)	59.9 (G_s)[d]
Doubling time	nd[e]
Source	Human faeces
References	(Dridi et al. 2012; Brugere et al. 2014)

[a]*TMA* trimethylamine
[b]*DMA* dimethylamine
[c]*$MeNH_2$* monomethylamine
[d]G_s genome sequencing
[e]*nd* not determined

3 Ecology of Methanogens

Methanogens are abundant in a wide variety of anaerobic habitats such as marine sediments, freshwater sediments, flooded soils, human and animal gastrointestinal tracts, anaerobic digestors, landfills, and geothermal systems (Liu and Whitman 2008). This cosmopolitan distribution of methanogens could be associated with their growth largely relied on only simple substrates such as H_2/CO_2, acetate, formate and other C1 compounds, which are widely available across ecosystems where complex substrates have to be degraded into simple substrates to drive the carbon cycle. A recent metagenomics survey has also predicted the presence of complex fermentation and β-oxidation pathways in the putative Bathyarchaeota methanogens, suggesting the ability of using complex substrates may be advantageous for methanogens that thrive in environments where degradation of complex substrates could be very slow (Evans et al. 2015). In addition, some methanogens as described in the taxonomy and phylogeny section can also survive extreme environmental conditions such as hyperthermophilic, psychrophilic, piezophilic, halophilic, alkaliphilic and acidophilic, which further expands their habitats.

In some natural habitats, methanogens are also present in microoxic environments. For example, members of *Methanobrevibacter* have been isolated from

large dental caries and subgingival plaque in the human mouth and gut periphery in termites. They are also somewhat oxygen tolerant, probably due to the presence of catalase activity and the protection by O_2-uptake aerobes (Brusa et al. 1987; Belay et al. 1988; Leadbetter and Breznak 1996). *Methanocellales* methanogens are prevalent in rice rhizosphere, which is transiently oxic, and their genomes encode a unique set of antioxidant enzymes, which may explain an aerotolerant life style (Erkel et al. 2006; Sakai et al. 2011; Lü and Lu 2012a; Lyu and Lu 2015, 2017).

In methanogenic habitats, electron acceptors such as O_2, NO_3^-, Fe^{3+}, and SO_4^{2-} are limiting. When electron acceptors other than CO_2 are present, methanogens are outcompeted by the bacteria that utilize them. This phenomenon occurs mainly because the reductions of these compounds are thermodynamically more favorable than CO_2 reduction to methane. However, because CO_2 is generated during fermentations, it is seldom limiting in anaerobic environments. Besides methanogens, homoacetogens are another group of anaerobes that can reduce CO_2 for energy production. However, acetogenesis with H_2 is thermodynamically less favorable than methanogenesis. Therefore, homoacetogens do not compete well with methanogens in many habitats. However, homoacetogens outcompete methanogens in some environments, such as the hindgut of certain termites and cockroaches. Possible explanations are their metabolic versatility as well as lower sensitivity to O_2. The ecology of each methanogen order is discussed below.

3.1 Methanobacteriales

Members of the *Methanobacteriales* are widely distributed in anaerobic habitats such as marine and freshwater sediments, soils, animal gastrointestinal tracts, anaerobic sewage digestors, and geothermal habitats. *Methanobacterium* has been cultivated from marine and freshwater sediments, groundwaters, soils, anaerobic digestors, and animal gastrointestinal tracts and has also been detected as endosymbionts in anaerobic ciliate (Embley et al. 1992). *Methanobrevibacter* has been isolated from rumens, feces, termite hindguts, human subgingival plaque, anaerobic digestors, and decaying wood tissues. *Methanosphaera* has only been isolated from animal gastrointestinal tracts but has been detected in anaerobic digestors (Weiss et al. 2008). *Methanothermobacter* has been cultivated from thermophilic anaerobic digestors and natural gas and oil fields (Nazina et al. 2006; Mochimaru et al. 2007). *Methanothermus* has only been isolated from solfarata hot springs.

3.2 Methanococcales

Members of the *Methanococcales* have all been isolated from marine environments. *Methanococcus* has been isolated from marine and salt marsh sediments.

Methanothermococcus has been isolated from coastal geothermally heated sea sediments, deep sea hydrothermal vents, and reservoir water from marine oil fields (Nilsen and Torsvik 1996) and has been detected in continental high-temperature oil reservoirs (Orphan et al. 2000) and tropical hypersaline coastal lagoons (Clementino et al. 2008). *Methanocaldococcus* has only been isolated from deep sea hydrothermal vents. *Methanotorris* has been isolated from shallow and deep sea hydrothermal vents. Environmental 16S rRNA sequences closely related to *Methanococcales* have also been detected in anaerobic granular sludge (Liu et al. 2002; Diaz et al. 2003). Quantitative real-time PCR assays have also recently shown possible presence of *Methanococcales* in forest and grassland soils, but how specific the primers were remain unknown (Hofmann et al. 2016). Since this finding is very much unexpected, sequence data is also needed to make conclusive taxonomy inference.

3.3 Methanomicrobiales

Members of the *Methanomicrobiales* are widely distributed in anaerobic habitats, including marine and freshwater sediments, anaerobic sewage digestors, rice paddies, oil fields, groundwaters, and animal gastrointestinal tracts. Anaerobic digestors and sewage sludge are common habitats of *Methanoculleus*, *Methanofollis*, *Methanocorpusculum*, *Methanospirillum*, and *Methanomicrobium*. From marine sediments, species belonging to *Methanoculleus*, *Methanogenium*, and *Methanolacinia* have been isolated. From freshwater sediments, species belonging to *Methanoculleus*, *Methanogenium*, and *Methanocorpusculum* have been isolated. From rice roots and rice-field soils, species belonging to *Methanoculleus* have been isolated, and environmental clone sequences closely related to *Methanoculleus* and *Methanogenium* have been identified (Kudo et al. 1997). *Methanomicrobium mobile* has been isolated from bovine rumen (Paynter and Hungate 1968). *Methanoplanus endosymbiosus* lives as endosymbiont of the marine ciliate *Metopus contortus* (Bruggen et al. 1986).

3.4 Methanosarcinales

Members of the *Methanosarcinales* are widely distributed in marine and freshwater sediments, anaerobic digestors, and animal gastrointestinal tracts. *Methanosarcina* has been isolated from marine and freshwater sediments, anaerobic digestors, and rumen and has been detected in rice paddies (Chin et al. 2004; Krüger et al. 2005; Lu et al. 2005). *Methanococcoides* and *Methanolobus* have been isolated from aquatic environments with salinity near that of seawater. The habitats of *Methanohalobium*, *Methanohalophilus*, and *Methanosalsum* are restricted to hypersaline environments. *Methanomethylovorans* has been isolated from freshwater sediments and bioreactors. *Methanosaeta* has been isolated from freshwater sediments and anaerobic digestors and has been detected in rice paddies (Chin et al.

2004; Krüger et al. 2005) and marine sediments (Purdy et al. 2002). *Methanimicrococcus* has been isolated from cockroach hindgut and has been detected in anaerobic digestors (Weiss et al. 2008).

3.5 Methanocellales

All members of the *Methanocellales* have been isolated from rice soils, but they are also widely distributed in terrestrial ecosystems such as wetland soils and freshwater sediments based on environmental DNA sequence surveys (Conrad et al. 2006; Sakai et al. 2008, 2010; Lü and Lu 2012b). *Methanocellales* have been studied extensively in rice soils both *in situ* and in microcosms, revealing the following unique ecophysiological features. (i) They are closely associated with rice roots where they can actively convert plant-derived carbon into biomass and methane (Lu and Conrad 2005); (ii) they are able to tolerate the microaerophilic conditions around the rice roots, probably due to a robust antioxidant system encoded in their genomes (Erkel et al. 2006; Conrad et al. 2008; Sakai et al. 2011; Lü and Lu 2012a; Lyu and Lu 2017); (iii) they tend to become more active under low H_2 but high temperature conditions (Lu et al. 2005; Wu et al. 2006; Peng et al. 2008; Sakai et al. 2009); and (iv) they frequently form syntrophic relationships with fatty acid degrading bacteria (Lueders et al. 2004; Liu et al. 2011; Rui et al. 2011; Gan et al. 2012). Additional ecophysiological features have also been revealed by studying *Methanocellales* in acidic peat soils, tank bromeliads and arid soils, suggesting that at least some members of *Methanocellales* could survive moderately acidic conditions, interact with plants other than rice such as *Sphagnum* in peat soil and tank bromeliads in neotropical forests, and tolerate desiccation (Sizova et al. 2003; Cadillo-Quiroz et al. 2010; Martinson et al. 2010; Angel et al. 2011, 2012).

3.6 Methanomassiliicoccales

Only one member of *Methanomassiliicoccales* has been isolated into pure culture from human feces (Dridi et al. 2012). Metagenomic analysis with human feces enrichment samples also revealed two new candidate species *Candidatus* 'Methanomassiliicoccus intestinalis' and *Candidatus* 'Methanomethylophilus alvus' (Borrel et al. 2012, 2013). This apparent common association with human suggests that *Methanomassiliicoccales* may play a role in human health. Due to their ability to metabolize trimethylamine into methane, it has been proposed that *Methanomassiliicoccales* may prevent or limit human diseases that are induced by trimethylamine (Brugere et al. 2014). However, distribution of *Methanomassiliicoccales* is not restricted to the human gut. An enrichment culture from anaerobic digester has led to the proposal of another candidate species *Candidatus* 'Methanogranum caenicola' (Iino et al. 2013). Environmental DNA sequence survey has suggested that *Methanomassiliicoccales* could be grouped into two clades, a

gastro-intestinal tract clade that is largely associated with animal samples, and an environmental clade which includes mainly aquatic and terrestrial samples.

3.7 Other Methanogen Candidates

Methanogenesis pathways have been predicted from a euryarchaeon, *Candidatus* 'Methanofastidiosa', members of the newly proposed archaeal phyla Verstraetearchaeota and Bathyarchaeota (Evans et al. 2015; Nobu et al. 2016; Vanwonterghem et al. 2016). *Candidatus* 'Methanofastidiosa' belongs to the uncultivated WSA2 or Arc I cluster, which has long been identified as a core euryarchaeal group in anaerobic digestion that was previously thought to use H_2/CO_2 or formate for methanogenesis (Hendrickson et al. 2004; Nakamura et al. 2013). However, genomic data has now proposed that WSA2 methanogens may conduct methylated thiol reduction with H_2 (Nobu et al. 2016). This suggests that they may be able to bridge the carbon and sulfur cycles, which may enable competition with CO_2 reducing methanogens and sulfate reducers. Previously loosely classified as the Terrestrial Miscellaneous Crenarchaeota Group or TMCG, members of Verstraetearchaeota methanogens also had their first metagenomes reconstructed from anaerobic digesters, but environmental DNA sequence survey could extend their distribution to wetlands, freshwater sediments, and hydrocarbon-rich environments (Vanwonterghem et al. 2016). Previously known as the MCG or Miscellaneous Crenarchaeotal Group, the recently proposed Bathyarchaeota have been found in deep ocean and freshwater sediments, and they are particularly present in high abundance within sulfate-methane transition zones (Vetriani et al. 1999; Inagaki et al. 2003; Gagen et al. 2013; Evans et al. 2015). Likewise, their first metagenomes were recovered from coal-bed methane wells in an ocean basin (Evans et al. 2015). Although those novel methanogen candidates suggest the diversity of methanogens would be much higher than previously anticipated, interpretation of their environmental distribution and ecophysiology should be cautious. This is because no pure cultures have been available so far, and it remains elusive if every member of the WSA2, Verstraetearchaeota and Bathyarchaeota could also be capable of methanogenesis as predicted from a limited number of metagenomes.

4 Research Needs

A few established methanogen orders are still underrepresented by cultivated members. *Methanocellales* is only represented by one genus, and both *Methanomassiliicoccales* and *Methanopyrales* are represented by just one species. Discovery and isolation of new strains will certainly add to our knowledge of the diversity of those orders. Isolations of new strains are also necessary to support the classification of *Methanimicrococcus blatticola* and *Methermicoccus shengliensis* as separate families within the order *Methanosarcinales* and expand our knowledge of the diversity of *Methanosarcinales*. On the other hand, since the *Methanosarcinales* can use a

relatively broad range of substrates for methanogenesis, isolation of new strains suitable for industrial purposes can be valuable.

Recent culture-independent studies have revealed the presence of novel phylogenetic groups of methanogens. Their isolation and characterization will also shed new insight into these organisms. For instance, investigations of rumen methanogens have found a novel lineage containing at least two families. The 16S rRNA gene sequences of this group have similarities closest to, but less than 80%, with those of *Methanosarcinales* (Nicholson et al. 2007). In addition, many novel methanogen candidates are still only represented by metagenomes, such as the *Candidatus* 'Methanofastidiosa' and members of the archaeal phyla Verstraetearchaeota and Bathyarchaeota (Evans et al. 2015; Nobu et al. 2016; Vanwonterghem et al. 2016).

Methanogens have fewer easily determined physiological characteristics than most bacteria. Comparative 16S rRNA gene sequence analyses are indispensable for determination of taxonomic levels higher than species. However, it is frequently insufficient for taxonomy of methanogens at species and subspecies levels. For instance, some isolates of *Methanobrevibacter* have >98% 16S rRNA gene sequence similarities but exhibit less than 50% DNA relatedness, suggesting that they belong to different species (Lin and Miller 1998; Keswani and Whitman 2001). The discovery of novel molecular markers is desirable. The methyl-coenzyme M reductase alpha-subunit (*mcrA*) gene has been applied as a phylogenetic marker for methanogens in addition to 16S rRNA genes (Springer et al. 1995) and as a target for the detection of methanogens in a wide range of environments (Ohkuma et al. 1995; Lueders et al. 2001; Luton et al. 2002; Earl et al. 2003; Kemnitz et al. 2004). Phylogenomic analyses based upon whole-genome sequences may lead to improvement of the taxonomy and better view of phylogenetic relationships. For instance, the genome-wide pairwise average nucleotide identity or ANI has been increasingly used to delineate species (Goris et al. 2007). However, convenient tools and methods will still need to be developed to meet the needs for analyzing large genome dataset. The Joint Genome Institute or JGI has been a pioneer in this filed, which has developed an Integrated Microbial Genome online pipeline to tackle the big data challenge (Markowitz et al. 2007a, b, 2009). Another grand challenge is to associate the environmental meta-data with the sequence data, which can provide enormous ecophysiological context for not only interpreting the sequence data from a single project but uncovering new trends across different projects.

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Hydrogenotrophic Methanogenesis

3

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Abstract

Massive amounts of methane are produced on Earth. Methane is useful as an energy source and as an energy storage material for H_2. However, there is increasing concern about methane concentrations in the atmosphere because it is a potent greenhouse gas. Methane is biologically produced primarily by methanogenic archaea, most of which produce methane hydrogenotrophically from H_2 and CO_2. Many enzymes involved in the hydrogenotrophic methanogenic pathway are shared in the methanogenic pathway from C1

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compounds or acetate. The methanogenic pathways contain unique enzymes and their prosthetic groups using unique electron and C1 carriers. Here, we describe an overview of the hydrogenotrophic methanogenic pathway, including the energy conservation and energy-coupling systems. The catalytic functions and mechanisms of the methanogenic enzymes are discussed based on their crystal structures.

1 Introduction

Hydrogenotrophic methanogens are archaea that can grow on H_2 and CO_2 with the production of methane, an important intermediate in the global carbon cycle. They have a unique biochemistry that has been unraveled over the last 40 years. Methane is produced mainly by the anaerobic decomposition of plant biomass in anoxic environments, where the concentrations of sulfate, Fe(III), Mn(IV), and nitrate are low, such as in freshwater sediments, wetlands, and the intestinal tract of animals (Thauer et al. 2008). In anoxic environments, methane is generated by methanogenic archaea, and via this action, approximately 1 Gt of methane is formed globally every year, which is approximately 1% of the net carbon fixed from CO_2 into plant biomass every year via photosynthesis. Most methane diffuses into oxic environments, where approximately 60% is oxidized to CO_2 with O_2 by methanotrophic bacteria. The remaining 40% escapes into the atmosphere, where most of it is photochemically converted to CO_2. The concentration of methane in the atmosphere has more than doubled in the last 100 years, indicating that the rate of methane release (from all sources) into the atmosphere has increased relative to the rate of methane oxidation (Thauer et al. 2008). This is of concern, since methane is a potent greenhouse gas considered to contribute significantly to global warming. Methanogenesis is also of biotechnological interest in sewage treatment plants and in biogas production plants. The microbial formation of methane from H_2 and CO_2 has also been discussed as a means of H_2 storage (Thauer et al. 2010).

Phylogenetic analysis indicated that methanogenic organisms are exclusively classified into archaea (Boone et al. 1993). Most methanogenic archaea are found in the lineage of Euryarchaeota, but recent metagenomic analysis has shed light on the presence of two lineages – Bathyarchaeota and Verstraetearchaeota – that are phylogenetically distant from Euryarchaeota (Fig. 1). Most orders of methanogenic archaea produce methane from H_2 and CO_2, from formate, or from H_2 and methanol; these are referred to as hydrogenotrophic methanogens. Only one order, the Methanosarcinales, can also produce methane from acetate and from the disproportionation of C1 compounds such as methanol, methylamines, and methylthiols; these are referred to as acetoclastic methanogens and methylotrophic methanogens, respectively. The hydrogenotrophic methanogens differ from the archaea in the order of Methanosarcinales, as they are devoid of cytochromes and

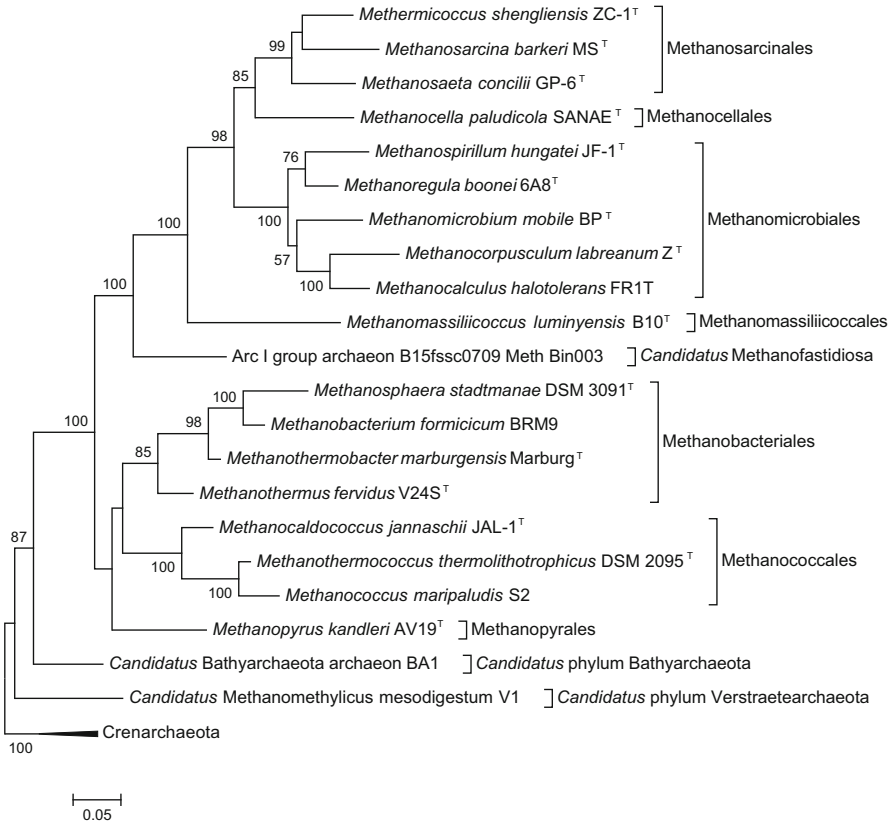

Fig. 1 Phylogeny of methanogenic archaea. Maximum likelihood tree was constructed from 16S rRNA sequences aligned by ClustalW. There were a total of 1396 comparable positions. Bootstrap values >50% based on 100 resamplings are indicated at the nodes. Crenarchaeota was used as the outgroup. Bar indicates 0.05 substitutions per nucleotide position. The tree consists of all known methanogenic families, orders, and phyla including *Candidatus* taxon

methanophenazine (Fig. 2) and use only sodium ions rather than protons for chemiosmotic energy conservation (Thauer et al. 2008).

2 Energy Metabolism on H_2 and CO_2

The standard free energy change of methane formation from $4H_2$ and CO_2 ($\Delta G^{\circ\prime}$) is −131 kJ/mol. Under physiological conditions where the partial pressure of H_2 is only approximately 10 Pa, the free energy change is only approximately −30 kJ/mol of methane formed. The biosynthesis of ATP from ADP and inorganic phosphate in vivo is estimated to be between −60 and −70 kJ/mol, although under energy

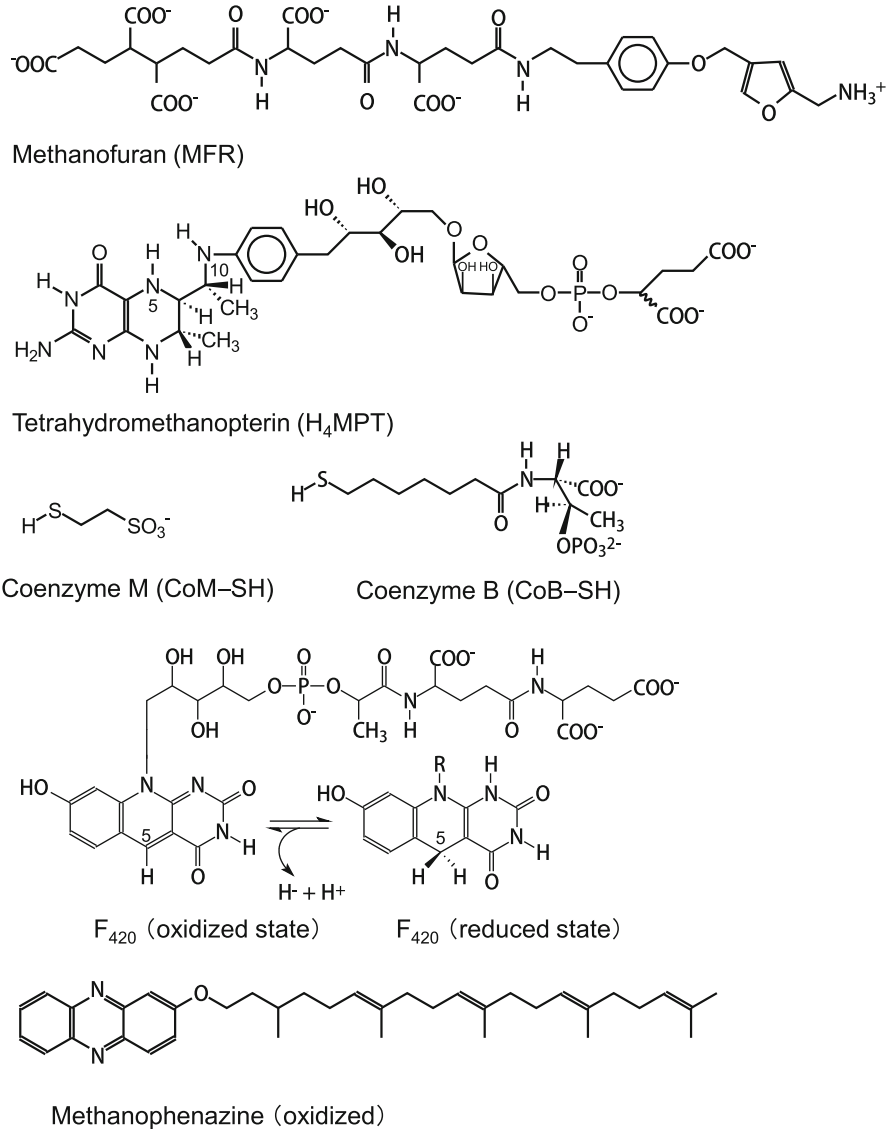

Fig. 2 Coenzymes involved in the hydrogenotrophic methanogenic pathways. In methanogens belonging to Methanosarcinales, tetrahydrosarcinapterin (H_4SPT) instead of H_4MPT is used as a C1 carrier. Methanophenazine is used only in Methanosarcinales (Abken et al. 1998; Beifuss et al. 2000)

limitations, the value might be considerably lower. It is likely that less than 1 mol ATP is formed per mol methane. The exact ATP gain (mol ATP/mol CH_4) is of general interest because it is an open question how close to thermodynamic equilibrium the energy metabolism of strict anaerobes can operate and how small the

minimal free energy change increment must be to sustain life in anaerobic environments such as the deep biosphere (Thauer et al. 2008).

3 Reactions Involved in Methanogenesis from H_2 and CO_2

From mainly the works of Wolfe (Dimarco et al. 1990; Wolfe 1991), Gottschalk (Gottschalk and Blaut 1990; Deppenmeier et al. 1996), and Thauer (Thauer et al. 2008) and their collaborators, methanogenesis from H_2 and CO_2 is known to involve five coenzymes (Fig. 2) and ten reactions (Fig. 3). The structure of methanopterin was elucidated by Keltjens and Vogels (van Beelen et al. 1984). The pathway begins with the reduction of CO_2 on methanofuran (MFR) with reduced ferredoxin (Fd_{red}) to formyl-MFR catalyzed by formylmethanofuran dehydrogenase; in most methanogens there are two isoenzymes, one containing molybdenum (Fmd) and

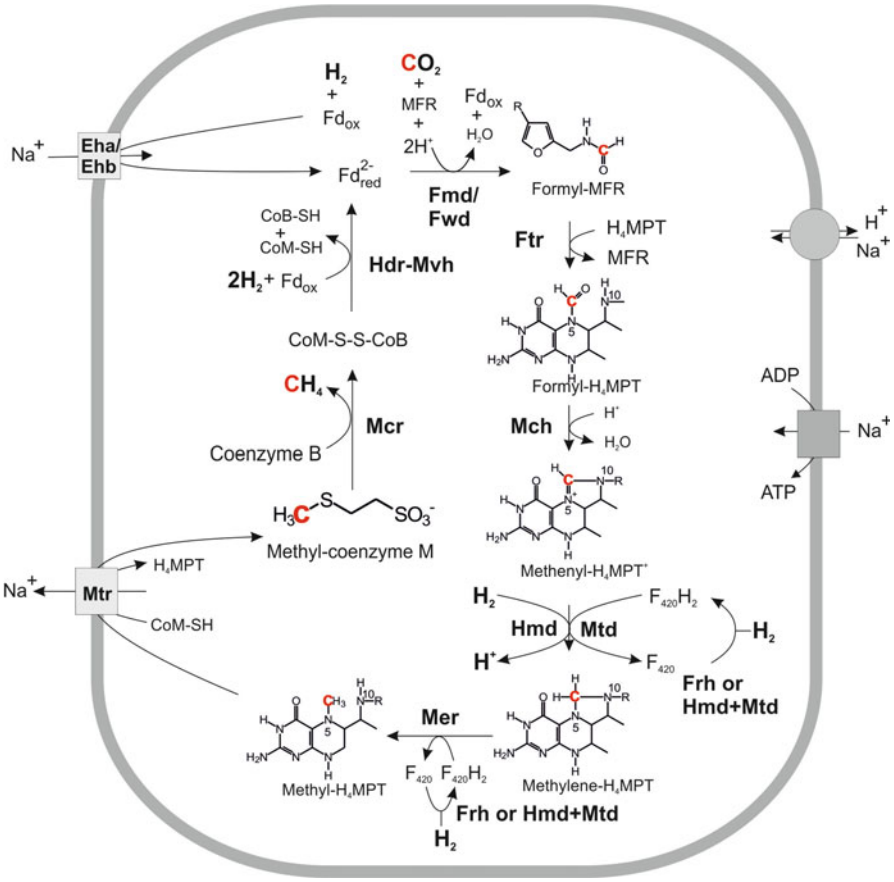

Fig. 3 Overview of the hydrogenotrophic methanogenic pathway (Bai et al. 2017)

the other containing tungsten (Fwd). Subsequently, the formyl group of formyl-MFR is transferred to tetrahydromethanopterin (H_4MPT) by formyltransferase (Ftr). N^5-Formyl-H_4MPT is subsequently converted in three steps to methyl-H_4MPT via methenyl- and methylene-H_4MPT as intermediates using methenyl-H_4MPT^+ cyclohydrolase (Mch), F_{420}-dependent methylene-H_4MPT dehydrogenase (Mtd), and F_{420}-dependent methylene-H_4MPT reductase (Mer). An alternate reaction to Mtd is catalyzed by H_2-forming methylene-H_4MPT dehydrogenase (Hmd or [Fe]-hydrogenase), which catalyzes the conversion of methenyl-H_4MPT^+ to methylene-H_4MPT using H_2 as an electron donor. F_{420} is a 5-deazaflavin that is converted to the reduced form ($F_{420}H_2$, Fig. 2) by H_2 catalyzed by F_{420}-reducing [NiFe]-hydrogenase (Frh). $F_{420}H_2$ is used as an electron donor for the two reduction steps in hydrogenotrophic methanogenesis and for other anabolic reduction reactions in the methanogenic archaea. Under nickel-limiting conditions, the [NiFe]-hydrogenase Frh is substituted by Hmd; Hmd is coupled with Mtd to reduce F_{420} with electrons from H_2. After methyl-H_4MPT is formed, its methyl group is transferred to coenzyme M (CoM-SH), yielding methyl-S-CoM in an exergonic reaction catalyzed by a membrane-associated methyltransferase complex (MtrA-H). The exergonic methyl-transfer reaction is coupled to endergonic sodium-ion translocation (Gottschalk and Thauer 2001). The sodium ion motive force thus generated is utilized by an A_1A_O-type ATP synthase to drive the phosphorylation of ADP (Vonck et al. 2009). In the next step, methyl-S-CoM is reduced with coenzyme B (CoB-SH) to methane and a heterodisulfide (CoM-S-S-CoB); this reaction is catalyzed by methyl-S-CoM reductase (Ermler et al. 1997a). CoM-S-S-CoB is reduced with H_2 to CoM-SH and HS-CoB, catalyzed by the electron-bifurcating [NiFe]-hydrogenase/heterodisulfide reductase complex (MvhADG-HdrABC). This complex couples the exergonic reduction of CoM-S-S-CoB with H_2 to the endergonic reduction of ferredoxin with H_2. The reduced ferredoxin thus generated is used in the first step of the hydrogenotrophic methanogenesis, the reduction of CO_2 to formyl-MFR.

4 Methanogenic Enzymes

4.1 Formylmethanofuran Dehydrogenase (Fmd and Fwd)

Hydrogenotrophic methanogenesis begins with the reductive bonding of CO_2 to the amino group of the C1 carrier methanofuran to form formylmethanofuran. This reversible reaction is catalyzed by Fmd or Fwd. The redox potential of the formylmethanofuran/methanofuran couple is very low ($E^{\circ\prime} = -530$ mV) (Bertram and Thauer 1994); therefore, the reduction requires high-energy electrons from reduced ferredoxin ($E' = \sim -500$ mV) (Kaster et al. 2011).

Crystal structure analysis of Fwd from *Methanothermobacter wolfeii* revealed a Fwd(ABCDFG)$_4$ organization (Fig. 4) (Wagner et al. 2016a). FwdA is similar to the amidohydrolases, i.e., urease, phosphotriesterase, and dihydroorotase/hydantoinase. The metal ligands, including the posttranslationally

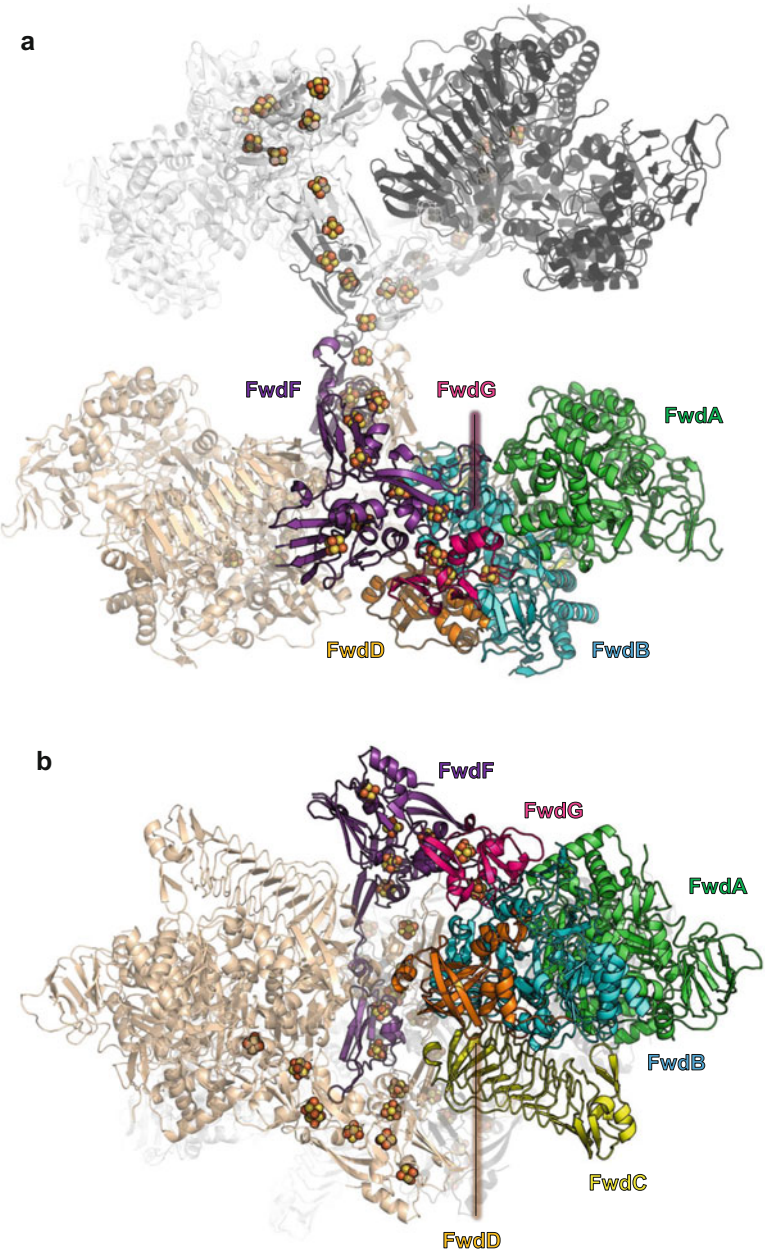

Fig. 4 Structure of tungsten-containing formylmethanofuran dehydrogenase (Fwd) from *Methanothermobacter wolfeii* (Wagner et al. 2016a). (**a**) The Fwd(ABCDFG)$_4$ complex. Four FwdABCDFG heterohexamers are shown in white, black, sand, and multiple colors of subunits. (**b**) The Fwd(ABCDFG)$_4$ complex in 90° rotated angle from the orientation in panel **a**

modified N6-carboxylysine and a catalytically crucial aspartate, are strictly conserved in FwdA. Crystals soaked with methanofuran led to the identification of the binding site: a cavity extending from the dinuclear metal center to bulk solvent (Fig. 5a, c). FwdB and FwdD form a formate dehydrogenase-like catalytic unit, which is a member of the molybdo-/tungstopterin-dependent DMSO reductase superfamily. The redox-active tungsten of FwdBD is coordinated to four dithiolene thiolates of two tungstopterin guanine dinucleotide molecules (Fig. 5b). The [4Fe-4S]-cluster, tungstopterin-binding, the active site residues, and tungsten ligation mode are essentially conserved between FwdBD and formate dehydrogenases. FwdC shares the highest structural similarities to a C-terminal glutamate synthase domain (Binda et al. 2000), which has an architectural function. FwdF is the first polyferredoxin to be structurally analyzed; it is composed of four fused similar ferredoxin modules, each carrying two [4Fe-4S] clusters that are arranged in a "T"-shaped conformation (Fig. 4).

The crystal structure of the FwdABCDFG complex provided evidence of the catalytic mechanism. The Fwd(ABCDFG)$_4$ complex can be subdivided into an electron-supplying core (FwdF and FwdG) flanked by four catalytic units formed by FwdABCD (Fig. 5a). Each catalytic unit hosts two spatially separated active sites for the dual reactions. First, CO_2 is funneled through a narrow 35-Å-long hydrophobic channel to the FwdBD tungstopterin center, namely, the formate dehydrogenase core (Fig. 5b). Previous biochemical studies indicated weak formate dehydrogenase activity for formylmethanofuran dehydrogenases (Bertram et al. 1994). The deeply buried redox-active tungsten center is connected to the [4Fe-4S] chains to efficiently transfer low-potential electrons to reduce CO_2 to formate. The produced formate is transferred via an internal, 27-Å-long, hydrophilic tunnel and reacts with the amino group of methanofuran to form formylmethanofuran at the binuclear metal center of FwdA (Fig. 5c). The Fwd(ABCDFG)$_4$ complex harbors 46 [4Fe-4S] clusters in the electron-supplying unit (Fig. 4), which is composed of iron-sulfur cluster chain links with short edge-to-edge distances for efficient electron transfer. The electron wires connect the redox-active tungsten sites of the 12-mer Fwd(ABCDFG)$_2$ and the 24-mer Fwd(ABCDFG)$_4$ over distances of ca. 188 Å and 206 Å, respectively.

4.2 Formyltransferase (Ftr)

The formyl group bound to methanofuran is transferred to H_4MPT to form N^5-formyl-H_4MPT. This formyl transfer reaction is catalyzed by Ftr. The crystal structures of Ftr from *Methanopyrus kandleri*, *Methanosarcina barkeri*, and *Archaeoglobus fulgidus* have been solved. *Methanopyrus kandleri* is a hyperthermophilic methanogen (optimum growth temperature, 98 °C), and its Ftr contains a homotetramer in the crystal structure (Fig. 6a, c) (Ermler et al. 1997b). Biophysical experiments using analytical ultracentrifugation indicated that Ftr from *M. kandleri*

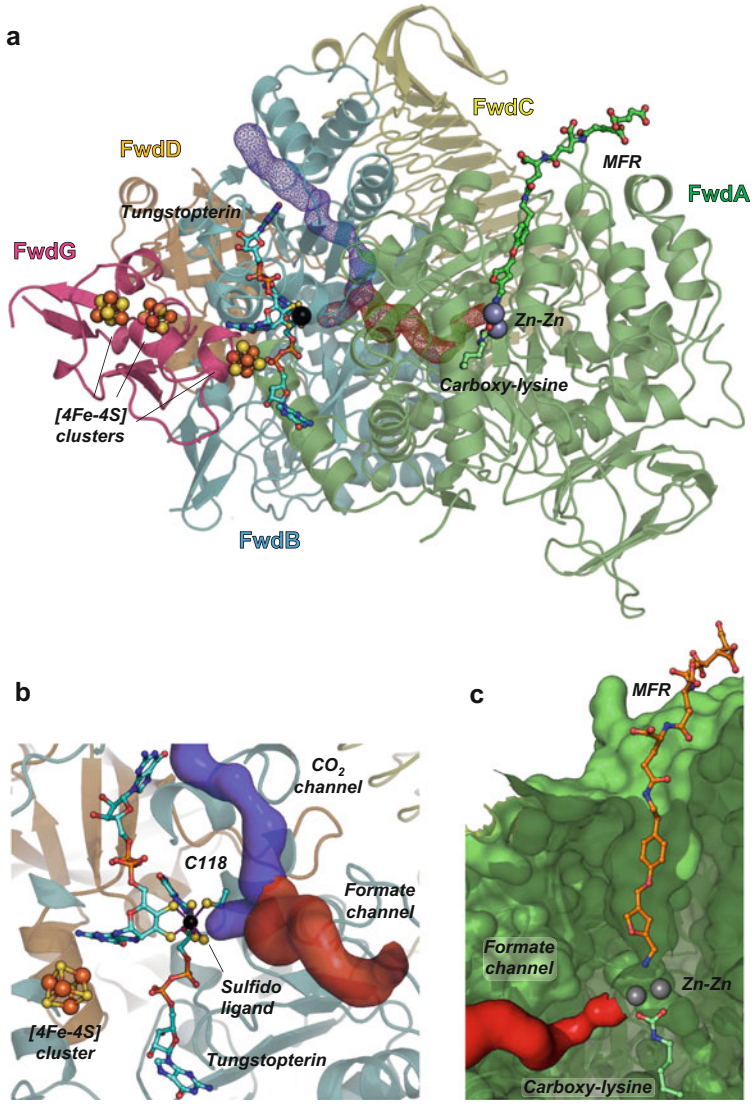

Fig. 5 Active site structures of Fwd. (**a**) FwdABD contains two metal active sites responsible for CO_2 reduction and condensation of formate on MFR. FwdBD harbors the tungstopterin (light blue stick model for the organic part and black ball model for tungsten), and FwdA has a dinuclear zinc site (gray ball model). Iron-sulfur clusters in FwdG and FwdB are depicted as brown and yellow ball. CO_2 entrance channel (blue surface) and formate transport channel (red surface) are shown, connecting the tungstopterin active site (**b**) and dinuclear zinc amidohydrolase active site (**c**)

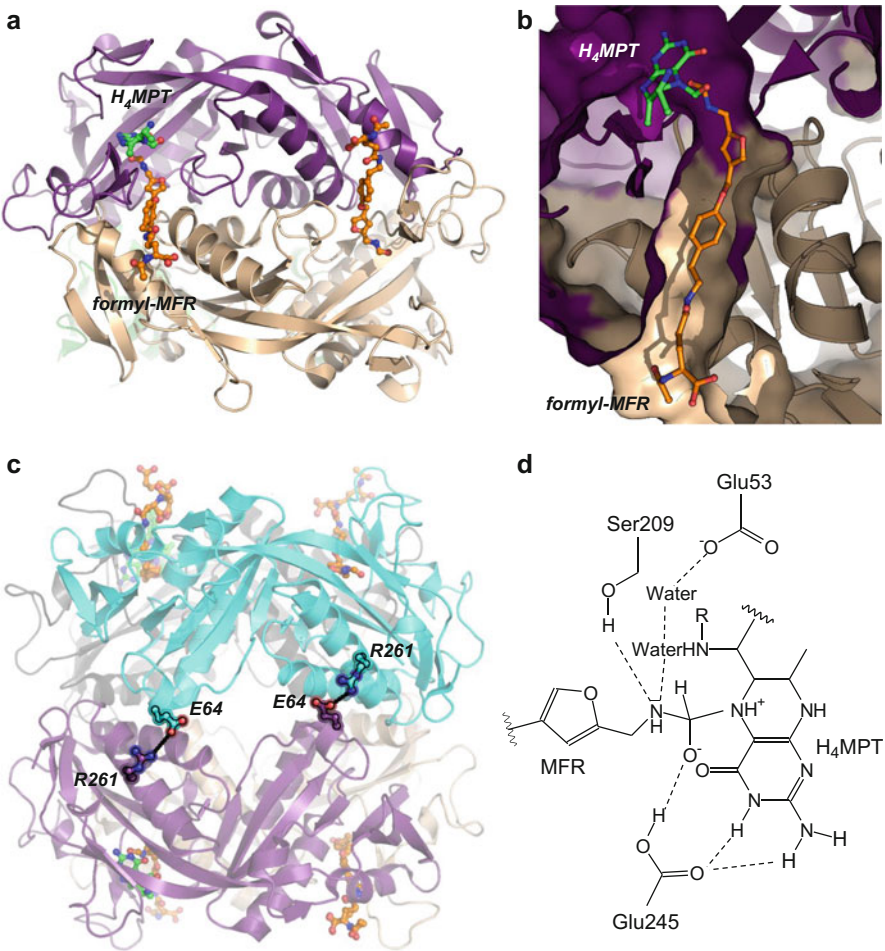

Fig. 6 Structure and function of formyl-MFR:H_4MPT formyltransferase (Ftr). (**a**) Cartoon model of the dimer of Ftr from *M. kandleri* in complex with its substrates formyl-MFR and H_4MPT (Acharya et al. 2006). The bulky coenzymes MFR (orange) and H_4MPT (green) are embedded into large surface clefts located between two monomers (purple and sand). (**b**) Surface model of the formyl-MFR and the H_4MPT binding site shows the substrate-binding cleft. Two monomers are colored in purple (H_4MPT binding) and sand (formyl-MFR binding). (**c**) In the tetrameric form, the contacts between two dimers (light blue and purple) involve salt bridges between Arg261 and Glu64. (**d**) A tetrahedral oxyanion intermediate proposed in the catalytic reaction

is in equilibrium of monomer/dimer/tetramer, which is dependent on the concentration of lyotropic salts (i.e., potassium phosphate and ammonium sulfate) in the enzyme solution (Shima et al. 1998). The larger oligomeric forms appear with increasing salt concentration. At low salt concentration, this enzyme is inactive (as a monomer). Ftr is activated at higher salt concentrations when it forms dimer or tetramer. This finding suggests that the active form is a homodimer. This hypothesis

was supported by the X-ray crystal structure analysis of Ftr in complex with the substrates formylmethanofuran and H_4MPT (Acharya et al. 2006). The structure shows that each substrate is bound to different subunits, as shown in Fig. 6a, b, which indicate localization of the active site at the dimeric interface. For *M. kandleri*, the major dimer/dimer interaction of Ftr is the salt bridges between Glu64 and Arg261 (Fig. 6c). The tetrameric form of Ftr from *M. kandleri* stabilizes this protein against heat rather than catalytic activity (Shima et al. 2000a).

A catalytic mechanism for Ftr was proposed based on the ternary complex of Ftr with formyl-MFR and H_4MPT (Acharya et al. 2006). Hydrogen bonds between Ser209 and formamide-N of formyl-MFR and between the formamide-O and the protonated carboxy-group of Glu245 increase the electrophilicity of the formamide-C. Nucleophilic attack of N5 of H_4MPT produces a tetrahedral oxyanion intermediate (Fig. 6d), which is then stabilized by protonation from the protonated carboxy of Glu245. A proton is transferred to the nitrogen of MFR and formyl-H_4MPT is finally formed.

4.3 Cyclohydrolase (Mch)

Mch reversibly catalyzes the condensation reaction of formyl-H_4MPT to methenyl-H_4MPT$^+$. The first crystal structure of Mch was solved using heterologously produced enzyme from *M. kandleri*. The catalytic reaction of Mch was studied based on the heterologously produced enzyme from the sulfate-reducing archaeon *Archaeoglobus fulgidus*, which has a C1 pathway containing H_4MPT (Klein et al. 1993). Mch is a homotrimeric enzyme (Fig. 7a), and the substrate N^5-formyl-H_4MPT binds to the cleft between domain A and B of each monomer (Fig. 7b, c) as observed in the catalytically inactive mutant E186Q. In the proposed catalytic mechanism, from methenyl-H_4MPT$^+$ to formyl-H_4MPT, the substrate water molecule trapped between Arg183 and Glu186 nucleophilically attacks the C14a of methenyl-H_4MPT$^+$ to form a tetrahedral imidazolidin-2-ol intermediate (Fig. 7d) (Upadhyay et al. 2012). A proton of the intermediate is transferred to N10 of H_4MPT via the carboxy group of Glu186, which preferentially selects N5 as the leaving group. A proton on the intermediate is finally transferred to Glu186, which forms N^5-formyl-H_4MPT.

4.4 F_{420}-Dependent Methylene-Tetrahydromethanopterin Dehydrogenases (Mtd)

Mtd catalyzes reversible hydride transfer from $F_{420}H_2$ to methenyl-H_4MPT$^+$ to form methylene-H_4MPT. The crystal structure of Mtd from *M. kandleri* was reported (Hagemeier et al. 2003) and indicated that Mtd is a homohexameric protein composed of a trimer of dimers (Fig. 8a). Mtd has no structural similarity to known proteins, including those binding F_{420} and the H_4MPT derivatives. Based on the ternary Mtd complex structure with $F_{420}H_2$ and methenyl-H_4MPT$^+$, a catalytic

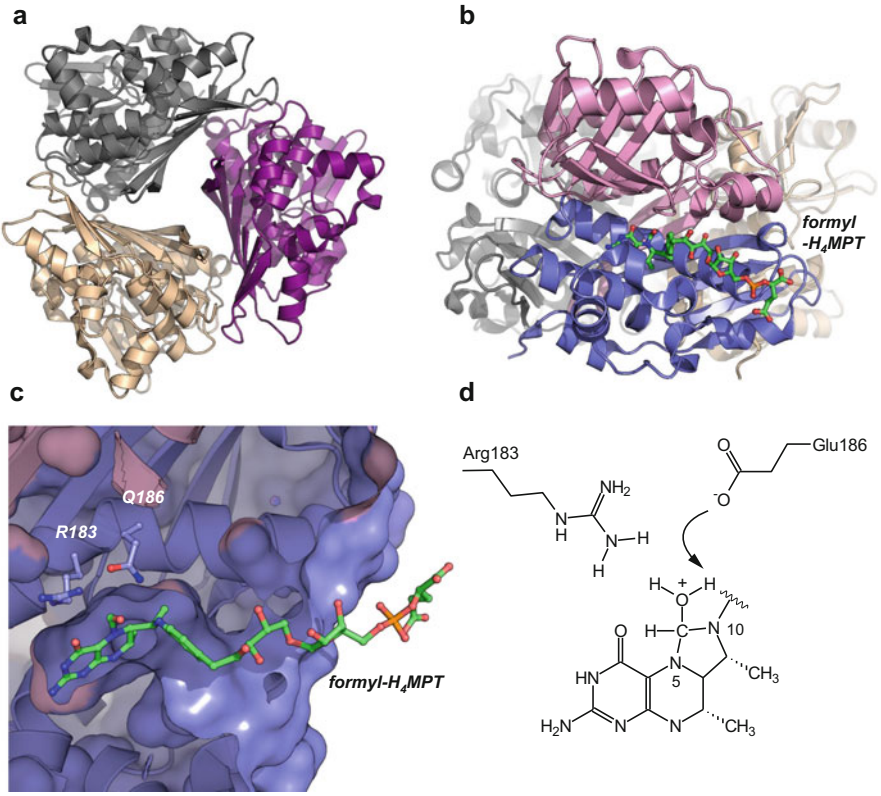

Fig. 7 Structure and function of methenyl-H_4MPT^+ cyclohydrolase (Mch) (Upadhyay et al. 2012). (**a**) Homotrimer of Mch from *M. kandleri*. (**b**) The substrate-binding site of the monomer built up between domains A (pink) and B (blue) from the Mch of *Archaeoglobus fulgidus*. (**c**) Location of the catalytic R183 and Q186 and formyl-H_4MPT binding site of the E186Q mutant. (**d**) A tetrahedral imidazolidin-2-ol intermediate proposed in the catalytic reactions

mechanism was proposed (Ceh et al. 2009). The substrates bind to the active site formed in the cleft on a subunit at the interface of two domains (Fig. 8b, c), in which both substrates face each other (Fig. 8b, c, d). This substrate arrangement indicated the direct hydride transfer between C5 of $F_{420}H_2$ and C14a of methenyl-H_4MPT^+, which allows stereospecific hydride transfer (Fig. 8d).

4.5 H_2-Forming Methylene-Tetrahydromethanopterin Dehydrogenase (Hmd)

Hmd ([Fe]-hydrogenase) catalyzes reversible hydride transfer from H_2 to methenyl-H_4MPT^+ (Shima and Ermler 2011). The products of this reaction are methylene-H_4MPT and a proton. This enzyme is found in the majority of *hydrogenotrophic*

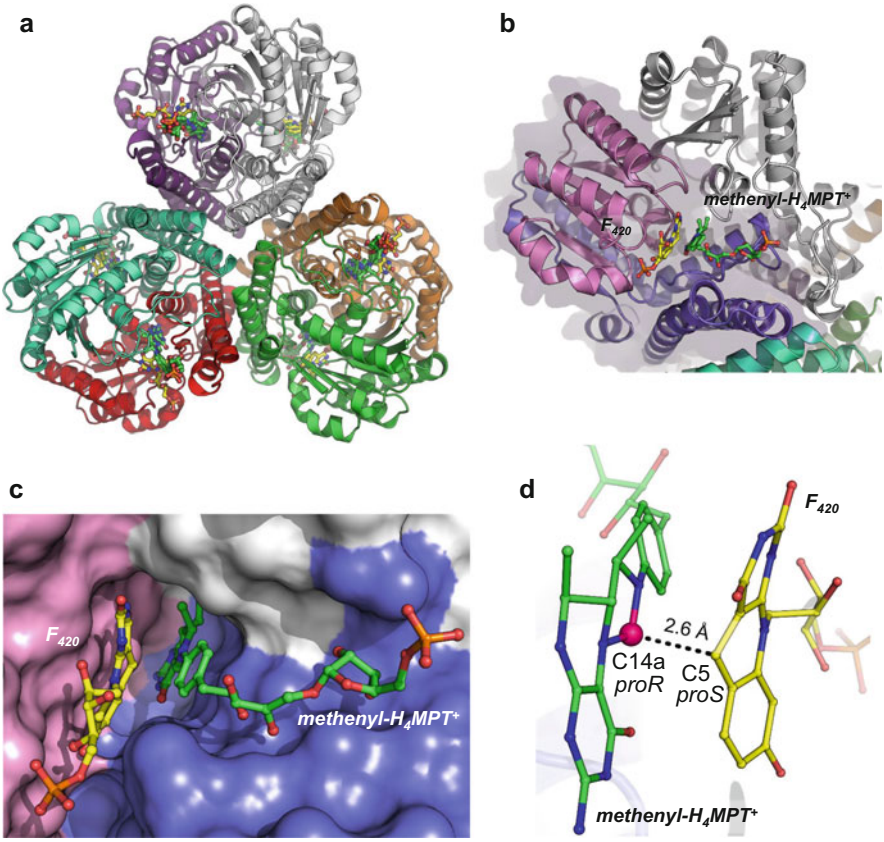

Fig. 8 Structure of F_{420}-dependent methylene-H_4MPT dehydrogenases (Mtd). (**a**) Structure of the homohexameric enzyme complex from *M. kandleri* in complex with methenyl-H_4MPT^+ (green stick) and F_{420} (yellow stick). (**b**) The binding site of methenyl-H_4MPT^+ and $F_{420}H_2$ is at the interface of the two domains (in pink and blue) from one monomer shown with transparent surface model. (**c**) Active site cleft (surface model) binding the substrates with the same color code than shown in panel b. (**d**) Stereospecific hydride transfer of the *proS* hydride bound to C5 of $F_{420}H_2$ to the *proR* side of the C14a atom of methenyl-H_4MPT^+

methanogenic archaea. Hmd contains a unique iron guanylylpyridinol (FeGP) cofactor (Fig. 9a, b). Crystallographic, spectroscopic, and chemical analyses of [Fe]-hydrogenase revealed that its iron center is ligated by Cys176-sulfur, two CO, one solvent molecule, an sp^2-hybridized pyridinol-nitrogen, and an acyl carbon in the substituent of the pyridinol ring. The FeGP cofactor is extractable from [Fe]-hydrogenase, and the active holoenzyme can be reconstituted from the isolated cofactor and the apoenzyme that is heterologously produced in *Escherichia coli* (Shima and Ermler 2011).

In the crystal structures, the apoenzyme (without the FeGP cofactor) and holoenzyme (with the FeGP cofactor) of [Fe]-hydrogenase have closed and open

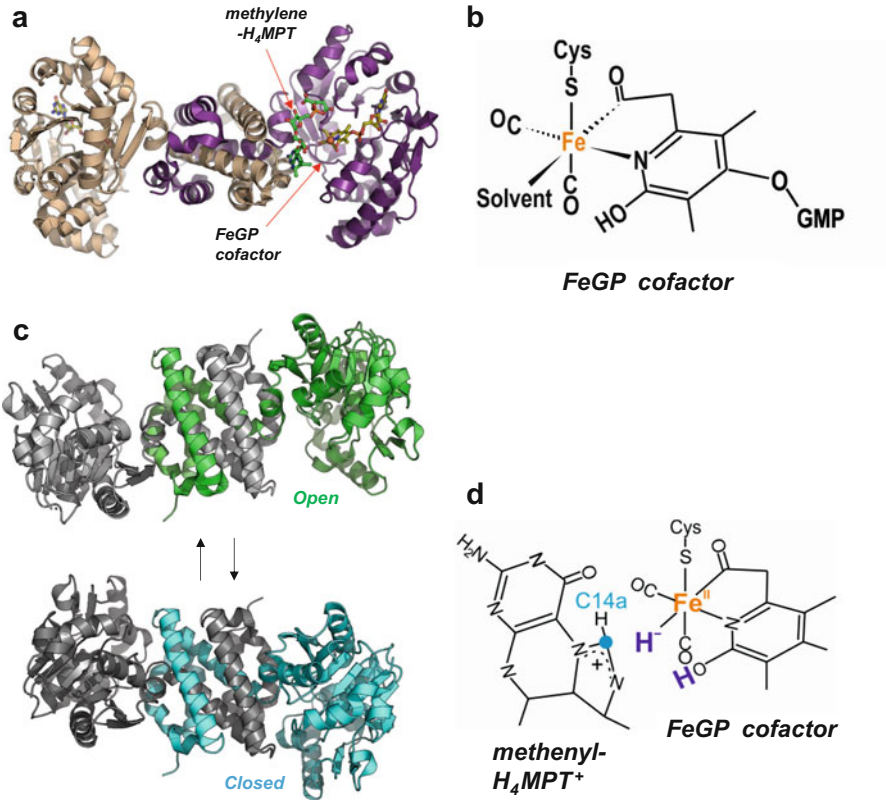

Fig. 9 H_2-forming methylene-H_4MPT dehydrogenase (Hmd). (**a**) C176A-mutated holoenzyme from *M. jannaschii* in complex with methylene-H_4MPT. The FeGP cofactor and substrate are indicated in stick models. (**b**) Chemical structure of the FeGP cofactor. The solvent-binding site was proposed as the H_2-binding site (Shima et al. 2015). (**c**) The closed and open conformations of the Hmd homodimer. Two monomers are shown in black and green (open)/light blue (closed) cartoon models. (**d**) An iron-hydride intermediate representation from the proposed catalytic mechanism

conformations in the active site cleft, respectively. The crystal structure of the C176A-mutated holoenzyme-substrate binary complex of [Fe]-hydrogenase was reported and revealed an open cleft with a distance of 9.3 Å between the iron and the C14a atom of the substrate (Fig. 9a). This distance is obviously too long for the direct transfer of hydride ions. To model a catalytically productive conformation, the closed conformation of the apoenzyme was used. The movement of the peripheral unit from the open to the closed form essentially corresponds to a rotation of 35° (Fig. 9c). A structure-based mechanism of [Fe]-hydrogenase has been proposed based on biochemical and biophysical studies (Vogt et al. 2008; Hiromoto et al. 2009; Yang and Hall 2009; Hedegard et al. 2015; Shima et al. 2015). The catalytic cycle is initiated by the binding of methenyl-H_4MPT^+ to the open form, which triggers the closure of the cleft. Subsequently, H_2 is supplied to the active site in the

closed form and is captured in the "open coordination" site (Fig. 9b) of the iron center. The H_2 molecule likely binds to the iron. The base of the reaction may be the deprotonated form of the pyridinol hydroxy group. Semisynthetic Hmd enzymes built up with heterologously produced apoenzyme and chemically synthesized mimics reveal that the deprotonated 2-hydroxy group is crucial for enzyme activity, which supports the base function of the 2-pyridinol hydroxyl group (Shima et al. 2015). Density functional theory (DFT) calculations support the catalytic mechanism including the iron-hydride intermediate (Fig. 9d). However, experimental evidence of the iron-hydride intermediate has not been reported.

4.6 Methylenetetrahydromethanopterin Reductase (Mer)

Mer catalyzes the reversible reduction of methylene-H_4MPT to form methyl-H_4MPT. The crystal structure of Mer was obtained using the purified enzymes from *M. marburgensis*, *M. kandleri*, and *M. barkeri* (Fig. 10) (Shima et al. 2000b; Aufhammer et al. 2005). Heterologous expression of Mer in *E. coli* was unsuccessful, likely because of the presence of a non-prolyl *cis*-peptide bond (Fig. 10c). Mer is organized as a TIM-barrel fold that forms a homodimer (for *M. marburgensis*) or homotetramer (*M. barkeri* and *M. kandleri*). The enzyme is homologous to F_{420}-dependent secondary alcohol dehydrogenase (Aufhammer et al. 2004) and bacterial luciferase family proteins (Fig. 10d) (Baldwin et al. 1995; Aufhammer et al. 2005). The crystal structure of Mer from *M. barkeri* was solved in the complex structure with F_{420} (Fig. 10a, b), but the crystal structure of the complex with methylene-H_4MPT or methyl-H_4MPT has not yet been reported.

4.7 Integral Membrane Methyltransferase (MtrA-H)

The membrane-associated MtrA-H complex catalyzes an exergonic cobalamin-dependent methyltransferase reaction and couples it to the electrogenic translocation of two sodium ions, as demonstrated by experiments using reconstituted ether lipid liposomes (Gottschalk and Thauer 2001). From the primary structure, it is predicted that MtrH is a peripheral protein without a membrane anchor; that MtrA, MtrB, MtrF, and MtrG are peripheral proteins with one transmembrane helix anchor; and that the three other subunits, MtrC, MtrD, and MtrE, are integral membrane proteins with at least six transmembrane helices (Fig. 11). MtrH has been shown to catalyze the methyl-transfer reaction from methyl-H_4MPT to the corrinoid prosthetic group of MtrA. From the methylated corrinoid, the methyl group is transferred to CoM-SH. The latter methyl-transfer reaction is dependent on the presence of sodium ion, which suggests its involvement in sodium-ion translocation.

The crystal structure of the MtrA soluble domain indicated that MtrA has a unique cobalamin-binding site (Fig. 12) (Wagner et al. 2016b). The cobalt coordination in the crystal structure is hexa-coordinated including an external histidine

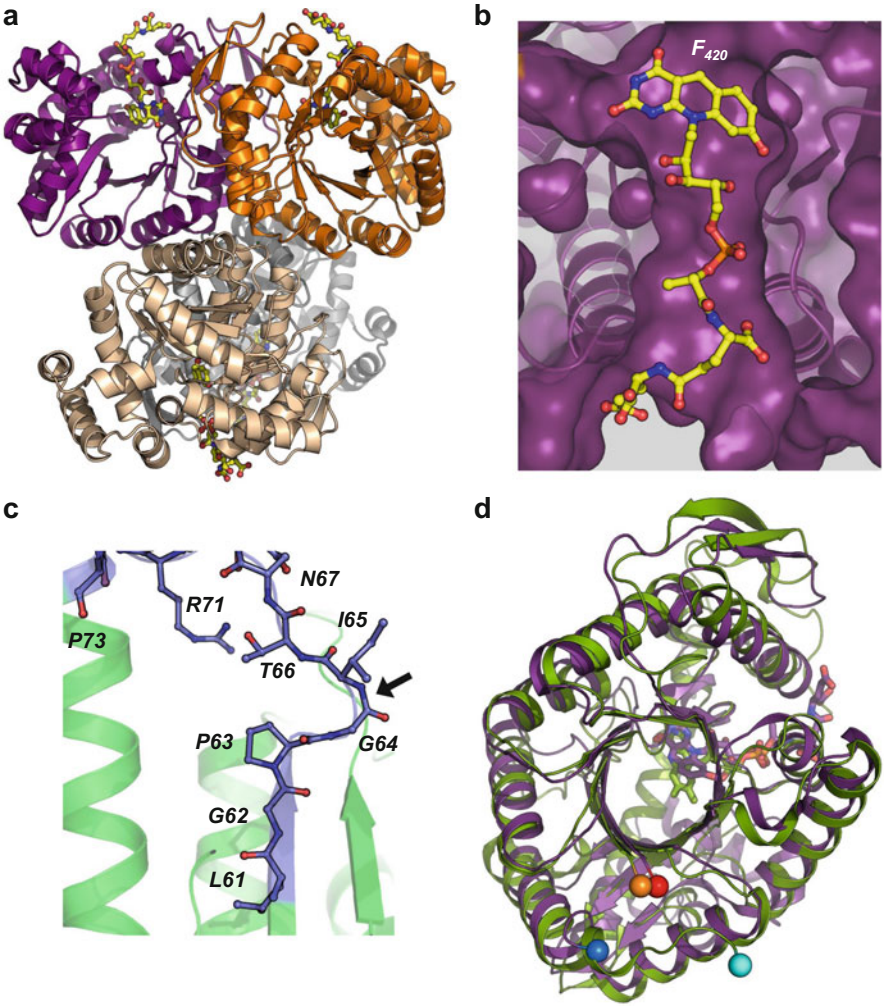

Fig. 10 Structure and function of F_{420}-dependent methylene-H_4MPT reductase (Mer) (Shima et al. 2000b; Aufhammer et al. 2004). (**a**) The tetrameric form of Mer from *M. barkeri*. Each monomer is shown in a different color. (**b**) F_{420} binding site. (**c**) A protein segment of Mer from *M. kandleri*, which contains *cis*-peptide bond between Gly64 and Ile65, indicated by a black arrow. (**d**) Superposition of Mer from *M. barkeri* (purple) F_{420} complex and bacterial luciferase (LuxA) from *Vibrio harveyi* with bound FMN (green). Spheres in cyan/blue indicate the N-terminal positions, and red/orange indicate the C-terminal positions

residue from another monomer. From the coordination chemistry of B_{12}, previous site-directed mutagenesis studies, and the crystal structure, it was predicted that in the reduced non-methylated Co(I) form, the histidine ligand will be decoupled from cobalt (tetra-coordination), and in the methylated Co(III) form, the histidine will

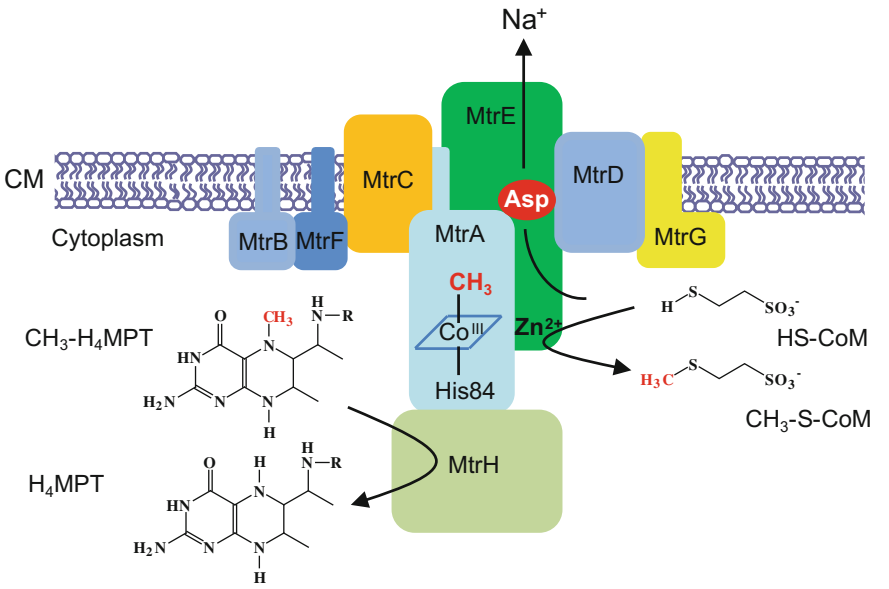

Fig. 11 Reaction and model of the membrane-associated MtrA-H complex catalyzing methyl transfer from methyl-H_4MPT to coenzyme M (HS-CoM). MtrH has been shown to catalyze methyl transfer from methyl-H_4MPT to Co(I) of the corrinoid bound to MtrA. MtrE is proposed to catalyze methyl transfer from CH_3-Co^{III}-MtrA to CoM-SH and to couple this reaction with the translocation of two sodium ions. Demethylation rather than methylation has been shown to be dependent on sodium ions (Gottschalk and Thauer 2001)

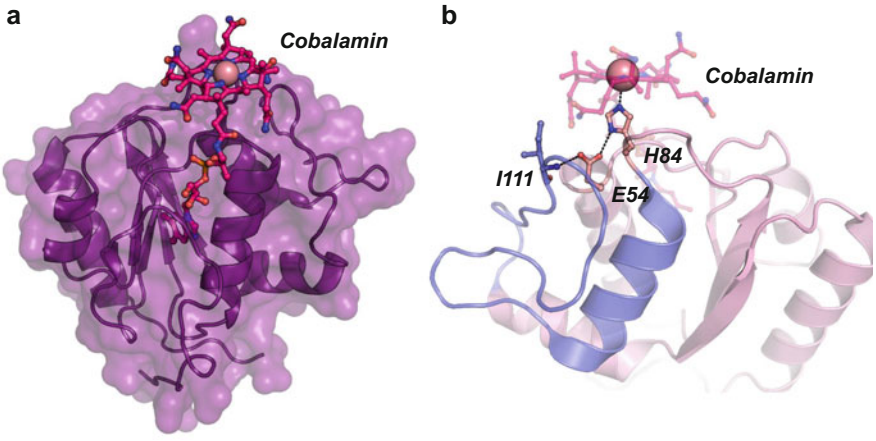

Fig. 12 Crystal structure of the cytoplasmic MtrA with cobalamin in the Co(III) oxidation state (Wagner et al. 2016b). (**a**) Structure of MtrA was shown by cartoon and surface models. Cobalamin is depicted as ball and stick model (carbons in magenta). (**b**) The lower axial ligand H84, E54, and I111 and cobalamin are shown as stick model

bind to cobalt (hexa-coordination). The switch to cobalt coordination upon demethylation would drive sodium-ion translocation using the conformational change in a protein segment (Gottschalk and Thauer 2001).

4.8 Methyl-coenzyme M Reductase (Mcr)

The common final step of all methanogenic pathways is the Methyl-coenzyme M reductase (Mcr) reaction (Thauer 1998). This enzyme catalyzes the reduction of methyl-coenzyme M (methyl-S-CoM) with coenzyme B (CoB-SH) to methane and heterodisulfide (CoB-S-S-CoM) (see Figs. 2 and 3). It is known that Mcr also catalyzes the reverse reaction, consisting of the anaerobic oxidation of methane, the first reaction in the metabolism involved in anaerobic methanotrophic archaea, coupled to the reduction of sulfate (Shima et al. 2012) and nitrate (Haroon et al. 2013).

Mcr is composed of α-, β-, and γ-subunits in an $(\alpha\beta\gamma)_2$ configuration. The crystal structures of Mcr from *M. marburgensis* (Fig. 13a), *M. kandleri*, *M. barkeri*, and *M. wolfeii* were solved in several inactive states (Ermler et al. 1997a; Grabarse et al. 2000, 2001; Wagner et al. 2016c). The active site of Mcr contains a nickel porphinoid F_{430} as a prosthetic group (Fig. 13b). The Ni(I), Ni(II), and Ni(III) states of F_{430} are involved in the catalytic reactions (Thauer and Shima 2007). Two F_{430} molecules are embedded in the protein core composed of the α-, α′-, β-, and γ-subunits. The catalytic core is connected to bulk solvent via a channel occupied by coenzyme B. The active site is mainly constructed with α-, β-, and γ-subunits, but the reverse side of F_{430} is ligated with glutamine oxygen from another α-subunit. This structural feature is of interest because biochemical experiments suggest that the active site of Mcr is coupled with the other active site to couple endergonic and

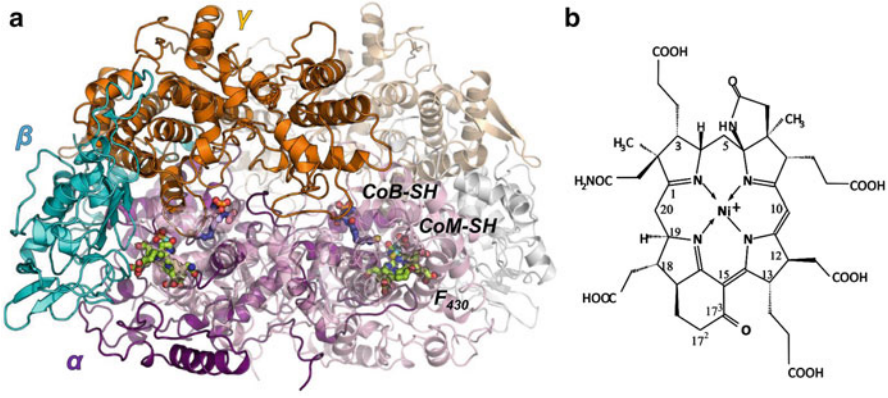

Fig. 13 Structure of methyl-coenzyme M reductase (Mcr). (**a**) Whole MCR structure from *M. marburgensis*. F_{430}, CoM-SH, and CoB-SH are represented in ball and stick. (**b**) Chemical structure of F_{430}

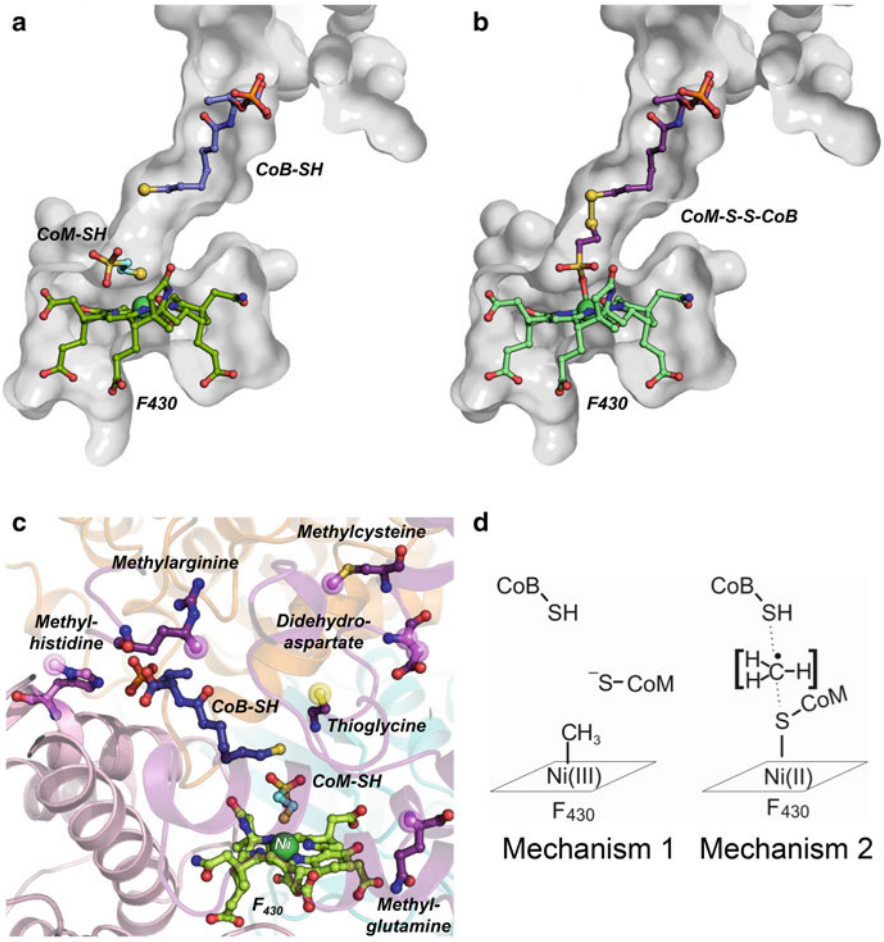

Fig. 14 The active site structure of methyl-coenzyme M reductase (Shima 2016). The active site structure of Mcr isoenzyme I from *M. marburgensis*; the active site of the $Mcr_{ox1\text{-}silent}$ form contains CoM-S-Ni (F_{430}) and CoB-SH (**a**) and that of Mcr_{silent} form contains the heterodisulfide (**b**). (**c**) The modified amino acid residues found near the active site. (**d**) Key intermediates of two proposed Mcr catalytic mechanisms. In mechanism 2, the methyl radical is shown in brackets as it is only transiently present

exergonic catalytic reaction steps (Thauer and Shima 2007). The crystal structures of Mcr in complex with coenzyme B and coenzyme M (MCR_{ox1}-silent form) (Fig. 14a) and with heterodisulfide (MCR_{silent} form) (Fig. 14b) were reported. The coenzyme B moiety of heterodisulfide in the MCR_{silent} structure and coenzyme B in the $MCR_{ox1\text{-}silent}$ structure are bound to the same site of the substrate entrance channel. By contrast, coenzyme M binding sites are different. In the MCR_{silent} form, the coenzyme M moiety is bound to the nickel site of F_{430} through its sulfonate oxygen. In the $MCR_{ox1\text{-}silent}$ form, coenzyme M is bound to the nickel site of F_{430} with its sulfur (Ermler et al. 1997a).

One of the intriguing features of Mcr is the posttranslationally modified amino acid residues near the active site (Fig. 14c) (Kahnt et al. 2007; Wagner et al. 2016c). In Mcr from *M. marburgensis*, four methylated amino acids, one thioglycine, and a didehydroaspartate were identified. In addition, in Mcr from the ANME-1 methanotrophic archaeon, a 7-hydroxy-tryptophan was found in the crystal structure (Shima et al. 2012), and most recently a 6-hydroxy-tryptophan was identified in *Methanotorris formicicus* (Wagner et al. 2017). Didehydroaspartate, methylcysteine, and hydroxytryptophan were not conserved in other Mcr (Wagner et al. 2016c), which suggests that these modifications are not necessary for catalytic activity but improve catalytic activity and/or stability.

Based on the crystal structure of the $MCR_{ox1\text{-}silent}$ form, the first catalytic mechanism was proposed (Ermler et al. 1997a; Grabarse et al. 2001), in which the Ni(I) of F_{430} attacks methyl-coenzyme M to make methyl-Ni(III) and CoM anion (Fig. 14d). After electron transfer from coenzyme M anion to methyl-Ni(III) forming methyl-Ni (II) and CoM thiyl radical, a hydrogen atom is transferred from CoB-SH to methyl-Ni(II) to produce methane (Ermler et al. 1997a). This mechanism is analogous to Co (I) chemistry in cobalamin-dependent enzymes, in which a methyl-cobalt intermediate is formed. The presence of Ni-H and Ni-S bonds is revealed by electron paramagnetic resonance (EPR) spectroscopic data (Harmer et al. 2005, 2008).

The second radical-based catalytic mechanism was proposed using a density function theory (DFT) calculation, again based on the crystal structure of $MCR_{ox1\text{-}silent}$. In the second mechanism, Ni(I) attacks methyl-S-CoM to produce methyl radicals and CoM-S-Ni(II) (Fig. 14d) (Pelmenschikov et al. 2002). Subsequently, the methyl radical accepts hydrogen atoms from CoB-SH to produce methane. To avoid rapid racemization of methyl radicals, C–S bond cleavage and C–H bond formation proceed in one step (Pelmenschikov et al. 2002; Scheller et al. 2017). Recently, Ragsdale and his colleagues have identified the CoM-S-Ni (II) intermediate of the reaction of Mcr using spectroscopic methods. Ultraviolet-visible spectroscopy, electron magnetic resonance spectroscopy, and magnetic circular dichroism spectroscopy were used to detect the intermediates trapped with a stopped-flow system using an analogue of CoB-SH containing a hexanoyl, instead of a heptanoyl side chain, which slows the reaction rate (Wongnate et al. 2016).

4.9 Heterodisulfide-Reductase/[NiFe]-Hydrogenase Complex (Hdr-Mvh)

The reduction of the heterodisulfide of coenzyme M and coenzyme B (CoM-S-S-CoB) with H_2 is an exergonic reaction ($\Delta G^{\circ\prime} = -49$ kJ/mol) (Thauer et al. 2010). Methanogens with cytochromes contain a membrane-associated heterodisulfide reductase (HdrDE) and a membrane-associated [NiFe]-hydrogenase (VhtAGC), which couple the exergonic reduction of the heterodisulfide with H_2 to the endergonic translocation of protons through the cytoplasmic membrane (Peinemann et al. 1990; Deppenmeier et al. 1992; Abken et al. 1998). By contrast, in methanogens

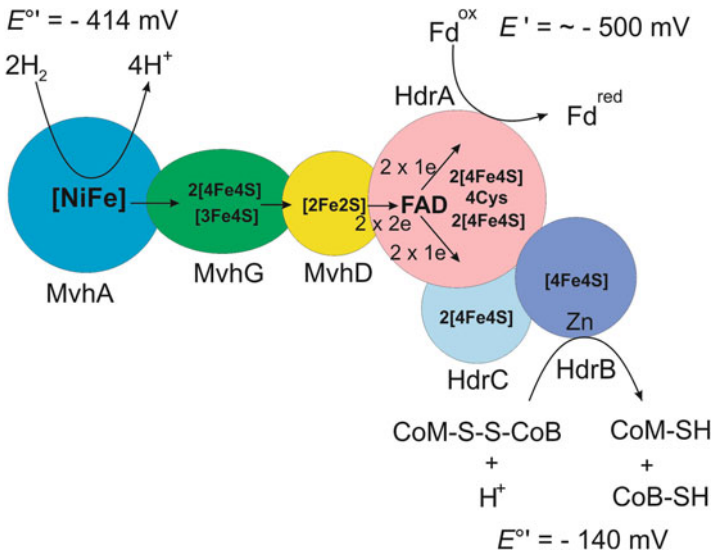

Fig. 15 Composition and reaction of the heterodisulfide-reductase/[NiFe]-hydrogenase complex (HdrABC-MvhAGD) (Buckel and Thauer 2013)

without cytochromes, a cytoplasmic electron-bifurcating heterodisulfide reductase/[NiFe]-hydrogenase complex (HdrABC-MvhAGD) couples the reduction of CoM-S-S-CoB with H_2 to the endergonic reduction of ferredoxin (Hedderich et al. 1989; Setzke et al. 1994; Kaster et al. 2011). Under physiological conditions, the redox potential E' of ferredoxin is near −500 mV and that of the $2H^+/H_2$ couple is near −400 mV. Therefore, ferredoxin can only be fully reduced by H_2 when it is coupled to an exergonic reaction (Buckel and Thauer 2013). Experimental observations indicated that the HdrABC-MvhAGD complex catalyzes the complete reduction of ferredoxin with H_2, but only in the presence of CoM-S-S-CoB (Kaster et al. 2011). The stoichiometry was found to be: $2H_2$ + CoM-S-S-CoB + Fd_{ox} = CoM-SH + CoB-SH + Fd_{red}^{2-} + $2H^+$. A model of the HdrABC-MvhAGD complex that considers these findings is illustrated in Fig. 15.

The HdrABC-MvhAGD complex is composed of the hydrogenase module (MvhAGD) and the heterodisulfide reductase module (HdrABC). MvhA (53 kDa) is the large subunit of [NiFe]-hydrogenase, which contains a [NiFe] catalytic center. MvhG (34 kDa) is the small subunit of the hydrogenase, which contains three iron-sulfur clusters. MvhD (16 kDa) contains one [2Fe-2S] cluster and is predicted to provide the electronic connection to HdrABC. HdrA (72 kDa) contains one flavin binding site, four [4Fe-4S] clusters, and four characteristically spaced conserved cysteines. HdrB (33 kDa) harbors a zinc-binding motif at the N-terminal domain and two copies of a cysteine-rich sequence, $CX_{31\text{-}39}CCX_{35\text{-}36}CXXC$, which is proposed to be involved in binding of an unusual [4Fe-4S] cluster. HdrB harbors the active site for heterodisulfide reduction, and HdrC (21 kDa) serves as an electron connector between HdrA and HdrB, which provides two [4Fe-4S] clusters.

In the complex, the six subunits are present in a 1:1:1:1:1:1 stoichiometry. The apparent molecular mass of the HdrABC-MvhAGD complex was found to be approximately 500 kDa, indicating that the heterohexamer forms a dimer. The dimer is in equilibrium with the heterohexamer and is composed of the subcomplexes MvhAGD (103 kDa) and HdrABC (126 kDa) (Setzke et al. 1994). The purified complex contained 0.6 mol nickel, 0.9 mol FAD, 26 mol non-heme iron, and 22 mol acid-labile sulfur per mol of heterohexamer. FAD is only loosely bound; therefore, FAD must be added to the buffers used for purification. In most methanogens, the genes encoding these proteins are organized into three transcription units, *mvhDGAB*, *hdrA*, and *hdrBC*. The gene *mvhB* encodes a polyferredoxin with 12 [4Fe-4S] clusters.

In the model shown in Fig. 15, one FAD of HdrA is assumed to be the site of electron bifurcation. The FAD is reduced by 2 × 2 electrons from H_2 and is oxidized by 2 × 1 electrons bifurcated to CoM-S-S-CoB and ferredoxin. However, the mechanism of flavin-based electron bifurcation requires that FAD is reduced with H_2 in a $2e^-$ reduction step (a hydride transfer). How this is achieved by only one FAD is difficult to envisage on the basis of the model, since iron-sulfur proteins generally transfer only one electron at a time.

4.10 [NiFe]-Hydrogenases

In the hydrogenotrophic methanogenic pathway of methanogens without cytochromes, three types of [NiFe]-hydrogenases are involved: F_{420}-reducing hydrogenase (Frh), heterodisulfide-reductase-associating hydrogenase (Mvh), and integral membrane energy-conserving hydrogenase (Eha and Ehb). In methanogens with cytochromes, integral membrane energy-conserving hydrogenase (Ech) homologous to Eha and Ehb, methanophenazine-reducing [NiFe]-hydrogenase (VhtAGC) is additionally involved (Thauer et al. 2010).

Frh is found in most methanogenic archaea. In the hydrogenotrophic methanogenic pathway, Frh uses electrons from H_2 to produce $F_{420}H_2$, which is used as hydride donor for the reactions catalyzed by Mtd and Mer and other reactions. In the methanogenic pathway from C1 compounds, $F_{420}H_2$ is generated from the oxidation of methyl-H_4MPT to CO_2 in the reverse reactions of those shown in Fig. 3. In methanogenesis, using formate, F_{420} can be reduced to $F_{420}H_2$ by F_{420}-dependent formate dehydrogenase, and $F_{420}H_2$ is used for the formation of H_2, which is catalyzed by Frh. However, Leigh et al. reported that F_{420}-dependent formate dehydrogenase forms a complex with heterodisulfide reductase, which suggests that electrons from formate could be directly transferred to the heterodisulfide reductase system (Costa et al. 2010, 2013). Thus, Frh is used for both, the oxidation and reduction of H_2 under physiological conditions. The catalytic unit of Frh appears to be the FrhAGB heterotrimer (Mills et al. 2013; Vitt et al. 2014) (Fig. 16a). The FrhA and FrhG subunits correspond to the large and small subunits of [NiFe]-hydrogenase, respectively. FrhA contains the [NiFe] dinuclear catalytic center, similar to that of other [NiFe]-hydrogenases, in which one CO and two CN ligands are coordinated to the iron site. FrhG contains three [4Fe-4S] clusters, which are slightly different from other [NiFe]-hydrogenase because the medial iron-sulfur

cluster of other [NiFe]-hydrogenases is a [3Fe-4S] cluster rather than a [4Fe-4S] cluster; in addition, one of the ligands of the proximal [4Fe-4S] cluster is substituted to aspartate (instead of cysteine). In addition, one of the ligands of the distal [4Fe-4S] cluster was substituted to cysteine instead of histidine, which is found in the standard [NiFe]-hydrogenases. The redox potentials of the three [4Fe-4S] clusters in FrhG are lower than −400 mV, which is close to the redox potential of the $H_2/2H^+$ couple ($E^{\circ\prime} = -414$ mV) and the $F_{420}/F_{420}H_2$ couple ($E^{\circ\prime} = -360$ mV). The redox potential

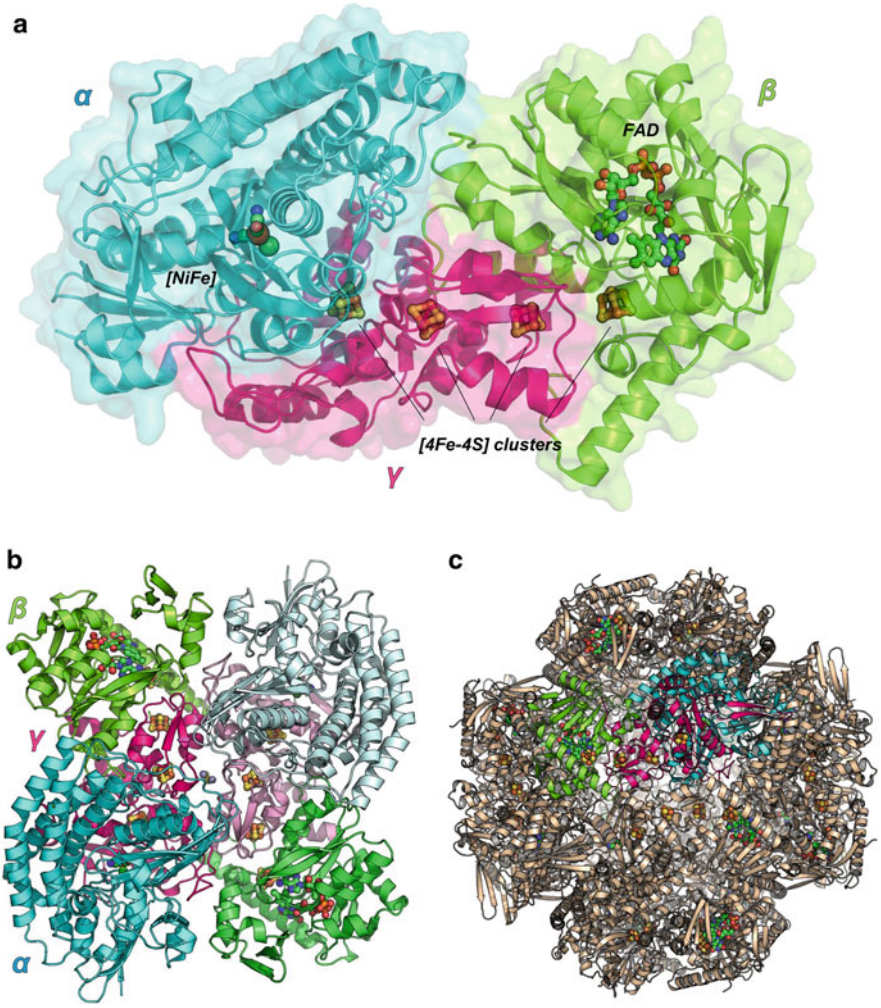

Fig. 16 Structure of F_{420}-reducing hydrogenase (Frh) (Vitt et al. 2014). (**a**) A FrhAGB heterotrimer; the FrhA, FrhB, and FrhG subunits are shown in cyan, magenta, and green, respectively. The Ni, Fe, and S of the [NiFe] center and the [4Fe-4S] cluster are shown as green, brown, and yellow spheres, and FAD is shown as stick model. (**b**) Dimer of the FrhAGB. The three subunits of one trimer are colored as panel a. (**c**) Cartoon model of "nanoball" structure of hexameric FrhAGB dimer. The three subunits of one trimer are colored as panel **a**

of the iron-sulfur clusters is substantially lower than those in other [NiFe]-hydrogenases, which might be responsible for the reversibility of Frh.

The plausible catalytic unit of FrhAGB forms a homodimer of heterotrimers (Fig. 16b), and the six molecules of the homodimer form a cubic hexamer (Fig. 16c). In the hexameric "nanoball," the entrances of the substrates H_2 and F_{420} are located on the surface of the nanoball, which indicates that the internal space of the nanoball appears to be not involved in catalytic reactions. Shielding of the electron chains and the [NiFe]-active site from bulk solvent and stabilization of the protein in the physiological cytoplasmic environments are discussed (Vitt et al. 2014).

In the hydrogenotrophic methanogenic pathway, reduced ferredoxin is regenerated by the electron-bifurcating heterodisulfide-reductase/[NiFe]-hydrogenase complex. However, a part of reduced ferredoxin is used for the other anabolic reactions, and an intermediate of the methanogenic pathway, methyl-H_4MPT, is consumed for anabolic metabolism. Therefore, the ferredoxin used for the anabolic reactions must be compensated by the other enzyme system. The integral membrane [NiFe]-hydrogenase complexes Eha and Ehb catalyze reduction of ferredoxin ($E' = \sim -500$ mV) with oxidation of H_2 ($E^{\circ\prime} = \sim -414$ mV). This endergonic reaction is driven by a sodium ion potential created by the integral membrane MtrA-H complex. EhaA-T and EhbA-Q are homologues of the energy-converting [NiFe]-hydrogenase EchA-F identified in methanogens with cytochromes. EchE is the large subunit containing a [NiFe] site, and EchC is the small subunit but contains only one [4Fe-4S] cluster. EchF contains two [4Fe-4S] clusters. EchD has no prosthetic groups, and its function is unknown. EchA and EchB are integral membrane proteins, which can mediate ion translocation. EhaA-T and EhbA-Q contain homologous subunits to EchA-G, and similar catalytic functions are predicted. However, 14 and 11 subunits with unknown function are additionally found in the gene cluster of Eha and Ehb, respectively (Tersteegen and Hedderich 1999).

5 Research Needs

Over the last two decades, the catalytic mechanism of methanogenic enzymes has been studied based on the crystal structures of the enzymes and enzyme complex with substrates and/or inhibitors. However, the crystal structures of some methanogenic enzymes have not yet been solved, and their catalytic mechanisms are not fully understood, as described below.

For instance, the catalytic mechanism of the heterodisulfide reductase/[NiFe]-hydrogenase complex (HdrABC/MvhAGD) using an electron bifurcation mechanism is unknown. The HdrB subunit, which is proposed to catalyze heterodisulfide reduction, contains a unique CCG motif, which might bind a new iron-sulfur cluster. This plausible iron-sulfur cluster-binding motif is conserved in more than 2,000 proteins involved in the three domains of life (Pereira et al. 2011). To unravel the catalytic mechanism of the enzyme complex, a high-resolution crystal structure of the HdrABC-MvhAGD complex is necessary. The unique energy conservation reaction of the MtrA-H complex using the methyl-transfer reaction to translocate

sodium ions through the membrane must be elucidated. The structure of these two complexes will open the way to understand these unique machineries.

The methyl-coenzyme M reductase reaction is still the major target of interest. The findings for the CoM-S-Ni(II) intermediate, together with the previous finding that secondary deuterium isotope effects are consistent with the formation of methyl radical (Scheller et al. 2013a, b, 2017; Wongnate et al. 2016), support the methyl radical catalytic mechanism (mechanism 2) (Fig. 14d). However, to unravel the reaction mechanism of MCR, further experiments are required. All proposed mechanisms are based on crystal structures of the inactive forms of MCR; therefore, a crystal structure of the active form must be solved. ^{19}F-ENDOR data for the active MCR in the presence of HS-CoM and CF_3-S-CoB indicated a shift in the 7-thioheptanoyl chain toward nickel by more than 2 Å (Ebner et al. 2010), which reflects the potential of MCR to undergo a major conformational change in the active enzyme states during catalysis. Furthermore, MCR contains many modified amino acids near the active site. Investigations to analyze the function of posttranslational modifications and their biosynthesis are of interest.

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Ecophysiology of Acetoclastic Methanogens

4

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Abstract

Acetate is the most important precursor for methane in the degradation of organic matter. Only two genera of methanogenic archaea, *Methanosarcina* and *Methanothrix* (former *Methanosaeta*), are able to grow with acetate as sole energy and carbon source. Phylogenetically, *Methanosarcina* and *Methanothrix* both belong to the *Methanosarcinales*. These two genera show besides morphological differences, interesting differences in physiology. *Methanosarcina* is a generalist that can grow on a variety of substrates, while *Methanothrix*

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specialized in growth on acetate. The acetate metabolism shows differences in acetate activation and energy conservation. At conditions that are less favorable for acetoclastic methanogens, syntrophic acetate oxidation may occur. This, however, is not further addressed here.

1 Introduction

In anaerobic environments where inorganic electron acceptors, such as nitrate, Fe(III), Mn(IV), or sulfate and sulfur, are limiting complex organic matter is decomposed to methane and carbon dioxide as main products (Stams and Plugge 2009). This involves a series of sequential conversions performed by communities of fermentative anaerobic bacteria and methanogenic archaea. Anaerobic bacteria degrade the organic compounds to products, typically hydrogen, carbon dioxide, formate, and acetate, which are the main substrates of methanogens. When organic matter is completely degraded, about 60–70% of the methane is formed from acetate, the remainder from H_2 + CO_2 and formate. This shows the quantitative importance of acetate in the formation of the hydrocarbon methane.

The fate of acetate in a methanogenic environment is largely dependent on the chemo-physical conditions, such as temperature, pH, salt, and presence of inhibitory compounds, e.g., ammonium. At circumneutral pH and at low or moderately high temperature, acetate is directly degraded by methanogenic archaea. Just two genera of acetotrophic methanogens, *Methanothrix* (former *Methanosaeta*) and *Methanosarcina*, are able to grow on acetate. Syntrophic acetate degradation can occur as well. This is particularly important at conditions where acetotrophic methanogens cannot grow well, which is typically the case in environments with a high ammonium concentration, high temperature (above 60 °C), and high salt/high pH. The first observation of syntrophic acetate oxidation was done by Zinder and Koch (1984). They enriched a culture with acetate at 60 °C and obtained a coculture of a bacterium and a hydrogenotrophic methanogen. Syntropic conversion was further demonstrated by using labeled substrates. Acetoclastic methanogens split acetate to methane and CO_2, where the methyl-group yields methane and the carboxyl-group CO_2. During syntrophic acetate oxidation, both C-atoms of acetate are first converted to CO_2 and the methanogen uses the formed CO_2 to produce methane. Consequently, during syntrophic acetate oxidation labeled methane is formed when unlabeled acetate and labeled CO_2 are provided. This strategy was regularly applied to demonstrate syntrophic acetate oxidation in other environments, e.g., in environments with ammonium concentrations that are inhibitory for acetoclastic methanogens (Schnürer et al. 1999).

2 Phylogeny and Taxonomy

The two known acetotrophic methanogenic genera are *Methanothrix* ("*Methanosaeta*") and *Methanosarcina*. These two genera belong to the order *Methanosarcinales* within the archaeal kingdom *Euryarchaeota*. Described

mesophilic and thermophilic *Methanosarcina* species include, *M. barkeri*, *M. mazei*, *M. acetivorans*, *M. baltica*, *M. frisia*, *M. horonobensis*, *M. lacustris*, *M. semesiae*, *M. siciliae*, *M. soligelidi*, *M. vacuolata,* and *M. thermophila*. *Methanothrix s*pecies include *M. soehngenii*, *M. concilii*, and *M. harundinacea*, which are mesophiles, and the thermophiles *M. thermoacetophila* and *M. thermophila*. The synonym for *Methanothrix* is *Methanosaeta*. Based on a long-standing discussion, the most recent opinion of the International Committee on Systematics of Prokaryotes (Tindall 2014), *Methanothrix* is appropriate and this name is used throughout this chapter, though *Methanosaeta* is most-often used in publications of the last decade.

In pioneering studies by Nicolaas L. Söhngen and later by Horace A. Barker, sarcina type methanogens were enriched (Söhngen 1906; Barker 1936). The first pure culture of *Methanosarcina barkeri* was obtained by Schnellen (1947), and since then many mesophilic and thermophilic *Methanosarcina* species have been isolated and described.

Methanothrix soehngenii was first described by Huser et al. (1982) and later *Methanothrix concilli* was described (Patel 1984). Based on a comparative analysis of *Methanothrix* strains, it was concluded that *M. concilli* is a synonym of *M. soehngenii* (Touzel et al. 1988). As the *M. soehngenii* culture was not pure, its name was found not be valid according to rule 31a of the International Code of Nomenclature of Bacteria stating that "*the name of a species or subspecies is not validly published if the description is based upon studies of a mixed culture of more than one species or subspecies*" and *Methanosaeta concilli* was proposed as the type strain of filamentous acetoclastic methanogens (Patel and Sprott 1990). Similarly, a thermophilic acetoclastic methanogen had been described, *Methanothrix thermoacetophila* (Nozhevnikova and Chudina 1985). However, as that name was never validated and the culture turned out not to be pure also that name was considered not to be valid, and *Methanothrix thermophila* was described by Kamagata et al. (1992) to represent thermophilic acetoclastic filamentous methanogens. That archaeon was proposed to be named *Methanosaeta thermophila* (Boone and Kamagata 1998), which was approved by the International Committee on Systematics of Prokaryotes (Tindall 2008). However, recently both *Methanothrix soehngenii* and *Methanothrix thermoacetophila* were reestablished as valid names based on a changed view of the interpretation of rule 31a, "*the name of a species or a subspecies is not validly published if the description is demonstrably ambiguous and cannot be critically identified for purposes of the precise application of the name of a taxon*" (Tindall 2014).

3 Physiological Properties

Methanosarcina and *Methanothrix* are morphologically and physiologically different. Morphologically, the sarcina-shape and thix-shape are represented in the genus names. The cell wall structure of the two types of methanogens is also different. As most methanogens, *Methanosarcina* and *Methanothrix* contain S-layers which are mostly composed of a single protein or glycoprotein which is associated with the cytoplasmic membrane (Albers and Meyer 2011). The cell wall of *Methanosarcina*

contains methanochondroitin, which is a fibrillar polymer composed of a trimer repeat of two *N*-acetylgalactosamines and one glucuronic acid. Its formation is associated with the aggregated form of *Methanosarcina* (Kreisl and Kandler 1986). *Methanothrix concilii* has a rather complex cell envelope. The filamentous chains are enclosed by a unique tubular paracrystalline proteinaceous sheath surrounding the S-layer and the cytoplasmic membranes.

Physiologically, *Methanosarcina* and *Methanothrix* show interesting differences. *Methanosarcina* has a broader substrate range. Besides acetate, *Methanosarcina* species can grow with H_2 + CO_2, methanol and methylated amines. It can be considered as a generalist. *Methanothrix* is a specialist that only uses acetate as growth substrate, though as discussed later it also has the ability to convert CO_2 to methane, without the involvement of hydrogenases. The growth behavior of the two types of methanogens is different. While *Methanosarcina* shows faster growth, *Methanothrix* has a higher affinity for acetate (Table 1). The higher affinity has been associated with the enzyme systems for acetate activation (Jetten et al. 1990). The difference in acetate transport was proposed to play a role as well (Smith and Ingram-Smith 2011). The differences in specific growth rate and affinity for acetate make *Methanosarcina* easily enriched using routine isolation procedures, while *Methanothrix* is often the most abundant acetoclastic methanogens in environments where a low acetate concentration is observed.

Methanosarcina is considered to be a rather robust methanogen in comparison with *Methanothrix* as it grows faster with acetate and can use other substrates. In addition, *Methanosarcina* can better resist and recover when exposed to stressors such as ammonium, chlorinated compounds, salt, and high acetate concentration (De Vrieze et al. 2012). In comparison with *Methanothrix*, *Methanosarcina* is most resistant to oxygen. Recently, even the cocultivation with aerobic methanotrophs was described (in ‘t Zandt et al. 2018). However, there are examples where *Methanothrix* seems to be more resistant. *Methanothrix* is more resistant to long-chain fatty acids (Silva et al. 2016) and humic substances (Azman et al. 2017) than *Methanosarcina*. The higher tolerance in these cases was thought to be related to the different cell wall structure.

In recent years, there is quite some attention for direct electron transfer in methanogenic microbial communities, with or without electron mediators (Lovley 2017; Martins et al. 2018). Acetoclastic methanogens have been described to accept

Table 1 Comparison of the physiological parameters of *Methanosarcina* spp. and *Methanothrix* spp. (Jetten et al. 1990)

	Methanosarcina	*Methanothrix*
Physiology	Generalist	Specialist
Substrates	Acetate, hydrogen, methanol, methylamines	Acetate
Specific growth rate (day^{-1})	0.3	0.1
Doubling time (days)	0.5–2	1–12
Yield (g/mol Ac)	2.1	1.4
Km (mM)	3.0	0.5

directly electrons provided by another bacterium or a solid surface to produce methane from CO_2. Morita et al. (2011) suggested the potential of direct interspecies electron transfer in a methanogenic bioreactor. In an ethanol-fed bioreactor, *Methanothrix* became the dominant methanogens, and *Geobacter* the most abundant and metabolically most active bacteria (Shrestha et al. 2013); metatranscriptomics revealed that the *Methanothrix* species in the digester were highly expressing genes for the reduction of carbon dioxide to methane (Rotaru et al. 2014b). These observations were quite remarkable as *Methanothrix* is not able to grow with H_2 + CO_2 and lacks the essential hydrogenases of typical hydrogenotrophic methanogens. In a coculture of *Methanosarcina barkeri* and *Geobacter metallireducens*, direct interspecies electron transfer was involved in ethanol conversion (Wang et al. 2016), while in a coculture of *M. barkeri* and *Pelobacter carbinolicus* interspecies hydrogen transfer played a role (Rotaru et al. 2014a). Unlike *Methanosaeta*, *Methanosarcina* is able to grow with H_2 + CO_2. *Methanosarcina* can perform a hydrogen-dependent relationship with other bacteria. When sulfate is present, *Methanosarcina* can transfer hydrogen formed in the conversion of methanol or acetate to a sulfate-reducing *Desulfovibrio* (Phelps et al. 1985). *Methanosarcina* can act as hydrogen scavenger when a *Desulfovibrio* is grown on lactate without sulfate (Bryant et al. 1977). This shows that *Desulfovibrio* and *Methanosarcina* can both act as hydrogen-producing and hydrogen-consuming microorganisms, which makes it an interesting coculture for further genome-based studies (Plugge et al. 2010; Scholten et al. 2007).

In anaerobic environments, acetoclastic methanogens may compete with sulfate-reducing bacteria for acetate. Research by Schönheit et al. (1982) showed that *Desulfobacter postgatei* outcompeted *Methanosarcina barkeri* for acetate. This was explained by differences in affinity for acetate; the Km values for acetate are 0.2 and 3.0 mM for the sulfate reduce and the methanogen, respectively. However, *Methanothrix* is often the most abundant acetoclastic methanogen and as explained, *Methanothrix* has a higher affinity for acetate than *Methanosarcina* (Jetten et al. 1992). In addition, *Desulfobacter* is a typical marine sulfate reducer, while in freshwater environments, *Desulfobacca acetoxidans* is an important sulfate reducer specialized in degradation of acetate (Oude Elferink et al. 1999). The Km for acetate of this bacterium is 0.1–1 mM, which is just slightly lower than that of *Methanothrix* (Km is 0.4–1.2 mM) (Oude Elferink et al. 1998; Stams et al. 2005). Also the threshold value for acetate consumption of *D. acetoxidans* is just slightly lower than that of *Methanothrix*. Thus, *Methanothrix* will be outcompeted by sulfate reducers, like *D. acetoxidans*. However, when a bioreactor with methanogenic sludge was fed with acetate and sulfate, it took a very long time before acetate was degraded by sulfate reducers, and it was calculated that it could take 200–500 days before methanogens and sulfate reducers became equally important in the conversion of acetate (Visser et al. 1993). This reflects the small differences in growth kinetic properties of the two types of microorganisms. An interesting feature in this respect is that acetoclastic sulfate reducers have a lower affinity for sulfate than hydrogenotrophic sulfate reducers (Laanbroek et al. 1984). This has important consequences. In environments

where the sulfate concentration is not sufficient for complete degradation of organic matter, acetoclastic methanogens are not easily outcompeted and acetoclastic methanogenesis still prevails (Sousa et al. 2009).

4 Pathway and Energetics

Methanogenesis from acetate yields little energy. At standard conditions, the Gibbs free energy change of the conversion of acetate to CO_2 + CH_4 is just −31 kJ/mol, which is much less than the free energy needed to synthesize one ATP; the standard Gibbs free energy change for the phosphorylation of ADP to form ATP is +45 kJ/mol (Thauer et al. 1977). By contrast, the standard Gibbs free energy change of methane formation from 4 H_2 + CO_2 is −136 kJ per mol. The pathway of acetotrophic methanogens was the subject of several excellent reviews (Ferry 2011, 2015; Schlegel and Müller 2013; Welte and Deppenmeier 2014). Available genome sequences are helpful to refine the insight of the pathways and energy conservation mechanisms. The initial step in the metabolism of acetate is the activation to acetyl-CoA (Fig. 1). Acetate activation in *Methanothrix* and *Methanosarcina* is different (Jetten et al. 1990). An acetate kinase/phospho acetyl transferase (AK/PAT) system is used by *Methanosarcina* species. This enzyme system has a high activity, but low affinity, which reflects the physiological features of *Methanosarcina*. The low-activity but high-affinity AMP-dependent acetyl-CoA-synthetases (ACS) is used

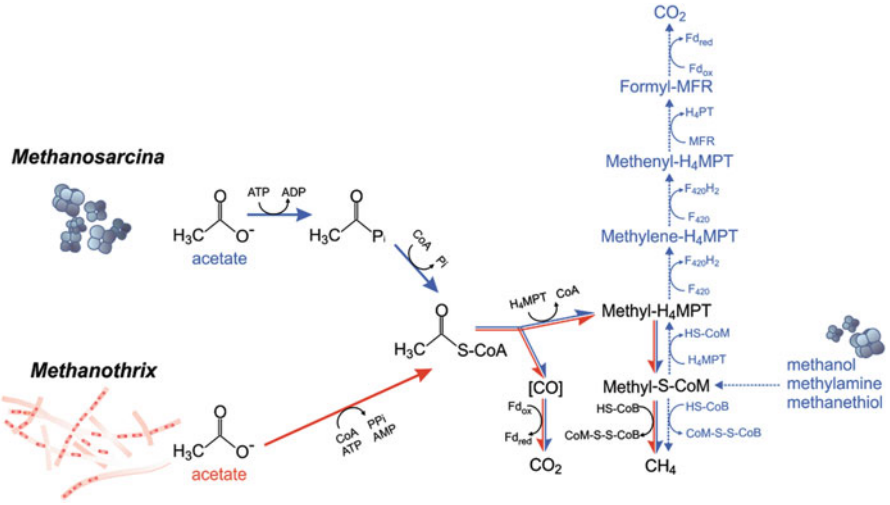

Fig. 1 Pathway of acetate conversion in *Methanosarcina* (blue arrows) and *Methanothrix* (red arrows). The pathway for the conversion of other methylated compounds by some species of *Methanosarcina* is also shown (text in blue and dashed arrows). Abbreviations: MFR, methanofuran; H_4MPT, tetrahydromethanopterin; HS-CoM, coenzyme M; HS-CoB, coenzyme B; $F_{420}H_2$, reduced form of the electron carrying coenzyme F_{420}; Fd, ferredoxin; CoA, coenzyme A. (Adapted from Welte and Deppenmeier (2014))

Table 2 Genes (locus tags) involved in acetate activation in some representatives of acetoclastic methanogens. (Data obtained from the KEGG database)

	Methanosarcina		*Methanothrix*	
	M. barkeri	*M. thermophila*	*M. thermophila*	*M. concilii*
	MS (DSM 800)	TM1 (1825)	PT (DSM 6194)	GP6 (DSM 3671)
Acetate kinase	msbrm 2524	mstht 1038		
Phosphoacetyltransferase	msbrm 2525	mstht 1037		
ADP-dependent ACS	msbrm 2523	mstht 1039		
AMP-dependent ACS			mthe 0155	mcon 0556
			mthe 1194	mcon 0558
			mthe 1195	mcon 0559
			mthe 1196	mcon 0561
			mthe 1413	mcon 0780
				mcon 2868
Pyrophosphatase	msbrm 0400	mstht 0033	mthe 0236	mcon 1906
	msbrm 0992	mstht 0782		
	msbrm 2200	mstht 1810		
		mstht 2185		
Adenylate kinase	msbrm 2589	mstht 1141	mthe 0311	mcon 1615
	msbrm 2695	mstht 2391	mthe 1504	mcon 1964

by *Methanothrix*. The AK/PAT system generates ADP, phosphate, and acetyl-CoA from ATP, CoA, and acetate, while the ACS converts ATP, CoA, and acetate to acetyl-CoA, AMP, and pyrophosphate (Jetten et al. 1990; Berger et al. 2012; Ferry 1992). The pyrophosphatase of *Methanothrix* is a soluble protein, which makes it unlikely that energy of pyrophosphate cleavage is conserved (Berger et al. 2012; Zhu et al. 2012). Remarkably, membrane-bound pyrophosphatase is present in *Methanosarcina*. Thus, the activation of acetate in *Methanosarcina* requires one ATP, while acetate activation in *Methanothrix* requires two ATP, as ATP + AMP is converted to ADP by adenylate kinase. Genes coding for enzymes in some acetoclastic methanogens are presented in Table 2. As acetoclastic methanogens grow with acetate as sole carbon and energy source, energy conservation mechanism should yield more than one and two ATP per molecule of acetate in *Methanosarcina* and *Methanothrix*, respectively.

Acetyl-CoA is converted to a methyl and carbonyl moiety by the action of a CO dehydrogenase/acetyl-CoA synthase (Fig. 1). At the enzyme, the carbonyl group is oxidized to CO_2 and electrons are transferred to ferredoxin. The methyl group is transferred to a methanogenic cofactor (tetrahydromethanopterin) and subsequently transferred to coenzyme M by a membrane bound sodium translocating methyltransferase. Reduction of the methyl group to methane with coenzyme B as electron donor leads to the formation of a heterodisulfide (CoM-S-S-CoB). In both methanogens, the heterodisulfide is cleaved (reduced) to coenzyme M and coenzyme B with reduced ferredoxin, a process that results in energy conservation (Welte and

Deppenmeier 2011; Feist et al. 2006). *Methanosarcina barkeri* employs an energy conserving hydrogenase (Ech) complex and F420 nonreducing hydrogenase, while *M. acetivorans* uses an Rnf-like complex (Li et al. 2006; Ferry 2015; Schlegel and Müller 2013). The involvement of these membrane bound enzyme systems results in the formation of an electrochemical gradient (protons, sodium) to drive ATP synthesis (Wang et al. 2011). In the genomes of *Methanothrix* species, the genes for Ech or the Rnf-like complex are not present (Barber et al. 2011; Zhu et al. 2012; Welte and Deppenmeier 2011). Instead, a multi-gene cluster encoding for a reduced F420 dehydrogenase, which is not present in obligate hydrogenotrophic methanogens, but which is found in *Methanothrix* and in *Methanosarcina,* seems to play an important role in the formation of a proton gradient in *Methanothrix* when grown on acetate. As discussed by Welte and Deppenmeyer (2014), with some assumptions about the number of protons and sodium ions exported, sufficient energy could be conserved for net ATP synthesis. Generally, it is assumed that three protons or sodium ions drive the synthesis of one ATP, but the stoichiometry for methanogens was found to be four (Deppenmeier and Müller 2007). However, the way *Methanothrix* conserves energy needs further study. Modeling can help in prioritizing which possible explanations may be most likely. One powerful approach is called genome-scale metabolic modeling.

5 Genome-Scale Metabolic Modelling

Genome scale metabolic models are in essence a computable inventories of all metabolic reactions that the gene products – proteins, i.e., enzymes – of the genome can carry out (Henson 2015). Many bioinformatic tools and databases are available for such a metabolic reconstruction, and also for acetoclastic methanogens such genome-scale metabolic models are made (Feist et al. 2006; Benedict et al. 2012; Hanemaaijer 2016). Knowledge of the kinetic properties of redox enzymes involved in methanogenesis can be used to obtain insight in the physiology and the bioenergetics of acetoclastic methanogens, as discussed above; genome-scale models add a quantitative bookkeeping of all ATP-generating and ATP-consuming reactions in the metabolic network required for growth. Such models can run scenarios that are very difficult to perform experimentally, and in such a way combine molecular and physiological data to test the conditions at which specific hypotheses or stoichiometries are feasible.

For example, for *Methanothrix concilii,* Hanemaaijer (2016) reconstructed the metabolic network and investigated quantitatively which adjustments in the current stoichiometries of acetate activation and membrane pump stoichiometries (protons, sodium) would allow *M. concilii* to grow on acetate. For example, as a function of ΔpH and specific metabolite levels, the maximum number of protons dissipated per ATP formed can be calculated, which is less than four (and likely more than three). Also if a membrane-bound, proton-pumping pyrophosphatase would be present (or engineered), the minimal number of protons will have to be two (1.8 in the model), again under some assumptions of unknown concentrations that determine the Gibbs free energy changes of the associated reactions. Despite that, in *Methanothrix* a

typical energy-conserving membrane-bound pyrophosphatase is not present, but its ability to contribute to energy conservation is still possible. Welte and Deppenmeier (2014) proposed that pyrophosphate cleavage might be linked to other energy-dependent reactions in the anabolism by which at least partially the energy of pyrophosphate hydrolysis is conserved. In the models, it can be calculated which combination of changes in stoichiometries are feasible and which not, and based on this dedicated experimental validations can be suggested.

Genome-scale metabolic models are also often used in biotechnology for strain and process optimization (Branco dos Santos et al. 2013; Gottstein et al. 2016). For acetoclastic methanogens, who seem to live on the edge of thermodynamic feasibility, it is important to integrate thermodynamic constraints based on metabolite levels. Recently, such a genome-scale metabolic modeling approach was developed to understand how microbes, among which acetoclastic methanogens cope with substrate concentrations that prevail in natural environments (Shapiro et al. 2018). Such approaches should in the future be combined with additional cellular constraints based on either resource allocation (Basan 2018) or thermodynamics (Kümmel et al. 2006) to become powerful predictors of growth phenotypes.

6 Concluding Remarks and Perspectives

Two genera of acetoclastic methanogens, *Methanosarcina* and *Methanothrix,* have been described. There are remarkable differences between these two genera in terms of morphology and physiology. Acetoclastic methanogenesis is energetically not a very favorable process, but nevertheless, it is very important for complete degradation of organic matter. Research done to understand the metabolism and energy-conservation mechanisms in acetoclastic methanogens showed differences in acetate activation and electron transfer mechanisms. Further genome-based transcriptome and proteome analyses, in combination with biochemical and modeling studies, will shed further light how these types of archaea cope with the energy constraints. Omics information will also help to understand better the interaction of acetoclastic methanogens with other microorganisms and with inorganic materials.

7 Research Needs

In methanogenic environments, acetate can be degraded by acetoclastic methanogens (*Methanothrix* and *Methanosarcina*) or by syntrophic associations of acetate-degrading bacteria and hydrogenotrophic methanogens. Further research is needed to define the exact environmental conditions stimulating direct and indirect methanogenesis from acetate.

In-depth genome-based analysis and genome-scale modeling offer the possibility to get further insight of the bioenergetic features of acetoclastic methanogens and to formulate hypotheses that can be tested. To completely resolve the bioenergetic

properties of acetoclastic methanogens, physiological and enzymatic studies are needed.

Advances in the development of genetic systems in acetoclastic methanogens are still modest, with some successful genetic modification trials of *Methanosarcina*, but no attempts on *Methanothrix*. The ability to insert genes and create knock-outs of acetoclastic methanogens will aid the study of their physiology and bio-energetic features.

The observation that acetoclastic methanogens are involved in mediated electron transfer (MET), direct electron transfer (DET), and direct interspecies electron transfer (DIET) is intriguing. A better picture of the quantitative importance of electron transfer processes linked to acetoclastic methanogens can be obtained by studying pure culture and defined mixed cultures.

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Methanogenesis from Carbon Monoxide

5

Christian Schöne and Michael Rother

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Abstract

The biological formation of methane, methanogenesis, constitutes the final step of biomass degradation in anaerobic environments where exogenous electron acceptors are scarce. It is therefore a fundamentally important aspect of the global carbon cycle. The organisms responsible are methanogenic archaea (methanogens), a diverse but monophyletic group within the Euryarchaeota. The major metabolic substrates for methanogenic energy metabolism are $H_2 + CO_2$, methylated compounds, and acetate. From a bioenergetic and

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biochemical standpoint, carbon monoxide (CO), a toxic, odorless, flammable gas, which accrues from incomplete combustion, could be considered an excellent source of energy and carbon for methanogens, but the capacity to grow on CO, i.e., carboxydotrophic growth, has been demonstrated only for a few methanogenic species. It appears that CO is not a well-suited methanogenic substrate due to its toxicity toward transition metal-containing enzymes and the negative reduction potential of the CO_2/CO couple. In this chapter, we will summarize current knowledge about the catabolic pathways of CO utilization in hydrogenotrophic and methylotrophic methanogens, how they are coupled to energy conservation, and how they cope with the unfavorable properties of CO.

1 Introduction

Methane (CH_4) is the most abundant hydrocarbon present in earth's atmosphere. With a current concentration of more than 1.8 ppm (Blasing 2016 and see http://www.epa.gov), methane contributes significantly to global warming because, despite of its "short" atmospheric lifetime of ca. 12 years (compared to 100–300 years for carbon dioxide, CO_2), it adsorbs infrared radiation (IR), which is thereby trapped in the atmosphere. Although the atmospheric concentration is less than 0.5% that of CO_2, CH_4 absorbs IR 24 times more effectively on a molar basis and is therefore 66 times more effective as a greenhouse gas on a mass basis (Wuebbles and Hayhoe 2002). Furthermore, CH_4 influences the concentrations of the hydroxyl radical, the primary tropospheric oxidizing agent, which in turn affects removal of CH_4 from the atmosphere. This oxidation is also a significant source of ozone, formaldehyde (CH_2O), carbon monoxide (CO), water vapor, and its eventual final product, CO_2 (Wuebbles and Hayhoe 2002).

Of the estimated 0.6×10^9 metric tons of CH_4 released annually into the atmosphere, about 70% can be attributed – directly or indirectly – to human activity, like agriculture, burning of biomass, or use of fossil fuels (Saunois et al. 2016), which explains why the atmospheric methane content has more than doubled since pre-industrial times. Nearly all of the biologically produced CH_4 derives from the metabolism of methanogenic archaea (methanogens). The process, methanogenesis, constitutes the last step in anaerobic biomass degradation occurring in the absence of exogenous electron acceptors, and an estimated 10^9 metric tons, which is about 0.7% of the net primary biomass production, are recycled annually through CH_4 (Thauer 2011). However, most of it never escapes into the atmosphere but fuels both anaerobic (Joye 2012) and aerobic (Murrell 2010) methanotrophic energy metabolism.

Compared to other microorganisms, methanogens use only a very limited number of energy substrates, which explains their ecological niches (this edition). The most widely used electron donor is molecular hydrogen (H_2), which is oxidized in order to reduce CO_2 to methane (see ▶ Chap. 3, "Hydrogenotrophic Methanogenesis" by Shima, in this edition). The quantitatively and, thus, ecologically most relevant methanogenic energy source is acetate, which is disproportionated to CH_4 and CO_2 (aceticlastic methanogenesis; Ferry 2010a). Methylated compounds such as

methanol, methylamines, and methylsulfides can also serve as methanogenic energy substrates, whereby 4 methyl groups are disproportionated to 3 CH_4 and 1 CO_2 (methylotrophic methanogenesis; Deppenmeier 2002).

A rather uncommon – or under-explored – energy source for methanogens is carbon monoxide (CO). Although its redox properties should make it a well-suited substrate for methanogenic energy metabolism, this gas is apparently toxic for methanogens in much the same way as for other (anaerobic) microorganisms. Still, a number of methanogenic strains have been shown to grow with CO as the sole energy source, and physiological, genetic, and proteomic analysis revealed peculiarities of CO-dependent energy metabolism, one of which is the observation that besides CH_4, other metabolites are produced. This chapter aims at summarizing current knowledge about CO-dependent physiology of methanogenic archaea and putting it in the context of the "classic" paths of methanogenesis; some emphasis will be given to the path of carbon and electrons (from CO oxidation) leading to energy conservation and biomass production.

2 General Aspects of CO-Dependent Energy Metabolism

2.1 Properties of CO

CO is a colorless, odorless, flammable gas and only little more soluble in water than H_2 (ca. 1.0 mmol l^{-1} for CO versus ca. 0.8 mmol l^{-1} for H_2 at 25 °C and ambient pressure) (Kaye and Laby 1986). Natural sources of atmospheric CO (today all of them affected by human activities) are, for example, incomplete combustion of organic matter (e.g., forest fires), volcanic activity, photoproduction from dissolved organic matter in the oceans, photochemical oxidation of CH_4 and other hydrocarbons with hydroxyl radicals, and enzymatic degradation of heme (Swinnerton et al. 1970; Weinstock and Niki 1972; Khalil and Rasmussen 1990; Boehning and Snyder 2003; Rivera and Rodriguez 2009). The CO molecule is isosteric and isoelectronic to cyanide (CN^-), and therefore many enzymes susceptible to inhibition by CN^-, e.g., heme-containing enzymes, are also inhibited by CO. Particularly hemoglobic animals (like humans) are sensitive toward CO because their capacity to transport oxygen is reduced (Haab 1990). However, CO is also a neurotransmitter acting in signaling during various cellular processes (Gullotta et al. 2012). In energy-intensive industrial processes, such as steel milling, fossil energy carriers like coke are used to generate heat and process gases. One of the main off-products there is syn(thesis) gas, which consists mainly of CO, H_2, and CO_2. Utilization of syngas from steel mills, from steam or oxygen reforming, or from gasification of lignocellulosic biomass or of municipal waste has become a promising alternative to fossil carriers for the production of fuels and other platform chemicals, as well as for remediation purposes (for reviews, see Sipma et al. 2006; Daniell et al. 2016). The estimated annual generation (via biotic and abiotic processes) of CO amounts to ca. 2.6×10^9 metric tons (Khalil and Rasmussen 1990), most of which, however, is abiotically oxidized to CO_2 by reacting with hydroxyl radicals in the troposphere, thereby

indirectly contributing to global warming (Conrad 1996; Daniel and Solomon 1998). Additionally, various microorganisms use CO as a source of energy and carbon, thus keeping its partial pressure near the earth's surface low.

2.2 Carboxydotrophs

Microorganisms using CO as the source of energy for growth are termed carboxydotrophic (Oelgeschläger and Rother 2008). Originally, the term was coined for microbes, which couple the oxidation of CO to the reduction of O_2 in a respiratory, chemolithoautotrophic fashion (Meyer and Schlegel 1983). However, both aerobic and anaerobic carboxydotrophs have important aspects of their energy metabolism in common, which are (i) the presence of carbon monoxide dehydrogenase and (ii) the coupling of CO oxidation to reduction of the electron acceptor via – at least partly – membrane-bound respiratory complexes leading to formation of an ion motive force used for synthesis of ATP by ATP synthase, i.e., electron transport phosphorylation (ETP). Rather well-known electron acceptors for carboxydotrophy are O_2 (aerobic respiration), nitrate (denitrification), protons (hydrogenogenesis), sulfate (sulfidogenesis), and CO_2 (acetogenesis and methanogenesis), but more unusual compounds like anthroquinone disulfonate, fumarate, or ferrihydrite were also shown to be employed (Henstra and Stams 2004; Slobodkin et al. 2006).

It was argued that CO-dependent hydrogenogenesis, acetogenesis, and methanogenesis could be viewed as fermentative energy metabolism, because in each case, a product of CO oxidation (protons in hydrogenogens and CO_2 in acetogens and methanogens) (Eq. 1) is subsequently reduced, which is a hallmark of fermentation (Diender et al. 2015). While true for CO-dependent acetogenesis (Eq. 2) and methanogenesis (Eq. 3), applying such definition to CO-dependent hydrogenogenesis is debatable, because the protons derive from the solvent. Furthermore, another hallmark of fermentation is that a respiratory electron transport chain is (largely) absent (or not very relevant for energy conservation) and, thus, that ATP synthesis mainly occurs by substrate-level phosphorylation (SLP) (Gottschalk 1986). While SLP occurs during acetogenesis, net energy is solely conserved via ETP (Ljungdahl 1994). Furthermore, no step in methanogenesis involves SLP (see below). As CO utilization in methanogenic archaea is the focus of this chapter, the reader is referred to previously published summaries (and the references therein) on the various other carboxydotrophic ways of life (Meyer et al. 1986; King and Weber 2007; Oelgeschläger and Rother 2008; Kim and Park 2012; Diender et al. 2015).

$$CO + H_2O \rightarrow CO_2 + 2H^+ + 2\,e^- \tag{1}$$

$$4\,CO + 2\,H_2O \rightarrow CH_3COO^- + 2\,CO_2 + H^+ \Delta G^{0'} = -43.6\,\text{kJ/mol CO} \tag{2}$$

$$4\,CO + 2\,H_2O \rightarrow CH_4 + 3\,CO_2 \Delta G^{0'} = -52.6\,\text{kJ/mol CO} \tag{3}$$

2.3 Carbon Monoxide Dehydrogenase

Despite the diversity of carboxydotrophic energy metabolism and the organisms capable of it, they all depend on the presence of carbon monoxide dehydrogenase (CODH, CO:acceptor oxidoreductase). Both soluble, e.g., ferredoxin (Fd) (Terlesky and Ferry 1988) or cofactor F_{420} (a 5-deazaflavin derivative functionally analogous to NAD^+; Jetten et al. 1989), and membrane-bound, e.g., cytochromes (Jacobitz and Meyer 1989), physiological electron acceptors of CODH are known. CODHs can be classified according to their sensitivity toward O_2 into aerobic and anaerobic, according to the (transition) metals they contain in the active site into molybdenum- and nickel-containing, or according to their subunit composition and (consequential) metabolic role, into monofunctional CODH and bifunctional CODH/acetyl-coenzyme A synthase (CODH/ACS) (Ragsdale and Kumar 1996; Lindahl 2002; Ragsdale 2004; King and Weber 2007; Jeoung et al. 2014). Its distribution among physiologically and phylogenetically diverse lineages of bacteria and archaea suggests that CODH is very ancient (Martin and Russell 2007), but whether its evolution is monophyletic is difficult to ascertain due to frequent lateral gene transfer and considerable diversification; while evolutionary history of aerobic CODH remains unclear (King and Weber 2007), evolution of the anaerobic CODH/ACS could be plausibly recapitulated (Lindahl and Chang 2001).

Monofunctional CODH catalyzes only the oxidation of CO to CO_2 (and the reverse reaction) and contains either molybdenum in enzymes (abbreviated Cox) from aerobic organisms or nickel in enzymes (abbreviated Coo) from anaerobic organisms. Bifunctional CODH/ACS (abbreviated Cdh or Acs), which is only found in strictly anaerobic bacteria and archaea, exhibits an additional activity: the formation (or cleavage) of acetyl-CoA from (into) a methyl group, coenzyme A (HS-CoA), and CO (ACS, CO:methylated corrinoid protein:CoA lyase). The fact that CODH/ACS contains distinct, spatially separated active sites for CODH and ACS activity, respectively (connected by a CO channel; Maynard and Lindahl 1999; Seravalli and Ragsdale 2000), and the fact that both CODH and ACS can be found independently from each other (Svetlitchnyi et al. 2004) demonstrate the bifunctionality of this enzyme complex.

The term acetyl-CoA decarbonylase/synthase (ACDS) has been used for the CODH/ACS of acetotrophic methanogens (see below), in order to emphasize that (i) the purified enzyme complex consists of five distinct subunits and contains the respective corrinoid-containing methyltransferase activity (Grahame and Demoll 1995), which in acetogenic bacteria constitutes a "separate" activity (the corrinoid iron-sulfur protein, CoFeSP; Svetlitchnaia et al. 2006), and that (ii) the "physiological direction" of the enzyme is acetyl-CoA cleavage. However, CODH/ACS and CoFeSP form a (more or less tightly bound) functional unit and are encoded in operons, both in bacteria and archaea. Furthermore, the ACS reaction is freely reversible (Grahame et al. 2005), making it unnecessary to stress a direction of the reaction. Most importantly, CODH/ACS from bacteria and archaea still catalyze the

"same" reactions (only differing in the nature of the methyl-donating/methyl-accepting cofactor) (Ragsdale and Kumar 1996), even if considerably diverged through evolution and serving different metabolic functions.

2.4 CO and Methanogenesis

For methanogenic archaea, CO could principally be considered an excellent source of energy since the reduction potential ($E^{0\prime}$) of the CO_2/CO couple (−524 to −558 mV) (Thauer 1988; Grahame and Demoll 1995) is lower than that of any redox-active cofactor employed in methanogenesis. However, utilization of CO appears to be not very compatible with methanogenesis, because growth on this substrate is generally slow and/or metabolism is shifted toward other products than CH_4. Reasons for this "incompatibility" in methanogens could be the toxic nature of CO or cellular redox imbalance(s) building up in the CO-utilizing pathways. In order to create context for these pathways, first, some general aspects of methanogens and their energy metabolism will be considered here.

3 General Aspects of Methanogenesis

3.1 Ecology of Methanogenesis

In anaerobic environments with limited supply of exogenous electron acceptors like NO_3^-, SO_4^{2-}, and Fe^{3+} (or other metal[loid] oxides), which can be used for respiratory metabolism, biomass is degraded in a fermentative manner. First, hydrolytic microorganisms convert the polymeric constituents (proteins, carbohydrates, fats) to monomers (amino acids, sugars, fatty acids) and partly oxidize them to typical fermentation products like short chain fatty acids, alcohols, H_2, and CO_2. Secondary (syntrophic) fermenting bacteria oxidize the primary fermentation products further, mostly to acetate, formate, H_2, and CO_2. However, this process is only sufficiently exergonic for the organism to thrive by, if the H_2 partial pressure is kept low (Plugge et al., this edition) (Schink 1997; McInerney et al. 2008). Thus, H_2-consuming metabolism is critical at this step and most often carried out by hydrogenotrophic methanogens. Acetotrophic (aceticlastic) methanogens, on the other hand, convert the acetate to CH_4 and CO_2, which results in complete degradation of the biomass to CH_4 and CO_2. This natural process is also applied in anaerobic digesters, operated to produce biogas from renewable feedstock and/or to treat biological waste. Biogas-producing digesters can also be supplemented with CO or syngas, which leads to an increased methane yield but also to alterations in the composition of the microbial community, which makes the effect CO has on the methanogens involved difficult to ascertain (Luo et al. 2013; Sancho Navarro et al. 2016).

3.2 The Different Types of Methanogens

Most known members of the currently established, phylogenetically classified, seven orders of methanogens (Methanobacteriales, Methanococcales, Methanopyrales, Methanomicrobiales, Methanosarcinales, Methanocellales, Methanomassiliicoccales) are able to grow with $H_2 + CO_2$, i.e., via hydrogenotrophic methanogenesis (Fig. 1). However, physiologically, hydrogenotrophic methanogens can be divided into obligate and facultative hydrogenotrophs. In fact, methanogens in toto may be classified, according to the principle architecture of their energy-conserving systems, into methanogens which employ cytochromes and membrane-dissolved electron carriers in "typical" respiratory chains (Fig. 1b) and methanogens which do not (for a comprehensive review, see Thauer et al. 2008) (Fig. 1a). Regardless of their energy substrate or the architecture of their energy-conserving systems, the process of methanogenesis is coupled to the generation of an ion motive force (H^+ and/or Na^+), which in turn is used to synthesize ATP via ATP synthase or to drive other energy-demanding processes (Deppenmeier and Müller 2008). Notably, only among cytochrome-free methanogens are hyperthermophilic and/or obligate hydrogenotrophs found. Conversely, the ability to grow with acetate or with methylated compounds and without an additional electron donor (e.g., H_2) is restricted to methanogens with cytochromes, all of which belong to the Methanosarcinales. As this group is also the last of the methanogens to branch off the euryarchaeal line of descent (Whitman et al. 2006), they may be considered "modern" in comparison with the "ancestral" (i.e., cytochrome-free) way of methanogenic life.

3.3 Hydrogenotrophic Methanogenesis

Although this topic is comprehensively covered elsewhere in this edition (Shima), the path of hydrogenotrophic methanogenesis is relevant to the following discussion and therefore mentioned here as well. Principally, CO_2 is reduced in this pathway with four pairs of electrons [in most cases derived from the oxidation of molecular hydrogen (H_2)] to CH_4 in seven steps and using three C1-carrying cofactors (Fig. 1). Methanofuran (MF; Leigh et al. 1984) is used for the reduction and simultaneous activation of CO_2 to formyl-MF (Wagner et al. 2016), tetrahydromethanopterin (H_4MPT, structurally similar to tetrahydrofolate), or the related tetrahydrosarcinapterin (H_4SPT, found in *Methanosarcina* species) for the reduction of the formyl-group to the oxidation state of methanol (i.e., methyl-H_4MPT) via that of formaldehyde (i.e., methylene-H_4MPT), and coenzyme M (HS-CoM, 2-thioethanesulphonate) for the reduction of the methyl group (methyl-S-CoM) to methane.

Prior to this last step, which is catalyzed by methyl-S-coenzyme M reductase (Mcr; Ermler et al. 1997), a membranous methyltransferase (N^5-methyl-H_4MPT:HS-CoM methyltransferase, Mtr) couples the exergonic transfer of the methyl group from H_4MPT to HS-CoM to the extrusion of Na^+ ions, i.e., the generation of a sodium motive force (Gottschalk and Thauer 2001). Coenzyme B (HS-CoB,

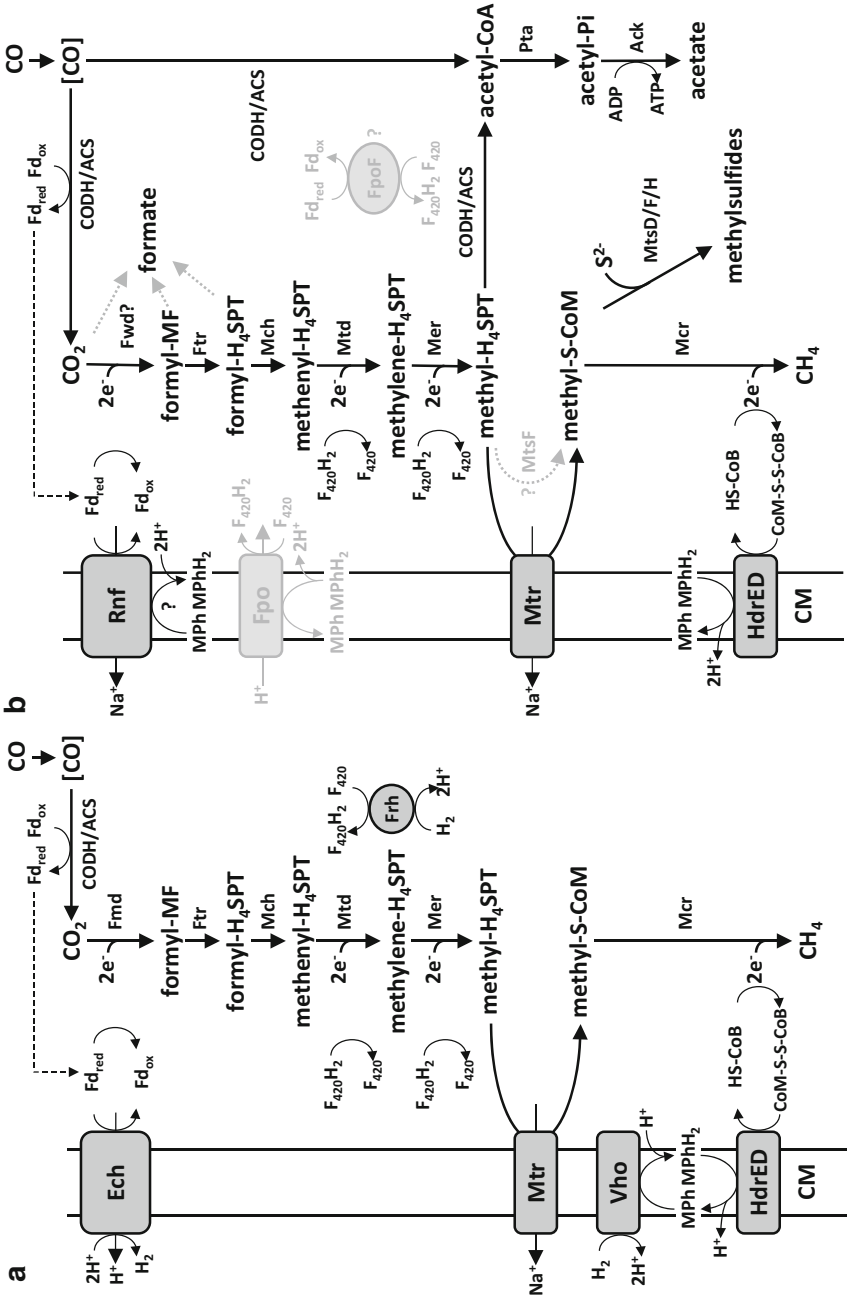

Fig. 1 (continued)

7-thioheptanoyl-*O*-phospho-l-threonine) is the electron donor for the Mcr-catalyzed reaction, which generates CH_4 and the heterodisulfide of HS-CoM and HS-CoB (CoM-S-S-CoB) that is in turn reduced by heterodisulfide reductase (Hdr), thus regenerating the free thiols.

The electron donor at each step is H_2, which is oxidized by different types of hydrogenases (reviewed in Thauer et al. 2010). Coenzyme F_{420} (Eirich et al. 1978) is reduced by F_{420}-reducing hydrogenases (Frh, Fru, Frc, depending on the organism). However, how the electrons from H_2 are funneled to other dedicated acceptors differs remarkably between cytochrome-free and cytochrome-containing methanogens. While catabolic H_2 oxidizing activity is cytosolic in the former, the latter employ two membrane-bound hydrogenases for catabolism. For providing reduced ferredoxin (Fd_{red}), which is required for the initial CO_2 reduction/activation step (Fig. 1b), an energy-converting hydrogenase, Ech (similar to hydrogenase 3 from *E. coli*), couples the endergonic reduction of ferredoxin (Fd_{ox}, $E^{0\prime}$ ca. −500 mV) with H_2 ($E^{0\prime}$ −414 mV) to the intrusion of H^+, i.e., uses the proton motive force (pmf) to drive this reaction (Hedderich and Forzi 2005). The other membrane-bound hydrogenase, Vht (or Vho), contains *b*-type cytochrome (*cyt b*) and catalyzes the H_2-dependent reduction of methanophenazine (MPh, functionally analogous to quinones) (Abken et al. 1998). Reoxidation of reduced MPh ($MPhH_2$) by the membrane-bound, *cyt b*-containing heterodisulfide reductase (HdrED) generates a pmf (Ide et al. 1999).

In contrast, cytochrome-free methanogens contain a cytoplasmic hydrogenase (Mvh, Vhu, Vhc, depending on the organism), which transfers the electrons from H_2 oxidation to a tightly bound, flavin-containing heterodisulfide reductase (HdrABC), in order to couple the exergonic reduction of CoM-S-S-CoB ($E^{0\prime}$ ca. −140 mV; Tietze et al. 2003) to the endergonic reduction of Fd_{ox} via a recently discovered mechanism, flavin-based electron bifurcation (Kaster et al. 2011). This mechanism could explain why in cell extracts of *Methanobacterium thermoautotrophicum* ΔH (originally named *Methanobacterium thermoautotrophicus* and later reclassified as *Methanothermobacter thermautotrophicus*; Zeikus and Wolfe 1972; Boone and Mah 1989) the reduction of CO_2 with H_2 to CH_4 proceeds only in the presence of catalytical amounts of methyl-S-CoM (the so-called RPG effect) (Gunsalus and

Fig. 1 Hydrogenotrophic methanogenesis in cytochrome-free (**a**) and in cytochrome-containing (**b**) methanogens. *CM* cytoplasmic membrane (right inside), *CoM-S-S-CoB* heterodisulfide, *Eha/b, Ech* energy-converting hydrogenase, F_{420} oxidized cofactor F_{420}, $F_{420}H_2$ reduced F_{420}, Fd_{red} reduced ferredoxin (2 e^-), Fd_{ox} oxidized ferredoxin, *Fmd* formyl-MF dehydrogenase, *Frh* F_{420}-reducing hydrogenase, *Ftr* formyl-MF:H_4MPT formyltransferase, H_4MPT tetrahydromethanopterin, H_4SPT tetrahydrosarcinapterin, *Hdr* heterodisulfide reductase, *HS-CoB* coenzyme B, *HS-CoM* coenzyme M, *Mch* N^5,N^{10}-methenyl-H_4MPT cyclohydrolase, *Mcr* methyl-S-CoM reductase, *Mer* N^5,N^{10}-methylene-H_4MPT reductase, *MF* methanofuran, *MPh* oxidized methanophenazine, $MPhH_2$ reduced methanophenazine, *Mtd* N^5,N^{10}-methylene-H_4MPT dehydrogenase, *Mtr* N^5-methyl-H_4MPT:CoM methyltransferase, *Mvh, Vho* F_{420}-non-reducing hydrogenase; for simplicity, some reactants (e.g., free HS-CoM) and ATP synthase are not shown; the number of ions translocated is not balanced; reactions in gray are presumed to be required for anabolism; see text for details

Wolfe 1977), i.e., how the first and the last step of methanogenesis are coupled. Principally, this coupling (via Fd) renders this linear pathway cyclic (Thauer 2012), a notion that is illustrated by the finding that in *Methanococcus maripaludis* the Vhu/Hdr complex is also bound in vivo by formyl-MF dehydrogenase (Costa et al. 2010).

3.4 Methylotrophic Methanogenesis

All known methanogens capable of growing solely with methylated substrates as the source of energy are members of the Methanosarcinales (Keltjens and Vogels 1993). However, the use of methylated compounds as methanogenic substrates together with the concurrent requirement of a reductant, like H_2 or formate, can be found outside the Methanosarcinales; the human commensals *Methanosphaera stadtmanae* (Methanobacteriales) (Fricke et al. 2006) and *Methanomassiliicoccus luminyensis* (Methanomassiliicoccales) (Borrel et al. 2014), as well as members of a recently discovered, extremely halophilic euryarchaeal class-level lineage, for which the name Methanonatronarchaeia was proposed, (Sorokin et al. 2017), are examples for methanogens employing this "mixotrophic," obligate "methyl reduction" (also called methyl-respiration) lifestyle.

During methylotrophic methanogenesis (Fig. 2), methyl groups from, e.g., methanol, methylamines, or methylsulfides are activated by two methyltransferases designated MT1 and MT2 (Ferry 1999). The substrate-specific MT1 consists of a corrinoid protein [MtaC for methanol, MtmC for monomethylamine (MMA), MtbC for dimethylamine (DMA), MttC for trimethylamine (TMA)] and a methyltransferase (MtaB for methanol, MtmB for MMA, MtbB for DMA, MttB for TMA) that transfers the methyl group to the corrinoid protein. A second methyltransferase, MT2 (MtaA for methanol, MtbA for methylamines), transfers the methyl group from the corrinoid protein to HS-CoM. For activation of dimethylsulfide, one methyltransferase (MtsA) is apparently sufficient (Tallant et al. 2001); in *Methanosarcina acetivorans* (MtsD) it is even fused with the corrinoid protein (Oelgeschläger and Rother 2009a; Fu and Metcalf 2015). Methyl-S-CoM is then disproportionated in a 3:1 ratio; one mole of methyl-S-CoM is oxidized to CO_2, through a reverse of the CO_2 reduction pathway, generating reducing equivalents [two moles reduced F_{420} ($F_{420}H_2$) and one mole Fd_{red} (two equivalents of electrons)] (Keltjens and Vogels 1993; Meuer et al. 2002), for every three moles of methyl-S-CoM reduced to methane. Some differences have been observed how methylotrophic methanogenesis is coupled to energy conservation. All organisms drive the endergonic transfer of the methyl group from methyl-S-CoM to H_4SPT with the sodium motive force via Mtr; $F_{420}H_2$ dehydrogenase (Fpo, similar to Nuo from *E. coli*), which couples reoxidation of $F_{420}H_2$ to the reduction of MPh and the generation of a pmf, is present in *Methanosarcina* species for which genome sequences are available. However, for methylotrophic growth, Fpo is dispensable in *M. barkeri* (Kulkarni et al. 2009), important but not essential in *M. mazei* (Welte and Deppenmeier 2011), and essential in *M. acetivorans* (Oelgeschläger 2009). Presumably, the different degrees to which formation of H_2 (from $F_{420}H_2$, via Frh)

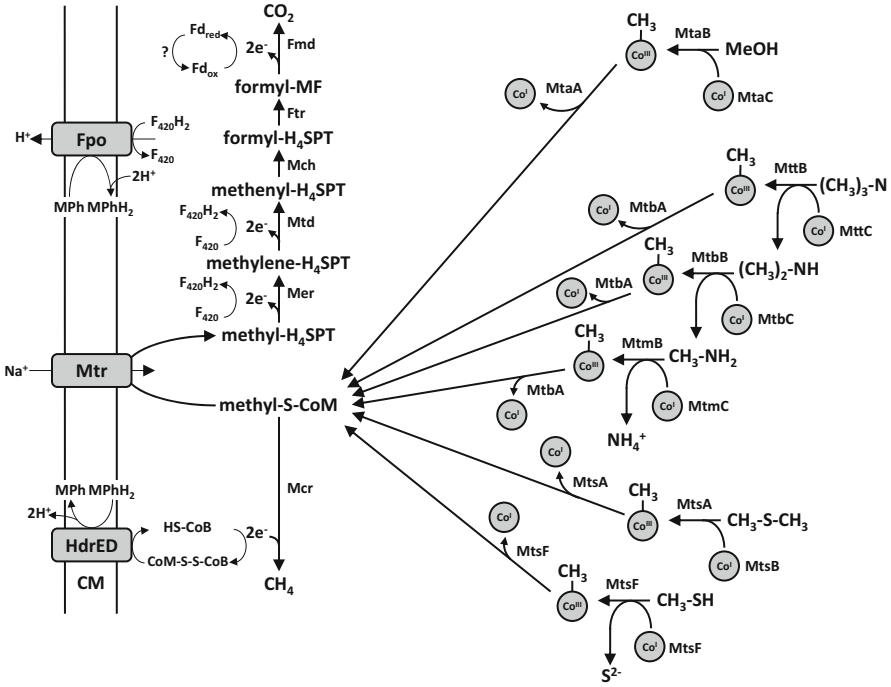

Fig. 2 Methylotrophic methanogenesis. *Fpo* $F_{420}H_2$ dehydrogenase, *MeOH* methanol, *MtaA, MtbA* methylcorrinoid:HS-CoM methyltransferase, *MtaB* methanol:cob(I)alamin methyltransferase, *MtsA* dimethylsulfide:HS-CoM methyltransferase, *MtsF* methanethiol:HS-CoM methyltransferase, *MtaC, MttC, MtbC, MtmC, MtsB* corrinoid protein of the corresponding methyltransferase, *MttB, MtbB, MtmB* (tri-, di-, mono-)methylamine:cob(I)alamin methyltransferase; for simplicity, some reactants (e.g., free HS-CoM) and ATP synthase are not shown; the number of ions translocated is not balanced; see legend of Fig. 1 for other abbreviations and text for details

can be employed to bypass Fpo, i.e., conserve energy through "hydrogen cycling" (Odom and Peck 1981), are responsible for this variability; while *M. acetivorans* cannot produce or utilize H_2 (Guss et al. 2009), *M. barkeri* apparently "favors" energetic coupling via H_2 (Kulkarni et al. 2009). How exactly electrons from Fd_{red} are funneled to HdrED in *Methanosarcina* is also somewhat uncertain; it was thought that Ech couples oxidation of Fd_{red} to H_2 formation and generation of a pmf (Hedderich et al. 1998), but a *M. barkeri* mutant lacking Ech generates methane and CO_2 from methanol with wild-type rates (Meuer et al. 2002). *M. acetivorans* not even encodes Ech, but instead, involvement of a membrane-bound complex homologous to Rnf from *Rhodobacter capsulatus* (see below) was assumed; but again, an *M. acetivorans* mutant lacking Rnf grows methylotrophically like the wild type (Schlegel et al. 2012b). It is therefore plausible to assume that even if Ech (in *M. barkeri*) and Rnf (in *M. acetivorans*) are involved in methylotrophic energy conservation, their proposed function can be effectively substituted in vivo.

3.5 Aceticlastic Methanogenesis

About two-thirds of the CH_4 produced during anaerobic biomass degradation in the absence of exogenous electron acceptors stems from the methyl group of acetate (Jeris and McCarty 1965), illustrating the ecological relevance of this type of methanogenesis. Intriguingly, only two genera of methanogens are known that are capable of aceticlastic methanogenesis, namely, *Methanosarcina* and *Methanothrix* (the latter also referred to as *Methanosaeta*, which has been rejected as a source of confusion; Garrity et al. 2011). *Methanothrix*, which is the only known genus of obligate acetotrophic methanogens, appears to be particularly well adapted to acetate utilization, exemplified by its threshold concentration (< 1 mM), which is considerably lower than that of *Methanosarcina* species (for reviews, see Jetten et al. 1992; Welte and Deppenmeier 2014). Of all methanogenic pathways, aceticlastic methanogenesis results in the lowest amount of free energy to be conserved ($\Delta G^{0\prime} = -36$ kJ/mol), which is close to the thermodynamic limitations for life (Müller et al. 1993).

In *Methanosarcina* species, acetate is activated to acetyl-phosphate by acetate kinase (Ack), and subsequently the acetyl group is transferred to HS-CoA by phosphotransacetylase (Pta) (Ferry 1997). Acetyl-CoA is cleaved by the ACS activity of CODH/ACS, and the methyl group transferred to H_4SPT via a corrinoid-containing subunit (Grahame 1991). The enzyme-bound CO is oxidized to CO_2 by the CODH activity of CODH/ACS using Fd_{ox} as the electron acceptor (Fischer and Thauer 1990). Methyl-H_4SPT is reduced to CH_4 via reactions of the hydrogenotrophic pathway (i.e., Mtr generating a sodium ion force and Mcr generating CoM-S-S-CoB). In facultative hydrogenotrophic *Methanosarcina* (e.g., *M. mazei* and *M. barkeri*), Ech hydrogenase couples reoxidation of Fd_{red} to generation of a pmf and H_2 (Welte and Deppenmeier 2014), the latter of which is oxidized by Vho hydrogenase; the electrons are funneled to HdrED (via an MPh/$MPhH_2$ loop) for CoM-S-S-CoB reduction and generation of a pmf (Fig. 3a; Ferry 2010a). *M. acetivorans* lacks Ech and Vho but contains a membranous multi-subunit enzyme complex homologous to the energy-converting NADH:Fd_{ox} oxidoreductase from *Rhodobacter capsulatus*, which is involved in nitrogen fixation (Schmehl et al. 1993) (therefore designated Rnf) (Galagan et al. 2002; Li et al. 2006). Rnf from *M. acetivorans* was proposed to couple oxidation of Fd_{red} to reduction of MPh and, based on the function of homologous enzymes in other organisms, concomitant generation of an ion motive force (Fig. 3b, Ferry 2010a). A *M. acetivorans* strain in which the operon encoding Rnf had been deleted is unable to grow with acetate as the energy source, which demonstrates its essentiality under this condition, but growth on methylated substrates (see above) and CO (see below) is not affected, which indicates that Rnf is either not important under the latter conditions or that its loss can be fully compensated by other factors (Schlegel et al. 2012b). The finding that Fd_{red}-dependent reduction of CoM-S-S-CoB by membranes of the *rnf* mutant was not abolished but only impaired by 50% supports this notion (Schlegel et al. 2012b). In contrast, Na^+ transport across the membranes (in inverted vesicles) coupled to the Fd_{red}-dependent reduction of CoM-S-S-CoB was completely

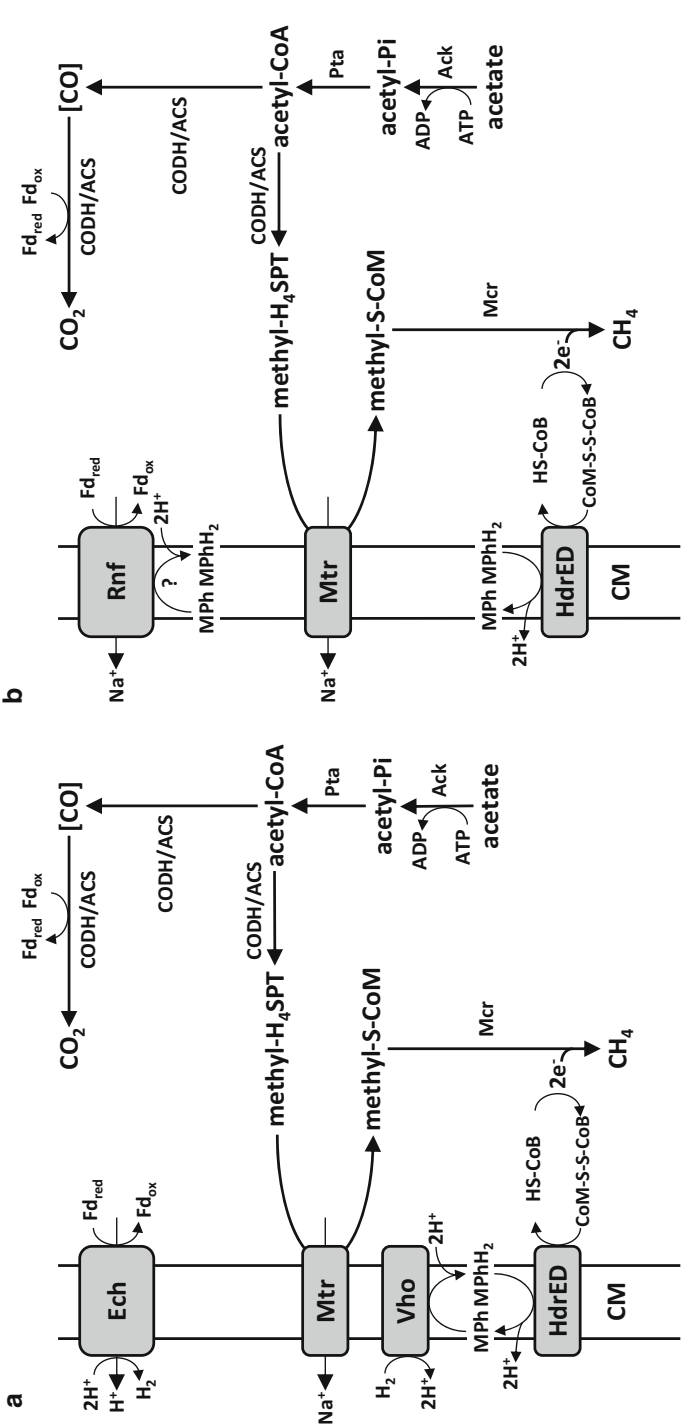

Fig. 3 Aceticlastic methanogenesis in facultative hydrogenotrophic *Methanosarcina* species (**a**) and in *M. acetivorans* (**b**). *Ack* acetate kinase, *[CO]* CODH/ACS-bound CO, *CoA* coenzyme A, *CODH/ACS* carbon monoxide dehydrogenase/acetyl-CoA synthase, *Pi* orthophosphate, *Pta* phosphotransacetylase, *Rnf* postulated Fd:MPh oxidoreductase; for simplicity, some reactants (e.g., free HS-CoM and HS-CoA) and ATP synthase are not shown; the number of ions translocated is not balanced; see legend of Fig. 1 for other abbreviations

abolished in the absence of Rnf, which demonstrated its role in generating a primary sodium motive force (Schlegel et al. 2012b).

Despite the numerous – and by now conveyed as "established" – statements about the mechanism of Rnf-mediated electron transfer in *M. acetivorans*, e.g. (Yan et al. 2017), direct experimental evidence for the notion that Rnf transfers electrons to MPh has so far not been published. The fact that the operon encoding Rnf in *M. acetivorans* contains a gene for a ca. 55 kDa multiheme *c*-type cytochrome (*cyt c*) and that membrane-bound *cyt c* is oxidized by 2-hydoxyphenazine (2OH-MPh, a soluble MPh analog) (Wang et al. 2011) suggests that MPh is indeed linked to *cyt c* in the respiratory chain. However, whether this link is direct or indirect remains unanswered since preparations of complete membranes (containing Rnf and all other membrane proteins) were used in these experiments and *M. acetivorans* synthesizes at least two distinct membrane-bound *cyt c* (Li et al. 2006) while encoding even three (Galagan et al. 2002).

Coupling Fd_{red} oxidation (with an assumed $E^{0\prime}$ of ± -500 mV) directly to the reduction of MPh ($E^{0\prime}$ −165 mV; Tietze et al. 2003) would be highly exergonic ($\Delta G^{0\prime}$ of −64.5 kJ/mol) and sufficient to translocate 4–5 Na^+ (per two electrons), assuming a transmembrane electrochemical ion potential of around −160 mV (Blaut and Gottschalk 1984). Translocation of only two Na^+ per two electrons by Rnf from *M. acetivorans* (as proposed for the Na^+-dependent Rnf from *Acetobacterium woodii*; Hess et al. 2013) would result in considerable loss of free energy, which, particularly when growing on acetate, *M. acetivorans* could not afford to waste. Therefore, it will be important to (i) assess Rnf-dependent reduction of MPh in a defined (in vitro) system and to (ii) determine the stoichiometry of Na^+ translocated per electron, in order to accurately define the role of this intriguing membrane complex in *M. acetivorans*.

4 Carboxydotrophy of Methanogens

All methanogens known employ a route analogous to the acetyl-CoA pathway (originally described in acetogenic bacteria) for CO_2 fixation (Stupperich et al. 1983; Ladapo and Whitman 1990). It proceeds by reduction of CO_2 to a methyl group (bound to H_4MPT or H_4SPT, via the hydrogenotrophic path, Fig. 1) and transfer of the methyl group to the ACS active site of CODH/ACS, where it is combined with an enzyme-bound CO (derived from reduction of CO_2 at the CODH active site of CODH/ACS and transferred to the ACS active site) and HS-CoA to form acetyl-CoA (i.e., part of the aceticlastic pathway in reverse, Fig. 5b). Thus, CODH/ACS-bound CO is an intermediate in autotrophic carbon fixation (and in aceticlastic energy metabolism) of methanogens. CO could, therefore, be considered a common methanogenic substrate, but the toxic nature of CO demands special equipment and procedures for its handling in the laboratory, leaving the carboxydotrophic capabilities of newly isolated or already described methanogens often un(der)-examined. Only few methanogens have been shown to grow carboxydotrophically in pure culture. All of them need rather long periods of

adaptation and grow more slowly on CO than on their common substrate(s), which indicates that beside the toxicity of CO, its seemingly beneficial features (i.e., having a low redox potential and constituting both energy and carbon source) actually challenge the organism's physiology. Recent studies suggest that these challenges manifest differently in cytochrome-free and in cytochrome-containing methanogens.

4.1 CO-Dependent Physiology in Cytochrome-Free Methanogens

Reduction of CO to CH_4 had been demonstrated early for complex environmental samples (Fischer et al. 1931) and for pure cultures of methanogens (Kluyver and Schnellen 1947). The first methanogen reported to grow on CO was *M. thermautotrophicus* (Daniels et al. 1977). With a doubling time of >200 h under this condition, it grows considerably slower than on $H_2 + CO_2$ (2.8 h). Interestingly, non-growing cells of *M. thermautotrophicus* accumulated considerable amounts of H_2 (and CO_2) in the presence of CO, while actively growing cultures produced CH_4 and CO_2. It appears that CO is first oxidized and protons reduced (Fig. 4) in a reaction sequence analogous to the water-gas shift reaction (Eq. 4, Graven and Long 1954) and employed by hydrogenogenic carboxydotrophs as their sole energy metabolism (Sokolova et al. 2009; Diender et al. 2015). Subsequently, CO_2 is reduced to methane using H_2 as reductant (Fig. 4), i.e., employing the hydrogenotrophic pathway (Eq. 5) for growth.

$$CO + H_2O \rightarrow CO_2 + H_2 \Delta G^{0'} = -20\ \text{kJ/mol CO} \tag{4}$$

$$CO_2 + 4\ H_2 \rightarrow CH_4 + H_2O \Delta G^{0'} = -131\ \text{kJ/mol } CH_4 \tag{5}$$

Because CO-dependent growth of *M. thermautotrophicus* ceased with concentrations above 60% (vol/vol), and hydrogenotrophic growth was increasingly inhibited in a dose-dependent manner above 10% in the gas phase (Daniels et al. 1977), methanogenesis appears to be inhibited by CO. Although most redox active enzymes containing transition metals could be affected through binding to CO, hydrogenases seem like the most likely candidates (e.g., through binding to Ni in [NiFe] hydrogenases). Still, Fe in the active site often contains (a) CO ligand(s) required for activity (Fontecilla-Camps 2009; Peters 2009; Shima et al. 2009). Furthermore, CO-dependent accumulation of H_2 requires the presence of active hydrogenase and may instead suggest that H_2 production and its utilization (via the CO_2 reduction pathway) are imbalanced.

Additional knowledge about CO metabolism in obligate hydrogenotrophs was gained from a study on the physiology and proteome of *Methanothermobacter marburgensis* (formerly *Methanobacterium thermoautotrophicum* strain Marburg) (Fuchs et al. 1978; Boone et al. 1993; Wasserfallen et al. 2000), which, after an initial lag phase of about 500 h, could be adapted to grow on CO as the sole energy source (Diender et al. 2016). Interestingly, CO-dependent methanogenesis only commenced

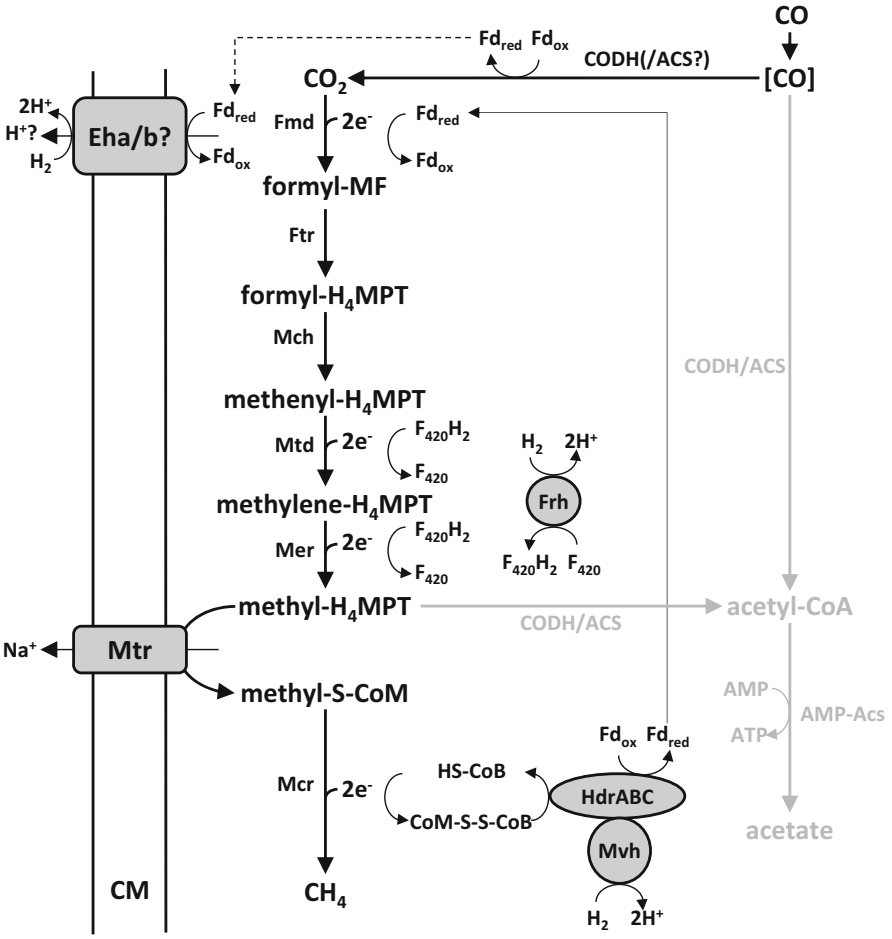

Fig. 4 Carboxydotrophic methanogenesis in cytochrome-free methanogens. *AMP-Acs* acetyl-CoA synthetase (AMP forming); reactions in gray were proposed for *M. marburgensis* (Diender et al. 2016); for simplicity, some reactants (e.g., free HS-CoM and HS-CoA) and ATP synthase are not shown; the number of ions translocated is not balanced; see legends of Figs. 1, 2, and 3 for other abbreviations and the text for details

after some H_2 had accumulated, which indicates that not only it is a required intermediate but that it needs to be present at a certain threshold concentration (Diender et al. 2016). The authors plausibly speculate that the basis for this requirement may lie in the obligate electron bifurcating nature of the Mvh-Hdr complex, which couples H_2 oxidation to the exergonic reduction of CoM-S-S-CoB and the concomitant endergonic reduction of Fd_{ox} (Kaster et al. 2011). CO oxidation by CODH is directly coupled to Fd_{ox} reduction leading to a high cellular Fd_{red}:Fd_{ox} ratio, which, at the very low H_2 partial pressure initially present, may render the Mvh-Hdr-catalyzed reduction of Fd_{ox} too endergonic to proceed, thereby impeding

the regeneration of the free thiols from CoM-S-S-CoB. Only once the H_2 concentration is sufficiently high can CoM-S-S-CoB be reduced and, thus, methanogenesis commence. Together with the sensitivity of hydrogenases toward CO, this scenario might also explain why cytochrome-free methanogens tolerate only limited amounts of CO. Fd_{red}-dependent H_2 formation probably stems from the activity of an Ech-type hydrogenase, thus generating a pmf. The notion that CO renders the cellular milieu of *M. marburgensis* "over-reduced," which can be considered a stress condition like H_2 limitation (Morgan et al. 1997), is supported by increased synthesis of redox-responsive, stress-related factors, like F_{420} oxidase, superoxide dismutase, superoxide reductase, and F_{390} synthetase in the presence of CO (Diender et al. 2016).

Another interesting observation, which has been made previously for *M. acetivorans* (Rother and Metcalf 2004), was the CO-dependent formation of acetate by *M. marburgensis* (Fig. 4). There, it coincided with increased levels in the presence of CO (than in its absence) of CODH/ACS, the tungsten-dependent formyl-MF dehydrogenase, and a putative (AMP-forming) acetyl-CoA synthetase (Diender et al. 2016). CO-dependent acetogenesis could be a means to conserve energy (via SLP) or to reduce carbon flux through methane formation in order to "avoid" the Mvh-Hdr-catalyzed reaction, which would only add (by Fd_{red} formation) to the "redox stress" of the cell.

4.2 CO-Dependent Physiology in Cytochrome-Containing Methanogens

Already 1947 it was noted that cultures of *M. barkeri* (pre-grown on methanol) converted only a fraction of the CO metabolized to methane and that this conversion proceeded via the generation of CO_2 and H_2 (Kluyver and Schnellen 1947). The transient production of H_2 has also been observed in *M. barkeri* cultures growing on CO (O'Brien et al. 1984). Thus, CO-dependent growth in this facultative hydrogenotrophic, (Fig. 5a) cytochrome-containing methanogen appears to be principally analogous to that observed in the cytochrome-free methanogens. The reported doubling time for *M. barkeri* of ±65 h under this condition is much longer than that for hydrogenotrophic growth (6–8 h), which suggests that methanogenic utilization of CO via H_2 production is unfavorable irrespective of being cytochrome-free or not. Still, *M. barkeri* grows in the presence of 100% (vol/vol) CO and the amount of H_2 accumulating rises with the amount of CO supplied (O'Brien et al. 1984), which may indicate that some reaction(s) of the CO_2 reduction pathway, rather than merely its hydrogenases, become inhibited in a CO-dependent fashion.

A *M. barkeri* mutant lacking Ech hydrogenase, which is unable to grow on $H_2 + CO_2$ or CO, is devoid of CO- and of Fd_{red}-dependent hydrogen evolution activity (Meuer et al. 2002). Furthermore, in this mutant (i) the F_{420}-dependent hydrogenase is as active as in the wild type (Meuer et al. 2002), (ii) CH_4 is formed when CO and H_2 (but not with H_2 alone) are provided (Stojanowic and Hedderich 2004), and (iii) the initial step of methanogenesis from CO_2 (i.e., reduction of CO_2

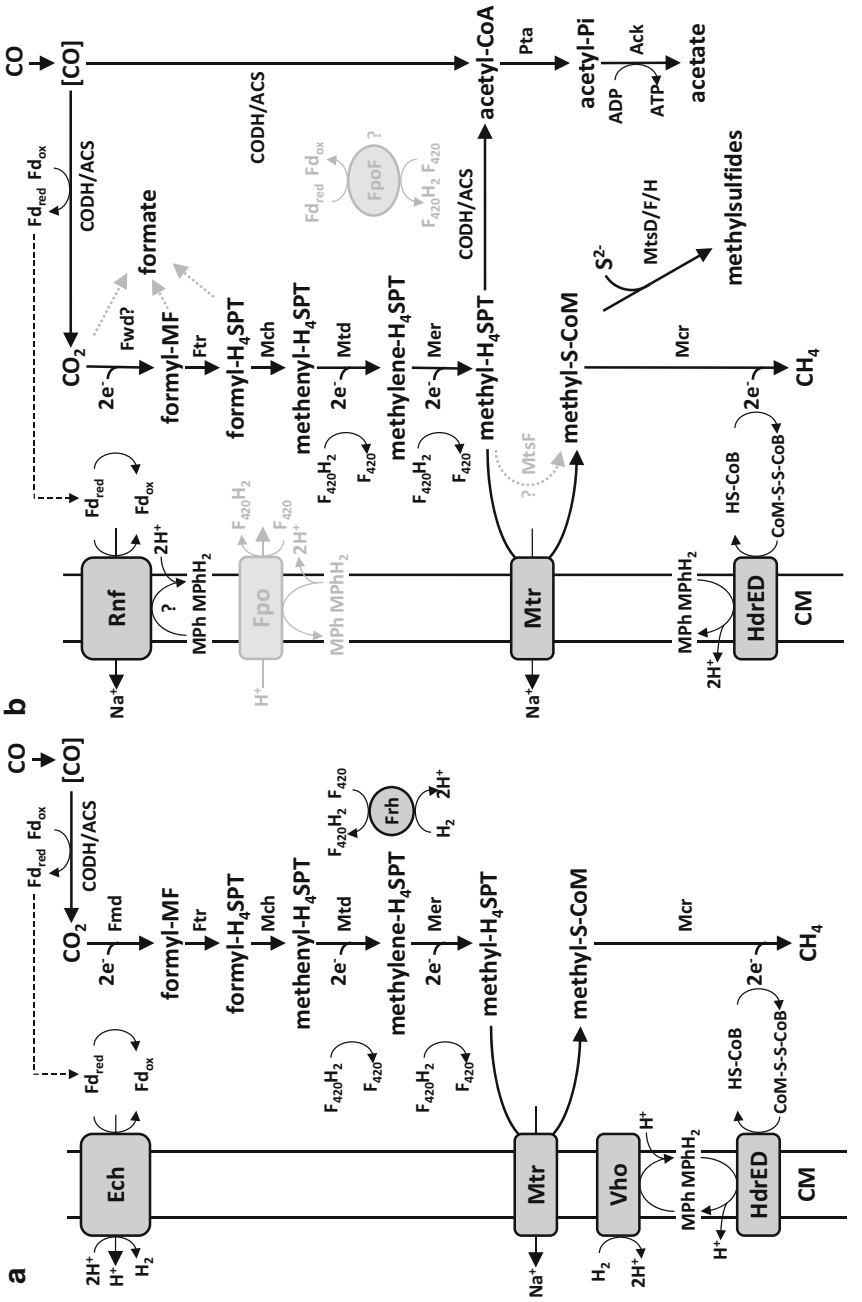

Fig. 5 (continued)

to formyl-MF) is principally independent of Ech (i.e., does not require H_2 and is not dependent on the membrane potential) (Stojanowic and Hedderich 2004). Taken together, these findings strongly suggest that in order for *M. barkeri* to grow with CO as the sole energy source, Ech-dependent H_2 formation has to occur; whether this requirement stems from the organism's bias toward "hydrogen cycling" (see Sect. 3.4) or from F_{420}-dependent hydrogenase being the only route toward $F_{420}H_2$ (required for the reactions catalyzed by Mtd and Mer, Fig. 5a) remains an open question.

Of all methanogens investigated thus far, *M. acetivorans* exhibits the most robust but also the most unusual, CO-dependent physiology. The organism was isolated from marine sediment off the coast of La Jolla, USA, based on its acetotrophic capabilities (Sowers et al. 1984). Unlike most methanogens it is unable to grow hydrogenotrophically due to the lack of any significant H_2 metabolism (Sowers et al. 1984; Lovley and Ferry 1985). *M. acetivorans* lacks genes for Ech but contains those for Frh and Vht (Galagan et al. 2002), the latter two of which are not expressed due to inactive promoters (Guss et al. 2009). *M. acetivorans* could be adapted to carboxydotrophic growth from methylotrophic conditions but not from aceticlastic conditions (Rother and Metcalf 2004). Although it would seem intuitive to shift *M. acetivorans* to CO from acetate as the growth substrate, because CODH/ACS is present at very high levels under this condition (Li et al. 2007), acetate-dependent growth is inhibited by even small amounts of CO (5 kPa) in the gas phase (Rother and Metcalf 2004), indicating that some part of the enzymatic machinery employed in aceticlastic methanogenesis is inhibited. As *M. acetivorans* does not synthesize hydrogenases and much of its CO_2 reduction path is downregulated under this condition (Li et al. 2007), of the "C1 reactions," only those catalyzed by Mtr, and Mcr, remain as plausible candidates for being inhibited by CO. Both growth rate and growth yield increase upon prolonged cultivation in the presence of up to 300 kPa CO, which indicates that acclimation to this difficult substrate occurs in *M. acetivorans* (Kliefoth et al. 2012). This acclimation manifests in substantial CO-dependent alterations of the protein inventory (Lessner et al. 2006; Rother et al. 2007; Kliefoth et al. 2012) and in an unprecedented shift from CO-dependent methane formation toward acetate as a major metabolite (Rother and Metcalf 2004). In addition, substantial amounts of formate, as well as small amounts of dimethylsulfide, were produced at high CO concentrations (Rother and Metcalf 2004; Moran et al. 2008). The decrease of methane formation from CO in *M. acetivorans* correlated to the amount of CO supplied, which was initially interpreted as CO-dependent inhibition of the CO_2 reduction pathway (Rother and Metcalf

Fig. 5 Carboxydotrophic methanogenesis in (**a**) *M. barkeri* and (**b**) *M. acetivorans*. Dotted arrows represent proposed reactions for formate formation; reactions or enzymes in gray have been suggested to be involved; for simplicity, some reactants (e.g., free HS-CoM and HS-CoA) and ATP synthase are not shown; the number of ions translocated is not balanced; for abbreviations see legends of Figs. 1, 2, and 3 and text for details

2004). However, it was later shown that the rate of methane formation in CO-adapted *M. acetivorans* did not change with the amount of CO supplied but was slow to begin with and that acetate and formate were formed at faster rates, resulting in only 10% of the CO metabolized ending up in methane (Oelgeschläger and Rother 2009b). Thus, it appears that a consequence of adaptation to carboxydotrophic growth in *M. acetivorans* is the reduction of carbon flux through the C1 pathway leading to methane. Still, methane formation is required because 2-bromoethane sulfonic acid, a potent inhibitor of methanogenesis, impairs carboxydotrophic growth of *M. acetivorans* (Rother and Metcalf 2004).

Mutants of *M. acetivorans* lacking Pta and Ack only grow on CO when low levels are supplied, indicating that acetate production is required in the presence of high levels. The data also indicate that acetate is generated via acetyl-CoA (Fig. 5b) and, thus, via reactions analogous to those of the Wood-Ljungdahl pathway employed by acetogenic bacteria. Therefore, *M. acetivorans* probably couples carboxydotrophic growth to SLP. The *M. acetivorans* genome contains five distinct loci encoding CODH (Galagan et al. 2002), which raised the question for their relevance in CO-dependent physiology. The two genes encoding monofunctional CODHs (CooS1F and CooS2) were shown (by targeted mutagenesis) to play only minor roles during carboxydotrophic growth of *M. acetivorans* (Rother et al. 2007). Conversely, of the two (highly homologous) CODH/ACS isoforms (Cdh1 and Cdh2), one had to be present in *M. acetivorans* to allow for efficient CO oxidation and carboxydotrophic (or aceticlastic) growth (Matschiavelli et al. 2012), which demonstrated that CODH/ACS constitutes the major CO oxidizing activity in *M. acetivorans* and also that both isoforms are bona fide CODH/ACSs, i.e., catalyze both acetyl-CoA formation as well as cleavage. Intriguingly, a stand-alone CODH/ACS α-subunit (CdhA3) apparently affects transcription of the *cdh1* encoding genes (Matschiavelli et al. 2012), but how it exerts this effect is unknown.

Proteome analysis of *M. acetivorans* revealed that the energy-converting methyltransferase Mtr (Fig. 5b) was eightfold less abundant in CO-adapted cells than in cultures growing on methanol, which might explain the shift toward acetate formation in the organism (Lessner et al. 2006). CO-grown *M. acetivorans* also contain very high levels of three isoforms of a putative enzyme with domains homologous to both methyl-carrying corrinoid proteins and methyltransferase proteins (Lessner et al. 2006; Rother et al. 2007). Targeted mutagenesis demonstrated that these proteins, named MtsD, MtsF, and MtsH, are involved and required for utilization of methylated thiols and for the CO-dependent generation of dimethylsulfide (Oelgeschläger and Rother 2009a; Fu and Metcalf 2015). However, it has also been proposed that the role of the Mts system is to bypass Mtr, i.e., to catalyze the transfer of methyl groups from H_4SPT to HS-CoM when Mtr is in low abundance (Lessner et al. 2006). Indeed, MtsF, when heterologously produced in inclusion bodies in *E. coli*, refolded and reconstituted in vitro in the presence of aquocobalamin (the protein naturally contains factor III, M. Stassen and M. Rother, unpublished) showed considerable methyl-H_4MPT:HS-CoM methyltransferase activity (Vepachedu and Ferry 2012), which argues in favor of this notion. It was also proposed that MtsF is only relevant in vivo as an Mtr bypass at low CO

concentrations (Vepachedu and Ferry 2012). However, if lowering the cellular concentration of Mtr as a response to (inhibition by) CO is assumed, such function for MtsF would appear counterintuitive, because the organism would forfeit a (Na^+) coupling site when it wouldn't need to. A strain lacking the Mts system showed no growth defect compared to the wild type and produced methane from CO, even at very high CO partial pressures, at wild-type rates (Oelgeschläger and Rother 2009a). Therefore, the physiological role of MtsF (and the Mts system as a whole) during carboxydotrophic growth remains controversial and needs to be reassessed, preferably in a strain lacking Mtr. However, whether Mtr is dispensable in *M. acetivorans* (like in members of the Methanomassiliicoccales; Kröninger et al. 2016) remains to be demonstrated.

The CO-dependent production of formate in *M. acetivorans* is also peculiar, because, unlike *M. barkeri*, the organism neither encodes a homolog of formate dehydrogenase (Fdh) (Galagan et al. 2002) nor does it contain Fdh activity (assessed as the formate-dependent reduction of viologen dyes) (Nelson and Ferry 1984; Rother and Metcalf 2004). Still, formate formation from CO proceeds via CO_2 (Matschiavelli 2015), and a potential Fd_{red}-dependent reduction of CO_2 to formate would not be detected under these assay conditions. Also, hydrolysis of formyl-MF and formyl-H_4SPT, both intermediates of the CO_2 reduction pathway (Fig. 5b), has been proposed as possible routes for formate production (Rother and Metcalf 2004) because an analogous reaction was observed in aerobic methylotrophic bacteria (Pomper et al. 2002). Circumstantial evidence suggests that a tungsten-dependent formyl-MF dehydrogenase (Fwd) might be responsible (Fig. 5b), because a mutant lacking the encoding operon did not produce any formate from CO (Matschiavelli and Rother 2015). However, whether formate is formed at a step "downstream" (in the reducing direction) of formyl-MF, whether coenzyme-bound formyl-intermediate is involved at all, or whether more than one formate generating activity is present in *M. acetivorans* is currently unknown. Regardless of the path for its synthesis, formate is a dead-end metabolite, as *M. acetivorans* is unable to utilize it (Sowers et al. 1984). The fact that CO-dependent formate formation increases with the CO partial pressure supplied and that this activity is higher in acetate- than in CO-grown cells (Matschiavelli and Rother 2015) suggests that *M. acetivorans* produces formate to "vent off" reducing equivalents, i.e., uses formate formation as an electron sink. Thus, formate formation is neither stoichiometrically coupled to methane and/or acetate formation nor are the latter to each other. Therefore, CO metabolism of *M. acetivorans* is considerably flexible in terms of the product stoichiometries and, thus, not easily represented in a reaction equation, like given in Ferry (2010b). Instead, the CO partial pressure seems to dictate which metabolites are formed and to what extent.

Rendering CO metabolism of *M. acetivorans* independent of hydrogenases (and of H_2 formation), potentially as an adaptation to elevated levels of CO in its natural marine habitat, might – at least partially – explain its robust and comparably fast (doubling times of 10–20 h) growth under this condition. However, the question arises how *M. acetivorans* obtains $F_{420}H_2$, which is required for two reactions (Mtd and Mer) in the CO_2 reduction path. It was suggested that during growth on CO of

M. acetivorans, Fpo might be involved in reducing F_{420} using $MPhH_2$ as electron donor, i.e., coupling this endergonic reaction to reverse electron transport (Diender et al. 2015). This rather counterintuitive scenario for electron transport, Rnf-mediated reduction of MPh with Fd_{red} coupled to generation of a sodium motive force and Fpo-mediated (endergonic) reduction of F_{420} with $MPhH_2$ coupled to the use of a proton motive force, would require (i) a higher number of Na^+ translocated by Rnf than H^+ translocated by Fpo (per electron pair) to still allow net conservation of energy and (ii) either an efficient way to couple ATP synthesis to a H^+ and a Na^+ gradient or an efficient way to exchange the sodium motive force for a proton motive force (e.g., by use of a Na^+/H^+ antiporter, i.e., secondary transport). While neither the direct physiological electron acceptor of nor the number of ions translocated by Rnf has been experimentally determined (see Sect. 3.5), which could argue for or against F_{420} reduction by reverse electron transport, both strategies for the interchangeability of Na^+ and H^+ as coupling ions appear to be realized by *M. acetivorans*: both primary Na^+ and H^+ transport across the membrane lead to ATP formation by ATP synthase (Schlegel et al. 2012a), and a member of the multi-subunit cation/proton antiporter 3 family, Mrp, appears to be relevant during acetate-dependent growth, i.e., when electrons are transferred from Fd_{red} to CoM-S-S-CoB in the respiratory chain (Jasso-Chavez et al. 2013). An alternative scenario for F_{420} reduction with Fd_{red} could involve FpoF, a subunit of the Fpo complex, which is also found detached from the membrane and shown to have Fd_{red}:F_{420} oxidoreductase activity in *Methanosarcina mazei* (ca. 0.2 μmol min^{-1} mg^{-1};Welte and Deppenmeier 2011). Also, HdrA2, which is part of a proposed soluble HdrA2B2C2 complex (similar to that employed by obligate hydrogenotrophs during methanogenesis from $H_2 + CO_2$; Kaster et al. 2011) exhibits (very low) Fd_{red}:F_{420} oxidoreductase activity (ca. 0.006 μmol min^{-1} mg^{-1}; Yan et al. 2017).

5 Research Needs

Much has been learned about the carboxydotrophic physiology of methanogens in recent years. The fact that some methanogens utilize CO for methanogenesis may invite research to add methanogens to the ever-expanding applications of syngas-based microbiology. As current models are not very efficient carboxydotrophic methane producers in terms of rates and yields, searching in the environment for more efficient "syngas-methanogens" might be a promising enterprise. Also, transferring of what is currently known to application might be a fruitful exercise for process engineers.

In terms of basic science, it would be highly desirable to test some of the more indirectly derived hypotheses for obligate hydrogenotrophs by genetic analysis. For example, if the electron bifurcating nature of the Mvh-Hdr complex was the reason for the observed requirement for H_2 accumulation, eliminating the Fd-reducing activity of this complex would release this requirement. To achieve this, either *Methanothermobacter* has to be developed into a genetic model organism or CO

metabolism of a genetically accessible obligate hydrogenotroph, like *Methanococcus*, has to be assessed.

Although CO metabolism in *M. acetivorans* has been unraveled to considerable resolution, some (controversial) issues remain. For example, it remains unclear how Rnf transfers electrons in the respiratory chain and where to. It is still unclear how *M. acetivorans* generates formate from CO. The exact physiological role of the Mts system (methylsulfide metabolism or Mtr bypass? Or both?) also remains a matter of dispute. Finally, it appears obvious that not only hydrogenases but (one) other step(s) in the CO_2-reducing pathway of methanogens is/are inhibited by CO and, what circumstantial evidence there is, points toward Mtr. Thus, assessing the effect CO has on this large membrane complex, as well as elucidating if a methanogen without Mtr can grow with CO, will be challenging tasks for the future.

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Environmental Constraints That Limit Methanogenesis

6

Tori Hoehler, Nathaniel A. Losey, Robert P. Gunsalus, and Michael J. McInerney

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Abstract

Methanogens are active in many different ecosystems, including habitats with biologically-derived organic matter as substrates such as aquatic sediments, wetlands, agricultural or natural soils subject to inundation, sewage digesters, and the anoxic portions of animal digestive tracts. Methanogens are also present in habitats with geochemically-supplied substrates such as hot springs, hydrothermal vents, volcanically-influenced habitats, and, potentially, the deep crustal subsurface. Methanogens as a group tolerate a broad range of physicochemical conditions, including temperatures from −2 °C to 122 °C, pH values of 3.0–10.2, salinities up to halite saturation, and pressures of at least 75 MPa. Globally, variations in methane emissions can be explained to a large degree by variations in temperature and water availability. The distribution and activity of methanogens are constrained by ecological interactions that can be stimulatory or competitive, and by physicochemical factors that act at the biochemical or bioenergetic levels. In addition to the constraints placed on methanogens by physicochemical extremes, methanogen distribution and activity are constrained by the availability of energy and nutrients, the presence of inhibitory molecules (most notably oxygen), and the seawater anion, sulfate, due to competitive ecological interactions. Although methanogen tolerances to individual extremes are documented in culture, and the corresponding biochemical adaptations are understood to varying degrees, the natural environment frequently presents combinations of extreme conditions and energy limitations that may limit methanogen distribution to less than the optimally tolerated range of a single parameter. Little is understood about the compound effects of such extremes, nor the commonalities among them that will ultimately form the basis for predictive models of environmental methanogen population distribution. Future work that targets these questions, through a combination of culture work, "omic" analyses, in situ studies, and conceptual and quantitative models, will be needed to better understand the physiological ecology of methanogens.

1 Introduction

Biological production of methane, termed methanogenesis, is a quantitatively important component of the global carbon cycle on the modern Earth (Hedderich and Whitman 2006) and has likely been so since the origin of microbial methanogenesis over 3.5 billion years (Ueno et al. 2006). Approximately 1–2% of the net photosynthetic carbon produced annually is ultimately processed through methanogenesis (Hedderich and Whitman 2006). About 1,100 Tg of biogenic methane is produced annually, of which about 450 Tg enters the atmosphere (Dlugokencky et al. 2011; Reeburgh 2007). The remainder of the methane is consumed through aerobic and anaerobic microbial processes. Of concern is the increase in the rate of methane efflux since 2006, possibly due to an increase in Artic methane emissions due to global warming (Dlugokencky et al. 2011). Newly recognized sources of biogenic methane emissions include methane production by aerobic bacteria from the cleavage of methyl phosphonate (Karl et al.

2008) and by terrestrial plants as a result of ultraviolet irradiation (Bloom et al. 2010a; Keppler et al. 2006). This chapter will focus on the environmental constraints that limit methane production by methanogenic Archaea (Zinder 1993).

Although the abundance of methane in the modern atmosphere is less than 1% of the abundance of CO_2, it is considerably more efficient, on a per-molecule basis, as a "greenhouse" gas (Ramanathan et al. 1985). Emissions of methane from wetlands and marine methane hydrates have been implicated as strong contributors to global warming historically (Nisbet and Chappellaz 2009). Because methane is an important component of the Earth's radiation budget, the global methane budget and its associated microbial cycles are essential to understand and quantify. The perspective of this chapter will be to provide mechanistic understanding on how physicochemical changes in the environment will affect methanogenesis in different ecosystems.

The methanogen biosphere encompasses a diverse array of ecosystem types (Zinder 1993; Liu and Whitman 2008) and a broad range of physicochemical conditions. The best known and the most common methanogenic niches are in oxygen-free, aqueous systems that contain organic matter, which is degraded by a complex array of microorganisms to methanogenic substrates such as H_2, formate, acetate, and various methylated compounds (Table 1). Such systems include marine, lacustrine, and riverine/estuarine sediments; wetlands, such as swamps, bogs, and periodically flooded forest soils; agricultural soils subject to inundation, such as rice paddies; sewage digesters; and the anoxic portions of animal digestive tracts (Chaban et al. 2006; Hedderich and Whitman 2006; Liu and Whitman 2008). Additionally, because H_2 is produced through the interaction of crustal rocks and water (Hoehler 2005), the potential exists for a methanogen biosphere that is supported by geochemical energy sources rather than by photosynthetic activity. Methanogenic activity is known to occur in environments with temperatures from -2 °C to 122 °C, at pH from 3.0 to 10.2, at salt concentrations from near 0 to >5 M NaCl, and at pressures greater than 75 MPa (Table 2).

The prevalence of methanogens in ecosystems with a wide range of physiochemical "extremes" may arise from a combination of factors, including: (i) the availability of methanogenic substrates in a wide variety of settings, including from geochemical sources; (ii) a relatively simple biochemical machinery where a smaller genome, fewer core enzymes, and less complexity in general may foster tolerance to a broader range of physicochemical conditions and/or more rapid adaptation to new conditions; and (iii) more than three billion years in which to adapt and evolve a variety of phenotypes around a simple core metabolism. Together, these factors have presented methanogens with the impetus, potential, and time to evolve and occupy a broad range of ecological niches. Despite this ecological plasticity, the distribution of methanogens in nature is quite limited in comparison with the distribution/availability of potential methanogenic substrates. In general, methanogen distribution is constrained by ecological interactions or physicochemical environmental factors that breach biochemical or bioenergetic limits. This chapter considers these limitations and the resulting major environmental controls on methanogenesis. The interested reader is also directed to (Zinder 1993; Liu and Whitman 2008; Thauer et al. 2008) for a thorough consideration of the physiological ecology of methanogens.

Table 1 Gibbs free energy changes of methanogenic reactions[a]

Reaction	$\Delta G^{o\prime}$ (kJ mol^{-1} of carbon substrate)	$\Delta G^{o\prime}$ (kJ mol^{-1} of methane)
Methanogenesis		
$4\ H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3\ H_2O$	−135.6	−135.6
$4\ HCOO^-$ (formic acid) $+ H_2O + H^+ \rightarrow CH_4 + 3\ HCO_3^-$	−32.5	−130.1
$4\ CH_3OH$ (methanol) $\rightarrow 3\ CH_4 + HCO_3^- + H_2O + H^+$	−78.6	−104.8
$CH_3OH + H_2 \rightarrow CH_4 + H_2O$	−112.5	−112.5
$2\ (CH_3)_2$-S (Dimethylsulfide) $+ 3\ H_2O \rightarrow 3\ CH_4 + HCO_3^- + 2\ HS^- + 3\ H^+$	−60.6	−40.4
$(CH_3)_2$-S $+ H_2 \rightarrow CH_4 + CH_3SH$	−161	−161
$4\ CH_3$-NH_2 (methylamine) $+ 3\ H_2O + H^+ \rightarrow 3\ CH_4 + HCO_3^- + 4\ NH_4^+$	−91.9	−122.6
$2\ (CH_3)_2$-NH (dimethylamine) $+ 3\ H_2O + H^+ \rightarrow 3\ CH_4 + HCO_3^- + 2\ NH_4^+$	−143.1	−95.4
$4\ (CH_3)_3$-N (trimethylamine) $+ 9\ H_2O + H^+ \rightarrow 9\ CH_4 + 3\ HCO_3^- + 4\ NH_4^+$	−190.9	−84.8
CH_3COOH (acetic acid) $\rightarrow CH_4 + CO_2$	−31	−31

[a]Calculated from the Gibbs free energy of formation from Thauer et al. (1977), and Wagman et al. (1968), except for the reaction involving dimethylsulfide and hydrogen, which was from Nobu et al. (2016)

2 Biochemical and Bioenergetic Considerations

Biochemical limitations are encountered principally through physical or chemical disruption of core metabolic molecules, structures, networks, or processes. Examples include the thermal destabilization of enzyme tertiary structure, enhanced chemical hydrolysis of biopolymer linkages, chemical inactivation of enzyme binding sites, or the inherent limitation of enzymes to take up and process substrates at levels needed to compete effectively or support metabolism. Specific biochemical effects on methanogens are considered below.

Bioenergetic constraints on environmental habitability arise from life's fundamental need to harness energy from the surroundings and use the energy to maintain what is, ultimately, a disequilibrium state – that is, the maintenance of complex biological molecules and structures that are thermodynamically unstable with respect to the general environment. For an environment to be habitable from an energetic standpoint, the provision of energy by that environment and the organism's capability to access and use that energy must balance or exceed the organism's demand for energy (Hoehler 2004, 2007; Shock and Holland 2007; Hoehler and Jørgensen 2013).

The biological demand for energy is manifest in two requirements, which are analogous to the voltage and power requirements of an electrical device. The analog to voltage (energy per unit of energy carrier) is the biological energy quantum

Table 2 Examples of extreme methanogens and syntrophic metabolizers

Physiological type	Organism	Environmental Condition		Reference
		Optimum	Range	
Hyperthermophilic Methangens	*Methanocaldococcus spp.*	82–90 C	50–92 C	Jeanthon et al. 1998, 1999; L'Haridon et al. 2003; Mehta and Baross 2006; Ver Eecke et al. 2012
	Methanopyrus kandleri	98 C 105 C at 40 MPa	84–122 C	Kurr et al. 1991; Takai et al. 2008
	Methanothermus fervidus	83 C	65–97 C	Stetter et al. 1981
	Methanothermus sociabilis	88 C	55–97 C	Lauerer et al. 1986
	Methanotorris igneus	88 C	45–91 C	Burggraf et al. 1990
Psychrophilic methanogens	*Methanococcoides burtonii*	23 C	1.7–30 C	Fransmann et al. 1992
	Methanogenium frigidum	15 C	0–18 C	Fransmann et al. 1997
	Methanogenium marinum	25 C	5–25 C	Chong et al. 2002
	Methanolobus psychrophilus	18 C	0–25 C	Zhang et al. 2008
	Methanomethylovorans hollandica	25–35 C	1–35 C	Simankova et al. 2003
	Methanosarcina baltica	21 C	−2-28 C	Singh et al. 2005; von Klein et al. 2002
	Methanosarcina lacustris	25 C	1–35 C	Simankova et al. 2001
	Methanosarcina soligelidi	28 C	0–54 C	Wagner et al. 2013
	Methanospirillum psychrodurum	25 C	4–32 C	Zhou et al. 2014
	Methanospirillum stamsii	20–30 C	5–37 C	Parshina et al. 2014
	Ca. "Methanoflorens stordalenmirensis"	NA[a]	NA	Mondav et al. 2014
Acidiphilic methanogens	*Methanobacterium espanolae*	pH 5.6–6.2	pH 4.7	Patel et al. 1990
	Methanococcus aeolicus	NR[b]	pH 4.3–7.5-7	Kendall et al. 2006b

(*continued*)

Table 2 (continued)

Physiological type	Organism	Environmental Condition		Reference
		Optimum	Range	
	Methanoregula boonei	pH 5.1	pH 4.5–5.5	Bräuer et al. 2011
	Methanosphaerula palustris	pH 5.7	pH 4.8–6.4	Cadillo-Quiroz et al. 2009
Halophilic methanogens	*Halomethanococcus doii*	3.0 M NaCl	>1.8 M NaCl	Yu and Kawamura 1987
	Methanohalobium evestigatum	4.3 M NaCl	2.6–5.1 M NaCl	Zhilina and Zavarzin 1987
	Methanohalophilus halophilus	1.2–1.5 M NaCl	0.3–2.6 M NaCl	Zhilina 1983
	Methanohalophilus mahii	1.0–2.5 M NaCl	0.5–3.5 M NaCl	Paterek and Smith 1988
	Methanohalophilus portucalensis	0.6–2.1 M NaCl	>1.4 M NaCl	Boone et al. 1993
Akalophilic methanogens	*Methanocalculus natronophilus*	pH 9–9.5 Na^{+c} 1.4–1.9 M	pH 8–10.2 Na^{+} 0.9–3.3 M	Zhilina et al. 2013
	Methanohalophilus zhilinae	pH 9.2 0.7 M NaCl	pH 8.0–10 0.2–2.1 M NaCl	Mathrani et al. 1988
	Methanolobus oregonensis	pH 8.6	pH 8.2–9.2	Liu et al. 1990
	Methanolobus taylorii	pH 8	pH 5.5–9.2	Oremland and Boone 1994
	Methanosalsum natronophilum	pH 9.5 Na^{+} 1.5 M	pH 8.2–10.2 Na^{+} 0.5–3.5 M	Sorokin et al. 2015
Piezophiles (Barophiles)	*Methanocaldococcus jannaschii*	75 MPa	<75 MPa	Miller et al. 1998
	Methanococcus thermolithotrophicus	50 MPa		Bernhardt et al. 1988
Thermophilic syntrophic metabolizers	*Desulfotomaculum thermocisternum*	62 C	41–75 C	Nilsen et al. 1996
	Pelotomaculum thermopropionicum	55 C	37–70 C	Imachi et al. 2002
	Syntrophothermus lipocalidus	55 C	45–60 C	Sekiguchi et al. 2000
	Thermosyntropha lipolytica	60–66 C pH 8.1–8.9	52–70 C pH 7.5–9.5	Svetlitshnyi et al. 1996
Psychrophilic syntrophic metabolizer	*Algorimarina butyrica*	15 C	10–25 C	Kendall et al. 2006a

(*continued*)

Table 2 (continued)

Physiological type	Organism	Environmental Condition		Reference
		Optimum	Range	
Akalophilic syntrophic metabolizers	"Ca. Syntrophonatronum acetioxidans"	pH 8.9 Na^+ 0.3–1.0	pH 8.9–10.2 Na^+ 0.5–3.0	Sorokin et al. 2016
	"Ca. Syntrophocurvum alkaliphilum"	pH 9.0 Na^+ 1.0	8.5–10 1–3	
	Tindallia spp.	pH 10 Na^+ 0.6–1	8–10.4 0.3–3.75	
	"Ca. Desulfonatronobulbus propionicus"	pH 10 Na^+ 1.0	8.5–10.3 0.3–4	Sorokin and Chernyh 2016

[a]NA, not applicable as information derived from metagenomic analysis only
[b]NR, not reported
[c]Na^+ indicates the sodium concentration with anions other than chloride

(BEQ), which is defined as the smallest Gibbs free energy change (ΔG) of a metabolic reaction that can still be used to drive ATP synthesis, which is needed to sustain metabolic activity (Schink 1997; Schink and Stams 2006). The analog to power (energy per unit time) is the maintenance energy (ME), which is the flux of energy needed to support a unit of biomass in a steady state at a net zero growth rate ($kJ \cdot mol^{-1} \cdot h^{-1}$) (Harder 1997; Tijhuis et al. 1993). Each requirement is characterized by a minimum value, below which sustained metabolism is not possible, and both requirements must be met in order for a given environment to be habitable by a given organism (Hoehler 2004, 2007) (Fig. 1).

The magnitudes of the BEQ and, in particular, the ME requirements are set in part by the biochemical and physiological characteristics of the organism in question and in part by the environment in which it lives. Nominally, the magnitude of the BEQ has been estimated at about -20 $kJ \cdot mol^{-1}$ for actively growing organisms (Schink 1997) and -12 to -15 $kJ \cdot mol^{-1}$ for organisms operating under energy-limiting conditions (Schink and Stams 2006). Measurements of energy yields associated with methanogenesis in various environments suggest that methanogens may be able to capitalize on energy yields as small as -10 $kJ \cdot mol^{-1}$ (Hoehler et al. 1998, 2001). The magnitude of the ME requirement may vary more substantially across different organisms, and is considerably less well constrained than that of the BEQ (Hoehler and Jørgensen 2013). Estimates of ME derived from culture-based and environmental studies, and from growing versus non-growing organisms vary over orders of magnitude (Morita 2000; Price and Sowers 2004; Tijhuis et al. 1993). Environmental deviations from biologically optimal physiochemical conditions may increase an organism's energy demands significantly. Indeed, natural systems can present multiple physicochemical extremes such as high temperature and low pH that may have compound effects in increasing cellular energy demands. For environments offering only limited fluxes of methanogenic substrates, energy demands due to physiochemical stresses may ultimately exceed the environmental energy supply,

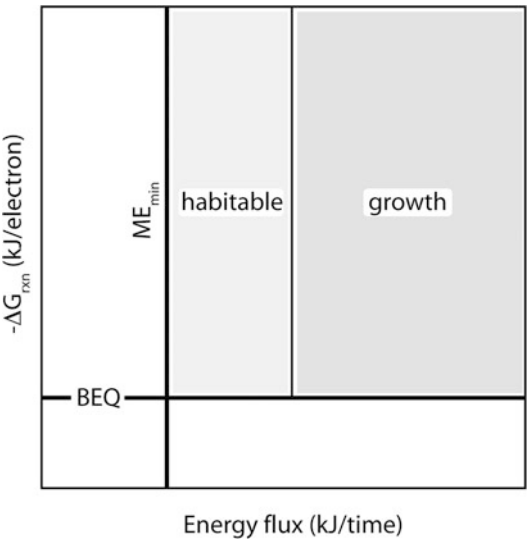

Fig. 1 The importance of energy yield and energy flux in determining the habitability of an environment (After Hoehler 2007). The free energy of the catabolic reaction (ΔG_{rxn}) must be more favorable (e.g., more negative ΔG) than the minimum amount of energy needed to make ATP (biological energy quantum, BEQ). The flux of energy must be larger than the minimum amount to maintain cellular functions and viability (maintenance energy minimum, ME_{min}). Growth will occur when the energy flux is large enough to supply sufficient energy for biosynthesis. If either requirement is not met by the energy available in the local environment, the system is uninhabitable (unshaded region). Note that the magnitude of both BEQ and particularly maintenance energy are highly dependent on the physicochemical environment and may, in the case of environmental extremes, rapidly exceed levels that can be sustained within a given system

and thereby render the environment uninhabitable (Hoehler 2007). Thus, in natural systems, bioenergetic factors may limit habitability before biochemical limitations are encountered.

While the bioenergetic considerations outlined above are applicable for all organisms, they are especially relevant for metabolisms with low energy yields where the Gibbs free energy change of the catabolic reactions under typical environmental conditions is close to the BEQ. Methanogenesis exemplifies this situation, and many aspects of the environmental distribution/limitation on methanogen activity are attributable to bioenergetic effects.

3 Ecological Interactions

Methanogens as an overall metabolic group are capable of using H_2/CO_2, formate, acetate, CO, and several methylated compounds as substrates for methane production (Table 1), although individual taxa may use only a subset (Zinder 1993; Liu and

Whitman 2008). While any of these may conceivably predominate in a given environment, the overall most quantitatively important methanogenic substrates are H_2/CO_2 (and/or formate) and acetate. Both occupy a central role in carbon and electron flow in anaerobic microbiology, and in the metabolic processes of a variety of microorganisms. As such, they present a basis for interactions, both stimulatory and inhibitory, between methanogens and other organisms.

3.1 Syntrophic Interactions

The ability of methanogens to grow autotrophically with H_2 as an electron donor potentially allows them to directly access geochemical sources of energy such as the H_2 produced by water-rock reactions. However, most of the known methanogenic ecosystems are instead fueled by the decomposition of complex organic matter (Chaban et al. 2006; Hedderich and Whitman 2006; Liu and Whitman 2008). In the absence of oxygen, the complete decomposition of complex organics requires the collective activities of a diversity of anaerobic microorganisms, each catalyzing individual steps in the overall process (Schink 1997). Only in the final steps of this process are the methanogenic substrates H_2, formate and acetate made. For this reason, methanogen activity in anaerobic environments fueled by the decomposition of complex organic matter is dependent on the activity of syntrophic partner organisms. The production of H_2 from NADH and $FADH_2$ generated during fermentative metabolism is unfavorable when the pressure of H_2 is high (> 200 Pa) (Schink 1997). Hydrogen-using methanogens rapidly use H_2 produced by fermentative bacteria, thereby maintaining low H_2 concentrations, which makes hydrogen production by fermentative bacteria favorable. The interaction between hydrogen-producing fermentative bacteria and hydrogen-using methanogens is called "interspecies electron transfer." Because of the strict interdependence between H_2-producing fermentative microorganisms and H_2-using methanogens, any environmental or ecological factor that influences one of the partners can also influence the overall rate and extent of methanogenesis in that ecosystem. Thus, the environmental distribution of methanogenesis may be limited by community-level effects that disrupt syntrophic interactions before the absolute physicochemical tolerances of methanogens are exceeded. This is principally a bioenergetic limitation on methanogen distribution, by virtue of disruption of substrate flow to methanogens at levels or rates needed to meet cellular energy demands.

3.2 Competitive Interactions

Acetate and H_2 are utilized in a wide range of microbial metabolisms, so that methanogen distribution may be limited by competition for these substrates. In anoxic systems driven by organic matter decomposition, the principal competitors for acetate and/or H_2 are organisms that oxidize these substrates using inorganic electron acceptors such as nitrate, Mn^{4+}, Fe^{3+}, and sulfate (Zehnder and Stumm

1988). Other organic and inorganic oxidants can serve the same function and may be important in specific environmental settings. Observations of anoxic sediments show that organic matter decomposition often proceeds via successive oxidants, in the order indicated above, with one oxidant (e.g., sulfate) being completely exhausted before another one (e.g., CO_2, in methanogenesis) is utilized. The order in which oxidants are used reflects the magnitude of the standard Gibbs free energy yield associated with oxidation of hydrogen or acetate by that oxidant, with the reduction of nitrate to N_2 by H_2 yielding the largest standard Gibbs free energy change ($\Delta G^{o'} = -560$ kJ • mol^{-1} of oxidant) and the reduction of CO_2 to methane by H_2 yielding the smallest standard Gibbs free energy change ($\Delta G^{o'} = -135$ kJ • mol^{-1} of oxidant) (Zehnder and Stumm 1988).

As suggested by the ordering of oxidants based on free energy yield, the competitive exclusion of methanogenesis is hypothesized to have a thermodynamic basis. Differences in standard Gibbs free energy yields by themselves, however, do not provide a mechanism for exclusion of one organism by another. Rather, a larger free energy yield *potentially* enables one organism to compete more effectively for a common substrate, such as H_2 or acetate, because it should be able utilize the substrate to a lower concentration than an organism that uses a less energetic oxidant and still extract a Gibbs free energy yield that meets the BEQ requirement. Complete inhibition of one metabolism by another will occur if one organism can actuate this potential and consume a common substrate down to a concentration that does not meet the BEQ or thermodynamic favorability requirement of its competitor. Complete inhibition of H_2-using methanogenesis by H_2-using sulfate reduction via this mechanism has been hypothesized or demonstrated in a variety of systems (Cord-Ruwisch et al. 1988; Lovley and Goodwin 1988; Hoehler et al. 1998) and is illustrated in Fig. 2. Whether or not the same potential is actuated in other microbial interactions that involve another electron donor or different oxidants depends on the energetically-advantaged organism having (i) enzyme kinetics that allow it take up the substrate at a concentration and rate that excludes its competitor and (ii) a supply rate of oxidant that exceeds the supply rate of the electron donor.

Enzyme kinetic considerations may limit the potential for competitive exclusion in the case of acetate. This is because the change in acetate concentrations required to actuate an energetic advantage is very large, and would require enzymes with capabilities to take up substrate at extremely low levels. For example, to actuate the >15 kJ·mol^{-1} advantage that sulfate reducers appear to exercise over methanogens in the marine sediments presented in Fig. 2 would only require a > 4.5-fold decrease in H_2 concentrations. Such a decrease in H_2 concentrations was observed in Cape Lookout Bight sediments shown in Fig. 2 (Hoehler et al. 2001). However, more than a 430-fold decrease in acetate concentrations would be needed for acetate-using sulfate reducers to exclude acetate-using methanogens. In practice, acetate concentrations are only a few-fold lower in sulfate-reducing sediments compared to methanogenic sediments; hence, it is not clear that the same bioenergetic basis for competitive exclusion exists for acetate as in the case of H_2. Nonetheless, methanogenesis including that from acetate is often completely excluded by sulfate reduction. These seemingly discrepant observations can be

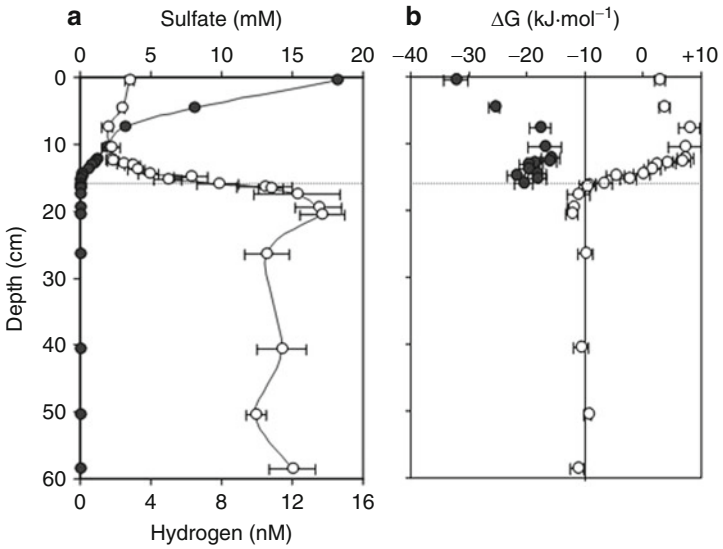

Fig. 2 Thermodynamic-based competitive exclusion of methanogenesis by sulfate reduction in a marine sediment (Cape Lookout Bight, North Carolina, USA). (**A**) Depth profiles of concentrations of sulfate (filled circles) and hydrogen (open circles). Note that hydrogen concentrations are maintained, by the activity of sulfate reducers, at 5- to 10-fold lower levels within the sulfate-containing zone. (**B**) Depth profiles of Gibbs free energies of reaction for H_2-based sulfate reduction (per mole sulfate; filled circles) and methanogenesis (per mole methane; open circles). By virtue of their control over H_2 concentrations, sulfate reducers limit methanogenic energy yields to values below the minimum bioenergetic requirement and, for the upper 13 cm of the sediment column, at thermodynamically unfavorable levels (right of the solid vertical line at $\Delta G = 0$). Below the depth of sulfate depletion (dashed horizontal line), methanogenesis yields about -10 kJ·mol^{-1}, consistent with lower-end estimates of the BEQ. In both (A) and (B), error bars represent the standard deviation about the mean of triplicate samples. (Figure modified from Hoehler et al. 2001)

reconciled if methanogenic metabolism of acetate is influenced by H_2 concentrations in the system and therefore by the actuated energetic advantage of sulfate reducers. Indeed, Finke et al. (2007) showed that methylotrophic methanogens can covert >95% of the substrate methyl carbon to CO_2 and H_2 rather than to methane when porewater H_2 concentrations are held at low levels by sulfate reducers. This hypothesis could explain the apparent competitive exclusion of acetate-based methane production from sulfate-containing sediments, despite the *superficial* lack of a thermodynamic basis, while also associating a potentially energy-yielding metabolic activity (Finke et al. 2007) with methylotrophic methanogens that appear to be present in some such sediments (Kendall et al. 2007).

By virtue of competition for common substrates, the presence of any of the oxidants mentioned above (e.g., NO_3^-, Mn^{4+}, Fe^{3+}, SO_4^{2-}) has potential to completely exclude methanogenesis. In practice, the presence or absence of sulfate, a major anion in seawater (~28 mM), is by far the most important among these oxidants in limiting the environmental distribution of methanogenesis. Specifically,

in the sediments of marine, estuarine, and hypersaline environments (which globally comprise a large repository of potential methanogenic fuel), methanogenic activity is largely restricted to deeper sediment layers where sulfate has been fully consumed by sulfate reduction or, in cases where sulfate persists throughout the sediment column, methanogenesis may be completely absent. Limitations in the supply of the oxidant may serve to limit the effectiveness of terminal electron-consuming processes other than sulfate reduction in excluding methanogenesis by competition for common electron donors. In many natural systems, nitrate is rapidly used and not readily replenished, so the potential for nitrate reducers to exclude other competitors for common electron donors is limited. However, in systems high in nitrate and largely lacking in sulfate such as fertilized agricultural soils or wetlands affected by agricultural run-off, nitrate may be an important agent for exclusion of methanogenesis. Mn^{4+} and $Fe^{3+}$are present as insoluble, particulate oxides, and, thus, may be in short supply if the organisms that utilize them depend on diffusive supply of the dissolved form. As a result, the capability of Mn^{4+}- and Fe^{3+}-respiring organisms to lower the concentrations of common substrates in accordance with their energetic advantage may be limited. Lovley and Goodwin (1988); Achtnich et al. (1995) observed decreased H_2 concentrations in the presence of these metal oxides, but this effect may be highly concentration dependent (Hoehler et al. 1998). Indeed, metal reduction in sediments is frequently accompanied by co-occurring sulfate reduction or methanogenesis, except at high metal oxide concentrations (Thamdrup 2000).

Although bioenergetic considerations appear to underlie much of the competitive exclusion of methanogenesis as illustrated by the competitive effects of sulfate reduction, other mechanisms may also be important. In particular, it has been suggested that low temperatures may favor autotrophic acetogenesis (production of acetate from CO_2 to H_2) over hydrogentrophic methanogenesis in some systems (Conrad 1999; Kotsyurbenko 2005; Nozhevnikova et al. 2007), even though methanogenesis is the more thermodynamically favored of the two processes under most environmental conditions. It is suggested that homoacetogens outcompete methanogens on the basis of having a greater maximum rate of hydrogen use at low temperatures than methanogens, rather than on a bioenergetic basis (Kotsyurbenko et al. 2001).

3.3 Substrate Preferences

The use of a non-competitive substrate such as trimethylamine (Table 1) (King 1983) may allow methanogenesis to co-occur with sulfate reduction in marine sediments where complete inhibition of H_2-consuming methanogenesis by H_2-consuming sulfate reduction would be predicted based on the bioenergetics model described above. Recently, methanogens with substrate utilization patterns different from those of cultured methanogens have been described, which would also change the bioenergetics considerations used for habitability analysis. *Candidatus* "Methanofastidiosum methylthiophilus" lacks pathways for CO_2-reducing and acetoclastic

methanogenesis, but has enzymes for reducing dimethylsulfide to methane (Nobu et al. 2016). At 10 Pa H_2, methanogenesis from H_2 and dimethylsulfide ($\Delta G' = -140$ kJ mol^{-1}) is much more favorable than that from H_2 and CO_2 ($\Delta G' = -18$ kJ mol^{-1}) (Nobu et al. 2016). Similar bioenergetic considerations hold for members of the order Methanomassiliicoccales (Dridi et al. 2012; Borrel et al. 2013, 2014) and the archaea phylum, Verstraetearchaeota (Vanwonterghem et al. 2016), whose members are known to require both H_2 and methylated compounds for methanogenesis. Two genomes in the archaeal phylum *Bathyarchaeota* indicate that methanogen members of this phylum may be capable of carbohydrate fermentation (Evans et al. 2015). Another example of novel substrate for methanogenesis is the use of methoxylated aromatic compounds present in coal by *Methermicoccus shengliensis* (Mayumi et al. 2016). The diversification of substrate use by some methanogens would allow them to occupy habitats that would exclude hydrogenotrophic or acetoclastic methanogens.

4 Physicochemical Environment

Physicochemical "extremes" can impact methanogens at both biochemical and bioenergetic levels, either directly or by affecting their ecological interactions with other organisms.

4.1 Oxygen

The ambient concentration of oxygen is an important determinant of the environmental distribution of methanogenesis. Methanogens are strict anaerobes and, in culture, will not grow or produce methane in the presence of even trace levels of oxygen (Zinder 1993). Given the high abundance of oxygen in Earth's atmosphere, this sensitivity has the potential to severely limit methanogenesis by methanogenic archaea, although not by aerobic, methyl phosphonate-cleaving bacteria (Karl et al. 2008). Two factors serve to mitigate oxygen inhibition of methanogenesis to some degree. First, while methanogens do not grow or metabolize in the presence of atmospheric levels of oxygen, they do exhibit some tolerance to oxygen exposure (Zinder 1993). This suggests that core methanogen enzymes may not be irreversibly damaged or, at least, can be reactivated, following oxygen exposure. Oxygen tolerance may allow methanogens to persist in environments that may fluctuate between oxic and anoxic conditions. Second, aqueous habitats containing particulate organic matter tend to endure limited permeation by oxygen because: (i) the solubility of oxygen in water is relatively low, which effectively reduces the potential mass flux of oxygen from an overlying gas phase to methanogens inhabiting the aqueous phase; (ii) organic-containing sediments provide a physical matrix that limits oxygen mass transfer to molecular diffusion, which is slow over spatial scale of more than a few millimeters; and (iii) sediments contain active microbes or reactive chemicals that reduce oxygen quickly. For ecosystems with large

amounts of organic matter, the slow diffusion and rapid consumption restricts oxygen penetration to a narrow surface zone that may range from microns to a few centimeters. Methanogenic decomposition of organic matter can then occur below the zone of oxygen penetration.

4.2 Temperature

Methanogens are represented across most of the known biologically-tolerated range of temperature from −2 °C to 122 °C (Table 2). The rates of methanogenesis increase more steeply with temperature than do other important biological processes such as heterotrophic respiration or photosynthesis (Yvon-Durocher et al. 2014). The sensitivity of the rates of methanogenesis to temperature demonstrates the importance of temperature in controlling methane flux globally. However, large site-to-site variations in methane emission exist showing that other variables such as water saturation and vegetation are also important (Turetsky et al. 2014). For example, methane emissions from wetlands are best explained when variations in surface temperature and water-table depth are both considered (Bloom et al. 2010b).

Psychrophily. Methanogens are found in many low temperature habitats. Methanogenic low temperature habitats include high latitude wetlands such as boreal fens, tundras, and bogs, marine and freshwater sediments underlying deep waters, and sediments in arctic and antarctic regions (Cavicchioli 2006). Collectively, these habitats also have significant differences in pH, salinity, pressure, and energy availability. Temperature ranges for cell growth in psychrophilic and psychrotolerant methanogens generally span from −2 °C to 54 °C (Table 2). The lower limit can extend below 0 °C when cells possess means to suppress ice formation (Cavicchioli 2006). Cell doubling times for *Methanococciodes burtonii* and *Methanogenium frigidum* are considerably below that described for the extreme thermophiles, and may be in the range of 0.1–0.3 generations per day (Cavicchioli 2006).

The vast proportion of Earth's surface, including most or all of the deep ocean basins, high latitude lakes and ocean shelves, and high latitude wetlands and soils, is subjected to periodically or permanently low temperatures. The presence of the significant reserves of organic carbon, which are sequestered in periodically or permanently cold environments, suggest that low temperature environments represent a vast potential habitat for psychrophilic methanogens. Nonetheless, relatively little is understood about the environmental diversity of the psychrohilic and psychrotolerant methanogens, or the mechanisms that allow them to adapt to low temperature niches, but environmental genomic analyses are beginning to yield insights. Metagenomic analyses revealed that partially thawed, Arctic permafrost samples were dominated by a single archaeal phylotype, *Candidatus* "Methanoflorens stordalenmirensis," which belongs to the uncultivated lineage "Rice Cluster II" (*Candidatus* "Methanoflorentaceae") (Mondav et al. 2014). Members of Ca. "Methanoflorentaceae" are globally distributed in habitats with diverse physiochemical conditions. Such metagenomic analyses are important tools for

identifying methanogens present in cold environments and characterizing their potential metabolic properties, given that the typical very slow growth of psychrophiles makes laboratory studies challenging.

Thermophily. Thermophilic and hyperthermophilic methanogens are found in fluid outflows from marine and fresh water volcanic seeps, hot springs, thermal mud pools, and solfataric fields (Huber et al. 2000). These habitats are typically rich in H_2 and minerals, low in organics, and may vary significantly in pH and salinity (from fresh to marine). The chemoautotrophic methanogens *Methanotorris igneus (Methanococcus igneus)* and *Methanothermus fervidus* were isolated from a shallow offshore submarine vent and a thermal terrestrial waterhole in the mountains of Iceland, respectively. *Methanopyrus kandleri*, currently the high temperature "record holder" among cultured methanogens, was isolated from a deep hydrothermal "black smoker" vent at 2000 m in the Gulf of California (Kurr et al. 1991; Takai et al. 2008). It has a growth optimum of 105 °C at 40 MPa and is capable of growth at 122 °C (Table 2). By virtue of the general enhancement of metabolic rate by increasing temperature, cell doubling times for these thermophilic methanogens can be less than one per hour (Jeanthon et al. 1998; Takai et al. 2004).

The mechanisms of high temperature limitation of methanogens can be biochemical, bioenergetic, and/or ecological. While some large organisms can maintain internal temperatures significantly above or below ambient, individual microbes, or microbes in small clusters, cannot. Biologically meaningful temperature gradients cannot be maintained at the scale of microbial cells, so the environmental temperature is, effectively, the intracellular temperature. Thus, temperature effects can act directly upon the biochemical machinery of the cell. The deleterious effects of high temperatures relate principally to the thermal destabilization of core biomolecules, with resulting impacts on functionality. For example, high temperatures disrupt the tertiary structure necessary for enzyme function as well as lipid membrane stability. The biochemical impacts of high temperature for organisms in general, which are applicable to methanogens, are thoroughly reviewed by Jaenicke and Sterner (2002). The impact of high temperature may set the ultimate upper limit on methanogen growth and metabolism in habitats where energy is in abundance and other growth parameters are optimal.

Temperature can also influence methanogen metabolism by factors other than biochemical mechanisms. As noted earlier, for systems driven principally by organic matter decomposition, methanogen activity is ultimately dependent on the collective function of a broader community of organic matter-degrading organisms, and is therefore subject to the physicochemical limitations of critical organisms within that population. Thus, elevated temperatures may limit methanogen distribution by inhibiting partner organisms before the biochemical temperature limits of methanogens are encountered. Consistent with this notion, the cultured methanogens representing the upper end of the tolerated temperature range are generally derived from environments in which the substrate, principally H_2, is provided by geochemical sources, rather than by community-enabled organic matter decomposition. The maximum growth temperature so far for a cultured syntrophic metabolizer is about 75 °C (Table 2).

Lastly, temperature has a strong effect on cellular maintenance energy, and may thereby serve to limit methanogenic activity via bioenergetic inhibition. The effect of temperature on maintenance energy has been quantified experimentally (Tijhuis et al. 1993) and conforms to an Arrhenius-type relationship (Harder 1997) (eq. 1):

$$ME = A \cdot e^{-Ea/RT} \quad (1)$$

where A is a positive constant, E_a is the activation energy (kJ • mol^{-1}), R is the universal gas constant, and T is temperature in °K. According to this relationship, the energy required to support a unit of biomass increases exponentially with temperature. Empirically determined values for E_a (Tijhuis et al. 1993; Harder 1997) predict that maintenance energy increases more than three-thousand fold as temperature increases from 0 °C to 100 °C. Thus, in environments offering limited substrate fluxes, increasing temperatures may rapidly lead to bioenergetic limitation of methanogen growth and maintenance before absolute biochemical limits are reached.

4.3 pH

Methanogenesis is common in marine and freshwater boreal fens, tundras, and bogs, where accumulation of plant tannins and organic acids can lower pH to values ranging from weakly acidic to 3.5 or less (Zinder 1993). Methane formation has been observed in peat samples at pH values as low as 3, although higher values were needed for optimal rates of methanogenesis (Williams and Crawford 1984; Bräuer et al. 2006; Cadillo-Quiroz et al. 2008). These habitats are frequently characterized by low temperatures, and potentially represent multiple "extremes" to microbial inhabitants. As a group, acidiphilic methanogens are relatively little studied. However, several acidiphilic methanogens have been isolated that grow and produce methane at pH values as low as 4.3 although optimal growth occurs at pH $\geq$ 5.0 (Table 2).

Some methanogens are also capable of growth or metabolism under alkaline conditions. Most of the studied alkaliphilic methanogens listed in Table 2 are associated with evaporitic basins such as Mono Lake, California, the Dead Sea, the Rift Valley Lakes of East Africa, or desert soda lakes and streams. In addition, methanogenic activity has been inferred in alkaline seeps where serpentinization yields waters with elevated pH (Kelley et al. 2005). Cultured representatives are moderately alkaliphilic (Table 2), with *Methanocalculus natronophilus* and *Methanosalsum natronophilum* having a pH growth optimum of 9.0–9.5 and a growth limit of 10.2 (Mathrani et al. 1988). Alkaline environments may present multiple extremes for methanogen activity. Alkalinity associated with evaporitic settings is frequently accompanied by concentrated salts. Mono Lake, for example, has sodium concentration of 1.3 M and carbonate concentration of 0.4 M (Oremland et al. 1993). Thus, some alkaliphilic methanogens are also halophilic. *M. natronophilus* and *M. natronophilum*, for example, are capable of growth at salinities up to 3.3–3.5 M NaCl, or about 5–6 times seawater salinity (Mathrani et al. 1988).

Alkaline environments also contain the requisite syntrophic partners needed for organic matter decomposition (Table 2). Alkalinity may also be associated with high temperatures, as in alkaline hot springs or, in particular, hydrothermal settings associated with serpentinizing host rocks. For example, some venting fluids at the Lost City hydrothermal field, which is located about 15 km away from of the Mid-Atlantic Ridge, reach pH values of 10–11 at temperatures of 70 °C (Kelley et al. 2005).

Significant deviations from neutral pH have the potential to adversely affect cellular biochemistry at a variety of levels, so that only modest variations in intracellular pH can be tolerated. However, habitation of environments with higher or lower than biochemically tolerable pH values is nonetheless feasible because the lipid bilayer membrane is an effective barrier to ionic species like H^+, OH^- or CO_3^{2-}. Thus, it is possible to maintain intracellular pH at more moderate levels than in the extracellular medium, through active regulation. The mechanisms associated with such regulation are understood biochemically (Krulwich 1995, 2000; Krulwich et al. 1996) and it is clear that actuation of these strategies such as active transport of protons must increase cellular maintenance energies. The effect of pH on maintenance energy of methanogens has not been quantified directly. However, the minimal energetic cost of pH regulation is determined by the rate of proton pumping necessary to maintain the appropriate internal pH. The internal pH, in turn, depends on the leakiness of the membrane, the presence of weak acids and bases that may diffuse across the membrane, and the energy required to pump a unit quantity of protons (Krulwich 2000). All of these factors can be expected to increase monotonically with increasing or decreasing extracellular pH, so that maintenance energy should increase as the environmental pH deviates from the biochemical optimum. Thus, bioenergetic effects may factor prominently in setting the practical environmental pH limits.

A secondary effect that may significantly constrain the habitability of alkaline or acidic environments with respect to methanogenesis is the speciation of methanogenic substrates in response to pH. Specifically, the conversion of methanogenic substrates into predominantly ionic forms that cannot diffuse across the cell membrane will require either energy expenditure for the active transport of these substrates or an increase in membrane permeability. The latter would be problematic as it would increase pH leakage and require higher rates of proton pumping to maintain the appropriate internal pH. Such effects are probably most important in limiting methanogenesis in alkaline environments, due to the deprotonation of acetic and carbonic acid, but could also conceivably be important for the methanogenic consumption of methylamines due to its protonation in acidic environments.

4.4 Salinity

Methanogens are found in environments with salinities ranging from that of freshwater to halite-saturated (> 5 M NaCl). Halophilic and extremely halophilic

methanogens are most commonly associated with hypersaline environments that include dead seas, solar salterns and halite crystallizing ponds, and alkali lakes. As mentioned above, these environments often have high concentrations of sodium chloride, magnesium chloride, sulfate, carbonate and other salts (Oren 2002; Ollivier et al. 1994). Notably, the conditions responsible for generating hypersaline conditions may also enhance alkalinity, and are the result of elevated temperatures, so that methanogenic inhabitants of hypersaline environments most likely face multiple extremes. Described halophilic methanogens include moderate halophiles such as *Methanohalophius mahii, Methanohalophilus halophilus*, and *Methanosalsus zhilinae* (Ollivier et al. 1994), and extreme halophiles, including *Halomethanococcus doii* and *Methanohalobium evestigatum* (Table 2). The former have optimal growth with 1–2.5 M NaCl, while the latter are able to grow in halite-saturated brines (over 5 M).

Elevated intracellular salt concentrations would directly and significantly impact cellular biochemistry. Some organisms, e.g., *Halobacteria,* employ a "salt-in" strategy in which biochemical accommodations are made to high intracellular salt concentrations (Oren 2001). However, all of the known methanogens are "salt out" strategists (Oren 2001) where intracellular salt concentrations are held below environmental levels by virtue of the barrier presented to ionic species by the lipid bilayer membrane, and through active transport of salts across the membrane. To compensate for the resulting differential in osmotic pressure, methanogens produce and concentrate intracellular osmolites such as glycine betaine, β-glutamate, β-glutamine, and *N*ε-acetyl-β-lysine (Lai and Gunsalus 1992; Lai et al. 1991). Production of these compounds at the rates and levels needed to compensate for high salinity, along with active regulation of ion transport across the cell membrane, constitutes significant and ongoing energy expenditure. Indeed, the energy expenditure to maintain osmotic balance and regulate intracellular salt concentrations can be expected to increase monotonically with the salinity of the environment. Thus, salinity-based limitations on methanogenic metabolism may act principally at a bioenergetic level (Oren 1999, 2001). Typically, methanogens that grow at high salinities are methylotrophic rather than H_2- or acetate-utilizing methanogens, and likely due to the abundance of methyl group-containing osmolytes like betaine. Oren (2001) noted that the standard Gibbs free energy change per substrate is greater for di- and tri-methylamines than H_2 or acetate (Table 1). The larger energy yields may serve to balance the higher rates of energy expenditure required for life at higher salinities. While higher overall rates of metabolic energy production will certainly serve to balance higher cellular maintenance energies, some caution is warranted in this interpretation. Specifically, larger standard Gibbs free energy yields are, by themselves, only one determinant of the total metabolic energy yield, and the rate of substrate flux/consumption must also be considered. For example, a methanogen consuming acetate with a 5-fold lower Gibbs free energy yield than a methanogen consuming methylamine will nonetheless have a higher overall rate of metabolic energy production if it receives a 10-fold higher flux of substrate. Importantly, however, methylotrophic substrates – some of which are among the breakdown products of osmoregulants – appear to be proportionately more abundant as

methanogenic substrates in hypersaline settings. The high substrate concentration in combination with the larger free energy yields associated with methylotrophic methanogenesis may account for the higher salinity tolerance of methylotrophic methanogens compared to their H_2- or acetate-utilizing counterparts. Regardless, energy balance appears to be a critical determinant of the methanogenic habitability of hypersaline environments.

4.5 Pressure

Elevated pressure is a characteristic of sediments underlying the deep ocean basins, and methanogen activity is documented to pressures of 75 MPa, equivalent to >7000 m water depth (Miller et al. 1988). However, the difficulties associated with conducting physiological studies at very high pressures have limited our direct understanding of the tolerance and adaptations of methanogens to high pressures. Because biochemical and metabolic reactions occur in aqueous solution, and because the partial molar volume changes associated with aqueous reactions are typically quite low, high pressures have only minimal effects on the thermodynamics of methane production. However, high pressures are known to affect key biochemical functions (Abe 2007). Pressures of 10–20 MPa can impair important cellular processes in mesophilic bacteria such as motility, cell division, nutrient uptake, and membrane protein function. Replication, transcription and protein synthesis are affected at pressures of 50 MPa and above. Thus, if methanogens respond to high pressure in a manner similar to that of bacteria, piezophilic (barophilic) methanogens must have evolved adaptive mechanisms to survive and thrive at high pressure. Elevated pressures also enhance the solubility of gaseous substrates and products of metabolism. The transport of hydrogen can thus be significantly greater at high pressure, which would allow for higher maximum rates of substrate conversion (energy production) by hydrogen-using methanogens. However, high pressure can impair nutrient uptake, which may increase maintenance energy requirements.

5 Research Needs

Culture-based microbiology has given us a snapshot of the methanogen tolerance to environmental extremes, but it is not clear how to translate these observations into a realistic predictor of their population distribution and activity in the natural world, where these organisms may function in the context of syntrophic and competitive interactions, and may face energy limitation and multiple physicochemical extremes. Advances in a variety of areas will ultimately help to address this question.

The study of methanogens in laboratory cultures has greatly enhanced our understanding of the physiological ecology of methanogenesis. Traditionally, however, culture work optimizes energy availability and other growth factors, while seeking to isolate individual physicochemical variables for study. To begin to probe the question of survival in complex environments, these studies must begin to

incorporate constraints – for example, energy limitation or combinations of physicochemical extremes – that realistically mimic the natural environment. Key areas requiring further study are how microbial energy metabolism, in particular, maintenance energy, responds to non-optimal growth conditions and what biochemical and regulatory mechanisms are used to adapt to extreme growth conditions. Such studies will also benefit from continued attempts to sample the full diversity of methanogens in environments presenting individual and combinations of extremes (including energy limitation), to ensure that the full range of environmental tolerances and mechanisms of adaptation are reflected in cultured organisms.

Beyond the petri dish, advances in characterizing microbial ecology and physiology in situ will significantly enhance our predictive capability regarding the environmental distribution of methanogenic activity vis-à-vis a variety of extremes. Key areas for advancement include accurate in situ rate measurements, especially in cases of low metabolic activity (e.g., in cold or low energy settings); methods for discerning and discriminating metabolic status (e.g., active growth vs. simple maintenance) and for obtaining accurate cell counts at low numbers; methods for linking geochemical function with genetic identity; and means for better resolving complex ecological interactions and associations involving the syntrophic partnerships and competitive interactions.

Combining genomic and transcriptomic analyses with comprehensive and quantitative in situ analyses will provide a much clearer picture of the phylogenetic and metabolic diversity of methanogens and how various physiochemical factors affect their activity. Improved bioinformatics tools are needed to provide a more complete understanding of the physiological properties of uncultured methanogens and the signaling and regulatory systems that they use to respond to changing environmental conditions. The combination of metagenomic, metatranscriptomic, metabolomic, bioinformatic and computational approaches is needed to understand the factors that govern interspecies interactions between methanogens and their syntrophic partners and how these organisms orchestrate their metabolisms into a coordinated catalytic unit.

Finally, theoretical work on microbial energy metabolism may aid in developing a quantitative framework in which to understand and predict the effects of multiple environmental forcing factors. The approach would combine with culture-based and environmental studies to assess the biochemical and bioenergetic impacts and adaptations associated with environmental extremes. Identifying common denominators such as the effects of diverse physicochemical extremes on cellular maintenance energies will lead to an improved ability to quantify and predict multifactorial effects on methanogen metabolism. Numerical models that include substrate transport and reaction at single-cell scales are needed to simulate and predict biological processes at spatial or temporal scales that are not currently accessible by experimental or observational means.

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Methanogens: Syntrophic Metabolism

7

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Abstract

Syntrophy is a mutualistic interaction in which two metabolically different types of microorganisms are linked by the need to keep metabolites exchanged between the two partners at low concentrations to make the overall metabolism of both organisms feasible. In most cases, the cooperation is based on the transfer of hydrogen, formate, or acetate from fermentative bacteria to methanogens to make the degradation of electron-rich substrates thermodynamically favorable. Syntrophic metabolism proceeds at very low Gibbs' free energy changes, close to the minimum free energy change needed to conserve energy biologically, which is the energy needed to transport one proton across the cytoplasmic membrane. Pathways for syntrophic degradation of fatty acids predict the net synthesis of about one-third of an ATP per round of catabolism. Syntrophic metabolism entails critical oxidation-reduction reactions in which H_2 or formate production would be thermodynamically unfavorable unless energy is invested. Molecular insights into the membrane processes involved in ion translocation and reverse electron transport revealed that syntrophs harbor multiple systems for reverse electron transfer. While much evidence supports the interspecies transfer of H_2 and formate, other mechanisms of interspecies electron transfer exist including cysteine cycling and possibly direct interspecies electron transfer as electric current via conductive pili or (semi)conductive minerals.

1 Introduction

Syntrophy is an energetically limited interaction between cells of different species, e.g., the fatty acid degrader and the methanogen (Table 1) (McInerney et al. 2008; Schink 1997; Schink and Stams 2013). The mutual dependence between the two

Table 1 Reactions involved in syntrophic metabolism

Reactions	$\Delta G^{o/a}$ (kJ/mol)	$\Delta G'^{b}$ (kJ/mol)
Methanogenic reactions		
$4\ H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3\ H_2O$	−135.6	−15.8
$4\ HCOO^- + H_2O + H^+ \rightarrow CH_4 + 3\ HCO_3^-$	−130.4	−11.8
Syntrophic oxidations		
$Acetate^- + 4\ H_2O \rightarrow 2\ HCO_3^- + H^+ + 4\ H_2$	+104.6	−1.5
$Propionate^- + 3\ H_2O \rightarrow Acetate^- + HCO_3^- + H^+ + 3\ H_2$	+76.1	−16.9
$Butyrate^- + 2\ H_2O \rightarrow 2\ Acetate^- + H^+ + 2\ H_2$	+48.6	−39.2
$Benzoate^- + 7\ H_2O \rightarrow 3\ Acetate^- + HCO_3^- + 3\ H^+ + 3\ H_2$	+70.1	−68.5

[a]Calculated from the data in Thauer et al. (1977) with the free energy of formation for benzoate given in Kaiser and Hanselmann (1982)

[b]Calculated on the basis of the following conditions observed in methanogenic ecosystems: partial pressures of H_2 of 1 Pa and of CH_4 of 50 kPa, 50 mM bicarbonate, and the concentrations of the substrates and acetate at 0.1 mM each

metabolic types of organisms is so extreme that neither one functions without the activity of its partner. Together, the partners perform functions that neither one can do alone. The degradation of the respective substrate, in this case, a fatty or aromatic acid (Table 1), is thermodynamically unfavorable if the product concentrations are at standard conditions (1 M concentration, or 1 atm for gasses). The function of methanogens is to consume hydrogen, for example, to low steady-state pressure (10^{-4}–10^{-5} atm) to make fatty and aromatic acid oxidation thermodynamically favorable (Table 1). This chapter focuses on obligate syntrophy where reverse electron transport is a key requirement in the energy budget.

2 Importance of Syntrophy

Syntrophic metabolism is an essential but the least energetically favorable step in the conversion of organic matter to methane and carbon dioxide in anoxic environments. Biological methane production, also termed methanogenesis, is an important process in the global carbon cycle, accounting for about 1–2% of the carbon fixed annually by photosynthesis (Hedderich and Whitman 2006). Annual global methane emissions into the atmosphere are large, about 550–650 Teragram (Tg) (1 Tg equals 10^{12} g), and more than 70% (400–450 Tg) of these emissions are due to microbial activity (Ehhalt et al. 2001; IPCC 2014). Syntrophic metabolism is often the rate-limiting step in methanogenesis (McCarty 1971; McInerney et al. 1981) and, thus, is an important process controlling the global carbon flux.

The degradation of natural polymers such as polysaccharides, proteins, nucleic acids, and lipids to CO_2 and CH_4 involves a complex microbial community (McInerney et al. 1981; Schink and Friedrich 1994). Fermentative bacteria hydrolyze the polymeric substrates such as polysaccharides, proteins, and lipids and ferment the hydrolysis products to acetate and longer-chain fatty acids, CO_2, formate, and H_2. Propionate and longer-chain fatty acids, alcohols, and some amino acids and aromatic compounds are syntrophically metabolized to the methanogenic substrates: H_2, formate, and acetate (Schink 1997; Schink and Stams 2013). Lastly, two different groups of methanogens, the hydrogenotrophic methanogens and the acetotrophic methanogens, complete the process, converting acetate, formate, and H_2 produced by other microorganisms to methane and carbon dioxide.

The syntrophic degradation of fatty and aromatic acids accounts for much of the carbon flux in methanogenic environments (McCarty 1971; Pavlostathis and Giraldo-Gomez 1991). Initial anaerobic transformations of aromatic compounds (Heider and Fuchs 1997a, b; Schink et al. 2000) generally lead to the conversion of diverse aromatic compounds into benzoyl-coenzyme A (CoA) (Merkel et al. 1989; Gallert and Winter 1994; Gibson et al. 1994, 1997; Breese and Fuchs 1998; Hirsch et al. 1998). In methanogenic environments, the reduction and cleavage of the aromatic ring are catalyzed by syntrophic associations of benzoate-degrading microorganisms and hydrogen- and/or formate-using methanogens (Ferry and Wolfe 1976; Mountfort and Bryant 1982; Szewzyk and Schink 1989).

3 Bioenergetic Considerations

Syntrophy is a fascinating process, especially from a bioenergetic perspective. Syntrophic metabolism releases very little free energy, which must be shared among the partner organisms involved (Schink 1997). Organisms capable of syntrophic metabolism operate at free energy changes very close to the minimum increment of energy required for ATP synthesis (Schink 1997; Hoehler 2004). This minimum amount of energy needed for ATP synthesis has been predicted to be about -20 kJ mol^{-1} (Schink 1997) but, depending on the H^+/ATP stoichiometry of the ATPases involved, may be as low as -10 to -15 kJ mol^{-1} (Spahn et al. 2015; Lever et al. 2015). Most of the free energy changes observed during syntrophic metabolism are in the range of -20 kJ mol^{-1} (Schink 1997) although some studies have found free energy changes less than -10 kJ mol^{-1} (Dwyer et al. 1988; Scholten and Conrad 2000). Coupling energy-transforming reactions at the cytoplasmic membrane with translocation of different ions, e.g., protons and Na^+ ions, of different energetic values may allow to operate an energy metabolism at such a low-energy yield. The recently discovered phenomenon of electron bifurcation and electron confurcation (Li et al. 2008; Buckel and Thauer 2013) may provide a further option for ATP synthesis at extremely low-energy yields. According to this concept, electrons at an intermediate redox potential can be shifted through a flavin carrier to a lower potential at the expense of a simultaneous transport of other electrons of the same potential to a higher one. Thus, a certain (minimal) potential difference can be achieved for a single electron by running two electrons over only half that potential difference. Electron bifurcation and confurcation reactions have been found repeatedly in the recent past as essential means to understand energy coupling in the metabolism of methanogens, sulfate reducers, and fermenting bacteria, including syntrophically fermenting ones (Sect. 6).

The second fascinating feature of syntrophic metabolism is the necessity for reverse electron transport. In syntrophic metabolism, there are critical oxidation-reduction reactions that are thermodynamically unfavorable. For example, the production of H_2 (E' of -261 mV at 1 Pa H_2) or formate (E' of -258 mV at 1 μM formate) (Schink 1997) from electrons generated from the oxidation of acyl-CoA intermediates to their respective enoyl-CoA intermediates (E' of -10 mV) (Sato et al. 1999) has a $\Delta E'$ of about -250 mV. A H_2 partial pressure of about 10^{-5} Pa would make this reaction thermodynamically favorable (Schink 1997). The syntrophic oxidation of propionate by *Syntrophobacter wolinii* through the methylmalonyl-CoA pathway (Houwen et al. 1990) involves the oxidation of succinate to fumarate ($E^{o\prime}$ of $+33$ mV) (Thauer et al. 1977). Here again, a very low H_2 partial pressure (10^{-6} Pa) is needed for H_2 production to be thermodynamically favorable (Schink 1997). Methanogens cannot generate such low H_2 partial pressures because hydrogenotrophic methanogenesis reaches thermodynamic equilibrium at 0.2 Pa H_2. H_2 or formate production up to concentrations that support the energy metabolism of methanogens requires an input of energy, a process called reversed electron transport. The most likely energy source for this energy input is an ion gradient that is provided by a membrane-bound ATPase. Consistent with the

requirement for an ion gradient for H_2 production, the protonophore (CCCP) and the ATP synthase inhibitor (DCCD) inhibited H_2 production from butyrate by *Syntrophomonas wolfei* and from benzoate by *Syntrophus buswellii* (Wallrabenstein and Schink 1994). Similarly, H_2 formation from glycolate by membrane vesicles of *Syntrophobotulus glycolicus* (Friedrich et al. 1996) required ATP or a proton gradient (Friedrich and Schink 1993, 1995). While it is clear that reversed electron transport is needed for syntrophic metabolism, the nature of such a system has been elucidated only in few cases so far (Sect. 6).

How do syntrophic microbial associations operate at these low-energy conditions? Do they have novel mechanisms for energy conservation, or are they more efficient at conserving energy than other microorganisms? We will analyze what is known about syntrophic metabolism in an attempt to answer these questions. Further details on the physiology of the organisms capable of syntrophic metabolism are available in several comprehensive reviews (Schink 1997; Schink and Stams 2013; McInerney et al. 2008; Sieber et al. 2012; Schink et al. 2017).

4 Interspecies Electron Transfer

Above, we defined syntrophy based on the exchange of H_2 between the syntrophic partners. However, there are also other mechanisms to transfer electrons. Most hydrogenotrophic methanogens use either formate or H_2 or both simultaneously (Hedderich and Whitman 2006; Liu and Whitman 2008). There is very little difference in free energy change for methane production when H_2 versus formate serves as the electron donor (Table 1). The conclusion of many studies is that syntrophic metabolism can involve either interspecies transfer of H_2 and/or formate. Syntrophic metabolism by H_2 transfer was shown for glycolate metabolism by *Syntrophobotulus glycolicus* (Friedrich et al. 1996); sugar metabolism by *Syntrophococcus sucromutans* (Krumholz and Bryant 1986); acetate metabolism by a thermophilic, syntrophic acetate-oxidizing strain AOR (Lee and Zinder 1988b); and ethanol metabolism by the S-organism (Bryant et al. 1967), by culturing these organisms with a methanogen that uses only H_2. In a similar fashion, syntrophic formate transfer was shown for an amino acid degrader with a sulfate-reducing partner that uses formate but not H_2 (Zindel et al. 1988). Syntrophic propionate degradation by *Syntrophobacter fumaroxidans* (Dong et al. 1994b; Dong and Stams 1995) and syntrophic butyrate degradation by *Syntrophomonas* (*Syntrophospora) bryantii* (Dong et al. 1994a) occurred only with a methanogen that used both H_2 and formate, and not with a methanogen that used only H_2, implicating the need for formate metabolism. Proteomic and enzymatic analyses showed high levels of formate dehydrogenase in both *S. fumaroxidans* and its methanogenic partner, arguing for formate as an important electron carrier (de Bok et al. 2002a,b, 2003). Transcriptomics of gene coding for formate dehydrogenases (fdh) and hydrogenases (hyd) in a coculture of *Syntrophobacter fumaroxidans* and *Methanospirillum hungatei* revealed that all *fdh* and *hyd* genes were transcribed and transcription levels of the individual genes varied significantly depending on the substrate and

growth conditions (Worm et al. 2011). This shows that both syntrophic partners tightly regulate their interspecies metabolism to grow together. Flux analysis of this coculture (de Bok et al. 2002a) and of a butyrate-degrading coculture (Boone et al. 1989) indicated that H_2 diffusion was too slow to account for the observed rates of syntrophic propionate oxidation. Also, syntrophic acetate oxidation by *Thermacetogenium phaeum* appears to use both formate and H_2 as electron carriers (Hattori et al. 2001). The use of H_2 and/or formate as the interspecies electron carrier provides an explanation why so many methanogens use both H_2 and formate. Genomic analyses support the involvement of both compounds, as the genomes of *Syntrophus aciditrophicus*, *Syntrophomonas wolfei*, and *Methanospirillum hungatei*, the methanogenic partner most often observed in syntrophic associations, have multiple formate dehydrogenase and hydrogenase genes (McInerney et al. 2007; Sieber et al. 2008, 2012). More recent evidence has revealed that while hydrogenase and formate dehydrogenase activity is present during syntrophic growth in whole cell assays of both *S. wolfei* and *S. aciditrophicus*, *S. wolfei* relies on hydrogen transfer predominantly, while *S. aciditrophicus* can use both hydrogen and formate at the same time when growing with *M. hungatei* (Sieber et al. 2014).

Molecules other than H_2 or formate may be involved in interspecies electron transfer, such as humic compounds with anthraquinone disulfonate as a defined representative (Lovley et al. 1998), activated carbon or biochar (Liu et al. 2012; Chen et al. 2014) or various iron oxides (Kato et al. 2012a, b; Viggi et al. 2014; Zhou et al. 2014). An acetate-oxidizing coculture of *Geobacter sulfurreducens* and *Wolinella succinogenes* used cysteine as the interspecies electron carrier (Kaden et al. 2002). Nonetheless, one has to keep in mind that the transferred electrons have to reach the methanogenic partner at a redox potential low enough to provide it with a minimum amount of energy, i. e., at $E_0' =$ ca. -270 mV (Schink et al. 2017). Certain iron oxides such as goethite, hematite, or magnetite have redox potentials low enough to accomplish this (Straub et al. 2001); others such as ferrihydrite or humic compounds can act as efficient electron carriers only in reduction of more positive acceptors such as ferric iron, fumarate, nitrate, and the corresponding acceptor-reducing bacteria (Schink et al. 2017).

Syntrophic methanogenesis has been shown to be promoted by the presence of (semi)conductive mineral particles (Kato et al. 2012a, b; Kouzuma et al. 2015). Interspecies electron transfer via electric currents through magnetite particles was calculated to be an intrinsically faster mechanism compared to interspecies H_2 transfer (Viggi et al. 2014). It remains to be revealed what the underlying mechanisms are and what this means for syntrophic methanogenesis in nature where (semi) conductive minerals are abundant.

Another option is direct electron transfer between syntrophic partners by electron-conductive pili or nanowires (Reguera et al. 2005; Gorby et al. 2006) or by direct cell-to-cell transfer (Shrestha et al. 2013; Shresta and Rotaru 2014; Li et al. 2015). Interspecies electron transfer by nanowires is difficult to prove in syntrophic associations because we cannot mutate the pilus genes in either of the syntrophic partners at present. Nanowire-like structures connecting the syntrophic propionate degrader *Pelotomaculum thermopropionicum* with its methanogenic partner have been

observed by electron microscopy (Ishii et al. 2005; Gorby et al. 2006), and scanning tunneling microscopy showed that these structures were electron transmissive (Gorby et al. 2006). Electron transfer via direct cell contact or nanowires would require close spatial associations between the cooperating partners. Some researchers point to aggregation of cells in cocultures as proof of direct electron transfer (Logan and Regan 2006), but aggregation also reduces the distance between the syntrophic partners and would increase the rate of H_2 or formate transfer as well (Conrad et al. 1985; Thiele and Zeikus 1988; Ishii et al. 2005). Whether adhesins produced by certain methanogens may help to establish interactions with fermenting partners specifically for interspecies electron transfer (Ng et al. 2016) remains to be examined.

5 Biochemical Pathways for Syntrophic Metabolism

The pathways for several syntrophic metabolisms are known, and an analysis of the bioenergetics of these pathways illustrates how small amounts of energy are conserved during syntrophic metabolism (Schink 1997).

5.1 Acetate Metabolism

Syntrophic acetate metabolism is a remarkable process that supports the concept that syntrophic metabolism is very energy efficient. A thermophilic organism, strain AOR, was found to produce acetate when grown axenically with H_2 and CO_2 and oxidizes acetate when grown syntrophically (Lee and Zinder 1988a; Hattori et al. 2000). Since then two thermophilic, *Pseudothermotoga lettingae* and *Thermacetogenium phaeum* (Hattori et al. 2000; Balk et al. 2002), and three mesophilic, *Tepidanaerobacter acetatoxydans*, *Clostridium ultunense*, and *Syntrophaceticus schinkii*, syntrophic acetate-oxidizing bacteria have been characterized (Schnürer et al. 1997; Westerholm et al. 2010, 2011). The known species are phylogenetically diverse but poorly understood as strict cultivation requirements, slow growth, and difficulties in reconstituting the thermodynamically unfavorable syntrophic acetate metabolism under laboratory conditions prevent the thorough investigation of their metabolism. Enzyme activity studies using crude cell extract and genome analysis indicate the involvement of the reversed Wood Ljungdahl (WL) pathway in syntrophic acetate oxidation for *T. phaeum*, *C. ultunense*, and *S. schinkii* (Oehler et al. 2012; Manzoor et al. 2016) (Fig. 1). The genome of *T. phaeum* encodes all enzymes of the WL pathway, and most of the WL enzymes were encoded only once, indicative of bidirectional catalysis. Although electron transfer mechanisms involved in acetate oxidation by *T. phaeum* still remain unknown, they might be similar or even the same as those used for acetate synthesis. In this context, the enzymes formate dehydrogenase, CO dehydrogenase, methylene-THF reductase, and hydrogenase are of special relevance as these may contribute to energy conservation in both directions.

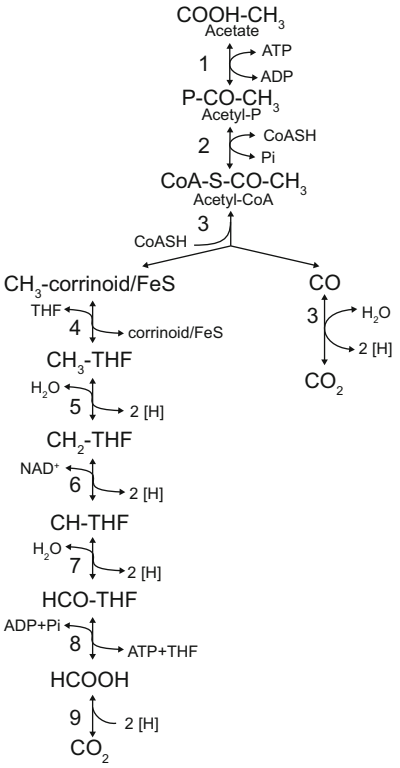

Fig. 1 Pathway for acetate oxidation and synthesis in syntrophic acetate oxidizers, adapted from Hattori (2008). The enzymes involved are as follows: 1, acetate kinase; 2, phosphotransacetylase; 3, carbon monoxide dehydrogenase; 4, methyltransferase; 5, methylene-THF reductase; 6, methylene-THF dehydrogenase; 7, methenyl-THF cyclohydrolase; 8, formyl-THF synthetase; 9, formate dehydrogenase. THF is tetrahydrofolate and [H] is reducing equivalents

The working draft genome of *Syntrophaceticus schinkii* indicates limited metabolic capacities with the lack of organic nutrient uptake systems, chemotactic machineries, carbon catabolite repression, and incomplete biosynthesis pathways (Manzoor et al. 2016). During syntrophic growth, Ech hydrogenase, [FeFe] hydrogenases, [NiFe] hydrogenases, F_1F_0-ATP synthase, and membrane-bound and cytoplasmic formate dehydrogenases were highly expressed, whereas Rnf and a predicted oxidoreductase/heterodisulfide reductase complex, both encoded in the genome, were not expressed. Remarkably, a transporter sharing similarities to the high-affinity acetate transporters of acetotrophic methanogens was also found to be expressed, suggesting that *S. schinkii* can potentially compete with methanogens for acetate. *S. schinkii* appears to be a niche-adapted microorganism specialized in, and consequently reliant on, syntrophic acetate oxidation. Its large set of respiratory complexes might contribute to overcome limiting bioenergetic barriers and drives efficient energy conservation from reactions operating close to the thermodynamic equilibrium, which might enable *S. schinkii* to occupy the same niche as the acetotrophic methanogens.

The genome of *T. acetatoxydans* lacks genes encoding formate dehydrogenase and the F_1F_0-ATP synthase. The WL pathway is organized into one operon but lacks formate dehydrogenase. The lack of an F_1F_0-ATP synthase may have implications

for harvesting the ATP coming from substrate-level phosphorylation during formyl-THF synthase activity and using it for acetate activation (Müller et al. 2015) As the genome encodes for all genes to perform an oxidative TCA cycle, in analogy with *Desulfobacter postgatei*, this pathway is postulated for acetate oxidation in *T. acetatoxydans* rather than the WL pathway (Möller et al. 1987). An alternative pathway, which bypasses the carbonyl branch of the WL pathway by combining the glycine cleavage system with the methyl branch of the WL pathway, has been hypothesized for a terephthalate-degrading *Mesotoga* community, which is dominated by the thermophilic syntrophic acetate oxidizer, *Pseudothermotoga lettingae* (Nobu et al. 2015). As *P. lettingae* does not contain genes for acetyl-CoA synthase/carbon monoxide dehydrogenase, the WL pathway is not encoded with a complete set of genes (Hattori 2008) and such a glycine shunt may be operational in *P. lettingae*.

Other organisms capable of syntrophic acetate metabolism include *Geobacter sulfurreducens* (Cord-Ruwisch et al. 1998), the haloalkaline "*Candidatus* Contubernalis alkalaceticum," and "Ca. Syntrophonatronum acetioxidans" (Zhilina et al. 2005; Sorokin et al. 2014). *G. sulfurreducens* metabolizes acetate through the citric acid cycle (Galushko and Schink 2000).

5.2 Propionate Metabolism

Two pathways for propionate metabolism are known, the methylmalonyl-CoA pathway and a dismutation pathway (Fig. 2). The methylmalonyl-CoA pathway is found in many syntrophic propionate oxidizers including *Syntrophobacter* species (Boone and Bryant 1980; Wallrabenstein et al. 1995; Harmsen et al. 1998; Chen et al. 2005), *Desulfotomaculum thermobenzoicum* subsp. *thermosyntrophicum* (Plugge et al. 2002), *Pelotomaculum thermopropionicum* (Imachi et al. 2002), and *Pelotomaculum schinkii* (de Bok et al. 2005). The dismutation pathway has been detected only in *Smithella propionica* (Liu et al. 1999; de Bok et al. 2001). *S. propionica* produces acetate and butyrate from propionate (de Bok et al. 2001). To explain the unusual labeling patterns observed in acetate and butyrate when different position-labeled propionate compounds were used, de Bok et al. (2001) concluded that two propionate molecules must condense to form a six-carbon intermediate, which is then rearranged to a 3-ketohexanoic acid intermediate before it is cleaved to form butyrate and acetate (Fig. 2a). The enzymes involved in these reactions are not yet known.

The methylmalonyl-CoA pathway (Fig. 2b), also called the randomizing pathway, involves the activation of propionate to propionyl-CoA by transfer of a CoA group from acetyl-CoA and the synthesis of methylmalonyl-CoA by transfer of a carboxyl group from oxaloacetate by a transcarboxylase (Houwen et al. 1990; Plugge et al. 2012). Methylmalonyl-CoA is then rearranged to form succinyl-CoA, which is oxidized via fumarate, oxaloacetate, and pyruvate to acetate. The pathway predicts that one ATP is generated by substrate-level phosphorylation per propionate degraded, and three electron pairs are released. Genomic and proteomic analyses

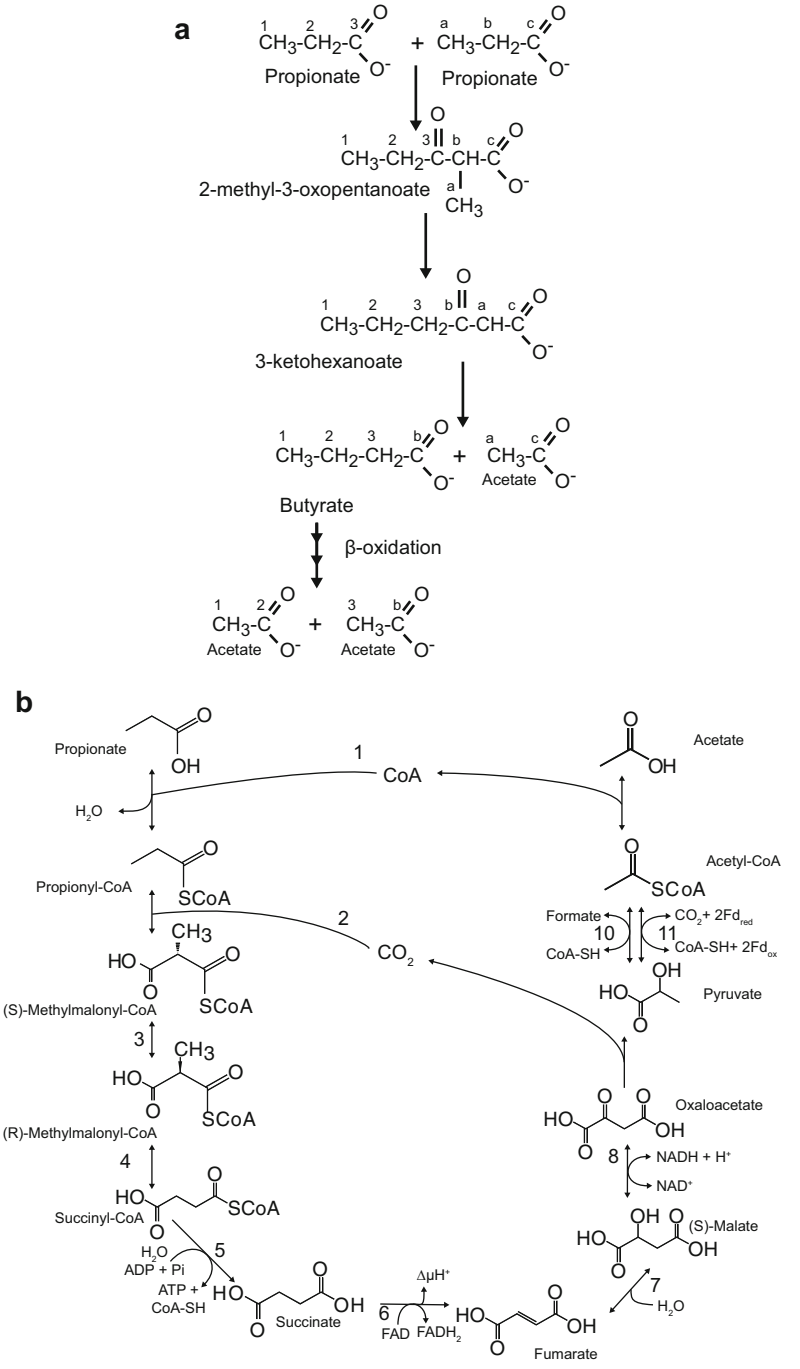

Fig. 2 (continued)

show that the methylmalonyl-CoA pathway is operative in *Syntrophobacter fumaroxidans* and *P. thermopropionicum* (Plugge et al. 2012; Kosaka et al. 2006). The production of H_2 or formate from electrons derived from the oxidation of succinate is energetically unfavorable. Succinate reduced cytochrome *b* in membranes of *S. fumaroxidans*, and 2-(heptyl)-4-hydroxyquinoline-*N*-oxide inhibited succinate oxidation, suggesting the involvement of a reversed electron transport (Van Kuijk et al. 1998) (Fig. 5c). Both *S. fumaroxidans* and *P. thermopropionicum* have a membrane-bound succinate dehydrogenase/fumarate reductase (Sdh1ABC) with Sdh1C as the heme-containing, transmembrane protein that interacts with menaquinone (Kosaka et al. 2006, 2008; Plugge et al. 2012). The binding of menaquinone to Sdh1C close to the outside of the cell membrane allows an inward movement of protons when menaquinone is oxidized on the cytoplasmic side of the membrane by membrane-bound formate dehydrogenases or hydrogenases (Müller et al. 2010; Plugge et al. 2012; Worm et al. 2014).

Electron confurcation, coupling the oxidation of reduced ferredoxin and NADH to make either H_2 or formate, has been proposed to explain H_2 or formate production from NADH (Müller et al. 2010; Sieber et al. 2012; Worm et al. 2014). This electron confurcation would allow continued substrate metabolism when H_2 and formate levels increase to a point where their production from NADH becomes unfavorable without energy input. All the genes for the major subunits of the hydrogenases and FDHs in *Syntrophobacter fumaroxidans* were expressed during growth in coculture and in pure culture, irrespective of the substrate (Worm et al. 2011). Significantly higher expression of the confurcating hydrogenase, a periplasmic FDH, and the hydrogen-formate lyase was observed during syntrophic growth versus axenic growth (details in Sect. 6).

Reducing equivalents generated in cytosolic reactions, such as the oxidation of malate to oxaloacetate and pyruvate to acetyl-CoA and CO_2, probably produce NAD(P)H and reduced ferredoxin, respectively. Several soluble cytosolic hydrogenases (Sfum_0844-46) and formate dehydrogenases (Sfum_2703-07) then probably catalyze H_2 or formate production with the above-reduced electron carriers via confurcation. The energetically favorable production of H_2 or formate with reduced ferredoxin can presumably provide the energetic input to enable the energetically unfavorable formation of H_2 from NADH. Malate oxidation to oxaloacetate ($E^{\circ\prime} = -176$ mV) is coupled to NAD^+ reduction ($E^{\circ\prime} = -320$ mV) (van Kuijk and Stams 1996).

Fig. 2 Two pathways for syntrophic propionate metabolism. (**a**) The pathway for the metabolism of propionate by *Smithella propionica*. The carbons in each original propionate are labeled. The enzymes involved in this pathway have yet to be described and CoA esters of the compounds shown may be involved. This figure was adapted from de Bok et al. (2001). (**b**) The methylmalonyl-CoA pathway for propionate metabolism, found in *P. thermopropionicum*, adapted from Kosaka et al. (2006). The enzymes involved are as follows: 1, propionate CoA transferase; 2, propionyl-CoA/oxaloacetate transcarboxylase; 3, methylmalonyl-CoA epimerase; 4, methylmalonyl-CoA mutase; 5, succinyl-CoA synthetase; 6, succinate dehydrogenase/fumarate reductase; 7, fumarate hydratase; 8, malate dehydrogenase; 9, pyruvate dehydrogenase; 10, pyruvate: formate lyase; 11, acetyl-CoA synthase; and 12, acetate kinase. Fd is ferredoxin and [H] is reducing equivalents

Molar growth yields indicate that *S. fumaroxidans* synthesizes two-thirds of an ATP per fumarate when H_2 is the electron donor (Van Kuijk et al. 1998). This observation suggests that *S. fumaroxidans* consumes two-thirds of an ATP to drive H_2 production from succinate when grown syntrophically with propionate; this leaves about one-third of an ATP available to support growth. The free energy change needed for irreversible ATP synthesis is estimated to be about -70 kJ mol^{-1} (Schink 1997). If three to five protons are used to make ATP by the ATP synthase, then the minimum free energy change needed to form ATP in increments is -23 to -14 kJ mol^{-1} (Schink 1997). This analysis predicts that syntrophic propionate metabolism should have a free energy change of about -20 kJ mol^{-1} to allow for the net synthesis of one-third of an ATP. Measured free energy changes during syntrophic propionate metabolism by *S. fumaroxidans* lower than -30 kJ mol^{-1} have been observed (Scholten and Conrad 2000), which is in agreement with the energetic model. However, under some growth conditions, the free energy available from syntrophic propionate metabolism was <-10 kJ mol^{-1}. Thus, we do not yet fully understand the bioenergetics of syntrophic propionate metabolism.

5.3 Butyrate Metabolism

Organisms capable of syntrophic butyrate metabolism include all species of *Syntrophomonas* (McInerney et al. 1981; Lorowitz et al. 1989; Zhang et al. 2004, 2005; Sobieraj and Boone 2006; Wu et al. 2006a, b, 2007; Sousa et al. 2007), *Syntrophus aciditrophicus* (Jackson et al. 1999), *Thermosyntropha lipolytica* (Svetlitshnyi et al. 1996), and *Syntrophothermus lipocalidus* (Sekiguchi et al. 2000). The most intensively studied model organism representing butyrate-oxidizing bacteria is *Syntrophomonas wolfei*, which is specialized on syntrophic degradation of four to eight carbon fatty acids but can also grow axenically with several unsaturated fatty acids, especially crotonate (McInerney et al. 1981; Sieber et al. 2010). No other growth-supporting substrates are known. Syntrophic butyrate metabolism proceeds via the β-oxidation pathway (Fig. 3) (Wofford et al. 1986). Similar to syntrophic propionate metabolism, butyrate is activated to butyryl-CoA by the transfer of the CoA group from acetyl-CoA; butyryl-CoA is then β-oxidized to two acetyl-CoA molecules (Wofford et al. 1986). One of the acetyl-CoA molecules is used to activate butyrate, and the other one is used for ATP synthesis. The oxidation of butyryl-CoA to crotonyl-CoA produces reduced electron transfer flavoprotein ($E^{o\prime}$ of -10 mV) (Sato et al. 1999) (Fig. 3), and the oxidation of L-3-hydroxylbutyryl-CoA to 3-oxobutyryl-CoA produces NADH. H_2 production (E' of -292 mV at 10 Pa H_2) from electrons derived from NADH ($E^{o\prime}$ of -320 mV) (Thauer et al. 1977) is favorable at the partial pressures maintained by methanogens (about 1–10 Pa) and occurs through an NADH-dependent hydrogenase or NADH-dependent formate dehydrogenase, depending on the cultivation conditions (Sieber et al. 2010, 2014; Schmidt set al. 2013) (Fig. 3). However, H_2 production with electrons derived from the oxidation of butyryl-CoA to crotonyl-CoA requires reversed electron transport (Wallrabenstein and Schink 1994). Reversed electron transport is fueled by a transmembrane proton

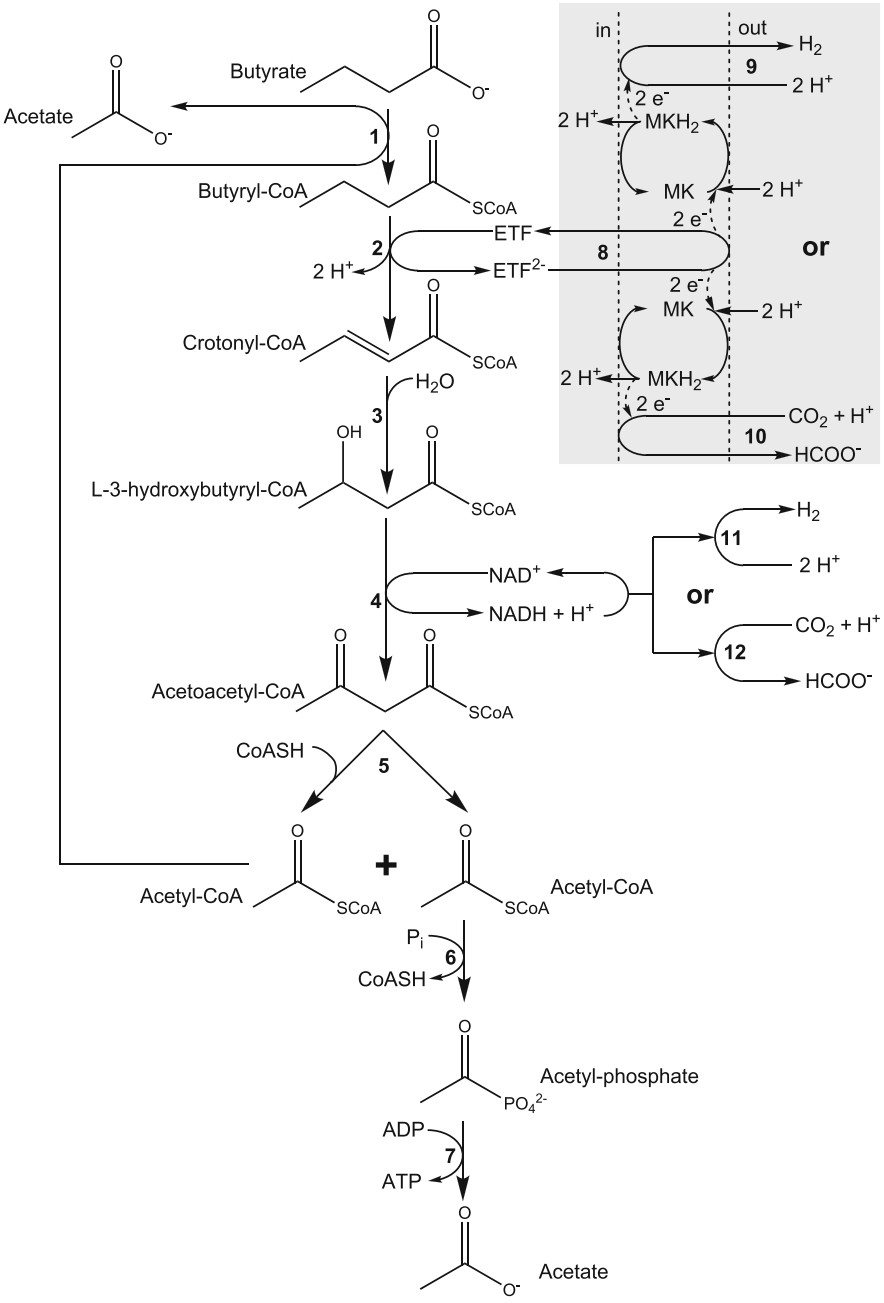

Fig. 3 The β-oxidation pathway for butyrate metabolism in *Syntrophomonas wolfei*, adapted from Wofford et al. (1986), Sieber et al. (2010), Schmidt et al. (2013), and Crable et al. (2016). The enzymes involved are as follows: 1, CoA transferase; 2, acyl-CoA dehydrogenase; 3, enoyl-CoA hydratase; 4, L-(+)-3-hydroxybutyryl-CoA dehydrogenase; 5, 3-ketoacyl-CoA thiolase;

potential generated by ATPase. A membrane-bound iron-sulfur oxidoreductase redox-linked to a membrane-bound hydrogenase or formate dehydrogenase forms a redox loop system via menaquinone (Crable et al. 2016; Schmidt et al. 2013). First, butyryl-CoA is oxidized to crotonyl-CoA, and two electrons are transferred to an electron-transferring flavoprotein (ETF), while two protons are being released into the cytoplasm. Reduced ETF is reoxidized at the membrane by an iron-sulfur oxidoreductase. Two protons from the periplasmic space drive the reduction of menaquinone to menaquinol with two electrons from butyryl-CoA oxidation located on the membrane-bound iron-sulfur oxidoreductase (Fig. 3). Menaquinol can be oxidized by either a membrane-bound hydrogenase or formate dehydrogenase to release H_2 or formate, most likely depending on the cultivation conditions (Müller et al. 2009; Schmidt et al. 2013; Sieber et al. 2014, 2015; Crable et al. 2016) or the available partner organism. Production of H_2 or formate consumes two protons from the exoplasmic space, respectively, which counterbalances the two protons released in the cytoplasm during oxidation of butyryl-CoA (Fig. 3). During reoxidation of menaquinol, two protons are released into the cytoplasm; thus the net amount of protons that need to be transported across the membrane per mol of butyryl-CoA oxidized amounts to two protons. This corresponds to about two-thirds of an ATP equivalent, which is needed to overcome this energy barrier, therefore leaving about one-third of an ATP available to support growth. The measured free energy changes available during syntrophic butyrate metabolism ranged from -5 to -17 kJ mol^{-1} (Dwyer et al. 1988; Jackson and McInerney 2002), somewhat lower than that predicted to be needed for ATP synthesis (see Sect. 3).

5.4 Benzoate Metabolism

Syntrophic benzoate degraders include three species of *Syntrophus*: *S. buswellii*, *S. gentianae*, and *S. aciditrophicus*, as well as *Sporotomaculum syntrophicum*, *Pelotomaculum terephthalicicum*, *Pelotomaculum isophthalicicum*, *and Syntrophorhabdus aromaticivorans* (McInerney et al. 2008; Nobu et al. 2014). The reduction of benzoyl-CoA represents a considerable energy barrier for anaerobic microorganisms because the midpoint potential of the first electron transfer is about -1.8 V (Heider and Fuchs 1997a; Boll and Fuchs 1998; Boll et al. 2000), which is well below that of any physiological electron donors (-0.4 V) (Boll and Fuchs 1998). In *Thauera aromatica*, benzoyl-CoA reduction requires the hydrolysis of two ATP molecules per electron pair to overcome this barrier (Boll et al. 1997). This ATP-dependent enzyme system is not found in the genomes of *S. aciditrophicus*

Fig. 3 (continued) 6, phosphotransacetylase; 7, acetate kinase; ETF, electron transport flavoprotein; ETF^{2-}, reduced form of ETF. The gray inset shows the membrane-bound reverse electron transport system: 8, iron-sulfur oxidoreductase; 9, hydrogenase; 10, formate dehydrogenase; MK, menaquinone, MKH_2, menaquinol. NADH reoxidation systems: 11, NADH-dependent hydrogenase; 12, NADH-dependent formate dehydrogenase

(McInerney et al. 2007) or *S. aromaticivorans* (Nobu et al. 2014). Both appear to employ an ATP-independent, type II benzoyl-CoA reductase similar to the tungsten-dependent benzoyl-CoA reductase, BamBC, found in *Geobacter metallireducens* (Kung et al. 2009; Wischgoll et al. 2005).

Previous studies detected 2-hydroxycyclohexane carboxylate, cyclohex-1-ene carboxylate, and pimelate in culture fluids of *S. aciditrophicus* grown with benzoate and the enzyme activities needed to convert cyclohex-1-ene carboxyl-CoA to pimelyl-CoA in cell-free extracts of *S. aciditrophicus* (Elshahed et al. 2001). The intermediates and enzyme activities detected were consistent with the metabolism of cyclohex-1-ene carboxyl-CoA to pimelyl-CoA by the pathway found in *Rhodopseudomonas palustris* (Harwood et al. 1998). However, genes homologous to those involved in benzoate metabolism in *R. palustris* were not detected in the *S. aciditrophicus* genome (McInerney et al. 2007). Interestingly, the genome of *S. aciditrophicus* contains genes with homology to those of the benzoyl-CoA degradation pathway found in *G. metallireducens* (Fig. 4) (McInerney et al. 2007). The genes for the cyclohex-1,5-diene carboxyl-CoA hydratase and the 6-oxocyclohex-1-ene carboxyl-CoA hydrolase of *S. aciditrophicus* have been cloned and expressed in *Escherichia coli* (Peters et al. 2007; Kuntze et al. 2008). Enzymatic analysis showed that the S. *aciditrophicus* cyclohex-1,5-diene carboxyl-CoA hydratase converts cyclohex-1,5-diene carboxyl-CoA to 6-hydroxycyclohex-1-ene carboxyl-CoA and that the *S. aciditrophicus* 6-oxocyclohex-1-ene carboxyl-CoA hydrolase makes 3-hydroxypimelyl-CoA from 6-oxocyclohex-1-ene carboxyl-CoA. 3-Fluorobenzoate-degrading cultures of *S. aciditrophicus* produced a metabolite with two double bonds, either 1-carboxyl-3-fluoro-2,6-cyclohexadiene or 1-carboxyl-3-fluoro-3,6-cyclohexadiene, consistent with an initial two-electron reduction of the benzoyl-CoA ring (Mouttaki et al. 2009). Thus, it appears that *S. aciditrophicus* uses a two-electron reduction reaction to convert benzoyl-CoA to cyclohex-1,5-diene carboxyl-CoA and the benzoyl-CoA degradation pathway as found in *G. metallireducens* (Wischgoll et al. 2005) to degrade benzoyl-CoA to acetyl-CoA (Fig. 4).

Cyclohexane carboxylate accumulates during syntrophic benzoate metabolism (Elshahed et al. 2001). Cyclohexane carboxylate and benzoate formation were observed when *S. aciditrophicus* was grown with crotonate (Mouttaki et al. 2007). Intermediates detected during crotonate metabolism were the same as those detected during syntrophic benzoate metabolism, which suggests that the pathway for benzoate metabolism is reversible. Cyclohexane carboxylate can be both utilized as a substrate and produced as a fermentation end product of crotonate or benzoate metabolism by *S. aciditrophicus* (Kung et al. 2013, 2014; Mouttaki et al. 2008). Cyclohexane carboxyl-CoA is converted to cyclohex-1,5-diene carboxyl-CoA by two consecutive redox reactions catalyzed by two different acyl-CoA dehydrogenases (Kung et al. 2013) (Fig. 4).

S. aciditrophicus utilizes a unique mechanism for substrate-level phosphorylation, an AMP-forming, acetyl-CoA synthetase (Acs), to form ATP from acetyl-CoA, AMP, and pyrophosphate rather than by phosphotransacetylase and acetate kinase that is used by almost all bacteria (James et al. 2016). Pyrophosphate needed for the Acs reaction can be made by ligase reactions involved in substrate activation

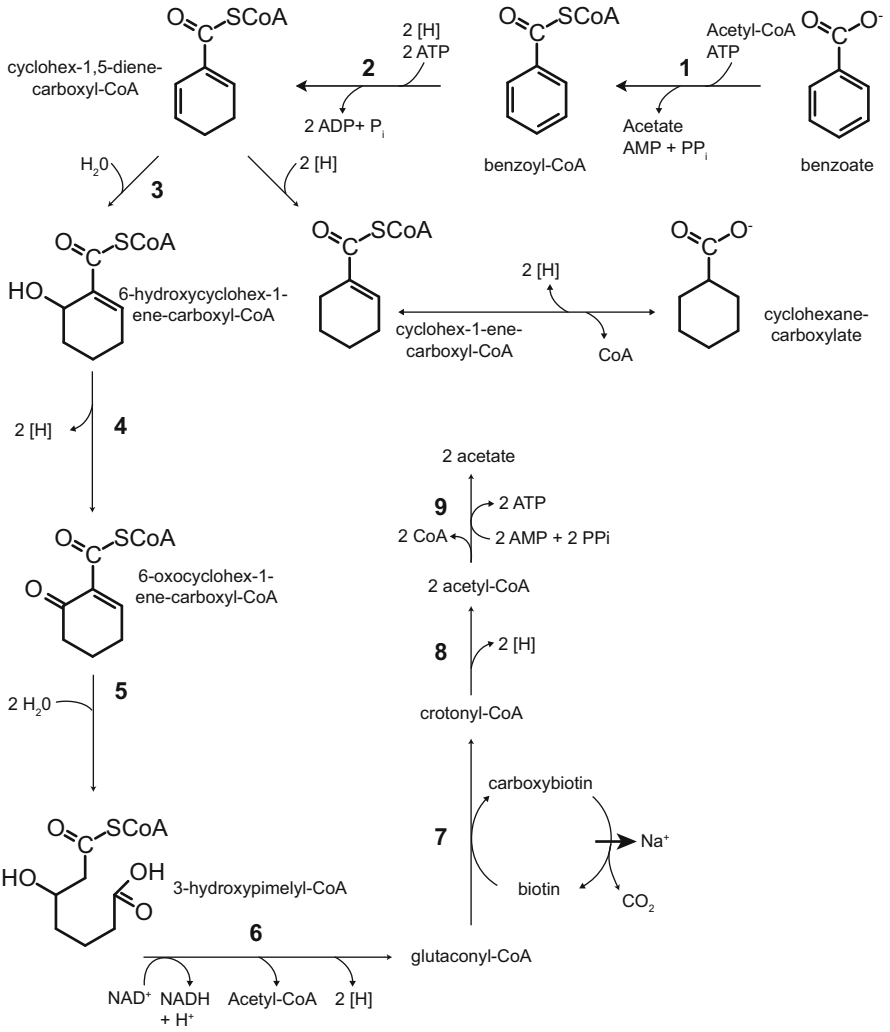

Fig. 4 Pathway for syntrophic benzoate metabolism adapted from McInerney et al. (2007). The enzymes involved are: 1, benzoyl-CoA ligase; 2, benzoyl-CoA reductase; 3, cyclohex-1,5-dienoyl-CoA hydratase; 4, 6-hydrocyclohex-1-ene-carboxyl-CoA dehydrogenase; 5, 6-oxocyclohex-1-ene-carboxyl-CoA hydrolase; 6, β-oxidation enzymes; 7, glutaconyl-CoA decarboxylase; 8, β-oxidation enzymes (see Fig. 3 for more detail); 9, AMP-forming, acetyl-CoA synthetase. [H] are reducing equivalents

(Elshahed et al. 2001; Schöcke and Schink 1998) and by membrane-bound pyrophosphatases (Schöcke and Schink 1998). The decarboxylation of glutaconyl-CoA by a sodium-linked membrane-bound decarboxylase (Beatrix et al. 1990; Schöcke and Schink 1998) would provide chemiosmotic energy needed for pyrophosphate synthesis. The reduction of benzoyl-CoA reduction requires a

low-potential electron donor such as reduced ferredoxin. One mechanism for the production of reduced ferredoxin in *S. aciditrophicus* is by the membrane-bound ion pump called Rnf, which uses the chemiosmotic gradient to drive the unfavorable reduction of ferredoxin with electrons derived from NADH oxidation (Fig. 5) (McInerney et al. 2007). Further work is needed on the energetics of ion translocation, pyrophosphate synthesis, H_2 and formate production, and benzoyl-CoA reduction to understand how net ATP synthesis occurs during syntrophic benzoate metabolism. The measured free energy changes during syntrophic benzoate metabolism range from about −30 to −45 kJ of energy (Warikoo et al. 1996; Schöcke and Schink 1997), which suggest that about one-third of an ATP or more could be formed per benzoate.

6 Mechanisms for Reverse Electron Transport

The oxidation of acyl-CoA intermediates ($E^{o\prime}$ of −10 mV) derived from syntrophic fatty acid and aromatic compound degradation coupled to H_2 or formate production (E' of about −261 to −260 mV at 1 Pa H_2 and −258 at 1 μM formate) is unfavorable ($\Delta E'$ of about −250 mV) (Schink 1997). Energy input in the form of reversed electron transport is needed to make this reaction favorable (Sieber et al. 2012; Schink 1997). The current model to explain reversed electron transport during syntrophic butyrate degradation is a quinone loop (Schink and Friedrich 1994) (Fig. 5a). Genomic analysis detected a gene for a membrane-bound iron-sulfur oxidoreductase with a DUF224 domain adjacent to genes for electron transfer flavoprotein (*etfAB*) (Sieber et al. 2012). During beta-oxidation, etfAB transfers electrons from acyl-CoA dehydrogenases to a membrane-bound electron transfer flavoprotein/menaquinone oxidoreductase. The co-localization of *etfA* and *etfB* with the gene for the membrane-bound iron-sulfur oxidoreductase suggests that the membrane oxidoreductase may serve as an EtfAB/menaquinone oxidoreductase to receive electrons from acyl-CoA dehydrogenases via EtfAB and subsequently reduce menaquinone to menaquinol (Crable et al. 2016; Müller et al. 2010; Narihiro et al. 2016; Schmidt et al. 2013; Sieber et al. 2010; Worm et al. 2014). Menaquinol can be reoxidized by either a membrane-bound hydrogenase (Crable et al. 2016; Sieber et al. 2014) or a membrane-bound formate dehydrogenase (Schmidt et al. 2013) depending upon the growth condition. The inward movement of protons by the quinone loop along with the consumption of protons on the outside of the membrane during H_2 or formate production would supply the chemiosmotic energy needed for reversed electron transport (Crable et al. 2016; Schmidt et al. 2013; Sieber et al. 2010).

In support of this model, electron transfer flavoprotein (EftAB2) and the membrane-bound iron-sulfur oxidoreductase (SWOL_RS03525 gene product) with a DUF224 domain were highly abundant in the *S. wolfei* proteome, suggesting that these enzymes provide a conduit for electron flow between acyl-CoA dehydrogenases and membrane redox carriers (Crable et al. 2016; Schmidt et al. 2013; Sieber et al. 2015). The SWOL_RS03525 gene product was detected in highly purified

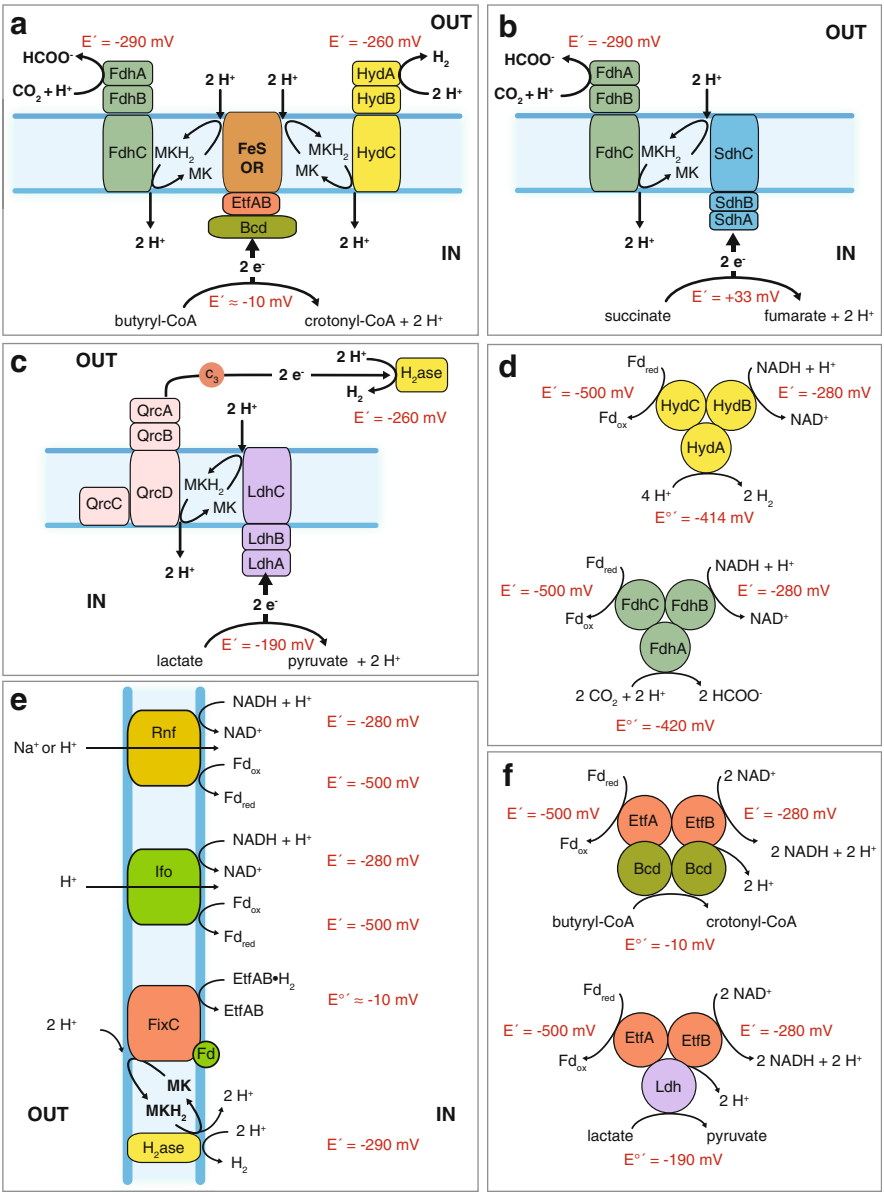

Fig. 5 Proposed models for reversed electron transfer. (**a**) syntrophic butyrate metabolism, (**b**) syntrophic propionate metabolism, (**c**) syntrophic lactate metabolism, (**d**) confurcating hydrogenases and formate dehydrogenases, (**e**) ion pumps, and (**f**) confurcating dehydrogenases. Redox values under physiological conditions are from Buckel and Thauer (2013) with H_2 at 10 Pa and formate at 10 μM. Abbreviations: *Bcd* butyryl-CoA dehydrogenase, *Etf* electron transfer flavoprotein, *FeS ox* iron-sulfur oxidoreductase with a DUF224 domain, *Fd* ferredoxin, *Fdh* formate dehydrogenase, *Hyd* hydrogenase, *Ifo* ion-translocating ferredoxin oxidoreductase, *Ldh* lactate dehydrogenase, *Hase* hydrogenase, *MK* menaquinone, *Rnf* ion-translocating NADH/ferredoxin oxidoreductase, *Sdh* succinate dehydrogenase, *in* cytoplasm, *out* outside the cytoplasmic membrane

preparations of butyryl-CoA dehydrogenase (Bcd) (Müller et al. 2009), consistent with a close interaction between the SWOL_RS03525 gene product and Bcd. Peptides of a membrane-bound formate dehydrogenase (Fdh2) (Schmidt et al. 2013) and transcripts of genes for a membrane-bound hydrogenase (*hyd2A*) (Sieber et al. 2014) were high in syntrophically grown *S. wolfei* cells.

The oxidation of succinate to fumarate ($E^{\circ\prime}$ of +33 mV) coupled to H_2 or formate production during syntrophic propionate degradation is also an unfavorable reaction that involves reversed electron transport (Müller et al. 2010; Sieber et al. 2012) (Fig. 5b). *S. fumaroxidans* and *P. thermopropionicum* both have a membrane-bound succinate dehydrogenase/fumarate reductase (Sdh1ABC) with Sdh1C being the heme-containing, transmembrane protein that interacts with menaquinone (Kosaka et al. 2006, 2008; Plugge et al. 2012). The binding of menaquinone to Sdh1C close to the outside of the cell membrane would allow the inward movement of protons when menaquinone is oxidized on the cytoplasmic side of the membrane by membrane-bound formate dehydrogenases or hydrogenases (Müller et al. 2010; Plugge et al. 2012; Worm et al. 2014). The genes for Sdh1 were highly expressed in propionate-grown cells of *P. thermopropionicum* (Kato et al. 2009), and Sdh1 was more abundant when *P. thermopropionicum* was grown in coculture on propionate than after growth on butanol (Kosaka et al. 2006). The genes for a periplasmic formate dehydrogenase (*fdh2*) were upregulated during syntrophic propionate growth of *P. thermopropionicum* and of *S. fumaroxidans* (Kato et al. 2009; Worm et al. 2011), consistent with the importance of interspecies formate transfer during syntrophic propionate growth (de Bok et al. 2002a).

The unfavorable production of H_2 or formate from electrons derived from syntrophic lactate oxidation ($E^{o\prime}$ of −190 mV) is also driven by a quinone loop mechanism (Fig. 5c) (Sieber et al. 2012). Transposon mutagenesis showed that a quinone-reducing complex (Qrc) and a periplasmic, tetraheme cytochrome c_3 were required for syntrophic growth of *Desulfovibrio desulfuricans* G20 on lactate (Li et al. 2011). A mutation in a periplasmic hydrogenase, *hydA*, impaired syntrophic growth of *D. desulfuricans* G20 on lactate.

Electron confurcation, which couples the oxidation of reduced ferredoxin and NADH to produce either H_2 or formate, has been proposed to explain H_2 or formate production from NADH (Müller et al. 2010; Sieber et al. 2012; Worm et al. 2014) (Fig. 5d). However, it is not clear that electron confurcation is needed for H_2 and formate production during syntrophic metabolism. The redox potentials for H_2 and formate production during syntrophic metabolism (E' of about −260 mV at 1 Pa H_2 and −258 at 1 μM formate) are close to the physiological redox potential of NADH oxidation (E' of −280 mV) (Buckel and Thauer 2013). However, electron confurcation would allow continued substrate metabolism when H_2 and formate levels increase to a point where their production from NADH becomes unfavorable. The genomes of a number of syntrophic metabolizers have NADH-linked hydrogenases and formate dehydrogenases (Sieber et al. 2012; Narihiro et al. 2016; Worm et al. 2014) that have high homology to known confurcating hydrogenases (Schut and Adams 2009) and formate dehydrogenases (Wang et al. 2013). A gene for NADH-linked confurcating hydrogenase was highly expressed, and the hydrogenase was abundant in the proteome when *S. wolfei* was grown syntrophically on butyrate

(Sieber et al. 2014, 2015). Another study (Schmidt et al. 2013) found both a NADH-linked formate dehydrogenase and a NADH-linked hydrogenase abundant in S. *wolfei*. Both *S. fumaroxidans* and *P. thermopropionicum* expressed genes for NADH-linked hydrogenases and formate dehydrogenases during syntrophic growth on propionate (Kato et al. 2009; Worm et al. 2011), and the genes for one NADH-linked formate dehydrogenase were upregulated in *P. thermopropionicum* during syntrophic growth on propionate (Kato et al. 2009).

Reduced ferredoxin needed to drive confurcation at high H_2 or formate concentrations can be made during syntrophic propionate and lactate metabolism by oxidizing pyruvate arising during the degradation of the growth substrate. It is not clear how other syntrophic fatty and aromatic acid degraders make reduced ferredoxin as the benzoyl-CoA degradation and beta-oxidation pathways form NADH and $EtfABH_2$ rather than reduced ferredoxin. One possibility is the use of ion pumps to produce reduced ferredoxin from electrons derived from NADH oxidation (Fig. 5e). Rnf is found in the genomes of many syntrophic metabolizers (Sieber et al. 2012; Worm et al. 2014) and uses the inward movement of sodium ions or protons to produce reduced ferredoxin from electrons derived from NADH. Another ion pump, Ifo, is believed to function in a similar manner (Nobu et al. 2014). Fix is another membrane complex that catalyzes reversed electron transport and is believed to use the chemiosmotic gradient to drive the unfavorable reduction of menaquinone with electrons derived from the oxidation of $EtfABH_2$ (Sieber et al. 2010, 2012). Peptides of the Fix system were in low abundance in the *S. wolfei* proteome, suggesting a biosynthetic role for Fix rather than serving as the main conduit of electrons derived from acyl-CoA oxidation (Sieber et al. 2015). Reduced ferredoxin made by ion pumps or by pyruvate metabolism could be used to drive H_2 and formate production from NADH when H_2 and formate concentrations increase. In addition, reduced ferredoxin could be used to drive the unfavorable reduction of NAD^+ (E' of -280 mV) with electrons derived from the oxidation of acyl-CoA intermediates (E_o' of -10 mV) or lactate ($E^{o\prime}$ of -190 mV) by confurcating butyryl-CoA dehydrogenases (Li et al. 2008) or lactate dehydrogenases (Weghoff et al. 2015) (Fig. 5f). However, experimental evidence suggests that the butyryl-CoA dehydrogenases in *S. wolfei* may not be confurcating (Müller et al. 2009). Whether other syntrophic metabolizers contain confurcating dehydrogenases remains to be shown.

7 Research Needs

The concept of a minimum free energy change for energy conservation provides the framework to understand how bacteria exploit small free energy changes. Pathways for syntrophic metabolism of fatty acids predict that ATP can be synthesized at increments of about one-third of an ATP, which is consistent with the measured free energy changes observed for the syntrophic metabolism of these compounds.

However, there is still much that we do not understand about how microorganisms exploit small free energy changes. The value for the minimum energy quantum depends on the proton or sodium stoichiometry of the ATP synthase, the membrane

potential, and the change in the free energy needed to make ATP. Our current understanding of the minimum energy quantum was developed with information from bacteria that use very exergonic catabolic reactions. Syntrophic metabolizers may have ATP synthases with different ion-to-ATP stoichiometries and maintain different membrane potentials and free energies of phosphorylation than other bacteria. Also, we need to understand the metabolome of syntrophic metabolizers to determine how the concentrations of reactants and products affect the equilibrium of key reactions involved in syntrophy. For example, the necessity of confurcation reactions in syntrophic metabolism could be determined by comparison of NADH/NAD^+ ratios relative to H_2 and formate levels. The ratios of enoyl-CoA to acyl-CoA intermediates and 3-hydroxyacyl-CoA to 3-oxoacyl-CoA intermediates may influence the equilibrium of key redox reactions during syntrophic fatty and aromatic acid metabolism. ATP synthesis is dependent on the presence of energy-rich compounds such as acetyl phosphate. However, ATP could be synthesized using an acyl-CoA metabolite, AMP, and pyrophosphate, depending on the internal concentrations of the reactants and products. We know very little about the enzyme systems involved in electron flow during direct electron transfer, which makes it difficult to determine from metatranscriptomic or metaproteomic data whether electrons are transferred directly or via interspecies H_2 or formate transfer.

Single-cell microbiology is an attractive approach as even isogenic populations of microorganisms have substantial cell-to-cell heterogeneity at cellular and gene levels. Until recently we have not been able to identify microbes and note their mostly invisible activities, such as nutrient consumption, at the level of the single cell, not even in the laboratory. This is currently changing with the rapid increase of new technologies for single-cell microbiology (Musat et al. 2012; Wessel et al. 2013) that enable to observe "who does what, where, when, and together with whom." Single cells taken from the environment can be identified and their genomes sequenced. Individual microbes can be observed in situ with a range of innovative microscopic and spectroscopic methods, enabling localization, identification, or functional characterization of cells in an environmental sample, combined with the detection of the uptake of labeled compounds. They can be placed into fabricated microfluidic environments, to study their interactions. These novel methods hold potential for testing under well-controlled conditions.

While the physiology of the syntrophic communities has been studied for several decades (Stams and Plugge 2009), relatively little is known about the genes and their expression dynamics associated with the syntrophic interactions, partially due to the lack of suitable methodologies for measurements of biological properties within mixed culture systems. Applying single-cell methods enables the detailed study of mechanisms underlying syntrophy. These mechanisms include choice of hydrogen and formate as interspecies electron compounds, localization of hydrogenases, and formate dehydrogenases.

Syntrophic partners regulate their metabolism to grow together at the limits of what is thermodynamically feasible (Stams and Plugge 2009). The methanogens are favored if H_2 and formate concentrations are high, while the syntroph requires low H_2 and formate concentrations. Metabolic flexibility to cope with these fluctuations

in H_2 and formate levels is essential and does occur. This suggests that syntrophic populations have numerous possibilities for interspecies electron transfer. In triplicate cocultures, the ribosomal activity of the methanogen varied up to ten times reflects this flexibility, though the trigger for it is not yet clear (Worm et al. 2011). To assess the level of metabolic flexibility of individual cells in a community, the specific environmental adaptation of syntrophic communities should be investigated further. To assess the level of metabolic flexibility of each individual in a syntrophic coculture, Qi et al. (2014) demonstrated gene-expression heterogeneity. A dual culture of *Desulfovibrio vulgaris* with *Methanosarcina barkeri* demonstrated very significant cell-to-cell gene-expression heterogeneity for selected *D. vulgaris* genes in both the monoculture and the syntrophic coculture.

Many syntrophic associations are highly organized, multicellular structures with the partners in close physical proximity to each other. We know very little about the molecular mechanisms involved in the formation and maintenance of these catalytic units. Regulatory mechanisms that control the development of attached consortia most likely are similar to those involved in biofilm formation. Transcriptomic analyses of each syntrophic partner would identify gene systems that respond to the syntrophic lifestyle and may provide clues as to the chemical signals that each organism uses to communicate with its partners. In addition, we do not understand the extent to which syntrophic metabolizers regulate their metabolisms in response to environmental stimuli such as H_2 concentration versus the global cellular status such as energy charge. The combination of metagenomic and metatranscriptomic analyses will allow us to interrogate the regulatory mechanisms involved in establishing and maintaining multispecies associations in order to quantify and predict the behavior of microorganisms and microbial communities in natural ecosystems. A thorough understanding of the formation and structure of dense microbial aggregates is essential for application of methanogenesis.

Many syntrophic associations still need to be discovered. Besides syntrophic oxidations of the compounds discussed here, syntrophic interactions may also play an important role in the degradation of compounds that are considered to be easily fermentable, e.g., sugars as shown by Krumholz and Bryant (1986) and Müller et al. (2008). Besides freshwater environments, sulfate-depleted marine sediments are also important methanogenic environments (Colwell et al. 2008). Syntrophic interactions in these marine methanogenic environments have not been studied thoroughly (Kendall et al. 2006). Meta-omics studies have discovered a large number of new phyla but only speculate about their metabolism. To identify still unknown syntrophic interactions, a holistic approach integrating physiology, ecology, and genomics can create a stepping stone in understanding microorganisms, microbial communities, and their potential application.

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Methanogenesis in Soils, Wetlands, and Peat

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Abstract

Soil is the naturally occurring rock particles and decaying organic matter (humus) on the surface of the Earth, capable of supporting life. It has three components: solid, liquid, and gas. The solid phase is a mixture of mineral and organic matter.

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Wetlands are areas on which water covers the soil or where water is present either at or near the surface of that soil. Wetlands often host considerable biodiversity and endemism. Their hydrological conditions are characterized by an absence of free oxygen sometimes or always. It favors the development of anaerobic microbial community. In the absence of electron acceptors other than bicarbonate, methane is the end product of organic matter degradation in wetland ecosystems. It makes wetlands important sources of the greenhouse gas CH_4 in the context of the problem of global climate changes. Peatlands are a type of wetlands and form when plant material is inhibited from decaying by acidic and anaerobic conditions.

Methane production in peatlands tends to vary tremendously both spatially and temporally and depends on environmental factors such as temperature, pH, and water table, as well as plant cover. In anaerobic peat, acetate and CO_2 are the most quantitatively important CH_4 precursors. Most studies suggest that acetoclastic methanogenesis is an important pathway for CH_4 formation in nutrient-rich fens covered with *Carex* sedges, whereas CO_2 reduction is an important methanogenic pathway in *Sphagnum*-dominated bogs. Such bogs, the predominant peatlands, are typically acidic (pH $<$ 5) with low concentrations of mineral nutrients. The *Sphagnum* bog microbes seem to have special metabolic mechanisms to cope with low-mineral and diluted nonbuffered solutions. As a whole, the soil microbial community in wetlands plays an important role in biogeochemical cycles and is crucial to the functions of wetland systems. Research on the diversity and abundance microorganisms in wetlands rapidly develops owing to the advantages of molecular biological methods. The insights into the microbial community functioning and adaptation mechanisms in wetlands provide a valuable background for studies on biotechnological applications of microorganisms inhabiting these ecosystems.

1 Introduction

The soil-microbe system is one of the most diverse components of the terrestrial ecosystem. Soil is an extremely complex, variable living medium performing many vital functions such as biomass production and storage and microbial transformation of various substances, including water and carbon. These processes are of particular

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interest in freshwater wetland ecosystems where nutrient cycling is highly responsive to fluctuating hydrology, and gases produced in soil may contribute to climate warming. Northern peatlands are one of the most typical environments located in Eurasia and North America. This type of environments has been proved to be one of the powerful sources of atmospheric methane (IPCC 2013). Besides low pH, other extreme conditions of such environments are high content of organic matter, low mineralization, and permanently low temperature in anoxic peat layers. Methane emission from such environments has been well established by various field measurements (Williams and Crawford 1984; Chen et al. 2008; Glagolev et al. 2012; Kelsey et al. 2016; Webster et al. 2018). The ecological, biogeochemical, and hydrological regimes in peatlands are complex, resulting in a wide temporal and spatial variability of CH_4 emissions. The rates of methanogenic degradation processes go down significantly under acidic conditions. In spite of rapidly developing molecular methods for study of microbial diversity, there is a lack of data to get a clear insight into the relationship between mechanisms of methane production and the functional structure of methanogenic microbial community. In anaerobic peat, CH_4 production is considered to be dominated by acetoclastic and H_2-dependent pathways and often occurs at acidic pH. Acidophilic microorganisms inhabiting peat wetlands can have peculiarities in the regulation of metabolism as well as in specific physiological adaptations to low pH (Russell 1991). Molecular biological studies of wetland anaerobic communities have revealed physiologically distinct bacterial and archaeal lineages also (Galand et al. 2002; Utsumi et al. 2003; Bräuer et al. 2011; Bodelier and Dedysh 2013; Dedysh and Ivanova 2019).

2 Anoxic Waterlogged Soils as a Habitat for the Methanogenic Community

Soil is defined as the top layer of the Earth's crust, capable of supporting life. It is the most complicated biomaterial on the planet and is formed by mineral particles, water, air, decaying organic matter (humus), and living organisms (Crawford et al. 2005). Soil is the greatest reservoir of biodiversity on the planet. Prokaryotes comprise more than half of the biodiversity on Earth, and their diversity in soil has been estimated to be about three orders of magnitude greater than in all other environments combined (Curtis et al. 2002). Soil microorganisms mediate many processes, providing the turnover of elements such as carbon, nitrogen, and sulfur and different metals that regulate ecosystem function and also feedback to influence atmospheric chemistry.

The waterlogging of biologically productive soils makes them anoxic and allows anaerobic microbial community to develop. Once the soil becomes anoxic, remineralization rates lower and organic matter can accumulate. Under anaerobic conditions in freshwater environments, CH_4 production becomes the most important terminal electron sink of anaerobic respiration.

Wetlands are areas on which water covers the soil or where water is present either at or near the surface of that soil. Water can also be present within the root zone all year or just during various periods of the year. The result is a hydric soil, one characterized by an absence of free oxygen sometimes or always. Wetland

ecosystems are characterized by hydrophilic plant communities and have fluctuating hydrology that gives rise to an interplay between aerobic and anaerobic processes (Gutknecht et al. 2006). Wetlands, because of their complex hydrology and nutrient cycling and presence in both urban and unmanaged areas, are uniquely positioned to influence biogeochemical cycling in many regions and at many scales. A wetland may be found in coasts, estuaries, floodplains, shallow lakes, and peatlands.

Wetlands are considered to be the largest natural source of atmospheric CH_4, an important contributor to global warming, and are responsible for the release of ca. 10–30% (50–150 Tg CH_4) of the total annual methane emission (Cicerone and Oremland 1988; IPCC 2013; Matthews and Fung 1987). Wetlands are not equally distributed across latitudinal zones, and therefore wetlands will have a different impact on the CH_4 budget at different latitudes. The greatest areal extent of wetlands is at higher latitudes of the Northern Hemisphere (>40°N) in temperate-cold climates, particularly in Russia, Canada, and the USA, occupied by peatlands (Aselmann and Crutzen 1989; IPCC 2013).

Peatlands are unbalanced wetland ecosystems where productivity normally exceeds biodegradation. This imbalance leads to the accumulation of organic deposits (peats), which are derived from dead and decaying plant material under conditions of permanent water saturation.

Although peatlands represent a relatively small area (~3% of the Earth's surface) and have low levels of primary productivity compared with other terrestrial ecosystems, the peat-accumulating wetlands are significant repositories of carbon. They store more than 30% of the world's terrestrial C pool and represent a large natural source of CH_4 to the atmosphere (Matthews and Fung 1987; Whalen 1993: Haddaway et al. 2014). Peatlands have an organic soil layer of at least 30 cm extending to 15–20 m depth with an estimated mean between 1.3 and 2.3 m for northern peatlands (Turunen et al. 2002).

Another type of wetlands situated at the intertidal zones along the tropical and subtropical coasts, particularly in Southeast Asia, are mangroves. They are diverse and productive ecosystems playing a very important role in shaping the coastal ecology and typical example of mesophilic and moderately halophilic environmental niches (Bhattacharyya et al. 2015).

Emissions of CH_4 from northern peatlands vary as a function of temperature, pH, substrate and nutrient availability, anoxia due to flooded conditions, the degree of CH_4 oxidation that occurs in the upper aerobic sediments of peatlands, production enhancement and transportation via certain vascular vegetation (Bellisario et al. 1999; Chen et al. 2008; Dijkstra et al. 2012; Sabrekov et al. 2014), and the presence of other microorganisms outcompeting the methanogens such as sulfate and iron reducers and homoacetogens (Frenzel et al. 1999; Kotsyurbenko et al. 1996, 2001; Hunger et al. 2015).

There are three primary mechanisms for CH_4 and CO_2 transport from anoxic soils to the atmosphere: diffusion, ebullition, and movement through plant aerenchymous tissues (Lansdown et al. 1992; Kutzbach et al. 2004). Plants may enhance the emission of CH_4 through root-leaf transport and by passing oxidation in the aerobic zone as well as by releasing carbon exudates from plant roots, which are labile substrates for methanogens (Ström et al. 2003; Kelsey et al. 2016).

Peatlands include a wide range of ecosystems: each with a characteristic peat soil derived from partially decaying plant material and with little or no rock-derived minerals. Types of peatlands mostly depend on geographic region, terrain, and vegetation type. A characteristic of many peatlands is that the plant species composition of surface vegetation can be quite different from the remains of plants in the peat that dominated in the past. Deep peat deposits occur in wetlands in northern latitudes and the depth of the peat decreases with a decreasing latitude due, in part, to climate (Yavitt et al. 1987).

Peatlands are characterized by surface heterogeneity and fluctuating water table position that results in differences in the thermal regime, nutrient cycling, plant community composition, and organic matter production on a scale of several meters (Whalen and Reeburgh 2000).

A major distinction in the types of peatlands is between bogs and fens (Aselmann and Crutzen 1989). Bogs are the most acidic peatlands, which are fed only by precipitation and are nutrient-poor. They are characterized by low-mineral nutrient concentrations and dominated by *Sphagnum* mosses and a few ericaceous shrub species. Fens are near-neutral pH peatlands, which are fed by surface and groundwater as well as precipitation and tend to be more nutrient-rich. An increasing pH is associated with less *Sphagnum* and a dominance by *Carex* sedges and graminoid plants.

Except for the surface water microlayer, wetlands are anaerobic environments. As such, they represent suitable habitats for all microbial groups from the methanogenic community (Conrad 1996).

Sulfate reduction zone can be also extensive in wetland soils, especially in marshes that are influenced by seawater. Methane production may be limited by microbial iron reduction (Metje and Frenzel 2005). Fe(III)-reducing microorganisms may suppress methanogenesis competing for H_2 or acetate (Roden and Wetzel 2002). However, most wetland soil is devoid of O_2 and also contains no electron acceptors other than CO_2 and H^+.

The main factors regulating methane turnover in peatlands vary along smaller-scale spatial gradients. Closely situated areas of the same peatland can become either carbon sinks (dry hummocks) or sources (hollows), due to an effect of microform variations such as water table level and vegetation on the microbial community that can either oxidize or produce CH_4 (Conrad 1996; Laanbroek 2010).

As a whole, wetlands are ecosystems of exceptional ecological and economical importance. Their hydrology and biogeochemistry affect downstream waters and impact the overall landscape providing important ecosystem services such as wildlife habitat, water purification, and flood control.

3 Methanogenesis in Peatlands

Methane is a major product of the anaerobic degradation of organic matter in peatlands and produced by methanogenic archaea. The activity of methanogens in peatlands can easily be detected, because methanogenic metabolic activity is directly related to the amount of CH_4 produced in peat. Measurements of potential methane

production show that methanogenic activity is restricted to the waterlogged layers of the peat, although it can also be observed in anaerobic microsites. The maximum CH_4 production in peat profile takes place at the depth where most of the anaerobic degradation occurs.

The methane production in acidic peats has been shown to be stimulated by increasing temperature (Kotsyurbenko et al. 2004; Metje and Frenzel 2007) and pH (Williams and Crawford 1984; Kotsyurbenko et al. 2007). The effect of these environmental parameters on methanogenesis indicates that peatland methanogens are metabolizing under suboptimal growth conditions that indicate the important role of microbial adaptation. The fact that microorganisms have growth optima that can never be achieved under in situ conditions is well established for different ecosystems. However, it has been also shown that peatlands contain endemic acid-tolerant microorganisms participating in methane cycle at a low pH (Dedysh et al. 2000, Dedysh 2002; Bräuer et al. 2011; Bodelier and Dedysh 2013; Serkebaeva et al. 2013).

There are large temporal and spatial variations of methane emissions from natural wetlands (Lin et al. 2015; Sabrekov et al. 2014; Li et al. 2019). The positive correlation exists between the diversity of methanogenic communities and rates of methane production (Yavitt et al. 2012). Besides, a greater genetic diversity potentially reflects a greater diversity of methane production and oxidation pathways (Stoeva et al. 2014).

The process of methanogenic degradation occurs stepwise and involves different microbial groups. Primary fermenters oxidize polymeric compounds via oligomers and monomers to acetate, H_2, CO_2, and other volatile fatty acids (VFAs). The VFAs are in turn converted to acetate and CO_2 by synthrophs that require hydrogenotrophic methanogens as partners for keeping the produced H_2 at a low, thermodynamically permissive, partial pressure (Kotsyurbenko 2005; Botsch and Conrad 2011). Methanogens have a very limited substrate range, and their activities are linked to the activities of other microbial groups of the community. In wetlands, methane is almost exclusively produced either from acetate or from H_2/CO_2 (Conrad 1999). H_2 and CO_2 can also be utilized by acetogens, and methanogens and acetogens may under certain conditions compete for the same substrates in fen and bog soils (Bräuer et al. 2004; Hunger et al. 2015). Methanol, which is released during the decomposition of pectin, a polymer of methoxylated galacturonic acid and a major cell wall component of plants, usually plays only a minor role (Hines and Duddleston 2001). The relative contribution of H_2/CO_2 versus acetate as methanogenic precursors can be quite different in various wetlands.

The predominance of one respiratory pathway over another is generally a result of either the availability or lability of the carbon substrate. The acetate fermentation pathway is thought to dominate over CO_2 reduction when fresh organic material is utilized as in sites with high plant productivity. Less productive plant communities with more recalcitrant material (*Sphagnum* dominate, sedges are scarce) tend to use the CO_2 reduction pathway (Galand et al. 2005; Keller and Bridgham 2007). Acetoclastic methanogenesis seems to predominate in fens populated by *Carex* sedges (due to the availability of root exudates supplied by the vascular plant

community), while CO_2 reduction was more important in *Sphagnum*-dominated bogs (Kelley et al. 1992). The acetate fermentation often exceeds CO_2 reduction in summer when decomposition of organic matter is most active (Avery et al. 1999).

Methane stable carbon isotope composition can be used to reveal active methanogenic pathways. Enriched $\delta^{13}C$-CH_4 isotopic signatures attributable to methane production pathway via acetate (Whiticar 1999; Conrad 2005) are often associated with sites exhibiting high rates of plant production and large CH_4 fluxes. This confirms that abundant fresh organic material at sites with the greatest plant productivity stimulates larger CH_4 emissions via the acetate fermentation pathway. Based on profiles of CH_4 stable isotope ratios in peat, it was also shown that the upper profile was dominated by acetoclastic and the lower profile by hydrogenotrophic methanogenesis (Popp et al. 1999; Hornibrook et al. 2000). Hydrogenotrophic methanogenesis can occur locally even when conditions are not conducive for this process in the bulk peat. The isotopic composition of emitted methane mostly resembled CH_4 of deeper soil layers (Popp et al. 1999).

The methanogenic pathways also have been shown to be dependent on pH and temperature. A low pH is advantageous to H_2-dependent methanogenesis, whereas a low temperature is favorable to acetoclastic methane pathway (Kotsyurbenko et al. 1996, 2005, 2007; Conrad 2002).

At lower temperatures (5 °C), addition of exogenous substrates (acetate, methanol, or H_2/CO_2) in incubation experiments has no stimulatory effect on either the rate of methanogenesis or methanogenic community structure. In contrast, at higher temperatures, substrate amendment enhances methane production in H_2/CO_2 amended microcosms and played a clear role in structuring methanogen communities in transiently cold environments; methanogen communities can rapidly respond to moderate short-term increases in temperature (Blake et al. 2015).

Acetate can even accumulate seasonally in northern peatlands (Hines and Duddleston 2001; Duddleston et al. 2002), followed by oxidation, and it has been proposed that aceticlastic methanogenesis may be absent or inhibited at these sites. Acetate also can be oxidized to CO_2 via aerobic respiration or other oxidative microbial processes (e.g., via the dissimilation of iron or nitrate) or oxidized syntrophically to CO_2 by the concerted activity of acetate-oxidizing anaerobes (Nüsslein et al. 2001; Hattori 2008). After diffusion into oxic environments, acetate can be also oxidized to carbon dioxide by aerobic microorganisms. Besides, the areas of active root growth of different plant species have played important roles in the improvement of soil redox and CH_4 consumption acting as a conduit for oxygen transportation into and out of the substratum (Wang et al. 2013). Supplemental acetate can be inhibitory to methanogenesis in acidic peat samples, whereas glucose and H_2 can be stimulatory (Williams and Crawford 1984). The negative influence of humic substances on methanogenesis has also been reported (Stewart and Wetzel 1982).

Acetic acid and other fatty acids are known to be toxic at a low pH (Russell 1991). Acetate and other volatile fatty acids inhibit methanogenesis in bog peat at pH 4.5, but not at pH 6.5 (Horn et al. 2003). The explanation is the abundance of undissociated acetic acid under low-pH conditions. At pH below 6.0, a greater fraction of

total acetate will be present as acetic acid ($pK_a = 4.7$), which can permeate cell membranes, causing acidification of the cell interior and acts as a decoupler of the proton motive force that can be lethal to the cell (Russell 1991; Beer and Blodau 2007). Acetate concentrations of 5–10 mM and higher are considered to be inhibitory at a low pH, while acetate may be utilized at its natural concentrations in the micromolar range (Bräuer et al. 2004).

In minerotrophic fens which are connected to the groundwater flow, ferric iron [Fe(III)] can be another potential electron acceptor. Fe(III) reduction appears to parallel CH_4 formation in northern acidic wetlands (Metje and Frenzel 2005; Küsel et al. 2008).

Methylotrophic methanogenesis is considered to be negligible in freshwater. However, methyl compounds especially methanol can play underestimated role as contributors to CH_4 production in wetlands (Jiang et al. 2010).

4 Methanogenic Diversity in Wetlands

Microbial processes leading to methanogenesis in wetlands are similar but facilitated by dissimilar microbial communities. They are different among wetland locations and microtopographies and associated with wetland soil properties.

Today for the ecological analysis of populations of methanogenic archaea as a part of complex microbial communities of wetland habitats, the whole spectrum of molecular methods is applied. Along with the commonly used 16S rRNA gene, the *mcrA* gene (encoding an alpha subunit of the methyl-coenzyme M reductase) (Friedrich 2005) is now successfully used as a molecular phylogenetic marker for analysis of methanogens. Using this functional gene as a marker and proper primer systems for its amplification (Narihiro and Sekiguchi 2011) allows for covering various non-monophyletic methanogenic groups while simultaneously eliminating from the analysis not methanogenic groups of microorganisms. Additionally, shotgun metagenome sequencing (SMS) (He et al. 2015) and qPCR (Cheema et al. 2015; Prasse et al. 2015) for the 16S rRNA gene and/or *mcrA* are successfully used for the analysis and quantifying methanogens in wetland habitats including new deep phylogenetic branches of methanogenic archaea. The most promising methodological direction for the study of methanogenic archaea in the complex microbial communities of wetland habitats is combinational methods of metagenomic, metatranscriptomic, and DNA and RNA stable isotope probing (DNA-SIP and RNA-SIP) (Hunger et al. 2011; Lin et al. 2015; Angle et al. 2017) with sediment slurry incubations and potential activity measurements.

The application of molecular techniques based on 16S rRNA and *mcrA* gene sequences to study wetlands has revealed a diversity of methanogens belonging to the Methanomicrobiaceae, Methanobacteriaceae, Methancoccaceae, Methanosarcinaceae, and Methanosaetaceae as well as recently discovered archaeal lineages within the Euryarchaeota (Upton et al. 2000; Galand et al. 2002, 2005; Basiliko et al. 2003; Horn et al. 2003; Kotsyurbenko et al. 2004; Hoj et al. 2005; Yavitt et al. 2006,

2012; Metje and Frenzel 2007; Tveit et al. 2012, 2014; Andersen et al. 2013; Narrowe et al. 2017).

The described representatives from these families are known to use both acetate and H_2/CO_2. It indicates that methanogenic community in peatlands contains all trophic groups of methanogenic archaea that are required to explain the formation of CH_4 via acetoclastic and H_2-dependent pathways. Nevertheless, methanogenic communities in bogs and fens are functionally different (McDonald et al. 1999; Galand et al. 2002, 2005). A bog has a more pronounced dominance of a few taxa, whereas a fen has a more even distribution among taxa. The methanogenic diversity at the bog is usually quite low (Galand et al. 2005). The peat pH, which is much lower in bogs, may be a factor in the selection of specific acid-tolerant microorganisms (Dedysh et al. 2000, Dedysh 2002; Bräuer et al. 2011). Methanogenic community composition has also been found to change vertically within a site (Galand et al. 2002, 2005).

The low concentration of acetate in mesotrophic peats favors *Methanosaeta* spp., which have a lower threshold for acetate than other acetotrophs belonging to the family Methanosarcinaceae (Galand et al. 2005). In ecosystems where acetate concentrations are high, *Methanosaeta* spp. are outcompeted by *Methanosarcina* spp. (Fey and Conrad 2000).

Essentially, a seasonal supply of fresh substrates in peatlands stimulates the growth of bacteria much more than the growth of archaea (Chan et al. 2005). The bacterial metabolism in turn stimulates methanogenesis, indicating the hydrolysis of polysaccharides and other complex organic materials as a first and rate-limiting step for the methanogenic degradation of organic matter production.

Temperature also plays a role in the archaeal composition (Fey and Conrad 2000). It can directly alter the community structure of methanogenic archaea and the methanogenic pathway (Kotsyurbenko 2005; Metje and Frenzel 2007). Hydrogenotrophic methanogens are more sensitive to a reduction of soil temperature than acetotrophic methanogens (Chin and Conrad 1995).

Acetate is considered to be the most important methanogenic substrate in near-neutral pH low temperature environments that favors acetoclastic methanogenesis and therefore enhances the contribution of acetoclastic methanogenesis to total methane production (Schulz et al. 1997; Kotsyurbenko 2005). In northern bogs and mires, a large proportion of sequences recovered had high homology with *mcrA* from known acetoclastic methanogens, i.e., >66% (Basiliko et al. 2003; Galand et al. 2005).

Methanogenic diversity appears to increase with an increasing temperature. The diversity of archaea among wetlands distributed across latitudinal gradients tends to increase with a decreasing latitude that also correlates with temperature (Utsumi et al. 2003). Nevertheless, methanogens are also abundant in cold environments and Methanosaetaceae, Methanosarcinaceae, Methanobacteriaceae, and Methanomicrobiales are often identified as the dominant methane producers in Arctic wetlands (Hoj et al. 2005; Stoeva et al. 2014; Kwon et al. 2017).

Another important parameter influencing the methanogenic community structure in wetlands is pH. Most of the detected cultured methanogens grow over a pH range

of 5–9 but prefer pH neutral conditions as they rely on a membrane proton gradient for energy conservation (Horn et al. 2003; Drake et al. 2013) and therefore must be well adapted to survive in these environments. An exception is *Methanoregula boonei*, a hydrogenotrophic methanogen that has a more acidic pH range (i.e., pH 4.5–5.5) (Bräuer et al. 2011). The properties of *M. boonei* are consistent with the observation that *Methanoregula*-affiliated species are the prevalent methanogens in surface peat (0–20 cm below water level) in low-nutrient and low-pH (3.5–4.5) *Sphagnum* bogs (Bräuer et al. 2011; Yavitt et al. 2012; Hunger et al. 2015). In a wide range of acidic peatlands, 'Methanoflorentaceae,' formerly known as rice cluster II, was also found (Kotsyurbenko et al. 2004; Yavitt et al. 2012; Juottonen et al. 2015).

Among acetoclastic methanogens, *Methanosaeta* is more abundant in the near neutral pH wetland soil, whereas *Methanosarcina* was detected in a wider pH interval in wetlands (Hunger et al. 2015).

Just recently, it has been found that *Methanomassilii coccales*, a new discovered order of methanogens dependent on two substrates for growth (H_2 and methylated compounds), also contributes to the high diversity of methanogens in wetlands (Söllinger et al. 2016).

Thus, the succession of methanogenic archaea communities is dependent on the changes in environmental conditions and the quantity and quality of substrate, which are in turn determined by the plant community structure.

Microorganisms inhabiting *Sphagnum* peat have specific growth requirements. They are sensitive to mineral composition in the environment and can only develop at low ionic strength (Bräuer et al. 2006a,b, 2011). The sensitivity of the methanogenic population from wetlands to nitrates and sulfates has been also reported (Scheid et al. 2003).

5 Modeling Methane Fluxes in Wetlands

The most important source of uncertainty on the methane budget is attributable to emissions from wetlands exhibiting large spatial variations at a field scale. Since it is impossible to cover all research sites by field measurements, modeling methane fluxes in wetland soils is proved to be crucial for estimating their contribution to global CH_4 cycle (Cao et al. 1995).

All the models presently applied for predicting methane production and emission are separated into three main groups:

1. Empirical (regression) models establishing direct links between methane emission and environmental factors like temperature, water table level, and some others. These models are well applicable if the values of input variables are close to those used for the model verification. In case of methane emission predictions, these values can be significantly different. It results in an enormously big deviation in emission rates obtained from the model estimations and the following experiments (Smagin and Glagolev 2001).

2. Process-based models describing both fundamental biological and chemical characteristics of methanogenesis (biokinetics) and physical basis of transfer processes (if available) in the system (Xu et al. 2016).
3. Intermediate models which combine features of both aforementioned models. In such models various processes can be described by both empirical and process-based categories of dependencies (Cao et al. 1995; Frolking and Crill 1994).

The accuracy of modern models and their well matching to experimental results is mostly achieved by the use of the model ensemble methodology that provides the final result of calculation by averaging the outputs of different ensemble models (Bloom et al. 2016).

A special attention is now paid for the use of satellite retrievals, an active field of methodological development, with special requirements on the sampling of the model and the treatment of data uncertainty (Houweling et al. 2017).

Additionally, attempts are made to couple sequence-based methods with biogeochemical and greenhouse gas measurements to contribute to modeling the microbial component in wetland methane fluxes. Methanogenesis gene abundance are negatively correlated with nitrate-, sulfate-, and metal-reducing bacteria and are most abundant at sampling sites with high peat accretion and low electron acceptor availability (He et al. 2015). Quantitative comparative analyses of sequence data can also provide molecular evidence explaining the spatial variations in biogeochemistry and methane production (He et al. 2015).

In sum, the perspective application of wetland methane flux models requires (1) explicit representation of the mechanisms underlying land-atmosphere CH_4 exchange, (2) proper simulation of CH_4 emissions across highly heterogeneous spatial and temporal scales, and (3) investing efforts to develop model benchmarking frameworks for easy working with data from molecular to global scales (Xiaofeng et al. 2016).

All these contributions to the models describing methane cycle in wetlands would be beneficial for the Earth System Models and further simulation of climate change feedbacks.

6 Research Needs

There has been much progress in understanding the mechanisms underlying methane production in wetlands. Nonetheless, further work is still needed to make adequate and precise predictions of greenhouse gas emissions on the global level and to explore new possible sources of methane such as plant matter and living plants (Vigano et al. 2008; Nisbet et al. 2009). A detailed knowledge of the structure and the functioning of methanogenic communities from the largest terrestrial methane-producing ecosystems would be beneficial for understanding the microbial ecology of methane production, which, in turn, may lead to interventions to ultimately control the factors of the global CH_4 turnover. The research is necessary that aims at integrating the analysis of environmental factors, biogeochemical processes, and

bacterial and archaeal community profiles in diverse wetland ecosystems. The effective concept of such study includes a consecutive consideration of interacting biosystems of different complexity levels (the systems biology approach). Accordingly, methane flux measurements in situ and in peat bog samples (ecosystem level) are followed by experiments on trophic microbial interaction, predominant methanogenic pathways (community level), and, finally, characterizing the microbial diversity and the metabolic potential in partial methanogens in the community (microbial group level) with an attempt to investigate the key microorganisms (microorganism level). The insights into the microbial community structure and the main controlling factors in wetlands provide a valuable background for further studies on biogeochemical processes in these ecosystems. Future studies should address the spatial and temporal heterogeneity of wetlands with special focus on the potential differences and similarities of anaerobic processes and associated microbial communities that drive methanogenesis in such research sites. Both cultivation-dependent and cultivation-independent approaches should be involved to characterize the true in situ microbial composition. The specific requirements of different groups of microorganisms living in mineral-deficient wetlands at low temperatures and an acidic pH should be analyzed to establish new protocols for getting active enrichments and maintenance of important, yet poorly culturable, microorganisms, which will significantly expand our knowledge on biodiversity and the limits to life under the harsh environmental conditions.

Microorganisms developing under such extreme conditions may exhibit novel adaptation mechanisms, which could lead to new bioproducts and extend the range of biotechnological applications. Investigating the functional features of anaerobic communities in transiently and permanently cold wetlands may contribute to the development of more economic and environmentally sustainable low-temperature microbially mediated waste treatment systems providing great low-temperature-adapted microbial resources (Blake et al. 2015).

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Methanogenesis in the Digestive Tracts of Insects and Other Arthropods

9

Andreas Brune

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Abstract

Termites, cockroaches, and scarab beetle larvae are the only insects known to emit methane, but they do so in impressive amounts. Methanogenesis occurs in the enlarged hindgut compartment and is fueled by hydrogen and reduced one-carbon compounds formed during symbiotic digestion of plant fiber and humus. The methanogens either colonize the hindgut wall or are associated with symbiotic protists. They comprise only a relatively small number of lineages from four methanogenic orders that are restricted to the intestinal tract of insects and millipedes. The host specificity of most lineages and the metabolic properties of the few isolates available to date indicate that they are well adapted to the microenvironment of their intestinal habitats. Methanogenesis is generally expected to stimulate symbiotic digestion, but benefits for the host are not well documented. Although the methane emissions of termites are mitigated by the methanotrophic activity of their mounds and the surrounding soil, their enormous biomass in the tropics makes them a significant natural source of atmospheric methane at the global scale.

1 Introduction

Many insects that thrive on a fiber-rich diet harbor microbial symbionts that participate in digestion. However, only termites, cockroaches, and the larvae of scarab beetles have been found to emit methane. Methane is the product of methanogenic archaea, which form the last link in a feeding chain of anaerobic microorganisms located in their enlarged hindguts – a microbial bioreactor that transforms plant fibers or other lignocellulosic matter to short-chain fatty acids, the major energy source for the host. Among other arthropods, methanogenesis seems to be restricted to certain millipedes and marine copepods.

This chapter will cover the diversity of methanogens associated with insects and other arthropods, structure, and location of the methanogenic communities in their digestive tracts, and their role in symbiotic digestion. The bias of this review towards termites reflects the limited number of studies on methanogenesis in the other arthropod groups and the interest that the methane emissions of termites received in the context of the global budget of this greenhouse gas. For additional information and more exhaustive surveys of the literature, readers should consult previous reviews focusing on specific aspects of the topic (e.g., Breznak 2000; Sugimoto et al. 2000; Purdy 2007; Brune 2010a; Hackstein and van Alen 2010; Hongoh and Ohkuma 2010; Brune and Ohkuma 2011; Ohkuma and Brune 2011; Brune 2014).

2 Methane as a Product of Symbiotic Digestion

In the intestinal tracts of insects, methanogenesis occurs exclusively in the hindgut (proctodeum), where a pronounced enlargement of the colon (hindgut paunch) and the oxygen consumption of the gut microbiota lead to anoxic conditions, at least in

the dilated compartments (Fig. 1). Negative redox potentials have been measured in the hindgut proper of scarab beetles (Bayon 1980; Lemke et al. 2003), termites (Ebert and Brune 1997; Kappler and Brune 2002), and cockroaches (Schauer et al. 2012; Bauer et al. 2015). Notable exceptions are the posterior hindgut compartments of soil-feeding termites (Bignell 1984a; Kappler and Brune 2002) and the larval hindgut of *Melolontha melolontha* (Scarabaeidae; Egert et al. 2005), which emit methane but show slightly oxidizing conditions.

2.1 Methanogenesis in Termite Guts

Emission of methane by termites had been suspected already more than 80 years ago. When Cook (1932) studied the respiratory gas exchange of *Zootermopsis nevadensis* using Warburg manometry, he found that these termites continued to form substantial amounts of an unidentified gas after the oxygen in the chamber was depleted. He was not able to analyze the gas but showed that its production depended on the presence of gut flagellates, which colonize the enlarged hindgut compartment of all phylogenetically "lower termites" (all families of Isoptera except Termitidae) and are essential for the digestion of cellulose and hemicelluloses (see Brune 2014). Inspired

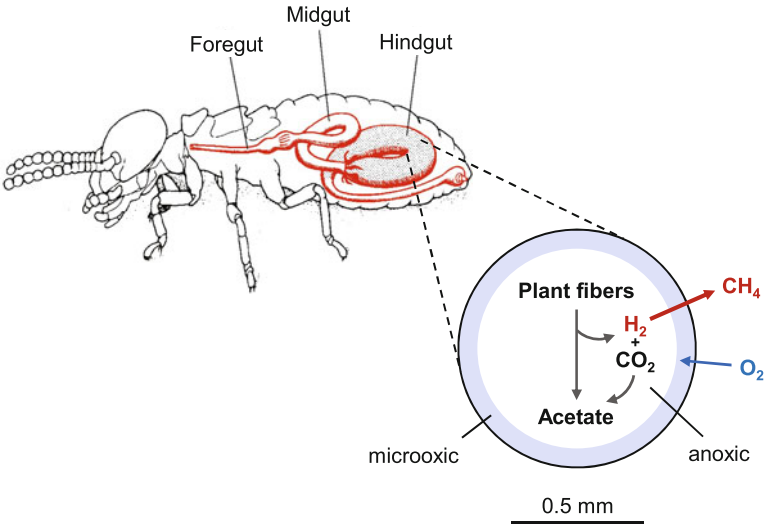

Fig. 1 Termites and other insects that feed on a fiber-rich typically possess a dense hindgut microbiota. The products of symbiotic digestion (acetate and other short-chain fatty acids) are an important carbon and energy source for the host. Hydrogen formed by the fermentative processes drives both reductive acetogenesis and methanogenesis. The microorganisms colonizing the hindgut periphery have to cope with a constant influx of oxygen and are responsible for maintaining the anoxic status of the gut lumen. Originally published in Brune (2010b), with kind permission of © Springer Science+Business Media New York. All rights reserved

by the situation in ruminants, he proposed that the gas was most likely hydrogen or methane, or a mixture of both.

A few years later, Hungate (1938, 1939) confirmed the observations of Cook and documented hydrogen formation both for the gut flagellates of *Zootermopsis* and for living termites. Gilmour (1940) noticed that the gas formed by the wood-feeding cockroach *Cryptocercus punctulatus*, a close relative of termites that harbors the same type of gut flagellates, could not consist exclusively of hydrogen because it produced some carbon dioxide when combusted. In the following years, Hungate (1943, 1946) conducted pioneering work on the fermentative metabolism of the gut flagellates of *Zootermopsis* species, which led to the first tenable concept of lignocellulose digestion in termite guts. Although he could show the conversion of cellulose to acetate, carbon dioxide, and hydrogen as major products, he never realized that part of the hydrogen formed by the flagellates is subsequently metabolized to methane by other members of the gut microbiota (Hungate 1977).

It took more than 40 years after Cook's initial observation until methane production in termite guts was finally recognized. The discovery was – as so often in science – a classic case of serendipity. While demonstrating nitrogenase activity in living termites and wood-feeding cockroaches with the acetylene reduction assay, Breznak et al. (1973, 1974) noticed an additional peak in the gas chromatograms and identified it as methane. Their work led to the realization that the amount of methane produced by termites is rather large and, based on body weight, in the same order of magnitude as that of ruminants (Breznak 1975). In the following years, methane emission was documented for almost all termite species investigated (e.g., Brauman et al. 1992; Shinzato et al. 1992; Wheeler et al. 1996; Bignell et al. 1997; Sugimoto et al. 1998b).

2.2 Methanogenesis in Other Arthropods

Subsequent surveys detected methane production only in two other groups of terrestrial arthropods: insects and millipedes (Bracke et al. 1978; Hackstein and Stumm 1994; Hackstein et al. 2006; Sustr et al. 2014). Phylogenetically, termites fall into the radiation of cockroaches. Although most cockroaches do not efficiently digest lignocellulose, they resemble termites in their general gut microenvironment and the presence of a dense gut microbiota in their enlarged hindguts that assists in the breakdown of their fiber-rich diet (Bignell 1984b; Schauer et al. 2012; Bauer et al. 2015). The same is true for the larvae of scarab beetles, which thrive on a wide range of vegetal matter in different stages of decay, ranging from living plant roots to humus. In both cases, symbiotic digestion involves the methanogenic fermentation of the plant polymers (Bayon and Etiévant 1980; Lemke et al. 2003; Egert et al. 2005). However, methanogens are lost during metamorphosis and are entirely absent from members of other coleopteran families, irrespective of life stage and diet (Hackstein et al. 2006). A report on the presence of methanogens in the anterior hindgut of a passalid beetle using a DNA microarray (PhyloChip) remains to be

substantiated (Ceja-Navarro et al. 2014); their 16S rRNA genes were not sequenced, and methane emission of the beetles has not been shown.

Among other soil arthropods, methanogenesis occurs only in millipedes. It was first detected in tropical species of the order Julida (Hackstein and Stumm 1994), but later also in temperate species and in members of other juliform orders (Sustr and Simek 2009; Sustr et al. 2014). Among marine arthropods, methane formation has been observed in certain copepods grazing on phytoplankton and in their fecal pellets (de Angelis and Lee 1994; Ditchfield et al. 2012); it may explain the enigmatic oversaturation of methane in the well-oxygenated mid-water column of open oceans (Karl and Tilbrook 1994).

Invertebrates other than arthropods do not emit substantial amounts of methane. Even in earthworms, whose gut and fresh casts provide transient anoxic niches in oxic soils (Horn et al. 2003), methane production and cultivable methanogens are typically absent (Drake and Horn 2007). However, there are notable exceptions. Borken et al. (2000) observed a transient emission of methane from *Lumbricus terrestris* and its fresh casts, and a well-developed methanogenic food web has been documented for the intestinal tract of the tropical earthworm *Eudrilus eugeniae* (Depkat-Jakob et al. 2012; Schulz et al. 2015).

The restriction of methanogenesis to certain groups of arthropods and the failed attempts to permanently establish methanogens in nonmethanogenic cockroaches lines by artificial infection suggest the presence of a genetic determinant and consequently a hereditary basis of the association (Hackstein and van Alen 2010). However, the nature of the specific interactions between methanogens and their hosts remains to be discovered.

2.3 Hydrogen as Central Intermediate

Methanogenic archaea form methane in two fundamentally different processes: the reduction of CO_2 or methyl groups to CH_4 (hydrogenotrophic methanogenesis) and the cleavage of acetate to CH_4 and CO_2 (aceticlastic methanogenesis) (Liu and Whitman 2008; Thauer et al. 2008). Hydrogen is a major fermentation product of the gut microbiota of termites and drives methanogenesis in the hindgut (Brune and Ohkuma 2011). Also acetate accumulates to high concentrations, but aceticlastic methanogens are generally absent from the intestinal tracts of all animals. It is assumed that they cannot cope with the short retention times of these habitats (Liu and Whitman 2008), but it remains puzzling why they do not avoid washout by attaching to intestinal surfaces (see below).

In the hindguts of lower termites, hydrogen is formed by the cellulolytic flagellates and can accumulate to substantial concentrations (Ebert and Brune 1997; Pester and Brune 2007; Desai and Brune 2012). In all other insects, hydrogen is the product of bacterial fermentations. In the hindguts of higher termites (family Termitidae), hydrogen accumulation is much stronger in wood-feeding *Nasutitermes* species than in soil-feeding *Cubitermes* species (Köhler et al. 2012; Schmitt-Wagner and Brune 1999). In cockroaches and scarab beetle larvae, hydrogen accumulates mostly in the

crop or midgut region, but not in all species, and concentrations can vary strongly among individuals (Lemke et al. 2003; Egert et al. 2005; Schauer et al. 2012; Bauer et al. 2015).

Regardless of the extent of hydrogen accumulation, methane production in insect guts is always limited by hydrogen. In all termites, cockroaches, and scarab beetle larvae investigated, the addition of hydrogen strongly stimulates methanogenesis in intact hindguts or hindgut homogenates (Brauman et al. 1992; Schmitt-Wagner and Brune 1999; Lemke et al. 2001; Lemke et al. 2003; Egert et al. 2005; Bauer et al. 2015). Methane production in lower termites strictly depends on the presence of hydrogen-producing gut flagellates (Odelson and Breznak 1983; Rasmussen and Khalil 1983; Messer and Lee 1989). Metronidazole, a drug that inhibits hydrogen formation in both anaerobic bacteria and protists, almost completely abolished methane emission by the cockroach *Periplaneta americana* (Bracke et al. 1978). Insects that do not emit methane and individuals from methane-emitting taxa that fail to produce methane often emit large amounts of hydrogen (Hackstein and Stumm 1994).

Already the early work of Odelson and Breznak (1983) on the gut microbiota of termites evidenced a competition for hydrogen between methanogens and hydrogen-consuming bacteria (homoacetogens). Subsequent studies corroborated that reductive acetogenesis from H_2 and CO_2 is the predominant hydrogenotrophic process in most wood-feeding termites, whereas the opposite is true for most fungus-cultivating and soil-feeding termite species, both in gut homogenates (Breznak and Switzer 1986; Brauman et al. 1992) and in intact guts (Tholen and Brune 1999; Tholen and Brune 2000; Pester and Brune 2007). Methanogens should always out-compete homoacetogens for hydrogen for thermodynamic reasons, but the explanation for this phenomenon was found in the spatial organization of the respective populations. While the majority of methanogens resides at the hindgut wall, which places them downstream in the hydrogen gradient, the highly motile homoacetogenic spirochetes are able to colonize the hydrogen-rich hindgut lumen (Fig. 2a). The situation has been investigated in detail with *Reticulitermes flavipes* and explains why methanogenesis is severely hydrogen limited but strongly stimulated by external hydrogen (Leadbetter and Breznak 1996; Ebert and Brune 1997), whereas reductive acetogenesis is not (Tholen and Brune 2000), and why the strong hydrogen sink at the gut wall of this termite is caused by an anaerobic process (Ebert and Brune 1997). A more detailed discussion of this topic can be found elsewhere (Brune 2010a).

Since methanogens located in the gut periphery are clearly hydrogen limited, whereas those in the lumen often are not (see above), Sugimoto et al. (1998b) suggested that the rates of hydrogen and methane emission of different termite species might depend on the particular location of methanogens relative to the hydrogen source. Nevertheless, termites (Zimmerman et al. 1982; Odelson and Breznak 1983; Ebert and Brune 1997; Sugimoto et al. 1998b; Schmitt-Wagner and Brune 1999; Cao et al. 2010; Yanase et al. 2013) and other methane-producing insects (Hackstein and Stumm 1994) simultaneously emit hydrogen in considerable amounts, which is most likely explained by the patchiness of the colonization of

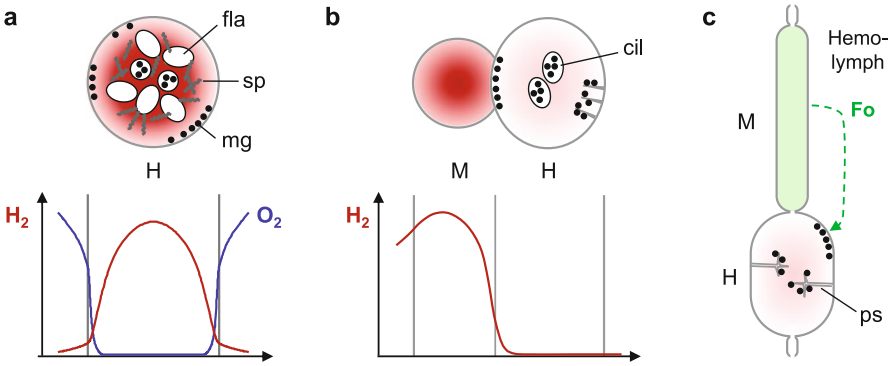

Fig. 2 Radial gut sections of a lower termite (a) and a cockroach (b), and axial gut section of a scarab beetle larva (c), illustrating the location of methanogens (mg), the corresponding concentration profiles of hydrogen and oxygen (in a, b), and the transfer of soluble reductants from midgut (M) to hindgut (H) via the hemolymph (arrow, in c). (a) In lower termites, hydrogen production by flagellates (fla) leads to steep hydrogen gradients from the anoxic lumen towards the microoxic hindgut periphery. While homoacetogenic spirochetes (sp) are distributed throughout the gut lumen, methanogens are associated with gut flagellates or attached to the hindgut wall. (b) In cockroaches, methanogens are either associated with hydrogen-producing ciliates (cil) or attached to the hindgut cuticle, whose surface is enlarged by cuticular hairs. Methanogenesis is driven in part by cross-epithelial transfer of hydrogen from the midgut (M) compartment. (c) In scarab beetle larvae, methanogens are attached to the gut epithelium or to tree-like epithelial invaginations (pseudosetae, ps) that extend into the lumen. Hydrogen does not accumulate strongly, and methanogenesis in the hindgut might be partially driven by formate (Fo) or other reductants that are produced in the midgut and transported to the hindgut via the hemolymph. Originally published in Brune (2010b), with kind permission of © Springer Science+Business Media New York. All rights reserved

the gut wall by methanogens and a spatial separation of hydrogen-producing and hydrogen-consuming processes in different gut compartments, which allows hydrogen to escape from the gut.

2.4 Association with Epithelial Surfaces

The discovery that most methanogens can be easily detected by light microscopy based on the autofluorescence of their coenzyme F_{420} allows their exact localization even in complex environments (Doddema and Vogels 1978). In most insects, microbial cells with the characteristic fluorescence of methanogens densely colonize the interior surface of the hindgut. At first glance, this appears surprising because of the constant influx of oxygen across the gut epithelium and the notorious oxygen sensitivity of methanogens in pure culture. However, the explanation rests in the capacity of methanogens to reduce molecular oxygen at astonishing rates. The genomes of all *Methanobrevibacter* species encode homologs of a coenzyme $F_{420}H_2$ oxidase (FprA), a flavoprotein that reduces molecular oxygen to water

(Seedorf et al. 2004; Poehlein and Seedorf 2016). This explains why pure cultures of *Methanobrevibacter* spp. and *Methanimicrococcus blatticola* growing in agar tubes with oxygen in the headspace are able to maintain anoxic condition a few millimeters below the meniscus (Leadbetter and Breznak 1996; Sprenger et al. 2007; Tholen et al. 2007), and why cell suspensions of *Methanobrevibacter* species remain metabolically active as long as hydrogen is available as a reductant and the oxygen flux does not exceed their capacity for its removal (Tholen et al. 2007). Obviously, the necessity to redirect electron flow from methanogenesis towards oxygen reduction will exacerbate the hydrogen limitation of methanogens at the hindgut wall.

An attachment to the hindgut cuticle prevents washout of the relatively slow-growing methanogens from the gut, which might compensate for the negative effects of hydrogen limitation and the exposure to inflowing oxygen (Breznak 2000). Adherence of F_{420}-fluorescent cells to cuticular structures projecting from the hindgut wall into the lumen of many higher termites, cockroaches, scarab beetle larvae, and millipedes (Bignell et al. 1980; Hackstein et al. 2006; Fig. 2b,c) might have similar explanations (see Hackstein et al. 2006 for a more detailed and excellently illustrated treatise of this topic).

2.5 Associations with Anaerobic Protists

An association of methanogens with intestinal protists was first observed with rumen ciliates (Vogels et al. 1980; Finlay et al. 1994) but subsequently detected also in termite gut flagellates (Odelson and Breznak 1985; Lee et al. 1987) and in anaerobic ciliates colonizing the guts of cockroaches and millipedes (Gijzen et al. 1991; Hackstein and Stumm 1994). The most obvious basis for the association is an exchange of molecular hydrogen, which is produced by anaerobic protists and consumed by their methanogenic symbionts (Fenchel and Finlay 1992).

Although an association of methanogens with gut flagellates is not uncommon in lower termites (Messer and Lee 1989; Shinzato et al. 1992; Radek 1994; Radek 1997; Tokura et al. 2000; Hara et al. 2004), it is not the rule. In *Zootermopsis* sp., only the smaller flagellates are colonized, whereas the larger species – the major hydrogen source – lack methanogenic symbionts (Lee et al. 1987; Messer and Lee 1989). Electron microscopy revealed that the flagellate-associated methanogens are not in direct contact with the hydrogenosomes (Radek 1994; Radek 1997), but in view of the high hydrogen concentrations throughout the hindgut lumen (Ebert and Brune 1997; Pester and Brune 2007), they should not be hydrogen limited even if their particular host itself does not produce any hydrogen (Fig. 2a). Also the small flagellates present in some cockroaches can harbor intracellular methanogens, whereas those in scarab beetle larvae are devoid of such symbionts (Hackstein and Stumm 1994).

An association of methanogens with anaerobic ciliates of the genus *Nyctotherus* and other clevelandellid species is common in cockroaches and certain millipedes (Hackstein and Stumm 1994; van Hoek et al. 2000; Bauer et al. 2015). Electron microscopy revealed that the endosymbiotic methanogens are in direct contact with

the hydrogenosomes of their protistan host (Akhmanova et al. 1998), which should help to overcome the low hydrogen partial pressure in the hindgut of cockroaches.

2.6 Intercompartmental Transfer of Hydrogen and Formate

While methanogenesis is always restricted to the hindgut of insects, microbial fermentations occur also in other gut compartments. In cockroaches, hydrogen can accumulate in the crop (*Panesthia angustipennis*; Bauer et al. 2015), midgut (*Blaberus* sp.; Lemke et al. 2001), or in both midgut and hindgut (*Shelfordella lateralis*; Schauer et al. 2012). In soil-feeding termites (*Cubitermes* spp.), hydrogen accumulates only in the anterior hindgut compartments, whereas methanogenic capacities are highest in the posterior, less alkaline gut regions (Schmitt-Wagner and Brune 1999; Kappler and Brune 2002). In all cases, methanogenesis in isolated hindgut compartments is strongly stimulated by an external supply of hydrogen, which indicates that the production of reducing equivalents by the microbial fermentations is a limiting factor.

An intercompartmental transfer of hydrogen from midgut to hindgut has been experimentally documented in a *Blaberus* sp. (Lemke et al. 2001). Methane production rates of isolated hindguts came close to the emission rates of living cockroaches only when the hindgut was incubated in the same vial as the hydrogen-producing midgut. When midgut and hindgut were placed in direct contact (mimicking the situation in vivo), steep concentration gradients indicated a strong flux of hydrogen across the adjoining borders of the two compartments (Fig. 2b). Also the stimulation of methanogenesis in the posterior hindgut compartments of *Cubitermes* spp. by external hydrogen can be explained by their close juxtaposition in the abdominal cavity of soil-feeding termites, which enables cross-epithelial transport of hydrogen between anterior and posterior gut segments (Schmitt-Wagner and Brune 1999).

Also the addition of formate to intact hindgut compartments stimulates methanogenesis in higher termites (*Cubitermes* spp.; Schmitt-Wagner and Brune 1999) and scarab beetle larvae (*Pachnoda ephippiata*; (Lemke et al. 2003). In this case, the compartments producing the methanogenic substrates (midgut and anterior hindgut) are not in direct contact with those harboring methanogens. The presence of considerable concentrations of formate in the hemolymph of soil-feeding termites (Tholen and Brune 1999) and scarab beetles (Stubblefield et al. 1966; Lemke et al. 2003) indicates that an intercompartmental transfer of reducing equivalents might occur via the hemolymph (Fig. 2c).

3 Diversity of Methanogens Associated with Insects

The methanogens that colonize insect guts belong almost exclusively to six genus-level lineages in the orders *Methanobacteriales*, *Methanosarcinales*, *Methanomicrobiales*, and *Methanomassiliicoccales*. Members of other euryarchaeotal orders (*Methanococcales*, *Methanocellales*, *Methanopyrales*,

and the recently discovered "Methanofastidiosa"; Nobu et al. 2016) have not been detected. Current knowledge rests mostly on cultivation-independent, 16S-rRNA-based surveys; only members of two genera have been isolated in pure culture. The lineages from insect guts are distinct from those that occur in ruminants or other mammals (Janssen and Kirs 2008), even when they fall into the same genus (Fig. 3).

3.1 Methanobacteriales

Almost all insects that emit methane are colonized by members of *Methanobacteriales*. Most of them fall into the radiation of the genus *Methanobrevibacter*. They are attached to the internal surface of the hindgut in termites, cockroaches, and scarab beetle larvae but are also associated with flagellate and ciliate protists that colonize the hindguts of these insects. Three *Methanobrevibacter* species from the lower termite *Reticulitermes flavipes* have been isolated in pure culture (Table 1). Like other *Methanobrevibacter* species isolated from the human gut or the rumen, they are exclusively hydrogenotrophic (Leadbetter and Breznak 1996; Leadbetter et al. 1998); their genomes have been sequenced (Poehlein and Seedorf 2016). Several isolates of hydrogenotrophic *Methanobacteriales* have been obtained from higher termites (Deevong et al. 2004). Although the strains were not deposited with a culture collection, they have been characterized to some extent. The *Methanobrevibacter* strain (from *Microcerotermes crassus*) was closely related to uncultured members of the invertebrate cluster (a sister group of *Methanobrevibacter arboriphilus*) and grew also on formate, whereas the *Methanobacterium* strains (from *Pericapritermes* sp., *Macrotermes gilvus*, and *Termes comis*) were close relatives of *Methanobacterium bryantii* and utilized also secondary alcohols.

The *Methanobrevibacter* lineages found in the guts of insects and millipedes are phylogenetically distinct from those in the rumen or colon of mammals (Fig. 3). Although phylotypes from the same host group often cluster with each other, the same host species can be colonized by distinct phylotypes, and closely related phylotypes are frequently present in distantly related hosts. This indicates that coevolution between the methanogens and their hosts is only diffuse, probably due to a mixed-mode transmission, as observed for members of the bacterial microbiota of termites (Bourguignon et al. 2018).

Interestingly, the endosymbiotic *Methanobrevibacter* phylotypes that colonize anaerobic ciliates of the genus *Nyctotherus* spp. in the hindgut of many cockroaches and certain millipedes are distinct from the *Methanobrevibacter* lineages present in termites or mammalian guts (van Hoek et al. 2000). Although the ciliates and their endosymbionts are vertically inherited by their respective hosts, there is ample evidence that they were repeatedly taken up from the environment (or exchanged) during their evolutionary history (Hackstein 2010).

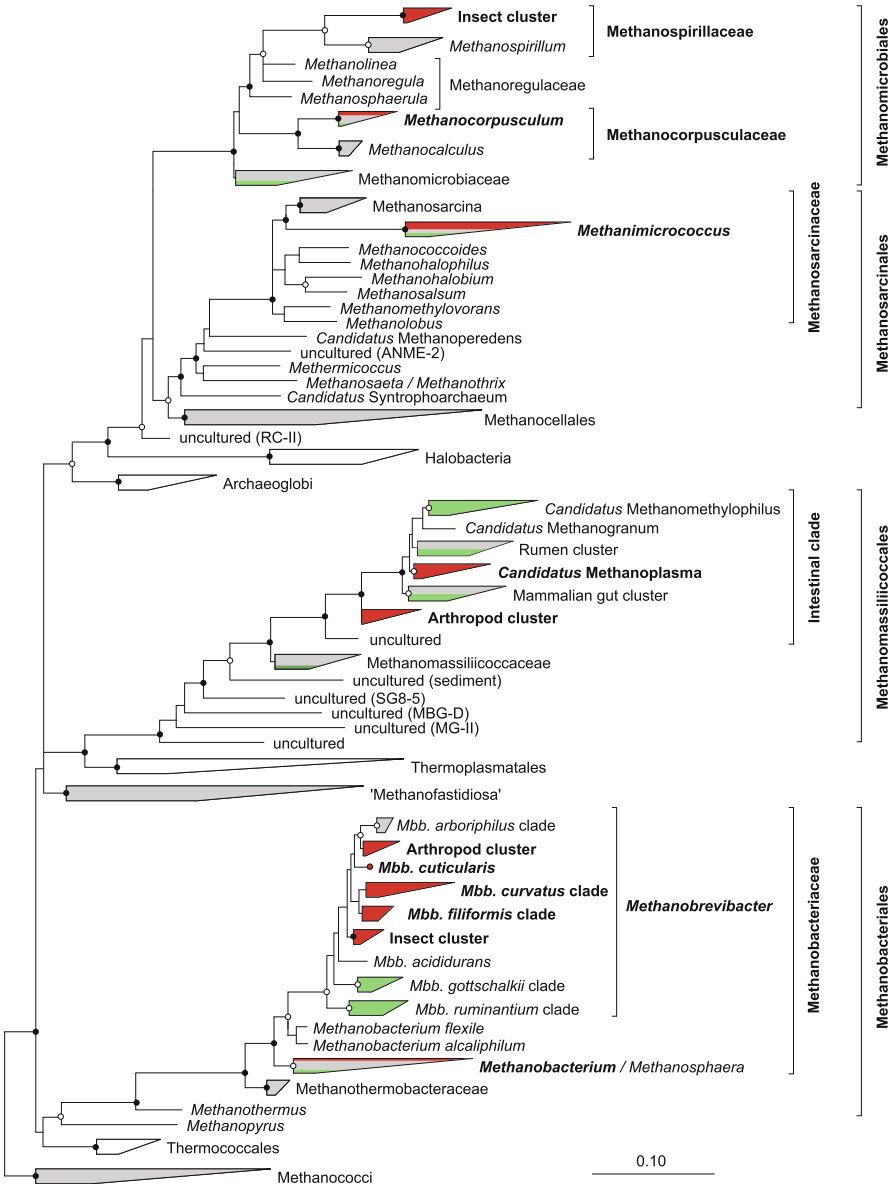

Fig. 3 Phylogenetic tree of methanogenic Euryarchaeota that illustrates the position of taxa that occur in the guts of insects (red) or mammals (green) relative to those from other environments (grey); nonmethanogenic clades appear in white. The names of the taxa that contain representatives from insect guts are in boldface. Simplified version of a larger maximum-likelihood tree, based on a manually curated alignment of near full-length 16S rRNA gene sequences; symbols indicate node support (●, >90%; ○, >70%)

3.2 Methanosarcinales

Most insects that emit methane also harbor members of the *Methanosarcinales*. They are not very diverse but fall into the radiation of the genus *Methanimicrococcus*, which comprises numerous phylotypes from cockroaches, higher termites, and scarab beetle larvae, but also several uncultured archaea recovered from the gut of mammals and from permafrost soil (Fig. 3). *Methanimicrococcus blatticola*, the only isolate of this group, is an obligately hydrogen-dependent methylotroph that reduces methanol or methylamines to methane (Sprenger et al. 2000); its genome remains to be sequenced (Table 1). The strict hydrogen requirement of methanogenesis from methanol is explained by its inability to generate reducing equivalents by the oxidation of methyl groups to carbon dioxide (Sprenger et al. 2005). The requirement of pure cultures for coenzyme M indicates a dependency on other methanogens in the hindgut. The substrate affinities of *M. blatticola* for hydrogen and methanol are higher than those of other methylotrophic methanogens (*Methanosphaera stadtmanae*, *Methanosarcina barkeri*), and since the use of methanol as the terminal electron acceptor is thermodynamically more favorable than the use of carbon dioxide, *M. blatticola* might have a competitive advantage over other methanogens at low hydrogen partial pressures (Sprenger et al. 2007).

3.3 Methanomicrobiales

Members of *Methanomicrobiales* colonize termites, cockroaches, and millipedes but have not yet been detected in scarab beetle larvae. They fall into two separate groups. One is a termite-specific lineage of uncultured *Methanospirillaceae* that represents a sister group of the genus *Methanospirillum* (Fig. 3). Members of this group have not been detected in lower termites or the fungus-cultivating Macrotermitinae, but a single clone was obtained from the cockroach *Salganea taiwanensis*. All other cockroach clones represent a lineage of uncultured archaea that falls into the radiation of the genus *Methanocorpusculum* but is well separated from the described species of this genus (which do not occur in intestinal environments) and other, uncultured relatives from mammalian guts. Isolates are not available for either group.

3.4 Methanomassiliicoccales

Analyses of archaeal diversity in the guts of termites (Shinzato et al. 1999; Friedrich et al. 2001), cockroaches (Hara et al. 2002), and scarab beetle larvae (Egert et al. 2003) revealed the presence of a deep-branching lineage that was distantly related to members of the nonmethanogenic *Thermoplasmatales*. The notion that members of the new lineage, which were subsequently detected also in the digestive tract of mammals, represent methanogenic Euryarchaeota was substantiated by a congruent phylogeny of the 16S rRNA genes and the corresponding *mcrA* genes (encoding

Table 1 Methanogens isolated from insect guts and a highly enriched culture with sequenced genomes

Order/Species	Source	Substrates	Type strain	Genome sequence	References
Methanobacteriales					
Methanobrevibacter cuticularis	*Reticulitermes flavipes*	$H_2 + CO_2$[a]	DSM 11139	LWMW00000000	Leadbetter and Breznak 1996; Poehlein and Seedorf 2016
Methanobrevibacter curvatus	*Reticulitermes flavipes*	$H_2 + CO_2$	DSM 11111	LWMV00000000	Leadbetter and Breznak 1996; Poehlein and Seedorf 2016
Methanobrevibacter filiformis	*Reticulitermes flavipes*	$H_2 + CO_2$	DSM 11501	LWMT00000000	Leadbetter et al. 1998; Poehlein and Seedorf 2016
Methanosarcinales					
Methanimicrococcus blatticola	*Periplaneta americana*	$H_2 + CH_3OH$[b]	DSM 13328	Not available	Sprenger et al. 2000
Methanomassiliicoccales					
Candidatus Methanoplasma termitum	*Cubitermes ugandensis*	$H_2 + CH_3OH$[c]	Enrichment culture	CP010070	Paul et al. 2012; Lang et al. 2015

[a]Also grows on H_2 + mono-, di- or trimethylamine. No growth on $H_2 + CO_2$
[b]Also grows on H_2 + monomethylamine. No growth on $H_2 + CO_2$
[c]Also grows slowly on formate

methyl-CoM reductase, a molecular marker for methanogenic archaea) recovered from the respective environments (Paul et al. 2012). The only isolate of the new order is *Methanomassiliicoccus luminyensis*, which was obtained from the human gut (Dridi et al. 2012), but members of a separate line of descent have been enriched from the guts of termites and millipedes (Paul et al. 2012), humans (Borrel et al. 2012), and an anaerobic digestor (Iino et al. 2013). They represent an intestinal clade, a family-level lineage of *Methanomassiliicoccales* that comprises uncultured archaea from both invertebrate and vertebrate hosts. The *Methanomassiliicoccaceae* represent an environmental clade, which (with the exception of *M. luminyensis* and its closest relatives) consists almost exclusively of phylotypes from peat soil (Lang et al. 2015; Söllinger et al. 2016).

The representatives from arthropod guts form two major lineages in the intestinal clade. One lineage comprises "*Candidatus* Methanoplasma termitum" and related phylotypes from termites, cockroaches, and millipedes (Paul et al. 2012) but lacks representatives from scarab beetle larvae. The other lineage is a phylogenetically distinct "arthropod cluster" that comprises phylotypes from all host groups (Fig. 3). Physiological characterization of the enrichment culture of "*Ca.* Methanoplasma termitum" and comparative analysis of its genome identified it as an obligately methyl-reducing hydrogenotroph (Lang et al. 2015; Table 1). This might explain the observation of Miyata et al. (2007), who found that members of the intestinal clade increased substantially in relative abundance when *Nasutitermes takasagoensis* was fed a diet consisting only of xylan, a substrate that contains substantial amounts of *O*-methylated glucuronic acid residues (Paul et al. 2012).

Although the morphology and ultrastructure of Ca. Methanoplasma termitum has been investigated in some detail (Lang et al. 2015), the exact location of these methanogens in the gut has not been clarified. Since all *Methanomassiliicoccales* lack coenzyme F_{420} (Lang et al. 2015), their visualization by epifluorescence microscopy will require in situ hybridization techniques.

4 Composition of the Methanogenic Communities

The methanogenic communities in insect guts have been investigated in a number of studies, using the 16S rRNA gene as a molecular marker. As in the case of the rumen, they consist only of a few genus-level taxa (Table 2). The clone libraries obtained in many of the earlier studies are quite small; therefore, relative abundances of individual lineages and their absence from certain hosts have to be interpreted with caution. Nevertheless, the phylogenetic framework provided by these sequences forms a robust basis for the classification of short reads in amplicon libraries generated by high-throughput sequencing approaches. However, the primer bias inherent to all PCR-based techniques will eventually be overcome by metagenomics datasets.

Although methanogens in arthropod guts are easily visualized by epifluorescence microscopy (with the exception of *Methanomassiliicoccales*, see above), their

Table 2 Genus-level lineages of methanogenic archaea represented in different groups of insects and their presence in julid millipedes. The table is based on information from clone libraries of 16S rRNA genes in hindgut DNA amplified with Archaea primers. Parentheses indicate that a lineage was not regularly encountered. The information was compiled from numerous references (for details, see text)

Lineage (order/genus)	Cockroaches[a]	Lower termites	Higher termites	Scarab beetles[b]	Millipedes[c]
Methanobacteriales					
Methanobrevibacter	+	+	+	+	+
Methanobacterium		(+)[d]	+[d]		
Methanomassiliicoccales					
Candidatus Methanoplasma	+	(+)[e]	+		+
Arthropod cluster (intestinal clade)	+		+	(+)[f]	+
Methanosarcinales					
Methanimicrococcus	+		+	(+)[f]	
Methanomicrobiales					
Methanocorpusculum	+	(+)[e]			
Insect cluster (*Methanospirillaceae*)	(+)[g]		+		

[a]Only two genera of Blaberidae (*Panesthia* and *Salganea* spp.) have been systematically studied
[b]Only two genera of Scarabaeidae (*Pachnoda* and *Melolontha* spp.) have been studied
[c]Short reads reamplified from a DGGE analysis of juliform millipedes (Sustr et al. 2014) and classified using the reference tree (Fig. 3). For additional taxa, see text
[d]Detected only by cultivation or high-throughput sequencing (Deevong et al. 2004; Rahman et al. 2015)
[e]Only a single clone has been recovered from *Reticulitermes speratus* (Shinzato et al. 1999)
[f]Occur only in the humivorous *Pachnoda ephippiata* (Egert et al. 2003)
[g]Only a single clone has been recovered from *Salganea taiwanensis* (Hara et al. 2002)

association with food particles, intestinal surfaces, and protistan cells makes them difficult to count. Most-probable-number estimations and viable counts of methanogens have been obtained only for the termite *Reticulitermes flavipes* (volume < 1 µl) and yielded about 10^6 methanogens per gut, which is equivalent to about 5% of the prokaryotic cells (Leadbetter and Breznak 1996; Tholen et al. 1997). Hybridization of RNA extracted from the guts of a wide range of termite species with domain-specific oligonucleotide probes indicated that the average proportion of archaeal rRNA to all prokaryotic rRNA was only 1.5%, with a higher fraction in soil-feeding species ($2.3 \pm 0.5\%$) than in wood-feeding and fungus-cultivating species ($0.9 \pm 0.5\%$) (Brauman et al. 2001). Although such numbers are inherently inaccurate, they underscore that methanogens are far outnumbered by bacterial cells.

This is in agreement with amplicon sequencing studies with both universal and prokaryote primers, where the numbers of archaeal reads obtained for *Reticulitermes* species ranged between 0.1 and 0.2% of the reads classified as

prokaryotes (Boucias et al. 2013; Rahman et al. 2015). However, also the accuracy of this method is put into question by astonishingly high numbers of archaea obtained for other termites (e.g., 21–33% in *Coptotermes*, 17–27% in *Schedorhinotermes*, 50–58% in *Porotermes*; Rahman et al. 2015), which, however, is not supported by substantially higher methane emission rates of these termite genera.

4.1 Lower Termites

The methanogenic communities in the hindgut of lower termites have been studied in representatives of several families (Archotermopsidae: *Hodotermopsis sjoestedti*; Kalotermitidae: *Cryptotermes* and *Neotermes* spp.; Mastotermitidae: *Mastotermes darwiniensis*; Rhinotermitidae: *Coptotermes* and *Reticulitermes* spp.; Ohkuma et al. 1995; Ohkuma and Kudo 1998; Ohkuma et al. 1999; Shinzato et al. 1999; Shinzato et al. 2001). They consist almost exclusively of *Methanobrevibacter* species (Table 2), which are attached to the hindgut wall (Fig. 4) but also associated with filamentous bacteria (Leadbetter and Breznak 1996) or certain gut flagellates (see above). Many lower termites harbor more than one lineage of

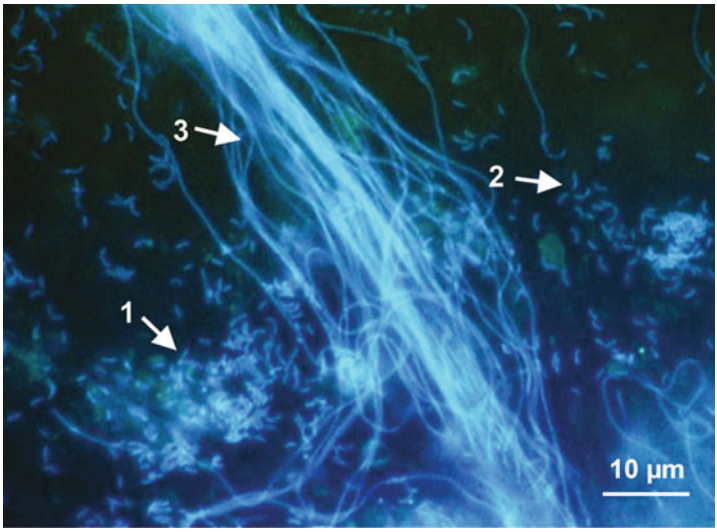

Fig. 4 Methanogens associated with the hindgut wall of *Reticulitermes flavipes*, visualized by the autofluorescence of their coenzyme F_{420}. The arrows point to the characteristic morphotypes of *Methanobrevibacter cuticularis* (1), *Methanobrevibacter curvatus* (2), and *Methanobrevibacter filiformis* (3). Microphotograph courtesy of J. R. Leadbetter and J. A. Breznak. (Originally published in Brune (2010a), with kind permission of © Springer Science+Business Media New York. All rights reserved

Methanobrevibacter. The phylotypes associated with gut flagellates are phylogenetically distinct from those attached to the hindgut cuticle (Tokura et al. 2000; Hara et al. 2004; Inoue et al. 2008), which suggests their specific adaptation to the intracellular habitat.

High-throughput amplicon sequencing of the gut microbiota of *Reticulitermes flavipes* (Boucias et al. 2013) and several other species (including *Heterotermes* and *Schedorhinotermes* spp.; Rahman et al. 2015) confirmed the prevalence of *Methanobrevibacter* in lower termites, but also revealed the occasional presence of lineages that had been previously encountered only in higher termites and other insects. The complete absence of archaeal reads from samples of certain Kalotermitidae (*Incisitermes*, *Glyptotermes,* and *Marginitermes* spp.) amplified with universal primers is in agreement with the absence of methanogenesis from certain dry-wood termites (see below).

4.2 Higher Termites

The hindgut of higher termites is typically more compartmented than those of lower termites, with a pronounced dynamics of physicochemical conditions along the gut axis, particularly in the soil-feeding species (Fig. 5a, b). The fungus-cultivating Macrotermitinae, which lack the hindgut compartmentation and alkaline pH characteristic for the other subfamilies, are an exception (Brune 2014). Clone libraries of methanogens are available for *Macrotermes*, *Odontotermes* (Macrotermitinae), *Nasutitermes*, *Trinervitermes* (Nasutitermitinae), *Cubitermes*, *Ophiotermes* (Cubitermitinae), *Alyscotermes* (Apicotermitinae), *Pericapritermes*, and *Microcerotermes* (Termitinae) (Ohkuma et al. 1999; Friedrich et al. 2001; Donovan et al. 2004; Miyata et al. 2007; Paul et al. 2012; Shi et al. 2015).

The methanogenic communities of higher termites are more diverse than those in lower termites and comprise representatives of *Methanomicrobiales*, *Methanosarcinales*, *Methanobacteriales*, and *Methanomassiliicoccales* (Table 2). Representatives of all four orders have been recovered from Termitinae and Cubitermitinae and were detected also in the short-read libraries obtained from *Syntermes wheeleri* (Syntermitinae) (Santana et al. 2015). Clone libraries of Macrotermitinae lack clones affiliated with the *Methanomicrobiales*, whereas those of Apicotermitinae and Nasutitermitinae yielded no *Methanosarcinales*. However, short-read libraries of wood-feeding *Nasutitermes* spp. yielded representatives of all four orders (Rahman et al. 2015). The results obtained for *Drepanotermes*, *Gnathamitermes*, *Macrognathotermes* (Termitinae), and *Tenuirostritermes* (Nasutitermitinae) differed between colonies and were not always consistent with those previously obtained for other members of these groups (Rahman et al. 2015). Obviously, the hypothesis that vertical inheritance is a major driver of community structure in higher termites remains to be seriously tested.

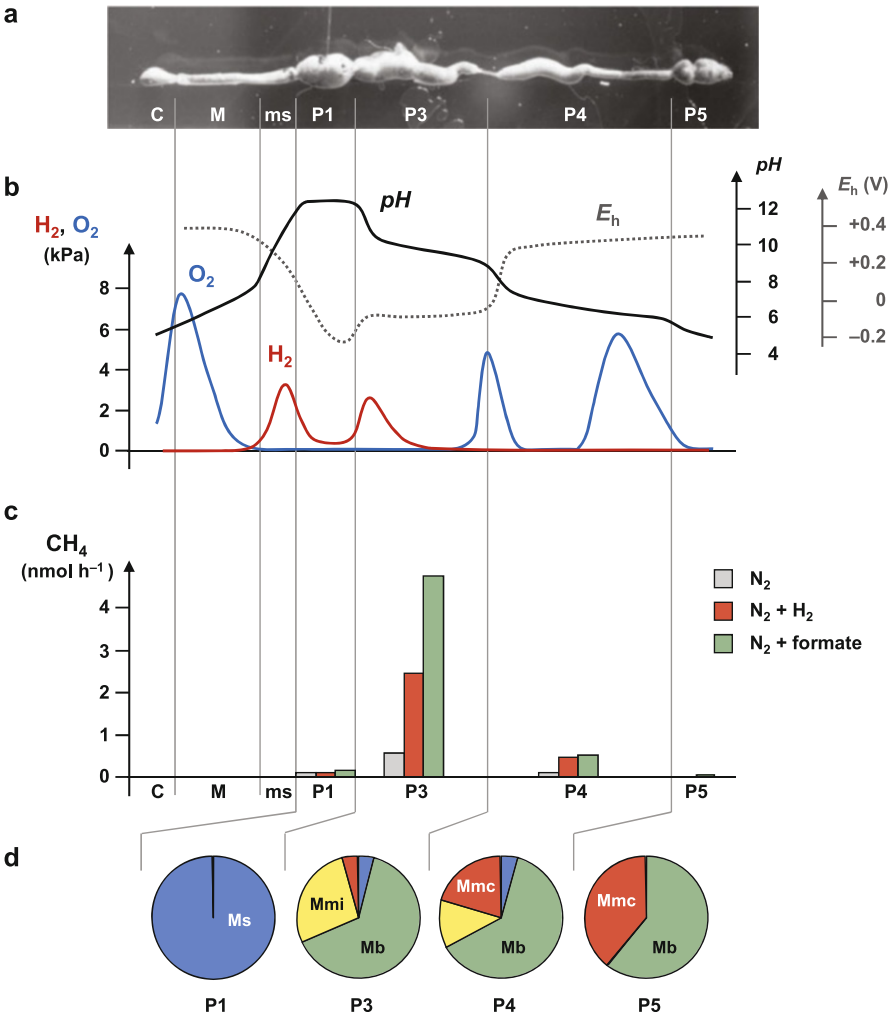

Fig. 5 Gut morphology (a) and microsensor profiles (b) of oxygen, hydrogen, and redox potential (E_h) along the gut axis of a soil-feeding termite (*Cubitermes* spp.). Methane emission rates (c) were determined with isolated gut sections incubated under a N_2 headspace with or without addition of H_2 or formate. Relative abundance of methanogens (d) in 16S-rRNA-based clone libraries of the respective gut sections (Ms, *Methanosarcinales*; Mb, *Methanobacteriales*; Mmi, *Methanomicrobiales*; Mmc, *Methanomassiliicoccales*). Vertical lines indicate the borders between the different gut regions. Scheme based on various studies (Brune and Kühl 1996; Schmitt-Wagner and Brune 1999; Friedrich et al. 2001; Kappler and Brune 2002). Originally published in Brune (2010a), with kind permission of © Springer Science+Business Media New York. All rights reserved

In soil-feeding *Cubitermes* species, methanogenic potential and community structure differ among the hindgut compartments (Fig. 5c, d). Many of the microbial cells attached to the gut wall or to cuticular spines projecting into the lumen show the characteristic autofluorescence of methanogens

(Schmitt-Wagner and Brune 1999) but remain to be assigned to the various phylogenetic groups.

4.3 Cockroaches

An association with methanogens has been reported for members of most cockroach families (Hackstein and Stumm 1994; Hackstein and van Alen 2010). However, community structure has been analyzed only in wood-feeding *Panesthia* and *Salganea* species (Blaberidae) (Hara et al. 2002), which harbor the same lineages of methanogens as higher termites (Table 2). In all other cockroaches, only the diversity of the endosymbiotic *Methanobrevibacter* lineages associated with the ciliate *Nyctotherus* has been studied in detail (van Hoek et al. 2000).

In the hindgut of *Periplaneta americana* (Blattidae), free-living microbial cells with the autofluorescence of methanogens comprised short rods, motile spirilla, and irregular cocci (Sprenger et al. 2000). The latter were localized at the hindgut wall and identified as *Methanimicrococcus blatticola*, an obligate methylotroph isolated from the same cockroach species. The predominance of *Methanimicrococcus blatticola* among the Euryarchaeota in *Periplaneta americana* was confirmed by short-read amplicon sequencing (Tinker and Ottesen 2016).

The addition of methanol strongly stimulated methanogenesis in hindgut homogenates of *Periplaneta americana*, and methanol concentrations in the homogenates were increased by the addition of pectin (which is rich in *O*-methyl residues) or bromoethanesulfonate (BES), a specific inhibitor of the methyl-CoM reductase of methanogenic archaea (Sprenger et al. 2007). Notably, the dominant phylotypes in the clone libraries of wood-feeding cockroaches, whose diet should be rich in pectin and xylan (another source of *O*-methyl residues), fall into the radiation of potentially methylotrophic lineages (*Methanimicrococcus* and the arthropod cluster of *Methanomassiliicoccales*; Hara et al. 2002; Paul et al. 2012).

4.4 Scarab Beetles

It has been reported that the larvae of numerous scarab beetles emit methane, and cells with F_{420}-autofluorescence are closely associated with the chitinous lobe-like structures protruding into the hindgut paunch (Hackstein 2010). The methanogens have been identified only in two species, namely, the humivorous larva of *Pachnoda ephippiata* (Egert et al. 2003) and the phytophagous larva of *Melolontha melolontha* (Egert et al. 2005). In both species, the community is dominated by members of the genus *Methanobrevibacter,* which belong to same lineages as those encountered in termites. The methanogenic community in the larvae of *M. melolontha* consists exclusively of a single phylotype of *Methanobrevibacter*, whereas clone libraries of *P. ephippiata* larvae yielded also phylotypes of *Methanimicrococcus blatticola* and an unclassified lineage in the intestinal cluster of *Methanomassiliicoccales* (Table 2).

4.5 Other Arthropods

Little is known about the methanogenic communities in millipedes. Unlike the paunch of wood- and litter-feeding insects, the hindgut is not dilated, but its inner surface is strongly developed, which offers opportunity for microbial colonization (Byzov 2006; Nardi et al. 2016). Positive redox potentials in the range of +200 mV were recorded in the hindgut of *Glomeris marginata* (Glomerida; Bignell 1984a), a species that does not emit methane but does emit hydrogen. However, when co-cultivated with methane-producing millipedes, a *Glomeris* sp. started to emit methane as well, which showed the potential to be colonized by methanogens (Hackstein and Stumm 1994).

All methane-emitting millipedes harbor ciliates that are associated with intracellular methanogens, and in *Orthoporus* and *Rhapidostreptus* spp., cells with the characteristic autofluorescence of coenzyme F_{420} were observed also outside of these protists (Hackstein and Stumm 1994). Fingerprinting analysis of short 16S rRNA gene fragments (DGGE) amplified from the hindguts of various juliform millipedes revealed that archaeal community structure differs between species (Sustr et al. 2014). A classification of the short sequences obtained by reamplification of individual bands excised from the DGGE gel confirmed the presence of phylotypes that belong to the same lineages in the genus *Methanobrevibacter* and the intestinal cluster of *Methanomassiliicoccales* encountered in insects (Table 2). Other phylotypes fell into the radiation of the genera *Methanosarcina*, *Methanocella*, and unclassified members of Rice Cluster II, which are represented also in clone libraries prepared from the food soil of humivorous scarab beetles (Egert et al. 2003) and soil-feeding termites (Donovan et al. 2004).

Although copepods are quite small, their intestinal tract can be entirely anoxic in the central metasome region (Tang et al. 2011). The methanogens identified in marine copepods and their fecal pellets belong to genera different than those colonizing terrestrial arthropods (Ditchfield et al. 2012). 16S rRNA gene sequences were most closely related to the saltwater isolates *Methanogenium organophilum*, *Methanolobus vulcani*, *Methanobacterium uliginosum*, and an uncultured member of the marine group II of *Methanomassiliicoccales*, which suggests a potential for both hydrogenotrophic and methylotrophic methanogenesis. The methanogens in the guts of the tropical earthworm *Eudrilus eugeniae* belong to the genera *Methanobacterium*, *Methanoculleus*, and *Methanosarcina*, which are typically encountered in soils but not in intestinal habitats and might not represent autochthonous populations (Depkat-Jakob et al. 2012).

5 Methane Emission by Soil Arthropods

5.1 Methane Emission Rates Differ Between Taxa

Methane production differs strongly between the major groups of arthropods (Table 3). High emission rates are generally encountered among termites and scarab beetle larvae. With the exception of several Kalotermitidae (e.g., Brauman et al.

Table 3 Methane emission rates of insects and other soil arthropods in comparison to that of cows and humans. Values are given in nmol g^{-1} h^{-1} and are based on fresh weight. *S* is the number of species tested (in the averages, only methane-emitting species were included). Data stem from studies using different experimental setups and were compiled from various sources (see footnotes)

Group	Diet	Range (nmol g^{-1} h^{-1})	Average (nmol g^{-1} h^{-1})	*S*
Isoptera				
Lower termites[a,b,c]	Wood	0–1300	200	17
Macrotermitinae[a,b,c,d]	Wood, plant litter	20–670	170	12
Termitinae (wf)[a,c]	Wood, grass	40–210	120	11
Termitinae (sf)[a,c,d]	Humus, soil	150–1090	440	23
Nasutitermitinae[a,b,c,d]	Wood, grass	40–240	140	10
Apicotermitinae[d]	Humus, soil	50–700	280	7
Blattodea[e]	Varied	0–268	46	9
Scarabaeidae[e]	Humus	0–741	255	7
Diplopoda[e]	Leaf litter	0–415	58	6
Cows[f]	Grass	1350–2230	1810	
Humans[g]	Varied	0–120	8	

Data from [a]Brauman et al. 1992; [b]Shinzato et al. 1992; [c]Sugimoto et al. 1998b; [d]Bignell et al. 1997; [e]Hackstein and Stumm 1994; [f]Kinsman et al. 1995; [g]Bond Jr et al. 1971 (calculated from pulmonary emissions, assuming a body weight of 75 kg and a colonic absorption of 20%)

1992; Sugimoto et al. 1998b; Yanase et al. 2013), all termites investigated emit methane, but rates vary strongly between taxa. The wide variation of emission rates between and within taxa and their seasonal and diurnal fluctuations (e.g., Wheeler et al. 1996; Sugimoto et al. 1998b) confound estimates of methane production for individual species. Almost all scarab beetles investigated formed methane, but not all subfamilies were tested (Hackstein and van Alen 2010). A discontinuous gas exchange in scarab beetle larva leads to a synchronous release of CH_4 and CO_2 several times per hour (Bijnen et al. 1996). Also the spiracular control of oxygen uptake, as shown for *Zootermopsis* species (Lighton and Ottesen 2005), should affect the efflux rates of methane and (via the influx of oxygen) also the activity of methanogens residing at the hindgut wall (Tholen et al. 2007).

Methane emission rates of cockroaches and millipedes are slightly lower than those of termites and scarab beetle larvae. Methane emission is widespread also among cockroaches, but not present in all lineages (Hackstein and van Alen 2010). In millipedes, methane formation is restricted to the juliform taxa (Hackstein and Stumm 1994; Sustr and Simek 2009; Sustr et al. 2014). The methane emission rates observed with juliform millipedes from temperate habitats (ranging from 0.4 to 3.2 nmol g^{-1} h^{-1} at 5 and 25 °C, respectively) are much lower than those of tropical species (Sustr and Simek 2009). The methane observed with methane emitting earthworms were in a similar range (Depkat-Jakob et al. 2012).

5.2 Influence of Diet on Methane Production

Methane production by arthropods seems to be restricted to those that thrive on fiber-rich food, but only a few studies tested the relationship between diet and methane production in insects. In the cockroach *Periplaneta americana*, methane emission varies under different feeding regimens (Gijzen et al. 1991; Zurek and Keddie 1998). Differences in the pattern of fermentation products indicated that methanogenesis is the major electron sink in the hindgut of individuals fed on high-fiber diet, whereas a low-fiber diet favors reductive acetogenesis and the accumulation of formate (Kane and Breznak 1991; Zurek and Keddie 1998). A pectin-rich diet did not strongly stimulate the methane emission of *P. americana*, which indicates that methanogenesis by the obligately methylotrophic *Methanimicrococcus blatticola* is hydrogen limited under in situ conditions (Sprenger et al. 2007).

It is more difficult to experimentally establish different feeding regimens in termites. Nevertheless, there is a clear trend in the methane emission rates of termites that naturally feed on different diets. Methane emission is lowest in termites that feed on sound wood and increases from other wood-feeding and fungus-cultivating species to humivorous wood–soil interface feeders and true soil feeders (Wandiga and Mugedo 1987; Brauman et al. 1992; Shinzato et al. 1992; Rouland et al. 1993; Bignell et al. 1997; Sugimoto et al. 1998b). Studies on the effect of artificial diets on methanogenesis are scarce, but the relative abundance of clones in the intestinal clade of *Methanomassiliicoccales* (Fig. 3) increased substantially when *Nasutitermes takasagoensis* was fed a diet consisting only of xylan (Miyata et al. 2007). This effect is most likely related to the increased supply of methanol released during the degradation of the *O*-methylated glucuronic acid residues of xylan (Paul et al. 2012). However, at that time the "uncultured Thermoplasmatales" were considered to represent a nonmethanogenic lineage, and the effect of the dietary shift on methanogenesis was not tested.

5.3 Do Methanogens Benefit Their Host?

Although the presence of methanogens should stimulate the fermentative breakdown of organic matter (Schink 1997), there is no evidence that colonization by methanogens increases the fitness of an insect. However, the number of studies addressing this question is limited. The inclusion of bromoethanesulfonic acid (BES) in the drinking water of insects eliminates the methanogens from their guts. However, such treatment had no effect on the survival of *Zootermopsis angusticollis* (Messer and Lee 1989) and did not affect the body weight and the number of *Nyctotherus ovalis* cells in the hindgut of *Periplaneta americana* (Gijzen and Barugahare 1992; Zurek and Keddie 1998). The wide variation in methane emission rates among termite colonies (e.g., Shinzato et al. 1992; Wheeler et al. 1996) and

individuals of the same cockroach species (Hackstein and Stumm 1994) suggests that the presence of methanogens does not provide a strong advantage to the host.

5.4 Absence of Methane Oxidation in Termites

In many environments, a large fraction of the methane produced in the anoxic zone is reoxidized at the anoxic–oxic interface. Although the counter-gradients of methane and oxygen in the periphery of the hindgut provide seemingly ideal conditions for aerobic methane oxidation (Brune et al. 2000), there is no evidence for the presence of methanotrophic bacteria or their activities in termite guts. Methane emissions of termite species from different families do not significantly increase when the animals are incubated under anoxic conditions (Messer and Lee 1989; Pester et al. 2007), and isotope tracer studies confirmed that $^{14}CH_4$ added to the headspace of vials containing live termites (*Reticulitermes flavipes* or *Cubitermes orthognathus*) is not converted to $^{14}CO_2$ (Pester et al. 2007). Members of methanotrophic lineages are not represented in any 16S rRNA clone libraries and are almost always below the detection limit in the short reads obtained by high-throughput amplicon sequencing. Attempts to amplify the *pmoA* gene, which encodes the particulate methane monooxygenase, a molecular marker for most methanotrophs, from DNA extracted from termite hindguts were unsuccessful (Pester et al. 2007).

While this eliminates the gut itself as a methane sink, the material of termite mounds and the surrounding soil proved to be an important factor mitigating the production of this important greenhouse gas at the environmental level. Field measurements indicated that net emissions of methane from termite nests into the atmosphere are often much smaller than methane production estimated on the basis of the termite population in the nest (e.g., Seiler et al. 1984; Khalil et al. 1990; Delmas et al. 1992; Bignell et al. 1997; MacDonald et al. 1998; MacDonald et al. 1999; Eggleton et al. 1999). The relationship between CH_4 and CO_2 fluxes from termite mounds and the internal concentration ratios of these gases vary greatly among termite species (Jamali et al. 2013). Up to 40% of the methane produced within mounds of *Coptotermes lacteus* is oxidized before it reaches the atmosphere (Khalil et al. 1990), whereas the mound material of *Macrotermes jeanneli* shows no significant methane oxidation capacity (Darlington et al. 1997).

Since many termites build either huge or diffuse nests, it is extremely tedious or even impossible to determine the population of termites in a colony and its net emission of methane into the atmosphere. It was therefore an important conceptual advance when Sugimoto et al. (1998a) compared the carbon isotope ratios ($^{13}C/^{12}C$) of methane produced by termites with that emitted from the mounds. Owing to the preferential conversion of the lighter isotope by methane-oxidizing bacteria, the residual methane becomes enriched in the heavier isotope, and the extent of this shift, combined with the fractionation factor of methane oxidation, allows an estimation of the proportion of methane oxidized *en route* to the atmosphere. The

results of this approach were striking because they convincingly documented that – contrary to the predictions from laboratory data – the net emissions of methane from the colonies of soil-feeding termites are much lower than those of wood-feeding termites (Sugimoto et al. 1998a; Sugimoto et al. 2000).

Since the activities of termites as ecosystem engineers also physically disturb the soil structure, it can be expected that the presence of termites will affect ecological niches and alter the community composition of methanotrophs (Kumaresan et al. 2011). The presence of termites stimulates the methane-oxidizing activity of soils and changes the structure of the methanotrophic community (Ho et al. 2013). Also the methane emissions of scarab beetle larvae might cause hot spots of methane oxidation in meadow soil (Kammann et al. 2009).

5.5 Insects as a Source of Atmospheric Methane

The first reports on methane production by termites immediately aroused the interest of atmospheric chemists. Initial estimates of the contribution of termites to global methane emissions were enormous (Zimmerman et al. 1982) but were criticized by other workers because of a nonrepresentative choice of termite species and an overestimation of termite biomass and food consumption (Rasmussen and Khalil 1983; Collins and Wood 1984). In the following years, increased taxon sampling, new data on termite biomass, and the introduction of scaling factors, which account for the ratio of wood-feeding to soil-feeding termites in a given region, increased the accuracy of the estimates but decreased the numbers by at least an order of magnitude (for critical reviews, see (Sanderson 1996; Bignell et al. 1997; Sugimoto et al. 1998a; Bignell 2010). Sugimoto et al. (2000) came to the conclusion that the contribution of termites to global methane emissions is probably less than 10 Tg per year (1.5–7.4 Tg) but almost certainly below 20 Tg per year – the number that is still used in the global budget of the Intergovernmental Panel on Climate Change (IPCC; Denman et al. 2007). Nevertheless, even these estimates are still confounded by a lack of data on most termite taxa, the wide variation of methane emission rates among the same termite species and their seasonal and diurnal fluctuations (see above), and the seasonal changes in termite biomass and behavior in a colony (Jamali et al. 2011a; Jamali et al. 2011b; Sawadogo et al. 2012). However, there is consensus that the contribution of termites to the total source strength of methane (ca. 600 Tg per year) is quite small (Bignell 2010) and dwarfed by the sources under anthropogenic influence (Kirschke et al. 2013).

The contributions of cockroaches, scarab beetles, and millipedes to global methane emission have received only little attention. Based on their laboratory measurements and on rough estimates of the biomass distribution of higher arthropod taxa in the tropics and subtropics, Hackstein and Stumm (1994) pointed out that millipedes and scarab beetles might represent another globally important source of methane. However, the methane emission rates of millipedes in temperate soils are too low to affect the regional budgets of this greenhouse gas (Sustr and Simek 2009). The fact

that most upland soils are sinks for methane indicates that methane emissions of soil macroinvertebrates are generally compensated by methane oxidation.

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Methanogenesis at High Latitudes

10

Xiuzhu Dong, Jianqing Tian, Lei Qi, and Lingyan Li

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Abstract

Methane (CH_4) is the second greenhouse gas after carbon dioxide (CO_2), and a quite portion of CH_4 is released from permafrost and cold wetlands at high latitudes and altitudes. Global warming is causing permafrost thawing that results in release of the permafrost stored ancient carbon by microbial degradation at elevated temperatures. Methanogenesis is exclusively implemented by methanogenic Archaea although thus far only a few of the psychrophilic or psychrotolerant methanogen species have been cultured. In this chapter, we present methanogenesis pathways prevalent in the cold regions at both the earth poles and the high altitude Tibetan Plateau, as well as information on cold adapted methanogens that are responsible for the methane production. At the last, we show the distinct cold adaptive mechanisms found in methanogenic Archaea.

1 Introduction

Methane (CH_4) has a high warming potential, so is an important greenhouse gas. During the last decade, the natural sources of CH_4 account for 35–50% of the decadal mean global emissions, and proximately 85% is biogenesis. Among which, wetlands contribute 177–284 Tg CH_4 yr^{-1} (Stocker et al. 2013). Permafrost presently covers approximately 25% of the earth's land area and contains vast amounts of biogenic methane (over 5000 Tg in the ice portion alone) (Bousquet et al. 2011) and organic carbon because of the slow microbial degradation at low temperature. Global warming causes permafrost melting and the temperature increasing could lead to a sudden release of methane into the atmosphere. Biological methanogenesis occurs in anoxic environments through microbial degradation of organic carbon complexes, like cellulose and starch, by multispecies through a food-chain mode, and the terminal process methanogenesis is limited to methanogenic archaea which affiliate with Euryarchaeota.

1.1 High Latitudes

High latitude regions refer to 60°N and 60°S to North and South poles, respectively, and are within either the Arctic or the Antarctic circles. These areas are characterized by a cold climate. Most of the Antarctic continent is permanently covered by ice and snow, while the Arctic region has seasonally varying snow and ice cover with the predominant treeless permafrost-containing tundra and peatlands. Avis et al. (2011) indicated that more than 50% of wetlands are located in the high northern latitudes, and the permafrost exerts a primary control of the wetland hydrology. Because of the inert microbial degradation at low temperature, the north high-latitudes store proximately one-third of the global organic carbon pool (Jungkunst 2010) and is identified as a region that is vulnerable to global climate change (Zhang et al. 2003; Trenberth et al. 2007; Jeffries et al. 2015) because the ancient soil carbons will be subject to an increased microbial degradation of organic matter to release CH_4 and CO_2 gases.

Arctic warming has been accelerated since the 1980s and driven an array of complex physical and ecological changes in the region (Smith et al. 2005). One likely ramification of this warming will be the increased vulnerability of the large carbon reservoirs in the Arctic and boreal permafrost (Schuur and Abbott 2011). Under the future high warming scenario, 9–15% of the top 3 m of permafrost are estimated to be degraded by the year of 2040 and with 30–63 billion tons of carbon released, and 67–79% of permafrost degradation with a concomitant 549–865 billion tons of carbon released by 2300 (Schuur and Abbott 2011). Those released carbon in form of climate-relevant trace gases from intensified microbial carbon turnover would further increase global warming and convert the Arctic tundra ecosystems from a carbon sink to a carbon source (Gao et al. 2013).

Methane emission from the arctic and subarctic regions is estimated to range from 17 to 42 Tg CH_4 $year^{-1}$ (Christensen et al. 1996; Corradi et al. 2005; van Huissteden et al. 2005) corresponding to about 25% of global CH_4 emission from natural sources (Fung et al. 1991). Therefore, many studies on methane release from permafrost have been carried out in Arctic region, and Ace Lake in Antarctica, a marine-derived meromictic lake. Studies on Antarctica methanogenesis were mainly from Cavicchioli laboratory on *Methanococcoides burtonii* isolated from Ace Lake, Vestfold Hills, where low methanogenesis rate of 2.5 μmol kg^{-1} day^{-1} at 20 m has been detected (Cavicchioli 2006). By using the psychrophilic methanogen *M. burtonii as a model*, the cold adaptive mechanisms of Archaea have been studied on the basis of cold responding omics.

1.2 High Altitude

Tibetan Plateau, though located at lower latitude (25°N~40 °N, 74 °E~104 °E) and having an average altitude of about 4000 m above sea level, is widely distributed of cold wetlands and permafrost lakes. Lakes on Tibetan Plateau cover a total area of more than 5.1×10^4 km^2 and are surrounded by 3.8×10^4 km^2 of wetlands. Zoige wetland at Tibetan plateau is located at a low latitude (33°56′N, 102°52′E) region but at a high altitude (average 3500 m above sea level); the annual temperature is around 1 °C, by the highest monthly mean temperature of 9.1–11.4 °C in July, and the lowest of −8.2–10.9 °C in January (Jin et al. 1999). This wetland is one of main CH_4 emission centers of China. Different from other high latitude wetlands, Zoige wetland is covered with flourished vegetation. Therefore, methanogenic pathways and the methanogen community in the wetland have been investigated in recent years.

2 Biological Methane Cycling in the Permafrost and Wetlands at High Latitudes

Under anaerobic conditions, organic matter is degraded in a four-step process: (1) hydrolysis of polymers by hydrolytic microorganisms, (2) acidogenesis by fermentative bacteria, (3) acetogenesis by homoacetogenic or syntrophic bacteria, and (4) methanogenesis by methanogenic Archaea (Garcia et al. 2000).

Methanogens are strict anaerobes which share a complex biochemistry for methane synthesis as part of their energy metabolism. They are widely distributed in natural habitats, such as rice paddy soil (Conrad 2009), sediments, hydrothermal vents and high saline environments (Garcia et al. 2000), permafrost soils, peatlands, and sediments (Karr et al. 2006; Rivkina et al. 2007; Kotiaho et al. 2010). Methanogens are capable to convert a limited number of carbon substances (e.g., carbon dioxide and hydrogen, acetate, formate, methanol, and methylated amines) to CH_4. According to the carbon substances they use, methanogens have been classified as three categories: (1) hydrogenotrophs use H_2 to reduce CO_2 to produce CH_4 and refer to hydrogenotrophic methanogenesis; (2) acetoclastic methanogens ferment acetate to CH_4 and CO_2 referring to acetoclastic methanogenesis; and (3) methylotrophs use methyl compounds to produce CH_4 and are defined as methylotrophic methanogenesis (Garcia et al. 2000). Relative contributions to CH_4 production of the three pathways vary in different ecosystems. In most cold environments, acetoclastic methanogenesis is prevalent which could contribute for 67% of total methane production (Conrad 1999), while methylotrophic methanogenesis is a predominant pathway in Zoige wetland at Tibetan Plateau, which has significant amount of permafrost around 35°N (Jiang et al. 2010). In northern acidic peatlands, hydrogenotrophic methanogenesis is dominant in methane production (Metje and Frenzel 2005; Kotsyurbenko et al. 2007; Yavitt et al. 2012). Studies on different types of boreal wetlands with acidic peat bogs in Finland have revealed distinct methanogenic pathways and different methanogenic communities (Galand et al. 2005), which is most likely related to the vegetation.

Although high-latitude environments are characterized by extreme climate conditions, the abundance and composition of the methanogens are similar to temperate soil ecosystems (Wagner et al. 2005). The highest cell counts of methanogens in the active layer of permafrost are up to 3×10^8 cells g^{-1} soil (Kobabe et al. 2004). High diversity of methanogens are also detected in northern high-latitude; those are Methanobacteriaceae, Methanomicrobiaceae, Methanosarcinaceae, Methanosaetaceae, uncultured Fen-cluster methanogens, and many other uncultured methanogens (Juottonen et al. 2005; Metje and Frenzel 2007; Rivkina et al. 2007; Juottonen et al. 2008; Wagner and Liebner 2010; Godin et al. 2012; Jassey et al. 2013).

3 Prevalent Methanogenic Pathways at High Latitudes

Cold regions at the high latitudes of earth contribute significantly to the global CH_4 budget. Many studies on the prevalent methanogenic pathways and the driving microbes in cold regions are from the boreal permafrost at high latitudes. Methanogenic pathways in a few of cold wetlands have been studied since 1990s; Kotsyurbenko et al. (2004) showed that Methanosarciaceae, Methanomicrobiaceae and Rice cluster II were dominant in an acidic Siberian peat bog. They have found that below 6 °C very little CH_4 emission is from this peat bog, but acetate accumulated, which is likely produced by homoacetogens from competition for hydrogen.

When the temperature is above 15 °C, acetoclastic methanogenesis becomes active and leads to ample CH_4 emission. This indicates that temperature is an important parameter restricting the three well-known methanogenic pathways. However, study in different types of boreal wetlands having acidic pH in Finland revealed distinct methanogenesis pathways and different methanogen communities (Galand et al. 2005), most likely correlated to the different vegetation. To clarify temperature-related microbes and metabolic alternation involved in methanogenic decomposition of organic matter, Tveit et al. (2015) have investigated the microbial community shift in the microcosms that were enriched from arctic permafrost (peat) by anaerobic culture at temperature ranging from 1–30 °C. By combination of the metagenomic, metatrancriptomic, and target metabolic profiles, they have found that the methane emission rate increases as elevated temperature, e.g., methane production rate at 25 °C is fourfold higher than that at 4 °C. However, they also found that below 7 °C, the rate-limitation of methanogenesis lies at the upstream syntrophic propionate oxidation. Microbial community is also altered concomitantly along with the temperature-related metabolic shift. At 7 °C, Methanomicrobiales replaces Methanobacterium as the predominant hydrogenotrophic methanogen, while *Methanosaeta* spp. becomes prevalent instead of *Methanosarcina* spp. This study suggests that the microbiomes in Arctic permafrost can be well adapted to cold and produce methane and rapidly adjust the community structure in response to temperature changes. Mondav et al. (2014) has obtained the complete genome of a novel uncultured methanogen Candidatus "*Methanoflorens stordalenmirensis*" gen. nov. sp. nov. by assembling the metagenome of permafrost soil at north Sweden. This genome encodes the entire suite of genes for hydrogenotrophic methanogenesis, and the isotopic data also support that the soil methane is from CO_2.These studies strongly suggest that the organic carbon stored in the permafrost can be degraded to CH_4 by the inhabited cold-adapted microbes including methanogens.

4 Prevalent Methanogenic Pathways in Tibetan Plateau

Because of the hypersensitivity to climate change, Tibetan Plateau is known as the climate controller and initiator of Northern Hemisphere. The glacier, permafrost, and high-altitude lakes and wetlands in low latitudes are mostly distributed in Tibetan Plateau. Although the cold temperature, the total CH_4 emission from the Tibetan Plateau wetlands has been estimated to be about 450 Gg $year^{-1}$ (Ding et al. 2004). Liu et al. (2013) have surveyed five Tibetan Plateau lakes at >4000 m above sea level for methanoarchaea community and methanogenic pathways through stable carbon isotope fractionation. They found that hydrogenotrophic methanogens are dominant in lake sediments, while acetoclastic methanogens are dominant in wetland soils; in addition, the 16S rRNA gene and the α subunit of methyl coenzyme M gene (mcr) specific sequences for *Methanosarcina* and *Methanolobus psychrophilus* R15 were found in the high-latitude lakes and wetlands. Methane isotope fractionation suggested that chemolithotrophic acetogenesis and methanol derived methanogenesis may also play a role in Tibetan Plateau. Furthermore, we found

that methanol derived CH_4 can contribute 17% of total CH_4 emission in Zoige wetland, a methane emission center of Tibetan Plateau (Jiang et al. 2010). We have isolated *M. psychrophilus* R15 homologous strains from sediments of four Tibetan high-latitude lakes, which suggested that methanol derived methanogenesis could be an important CH_4 emission pathway in cold regions. Through high-throughput sequencing, Liu et al. (2016) have found that *Methanosaeta* and *Methanolobus* are the predominant methanogens in the Tibetan saline lakes. Meanwhile, the 16S rRNA gene sequences of the uncultured Bathyarchaeota (Miscellaneous Crenarchaeota Group) were found at high abundance as well, implying that Bathyarchaeota could contribute to methane emission at Tibetan plateau. This archaeal phylum may be important in methanol fueled methane production according to the methanogenic genes that they have carried (see Sect. 6.1).

5 Correlation Between Vegetation and Methanogenic Pathways in the Cold Wetlands

Because of the highly specific methanogenic precursors used by the particular methanogen groups, methane emission rate and the prevalent methanogenic pathways in a given ecosystem should be reflected in the methanogenic community (Conrad 2005; Godin et al. 2012). In freshwater wetlands, the CH_4 production potential increases when the dissolved organic carbon concentration increases, and the dominant methanogens are also shifted from the acetotrophic Methanosaetaceae to the hydrogenotrophic Methanobacteriales (Liu et al. 2011). Higher CH_4 emissions have been found in Methanomicrobiales-dominated nutrient-rich fens than in the Methanosaetaceae-dominated nutrient-poor fens in Canada (Godin et al. 2012). Hence, dominant plant species in wetlands appear to determine the abundance and community of methanogens, which provide methanogenic substrates through root exudates and debris and plant litter (Rooney-Varga et al. 2007; Yuan et al. 2016). Thereby, vegetation may significantly control the northern high-latitude CH_4 budget (Bhullar et al. 2013; McEwing et al. 2015).

Vascular plants and mosses are the two predominant vegetation types in cold wetlands. Generally, vascular plants' root systems grow deeper than the non-aerenchymatous peatland plants. This is because they have a cortical oxygen-transport gas space (aerenchyma) to transport oxygen (O_2) to the roots situated in O_2-depleted layers of soil (Armstrong et al. 1991). The roots of vascular plants in anoxic layers provide methanogenic substrates, like acetate and the methanol-derived pectin, into the deeper peat layers (Galand et al. 2005) which lead to acetoclastic and methylotrophic methanogenesis dominant in methane production (Table 1). Whereas, the shallow rhizoid system and lack aerenchyma of mosses do not introduce labile carbons to the anaerobic peat layers and will not result in methane production (Galand et al. 2005). Consistent with plant physiology, hydrogenotrophic methanogenesis is dominant in the *Sphagnum*-dominated wetlands. The correlation of methanogenic pathways with plant species is more specifically determined in the Zoige wetland at Tibetan plateau. Methanol- and

Table 1 Summarized methanogenesis in different vegetation types in cold environments

	Study area	Study place	Dominant vegetation	Dominated pathway	References
1	48°21′N, 85°21′W		*Eriophorum vaginatum*, *Carex* sp.	Methanomicrobiales-dominated hydrogenotrophic methanogenesis	Godin et al. (2012)
			Sphagnum sp., *Vaccinium* sp., evergreen shrubs	Methanosaetaceae-dominated acetoclastic methanogenesis	
2	64°10′N, 19°33′E	Northern Sweden	*Sphagnum*-dominated	Methanomicrobiales-dominated hydrogenotrophic methanogenesis	Marti et al. (2015)
	57°16′N, 13°55′E	Southern Sweden	*Sphagnum*-dominated	Methanomicrobiales-dominated hydrogenotrophic methanogenesis	
	56°50′N, 10°11′E	Denmark	*Sphagnum*-dominated	Methanomicrobiales-dominated hydrogenotrophic methanogenesis	
3	68°21′N, 19°03′E	Northern Sweden	*Eriophorum* spp.	Rice Cluster II' (Candidatus '*Methanoflorentaceae*' fam. nov.) dominated hydrogenotrophic methanogenesis	Mondav et al. (2014)
4	45 ° 24′N, 75 ° 30′W	Mer Bleue bog, Canada	*Sphagnum* spp., heath shrubs	Methanosarcinaceae, Methanoregula-dominated hydrogenotrophic methanogenesis	Yavitt et al. (2012)
	43.24 ° N	Rome NY State	*Sphagnum* spp., heath shrubs	Methanoregula, RC-II-dominatedhydrogenotrophic methanogenesis	
	42.56 ° N	Chicago	*Sphagnum* spp., heath shrubs	Methanosarcinaceae, Methanoregula-dominated hydrogenotrophic methanogenesis	
	42.33 ° N	Mich. Holl	*Carex sedge*	Methanosaetaceae-dominated acetoclastic methanogenesis	
	39.56 ° N	Glades	*S. fallax*, mixed shrubs	Methanosarcinaceae, Methanoregula-dominated hydrogenotrophic methanogenesis	
	39.12 ° N	Big run	*S. fallax*, sedges	Methanoregula-dominated hydrogenotrophic methanogenesis	

(*continued*)

Table 1 (continued)

	Study area	Study place	Dominant vegetation	Dominated pathway	References
5	33°56′N, 102°52′E	Zoige wetland, China	*Eleocharis valleculosa*	Methylotrophic methanogenesis	Jiang et al. (2010)
6	61°48′N, 24°19′E	Lakkasuo Mire, Finland	*Carex spp.* and some herbaceous species	Acetoclastic and hydrogenotrophic methanogenesis	Galand et al. (2010)
		Lakkasuo Mire, Finland	*C. lasiocarpa* with some *Betula nana*	Hydrogenotrophic methanogenesis	
		Lakkasuo Mire, Finland	*Eriophorum vaginatum*, together with *Andromeda polifolia* and *Rubus chamaemorus*,	Hydrogenotrophic methanogenesis	
7	57°N, 83°E	West Siberia	*Sphagnum* moss	Hydrogenotrophic methanogenesis	Kotsyurbenko et al. (2007)
8	46°N, 89°W	Upper peninsula of Michigan	*Sphagnum* spp. and ericaceous shrubs	Hydrogenotrophic methanogenesis	Keller and Bridgham (2007)
			Carex spp., *Eriophorum* spp.,	Acetoclastic methanogenesis	
			Graminoids, *Carex* spp.	Acetoclastic methanogenesis	
9	42°30′N, 76°30′W	Ithaca, New York	*Sphagnum* mosses	Acetoclastic and hydrogenotrophic methanogenesis	Yavitt and Seidmann-Zager (2006)
			Carex lacustris	Acetoclastic and hydrogenotrophic methanogenesis	
			Sphagnum mosses	Acetoclastic methanogenesis	
			Sphagnum mosses	Acetoclastic and hydrogenotrophic methanogenesis	

10	77°36.570S, 163°08.969E	Lake Fryxell, Antarctica		Hydrogenotrophic methanogenesis	Karr et al. (2006)
11	61°48′N, 24°19′E	Lakkasuo, Finland	*Carex* spp. and *Eriophorum* spp.	*Methanosaeta*-dominated acetoclastic methanogenesis	Galand et al. (2005)
			Sphagnum	Hydrogenotrophic methanogenesis	
12	67°30′N, 86°36′E	North-Western Siberia		Acetoclastic methanogenesis	Metje and Frenzel (2007)
13	68°14.32′N, 27°11.44′E	Northern Finland		Hydrogenotrophic methanogenesis	Metje and Frenzel (2005)
14	33°56′ N, 102°52′ E	Zoige wetland	*Carex muliensis*	Acetoclastic methanogenesis	Tian et al. (2012)
15	61°35′–62°05′N, 23°50′ – 24°55′E	Lakkasuo, Central Finland	*Carex* spp.	*Methanosaeta*-dominated acetoclastic methanogenesis	Juottonen et al. (2005)
			Eriophorum vaginatum	Fen-cluster-dominated hydrogenotrophic methanogenesis	

acetate-derived methane emission is more prevalent in *Eleocharis valleculosa*-dominant soil, while this is not the case in *C. muliensis* soil (Jiang et al. 2010).

Terrestrial vegetation productivity and above-ground biomass have been decreasing in the Arctic since 2011 (Jeffries et al. 2015), which directly affect the below-ground biota (Jassey et al. 2013). These complex linkages between above- and below-ground communities regulate carbon sequestration in peatlands (Fenner and Freeman 2011). Vegetation supplies organic substrates for CH_4 production and transport CH_4 (Ström et al. 2003; von Fischer and Hedin 2007; McEwing et al. 2015). Hence, it is of importance to understand the methanogenic potentials in peatlands that will affect global warming by releasing the ancient carbon as a result of vegetation shift by temperature increase.

6 Methane Producing Organisms and Those Prevalent in High Latitudes and Altitudes

6.1 Methane Producing Organisms

Among the extensive studied methanogenic pathways, acetoclastic and hydrogenotrophic methanogenesis are the most common ones and contribute to the total CH_4 production at a theoretical ratio of 2:1 (Conrad 2005). It is generally accepted that methanogenesis is limited to methanogens that are affiliated with the Euryarchaeota phylum. Although methanogenic Archaea, the primary contributor of natural methane flux, are widely distributed, the methanogen populations and their abundance do not always account for the massive methane yield in a given environment. This is particularly the case in subsurface sediments and coalbeds, suggesting that other organisms outside the Euryarchaeota produce methane. Besides, methanogens represent less than 1% of microbial population in marine subsurface, whereas the uncultured Bathyarchaeota are prevalent. Meng et al. (2014) found the prevalence of a group of uncultured Bathyarcheaota, previously the Miscellaneous Crenarchaeota Group in deep ground biosphere. Evans et al. (2015) have obtained almost the complete genomes of Bathyarcheaota BA1 and BA2 by assembling the metagenome of a deep oil well ground water. The genomes encode the entire suite of genes for methylotrophic methanogenesis, but none for the subunits of Na^+-translocating CH_3-H_4MPT:CoM methyltransferase (MTR, *mtrABCDEFG*), the essential proteins involved in CO_2 reductive methanogenesis. In addition, the uncultured Bathyarcheaota enrichment uses amino acids or fermentation of maltose to produce reduced ferridoxin (Fd_{red}), which is then used to reduce methanol for producing methane. Therefore, Bathyarcheaota are predicted to implement methanogenesis using H_2 to reduce methanol and thereby a hypothesis is proposed that methane metabolism may be present in the last common ancestor of Euryarchaeota and Bathyarchaeota. This is the first non-Euryarchaeota member that produces methane and denotes that methanogenesis is more phylogenetically widespread than previously thought. Vanwonterghem et al. (2016) reported another novel uncultured archaeal phylum Verstraetearchaeota which is widely distributed in

anoxic ecosystems that release much CH_4, including wetlands, sediment of lakes, oil fields, and hot springs. In line with the presence of the methylotrophic methanogensis pathway in their metagenomes, a few of the Verstraetearchaeota species have been named as the Candidate new genera, such as Ca. "*Methanomethylicus mesodigestum*" gen. nov. sp. nov., and assigned to Methanomethyliaceae fam. nov, and Methanomethyliales ord. nov. Such naming is derived from the genome of Verstraetearchaeota that encodes genes of the enzymes involved in methylotrophic methanogenesis. In addition, the genome also contains the entire suite of genes for glycolysis, suggesting that Verstraetearchaeota possesses a similar metabolic potential as Bathyarcheaota. Distinct from the methanogens of the Euryarchaeota phylum, those in Bathyarcheaota and Verstraetearchaeota use glycolysis generated electrons to reduce methyl groups to methane, but not exclusively rely on methanogenesis, and thereby are categorized as facultative methanogens. Given that the wide distribution of the two novel uncultured archaeal phyla are predicted performing methylotrophic methanogenesis, the significant contribution of this methane production pathway could have been underestimated previously.

6.2 Cold Adaptive Methanogens Prevalent in High Latitudes and Altitudes

Though the majority of methanogenic species are mesophilic and thermophilic, some psychrotolerant methanogens have been isolated from the aquatic niches at boreal or polar region, and they grow optimally at 18–35 °C (Table 2) and at the lowest temperature of around zero. These psychrotolerant methanogens are thus far phylogenetically restricted to two of the six cultured methanogenic phyla, i.e., Methanosarcinaceae (*Methanococcoides* and *Methanosarcina*) and Methanomicrobiales (*Methanogenium*).

Members of Methanosarcinaceae comprise species with most diverse methanogenic pathways: acetoclastic, methylotrophic, and hydrogenotrophic methanogenesis, whereas, the methylotrophic pathway could be most efficient one at cold (Cao et al. 2014). Coincidently, *Methanococcoides burtonii* isolated form the Ace Lake, Antarctica (Franzmann et al. 1992) and its closely relative *Methanococcoides alaskense* (99.8% 16S rRNA identity) isolated from Skan Bay, Alaska, and *Methanolobus psychrophilus* R15 from Tibetan Plateau are all obligate methylotrophic methanogens. This is consistent with that methanol drives methane emission from Zoige wetland soil samples at lower temperature, even higher at 15 °C than at 30 °C (Jiang et al. 2010).

The psychrophilic and psychrotolerant hydrogenotrophic methanogens isolated from the high latitude and altitudes are restricted to Methanomicrobiales. *Methanogenium frigidum* is the first authentic psychrophilic archeaon growing optimally at 15 °C, but does not grow above 18 °C (Franzmann et al. 1997). *M. frigidum* was isolated from the Ace Lake, Antarctica, and exclusively uses H_2 and CO_2 to produce methane. The genome of *M. frigidum* encodes the entire suite of

Table 2 Methanogens isolated from cold environments

Species	Isolation site	In situ temperature (°C)	Growth temperature (°C)	Optimal temperature (°C)	References
Methanococcoides burtonii	Ace Lake, Antarctica	1~2	−2~28	23	Franzmann et al. (1997)
Methanosarcina lacustris	Soppen Lake	5	1~35	25	Simankova et al. (2001)
Methanosarcina baltica	Skan Bay, Alaska	1~6	5~28	21	von Klein et al. (2002)
Methanococcoides alaskense	Skan Bay, Alaska	1~6	5~28	24	Singh et al. (2005)
Methanolobus psychrophilus R15	Zoige Wetland	0.6~1.2	0~25	18	Zhang et al. (2008a)
Methanogenium frigidum	Ace Lake, Antarctica	1~2	0~18	15	Franzmann et al. (1997)
Methanogenium marinum	Skan Bay, Alaska	1~4	5~25	25	Chong et al. (2002)
Methanogenium boonei	Skan Bay, Alaska	1~6	5~30	19.4	Kendall et al. (2007)
Methanospirillum psychrodurum	Zoige Wetland	0.6~1.2	4~32	25	Zhou et al. (2014)

genes for hydrogenotrophic methanogenesis (Saunders et al. 2003) and a bacterial cold shock protein. However, the strain is difficult to culture in laboratory (Franzmann et al. 1997). Interesting, other cultured cold hydrogenotrophic methanogens all affiliate with Methanomicrobiales and mostly the genus *Methanogenium.* Such as *Methanogenium* AK-1 isolated from permanently cold marine sediments, 38–45 cm below the sediment surface at Skan Bay, Alaska (57° N, 167° W), located at the north-west side of Unalaska Island in the Aleutian Island chain. Strain SK1 grows fastest at 25 °C (ranged 5–25 °C). Like other H_2-using methanogens from saline environments, it is able to use hydrogen at very low concentration (< 1 Pa) (Chong et al. 2002). The psychrotolerant methanogenic strain, *Methanospirillum psychrodurum* X-18^T, is isolated from the Madoi wetland at Qinghai-Tibetan plateau and produces methane exclusively from H_2/CO_2 (Zhou et al. 2014). Methanomicrobiales harbors methanogenic species with a high affinity of H_2, which enables them to adapt to lower H_2 partial pressure. This prediction is supported by *Methanoculleus hydrogenotrophus*, a novel species affiliates with Methanomicrobiaeace, that has been isolated from Tibetan Plateau wetland through a syntrophic butyrate degradation consortium, which provides a lower H_2 partial pressure (Tian et al. 2010).

In addition, using culture-independent approach, Zhang et al. (2008b) found that a novel uncultured methanogen cluster, Zoige cluster I (ZC-I) affiliated to Methanosarcinales, is predominant in Zoige wetland by accounting for about 30% of the total Archaea in the soil. By combining enrichment with quantitative RT-PCR assay, the quantity of the ZC-I methanogens was determined to increase with acetate, H_2/CO_2, methanol, or trimethylamine as substrates, but not with formate, suggesting that ZC-I methanogens are affiliated with the genus *Methanosarcina*.

7 Characteristics of Cold Adaptation of the Methanogens Prevalent in Cold Wetlands

Since the cold climate of the high latitudes, methanogens and other prokaryotes have evolved a set of mechanisms to cope with cold stress. Cold shock response has been extensively studied in bacteria such as *E. coli* (Inouye and Phadtare 2007). Upon temperature downshift, cells change biological processes and macromolecules conformation, such as decrease in membrane fluidity, stabilization of nucleic acids secondary structures, reduced translation, and protein misfolding. Table 3 lists the molecular mechanisms of cold adaption employed by bacteria and methanogenic archaea. We will discuss the representative strategies adopted by psychrophilic methanogens as follows.

7.1 Change of Membrane Lipids

Decrease in membrane fluidity caused by low temperatures affects membrane-associated active transport and protein secretion. Increasing unsaturation of phospholipid ester-linked fatty acids is a common mechanism of cold adaptation for bacteria (Russell 1984). In constract, the unsaturation of membrane lipid in an Antarctic methanogen *M. burtonii* is achieved through incomplete

Table 3 Comparison of the cold adaptation mechanisms between bacteria and psychrophilic methanogens

Upon cold stress	Bacteria	Methanogens (*M. burtonii*; *M. psychrophilus*)
Decrease in membrane fluidity	Unsaturated fatty acids synthesis Desaturase	No desaturase Altered lipid biosynthesis
Stabilization of secondary structures of nucleic acids	RNA chaperone: CspA and its homologs Transcription antiterminator: NusA RNA helicase: SrmB, CsdA RNase: PNPase, RNase R	No CspA homologs; cold shock domain (CSD) protein: Csl4 Archaeal RNA chaperone: TRAM RNA helicase: RhlE, CsdA RNase: RNase J, exosome
Reduced translation	Cold shock ribosomal factor: RbfA and CsdA Translation initiation factor: IF2	Translation initiation factor: aIF2 tRNA modification
Protein misfolding	Trigger factor (TF) GroEL, GroES Caseinolytic proteases (Clps)	Peptidyl-prolyl *cis*/*trans* isomerase Small Hsp (sHsp) Proteasome

Note that all proteins listed in this table are upregulated at low temperature

reduction of the archaeol precursor rather than a desaturase mechanism (Nichols et al. 2004). A higher content of the unsaturated archaeol phospholipids is present in cells growing at 4 °C in comparison with cell grown at 23 °C.

7.2 Melting Nucleic Acids Secondary Structures

Low temperatures stabilize mRNA secondary structures, which often reduce transcription and translation by retarding movement of RNA polymerase and ribosomes (Phadtare et al. 2000). In *E. coli*, cold shock protein A (CspA) and its homologs act as RNA chaperones to unwind RNA secondary structure (Jiang et al. 1997) and act as transcription antiterminator to facilitate transcription (Bae et al. 2010). The common protein fold of CspA and its homologs is described as the cold shock domain (CSD). Methanogenic archaea do not possess CspA homologs except for one copy in *Methanogenium frigidum* (Saunders et al. 2003). However, a protein with a CSD fold in *M. burtonii* is examined to complement the cold-sensitive growth defect of *E. coli* (Giaquinto et al. 2007). It is surprising that this CSD fold protein, Csl4, is the RNA binding subunit of exosome, the multi-subunit RNA 3′-5′ decay machinery (Evguenieva-Hackenberg et al. 2003). Another RNA binding subunit of exosome Rrp4 shows increased abundance when growing at low temperature (Williams et al. 2011). These indicate that RNA binding proteins play an important role in cold adaptation of psychrophilic methanogen. In addition, three small proteins composed of a single TRAM domain in *M. burtonii* are upregulated at low temperature (4 °C versus 23 °C) and thereby are proposed as a RNA chaperone-like bacterial CspA (Williams et al. 2010) More recently, TRAM was reported to bind tRNA and 5S rRNA, further indicating it having RNA binding capacity (Taha et al. 2016). Indeed, we first confirmed that four TRAMs from *M. psychrophilus* behave like the bacterial CspA and they all complement the *cspA* mutation of *E. coli* (Zhang et al. 2017). In addition, the cold-inducible expression of TRAM is largely dependent on transcription activation. A putative cold responsive element bound by certain cold-inducible transcription factor was identified in the promoter region. TRAM mRNA shows a quick accumulation even more than 20-fold upon cold shock.

In addition to CspA and its homologs, the DEAD-box RNA helicase such as CsdA also contributes to cold acclimation through its involvement in mRNA decay (Prud'homme-Genereux et al. 2004). The two DEAD-box RNA helicase in *M. burtonii* exhibited higher abundances at 4 °C (Williams et al. 2010). Like the bacterial PNPase and RNase R, the recent discovered archaeal 5′-3′ exoribonuclease RNase J also elevates the abundance at low temperatures (Williams et al. 2010, 2011), suggesting that cold-induced structured RNA would have to be degraded.

7.3 Increased Translation Initiation

At low temperatures, ribosomes cease to translate most cellular mRNAs. In *E. coli*, cold shock ribosomal factors such as RbfA and CsdA bind ribosomes to convert the non-cold-adapted ribosomes to cold-adapted ribosomes (Jones and Inouye 1996). However, psychrophilic methanogens adapt another strategy by upregulating most of the ribosomal protein at low temperatures (Williams et al. 2010). Moreover, the translation initiation factor aIF2 β-subunit is upregulated at 4 °C. In contrast, translation elongation factors EF1 is less abundant at 4 °C. This indicates that translation initiation rather than elongation plays a pivotal role in modulating translation in response to low temperature. In addition to the translation machinery, *M. burtonii* tRNA is found to harbor a high proportion of dihydrouridine modification that can enhance tRNA flexibility (Noon et al. 2003).

7.4 Protein Folding and Degradation

Temperature downshift causes protein misfolding which exerts physicochemical constraints such as decrease in the folding rate (Piette et al. 2011). In *M. burtonii*, two peptidyl-prolyl *cis*/*trans* isomerase show upregulated at 4 °C while DnaK-DnaJ and thermosome subunits are upregulated at 23 °C (Williams et al. 2010, 2011). This divergence suggests that DnaK-DnaJ-GrpE and thermosome system response to heat stress. In *M. psychrophilus*, three small heat proteins (sHsp) showed 1.8–11-fold upregulation at low temperatures (Li et al. 2015). Given that DnaK-DnaJ-GrpE and thermosome system showed unchanged levels at low temperatures, sHsp may play a more important role in protein fold process in cold adaptation.

In contrast to protein chaperonin that rescue unfolded proteins, protein degradation systems hydrolyze denatured proteins. In *M. burtonii*, both subunits of the proteasome and several secreted proteins which predicted to participate in proteolysis are all induced at 4 °C (Williams et al. 2011). This in turn proves that protein folding is a temperature-sensitive process that restricts growth at low temperatures.

7.5 Metabolic Modulation

The two psychrophilic methanogens *M. burtonii* and *M. psychrophilus* are all methylotrophic that use trimethylamine or methanol as sole carbon source. Despite the different substrate (trimethylamine or methanol), methyl-CoM is the key intermediate under both conditions. Its oxidative branch of methyl-CoM, $F_{420}H_2$ dehydrogenase (Fpo) generates a proton motive force that is coupled to ATP synthesis via an ATPase (Deppenmeier 2002). Through transcriptomics and proteomics, the expression of the majority of the genes for methylotrophic methanogenesis and ATP synthesis was reduced at low temperature (Williams et al. 2010; Chen et al.

2012). Actually, genes involved in the trimethylamine/methanol-utilization pathway such as methyltransferases usually tended to decrease the expression at low temperatures (Williams et al. 2010; Chen et al. 2012). This explains the lower catabolism of the substrate which is required to maintain lower growth rates at low temperatures.

7.6 Oxidative Stress Resistance

Proteins against reactive oxygen species (ROS) are increased at low temperatures due to increased solubility of oxygen. In the cold-adapted transcriptome of *M. psychrophilus*, the superoxide dismutase (SOD), two catalases, two superoxide reductases (SOR), three rubrerythrins, and three hemerythrins are all upregulated for even more than 190-fold (Chen et al. 2012; Li et al. 2015). In contrast, catalase and SOR in *M. burtonii* were downregulated at low temperature (Williams et al. 2010). Moreover, *M. burtonii* is absence of SOD. This discrepancy is the most significant difference between the two psychrophilic methanogens. It can be attributed to their natural habitats that *M. psychrophilus* lives in the rhizosphere (Zhang et al. 2008), while *M. burtonii* inhabits at the methane-saturated and permanently anoxic bottom waters (Franzmann et al. 1992).

7.7 Long 5′ UTR Adds Another Layer of Post-Transcriptional Regulation

Recent progress of differential RNA-seq (dRNA-seq) that depicts the primary transcriptome of bacteria or archaea has broadly expanded our knowledge of post-transcriptional regulation. Using dRNA-seq, the genome-wide transcription start sites of several archaea have been determined (Sharma and Vogel 2014). Surprisingly, the length of 5′ UTRs of methanogens are exceptionally long with a mean length >50 nt, thus providing a platform for posttranscriptional regulation (Jager et al. 2009; Li et al. 2015). For instance, the 5′ UTRs of genes involved in methylotrophic methanogenesis (e.g., *mtaA*, *mtaB*, *mtaC*) are longer than that involved in acetoclastic methanogenesis (e.g., *pta*, *ack*), such as *mtaA1* and *mtaC1B1* harbor 270-nt and 238-nt long 5′ UTR, respectively, while a 27-nt 5′ UTR is present in the *pta-ackA* transcript. The long 5′ UTRs endow mRNA stability at low temperature (Cao et al. 2014), supporting the cold-adapted methylotrophic methanogenesis in the cold Zoige wetland in Tibetan Plateau.

8 Research Needs

Permafrost at higher latitudes and altitudes is a potential source of vast greenhouse gases, like methane and carbon dioxide, because of the huge storage of ancient organic carbons. Global warming caused permafrost melting, and temperature increasing could lead to a sudden release of methane into the atmosphere. Therefore, it is essential to

understand the methane metabolic pathways and the performers, like methanogenic Archaea affiliated with Euryarchaeota or non-Euryarchaeota like the uncultured Bathyarcheaota and Verstraetearchaeota. Understanding the unique mechanisms in adaptation of temperature changing employed by the permafrost, Archaea is another important matter. For example, previously we found that posttranscriptional regulation could play an important role in the cold adaptation of methanogenic Archaea. In contrast, we found that a previously isolated psychrophilic methanogen R15 becomes "mesophilic" after 10-year laboratory culturing. This provides an excellent model for interrogation into how Archaea adapt to elevated temperature, a climate changing scenario we are experiencing. These studies would equip us at advance of the knowledge in coping with the growing climate temperature.

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Methanogens and Methanogenesis in Hypersaline Environments

11

Terry J. McGenity and Dimitry Y. Sorokin

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Abstract

Methanogenesis is controlled by redox potential and permanency of anaerobic conditions; and in hypersaline environments, the high concentration of terminal electron acceptors, particularly sulfate, is an important controlling factor. This is because sulfate-reducing microbes, compared with methanogens, have a greater affinity for, and energy yield from, competitive substrates like hydrogen and acetate. However, hypersalinity is not an obstacle to methylotrophic

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methanogenesis; in many cases hypersaline environments have high concentrations of noncompetitive substrates like methylamines, which derive from compatible solutes such as glycine betaine that is synthesized by many microbes inhabiting hypersaline environments. Also, depletion of sulfate, as may occur in deeper sediments, allows increased methanogenesis. On the other hand, increasing salinity requires methanogens to synthesize or take up more compatible solutes at a significant energetic cost. Acetoclastic and hydrogenotrophic methanogens, with their lower energetic yields, are therefore more susceptible than methylotrophic methanogens, which further explains the predominance of methylotrophic methanogens like *Methanohalophilus* and *Methanohalobium* spp. in hypersaline environments. There are often deviations from the picture outlined above, which are sometimes difficult to explain. Identifying the role of uncultivated Euryarchaeota in hypersaline environments, elucidating the effects of different ions (which have differential stress effects and potential as electron acceptors), and understanding the effects of salinity on all microbes involved in methane cycling will help us to understand and predict methane production in hypersaline environments. A good demonstration of this is a recent discovery of extremely haloalkaliphilic methanogens living in hypersaline lakes, which utilize the methyl-reducing pathway and form a novel class "Methanonatronarchaeia" in the *Euryarchaeota*.

1 Introduction

Hypersaline environments are defined in different ways, but here we consider those environments with more than twice the salinity of seawater. Such environments are many and varied, in terms of their overall salinity and predominant ions (McGenity and Oren 2012). Coastal environments, both man-made and natural, are subject to desiccation, resulting in a wide variety of habitats from small, ephemeral salt pans within temperate salt marshes to large, permanently hypersaline sabkhas in subtropical regions (Hovorka 1987). Similarly, inland salt lakes can be as large as the Great Salt Lake or a tiny spring. Salt deposits, often several hundred meters in thickness, lie beneath about a quarter of the Earth's landmass and contain brines from a cubic micrometer in volume to many cubic meters.

Hypersaline environments are widespread and were more prevalent in former geological times, for example, much of Northern Europe was covered by the salt-saturated Zechstein Sea during the Permo-Triassic period (Zharkov 1981), and the Mediterranean Sea was desiccated more recently, with the first evaporites forming 5.96 million years ago (Roveri et al. 2014). Deep-sea anoxic hypersaline brines, which are derived from dissolution of such ancient evaporites, form large lakes on the floor of the Gulf of Mexico, Mediterranean Sea, and Red Sea. Oil and gas reservoirs are frequently associated with hypersaline environments, and many industrial waste streams are both anaerobic and hypersaline. Certain hypersaline environments, such as sabkhas, are considered to be Mars analogues (McKay et al.

2016), and martian (bio)methane is a key gas that could be detected by the NOMAD instrument on the recently launched Trace Gas Orbiter (Vandaele et al. 2015). Therefore, there is a lot of interest in understanding the influence of high salinity (as well as other extreme conditions) on methanogenesis in different types of hypersaline environment.

The inhabitants of hypersaline environments are generally termed halophiles, and the use and misuse of this term, together with all its qualifiers, as well as examples of the most ecologically important extreme halophiles, have been discussed (Oren 2008; McGenity and Oren 2012). The ability of microbes to tolerate hypersaline environments with different chemical compositions varies widely: Don Juan Pond, a $CaCl_2$-saturated brine, appears to support no life (see Oren 2002), whereas the African hypersaline soda lakes are among the most productive environments in the world (Grant and Tindall 1986). Salinity was found to be the main factor influencing microbial community composition in a synthesis of 111 studies (Lozupone and Knight 2007), and contrary to popular perception, microbial diversity can be extremely high in environments where the salinity is about two to three times greater than seawater and where redox and light gradients exist. For example, 42 of the main bacterial phyla and 15 novel candidate phyla were reported in a microbial mat in Guerrero Negro with a salinity of 8% (Ley et al. 2006; Harris et al. 2013). Also, where salinity gradients occur, microbial biodiversity, abundance, and activity can be greatly elevated owing to a cocktail of electron acceptors, electron donors, nutrients, and carbon sources in the ionic and redox gradient (Daffonchio et al. 2006).

In most environments methanogens are in competition with sulfate-reducing bacteria for the products of fermentation, particularly hydrogen, formate, and acetate, and it is well documented that where sulfate concentrations are sufficiently high, e.g., marine and hypersaline environments, sulfate reduction will be the dominant terminal electron-accepting process owing to the higher affinity for these competitive growth substrates (e.g., Lovley et al. 1982). Nevertheless, methanogenesis remains an important process in marine and hypersaline environments, for example, in sulfate-depleted zones in deeper sediments (Wilms et al. 2007), in areas with elevated hydrogen production (Hoehler et al. 2001; Buckley et al. 2008), and where carbon sources are available that cannot be used by sulfate reducers (Oremland et al. 1982a; Winfrey and Ward 1983). Such noncompetitive substrates include methanol, methylated sulfides, and methylated amines, of which the latter derive from salinity-induced compatible solutes. A scheme for the production of methylamines is indicated in Fig. 1. Dimethyl sulfide derives primarily from hydrolysis of its precursor molecule dimethylsulfoniopropionate, which, like glycine betaine, is a compatible solute and so is typically found at high concentrations in saline and hypersaline environments (Kiene et al. 1986; Kiene and Visscher 1987; Curson et al. 2011).

More recently, quaternary amines, such as choline and glycine betaine, have been shown to serve directly as substrates for methanogenesis in some marine *Methanococcoides* strains, allowing them to grow without the need for a syntrophic partner (Watkins et al. 2014; L'Haridon et al. 2014). One *Methanococcoides*

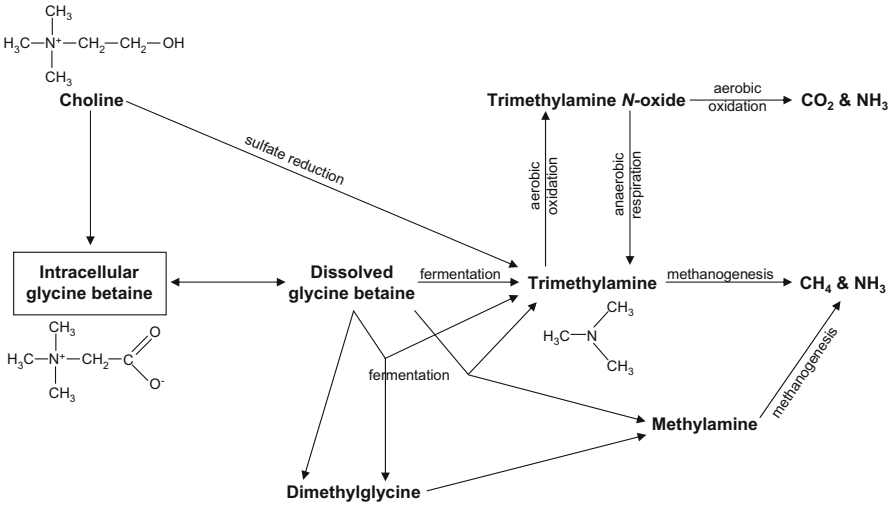

Fig. 1 Central importance of trimethylamine for methanogenesis in hypersaline environments. (Adapted from Welsh (2000), with permission from Wiley-Blackwell). Only the main pathways are shown, and many other scenarios have been illustrated and described by Oren (1990). In addition, glycine betaine and choline are used directly by some methanogens, removing the requirement for syntrophic microbes (see text for details)

strain studied in detail was shown to only partially demethylate glycine betaine to *N,N*-dimethylglycine, possibly because the product may also serve as a compatible solute and also because much more energy is gained from the first demethylation step than subsequent steps (Watkins et al. 2014). In hypersaline environments, the direct use of glycine betaine as a substrate for methanogenesis has not been demonstrated yet. Overall, methylotrophic methanogenesis is nearly always the dominant methanogenic pathway in hypersaline conditions, including salt-saturated environments (approx. 5.2 M NaCl), although there are exceptions (Oremland and King 1989). The relative importance of methylotrophic methanogenesis is well illustrated by an analysis of the upper salinity (in parentheses) at which pure methanogenic cultures have been shown to grow with various substrates (Oren 1999, 2011): methylamines (27%), hydrogen and carbon dioxide (12%), and acetate (4%). These salinities should not be considered as the upper limit of activity in situ, but indicative of the relative contribution to methanogenesis of these substrates at different salinities. Competition with sulfate reducers, and the consequent reduced pool of halophilic hydrogenotrophic and acetoclastic methanogens on which natural selection can act, partly explain the observed differences in salinity tolerance. However, this trend is also governed by the relative energy gain from different methanogenic reactions per mole of substrate (methylotrophic $>>$ hydrogenotrophic $>$ acetoclastic), especially since halophiles must expend a lot of energy to maintain an osmotically balanced and functional cytoplasm via the biosynthesis and/or uptake of organic compatible solutes and/or uptake of potassium ions (Oren 1999, 2011).

What has not been anticipated until very recently, is that there is a fourth (hybrid) pathway of methanogenesis, which is called methyl reduction, whereby C_1-methylated compounds are used only as electron acceptors and H_2 serves as an electron donor (Borrel et al. 2014). It turns out that methanogens using this pathway may out-compete those that use both the classical methylo- and hydrogenotrophic pathways in salt-saturated conditions (see Sect. 10).

This review will focus more on those long-term, large-scale hypersaline anoxic environments, which have been studied in much greater depth, with emphasis on contemporary work that builds on studies outlined in some excellent reviews (e.g., Oremland and King 1989; Ollivier et al. 1994; Oren 1999, 2002, 2011; Marvin diPasquale et al. 1999) and expands on a previous chapter in this series by McGenity (2010). In order to understand the role of methanogenesis in carbon cycling in hypersaline environments, evidence will be drawn from methane fluxes from field measurements, methane production rates (often from slurry experiments), cultivation, and investigation of uncultivated methanogenic communities. Methanogens are frequently studied without cultivation, owing to a generally good correspondence between phylogeny and phenotype that is less typical of other microbial groups. Also, the *mcr*A gene, coding for methyl-coenzyme M reductase subunit-A, has proven to be a valuable functional molecular marker for investigating methanogens, and there is (with a few exceptions) good correspondence between the phylogenies obtained with *mcr*A and the ribosomal RNA gene-based phylogeny. This has resulted in widespread application of these gene markers to investigate methanogens in hypersaline (and many other) environments. However, we now know that this phylogeny-function relationship is not absolute, and there is uncertainty over the phenotype of uncultivated organisms, giving rise to 16S rRNA gene sequences that cluster within the Euryarchaeota but outside of known methanogens. Moreover, there is now evidence using genomic reconstruction from metagenomes to suggest that methyl-reducing methanogenesis occurs in non-euryarchaeal candidate phyla within the Archaea, such as the "Bathyarchaeota" (Evans et al. 2015) and "Verstraetearchaeota" (Vanwonterghem et al. 2016).

2 Sedimentary Rocks

Waldron et al. (2007) exploited a natural salinity gradient from 8 mM to 3.5 M Cl^- in the subsurface Antrim Shale, rich in methane derived from biodegraded hydrocarbons, to understand the salinity constraints on different types of methanogenesis. Methanogenesis was an important process in the shales, owing to a lack of competition with other terminal-electron-accepting processes, and it was evident that methanogens were capable of a high level of activity at their in situ salinities, with the exception of the brine with 3.5 M Cl^-, in which there was no methane production. It is difficult to ascertain the percentage salinity of the two most saline brines (2.3 and 3.5 M Cl^-), because the Na^+ concentrations are 1.1 and 1.4 M, respectively, implying the presence of other cations (perhaps K^+, Mg^{2+}, Ca^{2+}) that were not measured. Based on most-probable-number enrichments, there was a clear change

in methanogenic processes, with a mixture of hydrogenotrophic, acetoclastic, and methylotrophic methanogenesis (primarily hydrogenotrophic) at lower salinities and a predominance of methylotrophic methanogenesis at higher salinities (Waldron et al. 2007). This finding was supported by 16S rRNA gene clone libraries, in which all clone sequences were from *Methanocorpusculum* spp. at 12.8 mM Cl^-, while most were from the methylotrophic *Methanohalophilus* spp. at 2.3 M Cl^-. The second most abundant group from the high-salinity well was closely related to *Methanoplanus petrolearius*, previously shown to tolerate 0.86 M Cl^- and able to use a range of compounds including hydrogen and carbon dioxide (Ollivier et al. 1997), hinting that hydrogenotrophic methanogenesis may be possible at this very high salinity. Indeed, Ollivier et al. (1998) isolated from a hypersaline oil reservoir the species *Methanocalculus halotolerans*, a hydrogenotrophic methanogen capable of growth up to 12% NaCl (=2 M Cl^-). Perhaps the long-term stability of the shale (>7000 years) and the absence of competing processes have enabled hydrogenotrophic methanogens to adapt to a higher salinity (Waldron et al. 2007). Wuchter et al. (2013) also investigated the Antrim Shale, focusing on formation water from wells that had been hydraulically fractured, drawing similar conclusions as Waldron et al. (2007), but also noting that methanol present in the fracturing fluid is likely to stimulate methane formation, a clear benefit for the fracking process. Gray et al. (2009) found hydrogenotrophic *Methanothermobacter* as the dominant methanogen in H_2/CO_2 enrichments from high-temperature gas-field formation waters with a salinity of around 9% but with a relatively low concentration of sulfate (< 0.1 M). Methanogenesis occurred over a relatively wide range of NaCl concentrations, down to 0.1% but not at 11% or above.

By using metabolic and metagenomic analysis, Daly et al. (2016) largely confirmed that the methanogenic processes proposed by both Waldron et al. (2007) and Wuchter et al. (2013) were also dominant in the deep, hypersaline Marcellus and Utica Shales, i.e., glycine betaine fueled methylotrophic methanogenesis with trimethylamine as an intermediate, carried out by *Methanohalophilus* and *Methanohalobium* species. To a lesser extent, methanol and other fracturing-fluid additives encouraged methylotrophic methanogenesis. Two representatives of the *Halanaerobiales*, *Halanaerobium* spp. and *Candidatus* Frackibacter, were identified as the most likely halophilic bacteria responsible for anaerobically converting glycine betaine to trimethylamine (Daly et al. 2016).

Kirk et al. (2015) demonstrated production of methane from coal beds, with an inferred dominance, based on 16S rRNA gene sequence analysis, of hydrogenotrophic methanogens, with an increasing contribution from acetoclastic *Methanosaeta* as the salinity increased to the maximum of 9.13%. The authors propose that in this habitat methylotrophic methanogens were out-competed by acetoclastic methanogens, which is surprising because, as previously discussed, acetoclastic methanogenesis is energetically unfavorable in highly saline conditions. An alternative explanation may be syntrophic associations of reverse-acetogens coupled with hydrogenotrophic methanogens.

Many microbes, most notably haloarchaea, have been isolated and their 16S rRNA gene signatures detected in salt crystals from buried salt deposits (see

McGenity et al. 2000; Gramain et al. 2011), but to date methanogens have evaded detection. Nevertheless, in salt mines naked flames are not permitted, and there are records of methane-induced explosions; also gas-induced popping salt is a common threat to mine workers. The redox potentials of fluid inclusions in halite are generally negative, usually from −10 to −130 mV (summarized by Roedder 1984), and methane, among other gases, is commonly detected (Roedder 1984). Perhaps methanogens lack the capacity for long-term survival as seen in haloarchaea (their close cousins who have adapted to aerobic conditions), in which case, the presence of methane can be explained by trapping of the gas during initial halite precipitation or more typically during recrystallization of buried halite (Pironon et al. 1995a, b). Alternatively, methane may be produced in young halite crystals by co-entombed methanogens which subsequently die, and over geological time their necromass serves as a source of carbon and energy for heterotrophic extremely halophilic microbes.

3 Deep-Sea Hypersaline Anoxic Brine Lakes

There are numerous locations in the deep sea and the deep subsurface where dissolution of rock salt has resulted in hypersaline brine seeps and lakes, which are often associated with methane seeps. In the case of the deep-sea hypersaline brine lakes, the density gradient between the hypersaline brine and overlying seawater, coupled with weak currents at depth, restricts mixing, which results in the hypersaline brines becoming anoxic. Such pools and lakes have been discovered on the floor of the Gulf of Mexico, Red Sea, and Mediterranean Sea. These hypersaline brines have commonly been shown to be a source of biogenic methane, often mixed with geogenic methane (Charlou et al. 2003; Joye et al. 2005). A small brine pool, called NR-1, in the Gulf of Mexico is even surrounded by dense beds of *Bathymodiolus* mussels, which house chemolithoautotrophic bacteria presumably fed by sulfide or methane from the anoxic brine lake (MacDonald et al. 1990). In sediments fed by a sulfate-depleted hypersaline brine seep, Paull et al. (1985) found that the carbon in mussel tissue was isotopically light and hence presumably derived (via endosymbiotic methanotrophs) from brine-derived methane. Therefore, there is good evidence that food webs are stimulated by reduced compounds derived from microbial activity in hypersaline brines analogous to hydrothermal vent communities (Martens et al. 1991).

Zhuang et al. (2016) investigated the processes responsible for methane production in methane-rich sediments in two sub-basins of the Orca Basin (Gulf of Mexico), with porewater salinities of 26.1–27.3% and 22.2–25.5%, using a range of approaches, including: (1) ^{13}C stable isotope analysis, e.g., of methane, putative precursors, lipids, and in tracer experiments with different potential substrates, (2) radiotracer incubations with precursors, (3) energetic calculations, (4) 16S rRNA-based phylogenetic analysis, and, importantly, (5) direct measurement of the concentrations of precursors including methylated compounds. Hydrogen concentrations were very low, and in contrast to other studies of brines and sediments from

deep-sea hypersaline anoxic basins of the Gulf of Mexico (Joye et al. 2009) and Mediterranean (Lazar et al. 2011), acetate concentrations were very low (Zhuang et al. 2016). However, methanol, TMA, and DMSP concentrations were high (Zhuang et al. 2016). The evidence points toward methylotrophic methanogenesis as the dominant process, with *Methanohalophilus* spp. contributing to methane production. Rates of methane production were higher in the less saline sub-basin and approximately a hundredfold lower than in the Napoli mud volcano sediments, potentially due to the latter's lower salinity (its chlorinity was 60% of that found in the Orca Basin sediments; Lazar et al. 2011).

In hypersaline brines on the floor of the Red Sea, such as the Kebrit and Shaban Basins, there were originally hints of the presence of methanogens from archaeal biomarkers (Michaelis et al. 1990) and euryarchaeal 16S rRNA gene amplicons (Eder et al. 2002), both of which could derive from non-methanogenic Archaea. More direct evidence came from four of the Eastern Mediterranean hypersaline basins, where van der Wielen et al. (2005) detected methane production rates (μM CH_4 d^{-1}) of 85.8 (Urania), 16.9 (L'Atalante), 4.2 (Bannock), and 2.6 (Discovery). The most abundant uncultivated archaeal clones (termed MSBL-1) in most of these basins (van der Wielen et al. 2005) and in a hydrothermal mud vent beneath Urania hypersaline brine (Yakimov et al. 2007a) branched most closely to methanogens. Very similar 16S rRNA signatures have been found in many anoxic, hypersaline environments. MSBL-1 Archaea probably represent a novel class of Euryarchaeota mostly related to Thermoplasmata, but a recent metagenomic analysis did not confirm their methanogenic nature, rather hinting at acetogenic metabolism (Mwirichia et al. 2016).

Yakimov et al. (2007b) found a change in the main archaeal community across the approximately 2-meter halocline from oxic Mediterranean seawater (depth 3498.5 m) to almost NaCl-saturated, anoxic L'Atalante brine, with a group related to ANME-1 (putative anaerobic methane-oxidizing group) co-existing with the aforementioned MSBL-1 group near the top of the interface, i.e., where salinity is not extremely high but where oxygen is highly depleted. *Methanohalophilus*-related 16S rRNA sequences were most abundant in the deeper parts of the halocline and in the hypersaline brine. Messenger RNA coding for methyl-coenzyme M reductase from *Methanohalophilus* spp. was detected in the L'Atalante hypersaline brine (Hallsworth et al. 2007), and we isolated strains from L'Atalante sediment with 99% 16S rRNA sequence similarity to *Methanohalophilus mahii* using sulfate-free medium and noncompetitive growth substrates (Sass et al. unpublished), and a similar strain has been isolated from Thetis brine (La Cono et al. 2015). This suggests that methanogenesis is occurring throughout the hypersaline brine of L'Atalante basin and is primarily mediated by *Methanohalophilus* spp. using methylamines. In Bannock basin, which is chemically similar to L'Atalante but located on the opposite side of the Mediterranean trench, methane production was observed throughout the halocline from deep Mediterranean seawater to the hypersaline brine and increased to 3.5 μM CH_4 d^{-1} in the near-salt-saturated brine (Daffonchio et al. 2006). No 16S rRNA sequences from *Methanohalophilus* spp. and relatives were found in Bannock

interface or brine, whereas MSBL-1 was detected in both compartments and ANME-1 in the interface (van der Wielen et al. 2005; Daffonchio et al. 2006), raising interesting questions about the factors influencing the distribution of methanogens.

Three of the Eastern Mediterranean brine lakes are dominated by NaCl, but the Discovery brine is unusual in that it derives from the dissolution of bischofite and so is an almost pure, 5 molar $MgCl_2$ brine, with a water activity (a_w) of 0.37, the lowest recorded in a marine environment and far below the current known limit of life (0.605) (see Hallsworth et al. 2007; Stevenson et al. 2015). Moreover, $MgCl_2$ destabilizes biological macromolecules at high concentrations and above a concentration of 2.3 M (in the absence of compensating solutes) appears to be inhibitory to life (Hallsworth et al. 2007). In support of this notion is the detection of mRNA, a highly labile indicator of active microbes, from sulfate reducers and methanogens, only in the upper half of the halocline from seawater to Discovery brine (<2.23 M $MgCl_2$; a_w of <0.801), whereas methanogen mRNA was detected in the NaCl-rich L'Atalante brine at a lower water activity of 0.741 (Hallsworth et al. 2007). Although mRNA was not detected in the lower half of Discovery interface, 16S rRNA from both sulfate reducers and methanogens was found and can be attributed to the exceptional preservation properties of $MgCl_2$ (Hallsworth et al. 2007). Marvin diPasquale et al. (1999) found that rates of methanogenesis were very low in the Dead Sea sediments (salinity of 30%, a_w of 0.669 (Krumgalz and Millero 1982) and highly enriched in divalent cations, especially $MgCl_2$), with only methanol additions in some samples permitting low levels of methane production. This is in contrast to the lower salinity (<18%) and NaCl-dominated Solar Lake sediments where rapid methanogenesis was observed with a wider range of noncompetitive substrates. The relative influence of salinity/low water activity and divalent cation concentration on biogeochemical processes like methanogenesis warrants further investigation.

Several new Eastern Mediterranean deep-sea brine lakes have been discovered and investigated: Lake Thetis (La Cono et al. 2011), Lake Medee (Yakimov et al. 2013), and Lake Kyros (Yakimov et al. 2015). In the $MgCl_2$-rich Lake Kyros, the most abundant *mcrA* transcripts from the brine-seawater interface belonged to *Methanohalophilus*, similar to the aforementioned Discovery interface. In addition, sequences similar to *Methanomassiliicoccales* were detected (Yakimov et al. 2015). Several studies from the Red Sea now report the presence of methanogens in the brines and their interface with overlying seawater (Antunes et al. 2011). For example, Guan et al. (2015) surveyed *mcrA* genes from several of the interfaces, finding the usual halophilic methylotrophic genera *Methanohalophilus* and *Methanococcoides* and also phylotypes that were related to *Methanomassiliicoccales*. *Methanomassiliicoccales* is a recently discovered order of methanogen in the Thermoplasmata that can reduce methanol and methylamines with hydrogen (Borrel et al. 2014), although, currently, only non-halophilic members of this methanogenic order have been characterized. The importance of this pathway for methanogenesis in hypersaline environments is considered in Sect. 10.

4 Hypersaline Microbial Mats

Microbial mats represent a much more temporally dynamic ecosystem than those ecosystems discussed previously, as they are subjected to, for example, changes in light and temperature, and many are intertidal. Therefore, the microbial communities within mats are probably more versatile in terms of salinity and oxygen tolerance and perhaps also more nutritionally versatile. Alternatively or additionally, this environmental variability means that mats are likely to house microbes that are inactive for long periods of time, returning to activity as conditions, including salinity, become optimal for them. It is pertinent to note here that the presence of a DNA signature alone in any of the exposed systems discussed in this chapter must be considered as preliminary evidence for the autochthonous nature of the detected microbe, and further work is required to attest to its in situ activity.

As mentioned earlier, the Guerrero Negro mats (Baja California Sur, Mexico) are hypersaline yet highly biodiverse. Hoehler et al. (2001) observed that, along a vertical transect of a subtidal microbial mat, high levels of methane production coincided with unusually high hydrogen concentrations (a consequence of cyanobacterial activity in the upper few millimeters of the mat) and concluded that competition with sulfate-reducing bacteria and hence hydrogen limitation for methanogenesis was reduced. Consistent with this idea, Skyring et al. (1989) had shown previously that inhibition of sulfate reduction left hydrogen consumption unaffected. However, no analysis of the methanogenic community was made to support this observation, and the fact that the surface of the mat also harbored the highest density of cyanobacteria that may be leaking the compatible solute, glycine betaine, giving rise to of methylamines (Fig. 1), could also explain high levels of methanogenesis, as observed by King (1988) in a different hypersaline, cyanobacteria-dominated mat and explained in detail by Oren (1990). Indeed, Smith et al. (2008) investigating the Guerrero Negro mats found that methylated amines were the primary route to methane formation and that methylotrophic members of the *Methanosarcinales* were the only methanogens detected by *mcrA* amplification and sequencing. In their artificial system with a salinity of 8.5%, in which sulfate was depleted more than 50-fold over 11 months (to analyze the archaeal community) and 17 months (to measure methane concentration), hydrogenotrophic methanogens appeared in clone libraries but were still less abundant than methylotrophic methanogens, and porewater methane concentration increased approximately a hundredfold (Smith et al. 2008). These observations, together with data from the inhibition of sulfate reduction, suggest that, in situ, sulfate reducers competitively inhibited hydrogenotrophic methanogens. However, they remain viable, and at low sulfate concentrations, a mixed community of hydrogenotrophs and methylotrophs is responsible for methanogenesis (Smith et al. 2008). Back-to-back studies on Guerrero Negro mats with salinities of 7–7.5% (Jahnke et al. 2008; Orphan et al. 2008) confirmed that methylotrophic *Methanosarcinales* were the dominant methanogens, although in most parts of the mat, methanogens were only a small fraction of the archaeal community. Also, various perturbations and analyses revealed a stratification of *Methanosarcinales*,

with the following trend: *Methanolobus* spp. in the photic zone, *Methanohalophilus* spp. in the middle, and *Methanococcoides* in the unconsolidated sediment below the mat, extending the salinity at which *Methanococcoides* spp. have been found by 3.5% (Orphan et al. 2008). Importantly, both studies revealed lipids that could serve as possible biomarkers for methanogenesis in microbial mats, both modern and ancient (Jahnke et al. 2008; Orphan et al. 2008). In a more hypersaline series of microbial mats, Mouné et al. (2003) did not detect methanogens at a salinity of 25–32%, but *Methanosarcinales* represented 1% of the prokaryote community in a mat with a lower salinity of 15–20%, thus further emphasizing the contribution of *Methanosarcinales* to methanogenesis in hypersaline microbial mats.

García-Maldonado et al. (2015) investigated five hypersaline microbial mats from Baja California Sur. Methane production was found in microcosms at 17% salinity, but not at 28% salinity (although methane was detected in situ), except after trimethylamine addition, which activated methanogenesis in the 28% salinity microcosm. An *mcrA* gene survey from these five hypersaline mats revealed the presence of typical halophilic methanogens: *Methanohalophilus*, *Methanohalobium*, and *Methanolobus* (the latter also found in soda lakes) and two distinct groups (probably genus-level differentiation) from the *Methanosarcinales*, as well as *Methanomicrobiales*. Activity of these putatively hydrogenotrophic *Methanomicrobiales* phylotypes in a mat of 6% salinity was demonstrated by reverse-transcriptase QPCR, but their *mcrA* transcript abundance was at least an order of magnitude lower than that of the methylotrophic genus, *Methanohalophilus* (García-Maldonado et al. 2015).

In a microbial mat in a saltern pond in Eilat with a salinity of 21.5%, Sørensen et al. (2004) estimated methane flux from the mat of 1.6×10^{-5} nmol cm^{-2} s^{-1}, one order of magnitude lower than in a pond of lower salinity (13.8%) studied previously. Slurry experiments with salinities from 5% to 32% revealed that the methane production rates increased between 15% and 25% salinity, coinciding with a decrease in the sulfate reduction rate. When molybdate was added to prevent sulfate reduction, methanogenesis also occurred at 5–15% salinity, proving that sulfate reducers were out-competing methanogens at the lower salinities. Despite this, at the in situ salinity, the rate of methanogenesis was only about 5% of sulfate reduction and thus contributes marginally to carbon mineralization (Sørensen et al. 2004). Sørensen et al. (2009) compared two ponds from the Eilat saltern with salinities similar to those in the above study, showing that methanogens (based on *mcrA* gene abundance) were largely restricted to the deepest layers of the phototrophic mats and more abundant in the 15.6% pond than the 20.6% salinity pond.

Giani et al. (1989) investigated methanogenesis as a function of depth and salinity in Kervalet saltern ponds (Bretagne) by placing gas domes on top of microbial mats. Methanogenesis was enhanced by the addition of methylated amines and methanol but not by acetate, formate, H_2/CO_2, and glycerol. Surprisingly, rates of methane evolution were almost as great from the highest-salinity ponds (20–33%) as from the 5% and 19% salinity ponds and were up to a hundred times higher than those reported for typical marine environments, despite elevated sulfate concentrations. Methane production decreased at intermediate salinities (7–12%), possibly due to increased anaerobic methane oxidation (Giani et al. 1989).

Fine-scale analysis of intertidal mats in hypersaline Shark Bay, Australia, revealed higher rates of methanogenesis and more immediate production of methane, in a lower-tidal mat compared with a higher mat (Wong et al. 2017). The water had a salinity of 6.8% at the time of sampling, but the degree of aerial exposure, and thus variation in desiccation and salinity, was much greater in the mat from the high-tide zone, which was dominated by extremely halophilic haloarchaea. Anaerobic slurries from the oxic and anoxic parts of the mats showed rapid methane production, with a shorter lag in the oxic mat incubations, probably reflecting higher substrate concentration and anoxic microsites within the mat surface. The lower-tide mat housed hydrogenotrophic and methylotrophic methanogens, while in the bottom part of the higher-tide mat methylotrophic methanogens were detected (Wong et al. 2017). *Methanomassiliicoccales* DNA signatures were detected in the lower-tide mat (Wong et al. 2017) and have also been detected in microbial mats in Salar de Llamara, Chile, which has a salinity of 4.3–6.6% (Saghaï et al. 2017). However, the salinity range over which representatives of this order function has not been tested. Wong et al. (2017) speculate that spatial co-existence of hydrogenotrophic sulfate reducers and methanogens near the surface of low-tide mats is due to tidal dynamics and consequent large fluctuations in salinity, resulting in temporal division of sulfate reduction and methanogenesis, with the latter dominant at higher salinities. Alternatively, they propose that sulfate reducers consume hydrogen at high concentrations, leaving lower concentration for *Methanomicrobiales.*

5 Soda Lakes

Soda lakes represent a specific type of salt lake where soluble sodium carbonate accumulates at molar concentrations along with NaCl, resulting in highly saline and alkaline brines with stable pH above 9. Methanogenesis has been observed in many soda lakes with high pH and salt concentration. The first evidence was obtained by the group of R. Oremland in North America, such as Big Soda Lake and Mono Lake (Oremland and Des Marais 1983; Oremland and Miller 1993; Oremland et al. 1982b, 1987, 1993; Iversen et al. 1987). The stable isotope and activity measurements indicated dominance of the methylotrophic pathway, but also the presence of hydrogenotrophic methanogens. However, the direct amplification of the *mcr*A gene from the hypolimnion brines of Mono Lake was not successful at that time, probably because primers were not optimized (Scholten et al. 2005). Later, in slurries from Searles Lake (30% salinity) and Mono Lake (9% salinity) incubated at 25 °C and pH 9.8, methane production rates decreased from 20–100 to 0.5–1 μmol cm^{-3} d^{-1} with an increase in salinity from 2.5% to 35% (Kulp et al. 2007), implying that many of the methanogens in these lakes survive periods of elevated salinity but are better adapted to lower salinity and are primed to become more active during wetter periods. It is notable, therefore, that in relatively recent history (thousands of years), both lakes were much fresher than currently.

Methanogenesis has also been investigated recently in soda lakes of two regions in Central Asia. In the southeastern part of Siberia (Tuva Republic and Transbaikal region, Russia), in moderately saline soda lakes, methanogenesis occurred either via

methylotrophic or hydrogenotrophic pathways, respectively, with no evidence for acetoclastic methanogens (Zavarzin et al. 1996; Namsaraev et al. 1999). However, more focused investigation of methanogenesis in hypersaline soda lakes of Kulunda Steppe in southwestern Siberia (Altai region, Russia) revealed a fundamental difference with hypersaline salt systems at neutral pH: all three methanogenic pathways were found to be active (Nolla-Ardèvol et al. 2012; Sorokin et al. 2015a). Methylotrophic methanogenesis was still the most active pathway, but hydrogenotrophy and even acetate-dependent methane formation were observed up to nearly salt-saturating conditions in hypersaline soda lakes of Kulunda Steppe. Screening the *mcr*A gene in sediment incubations and further microbiological analysis revealed that the hydrogen-/formate-dependent methanogenesis in soda lakes is performed by new representatives of the genus *Methanocalculus*, while acetate-dependent methane formation at low salt conditions is a function of a novel alkaliphilic *Methanothrix* species (Sorokin et al. 2015a, b). On the other hand, methanogenesis on acetate at high salinity and from propionate, butyrate, benzoate, and primary alcohols is driven by haloalkaliphilic syntrophic associations with *Methanocalculus* as a universal H_2-consuming partner (Sorokin et al. 2016).

6 Other Environments

In addition to the MSBL-1 group discussed previously, there are several reports of methanogenesis in hypersaline environments in which the associated archaeal clone libraries contain Euryarchaeota that do not fall within clusters of known methanogens, e.g., Solar Lake hypolimnion (salinity >15%; Cytryn et al. 2000) and Qinghai Lake, China (salinity, 12.5%; Dong et al. 2006). Studying a soil salinity gradient, Walsh et al. (2005) detected *Methanosarcinales*, including *Methanohalophilus*, as a minor component of the archaeal clones from the least saline (7%) soil, whereas at this salinity and higher there were numerous Euryarchaeota of unknown function.

Low temperatures are also not a barrier to methanogens. For example, in the Arctic Gypsum Hill spring (7.5% salinity; 5–7 °C), 4% of the archaeal community was represented by close relatives of methylotrophic, psychrotolerant *Methanococcoides* spp.; however, they were not detected in Colour Peak spring (salinity 15.5%; Perreault et al. 2007). Instead, in the higher salinity spring, there was an increase in putatively methanogenic Euryarchaeota (based on 85% 16S rRNA sequence similarity to various *Methanosarcinales*) to 16% of the archaeal community compared with 8% in the lower salinity spring (Perreault et al. 2007), again raising the important issue of understanding the true ecological roles of the Euryarchaeota giving rise to these 16S rRNA sequences.

7 Cycling of Methane

In most hypersaline environments, methanogenesis is quantitatively far less important than sulfate reduction in the cycling of carbon. Nevertheless, since aerobic methane-oxidizing bacteria are inhibited by high salt to a much greater extent than

methanogens, the overall methane flux to the atmosphere could be significant. Using a suite of incubation and inhibition techniques and measuring with gas chromatography, Conrad et al. (1995) found no evidence of methane oxidation in Solar Lake or in mats from the Eilat salterns, with salinities of 9% and 13%, respectively. By contrast, using ^{14}C-labeled methane, methanotrophy was observed in Crimean microbial mats with salinities up to 33% (Sokolov and Trotsenko 1995). There are also reports of pure methanotrophic cultures that can grow up to 15% NaCl (Heyer et al. 2005), and in situ methanotrophy has been observed in the alkaline-saline Mono Lake (salinity 9%) where both type I and type II methanotrophs were shown to be responsible and rates of aerobic methane oxidation exceed those found in many marine environments (Carini et al. 2005; Lin et al. 2005).

There is also geochemical evidence of anaerobic methane oxidation in Mono Lake (Joye et al. 1999) and Big Soda Lake (Iversen et al. 1987). Lloyd et al. (2006) examined sediments overlying a brine-pool methane seep in the Gulf of Mexico and showed that the archaeal community consisted predominantly of ANME-1b methane oxidizers (i.e., a phylogenetically distinct group of methanogens previously shown to oxidize methane by reverse methanogenesis in cooperation with sulfate-reducing bacteria), particularly concentrated around the methane-sulfate interface, where the salinity reaches 13%. Maignien et al. (2013) demonstrated anaerobic methane oxidation by ANME-1 Archaea at a salinity of 29% in cold-seep sediments of Mercator mud volcano. In lower salinity environments where anaerobic methane oxidation is occurring, other ANME groups are usually present, and ANME-1 sequences are also found. ANME-1-related sequences were also relatively abundant in Mediterranean deep-sea hypersaline anoxic basins (Daffonchio et al. 2006; Yakimov et al. 2007a, b; La Cono et al. 2011). This distributional data coupled with data derived from metabolic pathway reconstruction (see Cui et al. 2015) suggest that the ANME-1 group is better adapted to hypersaline conditions than other anaerobic methane oxidizers. How ANME-1 Archaea gain sufficient energy for growth under such energy-requiring hypersaline conditions (Oren 2011) deserves more detailed investigation.

It is well known that at marine cold seeps the increase in alkalinity, induced by anaerobic oxidation of methane coupled with sulfate reduction, leads to the precipitation of authigenic carbonates (Naehr et al. 2007). In addition, under hypersaline conditions, the following, intriguing, and potentially widespread phenomenon is also believed to be driven by anaerobic oxidation of methane (or other hydrocarbons). Ziegenbalg et al. (2012) investigated secondary carbonates and native sulfur from beneath a gypsum ($CaSO_4.2H_20$) layer that formed during the evapo-concentration of the Mediterranean during the Messinian Salinity Crisis. The biogenic limestones contained pseudomorphs after gypsum and were depleted in $\delta^{13}C$, suggesting that the carbonates were formed by archaeal methane oxidation coupled with sulfate reduction, whereby the terminal electron acceptor derived from dissolution of gypsum.

As mentioned previously, methylamines are probably the main carbon and energy source for halophilic methanogens, and these largely derive from compatible solutes such as glycine betaine, which enter the environment upon cell death. Zhilina and Zavarzin isolated the first halophilic homoacetogen, *Acetohalobium arabaticum*,

which converted glycine betaine to acetate and trimethylamine. When grown in co-culture with a *Methanohalophilus* strain, only acetate and methane accumulated in the medium, demonstrating the trophic link between an abundant compatible solute and methanogenesis in hypersaline environments (Zhilina and Zavarzin 1990). Subsequently several other members of the *Halanaerobiales* able to produce methylamines from betaine have been described (see Oren 2002). *Methanohalophilus* spp. and members of the *Halanaerobiales* frequently co-occur in hypersaline environments (e.g., Daffonchio et al. 2006; Borin et al. 2009; La Cono et al. 2015; Yakimov et al. 2015; Daly et al. 2016). For example, La Cono et al. (2015) obtained a stable three-member consortium at 24% salinity, including a glycine betaine-degrading *Halobacteroides* sp., a methylotrophic *Methanohalophilus* sp., and a *Halanaerobium* sp. that ferments other substrates, perhaps benefiting the consortium by regenerating reducing equivalents.

From the above discussion it is clear that the relationship between methanogenesis and salinity is far from simple and is dependent on the geological, historical, and current physico-geochemical situation of the environment. Methanogenesis may decrease with increasing salinity owing to a concomitant increase in sulfate concentration, which in turn provides an abundant supply of terminal electron acceptor for sulfate-reducing bacteria that out-compete methanogens for common substrate, such as acetate or hydrogen. Alternatively, methanogenesis may increase as salinity rises, because many microbes use glycine betaine as a compatible solute to cope with high salinity; and upon release from the cell, fermentative organisms can convert glycine betaine to methylamines (Fig. 1), which provide an energy-rich compound that can be used by many halophilic and halotolerant methanogens, but not by sulfate-reducing bacteria. Additionally, competition may be diminished by sulfate being used up, e.g., at depth or by precipitation of sulfate minerals. Nevertheless, growth at very high salinities is energetically demanding and so often serves to inhibit methanogenesis. Another important conclusion is that the difference in major salt composition might result in a very different impact of hypersalinity on growth and activity of the low-energy-generating anaerobic prokaryotes, involved in methanogenesis, including syntrophic bacteria and methanogenic Archaea. In particular, in soda lakes, whereby the weak electrolytic sodium carbonates are the dominant salt, the upper salinity barrier for hydrogenotrophic methanogens seems to be twice as high as in the NaCl-dominated habitats. A plausible explanation for this, as suggested by Sorokin et al. (2015c), is that sodium carbonates impose exactly two times less osmotic pressure in comparison with NaCl with the same molarity of Na^+.

8 Cultivated Halophilic Methanogens

Cultivated, taxonomically described halophilic methanogens are shown in Table 1, and in addition there are several interesting halophilic strains whose names have not been validly published, especially in the genera *Methanohalophilus* and *Methanohalobium* (see Zhilina 2001). Strain OCM 283, from an oil-reservoir brine, has a specific requirement for calcium and magnesium ions, reflecting the ionic

Table 1 Cultivated, taxonomically described halophilic methanogens

Order/species	Strain	Habitat[a]	pH opt [b]	Na^+ range[c]	Na^+ opt [c]	Substrate[d]	Original publication
Methanosarcinales							
Methanohalophilus portucalensis	OCM 59	Saltern sediments, Portugal		0.3–4.3	0.5–2	Methanol, MA	Boone et al. (1993)
Methanohalophilus halophilus	OCM 160 = DSMZ 3094	Shark Bay, Australia, microbial mat		0.3–2.5	1.2–1.6	Methanol, MA	Zhilina (1983)
Methanohalophilus mahii	OCM 68 = ATCC 35705	Great Salt Lake, sediment		0.5–3.4	1–2.5	Methanol, MA	Paterek and Smith (1988)
Methanosalsum zhilinae	OCM 62 = DSMZ 4017	Bosa Lake Wadi Natrun		0.2–2.2	0.7	Methanol, MA, DMS	Mathrani et al. (1988)
Methanosalsum natronophilus	DSMZ 24634	Soda lakes, Kulunda Steppe	9.5	0.5–3.5		Methanol, MA, DMS	Sorokin et al. (2015b)
Methanohalobium evestigatum	OCM 161 = DSMZ 3721	Sivash Lake, microbial mat		2.5–5	4.1	Methanol, MA	Zhilina and Zavarzin (1987)
Methanomicrobiales							
Methanocalculus halotolerans	OCM 470	Oil field, Alsace		0–2	0.8	H_2 and CO_2, formate	Ollivier et al. (1998)
Methanocalculus alkaliphilus	DSMZ 24457	Soda lakes, Kulunda Steppe	9.5	0.3–2	0.6	H_2 and CO_2, formate	Sorokin et al. (2015b)
Methanocalculus natronophilus	DSMZ 22006	Soda lakes, Kulunda Steppe	9–9.5	0.9–3.3	1.6	H_2 and CO_2, formate	Zhilina et al. (2013)

[a]Habitat from which the type strain was isolated
[b]pH optimum for growth shown only for alkaliphiles
[c]NaCl range in which the species can grow, and opt = optimal salt concentration for growth; concentration in M Na^+
[d]*MA* methylamines, *DMS* dimethyl sulfide

composition of the oil-field brine from which it was isolated (Obraztsova et al. 1988). It was originally named "Methanococcoides euhalobius" (Obraztsova et al. 1988), but based on its wide NaCl tolerance (1–14%, optimum 6%), growth with methanol and methylamines, and 16S rRNA sequence comparison, it was proposed to transfer it to the genus *Methanohalophilus* (Davidova et al. 1997); however this name has never been validated. A strain whose name was validly published as *Halomethanococcus doii* (Yu and Kawamura 1987), but which probably belongs to the genus *Methanohalophilus*, has been lost (Boone 2001). Nakatsugawa (1991) described strain NY-218 which grew optimally at 14–18% NaCl and grew well on methylamines and methanol and moderately on acetate. *Methanocalculus halotolerans* is the most halotolerant strain known that uses hydrogen and carbon dioxide, as well as formate, as growth substrates (Ollivier et al. 1998). The genus *Methanolobus* has several slightly halophilic strains, such as *Methanolobus oregonensis* DSMZ 5435, which grows up to 9% NaCl, which, in addition to using methanol and methylamines, can use dimethyl sulfide and methanethiol (Liu et al. 1990). The combined properties of halophily and alkaliphily and the ability to convert methanethiol to methane by *Methanolobus oregonensis* and related species are actively being tested for desulfurization of petroleum and gas (van Leerdam et al. 2008).

Methanogens cope with elevated salinity by the accumulation of organic compatible solutes, often together with potassium ions which serve to counter the charge of anionic solutes. Additionally, enhanced expression of the gene coding for ClpB, a chaperone of the AAA+ superfamily, was identified in response to hyper- and hypoosmotic stress (Shih and Lai 2007). However, addition of glycine betaine reduced expression of *clpB* upon hyperosmotic shock, indicating that it has an important role in protein protection at high salinity. The common osmolyte and compatible solute, glycine betaine, can be synthesized and accumulated by many methanogens, but they also use a variety of uncommon solutes, for example, *Methanohalophilus* spp. use *N*ε-acetyl-β-lysine, β-glutamine, Lα-glutamate, and α-glucosyl-glycerate (Lai et al. 1991; Roberts 2005). Biosynthesis of glycine betaine is by stepwise methylation of glycine (Lai et al. 1991; Roberts 2005). Phosphoproteomic analysis of *Methanohalophilus portucalensis* demonstrated the importance of protein phosphorylation in regulating the synthesis of glycine betaine, its main compatible solute, in response to high salinity (Wu et al. 2016). Thus, on the one hand, not only does glycine betaine serve as a source of carbon and energy after fermentation to methylamines by other organisms (or sometimes directly), it is also an important osmoprotectant. It would be interesting to learn the extent to which halophilic methanogens "monitor" the concentration of dissolved glycine betaine in the environment and adjust its uptake and metabolism accordingly, on the one hand taking advantage of a premade compatible solute while on the other retaining sufficient for future growth.

Further insights into environmental adaptation are starting to come from genomic analysis of halophilic methanogens. For example, *Methanohalophilus mahii,* relatives of which often inhabit photosynthetic mats and are thus exposed of liberated oxygen and ultraviolet light, has been shown to possess an array of enzymatic defenses against oxidative stress and biomolecule repair mechanisms (Spring et al. 2010). Numerous other genomes are available, providing the basis for functional analysis to investigate the adaptation of methanogens to hypersaline environments.

9 Research Needs

Few halophilic methanogens have been isolated recently, and publication dates in Table 1 indicate that none has been taxonomically described in the last decade, except for the soda lake haloalkaliphilic species. Also, the previous discussion highlights the presence of numerous Euryarchaeota that may be halophilic methanogens, but which remain to be cultivated. Bringing such organisms into culture should be a research priority, as was done for peat-bog methanogens, now represented by *Methanoregula boonei* (Bräuer et al. 2006, 2011) and with a fully sequenced genome. The explosion of metagenomic approaches has shown highly unexpected results concerning the presence of putative methanogens in several very deep phylogenetic lineages, even outside of the Euryarchaeota, but it still needs confirmation by cultivation or, at the very least, activity tests (Borrel et al. 2014; Evans et al. 2015; Nobu et al. 2016; Vanwonterghem et al. 2016). For methanogens that evade cultivation, metagenomics, single-celled genomics, and stable-isotope probing, following ^{13}C (e.g., from methylamines and other growth substrates) into lipids and nucleic acids can provide a link between function and phylogeny.

The isolation of methanogens with different modes of methanogenesis, including in hypersaline environments (see Sect. 10), allows new probes and primers to be applied to assess their abundance, diversity, and gene expression. Moreover, it is important to determine how their mode of methanogenesis affects the stable-isotopic composition of methane, as many inferences about the dominant mechanism of methanogenesis are made using this approach (e.g., Kelley et al. 2012, 2014).

There are numerous other areas that need further research if we are to truly understand carbon cycling in the past, present, and future: it is important to understand how methanogens cope with desiccation (from rice paddies to salt marshes) because not only must methanogens tolerate low water activity but also increased exposure to oxygen. What are the constraints of growth at high salinity – energetic (e.g., cost of making compatible solutes), competition, nutrient uptake, etc. – and how do these affect methane producers and consumers over space and time? Research into these areas may help us to learn whether and how aerobic haloarchaea evolved from strictly anaerobic methanogens and direct the search for putative missing links between the two archaeal groups. Some of those questions now have a chance of being answered with the discovery of a new lineage of extremely halophilic methanogens in salt-saturated lakes.

10 Extremely Halo(alkali)philic Methyl-Reducing Methanogens Present in Hypersaline Lakes

During methanogenic incubations of sediment slurries from hypersaline soda lakes, the screening of the *mcr*A gene resulted in the detection of two obscure deep-lineage sequences unrelated to any known halophilic methanogens (Sorokin et al. 2015a). Further attempts to activate this lineage demonstrated that it favored a combination of salt-saturating conditions, high pH, and elevated temperature while being fed with

a mixture of substrates used by both methylotrophic and hydrogenotrophic methanogens – i.e., methanol or trimethylamine + formate or H_2 (Sorokin et al. 2017). The latter indicated that these methanogens utilized the fourth methanogenic pathway known as "methyl reduction," whereby the C_1-methylated compounds are only used as electron acceptors, while H_2 is used as an external electron donor. This pathway, until now, has been found only in two cultured mesophilic methanogens, but recently it has attracted much attention because all the novel methanogenic deep lineages (see above) appear to be methyl-reducers (Borrel et al. 2014; Evans et al. 2015; Vanwonterghem et al. 2016). However, utilization of formate as the electron donor is reported for none of them. The combination of abovementioned conditions eventually resulted in isolation of 11 pure cultures of the extremely halo(alkali)philic methyl-reducing methanogens from hypersaline soda lakes and three highly enriched cultures of extremely halophilic methyl-reducers from hypersaline salt lakes (Sorokin et al. 2017). On the basis of the 16S rRNA gene analysis, both groups were identified as members of the uncultured SA1 group of Euryarchaeota found previously in several hypersaline habitats, such as deep-sea brines of the Red Sea (Eder et al. 2002) and the Mediterranean (Yakimov et al. 2013), terrestrial Solar Lake (Sørensen et al. 2004), and Lake Chaka (Jiang et al. 2007). The soda and salt lake isolates form a family group with two genera having 90% 16S rRNA gene similarity to each other but only distantly related to the other Euryarchaeota (81–82% to its classes). Based on its ribosomal protein phylogeny, the novel methanogens are most closely related to the class *Halobacteria* (commonly referred to as haloarchaea), which is consistent with their extreme halophily (optimal growth and activity is at 4 M total Na^+). Furthermore, the organic osmolytes seem to be absent in the cells. Instead, high intracellular concentrations of potassium are detected, which differentiates the novel lineage from the known halophilic methanogens even further. They are currently suggested to form a novel class "Methanonatronarchaeia" (Sorokin et al. 2017). One of the interesting ecological implications of this finding is that, with the emergence of this extremely halophilic lineage, the previously established fundamentals on the methanogenesis in hypersaline conditions might be compromised. These organisms are not truly methylotrophic and not truly hydrogenotrophic – they are something between, which means that the dominance of classical methylotrophic methanogenesis in salt-saturating habitats is becoming questionable. For example, in many incubations of anaerobic sediments from salt-saturated lakes, both soda and salt, at elevated temperatures (50–60 °C), we observed that methane formation under methyl-reducing conditions rapidly out-competed the classical methylotrophic process (in the complete absence of hydrogenotrophy) (Sorokin et al. 2017). But even when the process was seemingly classically methylotrophic (addition of only methylated substrates) at elevated temperature, further enrichment was only possible after addition of an external electron donor, such as formate, to the original methyl substrate. This indicates that in the absence of an externally added electron donor, the sediments still produced a low level of H_2 or formate from endogenous fermentation to support a low-level activity of the methyl-reducers. But this effect disappeared as soon as the original sediments were diluted out (Sorokin et al. 2017). The implication here is that it might be that the evidence for

the prevalence of the classical methylotrophic pathway in hypersaline habitats, especially at salt-saturating conditions, might be overestimated. Another question is what to consider "methylotrophic" and "hydrogenotrophic" if the methyl-reducers are both? And yet another question, currently unclear, is why this pathway is outcompeting the classical methylotrophy at salt-saturating conditions despite the fact that energetically they are almost equal? We hope to solve these questions in the future, since, in contrast to most of the recently discovered deep-lineage methyl-reducers, these extremely halophilic ones have been cultured (Sorokin et al. 2017).

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Metagenomics of Methanogenic Communities in Rice Paddy: The Importance of *Methanocella*

12

Yahai Lu

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Abstract

Methane is a potent greenhouse gas in the atmosphere that has shown nearly tripled increase since the preindustrial era. Paddy fields represent an anthropogenic source contributing about 5% of annual global CH_4 emission. It is important to understand the mechanism of CH_4 production and emission in order to understand carbon cycling and develop mitigation technology for CH_4 emissions. In this chapter, I review the research advances of methanogenesis in association with rice roots with an emphasis on the finding and characterization of

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Methanocellales methanogens. The importance of root-derived C as a major C source for CH_4 production, the identification of *Methanocellales* as the key methanogens responsible for CH_4 production in rice rhizosphere, and the genomic insights into the adaptation of the *Methanocellales* methanogens to paddy field environments have been discussed. Mechanistic understanding of *Methanocellales* ecophysiology shall not only shed a light on methanogen evolution and ecology but also pave a way towards the development of biotechnology for control of methane emissions from paddy fields.

1 Introduction

Rice is cultivated on approximately 155 million hectares worldwide, accounting for 14% of total arable land (Haefele et al. 2014). More than half of rice cultivation is under irrigated conditions. As a result, paddy fields are one of major anthropogenic sources for atmospheric methane (CH_4), a potent greenhouse gas that has increased from about 715 ppb in the preindustrial times to 1850 ppb in 2015 (Saunois et al. 2016; Schaefer et al. 2016). The Intergovernmental Panel of Climate Change (2013) reported an annual emission of 33–40 Tg CH_4 from the world paddy fields, equivalent to 12.5% of anthropogenic CH_4, or 5.0% of annual global CH_4 emission (IPCC 2013).

A campaign of CH_4 flux measurements and observations in paddy fields initiated in the late 80s of last century. The consensus of numerous measurements from Europe, America, and across Asia revealed that the seasonal pattern of CH_4 emissions from paddy fields comprises two or three peaks, with the first occurring in a few weeks after flooding and the second or third in the later season. The emission of CH_4 is the result of three processes, namely, production, oxidation, and transportation. The production is processed by methanogens living in anoxic niches of paddy fields. Rice plants have the well-developed aerenchyma system that serves as the major conduit for the transportation of CH_4 into the atmosphere. This aerenchyma system also allows the diffusion of O_2 from the atmosphere to the roots and the surrounding soil, i.e., the rhizosphere. Thereby, the roots of rice plants and the rhizosphere become partially oxic and allow aerobic activity and especially the oxidation of CH_4 that consumes on average a half of CH_4 produced in the anoxic soils before emission (Conrad 2004; Liesack et al. 2000).

In correspondence to the seasonal pattern of CH_4 emissions, the production of CH_4 in paddy fields is considered comprising two phases. In the first phase, the soil organic matter, plant residues, and/or manures deposited from previous season provide the substrates for methanogenesis (Cicerone et al. 1992; Sass et al. 1991; Yagi and Minami 1990). In the later phase, the methanogenic substrates results mainly from the newly-generated plant materials, namely, the root exudates and sloughed-off root cells and debris (Holzapfelpschorn et al. 1986; Lindau et al. 1991). These two phases, often overlapping in reality, are assumed to correspond to the first and the second (and/or third) peak of CH_4 emissions from paddy fields (Kimura 1997; Vandergon and Neue 1995). Obviously, the roots of rice plants play very

important roles in CH_4 emissions from paddy fields. In this chapter, I review the research advances of methanogenesis in association with rice roots with an emphasis on the finding and characterization of *Methanocellales* methanogens. Four aspects will be highlighted in particular: (1) the importance of root-derived C as a major C source for CH_4 production in paddy fields; (2) identification of *Methanocellales* as the key methanogens responsible for CH_4 production from the root-derived C; (3) phylogenetic and genomic characterization of the *Methanocellales* methanogens; and (4) mechanistic understanding of ecophysiology of *Methanocellales* in rice rhizosphere.

2 Importance of Root-Derived C in Methane Formation

Rhizosphere is the critical interface in terrestrial ecosystem. Through this interface, plants take up nutrients from soil and in return release photosynthesized products into the soil, feeding soil microbes. Microorganisms in the rhizosphere are actively involved in biogeochemical cycling of C, N, S, Fe, and many other elements (Arth et al. 1998; Lu et al. 2002; Neubauer et al. 2002; Scheid and Stubner 2001). It has been estimated that 30–60% of the net photosynthesized carbon is allocated to the roots, and 40–90% of this fraction is released into soil in the forms of root exudates, sloughed-off cells, and root debris or rhizodeposition to name together (Lynch and Whipps 1990). The rhizodeposition in paddy soils serves as a major carbon source for CH_4 production. By using a ^{13}C tracer approach, Minoda and Kimura (1994) revealed that part of photosynthesized ^{13}C was transported to the rhizosphere, transformed to CH_4, and emitted to the atmosphere just a few hours after the commencement of $^{13}CO_2$ application (Minoda and Kimura 1994). Dannenberg and Conrad (1999) reported that about 3–6% of the assimilated radioactivity ($^{14}CO_2$) by rice plants were emitted as $^{14}CH_4$ within 16 d after labeling (Dannenberg and Conrad 1999).

The rhizodeposition can be separated into different groups such as water-soluble exudates, secretions, lysates, mucilages, sloughed-off cells, decaying root debris, and gases (Bolton Jr. et al. 1993). It can contain all kinds of chemicals found in a plant cell, from sugars, amino acids, organic acids to more complex components such as proteins, polysaccharides, lipids, hormones, and vitamins. To evaluate the effect of rhizodeposition in methanogenesis, a comparative experiment was conducted using acetate and glucose as controls (Lu et al. 2000c). The effect of root exudates was found to be similar to acetate and glucose. But the addition of acetate and glucose yielded a significant priming effect on the decomposition of soil organic matter leading to a higher CH_4 production, while root exudates caused only a moderate priming effect (Lu et al. 2000c).

To directly evaluate the effect of rhizodeposition on CH_4 production and emission, the experiments under in situ conditions were conducted by observing the spatial variation of dissolved organic carbon (DOC) and dissolved CH_4 in soil porewater along with a distance from rice roots (Lu et al. 2000a, b). These studies revealed that DOC in root zone soil, i.e., the rhizosphere, increased substantially

with plant growth while that in the nonroot zone soil did not show significant change. Since no external organic materials were added, the increase of DOC in the root zone soil reflects the release of organic C from plant roots. The maximal concentration of DOC occurred between rice flowering and maturation, in consistence with the observation that root exudation of rice plants reached maxima during these stages (Lu et al. 1999). Dissolved organic C represents a mobile and labile form of soil organic matter and is expected to be easily degradable.

Correspondingly, the dissolved CH_4 in the root zone soil began to increase at the maximum tillering stage of rice plants and reached to the maxima at the maturing stage (Lu et al. 2000a, b). In the nonroot zone, CH_4 concentrations also increased gradually to the levels comparable with those in the root zone. But a lag period of 1–3 weeks was consistently detected. Higher dissolved CH_4 in the root zone soil compared with nonroot zone soil (Fig. 1) suggests that CH_4 in the root zone soil was produced locally from the decomposition of DOC pool derived from plant photosynthesized C. In correspondence with the concentration of dissolved CH_4, the rate of CH_4 emission increased significantly during the period from rice flowering to

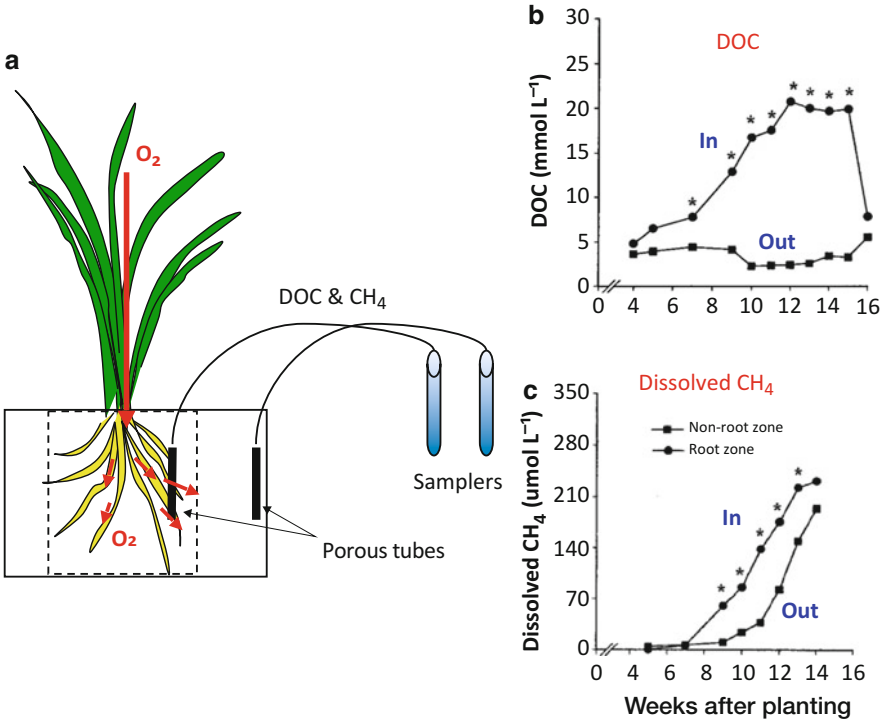

Fig. 1 Rice-plant microcosm for observing the dissolved organic C (DOC and dissolved CH_4 in paddy soil. (**a**) Two sampling ceramic tubes are buried vertically beneath the soil surface with one close to root zone (i.e., rhizosphere) and another outside root zone; (**b**) Seasonal change of DOC in root zone (In) and outside root zone (Out); (**c**) Seasonal change of dissolved CH_4 in root zone (In) and outside root zone (Out). (Taken from Lu et al. 2000b)

maturation. The statistical analyses revealed significant positive linear correlations between the porewater DOC, dissolved CH_4, and the rate of CH_4 emission over the growing season of rice plants (Lu et al. 2000a, b). These results support that the late season peaks of CH_4 emission are due to the supply of plant-borne C through rhizodeposition (Neue et al. 1997).

Collectively, the pioneering studies demonstrated that the DOC pool in the rice rhizosphere was continuously enriched by plant-borne C during plant growth and this DOC pool is easily available for methanogenesis. The rice rhizosphere is probably a very important place for methanogenic activity. This finding however is apparently in conflict with the conventional theory that methanogens are known to be strictly anaerobic while the root surface and the closely connected rhizosphere is partly oxic due to O_2 leaks from the plants. It remains elusive why certain methanogens can survive and even thrive in the rice rhizosphere.

3 *Methanocellales* as the Key Methanogens in Rice Rhizosphere

Two hypotheses were proposed to explain the activity of methanogenesis on rice roots and the rhizosphere: (i) methanogens colonizing rice roots are probably O_2 resistant; (ii) they may develop a spatial strategy, inhabiting where O_2 does not exist, for example, the old root segments where O_2 release is lacking (Conrad 2004; Grosskopf et al. 1998b). Indeed, the community composition and activity of methanogenic archaea in association with rice roots differ greatly from those in the soil distant from roots. Specifically, the CO_2 reduction pathway was found to be prevalent in CH_4 production in rice root preparations (Chin et al. 2004; Conrad and Klose 1999, 2000; Lehmann-Richter et al. 1999), whereas the aceticlastic pathway usually accounted for over 65% of total CH_4 production in the anoxic bulk soil (Conrad 1999; Conrad et al. 2002; Wind et al. 1999). Both environmental detection and enrichment cultivation from the excised rice roots revealed a dominance of an uncultured archaeal linage Rice Cluster I (Grosskopf et al. 1998b; Lehmann-Richter et al. 1999), which was later characterized as hydrogenotrophic methanogens and finally isolated into pure culture as a novel methanogen order *Methanocellales* (Lu and Lu 2012b; Sakai et al. 2008, 2010). The *Methanosaeta* spp. that often dominated in the bulk soil (Chin et al. 1999; Fey and Conrad 2003; Grosskopf et al. 1998a) was rarely detected on rice roots (Chin et al. 2004). These preliminary studies suggest that a very different population of methanogens are selected by rice roots. To identify the active methanogenic organisms responsible for CH_4 production in rice rhizosphere, several experiments using molecular and isotopic labeling approaches were conducted (Lu and Conrad 2005; Lu et al. 2005).

3.1 *Methanocellales* on Rice Roots

In an incubation experiment using the excised rice roots as inoculants, the ^{13}C fully labeled CO_2 was applied with H_2 or N_2 in the headspace of incubation vessels

(Lu et al. 2005). Two pH buffer systems based on carbonate or phosphate were prepared for incubation. The conditions thus created included the combination of buffer system phosphate (P) or carbonate (C) and the headspace composition H_2 or N_2. The $^{13}CH_4$ was detected immediately after the anaerobic incubation of rice roots, indicating the readily activity of methanogens from rice roots. The production of CH_4, however, was faster in C buffer than in P buffer. Strikingly, the rate of CH_4 production was greater with N_2 than with H_2 in the headspace during the initiation period of methanogenesis. An estimate based on ^{13}C labeling under C–N_2 combination indicated that approximately 100% and 65% of CH_4 were produced from CO_2 reduction during the early and late periods, respectively. These estimates were consistent with previous reports showing the prevalence of hydrogenotrophic methanogenesis on rice roots (Conrad and Klose 1999; Conrad et al. 2002; Lehmann-Richter et al. 1999). The higher CH_4 production under C–N_2 compared to C–H_2 combination indicates that the supply of H_2 resulted in a negative effect on CO_2-reducing methanogenesis in the incubations. This was somewhat surprising as H_2 was the energy source for hydrogenotrophic methanogens to reduce CO_2 for CH_4 production.

The analysis of archaeal 16S rRNA gene abundances revealed a significant difference in community composition among different conditions. Under C–N_2 condition, the *Methanocellales*, the yet-uncultured archaeal lineage by the time, showed a significant increase of 16S rRNA gene abundances in the ^{13}C-labeled DNA, indicating that these methanogens were more active than others. Apparently, the *Methanocellales* were responsible for CH_4 production from H_2/CO_2, the dominant pathway of CH_4 production in rice root preparations. The relative abundance of *Methanosarcinaceae* also increased in the late stage, indicating the increasing contribution of acetate-dependent methanogenesis towards the end of incubation (Chin et al. 2004; Conrad et al. 2002). When H_2 was supplied (i.e., under C–H_2), the *Methanosarcinaceae* became exclusively dominated, whereas *Methanocellales* were detected only at low abundance. The high H_2 condition apparently favored the growth of hydrogenotrophic *Methanosarcina* spp. over *Methanocellales*. Under P buffer conditions, the *Methanobacteriaceae* and *Methanosarcinaceae* were selected, while *Methanocellales* were present only marginally.

The *Methanocellales* had been repeatedly detected in different environments including rice roots, anoxic rice soils (Chin et al. 2004; Grosskopf et al. 1998a; b; Lueders and Friedrich 2000), and wetlands (Galand et al. 2002; Jurgens et al. 2000; Sizova et al. 2003). Little had been known however about their physiology. The above DNA-SIP experiment revealed that these methanogens were remarkably suppressed when H_2 was supplied to either P or C buffer systems. A previous enrichment study showed that phosphate was not toxic to *Methanocellales* (Lehmann-Richter et al. 1999). Therefore, application of H_2 appeared the only reason for the depression of *Methanocellales* in root preparations. This finding increased the clouding in understanding methanogenesis associated with rice roots. It was speculated that *Methanocellales* were probably adapted to low H_2 condition and were less selective under the artificially H_2-enriched conditions. It has been reported that the H_2 partial pressure can indeed regulate the expression of genes involved in methanogenesis (Luo et al. 2002) that can vary depending on methanogen identity.

3.2 *Methanocellales* in Rice Rhizosphere

The unculturability of vast microbial species in environments demands culture-independent approaches to understand their activity and functioning. The development of stable isotope probing (SIP) in combination with molecular fingerprinting based on DNA and RNA provided such a powerful approach (Lu and Conrad 2005). This technique has been used to detect methanogens in rice root preparations as described above (Lu et al. 2005). To identify the active methanogens in rice rhizosphere under in situ conditions, RNA-SIP approach was applied to in an intact rice-soil system, in which rice plants were supplied with the ^{13}C-labeled CO_2 for plant photosynthesis and the photosynthesized ^{13}C was tracked for its distribution from the plant top to the rhizosphere and the assimilation by soils microbes.

In this plant-soil microcosm, CH_4 in soil pore water as well as that emitted into the air was found to be rapidly labeled with ^{13}C (Lu and Conrad 2005), suggesting that methanogenesis in the rice rhizosphere was active and closely linked to plant photosynthesis under in situ conditions. The ^{13}C labeled RNA retrieved from rice rhizosphere revealed a signature fingerprint associated with methanogenic archaea (Fig. 2). Specifically, a characteristic terminal restriction fragment (394-bp) was significantly enriched with ^{13}C out of seven fragments belonging to different archaeal lineages (Lu and Conrad 2005). By comparison, no specific signature fingerprint was revealed in the control microcosm without $^{13}CO_2$. Undoubtedly, the methanogenic archaeal lineage characterized by the signature fragment 394 bp

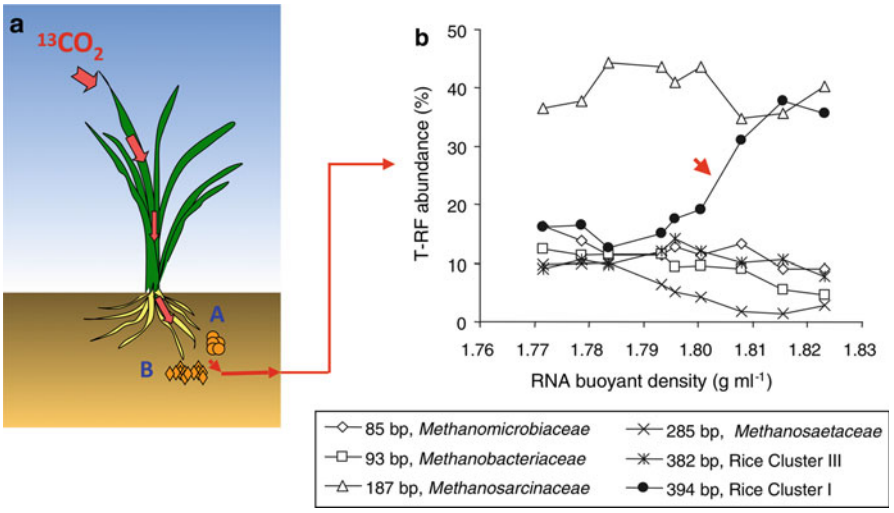

Fig. 2 Rice-plant microcosm for RNA-SIP detection of active methanogens in rice rhizosphere. (**a**) Rice plants were fed with $^{13}CO_2$ in a closed chamber and microbial RNA were extracted from rice rhizosphere for RNA-SIP dissection; (**b**) Fingerprinting of the density resolved RNA revealed that a signature fragment (394 bp), representative of Methanocellales (Rice Cluster I), was 13C labeled. (Taken from Lu and Conrad 2005)

assimilated the ^{13}C derived from organic substances that were deposited into the rhizosphere after photosynthesis. To characterize phylogenetic affiliation of this active methanogen in rice rhizosphere, the 16S RNA clone libraries were constructed, which revealed that out of seven methanogen lineages, the *Methanocellales* methanogens (i.e., uncultured RC-I by that time) was characterized with the signature fragment 394-bp (Lu and Conrad 2005). Thus, the *Methanocellales* were identified as the most active methanogens in rice rhizosphere where the release of organic substrates and O_2 leaks occur simultaneously. These results are in line with earlier studies showing that CH_4 production in excised rice root preparations is mainly due to the activity of *Methanocellales* (Lehmann-Richter et al. 1999; Lu et al. 2005; Lueders et al. 2001). Given the fact that paddy fields are an important source of methane emission (Conrad 2009; IPCC 2013) and plant-photosynthesized carbon provides a major source for CH_4 production in paddy soil (Lu et al. 2000a, b; Minoda and Kimura 1994), the identification of *Methanocellales* as the key player in rice rhizosphere opens a new window for further investigation and deeper understanding of methanogenesis in paddy fields.

4 Metagenomic Insights into *Methanocellales*' Adaptation to Rice Rhizosphere

After the discovery of *Methanocellales* as the key player of CH_4 production in paddy soils, it was highly demanding to elucidate the physiological mechanisms of their activity, particularly in a way associated with rice roots. Due to the nature of difficulty in isolating them into pure cultures, enrichment cultivations were intensively tried in the Max-Planck Institute for Terrestrial Microbiology that finally resulted in an enrichment, named MRE50, in which *Methanocellales* were the only archaeal component (Erkel et al. 2005). This enrichment was then served as a genomic source for constructing fosmid clone library in order to pinpoint the *Methanocellales* metagenome (Erkel et al. 2006). A complete genome sequence of a single *Methanocellales* representative (RC-I_{MRE50}) was reconstructed that offers the path to look into the putative metabolic capacity of *Methanocellales* methanogens.

The RC-I_{MRE50} genome has a size of about 3.18 Mb with 3103 predicted coding sequences. The genome reveals a series of unique features for energy conservation, biosynthesis, C and N metabolisms that are distinct from many known methanogens (Erkel et al. 2006). The central energy metabolism with CH_4 production from CO_2 reduction appears related to the hydrogenotrophic *Methanosarcina*, containing a membrane-bound hydrogenase with cytochrome b, a trait found only in the members of *Methanosarcinales* by the time (Thauer 1998). However, unlike *Methanosarcina* spp., RC-I_{MRE50} also encodes a system of using formate and formaldehyde for methanogenic growth, which is the typical trait of obligately hydrogenotrophic methanogens (Erkel et al. 2006). RC-I_{MRE50} harbors adenosine 5′-monophosphate-forming acetyl-coenzyme A (CoA) synthetase (ACS) for acetate assimilation and the carbon monoxide dehydrogenase complex

for acetyl-CoA biosynthesis from CO_2 that are common to most obligately hydrogenotrophic methanogens. But RC-I_{MRE50} additionally encodes a membrane-bound pyrophosphatase that can help these methanogens to recover a portion of the energy invested in acetate activation, which is not available in other methanogens that use ACS for acetate assimilation.

The pyruvate metabolism encoded in RC-I_{MRE50} includes ethanol production from acetaldehyde, acetoin production from acetolactate, and two pathways for acetyl-CoA formation from pyruvate. Most anaerobes including methanogens use the pyruvate-ferredoxin oxidoreductase that is oxygen-sensitive for the decarboxylation of pyruvate and acetyl-CoA production. By comparison, aerobes usually use the pyruvate dehydrogenase (PDH) for similar function. Interestingly, the RC-I_{MRE50} genome encodes both pathways. The PDH complex has been typically found in aerobic and facultatively anaerobic microorganisms but is lacking in all known methanogens by the time. It was therefore speculated that RC-I_{MRE50} likely uses the glycolytic pathway to survive the oxic periods (Erkel et al. 2006). Energy for maintenance may result from pyruvate and acetate production. Reducing equivalents generated from glucose and pyruvate oxidation can be recycled through the fermentation of pyruvate to ethanol. The allosteric control of the glycolytic pathway may allow RC-I_{MRE50} to respond quickly to the environmental changes in redox states.

The RC-I_{MRE50} genome appeared to contain biosynthetic pathways for all amino acids except glutamate (Erkel et al. 2006). But the glutamate synthesis was later found to be present in the genome analysis of *Methanocella* pure cultures (see details below). Nevertheless, RC-I_{MRE50} encodes a candidate ABC-type glutamate import system. The ability of RC-I_{MRE50} to take up glutamate from environments and to incorporate it into enzyme synthesis was experimentally confirmed (Erkel et al. 2006). This feature might confer an advantage for *Methanocellales* to live near rice roots as glutamate may be available in root exudates and/or decomposing plant root materials. Besides the glutamate uptake, RC-I_{MRE50} genome reveals two additional mechanisms for nitrogen acquisition via ammonium assimilation and dinitrogen fixation (nitrogenase). These combined traits indicate the metabolic flexibility of RC-I_{MRE50} in nitrogen acquisition. In addition, RC-I_{MRE50} also reveals an unique sulfur assimilation through the reduction of sulfate to sulfide. It contains genes coding for sulfurylase and adenylylsulfate kinase that are lacking in all methanogen genomes sequenced by the time. Most methanogens depend on sulfite, sulfide, or sulfur-containing amino acids as sulfur source for assimilation. The ability of RC-I_{MRE50} to use sulfate may confer *Methanocellales* another advantage to adapt the rhizospheric environment, where sulfate instead of the reduced sulfur forms may be available due to oxic conditions.

Since O_2 is diffused from the top of rice plants down to roots and released into the rhizosphere, the transient anoxic/oxic conditions prevail on root surface and in the rhizosphere soil. In addition, paddy fields often experience wet-dry cycling due to field management requirement (Liu et al. 2015). The key for methanogens to inhabit rice rhizosphere is therefore dependent on the capacity of resisting oxidative stresses. Aerotolerant systems were previously found in the aceticlastic *Methanosarcina* spp. The obligately hydrogenotrophic methanogens however acquire only a limited set of

antioxidant enzymes. Strikingly, the RC-I_{MRE50} genome encodes multiple sets of genes coding for antioxidant enzymes, including the mono-functional large subunit heme catalase that is most ancient and robust of all known catalases (Chelikani et al. 2004). Three different reactive oxygen species (ROS) scavengers are present that can be used to remove both external and internal superoxide anions. In particular, the exogenous superoxide anions can be scavenged by a periplasmic Cu, Zn-dependent superoxide dismutase (SodC) (Fournier et al. 2003), while the cytoplasmic superoxide anions be removed by two types of superoxide reductase (SOR) containing rubredoxin and desulfoferrodoxin, respectively. SORs are considered the most important oxygen defense systems in anaerobes (Jenney et al. 1999), especially under strong oxygen exposure (Fournier et al. 2003). In addition, the RC-I_{MRE50} also encodes bacterial-type enzymatic systems with repair mechanisms for oxidative lesions of DNA, such as formamidopyrimidine-DNA glycosylase (MutM), 3-methyladenine-DNA glycosylase (MPG), and the Holliday junction resolvasome (RuvABC) (Erkel et al. 2006). Possessing these multiple antioxidant and repair systems confers *Methanocellales* the extraordinary ability to be aerotolerant. Thus, *Methanocellales* are genetically equipped with competitive advantages over obligately hydrogenotrophic methanogens in the rice rhizosphere. Together with the potentials of acquiring alternate sulfur and nitrogen nutritions, *Methanocellales* appear to have evolved the methanogenic life well-fitting to the rice rhizosphere.

5 Isolation of *Methanocella* Species into Pure Culture

Despite the metagenomic insights into their adaptation to rice rhizosphere and more generally to oxic conditions, deeper understanding of their physiology and ecology is impossible without isolation of *Methanocellales* into pure culture. The efforts to isolate them therefore have never been stopped though the difficulty. The first pure culture of *Methanocella* were obtained from a Japanese rice field soil using a syntrophic cultivation approach. The formal order name, *Methanocellales*, was then given based on the phylogeny of this pure culture, and the strain itself was named as *Methanocella paludicola* strain SANAE^T (Sakai et al. 2007, 2008). The second isolate, a thermophilic methanogen, *Methanocella arvoryzae* strain MRE50^T, was later purified from the enrichment established for the metagenomic investigation (Lueders et al. 2001; Sakai et al. 2010). The isolation of these two strains would have offered a chance to address many ecophysiology questions. Unfortunately, despite the successful isolation of strains SANAE^T and MRE50^T, the maintenance and cultivation of these strains in lab require some extraordinary techniques, which impede the further investigations. Therefore, more isolates particularly with the fast-growing trait are still needed. Such a strain, *Methanocella conradii* strain HZ254^T, named after Ralf Conrad, a pioneering scientist on this methanogen lineage, was finally obtained from a Chinese paddy field soil (Lu and Lu 2012b). A moderate high temperature has been an effective strategy to isolate this strain, in line with early enrichment studies (Fey et al. 2001; Peng et al. 2008).

The third strain was phylogenetically closer to *M. paludicola* SANAE^T (16S rRNA gene similarity of 95.0% and mcrA gene similarity of 87.5%) than to *M. arvoryzae* MRE50^T (92.4% and 86.5% for the 16S rRNA and mcrA similarity, respectively) (Fig. 3) (Lu and Lu 2012b). Though three strains share some common phenotypic features, such as the rod-shaped morphology, they differ in formate utilization, flagellum formation, temperature optimum, pH range, and salinity susceptibility. In contrast to the phylogenetic relationship, strain HZ254^T seems to be closer to MRE50^T than SANAE^T in major phenotypic traits including temperature optimum, flagellum formation, and salinity susceptibility. The 16S rRNA gene sequence divergence of 5% between HZ254^T and SANAE^T implies that strain HZ254^T potentially represents a new genus instead of new species (Lu and Lu 2012b).

6 Comparative Genomics and Comprehensive Understanding of *Methanocellales*

6.1 Phylogeny and Taxonomy

Thus far three *Methanocella* strains have been available, namely *M. paludicola* SANAE^T, *M. arvoryzae* MRE50^T and *M. conradii* HZ254^T (Lu and Lu 2012b; Sakai et al. 2008, 2010). Though they have been classified together as a genus *Methanocella*, they could potentially represent multiple genera and even families due to low similarities of their 16S rRNA and mcrA genes (Lu and Lu 2012b; Sakai et al. 2010). In consistence with the analyses of 16S rRNA and mcrA, the global nucleotide identities calculated based on whole genome alignments suggest that *M. conradii* and *M. paludicola* are more closely related each other than to *M. arvoryzae*. The phylogenetic trees constructed based on multiple markers (i.e., 16S rRNA, mcrA and ribosomal proteins) also placed *M. conradii* closer to *M. paludicola* than to *M. arvoryzae* (Borrel et al. 2013; Lu and Lu 2012b). The Average Amino Identity (AAI) that can be more relevant to infer genetic relationship at high taxonomic levels indicated that *M. conradii* and *M. paludicola* together represent a genus, while *M. arvoryzae* alone represents a separate genus (Lyu and Lu 2015), according to the consensus criterion of AAI (Konstantinidis and Tiedje 2007).

Before the proposal of order *Methanocellales*, methanogens had been classified into five characterized orders, i.e., *Methanopyrales*, *Methanococcales*, *Methanobacteriales*, *Methanomicrobiales,* and *Methanosarcinales* (Liu and Whitman 2008). Comparative genomic analyses have grouped these orders into Class I (consisting of *Methanopyrales*, *Methanococcales* and *Methanobacteriales*), Class II (the *Methanomicrobiales*), and Class III (the *Methanosarcinales*) methanogens, respectively (Anderson et al. 2009). Phylogenetically, the *Methanocellales* can be placed between Class II and III methanogens (Lu and Lu 2012b; Sakai et al. 2008, 2010). Although the physiological relationships remain unclear, *Methanocellales* do share some ecological features with either Class II or III. For instances, both *Methanocellales* and Class II methanogens are detected in rice soils and wetlands where H_2 partial pressure is low (1–10 pa), whereas *Methanocellales* also share

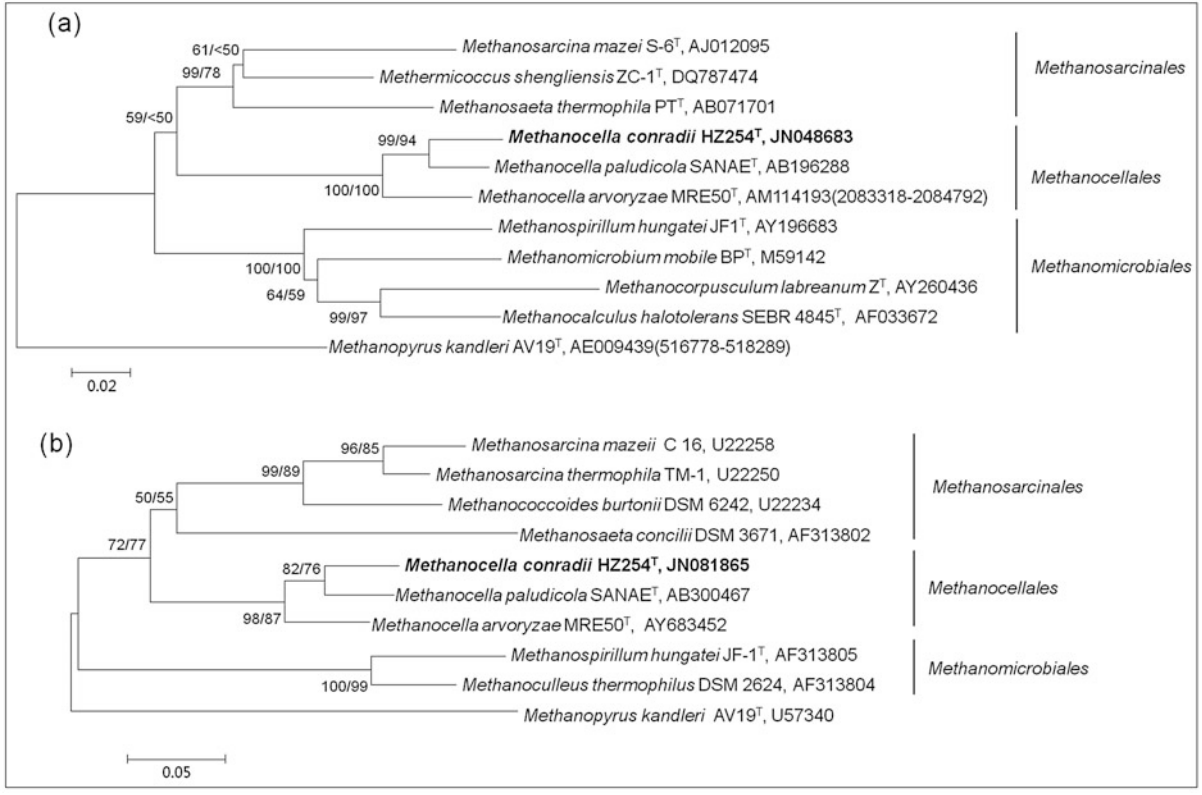

Fig. 3 Phylogeny of Methanocellales based on (**a**) 16 S rRNA genes and (**b**) Deduced methyl-coenzyme reductase subunit A amino acid sequence (McrA). (Taken from Lu and Lu 2012b)

common habitats with Class III methanogens, such as upland soils where aeration and desiccation occur periodically (Angel et al. 2012; Angel et al. 2011; Aschenbach et al. 2013; Conrad et al. 2006).

Genome sequences of three *Methanocella* strains and their comparative analysis offer an opportunity to elucidate the basic ecophysiology traits of this novel type of methanogens. A detailed reannotation of SANAE^T and MRE50^T was performed using the same annotation pipeline used for the third strain HZ254^T to ensure the consistency in comparison (Lyu and Lu 2015). The reannotation of SANAE^T and MRE50^T genomes revealed several new genes, pseudogenes, and some CRISPR region(s) that were not identified previously. Analyses of COG, Pfam, and TIGRfam classifications also revealed more functional insights into many genes not assigned before. Whole genome alignments revealed the extensive rearrangements of genomic regions among three strains. Three *Methanocella* strains share a core genome comprised of 1187–1245 ortholog groups, depending on the threshold set for amino acid identity (Lyu and Lu 2015). More orthologs are shared between *M. conradii* and *M. paludicola* than to *M. arvoryzae*, consistent with the phylogenetic relationship among them.

6.2 Novel Features of Core Metabolisms for Methanogenesis

All three strains possess a complete gene set for the typical hydrogenotrophic methanogenesis characterized as the closed Wolfe cycle (Thauer 2012). The major differences among three genomes are the copy numbers of several genes in the pathway, specifically the genes coding for the B subunit of F420-reducing hydrogenase (*frhB*), the D subunit of F420-nonreducing hydrogenase (*mvhD*), and the E subunit of energy-converting hydrogenase (*echE*). The ecological insights into these differences have yet to be evaluated. Two novel features, however, were identified that are shared by all three strains (Lyu and Lu 2015). The first is the gene organization related to the Wolfe cycle and the second is the presence of a putative [NiFe] hydrogenase complex that was not found in other methanogens.

Hydrogenotrophic methanogens are known to employ a multienzyme complex to perform the flavin-based electron bifurcation for the energy conservation from oxidation of H_2 or formate (Costa et al. 2010; Lie et al. 2012). This complex consists of formylmethanofuran dehydrogenase (Fwd), heterodisulfide reductase (Hdr), and Mvh (Fwd/Mvh/Hdr in short). The formate dehydrogenase (Fdh) may also join with the formation of the Fwd/Mvh/Fdh/Hdr supercomplex. In Class I methanogens, though the formation and functioning of Fwd/Mvh(Fdh)/Hdr multienzyme complex, the genes coding for these components are located separately in their genomes (Hendrickson et al. 2004; Kaster et al. 2011; Thauer et al. 2010). In contrast, *Methanocella* as well as many of Class II methanogens organize most of those genes into large gene clusters. A 10-gene cluster consisting of whole sets of *fwd* and *hdr* genes and a gene for the subunit D of Mvh (*mvhD*) was identified in all three *Methanocella* stains (Mtc_2477–2468, MCPlv_2811–2802, and MRE50lv_2189–2180) (Lyu and Lu 2015). There exists even a second larger gene cluster comprising the above 10 genes

together with two *fdh* genes in *M. arvoryzae* and *M. paludicola* (MCPlv_1593–1604 and MRE50lv_0274–0285). This unique organization of large gene cluster may facilitate the assembly of multienzyme complex with less biological cost and preventing the transcriptional resource waste (Anderson et al. 2009; Lie et al. 2012). The inclusion of *fdh* in the gene cluster may allow *M. arvoryzae* and *M. paludicola* to grow on formate as the sole carbon and energy source, whereas *M. conradii* is not known to have this ability (Lu and Lu 2012b).

Methanocella seem to be exceptionally adapted at low H_2 concentrations. This feature is initially illustrated in root preparation experiment (Lu et al. 2005). The isolation of the strain *M. paludicola* SANAE^T by using the syntrophic coculture technique confirmed that low H_2 condition favors *Methanocella* over other hydrogenotrophic methanogens (Sakai et al. 2007). More evidences are illustrated with the detection of *Methanocellales* in association with different bacteria syntrophs that syntrophically oxidize short-chain fatty acids in paddy soils (Gan et al. 2012; Liu et al. 2011; Lueders et al. 2004; Rui et al. 2011). Therefore, though the Class I methanogens use the similar Wolfe cycle and perform the flavin-based electron bifurcation for the core metabolisms, *Methanocellales* appear to possess a specific capacity to perform these functions at H_2 level close to the thermodynamic limit. The reason for this unique feature is possibly related to the presence of the large gene cluster coding for Fwd/Mvh/Hdr complex, which can confer a better efficiency in energy conservation through facilitating the assembly of multienzyme complex for electron bifurcation. Gene clustering is considered a common strategy used by prokaryotes to increase efficiency in forming protein complexes (Sneppen et al. 2010). A global transcriptional analysis for HZ254^T indeed illustrated the elevated expression of this gene cluster under limited H_2 condition in syntrophic coculture compared with high H_2 in monoculture (Liu et al. 2014).

The second unique feature of *Methanocella* genomes is the presence of a putative [NiFe] hydrogenase complex. The coding genes for this complex are organized into a 8-gene cluster (Mtc_0479–0486, MRE50lv_2279–2272 and MCPlv_2682–2674) including *echE* (energy-converting hydrogenase subunit E) and *hdrB* homologs (heterodisulfide reductase subunit B) (Lyu and Lu 2015). The *EchE* homologs possess the [NiFe] binding motifs and are phylogenetically more closely related to the bacterial Coo hydrogenase (carbon monoxide-induced hydrogenase) in the sulfate-reducing bacteria than to the canonical Ech hydrogenase in methanogens. A significant divergence from Coo and Ech is that the novel hydrogenase does not encode the Na^+/H^+ translocating subunit (i.e., CooM or EchA), while all other subunits essential for the oxidation of H_2 and electron transfer are present (Lyu and Lu 2015). Similar to the phylogeny of Ech, the HdrB homologs are phylogenetically more closely related to homologs in sulfate-reducing prokaryotes than to those in methanogens. Compared to the canonical form that catalyzes CoB-S-S-CoM reduction in methanogens, HdrB homologs in sulfate-reducing prokaryotes are involved in sulfite reduction and presumably reduce the intramolecular disulfide bridge of the DsrC (Dissimilatory sulfite reductase subunit C) (Grein et al. 2013). Based on phylogeny and traits described above, the novel hydrogenase is tentatively named as the Disulfide Reducing Hydrogenase (Drh) complex (Lyu and Lu 2015).

Due to the absence of the Na^+/H^+ translocating subunit, Drh would be unable to conserve energy from H_2 oxidation. It was speculated that the HdrB subunit in the Drh complex may use the disulfide of an unknown enzyme or compound as the electron acceptor (Aslund et al. 1997).

Methanocellales appears to have exceptional aerotolerant abilities, and all three strains encode a substantial number of genes involved in antioxidant resistance (Erkel et al. 2006) (and see below for further information). However, a robust antioxidant system would need to consume a number of reducing equivalents (Imlay 2008). The Wolfe cycle is unlikely to provide such a source, because its activity shall be severely repressed under oxic conditions. Given the close phylogenetic relationship of Drh to Coo and Fhl (formate-hydrogen lyase) that are known to be involved in CO detoxification and stress resistance (Bonam et al. 1989; Rossmann et al. 1991), Drh in *Methanocella* is probably involved in the antioxidant tolerance. It has been revealed that methanogens tend to develop their antioxidant systems around thioredoxins using the thio/disulfide redox cycling mechanism (Susanti et al. 2014). The oxidation of thio mosaics into disulfide in cells would be expected under air exposure. *Methanocella* perhaps use Drh to couple the H_2 oxidation (i.e., electron supply) to thio/disulfide redox cycling (i.e., via the HdrB) and channel the electrons into repairing machinery for oxidation damages.

6.3 Carbon Metabolisms

All genes for the Embden-Meyerhof-Parnas (EMP) pathway except hexokinase or glucokinase are present in three *Methanocella* strains, indicating that they are able to convert glucose-1-phosphate into pyruvate via glycolysis. The presence of *ppsA* (phosphoenolpyruvate synthase) and *suhB* (D-fructose 1,6-bisphosphatase) indicates that they also have the ability of synthesizing glucose-1-phosphate from pyruvate through gluconeogenesis that may further lead to the synthesis of glycogen, a reserve material in many methanogens (Yu et al. 1994). Thus, under certain circumstances *Methanocellales* may use gluconeogenesis to store energy and switch to glycolysis under starvation.

Pyruvate plays a pivotal role in cellular chemistry. *Methanocella* appear to have diverse pathways for pyruvate metabolisms. Firstly, all three strains could reversibly oxidize pyruvate to acetyl-CoA using pyruvate ferredoxin oxidoreductase (Por) and/or pyruvate dehydrogenase (Pdh). Acetyl-CoA can then be converted to acetate by acetyl-CoA synthase (Acd) or vice versa by acetyl-CoA synthetase (Acs). The presence of Ppa (inorganic pyrophosphatase) would allow *Methanocella* to recover a portion of energy via proton translocation during the acetate activation for biosynthesis. Though physiological tests indicate that acetate is needed for growth by all three strains (Lu and Lu 2012b; Sakai et al. 2008, 2010), *M. arvoryzae* may use the Codh/Acd (CO dehydrogenase/acetyl-CoA synthase) for autotrophy. As indicated earlier, Pdh is known to operate mainly in aerobic and facultatively anaerobic microorganisms while Por is oxygen sensitive. Comparative genomic analysis confirms that Pdh is present in all three strains (Lyu and Lu 2015). Possessing of

both For and Pdh by *Methanocella* possibly offers them an adaptive strategy to the alternating anoxic/oxic conditions. Specifically, Pdh is probably activated for pyruvate metabolism under oxic conditions (Erkel et al. 2006; Sakai et al. 2011). Secondly, all three strains possess the coding genes for acetolactate synthase, which could be used in biosynthesis of branched-chain amino acids from pyruvate (Bowen et al. 1997). A third potential pathway of pyruvate metabolism probably uses Pdc (pyruvate decarboxylase) to ferment pyruvate into either ethanol to recycle NAD or into acetate to generate reduced ferredoxin, which however was detected only in *M. arvoryzae* and the annotation for the coding genes was putative due to the low identity to known *pdc*. Further experimental studies are necessary to verify different pathways of pyruvate metabolisms in *Methanocella*.

Initial metagenomic and genomic surveys indicated that only the coding genes for isocitrate dehydrogenase and fumarase were present in *Methanocella*, leading to the assumption that neither the oxidative nor the reductive tricarboxylic acid (TCA) cycle was operated in *Methanocellales* (Erkel et al. 2006; Lu and Lu 2012a; Sakai et al. 2011). Due to the possible lacking of 2-oxoglutarate (2-OG) that is needed in glutamate synthesis, *Methanocella* may need to acquire glutamate from environments. A careful manual annotation of three *Methanocella* genomes, however, revealed that all three strains possess the (Re)-type citrate synthase homologs (Mtc_1389, MRE50lv_1257, and MCPlv_0455), sharing an identity of ~33% to that of *Clostridium kluyveri* (Lyu and Lu 2015). The manual annotation also identified a putative aconitase in all three strains encoded by two genes belonging to COG1679 and COG1786. These two genes located in a same cluster would presumably produce the functional motifs in one type of aconitate hydratase, aconitase A. Two types of aconitate hydratase are known: aconitase A widespread in all three domains of life while aconitase B found only in Proteobacteria (Makarova and Koonin 2003). Collectively, the manual reannotation suggests that *Methanocella* encode the nonconventional citrate synthase and aconitate hydratase, and together with the isocitrate dehydrogenase, a partial oxidative TCA from citrate to 2-oxoglutarate (2-OG) would be possible for *Methanocella* (Lyu and Lu 2015).

6.4 Nitrogen Metabolisms

Methanocella encode diverse nitrogen assimilation and regulation systems with a few differences among three strains (Lyu and Lu 2015). They all encode Amt (ammonia transporter) for ammonia uptake, which can then be assimilated via the GS (glutamine synthetase) and GOGAT (glutamate synthase) systems. GDH (glutamate dehydrogenase) that usually operates at high ammonium concentration is also present in *M. arvoryzae* and *M. paludicola*, increasing their flexibility for ammonium assimilation. At least one amino acid ABC transporter is identified in each strain, allowing them to uptake organic nitrogen sources. A complete *nif* operon for nitrogen fixation is present in *M. conradii* and *M. arvoryzae*, but not in *M. paludicola* (Lyu and Lu 2015). Thus, nitrogen fixation may operate in some but not all *Methanocellales* methanogens.

A 2-OG (2-oxoglutarate) based nitrogen regulation system is predicted in three strains (Lyu and Lu 2015). This system senses nitrogen level using 2-OG as a trigger as having been revealed in *Methanococcus* and *Methanosarcina* (Leigh and Dodsworth 2007). When nitrogen is limiting, 2-OG accumulates that removes the inhibitory effects of GlnK (nitrogen regulatory protein P-II) on Amt and GS and of NifI1I2 on Nif (nitrogenase), hence promoting both ammonium uptake and N_2 fixation. In addition, the enhancement of GS activity by 2-OG accelerates nitrogen assimilation. In addition, NrpR is also found in three strains. NrpR is a transcription repressor that is found mainly in Archaea (Lie et al. 2007; Lie and Leigh 2007). In nitrogen-starved cells, 2-OG would prevent NrpR from binding to the operators in the promoter regions of *nif* and *glnA*, hence facilitating transcription of these nitrogen assimilation genes. Though the identification of the 2-OG based nitrogen regulatory system, whether it functions and plays a role in N nutrition has yet to be determined by experimental studies.

6.5 Sulfur Metabolisms

The metagenomic analysis of RC-I_{MRE50} has revealed the presence of a complete set of genes for sulfate assimilation, namely, the *cysC* (adenylylsulfate kinase), *cysH* (PAPS reductase), and sulfite reductase (Erkel et al. 2006). This prediction is reconfirmed in the genomes of *M. arvoryzae* and *M. paludicola* (Sakai et al. 2011). But the gene coding for sulfite reductase is missing in *M. conradii* (Lu and Lu 2012b). Nevertheless, all three strains encode a PiT family transporter for the uptake of phosphate or sulfate, and *M. arvoryzae* additionally encodes a putative sulfate permease. Thus, at least *M. arvoryzae* and *M. paludicola* are likely able to use sulfate as a sulfur source. For FeS cluster assembly, sulfite is often the only sulfur source for many methanogens due to the lack of cysteine desulfurase, whereas the genes coding for this enzyme are present in *Methanocella* (Lyu and Lu 2015). In addition, three strains encode two iron sulfur assembly systems which enable them to explore alternative sulfur sources for FeS synthesis. The first uses ApbC type FeS carrier and SufBCD type synthesis system, which is present predominantly in Class I methanogens with sulfide as sulfur source, while the second uses the A-type FeS carrier and IscSU synthesis system with cysteine as sulfur source (Liu et al. 2012). This may allow *Methanocella* to switch between two systems in concert with redox changes in environment, using sulfide at low and cysteine at high redox potentials, securing sulfur nutrition. The putative use of sulfate and the presence of two iron sulfur assembly systems reinforce the adaptation of *Methanocellales* to oxidative conditions.

6.6 Understanding of Oxidative Adaptation

Methanogenic analysis has indicated that *Methanocellales* contain multiple sets of genes coding for antioxidant systems that is the key for surviving and thriving in

alternate anoxic/oxic habitats like rice rhizosphere. To confirm this capacity, an extensive comparative genomic analysis was conducted for three strains (Lyu and Lu 2018). Theoretically, three lines of antioxidant strategies could have been evolved in microbes to defend the oxygenation challenge: (i) avoiding the production of reactive oxygen species (ROS), (ii) reducing accumulation of ROS within the cell, and (iii) repairing self for ROS damage. Studies have revealed that these strategies are essential for both aerobes and anaerobes to survive oxidative stress (Imlay 2008, 2015). The comparative genomic analysis therefore has been focused on identifying these strategies in *Methanocella* genomes (Lyu and Lu 2018).

The methanogenesis pathway where redox reactions are most active inside the cell of methanogens is assumably the main place for ROS production. Specifically, the flavin-based electron bifurcation system that requires the formation of flavosemiquinone could react with oxygen to form O_2^- and H_2O_2 (Buckel and Thauer 2013). This electron bifurcation mechanism has been proposed to operate in *Methanocella* (Liu et al. 2014; Liu and Lu 2018). The comparative genomic analyses indicate that the number of [4Fe-4S] motifs involved in the electron bifurcation-based methanogenesis was reduced by about 70% in *Methanocella* compared to the Class I methanogens (Lyu and Lu 2018). This change in electron transfer machinery could reduce the chance for HO· production through the Fenton reaction.

The second major strategy lies on the capacity of O_2/ROS elimination that is catalyzed by a variety of antioxidant enzymes in microbes. The enzymes known to reduce O_2 to H_2O and transform H_2O_2 and O_2^- to less toxic O_2 have been characterized (Imlay 2008). Many of O_2/ROS eliminations depend on redox reactions and require reducing power to proceed. Small redox proteins play an important role in supplying such a reducing power (Lu and Holmgren 2014). These proteins also serve as a buffering system to keep cellular redox system from becoming over oxidized (Susanti et al. 2014). Though the presence of O_2/ROS elimination enzymes in many methanogens, *Methanocella* possess statistically more genes encoding these enzymes than the Class I (hydrogenotrophic) methanogen counterparts (Lyu and Lu 2018). These observations suggest that *Methanocella* are equipped with a higher capacity for O_2/ROS elimination (Fig. 4).

A closer examination of the O_2/ROS elimination systems indicates the evolutionary robustness of this elevated capacity in *Methanocellales*. First, NO/O_2 reductase is more abundant than $F_{420}H_2$ oxidase in *Methanocella*. Both enzymes can oxidize O_2 into H_2O, but the latter is deactivated when cells are exposed to air (Seedorf et al. 2004), while the former has a higher *Km* for O_2 (Silaghi-Dumitrescu et al. 2005). In addition, NO/O_2 reductase detoxifies NO, a product of denitrification that can be produced at the oxic-anoxic interface (Kluber and Conrad 1998). A shift from $F_{420}H_2$ oxidase to NO/O_2 reductase could suggest an evolutional adaptation of *Methanocellales* to the severer oxidative conditions. Second, rubredoxin and thioredoxin are the major small redox proteins found in methanogens. Thioredoxin operates at much lower redox potentials than rubredoxin, transferring electrons at around -300 to -120 mV versus 0 ± 100 mV, respectively (Aslund et al. 1997; Lin et al. 2005). In comparison with other hydrogenotrophic methanogens,

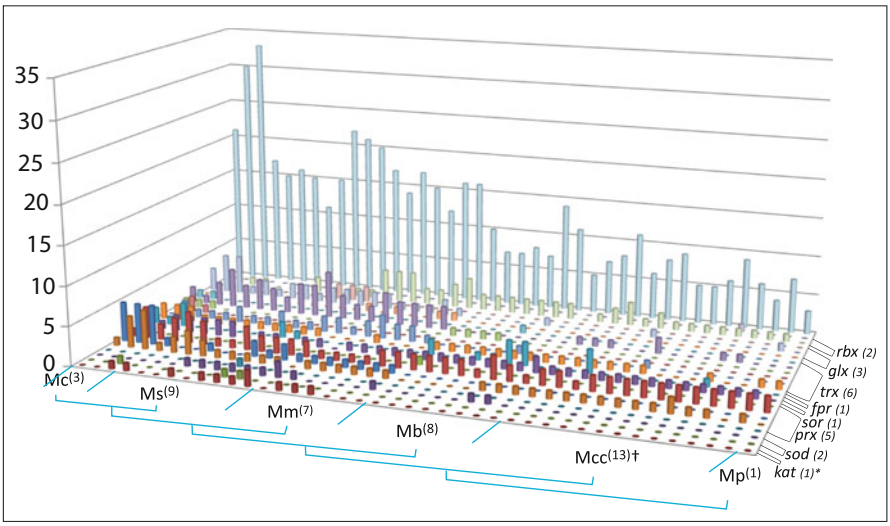

Fig. 4 Methanocellales contain on average highest numbers of genes encoding antioxidant systems. Included for the comparative analysis are three genomes of Methanocellales [Mc(3)], nine genomes of Methanosarcinales [Ms(9)], seven genomes of Methanomicrobiales [Mm(7)], eight genomes of Methanobacteriales [Mb(8)], thirteen genomes of Methanococcales [Mcc(13+)] and one genome of Methanopyrales [Mp(1)]. The coding genes for analysis consist of catalase (kat), superoxide dismutase (sod), peroxiredoxin (prx), superoxide reductase (sor), F420H2 oxidase (fpr), thioredoxin (trx), glutaredoxin system (glx) and rubredoxin (rbx). The number in parentheses indicates the COGs of the respective genes

Methanocella contain similar number of rubredoxin proteins, but the thioredoxin proteins are substantially increased (Lyu and Lu 2018), indicating a potential enhancement of the redox buffering system in *Methanocellales*. Third, transmembrane thioredoxin proteins are present in *Methanocella*, but rare in other hydrogenotrophic methanogens (Table 2). In addition to the presence of thioredoxin domain both in the cytoplasmic and periplasmic side, these transmembrane proteins have two or three cysteine residues in the transmembrane region. These transmembrane thioredoxin proteins may enable electron shuffle between the cytoplasmic and periplasmic spaces, which may help with redox recovery around the cellular membranes under oxidative stress. Fourth, while the classical hydrogenotrophic methanogens appear to use $F_{420}H_2$ to regenerate the reduced thioredoxin, *Methanocellales* probably use NADPH or NADH. NADPH or NADH are more stable electron carriers than $F_{420}H_2$ in an oxygenated Earth environments. These changes in oxidant-detoxifying systems of *Methanocellales* appear systematic and holistic.

The third strategy for oxidative tolerances is the self-repairing. Metagenomic analysis already revealed the repairing system is enriched in *Methanocellales* (Erkel et al. 2006). The analysis of pure culture genomes expanded these mechanisms with more details (Lyu and Lu 2018). ROS once formed can cause extensive damages to

cell components. For instances, DNA mutation or dysfunction may occur due to the oxidation of purines and pyrimidines (Dalhus et al. 2009). The membrane lipids can be oxidized into phospholipid hydroperoxides (PLOOH). The proteins containing sulfur amino acids can be deformed with the formation of disulfide bonds or methionine sulfoxide, leading to disorder of protein structures (Manevich et al. 2002). In addition, ROS may disrupt the iron-sulfur (FeS) clusters which are the prosthetic groups of many enzymes in methanogens. Genes coding for DNA base repairing and S–S or S=O group-reducing enzymes were moderately or strongly enriched in *Methanocella* compared with the Class I methanogens (Lyu and Lu 2018). The enrichment of cytoplasmic S–S reduction enzymes in *Methanocella* is consistent with the elevated abundance of thioredoxins relative to other hydrogenotrophic methanogens. The genes coding for PLOOH reduction (peroxiredoxins) are also enriched in *Methanocella*.

7 Conclusive Remarks

Methanocellales represent a novel type of methanogens initially discovered with DNA fingerprinting of paddy soils. These methanogens were often detected in rice rhizosphere or in association with rice roots. Earlier studies demonstrated that a considerable fraction of the plant-photosynthesized C is allocated to rice roots, released into rice rhizosphere and thereby the DOC pool serves as a major carbon source for methanogenesis, leading to the seasonal maxima of CH_4 emissions. Strikingly, methanogenesis appears to occur close to rice roots. This methanogenic activity was not very expected because rice plants have a well-developed aerenchyma system where O_2 can diffuse from the plant top to roots and released into the rhizosphere. As a result, rice roots and rhizosphere are partly oxic. The dilemma of active methanogenesis in the rice rhizosphere and the nature of strictly anaerobic lifestyle of methanogens causes a huge curiosity to look into the biological logic and mechanism.

Due to the nature of difficult-to-cultivation, intensive studies using molecular techniques were conducted with a focus on the ecophysiology of methanogens in paddy soils. Meanwhile, multiple efforts for enrichment and cultivation were undergone. Strikingly, molecular techniques including DNA-SIP approach revealed that albeit as hydrogenotrophic methanogens *Methanocellales* dominated over other methanogens when H_2 partial pressure was low, indicating that out of the vast methanogenic populations in paddy soil *Methanocellales* might be better adapted under low H_2 condition. This trait is possibly a reason why they escaped the isolation albeit existing widespread in environments. The exploration under in situ conditions using RNA-SIP technology revealed that *Methanocellales* play the key role in CH_4 production in rice rhizosphere. Further studies were then focused on why they can adapt to low H_2 condition and thrive in the rhizosphere where O_2 leaks can occur.

Metagenomic investigation revealed a series of traits that support the adaptation of *Methanocellales* to rhizosphere environment. They possess multiple sets of antioxidant systems and repair systems. They are versatile to assimilate various

sources of N and S and they may activate different core metabolisms to facilitate biosynthesis and survival during environment shift to oxic conditions. After continuous efforts for years, three strains were finally isolated into pure culture. Extensive genomic analyses were conducted to reveal the taxonomic, evolutional, and ecological properties of *Methanocellales*. The phylogenetic analyses using multiple marker genes in combination with genome alignment and AAI analyses consistently suggest that *M. conradii* and *M. paludicola* are closely related each other and together can be classified as a new genus while *M. arvoryzae* may belong to another genus. Comparative genomic analyses reveal that metabolic features for *Methanocellales* appear to be more diverse than previously predicted from metagenomic investigation. Three strains share close resemblance as well as novel features on the core metabolisms, such as specialization in utilizing H_2 at low concentrations. For the adaptation to oxic condition that is key for their activity in rice rhizosphere, at least three general evolutionary mechanisms have been acquired and enriched in *Methanocellales*. The first is the usage of enzymes producing less ROS in the central methanogenesis pathway, particularly the flavin-based electron bifurcation system has been modified from classical hydrogenotrophic methanogens toward a less possibility of ROS production. The second is the expansion and diversification upon a core antioxidant system for the O_2/ROS elimination. And the third is the occurrence of multiple self-repairing pathways from O_2/ROS damages. Further studies are necessary to explore these novel genomic features, which would not only contribute to a deeper understanding of *Methanocellales* and methanogens in general but pave a way towards the development of biotechnology for control of methane emissions from paddy fields.

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Metagenomics of Methanogenic Communities in Anaerobic Digesters

13

Sabine Kleinsteuber

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Abstract

Anaerobic digestion relies on complex microbial communities that closely interact in the anaerobic degradation of biomass and organic waste material to methane and carbon dioxide. The adoption of high-throughput molecular methods and the holistic "omics" approach in applied microbial ecology has greatly extended our view on the manifold metabolic diversity and trophic networks in the microbiomes thriving in anaerobic bioreactors. In this chapter, current concepts in metagenomics and microbial ecology of anaerobic digestion are described. Recent advances in gene-centric and genome-centric approaches and their application on lab-scale and production-scale biogas reactors have paved the way to a knowledge-based microbial resource management in anaerobic bioreactors. The adoption of systems biology principles in systems ecology of reactor microbiomes will open up new perspectives in process control and optimization of biotechnological processes relying on complex open mixed cultures.

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1 Introduction

Anaerobic digestion (AD) is an established biotechnological process for the treatment of organic waste and the production of biogas as a renewable energy carrier. It relies on complex microbial communities of hydrolytic and fermentative bacteria, syntrophic bacteria, and methanogenic archaea, which closely interact in the anaerobic degradation of organic matter to methane and carbon dioxide. Notwithstanding AD is widely used in various sectors, the process has been considered as a black box system by process engineers and plant operators for a long time, and a detailed understanding of the methanogenic communities driving the process was lacking. In the last decades, the advent of cultivation-independent molecular methods in microbial ecology as well as technical innovations in high-throughput techniques for the analysis of complex microbial communities has opened the black box and paved the way to a knowledge-based microbial resource management in AD technology. Next-generation sequencing (NGS) techniques and advanced bioinformatics tools have provided novel insights into the diversity, dynamics, and functionality of methanogenic communities in biogas reactors in a similar way as they caused a paradigm shift in microbial ecology of natural environments and other complex ecosystems such as the human gut microbiome. In this chapter, recent advances in analyzing AD microbiomes by means of the metagenome approach are described.

2 Overview and Paradigms of Anaerobic Digestion

As illustrated in Fig. 1, the overall process of converting complex biomolecules such as lipids, carbohydrates, proteins, and nucleic acids to methane under anaerobic conditions comprises the metabolic steps of (1) hydrolysis, i.e., the cleavage of polymeric compounds to oligo- and monomers by hydrolytic exoenzymes; (2) acidogenesis, i.e., the fermentation of sugars and amino acids to short-chain carboxylic acids, alcohols, hydrogen, and carbon dioxide; (3) acetogenesis, i.e., the syntrophic oxidation of organic fermentation products to acetate, hydrogen, and carbon dioxide; and (4) methanogenesis, which is the conversion of the methanogenic substrates (hydrogen and carbon dioxide or formate, acetate, methyl compounds) to methane and carbon dioxide. Three methanogenic pathways are known: (1) hydrogenotrophic methanogenesis that reduces carbon dioxide to methane with hydrogen as electron donor; (2) acetoclastic methanogenesis, which is the disproportionation of acetate to methane and carbon dioxide; and (3) methylotrophic methanogenesis that converts methyl compounds such as methanol, methylamines, or methyl sulfides to methane.

Hydrolysis and acidogenesis are catalyzed by facultative or strict anaerobic fermentative bacteria of various phylogenetic groups, whereas methanogenesis is a strict anaerobic respiratory pathway exclusively employed by archaea affiliated to seven different orders of Euryarchaeota (Methanobacteriales, Methanococcales,

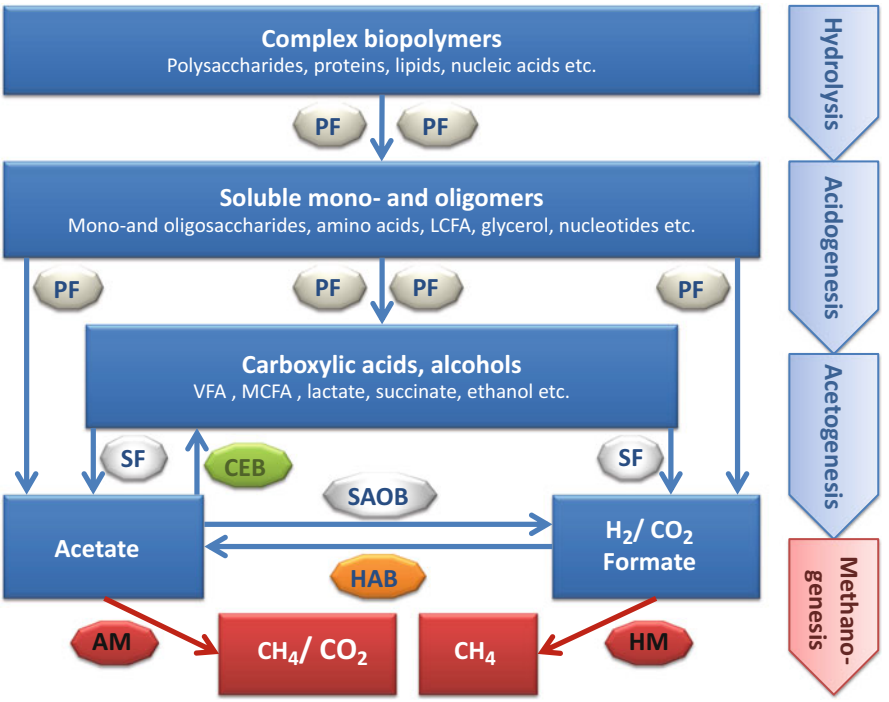

Fig. 1 Process steps of anaerobic digestion and involved functional groups of microbes. Process steps performed by bacteria and their products are indicated in *blue*; process steps performed by archaea and their products are indicated in *red*. *PF* primary fermenters (hydrolytic and acidogenic bacteria), *SF* secondary fermenters (syntrophic proton-reducing bacteria), *SAOB* syntrophic acetate-oxidizing bacteria, *HAB* homoacetogenic bacteria (bacteria performing reductive acetogenesis via the Wood-Ljungdahl pathway), *CEB* chain-elongating bacteria (e.g., *Clostridium kluyveri*), *AM* acetoclastic methanogens, *HM* hydrogenotrophic methanogens. For the sake of clarity, methylotrophic methanogenesis is not shown.

Methanomicrobiales, Methanosarcinales, Methanocellales, Methanopyrales, Methanomassiliicoccales). The oxidation of volatile fatty acids (VFA), alcohols, and other fermentation products to acetate, hydrogen, and carbon dioxide (acetogenesis) is performed by syntrophic bacteria and thermodynamically linked to hydrogenotrophic methanogenesis by interspecies electron transfer, mostly via hydrogen or formate that is formed by proton-reducing bacteria and consumed by hydrogenotrophic methanogens, thereby keeping the hydrogen partial pressure low and shifting the reaction equilibrium toward thermodynamic feasibility. A specialized group of syntrophic bacteria are syntrophic acetate-oxidizing bacteria (SAOB) that convert acetate to hydrogen and carbon dioxide in syntrophy with hydrogenotrophic methanogens. Syntrophic acetate oxidation (SAO) is an alternative acetate sink in AD when acetoclastic methanogenesis is impaired by adverse process conditions such as high ammonia load, VFA accumulation, or elevated temperature (Hattori 2008).

Depending on the characteristics of the input material, different metabolic steps of the AD process determine the overall conversion rate to methane. Structure-lending biomass components such as cellulose, hemicellulose, chitin, or keratin are slowly hydrolyzed due to the low bioavailability of these complex polymers for hydrolytic bacteria and exoenzymes. In particular, AD of lignified plant material is hampered by the fiber structure in which cellulose and hemicellulose components are protected by lignin biopolymers that are persistent under strict anoxic conditions. The pivotal role of effective hydrolysis, particularly in biogas production from fiber-rich material (e.g., energy crops, manure, agricultural residues such as straw), has drawn attention to hydrolytic (particularly cellulolytic) bacteria and their enzymes in metagenome studies on agricultural biogas plants (Güllert et al. 2016; Lebuhn et al. 2014).

In contrast to such AD processes where hydrolysis is the rate-limiting step, the methanation rate of easy to degrade organic material (e.g., municipal wastewater, dairy waste, stillage, food waste, sugar-rich energy crops) is limited by the rather low growth rates of methanogenic archaea and syntrophic bacteria compared to the acidogenic bacteria. Moreover, the groups of acetoclastic methanogens and syntrophic VFA-oxidizing bacteria lack functional redundancy and thus represent the most vulnerable elements of the AD food web. They are particularly affected by adverse process conditions such as high ammonia or salt load, high sulfide concentrations, inhibitory concentrations of long-chain fatty acids (LCFA), hydrocarbons or heavy metals (Chen et al. 2008), or deficiency of essential trace elements (Thanh et al. 2016). Hence, AD process design and control have to consider the challenges posed by the ecophysiological peculiarities of the different groups of methanogens, syntrophic fatty acid-oxidizing bacteria, and SAOB. Consequently, these groups have been also preferred targets for metagenome studies (Frank et al. 2016; Kougias et al. 2016).

3 Metagenomics Concepts and Application on AD Systems

The standard approach for microbial community analyses, which is also widely used to study AD microbiomes, involves the extraction of total nucleic acids from the reactor sample, followed by polymerase chain reaction (PCR) amplification of universal phylogenetic marker genes such as the 16S rRNA gene encoding the small subunit of the ribosomal RNA. Subsequently, the PCR products are profiled by fingerprinting techniques, cloned and sequenced or, using NGS techniques, directly sequenced without an additional cloning step. For the analysis of methanogenic communities, also the *mcrA* gene, encoding the α-subunit of the methyl-coenzyme M reductase, is commonly employed as a specific functional and phylogenetic marker for methanogenic archaea (Luton et al. 2002). The more recent but meanwhile widely used amplicon sequencing approach employing NGS techniques avoids the cloning bias, facilitates high-throughput analyses of multiple samples, and provides a much higher resolution of the community composition due to the high parallel read numbers covering also rare sequence types. Frequently, this

approach has been referred to as metagenomics in the literature or by sequencing companies, which is a wrong interpretation of the term metagenomics as PCR-based approaches lack the holistic concept of "omics." The term metagenome was coined by Jo Handelsman and colleagues who described for the first time the concept of cloning environmental DNA to access the genomes of uncultured microbes and their metabolic potential without any enrichment, isolation, or PCR step (Handelsman et al. 1998). In this sense, the metagenome comprises the entire genetic information of a certain environmental compartment or, in case of a bioreactor, the reactor microbiome. Thus, metagenomics circumvents the PCR bias and is a generic approach that does not require a priori knowledge on the system studied, in contrast to PCR-based studies that depend on sequence information for primer design.

As long as metagenome analyses were dependent on Sanger sequencing, environmental DNA was cloned in plasmid, cosmid/fosmid, or BAC (bacterial artificial chromosome) vectors to be directly analyzed for heterologous gene expression (function-driven metagenomics) or to be sequenced and annotated for predicting putative functions (sequence-driven metagenomics), as reviewed in detail by Handelsman (2004). With the advent of NGS methods, the sequence-driven approach switched to direct shotgun sequencing of community DNA without cloning step, which facilitated much higher throughput rates but posed new challenges for bioinformatics due to the short-read lengths of NGS methods. For instance, de novo assembling of contigs from NGS metagenome reads with assembly tools that were originally designed to deal with clonal populations in microbial genome projects, or compositional-based binning algorithms are not reliable for short reads [for a review, see Thomas et al. (2012)]. Meanwhile, read lengths of most recent NGS platforms have increased, and novel bioinformatics tools have been developed to cope with the specific problems of handling NGS data and metagenome reads even from highly complex samples [for recent reviews, see Ju and Zhang (2015); Kumar et al. (2015); Thomas et al. (2012)], thus facilitating metagenome studies on AD systems. Table 1 compiles examples of metagenome studies on full-scale biogas plants performed on different NGS platforms, while Table 2 lists metagenome studies on lab-scale biogas reactors.

Although the ultimate aim of (meta)genomics is to reconstruct complete species or population genomes and hence the complete genetic blueprint of microbial metabolism in a species or a community, this so-called genome-centric metagenomics approach was previously only possible for low complexity samples as demonstrated in a pioneering study by Tyson et al. (2004). Due to the limitations in sequencing depth (coverage of genomes) and reliability of bioinformatics tools, the alternative approach of gene-centric metagenomics has been more frequently employed in metagenome studies on AD systems, which harbor rather complex microbial communities. In the gene-centric approach, reads or contigs from assembled reads (so-called environmental gene tags, EGT) are assigned to phylogenetic or functional genes without sorting them to taxonomic units (binning), which on the one hand provides information on the overall metabolic pathways and on the other hand the overall taxonomic composition of the community, but contributions of

Table 1 Metagenome studies on full-scale anaerobic digesters

Reactor type/ temperature range	Feedstock	Sequencing platform	Dominant bacterial taxa	Dominant archaeal taxa	References
Agricultural CSTR[1] (dry fermentation, HRT[2] 59 d)/ mesophilic	Maize silage (63%), green rye (35%), chicken manure (2%)	454 pyrosequencing	Clostridia Bacteroidia	Methanomicrobiales	Jaenicke et al. (2011), Krause et al. (2008), Kröber et al. (2009), Schlüter et al. (2008), Stolze et al. (2015)
Agricultural CSTR[1] (wet fermentation, HRT[2] 55 d)/ mesophilic	Maize silage (72%), pig manure (28%)	454 pyrosequencing	Clostridia Bacteroidia	Methanomicrobiales	Stolze et al. (2015)
Agricultural CSTR[1]/ mesophilic	Maize silage (69%), cattle manure (19%), chicken manure (12%)	Illumina HiSeq	Clostridia Bacteroidia	Methanomicrobiales Methanosarcinales	Güllert et al. (2016)
14 full-scale reactors (HRT 3–32 d) /meso- or thermophilic (36–53 °C)	Manure (mainly cattle) or sewage sludge	Illumina HiSeq	Firmicutes, (manure-fed reactors); Proteobacteria (sludge-fed reactors)	Methanomicrobiales Methanosarcinales Methanobacteriales	Luo et al. (2016)
Wastewater treatment plants/ mesophilic	Municipal sewage sludge	Illumina HiSeq	Proteobacteria Bacteroidetes Firmicutes	Methanosarcinales	Yang et al. (2014)
Wastewater treatment plants/ mesophilic	Industrial wastewater or municipal sewage sludge	Illumina HiSeq	Proteobacteria Firmicutes Actinobacteria Bacteroidetes	Methanomicrobiales	Cai et al. (2016)

individual species are largely ignored (Kunin et al. 2008). The genome-centric approach aims at the reconstruction of complete or partial population genomes to reveal the ecophysiological function of individual populations and their interactions within the microbiome under study (Sales and Lee 2015).

Table 2 Metagenome studies on lab-scale and pilot-scale anaerobic digesters

Reactor type/temperature range	Feedstock	Sequencing platform	Dominant bacterial taxa	Dominant archaeal taxa	References
100 L /30 L two-phase leach-bed reactor/thermophilic	Rye silage, winter barley straw	454 pyrosequencing	Clostridia Thermotogae	Methanomicrobia, Methanobacteria	Rademacher et al. (2012)
5 L CSTR[1]/mesophilic	Maize silage (68% oTS[2]), pig manure slurry	SOLiD™	Clostridia Bacilli	Methanomicrobiales	Wirth et al. (2012)
1 L semi-continuous flow reactors with granular sludge/mesophilic	Waste activated sludge, synthetic wastewater	454 pyrosequencing	Proteobacteria Bacteroidetes Firmicutes Actinobacteria Chloroflexi	Methanosarcinaceae Methanobacteriaceae Methanosaetaceae	Wong et al. (2013)
5 L fed-batch stirred tank reactors/ mesophilic	Casein or pig blood as mono-substrates	SOLiD™	Clostridiales Bacillales Thermoanaerobacterales	Methanomicrobiales	Kovács et al. (2013)
Four parallel CSTR[1] (10 L)/ mesophilic	Cattle manure (87%), fish waste (13%)	454 pyrosequencing	Firmicutes Bacteroidetes WWE1 Proteobacteria	Methanomicrobiales	Solli et al. (2014)
S-CSTR[3]/mesophilic	*Spirulina* biomass	Ion Torrent PGM[4]	Clostridiales Bacteroidales Thermoanaerobacterales Bacillales	Methanobacteriales Methanosarcinales	Nolla-Ardevol et al. (2015a)
CSTR[1]/mesophilic	*Scenedesmus* biomass (mono- or co-digestion with maize silage)	Ion Torrent PGM[4]	Bacteroidetes Firmicutes Proteobacteria	Methanosarcinales Methanomicrobiales	Wirth et al. (2015)
Two parallel CSTR[1] (5 m^3)/38 °C ➔ 55 °C	Sugar beet pressed pulp	Ion Torrent PGM[4]	Bacteroidetes (37 °C) Firmicutes Synergistetes (55 °C) *Thermotoga* (55 °C)	*Methanosaeta* (37 °C) *Methanosarcina* (55 °C) *Methanothermobacter* (55 °C)	Tukacs-Hájos et al. (2014)
Three parallel CSTR[1] (10 L)/ 37 °C ➔ 55 °C	Maize silage	Ion Torrent PGM[4]	Firmicutes Bacteroidetes (37 °C) Synergistetes (55 °C)	*Methanosaeta* (38 °C) *Methanosarcina* (55 °C) *Methanoculleus* (55 °C)	Pap et al. (2015)

3.1 Gene-Centric Approach

The first metagenome studies on AD systems were performed with samples from a production-scale agricultural biogas plant mainly fed with energy crops (63% maize silage and 35% green rye) and 2% chicken manure (Krause et al. 2008; Kröber et al. 2009; Schlüter et al. 2008) as listed in Table 1. Analysis of gene content and phylogenetic classification of 16S rRNA genes as well as Pfam protein family members identified Clostridiales as the predominant bacterial order, while the methanogenic community was dominated by Methanomicrobiales (Krause et al. 2008; Schlüter et al. 2008). Mapping of metagenome reads and assembled contigs to reference genomes revealed major functions of Clostridiales in cellulose hydrolysis and sugar fermentation and of the genus *Methanoculleus* (Methanomicrobiales) in hydrogenotrophic methanogenesis, which seemed to be the prevailing methanogenic pathway in this reactor. By using a combined approach of 16S rRNA gene and *mcrA* clone libraries and shotgun pyrosequencing of metagenomic DNA to analyze the phylogenetic composition of the same sample, Kröber et al. (2009) confirmed Clostridiales and Bacteroidales as the predominant bacterial orders and *Methanoculleus* as the prevailing methanogen. With an improved sequencing technique yielding longer read lengths, Jaenicke et al. (2011) were able to identify additional bacterial genera as abundant community members in the same sample. While the leading roles of Clostridia in hydrolysis/acidogenesis and Methanomicrobiales in methanogenesis were confirmed, additionally major contributions of the classes Bacilli, Bacteroidia, and Gammaproteobacteria in polysaccharide degradation were revealed. In a follow-up study, Stolze et al. (2015) compared the metagenome of this dry fermentation reactor to that of another production-scale agricultural biogas plant performing wet fermentation of maize silage (72%) and pig manure (28%). The bacterial community composition was similar in both reactors, in particular at higher taxonomic ranks. In the wet fermentation reactor, Erysipelotrichaceae, Fibrobacteraceae, Succinivibrionaceae, and Clostridiaceae were more abundant, whereas more sequences were assigned to Acholeplasmataceae and Candidatus *Cloacamonas* in the dry fermentation reactor. Methanogenic communities in both reactors were dominated by the hydrogenotrophic genus *Methanoculleus*.

AD systems treating sewage sludge from municipal wastewater treatment plants (WWTP) are known to harbor slightly different microbial communities than agricultural biogas reactors (Riviere et al. 2009). Accordingly, Yang et al. (2014) detected Proteobacteria (10–14%) as dominant phylum, followed by Bacteroidetes (7–11%) and Firmicutes (8–9%) in metagenomes of two full-scale digesters treating municipal sewage sludge. The methanogenic communities were dominated by Methanomicrobia, with *Methanosarcina* and *Methanosaeta* as the dominant genera. At the class level, some groups showed seasonal differences in abundance when samples taken in September and March were compared. For instance, Gammaproteobacteria, Flavobacteria, and Anaerolineae were less abundant in the spring samples from both reactors compared to the fall samples. However, a general problem in metagenome analyses is that mostly only one or few samples are taken so that

community dynamics and seasonal effects are neglected, making quantitative conclusions questionable.

While most metagenome studies on full-scale AD systems focused on agricultural biogas plants digesting energy crops and manure or on municipal WWTP, Cai et al. (2016) compared the metagenome of a digester treating high-strength industrial wastewater from beverage manufacturing to that of a digester treating municipal sewage sludge. The most abundant bacterial phyla were Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes. Functional annotation revealed that genes related to carbohydrate transport and metabolism were overrepresented in the digester treating industrial wastewater, while lipid transport and metabolism genes were overrepresented in the reactor digesting municipal sludge.

Going beyond the analysis of single biogas plants, Luo et al. (2016) analyzed the metagenomes of 14 full-scale biogas reactors, either treating sewage sludge and operated at mesophilic temperatures or treating cattle manure and operated at mesophilic or thermophilic temperatures. The prevailing methanogenic pathways were determined by radioisotope analysis. Firmicutes (55–76%) and Bacteroidetes (4–20%) dominated in the manure-treating reactors, whereas Proteobacteria (26–34%), Firmicutes (9–15%), and Bacteroidetes (9–22%) were the predominant phyla in the sludge-digesting reactors. Regarding the methanogenic communities, Methanomicrobiales was the dominant order in all but one of the thermophilic manure-digesting reactors. Methanogens in one thermophilic manure-based reactor as well as most of the sludge-based reactors were dominated by Methanosarcinales, but the dominant genus was different for the manure-based (*Methanosarcina*) and sludge-based reactors (*Methanosaeta*). Methanobacteriales dominated in two of the mesophilic manure-based reactors. Regarding functional annotation, genes related to cellulose and hemicellulose degradation were more abundant in manure-based reactors compared to sewage sludge-based samples, which was consistent with the high-fiber content of manure. In the samples dominated by Methanomicrobiales, genes related to hydrogenotrophic methanogenesis were also dominant, which was in accordance with a high relative abundance of SAOB-related genera such as *Syntrophaceticus* in these reactors. Multivariate statistical analysis indicated that the functional patterns were identical to the taxonomic patterns and that temperature and free ammonia were the most important environmental variables shaping both taxonomic and functional patterns.

Metagenome studies on full-scale AD systems mostly have provided snapshot pictures of the microbiome in one or few reactors but only limited information on the effects of operational parameters and environmental factors on the reactor microbiome, its dynamics, and functionality. The reason is that full-scale reactors can hardly be manipulated intentionally, and detailed information on process parameters is frequently lacking when researchers depend on the data provided by the plant operator. Disturbed AD processes and impaired reactor microbiomes in full-scale reactors can only be analyzed if process failures happen accidently. Therefore, lab-scale reactor experiments are inevitable to study the relationships between AD microbiome and process performance in a targeted way. Lab-scale AD systems have been frequently investigated with respect to different substrates and feeding

regimes, process temperatures, reactor configurations and operational parameters, and disturbances followed by process recovery or complete process breakdown. Most of these studies employ PCR-based methods to analyze the composition, dynamics, and activity of the reactor microbiome, but several metagenome studies on lab-scale biogas reactors have been published in recent years (Table 2).

Rademacher et al. (2012) investigated a two-phase leach-bed AD system fed with rye silage and barley straw and operated at 55 °C. They analyzed the cellulolytic biofilm grown in the first phase hydrolysis reactor as well as the methanogenic biofilm grown in the second phase anaerobic filter reactor. Clostridia (27%), Methanomicrobia (2%), and Thermotogae (1%) were the most abundant classes in the cellulolytic biofilm sample, while the methanogenic biofilm was mainly composed of Clostridia (18%), Methanomicrobia (7%), and Methanobacteria (4%). The assignment of Pfam categories to taxonomic groups indicated Clostridia, Bacilli, Flavobacteria, and Gammaproteobacteria to be mainly involved in carbohydrate degradation and the genera *Methanothermobacter*, *Methanosarcina*, and *Methanoculleus* in methanogenesis. However, the authors noted that the major number of metagenome reads remained unidentified in this study, showing the limitations of databases and bioinformatics tools at that time.

Wirth et al. (2012) employed the SOLiD™ short-read DNA sequencing platform to investigate a lab-scale biogas reactor fed with maize silage and pig manure slurry and operated under mesophilic conditions. The MG-RAST server (Meyer et al. 2008) was used to assign the resulting short reads (50 nucleotides) to taxonomic and functional categories without assembling contigs. Clostridia (36%) and Bacilli (11%) were the most abundant bacterial classes, while the methanogenic community was dominated by hydrogenotrophic methanogens of the order Methanomicrobiales, mostly assigned to the genus *Methanoculleus*. The results obtained with this short-read approach compared well with those from previous metagenome studies on agricultural biogas plants employing the 454 pyrosequencing platform.

Wong et al. (2013) investigated the effect of alkaline pretreatment (pH 10 for 8 days) on the anaerobic digestion of waste activated sludge. The microbiomes of reactors fed with pretreated or untreated sludge were compared by metagenomics. Sludge pretreatment led to enhanced methane production and a slightly different bacterial community composition. Alpha-, Beta-, and Gammaproteobacteria as well as Flavobacteria, Sphingobacteria, and Cytophagia increased in relative abundances due to the sludge pretreatment, indicating that these classes were involved in more efficient hydrolysis and fermentation of sludge compounds to VFA and hydrogen.

The start-up of a biogas reactor is a critical phase when the inoculum has to adapt to feedstock that potentially causes process disturbances, such as nitrogen-rich substrates leading to high ammonia load. Solli et al. (2014) studied four parallel lab-scale continuous stirred tank reactors (CSTR) co-digesting fish waste and cattle manure. Samples for metagenome analysis were taken from the inoculum (originating from a reactor with similar substrate) and from the four reactors on day 59, after the reactors had been operated at a fixed feeding rate for 28 days with a hydraulic retention time (HRT) of 30 days. The most abundant phyla in all reactors were Firmicutes followed by Bacteroidetes and the candidate phylum Cloacimonetes

(WWE1). The reactor communities differed largely from the inoculum that harbored higher percentages of Firmicutes, Proteobacteria, and Actinobacteria, whereas Cloacimonetes were of minor abundance. The parallel reactors had similar taxonomic profiles except for one reactor that showed a very distinct community profile, mainly due to the lower abundance of Firmicutes and higher abundance of Cloacimonetes. This deviation of one reactor in community profile shows the importance of replication in lab-scale experiments to compensate for stochastic effects in community assemblage. This refers to biological replicates as it was done in this study, but also more sampling times are required to gain information on community dynamics during reactor start-up and stabilization phase, which usually takes several times the HRT. Candidatus *Cloacamonas* (of the candidate phylum WWE1) was the most abundant genus in the reactor samples with abundances of 3–10% of the reads; also *Syntrophomonas* and *Clostridium* were among the most abundant genera. The predominant methanogen was affiliated to the hydrogenotrophic genus *Methanoculleus*. Regarding the functional annotation, a high number of reads were assigned to enzymes involved in amino acid metabolism, which is consistent with the high protein content in the substrate.

AD of protein-rich substrates and the involved microbiomes were also investigated by Kovács et al. (2013) who studied the microbiomes involved in AD of casein or pig blood as mono-substrates in fed-batch reactors, Wirth et al. (2015) who studied *Scenedesmus* biomass in mono- or co-digestion, and Nolla-Ardevol et al. (2015a) who used *Spirulina* microalgae as feedstock and compared the obtained metagenome to a metagenome from a full-scale biogas plant fed with cellulosic material. In the lab-scale digester fed with the microalgal biomass, *Tissierella*, which is known to grow on proteinaceous substrates, was the predominant bacterial genus. Compared to the cellulose-fed digester, Pfam domains related to protein degradation were more frequently detected, and Pfam domains related to cellulose degradation were less frequent in the sample from the *Spirulina*-fed reactor. The protein-rich substrate had a selective impact on the bacterial community, whereas a direct influence of the substrate on the selection of specific methanogenic populations was not observed.

Besides the substrate, process temperature is a decisive environmental variable shaping AD reactor microbiomes. Pap et al. (2015) studied the effect of a gradual temperature shift from 37 °C to 55 °C at a rate of approximately 1 K per day. The metagenomes of the three parallel CSTR fed with maize silage were analyzed before starting the temperature increase, after 55 °C were reached, and 60 days later (twice the HRT) when the process parameters had reached quasi-steady state again. Pronounced community shifts upon the temperature increase were observed. At mesophilic conditions, the bacterial communities were dominated by Bacteroidetes (45% of all bacterial reads), Firmicutes (25%), and Proteobacteria (10%). After completed community shift, a clearly thermophilic community was established with Firmicutes as the predominant phylum (66%), while proportions of Bacteroidetes and Proteobacteria dropped to 3%. Additionally, Synergistetes increased tenfold in their relative abundance, mainly due to the rise of the genus *Anaerobaculum* (11% of all bacterial reads). Also the methanogenic communities underwent a complete

reorganization due to the temperature shift. *Methanosaeta* was dominant at 37 °C (64% of total archaea), while *Methanosarcina* became dominant at 55 °C with 28%, together with *Methanoculleus* (20%) and *Methanothermobacter* (19%). In accordance with the abundance shift from acetoclastic to hydrogenotrophic methanogens, the functional annotation of the metagenome reads revealed an increase of genes involved in hydrogen metabolism. The number of reads assigned to Fe-hydrogenase genes increased significantly, and the assigned taxa involved in hydrogen metabolism became more diverse, which is consistent with a decrease of acetoclastic methanogenesis and a higher share of SAO under thermophilic conditions. A similar reorganization of the reactor microbiome upon temperature shift was observed by Tukacs-Hájos et al. (2014) in pilot-scale reactors digesting sugar beet pressed pulp and shifted from 38 °C to 55 °C at a rate of 2 K per day. Firmicutes and Synergistetes increased, while Bacteroidetes and Proteobacteria decreased in their relative abundances. At a later stage of stable operation under thermophilic conditions, Thermotogae partially replaced Firmicutes, and Bacteroidetes disappeared almost completely. The methanogenic community shifted from the acetoclastic genus *Methanosaeta* toward a higher proportion of hydrogenotrophic genera (*Methanothermobacter*, *Methanoculleus*).

To address the controversial discussion about the potential role of biogas plants in dissemination of pathogens, metagenomes were analyzed for the detection of putative pathogenic bacteria such as *Clostridium botulinum* and *Escherichia coli* (Eikmeyer et al. 2013). Only very few sequences were predicted to originate from potentially pathogenic Clostridia, and known virulence determinants could hardly be detected. The authors concluded that the risk of pathogen dissemination by application of digestate as fertilizer is low. Another biosafety issue discussed in the context of AD technology is the dissemination of antibiotics resistance genes (ARGs). In a recent study, Luo et al. (2017) compared abundance and diversity of ARGs in the metagenomes of the 14 full-scale biogas plants described previously (Luo et al. 2016). Three groups of ARG subtypes were identified in sludge reactors, mesophilic and thermophilic manure reactors, respectively, and only few subtypes were shared between the three groups. Tetracycline resistance genes were the most prevalent ARG subtypes, and ARG abundance was lower in thermophilic reactors. This study has important implications for ARG management in biogas plants and demonstrates the need for more comprehensive and systematic studies on the dissemination of ARGs by AD microbiomes.

As mentioned above, the importance of efficient hydrolysis in biogas reactors digesting plant biomass has drawn special attention to hydrolytic enzymes, especially those involved in breakdown of lignocellulose such as cellulolytic glycoside hydrolases (GH), with potential application for the enhancement of lignocellulose degradation in AD systems (Pandit et al. 2016; Xia et al. 2013; Yan et al. 2013; Yang et al. 2016). Gene mining of lignocellulose-degrading microbiomes can be employed by heterologous expression of novel GH genes retrieved from metagenome datasets and biochemical characterization of the gene products to find novel applications of such enzymes in other biotechnological processes (Jabbour et al. 2013; Wang et al. 2015; Wei et al. 2015).

3.2 Genome-Centric Approach

In very recent years, there is an increasing trend toward genome-centric metagenomics in more complex microbial systems due to the availability of more advanced bioinformatics tools (Sales and Lee 2015). In a pioneering study, Lykidis et al. (2011) investigated the metagenome of a lab-scale anaerobic bioreactor treating terephthalate (TA) and retrieved partial population genomes by composition-based binning. The metabolic reconstruction of the major genome bins revealed multiple syntrophic interactions in the TA-degrading methanogenic consortium. Besides the population genomes of the syntrophic TA-degrading *Pelotomaculum* sp. and its methanogenic partners – the hydrogenotrophic genus *Methanolinea* and the acetoclastic *Methanosaeta* – an additional genome bin affiliated to the candidate phylum OP5 (meanwhile assigned as phylum Caldiserica; Mori et al. 2009) was retrieved. This bacterium was proposed to consume hydrogen from syntrophic TA degradation and to fix CO_2 via the Calvin-Benson-Bassham cycle while producing butyrate and thus fueling secondary syntrophic interactions of butyrate-degrading syntrophs and methanogens. Such a meandering carbon and electron flow is a striking feature of methanogenic consortia and might increase the overall metabolic flexibility and thus the process stability in AD systems.

Candidate phyla (i.e., phyla that are not represented by any cultured species but merely by their 16S rRNA sequences) are widespread in anaerobic environments such as methanogenic reactors. Genome-centric metagenomics is a promising approach to get clues on the ecophysiology of these uncultured species by assembling population genomes. A prominent example is "*Candidatus* Cloacamonas acidaminovorans," which is the only species of the candidate phylum Cloacimonetes (previously referred to as WWE1) and has been detected in various AD systems. Its genome was assembled from a metagenomic fosmid library of a WWTP (Pelletier et al. 2008).

Compared to the TA-degrading bioreactor studied by Lykidis et al. (2011), which was fed by a single compound as the only organic carbon source, agricultural and industrial biogas reactors are generally much more complex with regard to the substrates. Consequently, the bacterial communities involved in hydrolysis, acidogenesis, and acetogenesis are more diverse in most biogas reactors, and genome-centric metagenomics is more challenging regarding sequencing depth and binning algorithms. As recently as the last 2 years, numerous genome-centric metagenome studies on AD systems were published. Stolze et al. (2016) analyzed three mesophilic and one thermophilic production-scale biogas plants and retrieved four genome bins from the mesophilic reactors representing novel species of the phyla Cloacimonetes, Spirochaetes, and Fusobacteria, respectively, and one genome bin from the thermophilic reactor, which was affiliated to the phylum Thermotogae.

Campanaro et al. (2016) analyzed the metagenomes of 8 thermophilic lab-scale CSTR digesting cattle manure and extracted 106 genome bins from the metagenome dataset by employing a novel binning strategy based on differential coverage binning (Albertsen et al. 2013). However, approximately 70% of the

assembly could not be assigned to a specific genome bin, which gives an estimate on the unexplored microbial diversity in the reactors. The dataset of these 106 genome bins was used to map metagenome reads from lab-scale reactors exposed to LCFA shock loads (Kougias et al. 2016). LCFA are problematic AD intermediates that can accumulate and inhibit the biogas process if the microbiome is not adapted to the degradation of lipids, which depends on syntrophic interaction of fatty acid-oxidizing bacteria and methanogens. From the 106 genome bins, 45 presented distinct abundance changes in response to the LCFA pulse. Metagenome analysis further indicated that microbes involved in LCFA degradation were associated to specific traits such as chemotaxis and motility. Additionally, the authors suggest that interspecies electron transfer between LCFA-oxidizing bacteria (*Syntrophomonas*) and methanogens (*Methanosarcina*) was related to menaquinone electron carriers. The study also confirmed that an inoculum previously adapted to lipid-rich substrate is more efficient in LCFA degradation due to the specialization of the microbiome.

Following the bioinformatics workflow described by Campanaro et al. (2016), Treu et al. (2016b) extracted 236 genome bins from mesophilic and thermophilic two-stage reactor systems, which were operated in lab-scale to establish in situ upgrading of biogas by feeding hydrogen into the second stage reactor. From these genome bins, 157 were novel compared to the previous dataset described by Campanaro et al. (2016). Based on this extended dataset, the authors suggest a subset of common microbes that could be considered as the core essential group in anaerobic digesters. The concept of an AD core microbiome has been suggested previously for sludge-digesting reactors (Riviere et al. 2009). With the growing genome database from various types of AD systems, this concept can be revisited and refined. Another recent genome-centric metagenome study made a substantial contribution to this endeavor by assembling 101 population genomes assigned to 19 phyla from a metagenome dataset obtained from triplicate lab-scale biogas reactors fed only with cellulose as model substrate but inoculated with a diverse inoculum consisting of samples taken from various environments (Vanwonterghem et al. 2016b). Classification of the genome bins into functional guilds revealed metabolic networks with a high level of functional redundancy as well as niche specialization. A striking result was the discovery of a divergent *mcrA* gene, which was used to screen the public sequence databases for similar *mcrA* sequence types that are only distantly related to those of known methanogenic orders. A divergent *mcrA* cluster containing two sequences originating from AD systems was detected, and based on the according population genomes, the novel archaeal phylum Verstraetearchaeota was proposed (Vanwonterghem et al. 2016a). After the discovery of methane metabolism in the archaeal phylum Bathyarchaeota revealed by genome-centric metagenomics (Evans et al. 2015), this recent discovery further challenges the long-standing dogma that methanogenesis is restricted to Euryarchaeota. While Bathyarchaeota were so far not detected in AD metagenomes, the discovery of the novel phylum Verstraetearchaeota extends our view on methanogenic diversity in AD systems.

4 From Genetic Potential to Activity

The metagenome analyses described above have provided detailed knowledge on the genetic makeup of AD microbiomes on the community and single population levels. However, the genome information of an organism or a population defines just the limits of potential traits but does not reflect the actual metabolic activity, i.e., the expression of distinct genes and pathways. Realization of the genetic information needs to be studied on the level of gene products, i.e., transcripts and proteins. Accordingly, the holistic approach of metagenomics is extended to metatranscriptomics and metaproteomics in microbial community ecology.

Metatranscriptome analyses have the advantage that they provide information on the metabolically active community members and their actually expressed pathways while basically relying on the same sequencing technology and bioinformatics tools as metagenome analyses. By combining metatranscriptomics with genome-centric metagenomics, population genomes can be used as scaffold to map metatranscriptome reads to reference genomes from the same sample and thus reveal the dynamics of metabolic activity on the community level. Methodological challenges of metatranscriptomics are associated with RNA isolation, overrepresentation of ribosomal RNA, and the short half-life of mRNA.

The first metatranscriptome analysis of a biogas reactor was performed by Zakrzewski et al. (2012). The sample for RNA extraction was taken from the same agricultural biogas plant for which a metagenomics approach was previously carried out (Jaenicke et al. 2011; Krause et al. 2008; Schlüter et al. 2008). The study revealed Firmicutes as the dominant active bacterial phylum followed by Bacteroidetes, Actinobacteria, and Synergistetes. Transcripts for enzymes functioning in methanogenesis were more abundant than it was deduced from the 16S rRNA sequence tags. This result emphasizes that key enzymes of the methanogenesis are highly expressed, and despite the low relative abundance of methanogenic archaea compared to bacteria, they can be highly active as terminal key players of the AD process.

Bremges et al. (2015) demonstrated the combined application of metagenomics and metatranscriptomics with increased sequencing depth on samples from the agricultural biogas plant that was previously analyzed for its metagenome by Stolze et al. (2015). Sequencing at least one order of magnitude deeper than in previous studies enabled the mapping of transcripts to metagenome contigs and, hence, the identification of active metabolic pathways in target organisms. For instance, the reconstruction of methanogenic pathways was demonstrated, although not all key gene transcripts were detected in the metatranscriptome dataset.

A combined approach of genome-centric metagenomics and metatranscriptomics was employed to reveal the differences in lignocellulose digestion efficiency among the microbiomes of an agricultural biogas plant, cattle rumen, and elephant gut (Güllert et al. 2016). The authors suggested that the lower abundance of genes affiliated to certain GH families in the metagenome from the biogas plant was attributed to a partial lack of genes originating from Bacteroidetes and Fibrobacteres.

These phyla comprise important polysaccharide-degrading bacteria in animal gut and rumen systems, and an underrepresentation of GH genes derived from these phyla implies a potential limitation of lignocellulose hydrolysis in biogas reactors. Moreover, highly transcribed GH genes in the biogas plant samples were four times more often affiliated with the phylum Firmicutes compared to the Bacteroidetes, while an equal distribution was observed in the elephant feces sample. Thus, comparative metagenome and metatranscriptome studies of AD systems and gut microbiota from herbivorous animals deliver the foundations for novel bioaugmentation strategies in AD.

Further studies employing the combined metagenomics and metatranscriptomics approach investigated AD of *Spirulina* microalga at haloalkaline conditions (Nolla-Ardevol et al. 2015b) and the response of the AD microbiome in thermophilic manure-fed biogas reactors to LCFA (oleate) addition (Treu et al. 2016a). In the latter study, transcripts were mapped to the 106 genome bins compiled in the database by Campanaro et al. (2016).

To get even closer to the realized genetic information and metabolic activity, the metaproteome of AD microbiomes can be investigated by total protein extraction and fractionation followed by chromatographic separation and tandem mass spectrometric analysis. However, to fully exploit the potential of the metaproteomics approach, a comprehensive genome database related to the system under study is needed as generic public sequence databases are badly annotated and inflated with hypothetical proteins of unknown functions. To understand the physiological function of proteins identified in metaproteome studies, supportive metagenome databases are suitable as scaffold as demonstrated by Hanreich et al. (2013). Similarly, Ortseifen et al. (2016) found out during an integrated metagenome and metaproteome analysis of a biogas plant that public databases yielded insufficient identification rates compared to a corresponding metagenome database from the same sample. The application of metaproteomics for the analysis of AD microbiomes was recently reviewed by Heyer et al. (2015) including an overview of the workflow and potential pitfalls.

At least annotation of proteins involved in key functions of methanogenesis is mostly possible in metaproteome studies as methanogenic genera are comparably well represented in genome databases. However, comparison of a metaproteome with its corresponding metagenome can provide quantitative insights into the metabolic activities of different functional guilds. Hanreich et al. (2013) compared metagenome and metaproteome datasets from lab-scale batch reactors digesting straw and hay and followed the community dynamics on the proteome level. They observed that methanogens represented less than 4% of the community on the genome level, while 20–30% of the identified proteins were of archaeal origin, suggesting that methanogens are disproportionally active in biogas reactors as previously observed on the transcriptome level by Zakrzewski et al. (2012). Differential gene expression of methanogens at low temperatures (7 °C and 15 °C compared to 37 °C) was studied on the proteome level by Gunnigle et al. (2015).

The study by Frank et al. (2016) is an example for the combined application of genome-centric metagenomics and metaproteomics to go beyond the simple

description of the community and its metabolic potential. They investigated a production-scale ammonia-tolerant biogas reactor fed with slaughterhouse and municipal waste and reported the discovery and dominance of a novel uncultured phylotype (unFirm_1). The unFirm_1 population genome was reconstructed from the metagenome dataset. Quantitative metaproteome analysis implied a function of unFirm_1 in SAO. Although other genomes related to known SAOB were also identified in the metagenome, their limited proteomic representation suggested that unFirm_1 plays the major role in converting acetate to methane in syntrophic interaction with hydrogenotrophic methanogens.

A recent study used multiple meta-omics approaches including quantitative metaproteomics to characterize an industrial biogas reactor treating food waste at 60 °C and elevated free ammonia levels (Hagen et al. 2017). Multiple strains affiliated to *Coprothermobacter proteolyticus* were detected, introducing an additional level of complexity seldom explored in AD microbiome studies. Genome reconstructions provided metabolic insight into the microbes that performed biomass deconstruction and fermentation under these extremely thermophilic conditions, including the deeply branching bacterial phyla Dictyoglomi and Planctomycetes and the candidate phylum "Atribacteria." Metaproteomics data also suggested acclimatization and activity of a *Methanosaeta* species even at high ammonia levels. A metabolic scenario was drafted suggesting that multiple so far uncultured syntrophic bacteria are capable of SAO as well as syntrophic oxidation of other fatty acids via β-oxidation and the Wood-Ljungdahl pathway, respectively, to hydrogen and carbon dioxide.

A recent large-scale metaproteomics study investigated 35 different industrial biogas plants (Heyer et al. 2016). Microbial key players and major AD pathways were identified in this study, and it was confirmed on the proteome level that high ammonia loads in biogas reactors promote the dominance of SAO and hydrogenotrophic methanogenesis. Similarly, SAO coupled to hydrogenotrophic methanogenesis was previously confirmed as the dominant acetate sink at high temperatures, as observed in a metaproteome study on thermophilic cellulose-degrading biogas reactors (Lu et al. 2014). Further large-scale metaproteome studies on various AD reactors can complement metagenome surveys underpinning the AD core microbiome concept.

5 Research Needs

Meta-omics studies on methanogenic consortia in anaerobic digesters unveiled that most of the microorganisms are still unexplored and not represented by pure cultures. Consequently, only limited functional information can be derived from public genome databases at least for certain functional guilds due to missing reference genomes. Future metagenome studies should focus on the genome-centric approach and put further efforts on reconstructing high-quality population genomes to extend the present genome databases and underpin the Candidatus species concept for uncultured microbes (Konstantinides and Rosselló-Móra 2015). Complementary to the genome-centric metagenomics approach, single cell genomics should be

applied to biogas reactors as it has been done for various natural environments (Rinke et al. 2013). An extended database of reference genomes from AD systems will better support functional annotation in metaproteome surveys.

Technical advances in the field of metagenomics can be expected through further improved sequencing technologies with higher throughput and longer read lengths, thus enabling substantial cost reduction per metagenome sample. As the amount of data generated will increase accordingly, the further improvement of bioinformatics tools including data management remains an important research need. Metagenomics will become a widespread approach in applied microbial ecology similar to the currently established PCR-based high-throughput methods. By using metagenomics in combination with operational parameters and process performance data, functional redundancy in AD microbiomes and the level of metabolic diversity required to maintain process stability can be determined (Vanwonterghem et al. 2014).

To gain meaningful and statistically sound data, replication in terms of biological and technical replicates but also time series in sampling is inevitable. The first metagenome studies on AD systems suffered from the limitation that only one or a few samples from one reactor were analyzed, no biological replicates were included to account for stochastic effects in community assemblage, and time series were not done, thus neglecting short-term or seasonal fluctuations in the microbiome. Future metagenome studies should go beyond snapshot analyses and need to support complex experiments carefully designed to answer specific ecological questions (Prosser 2015).

Whenever possible, conclusions drawn from meta-omics studies on AD systems should be underpinned by experimental data gained with complementary methods, such as stable isotope probing (SIP) to analyze the carbon flow within the microbiome (Mosbæk et al. 2016). Although SIP approaches in anaerobic consortia are hampered by the generally low assimilation rate of anaerobes, at least the overall carbon flow based on labeled metabolites can be unveiled.

Future metagenome studies on AD microbiomes should also consider the role of other entities beyond bacteria and archaea, such as eukaryotes and viruses, and their impact on the community function. A recent survey on viromes in various full-scale AD systems (Calusinska et al. 2016), which greatly extends the existing view of viral genetic diversity in methanogenic environments, revealed that AD viromes are distinct not only among different reactor types but also from other environments already studied for their viral diversity.

Last but not least, systems biology approaches such as metabolic network modeling should be extended to more complex microbial communities such as AD microbiomes. The extension of constraint-based stoichiometric modeling such as flux balance analysis from single species or simple artificial consortia represented by reference genomes to natural methanogenic consortia in AD systems is a challenging but worthwhile endeavor that will greatly benefit from the progress in metagenome approaches (Gottstein et al. 2016; Perez-Garcia et al. 2016). The adoption of systems biology principles in systems ecology of open mixed cultures such as AD reactor microbiomes will open up new perspectives in a knowledge-based microbial resource management.

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Anaerobic Digestion as Key Technology in the Bio-based Economy

14

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Abstract

In our current society, there is a pressing need to shift from a fossil fuel-based to a bio-based economy in which renewable resources are used for the recovery of energy and production of bio-based chemicals for the industry. For several decades, anaerobic digestion has been the technology *par excellence* to deal with organic waste streams for waste stabilization and energy recovery in the form of biogas. This contributed a central role to anaerobic digestion in the biorefinery to deal with the massive amounts of waste streams that are generated during the production of bio-based chemicals. Due to their complex nature, these wastewaters often pose a challenge for anaerobic digestion, thus, integration with other technologies is needed to sustain its central role. The potential of anaerobic digestion can be extended beyond mere on-site energy recovery. Biogas

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upgrading to biomethane can be considered an interesting alternative for natural gas, yet, the economic viability will strongly depend on the future market value of electricity. Nutrient recovery and carboxylate production can also be targeted through the anaerobic digestion process, enabling the recovery of higher-value products, compared with biomethane. The potential integration of anaerobic digestion with other technologies for transport fuel production, integrated manure management, and carbon sequestration further emphasizes the versatility of anaerobic digestion and central position in the bio-economy.

1 Introduction: Anaerobic Digestion in the Bioeconomy: Past and Present

A first case in which biogas was used was suggested to originate from the tenth century B.C. in Assyria, where it was implemented to heat bath water (Bond and Templeton 2011). In ancient China, covered sewage tanks were already used 2000–3000 years ago (He 2010). In the seventeenth century, the potential of decaying organic matter to generate an inflammable gas was discovered, and in the eighteenth century, a correlation between the amount and type of organic matter and the volume of biogas produced was established (Abbasi et al. 2012). In the nineteenth century, the chemical composition of CH_4 was determined, and a high degree of similarity between coal gas and marsh gas, produced from organic matter by microorganisms, was confirmed (Abbasi et al. 2012). At the end of the nineteenth century, the stoichiometric conversions of (1) acetate to CH_4 and CO_2 (Gunnerson and Stuckey 1986), and (2) H_2 and CO_2 to CH_4 were identified (McCarty 1981).

A first technological application of anaerobic digestion (AD) can be situated in 1881, with the first version of a septic tank for the treatment of wastewater (McCarty 1981). Following some necessary updates to improve the overall efficiency, this process was applied to treat the wastewater of the city of Exeter in 1897 (Abbasi et al. 2012). The CH_4 produced during this process was used for on-site lightening and heating (Gunnerson and Stuckey 1986). During the following decades, however, further optimization of the AD process was directed almost exclusively toward the stabilization of organic waste streams, rather than renewable energy recovery.

In the twentieth century, numerous innovative AD technologies were developed and optimized, for liquid (Lettinga and Hulshoff Pol 1991; Lettinga et al. 1980) and solid (Mata-Alvarez et al. 2000) waste streams. To increase process control and to maximize CH_4 production, the key parameters and their optimal ranges were set of which pH, temperature, volatile fatty acid (VFA) concentration, salinity, and free and total ammonia concentration can be considered the most important ones (Appels et al. 2008; Chen et al. 2008; De Vrieze et al. 2012, 2015b). The number of farm-based AD installations strongly increased in the USA, Canada, and Europe, especially France and Germany, during the mid-1950s to treat organic waste streams, mainly manure (Abbasi et al. 2012). Initially, simple covered tanks or lagoons were used to stabilize these waste streams, whether or not in combination with biogas recovery, but gradually a more efficient approach in the concept of the continuous

stirred tank reactor (CSTR) was used, which, at present, is still prone to further optimization (Boe and Angelidaki 2009). In the past decades, biogas technology implemented in industrial and urban sectors increased considerably, related to the use of different types of feedstocks (Weiland 2010), both in developed and developing countries (Bond and Templeton 2011).

Both domestic and industrial wastewater treatments plants nowadays host a central role for AD, either for direct wastewater treatment via an upflow anaerobic sludge blanket (UASB) reactor (Seghezzo et al. 1998) or indirectly via a CSTR for waste activated and/or primary sludge digestion (Sundberg et al. 2013). If properly managed, this can result in energy-neutral wastewater treatment plant (De Vrieze et al. 2016c). The role of AD in the present bioeconomy, however, exceeds the boundaries of on-site electricity and heat production, as it can serve as a process for (1) renewable energy recovery, (2) nutrient recapture, and (3) the production of bio-based chemicals.

2 Anaerobic Digestion in the Biorefinery

2.1 Waste Streams in the Biorefinery: Problems and Potentials

One of the main requirements for the anticipated sustainable bioeconomy is the shift from a fossil fuel-based to bio-based production of chemicals for the industry. The first-generation plants for, e.g., bioethanol and biodiesel production make use of feedstocks rich in sugar or starch, such as sugar cane, sugar beet, wheat, or maize (de Vries et al. 2010; Dias et al. 2011). The sustainability of these plants can be considered highly questionable, because their production process (1) is in direct competition with food and animal feed production and (2) coincides with the generation of massive amounts of unused side streams (Naik et al. 2010). The second-generation plants or so-called biorefineries apply a more integrated approach in which not only the feedstocks are used, but also side streams such as sugar cane bagasse and vinasse are valorized (Moraes et al. 2015; Parajuli et al. 2015; Rabelo et al. 2011). Other feedstocks with a high lignocellulosic content that are not in competition for food or feed, such as agricultural residues, forestry wastes, and even industrial and municipal wastes, can be used to produce bio-based chemicals (Maity 2015; Menon and Rao 2012; Sarkar et al. 2012). This also opens the possibility to shift from bioethanol and biodiesel of which the long-term sustainability is controversial (DeWulf et al. 2005; Kim and Dale 2005), to other market-demand driven non-fuel chemicals, such as succinic acid, lactic acid, caproic acid, and bioethanol derivatives (Agler et al. 2012; Choi et al. 2015; Posada et al. 2013).

Although the integrated approach of the biorefinery leads to an optimal usage of the different feedstocks, the generation of massive amounts of (diluted) waste streams that require adequate treatment cannot be avoided (Ryan et al. 2009). The production of bioethanol, for example, coincides with the generation of 10–20 liters of wastewater per liter of bioethanol, and this wastewater contains high COD (chemical oxygen demand) and BOD (biological oxygen demand) concentrations,

up to 100 and 50 g L^{-1}, respectively (Pimentel and Patzek 2005; Satyawali and Balakrishnan 2008). These wastewaters often have a low pH in the range of 4–4.5 and can be highly saline, due to elevated nitrogen, phosphorus, and potassium concentrations (Satyawali and Balakrishnan 2008), which is the case for, e.g., vinasse wastewaters (Moraes et al. 2015). These characteristics pose several issues for biological treatment and imply the need for specific techniques.

Aerobic treatment of such wastewaters can be carried out by means of the *Penicillium decumbens* fungus, due to its tolerance to high concentrations of salt and phenolic compounds (Jimenez et al. 2005). The aerobic treatment, however, consumes energy, due to the required aeration, instead of recovering it, which questions its long-term sustainability, especially since additional anaerobic treatment is necessary (Jimenez et al. 2003). Anaerobic digestion is a second option to treat these wastewaters, and it allows the recovery of energy through the production of biogas. The high salinity and/or nitrogen content might inhibit the methanogenesis, as this is considered the most susceptible step in the AD process (Chen et al. 2008; De Vrieze et al. 2012). This implies the need for alternative strategies, such as co-digestion (Fang et al. 2011a, b; Misi and Forster 2001) or desalination (Zhang and Angelidaki 2015a) to reduce toxicity. To avoid the methanogenesis inhibition issue, fermentation of these wastewaters to produce additional renewable chemicals such as butyric and caproic acid could a suitable third option (Agler et al. 2014; Andersen et al. 2015; De Vrieze et al. 2016a), as long as the resulting production rates and concentrations are sufficiently high to obtain energy-efficient product extraction (Andersen et al. 2014).

2.2 Anaerobic Digestion as Central Technology in the Biorefinery

The success of the biorefinery depends on its potential to generate bio-based chemicals for the industry at high production rates, yields, and purities and acceptable costs, not exceeding the current market value of these chemicals. This implies that also the energy required to produce these chemicals should originate from a renewable source. Anaerobic digestion can be considered the technology *par excellence* to kill three birds with the same stone, as it not only deals with the treatment of side streams and wastewaters, but it also engages the production of renewable energy through biogas (Appels et al. 2011; Holm-Nielsen et al. 2009) and the potential for nutrient recovery (Batstone et al. 2015; Kjerstadius et al. 2015; Rodriguez-Garcia et al. 2014).

The centrality of AD in the integrated biorefinery is a consequence of the integration of several separation and concentration technologies that are needed to ensure adequate treatment of the concentrated organic waste streams and to maximize energy and nutrient recovery (Fig. 1). The process chain of the integrated biorefinery starts with the supply of biomass and the production of bio-based chemicals. In the framework of the second generation biorefinery, at least part of the side streams is valorized, and the remaining waste streams are sent for further treatment by means of AD (Cherubini 2010). Anaerobic digestion can take place

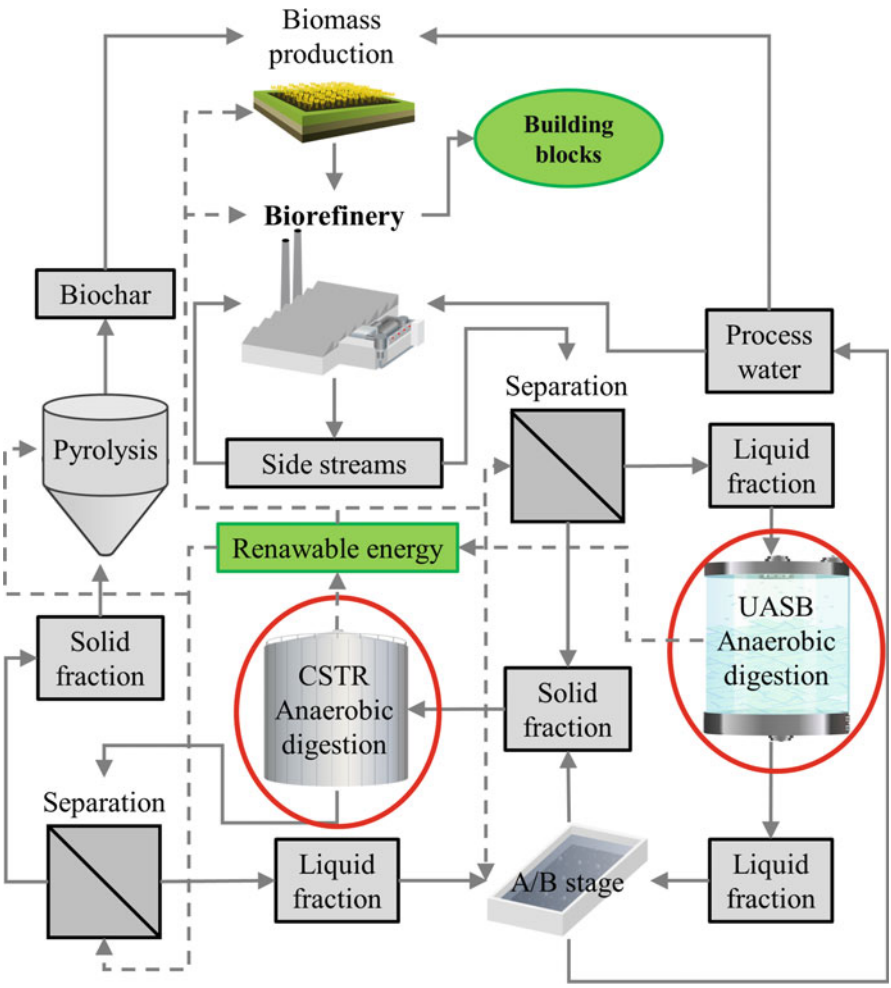

Fig. 1 Visualization of the central role of anaerobic digestion in the biorefinery, integrated with several separation and concentration technologies to obtain adequate treatment of the concentrated and diluted waste streams and to maximize energy and resource recovery. *Full lines* represent material flows, while *dashed lines* represent energy flows

through two parallel lines for the more solid, by means of a CSTR, and liquid, by means of an UASB digester, waste stream. This can be combined with a separation step, e.g., sedimentation or centrifugation, to avoid solids from blocking the UASB or to increase the solids content in the CSTR, respectively. The UASB effluent and CSTR supernatant will require additional polishing to reach the desired discharge or process water quality parameters (Khan et al. 2011), which can be obtained through the A-stage of the "Adsorptions-Belebungsverfahren" or A/B approach for wastewater treatment to maximize energy recovery (Boehnke et al. 1997; Ge et al. 2013; Meerburg et al. 2015). Depending on the nitrogen content, additional nitrogen

removal in the B-stage will be necessary via either conventional nitrification/denitrification or partial nitritation/anammox (Lackner et al. 2014). The effluent of the A/B process can be used as process water for, e.g., irrigation. The A-stage sludge is a suitable co-substrate to be used in the CSTR digester, as it has a high biodegradability, and it can stabilize the digestion process of the salt- and nitrogen-rich biorefinery waste streams (De Vrieze et al. 2013, 2015a). The solid fraction of the digestate of the CSTR and, to a lesser extent, the UASB can be used directly as fertilizer (Vaneeckhaute et al. 2013) or subjected to pyrolysis to produce biochar that can be used as a soil amendment (Monlau et al. 2016). The production of bio-oil and/or syngas during pyrolysis can further increase energy recovery in the biorefinery (Hubner and Mummea 2015; Monlau et al. 2016).

3 Future Potentials

3.1 Biogas Upgrading to Biomethane: Possible but Not Feasible?

The original purpose of AD was to stabilize organic waste streams, but throughout the years it has evolved from a clean-up technology to renewable energy factory. On-site valorization of the biogas through a combined heat and power (CHP) unit has been essential to guarantee the economic viability of the process. The electrical efficiency of an optimized CHP unit for biogas, at present, reaches a maximum of 40% (De Vrieze et al. 2016c; Deublein and Steinhauser 2008; Szarka et al. 2013). To maintain this value, regular maintenance is required to avoid incomplete combustion or the so-called "methane slip" (Meyer-Aurich et al. 2012; Pucker et al. 2013). When on-site electricity production surpasses the demand, the surplus electricity can be put on the grid (Holm-Nielsen et al. 2009). The fluctuating nature of (1) renewable energy production and (2) energy consumption requires the need to shift from a smart electricity grid to a smart energy grid, integrating multiple aspects of renewable energy production (Junne and Kabisch 2017; Lund et al. 2012).

This opens the potential for AD to shift its position from decentral producer of electricity and heat to a supplier of natural gas that can be put on the gas grit, eliminating the need for a CHP unit. The main advantage of this approach lies in the fact that the inherent low efficiency of the CHP is no longer a lingering issue, but it does imply the need to upgrade the biogas from a conventional 60–70% to a 95–97% CH_4 content (Jury et al. 2010; Ryckebosch et al. 2011; Weiland 2010). This has led to a recently strong increase in the availability of novel technologies for biogas upgrading to biomethane. These technologies range between physico-chemical technologies, such as water scrubbing, solvent scrubbing, chemical scrubbing, pressure swing adsorption, membrane separation, and cryogenic separation, and biological technologies, such as chemoautotrophic biogas upgrading and photosynthetic biogas upgrading (Munoz et al. 2015).

The feasibility of these technologies strongly depends on their energetic or operational and construction costs, as the cost of the upgrading process should not exceed the increased value of the biomethane compared to biogas. Based on the

general gas law, 1 kg of COD converted to biogas corresponds with 350 L of CH_4 at standard temperature (273 K) and pressure (101,325 Pa) conditions. An electrical efficiency of 40% can be assumed for the CHP unit, and an estimated market price of € 0.10 kWh^{-1} for electricity and € 0.05 kWh^{-1} for heat can be set (Verstraete and Vlaeminck 2011). Hence, 1 kg of COD converted corresponds with € 0.20–0.25, based on an energy content of 10 kWh m^{-3} for CH_4. In contrast, the market value of natural gas can be assumed at € 0.25–0.30 m^{-3} in Europe (Kleerebezem et al. 2015), which then corresponds with a market price of € 0.10 per kg of COD converted to biogas for biomethane. Hence, the market value of 1 kg of COD can be increased with a factor 2.5 when the biogas is burned in a CHP unit. This value does not consider the biogas desulfurization process operational and capital costs, but this is needed both for biogas burning in the CHP unit and injection in the gas grid. Injection of biomethane in the gas grid does not require the construction of a CHP unit, yet it does coincide with the construction of a biogas upgrading unit for which the capital costs can be considered similar as for a CHP unit. The operational costs of a CHP unit are negligible, while the biogas upgrading operational costs account for € 0.03–0.07 per kg of COD converted, depending on the technology (Munoz et al. 2015).

These results emphasize that, at present, it is more economically feasible to use the biogas for on-site burning in a CHP unit, rather than putting it on the grid as biomethane. The preferred strategy, however, strongly depends on the timely market value of electricity, as this depends on the fluctuating nature of the supply of electricity, due to its dependence on, e.g., wind and solar energy. Biogas upgrading to biomethane and subsequent injection on the gas grid could be considered a more economically sustainable investment on the long term.

3.2 Anaerobic Digestion Beyond Biogas Production

Biogas has been the sole compound of interest after the transition of AD from clean-up technology to renewable energy factory. The potential of the thick fraction of the digestate to be used as fertilizer has been demonstrated in numerous studies (Moller and Muller 2012; Tambone et al. 2010; Vaneeckhaute et al. 2013; Walsh et al. 2012), yet the potential presence of heavy metals (Jin and Chang 2011) and pathogenic bacterial species (Kjerstadius et al. 2013) limits its direct applicability. For the AD process to meet the renewable and sustainable settings of the current bioeconomy, it needs to expand its potential beyond the borders of merely biogas and digestate.

A first potential can be found in the transition of AD to fermentation in the framework of shift from recovery of energy to recovery of chemicals. Numerous organic waste streams, such as molasses wastewaters (De Vrieze et al. 2016a), thin stillage (Andersen et al. 2017), grass (Jagadabhi et al. 2010; Khor et al. 2016), and high-rate activated A-sludge (Cagnetta et al. 2016), have been evaluated for their potential to produce carboxylates in the so-called carboxylate platform through mixed culture fermentation (Agler et al. 2011). This allows the production of a

diverse gamma of carboxylates, such as acetate, propionate, butyrate, and lactate (Agler et al. 2011), which can be subjected to chain elongation to caproate and caprylate to obtain higher-value carboxylates (Agler et al. 2012; Spirito et al. 2014).

These medium chain carboxylates have a higher economic value compared with biomethane, with, for example, € 0.40 per kg of COD converted to caproate (Kleerebezem et al. 2015), while this was only 0.10 per kg of COD converted for biomethane. The disadvantage of the production of water-soluble carboxylates lies in the necessity to provide an efficient extraction step to obtain a clean concentrated product from the fermentation mixed liquor. Several technologies have been applied successfully for selective carboxylate extraction, such as liquid-liquid extraction, membrane-based solvent extraction, pertraction, membrane separation, and membrane electrolysis, often in combination (Andersen et al. 2014; Singhania et al. 2013; Xu et al. 2015). Each of these technologies, however, requires a capital investment and is also accompanied with operational costs, which will decrease the gap of the value per kg of COD between CH_4 and carboxylates.

A second potential lies in the recovery of nutrients, mainly N, P, and K, from the digestate to recover clean and concentrated nutrient streams. One of the most well-known examples for combined N and P recovery is struvite, which can be used both for the liquid and solid fraction of the digestate (Cusick et al. 2014; Munch and Barr 2001; Shu et al. 2006). The presence of heavy metals or toxic organic pollutants, especially in the case of waste activated sludge digesters, limits its usage for agricultural applications (Desmidt et al. 2015). This implies the need for alternative strategies to recover N, P, and K as an unpolluted concentrated stream. Ammonia stripping and subsequent absorption is an often used strategy for ammonia recovery from AD, and this can be applied either as a pre-treatment (Bonmatí and Flotats 2003; Zhang et al. 2012), on-line system (Serna-Maza et al. 2014), or a post-treatment (Gustin and Marinsek-Logar 2011). Electrochemical extraction can be an alternative approach to recover both N and K, and also this technology can take place either as a pre-treatment (Ippersiel et al. 2012; Mondor et al. 2008), on-line system (Desloover et al. 2015; Zhang and Angelidaki 2015b), or post-treatment (Desloover et al. 2012). The recovery of phosphate is also possible via electrochemical extraction, which provides a possible alternative for struvite (Ebbers et al. 2015; Mondor et al. 2008).

The techno-economic potential of nutrient recovery depends on (1) the cost per unit of product recovered and (2) the effect on the AD process. The market value of nitrogen can be assumed at € 1.0 kg^{-1} N, which provides a potential for recovery, especially given the fact that a reduction in the ammonia concentration can also lead to an increase in biogas production (Angelidaki and Ahring 1993; Rajagopal et al. 2013). In contrast, the market value of phosphate amounts only € 0.42 kg^{-1} P, and this, conventionally, does not increase biogas production, which complicates cost-efficient P-recovery from digestate. The overall feasibility of N, P, and K recovery strongly depends on each specific case, but has been demonstrated to be economically feasible in full-scale wastewater treatment plants (De Vrieze et al. 2016c).

3.3 Integration with Emerging Technologies

3.3.1 Hydrogen Production and Anaerobic Digestion

Hydrogen gas has been targeted as a clean energy carrier, due to its high energy content of 142 MJ kg^{-1} (Guwy et al. 2011). The dark fermentative H_2 production process is very similar to AD, and it is mediated by hydrogenase enzymes of anaerobic microorganisms (Kovács et al. 2004). Full-scale biohydrogen production, however, can be economically viable, only if integrated with a process that can use the fermentation by-products, mainly VFA. Anaerobic digestion is the most suitable technology for integration in this case, as it can be used to convert the VFA to CH_4 or it can be operated in fermentation mode to produce carboxylates (Sect. 3.2). Hydrogen and biomethane could be mixed and upgraded to provide a gaseous transport fuel (Bauer and Forest 2001; Guwy et al. 2011). The combined production of H_2 and CH_4 has been evaluated in multiple reactor configurations, but it appeared that biohydrogen production was not feasible from an energy point of view (DiStefano and Palomar 2010). A more suitable approach would be to combine the AD plant with an electrochemical cell to supply H_2 through direct water electrolysis for which the electricity can be provided, either from the CHP unit or via solar cells (Fig. 2). A biohydrogen fermentation step can be included prior to the digester, yet it would mainly serve as way to safeguard the AD process, and the resulting H_2 can be considered as a valuable side product (DiStefano and Palomar 2010; Guwy et al. 2011).

3.3.2 Integrated Manure Management

Intensive livestock farming in densely populated areas results in the production of massive quantities of animal manure that require adequate treatment for

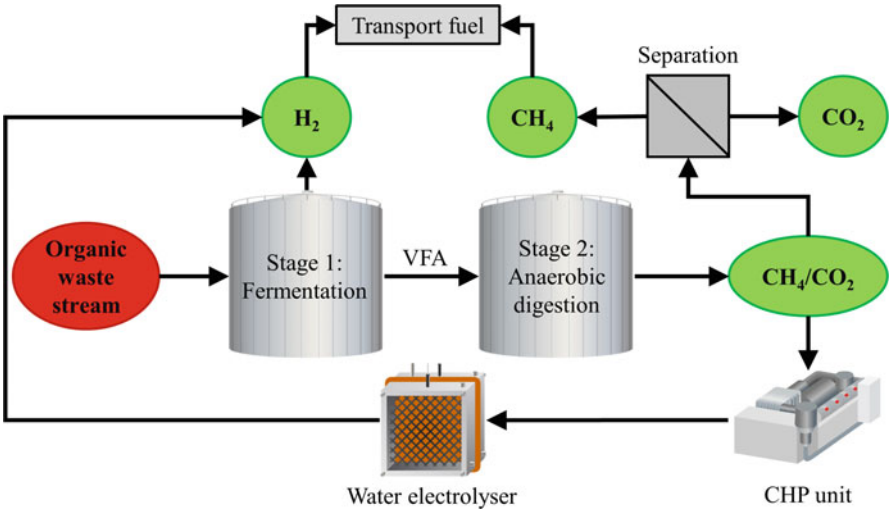

Fig. 2 Schematic representation of the combination of a two stage anaerobic digestion plant with an electrochemical cell to obtain a mixture of CH_4 and H_2 that can serve as transport fuel

which AD has been the most appropriate technology (Holm-Nielsen et al. 2009). The high salinity and nitrogen content often provoke methanogenesis inhibition, which results in process failure (Angelidaki and Ahring 1993; Hansen et al. 1998). One of the main strategies to prevent inhibition is co-digestion of manure with other waste streams with low nitrogen content, such as food waste, sewage sludge, and crude glycerol (Astals et al. 2012; Borowski and Weatherley 2013; Usack and Angenent 2015; Ye et al. 2015). The high nutrient content of manure also offers a huge potential in terms of recovery, as it is not only rich in nitrogen, but also potassium and phosphorus. Several recovery strategies have been suggested of which electrochemical extraction (Desloover et al. 2015; Desloover et al. 2012; Ippersiel et al. 2012; Mondor et al. 2008; Sotres et al. 2015), ammonia stripping (Bonmatí and Flotats 2003; Gustin and Marinsek-Logar 2011; Zhang et al. 2012), and struvite formation (Kataki et al. 2016) have been the most prominent ones. Such an integrated approach was applied in the ManureEcoMine pilot installation in which swine manure was co-digested with a mix of vegetable residues (Pintucci et al. 2017). The thermophilic pilot-scale digester was coupled to a side-stream ammonia stripping and acid scrubbing column to avoid ammonia inhibition and to recover nitrogen as ammonium sulfate.

3.3.3 Pyrolysis and Anaerobic Digestion

The residual organic fraction of AD or digestate, which consists of nondegraded organic matter and microbial biomass, needs to be dealt with in the most environmentally and economically feasible way. Digestate can be applied as alternative to synthetic fertilizer in agriculture (Tambone et al. 2010; Vaneeckhaute et al. 2013), yet there is a potential presence of pathogens (Kjerstadius et al. 2013; Viau and Peccia 2009) and heavy metals (Jin and Chang 2011). Digestate can be subjected to pyrolysis, which will eliminate pathogens and immobilize heavy metals, resulting in, depending on the operational conditions, syngas, bio-oil, and biochar (Beesley and Marmiroli 2011; Inyang et al. 2010; Monlau et al. 2015, 2016). These products obtained by digestate pyrolysis could be reintegrated in the anaerobic digester (Hubner and Mummea 2015; Monlau et al. 2016). Syngas can be used to produce biomethane or directly as fuel (Guiot et al. 2011). Biochar can be introduced in AD reactors (1) as an adsorbent to prevent process inhibition (Mumme et al. 2014; Torri and Fabbri 2014), caused by, e.g., heavy metals (Inyang et al. 2012) and (2) as a carrier material for microbial growth, mainly methanogens (De Vrieze et al. 2016b; Lu et al. 2016).

The end-use of the products from AD is not limited to energy recovery in a CHP for biogas and usage as fertilizer for digestate. There are a lot of alternative applications in which integration of different technologies is essential. Finding the optimal combinations to obtain the required end-products, which strongly depends on the feedstock composition, is highly case-dependent, and requires in-depth research to obtain the most optimal system from an energetic and environmental point of view.

4 Can Anaerobic Digestion Sustain Its Central Role in the Bioeconomy?

The central role of AD as main process for the treatment of organic waste streams and on-site provider of renewable energy in the biorefinery is apparent (Fig. 1). Municipal wastewater treatment facilities also heavily depend on AD for the stabilization of the activated sludge and partial recovery of the energy contained in the wastewater. The transition from conventional activated sludge to high-rate activated A-sludge to obtain a sludge with a higher biodegradability (De Vrieze et al. 2013; Meerburg et al. 2015) in combination with a shift from mesophilic to thermophilic digestion could even result in energy neutrality of the wastewater treatment plant (De Vrieze et al. 2016c).

The potential of AD, however, exceeds the stabilization of organic waste and recovery of renewable energy. Anaerobic digestion should evolve from a provider of renewable energy to a beacon of bio-based chemicals and recovered nutrients, thus maximizing its potential. The temperature-phased anaerobic digestion (TPAD) process (Han and Dague 1997) can serve as a suitable concept to reach this potential. The TPAD system contains 2 stages of which the first stage is operated at thermophilic conditions (50–70 °C), a low hydraulic retention time (HRT) of 2–5 days and high organic loading rate (OLR) of 10–15 g COD L^{-1} d^{-1}. In contrast, the second or mesophilic stage is operated at a high HRT of 10–20 days and an a low OLR of 2–5 g COD L^{-1} d^{-1} (Bolzonella et al. 2009; Ge et al. 2011; Lv et al. 2010; Oles et al. 1997; Riau et al. 2010).

This TPAD process can be considered for an integrated energy and resource recovery approach (Fig. 3). The first stage can be operated as a fermentation process in which even higher OLR values, exceeding 50 g COD L^{-1} d^{-1}, could be applied for high-rate carboxylate production, as demonstrated for lactic acid fermentation (Ahring et al. 2016). After on-line extraction of the produced carboxylates via the most appropriate technique (Singhania et al. 2013), the mixed liquor can be sent to the second stage for conversion of the residual organic matter to biogas. A nutrient (N, P, and K) recovery technology can be integrated either between the two stages or on-line in the second stage to obtain a clean nutrient stream. This has a dual

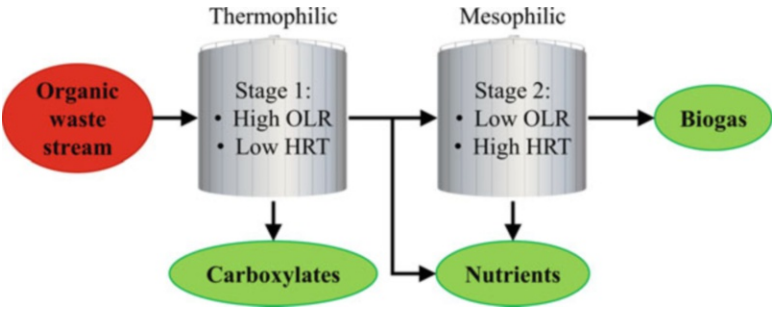

Fig. 3 Temperature-phased anaerobic digestion process scheme to obtain the integrated production of carboxylates and recovery of nutrients and biogas

advantage, as it does not only result in the recovery of nutrients, but it also prevents inhibition of the AD process in stage two, related to ammonia and salt toxicity (Zhang and Angelidaki 2015a).

Such an integrated approach combines the different possibilities of AD to maximize the potential of energy, carboxylates, and nutrients recovery. Combined with advanced community engineering, the polyvalent character of AD, which is the main strength of this microbial technology, will ensure the continuation of its central position in the bioeconomy.

5 Research Needs

Anaerobic digestion has been studied for several decades now, and this resulted in a well-described food web (Angenent et al. 2004) and a clear overview of the key microorganisms involved in the different steps (De Vrieze et al. 2015b; Sundberg et al. 2013; Zhang et al. 2014). This is reflected in the high number of full-scale applications in different reactor technology configurations (e.g., CSTR, UASB, and membrane-based systems) using different feedstocks. The production of massive amounts of complex waste streams in the biorefineries pushes for alternative approaches, from a technological and microbiological point of view, to tackle to issues of high salinity wastewaters, ammonia toxicity, and the presence of recalcitrant compounds to maximize energy recovery. The production of bio-based chemicals and the recovery of nutrients are already possible from a technological point of view, but the aspects of (1) economic feasibility and (2) product specificity require extra attention, before wide full-scale application can be targeted. The potential of biomethane as an alternative for natural gas resulted in multiple biogas upgrading technologies (Munoz et al. 2015), yet their full-scale potential will strongly depend on future energy prices, which implies that innovative technologies with a lower cost per unit of biomethane should be developed. Overall, the endurance of AD as central technology in the bioeconomy will strongly depend on continuous innovation and adaptability to the societal demands.

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Oxic Methane Cycling: New Evidence for Methane Formation in Oxic Lake Water

15

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Abstract

In contrast to common belief, the potent greenhouse gas methane can be produced and emitted from oxygenated water bodies. This has been shown for both marine and freshwater systems over the last decades and has been named "**the methane paradox**." The concentration of methane in anoxic sediments is orders of magnitude higher than in the oxic water layers; nevertheless, in most cases, methane from the sediment is oxidized by methanotrophic *Bacteria* and *Archaea* near the sediment. In contrast, the methane-rich oxic surface waters are in direct contact with the atmosphere and can be a significant source of atmospheric methane. Several biotic and abiotic mechanisms have been proposed to explain the "methane paradox." These include the formation of microenvironments suitable for classical anaerobic methanogenesis as well as novel pathways. Among the latter demethylation of methylphosphonates has been proposed as an important pathway in both marine and freshwater systems. We used the meso-oligotrophic Lake Stechlin in northeastern Germany as a model system for methane-emitting freshwater lakes. We showed that oxic methane production was seasonal, occurring mostly in spring and summer. A mass balance of the methane budget suggests minimal methane input from the littoral zone to the oxic pelagic waters and that in situ biological production was the main source of methane in the oxic epi- and metalimnion. Using metagenomic and metatranscriptomic analyses, we showed that *Archaea* in general as well as key methanogenesis genes were entirely absent from the epi- and metalimnion of the lake. Using incubation experiments, we showed that demethylation of methylphosphonates was a potential mechanism for methane formation in oxic Lake Stechlin water, but it likely was not the most significant process based on gene counts. Addition of trimethylamine, a known precursor to methane in anoxic environments, to lake water also resulted in oxic methane formation. A survey of gene databases revealed that most genes for methanogenesis were present in *Bacteria* from the lake, suggesting that analogs or paralogs of missing genes may still be identified. We propose that oxic methane formation in Lake Stechlin and other aquatic systems is a result of multiple sequential and parallel pathways.

1 Introduction

Methanogenesis is still regarded by many as a process limited to anoxic environments (e.g., sediments, shallow eutrophic water bodies, rice paddies, wastewater plants, animal and insect guts). Nevertheless, over the past few decades, increasing evidence of methane accumulation and production in oxygen-saturated marine (Scranton and Farrington 1977; Karl et al. 2008; Damm et al. 2010) and freshwater (e.g., Grossart et al. 2011; Tang et al. 2014) environments has emerged. Accordingly, this phenomenon has been named "the methane paradox" (reviewed in Tang et al. 2016). In general, the concentrations of methane in the oxic layers of freshwater ecosystems range between 0.02 and 280 μM and are on average orders of magnitude higher than in the marine environments where peak values reach 0.002–0.014 μM

(as reviewed in Tang et al. 2016), making freshwater lakes equally important for methane release to the atmosphere, despite their relatively low total surface area as compared to the seas.

The relatively high methane concentrations in the pelagic area of lakes have been suggested to be a result of lateral transport from the littoral zone (Fernández et al. 2016). Nevertheless, *in situ* incubation experiments (Grossart et al. 2011) and floating mesocosm study (Bogard et al. 2014) both showed strong evidence of direct methane production in oxic freshwater. On the other hand, DelSontro et al. (2010) showed in an oxic reservoir that high outgassing of methane is the result of bubble ebullition from the sediment. Their observations are highly relevant to shallow, organic matter-rich reservoirs and demonstrate that oxic reservoirs can be a potent methane source, although not necessarily related to methane production within the oxic water column. Supporting the latter, Donnis et al. (2017) conclude that bubble ebullition from the sediment in Lake Hallwil, Switzerland, can only partly explain the pelagic methane peak in the oxic water layer but does not account for the whole amount. This finding is supported by a recent study by DelSontro et al. (2017) comparing a number of Canadian lakes.

Several mechanisms have been proposed to explain methane production in oxic marine environments. Karl et al. (2008) proposed demethylation of methylphosphonates (MPNs) by marine microbes as a main source. Carini et al. (2014) later demonstrated the ability of the ubiquitous SAR11 to produce methane by degrading methylphosphonate. Phosphonates are common products of many prokaryotes and eukaryotes (Yu et al. 2013; Ju et al. 2015), yet the presence and production of MPNs remained elusive until Metcalf et al. (2012) showing their synthesis by the abundant marine *Thaumarchaeota*, which usually reside in the deep ocean. Subsequent studies have shown that the degradation of phosphonates other than methylphosphonates can also result in methane formation (Repeta et al. 2016).

Damm et al. (2010, 2015) proposed that DMSP (Dimethylsulfoniopropionate) acts as another precursor to oxic methane and suggested that DMSP-utilizing methane-producing bacteria have an anoxic cytoplasm, thus providing a suitable environment for the reduction of the methyl groups in DMSP, an abundant methylated compound produced by marine algae as an osmolyte (Stefels 2000; Tang et al. 1999). Once it is released, it can be taken up as a sulfur source by several marine bacteria including the abundant *Synechococcus* and *Prochlorococcus* (Vila-Costa et al. 2006); however, it is unknown whether this leads to the release of methane.

Thus far, no mechanism has been convincingly demonstrated to fully explain methane production in oxic freshwater. Recently, phosphonate demethylation by various bacterial groups was suggested as a possible mechanism in freshwater systems as well (Yao et al. 2016; Wang et al. 2017), especially when one considers the fact that the phosphonate operon (*Phn*/*Pho* genes), first recognized in *Escherichia coli.* (Wackett et al. 1987; Metcalf and Wanner 1993), is common in the bacterial domain. Nevertheless, while phosphonates are known to be produced by numerous organisms (Yu et al. 2013), their ambient concentrations have not been measured in lakes with an oxic methane peak. Additionally, not all degradable phosphonates lead to methane production in oxic waters (Gomez-Garcia et al.

2011). Hence, although this is a plausible mechanism that contributes at times to oxic methane formation, its quantitative significance remains questionable.

In contrast to the marine and other saline environments, DMSP is nearly absent from freshwater systems (Caron and Kramer 1994), and Yoch (2001) showed that DMS formation from DMSP in river water occurs at a rate of 1% of that in marine systems. Nevertheless, Carrión et al. (2015) found evidence for the direct production of DMS by sediment bacteria that typically occur in freshwater environments. Demethylation of DMS yields methanethiol (Visscher and Taylor 1993); subsequent conversion of methanethiol to methane is energetically feasible (Damm et al. 2010) and has been demonstrated at least in anaerobic bacteria (Tallant and Krzycki 1997). Therefore, sequential degradation of DMS is another possible pathway for the formation of the oxic methane peak also in freshwater.

Some studies suggest that within oxic environment, there are "methanogenesis-safe" anoxic microenvironments such as on large (>500 μm) particulate organic matter aggregates (Ploug and Jorgensen 1999; Ploug 2001). Additionally, zooplankton's gastrointestinal tracts were identified as anoxic microenvironments suitable for methanogenesis (De Angelis and Lee 1994). Recently, Schmale et al. (2017) showed the methane enrichment in the oxic layer of the central Baltic Sea overlaps with the density maxima of mesozooplankton. However, they conclude that the methane production rates obtained in their incubations are too low to explain methane formation in the oxic layer exclusively by zooplankton.

Oxic methane peaks have been found to be closely associated with phytoplankton dynamics across multiple lakes (Tang et al. 2014). It was postulated in the 1970s (Bothe et al. 1978) and later experimentally shown by Berg et al. (2014) that hydrogen produced during nitrogen fixation can be consumed by methanogens, pointing to the possibility for a photosynthetically regulated pathway for methane production. Grossart et al. (2011) found potentially methanogenic *Archaea* associated with *Cyanobacteria*. Thus, given the low oxygen concentration around the heterocysts of filamentous nitrogen fixing *Cyanobacteria*, this may form a suitable microenvironment for methanogenesis. Furthermore, Angel et al. (2012) have shown that methanogenic *Archaea* are able to detoxify the "harmful" oxygen at least to a certain extent. Methanogenic *Archaea* may indeed contribute to oxic methane production in different environments (Aben et al. 2017), but the precise mechanisms and ecological relevance still remain to be studied. More interestingly, Keppler et al. (2006) and later Lenhart et al. (2012, 2016), respectively, reported active release of methane under oxic conditions by terrestrial vegetation, saprophytic fungi, and the abundant marine coccolithophore *Emiliania huxleyi*. Such studies showing "spontaneous" release of methane under oxic conditions point to new and diverse possibilities in solving the methane paradox.

Because methane is a highly potent greenhouse gas, it is important to understand the processes leading to its production in oxic lake waters and its eventual emission to the atmosphere and the overall contribution of freshwater systems to the global methane budget. This missing information is urgently needed particularly in light of global climate change and increasing anthropogenic disturbance to freshwater habitats worldwide.

2 Results and Discussion

2.1 Oxic Methane in Lake Stechlin

Methane in the oxic meta- and epilimnion of Lake Stechlin has been documented on several occasions (Grossart et al. 2011; Tang et al. 2014; McGinnis et al. 2015). Following the methane concentrations in the water column of the lake between July 2014 and November 2015, it is evident that the evolution of methane in the oxic layer is a dynamic process with multiple peaks that occur in the late spring and early summer (Fig. 1). The peaks are mostly localized within and above the thermocline reaching concentrations above 1 μmol L^{-1}, with methane being redistributed in the water column due to wind-induced mixing or late autumn cooling of the lake. Such high concentrations are at least ten times higher than those measured in oxic marine open water (Tang et al. 2016), making freshwater lakes an equally important methane source despite their overall lower surface area. Evidently, the methane peaks coincide with peaks in oxygen supersaturation, suggesting a link to the activity of oxygenic phototrophs such as *Cyanobacteria* and algae (Grossart et al. 2011; Tang et al. 2014).

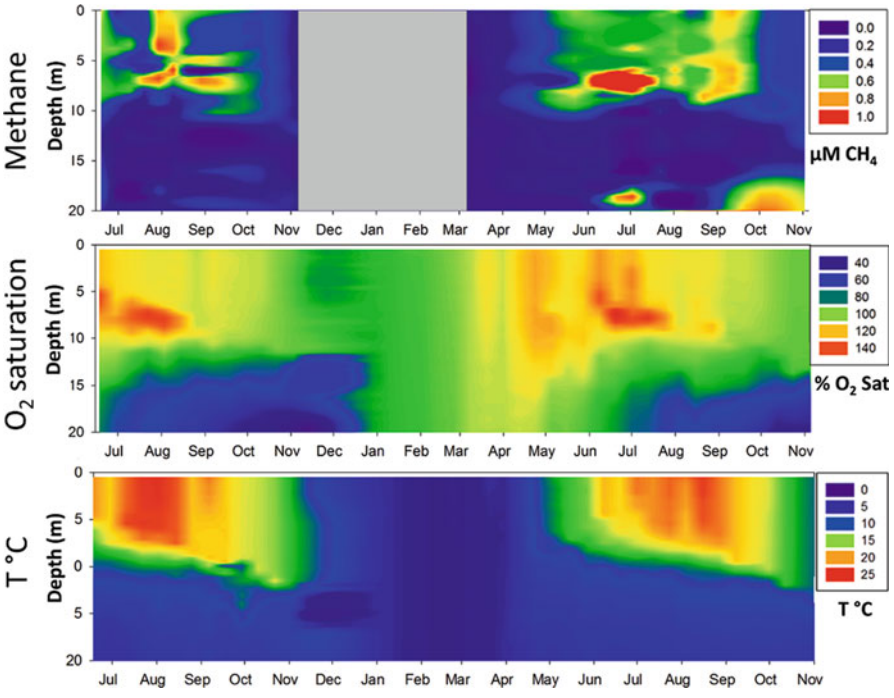

Fig. 1 Upper panel: Interpolated methane profiles collected every 2–4 weeks in Lake Stechlin between July 2014 and November 2015. Profiles were not available for the winter time. The water depth at the point of measurement was 20 m. Middle panel: interpolated oxygen profiles measured hourly by automated profilers situated at the point of methane measurements. Lower panel: interpolated temperature profiles measured hourly by automated profilers situated at the point of methane measurements

Elevated methane concentrations are also present near the sediment in the shallower southwestern bay of the lake, most likely resulting from classical *Archaea*-based methanogenesis. Nevertheless, it is evident from our long-term data that methane originating from the sediment is rapidly consumed far below the thermocline, most likely by methanotrophs. Thus, even in relatively shallow water bodies (in comparison to oceans) such as Lake Stechlin (max depth 69 m), the process of oxic methane production is disconnected from methane formation in the underlying sediment.

Several hypotheses have been brought forward regarding the source of oxic methane in lakes. These can be divided into biotic and abiotic with the latter favoring shore-based methane as the source (e.g., Fernández et al. 2016 and references therein). As lakes are smaller in size compared to oceans, it is suggested that methane produced in anoxic coastal sediments penetrates the oxic water column from the shore along density gradients, e.g., at the thermocline. This density-driven flow can occur due to groundwater inflow at the shoreline, water pumping by littoral plants, or differential cooling of shallow water in the littoral zone compared to the pelagic zone in lakes (Rudd and Hamilton 1978; Hofmann 2013; Bastviken et al. 2004; Murase et al. 2003; Fernández et al. 2016). To evaluate the possibility of transport from littoral areas, methane was measured in Lake Stechlin at different proximities to

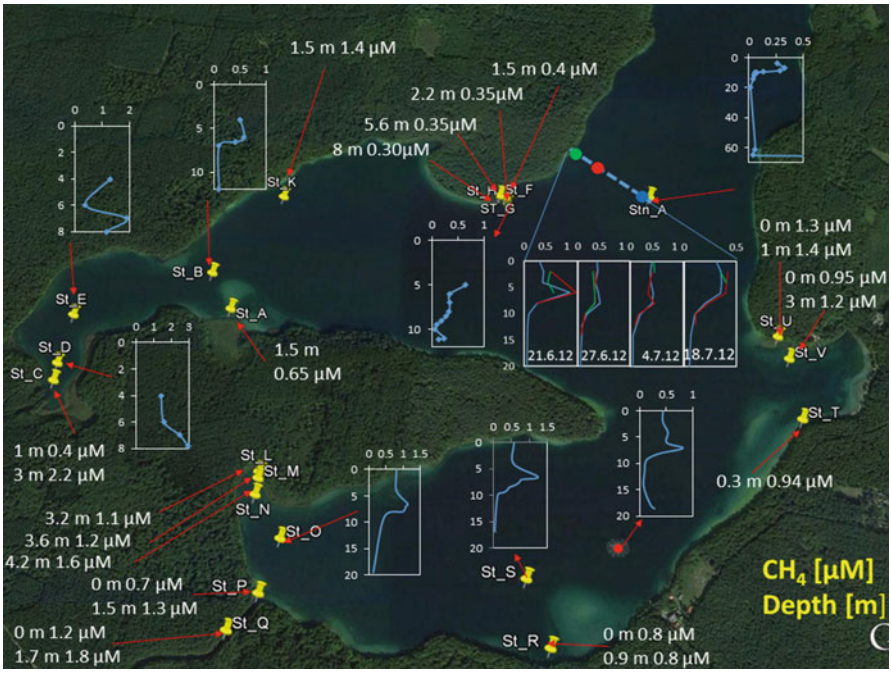

Fig. 2 Distribution of methane in littoral and pelagic areas of Lake Stechlin. The data were collected over 2 days in July 2015. The transect from the shore to station A was measured in June and July 2012. The background image was produced using Google Earth software

the shore (Fig. 2) including several transects. The near-bottom concentrations were higher than those measured at the pelagic peaks only at some locations (e.g., St-C, St-N), specifically in secluded and shallow bays. This negates the first condition for lateral transport as postulated by Fernández et al. (2016). Additionally, considering dilution during transport with methane-depleted lake water, as well as methane oxidation by methanotrophs, it is unlikely that transport from the shore is the sole contributor to pelagic methane in the oxic epilimnion of the lake. Data (as detailed below) from micro- and mesocosm experiments (Grossart et al. 2011; Bogard et al. 2014) have shown the production of methane under oxic conditions while being disconnected from potential external sources, e.g., the littoral zone. Nevertheless, lateral transport cannot be ruled out entirely, and its contribution is likely depending on local conditions (i.e., closed or open bays).

3 Estimates of Oxic Methane Production Rates

Between March and June 2015, the concentration of methane in the oxic layer of Lake Stechlin increased (Fig. 3a), suggesting that the production was higher than consumption and outgassing to the atmosphere. Assuming methane movement by diffusion only (i.e., no advective and/or convective flow), volumetric consumption and production rates were calculated from each profile, typically showing a net production above the thermocline and a net consumption in and below it (Fig. 3b). Overall, throughout March to August 2015, there was a net methane production in the lake (Fig. 3a). However, the intermittent decrease in methane concentration in June 2015 suggests that the methane dynamic of the lake was not entirely captured by our biweekly-to-monthly measurements.

Integrating the water column methane profiles taken between March and June (2015) (Fig. 3c) suggests that 5 mmol of methane were added to a 20 m^3 water column in 72 days. Accounting for an outgassing of 0.3 mmol m^{-2} day^{-1} as measured in a comparable period from March to June in 2016, 21.6 mmol of methane m^{-2} were lost to the atmosphere. Using the in situ and in vitro rates published in Grossart et al. (2011), we estimate the methane production rates in the upper 8 m of the water column to be 12–71 μmol m^{-3} day^{-1}. This results in an estimated production of 7–41 mmol methane in 0–8 m water depth for the entire sampling period. Methane oxidation measurements suggest that methane consumption was localized at depths between 8 and 12 m and ranged between 31 and 55 μmol m^{-3} day^{-1}. This is consistent with low methane oxidation due to photoinhibition and high oxygen concentrations (Murase and Sugimoto 2005) and low abundance of methane oxidizers' gene sequences in the Stechlin water (see Grossart et al. 2011 and below). These rates sum up to 9–16 mmol methane consumed during the entire 72-day period. To balance the budget, it would require a lateral transport of 1–35 mmol methane from the littoral to a 20 m^3 water column in the pelagic zone. The lack of any lateral concentration gradient between the littoral and the pelagic zones (Fig. 2) suggests that the littoral contribution is on the lower end of these estimates. Overall, the production and consumption rates are in the same range as calculated by

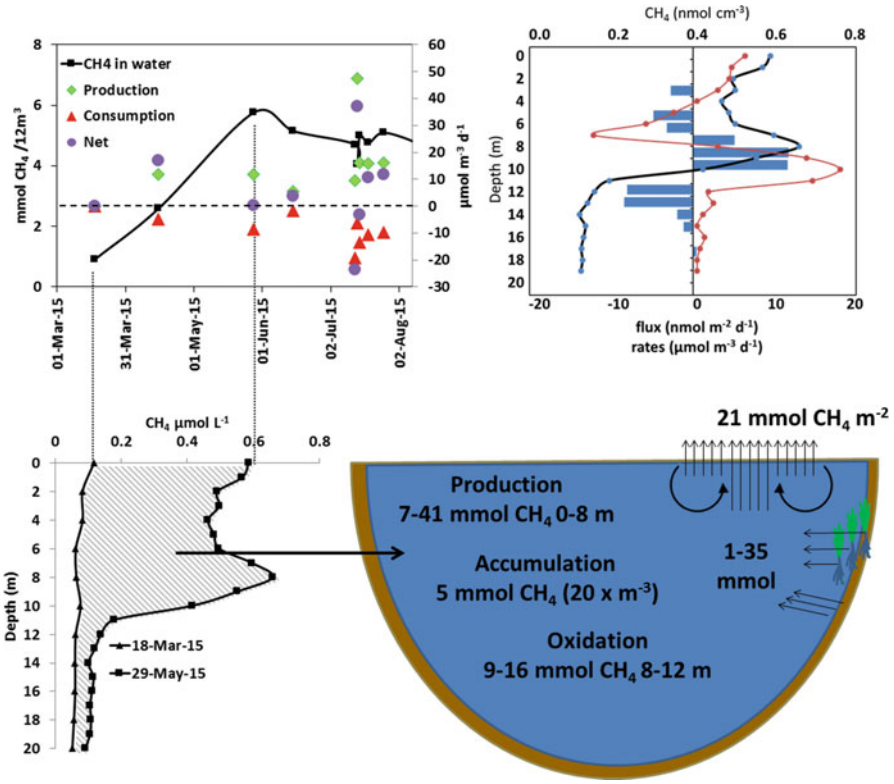

Fig. 3 (**a**) Methane accumulation in Lake Stechlin between March and August 2015. Production, consumption, and net rates were calculated based on diffusive profiles (as in panel **b**) and were summed up over a 20 m^3 water column. (**b**) Methane profile, diffusive flux, and volumetric rates of methane consumption and production. The fluxes and rates were calculated according to Fick's 1st and 2nd law assuming diffusive flux alone. (**c**) Methane accumulation in the water column based on the difference in profiles between the 18th of March and 29th of May 2015. (**d**) A hypothetical methane budget. Average fluxes to the atmosphere were directly measured via flux chambers from March to June 2016 and potentially include diffusive and advective (wind driven) methane fluxes to the atmosphere

diffusive flux alone from the methane profiles measured in this period (Fig. 3a, b). The discrepancies, however, suggest that other processes, such as convective mixing and wind turbulence, influence methane dynamics in the lake.

4 Exclusion of Classical Archaea Methanogens

The biological production of methane under oxic conditions can be explained either by the activity of aerobic organisms employing novel biochemical pathways or by anaerobic methanogens that are able to protect themselves against the toxic molecular oxygen. The latter can include methanogenesis in anoxic/hypoxic

microenvironments (Ploug and Jorgensen 1999; Ploug 2001; Schulz et al. 2001; Schmale et al. 2017) such as the inner parts of organic matter particles, gastrointestinal tracts of zooplankton, or around the heterocysts of N-fixing filamentous cyanobacteria. We tested for the presence of methanogens in the water column in and around the oxic methane peak: Microbial community composition was reconstructed from metagenomic data from June 2013 and August 2014 (six metagenomes) and metatranscriptomic data from August 2014 (two metatranscriptomes). In these datasets, no *Archaea* gene sequences were found. Fourteen additional metagenomes from Lake Stechlin, sampled as part of other studies, were searched as well. All in all, over 300,000,000 metagenomic reads were scanned for *Archaea* rRNA (16S or 23S) genes without any positive match. Occasional reads annotated as *Archaea* were identified but could not be related to any known methanogens. Long-term community monitoring of Lake Stechlin (2002–2012) analyzed as part of the Earth Microbiome Project (Thompson et al. 2017) revealed that *Archaea* in the lake water appear periodically every few years in low abundance and consist mostly of the members of the order *Thermoplasmatales*. Methanogenic archaea follow these trends but not exceeding 0.2% of the reads per sample. Such low abundance suggests that these archaea are insignificant to the oxic methane peak and deems these organisms undetectable in our metagenomic libraries if present at the time.

5 Methane-Producing Organisms Other Than Methanogenic *Archaea*

Microbial communities from 2013 and 2014 did not differ drastically from each other (Fig. 4). There was a remarkable presence of *Methylacidiphilum*, a known methane oxidizer from hyperthermal environments (Bodrossy et al. 1999; Sharp et al. 2012). Other members of the *Verrucomicrobia* phylum also contributed to a significant part of the microbial community in both years. This phylum is known to contain methane-oxidizing bacteria as well. Nevertheless, the methane monooxygenase gene (e.c.:1.1.13.25) was not found in any of the metagenomes or metatranscriptomes. Sequences affiliated to methanotrophs other than *Methylacidiphilum* were found only in the metatranscriptomes, which were affiliated with *Alpha-*, *Beta-*, and *Gammaproteobacteria* – mostly with *Methylophilaceae* (*Betaproteobacteria*), but no taxa made up more than 1% of the reads.

Nitrogen-fixing cyanobacteria have been suggested to play a role in oxic methane production. This is mainly due to formation of favorable microenvironments around heterocysts (Lupton and Marshall 1981) and by supplying H^+ produced by the nitrogenase enzyme to methanogens (Berg et al. 2014). Interestingly, while the cyanobacterial community in 2013 was dominated by N-fixing filamentous strains, in 2014 the community was dominated by unicellular *Cyanobacteria*. The latter do not form heterocysts and are unlikely to form a hypoxic microenvironment around the cells. Therefore, given the alternating composition of the cyanobacterial community and the lack of methanogen sequences in the datasets, it is unlikely that

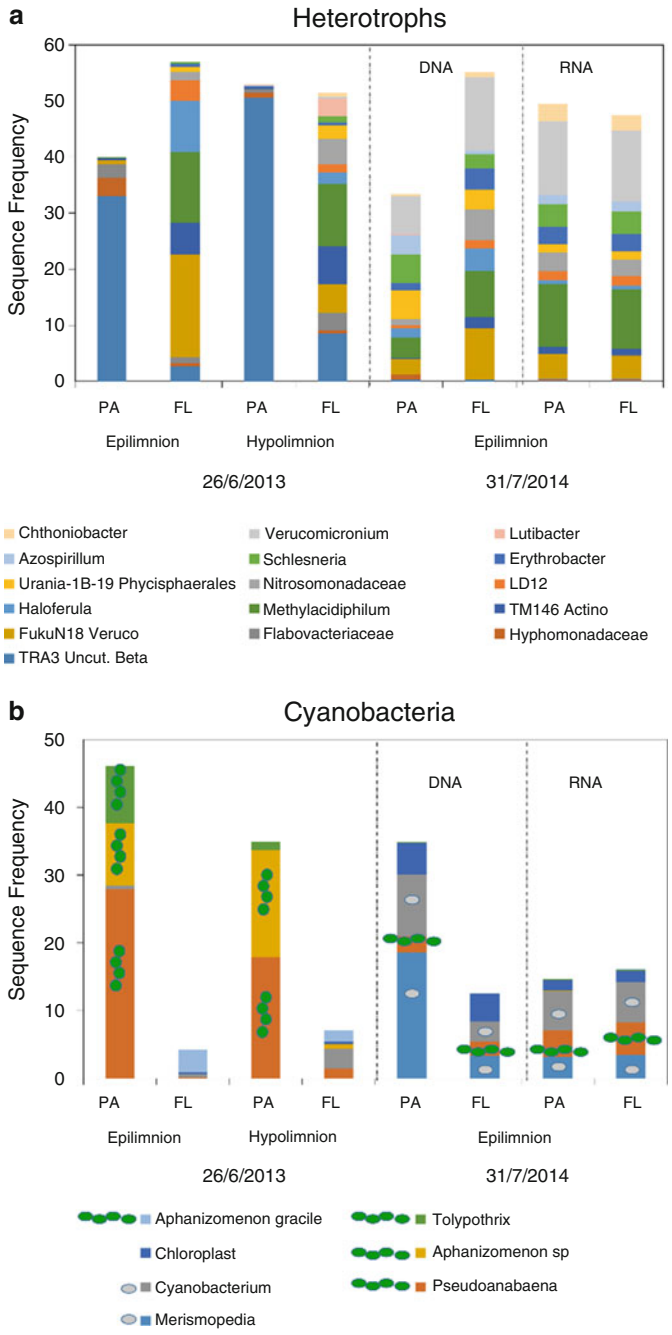

Fig. 4 Bacterial community associated with the oxic methane peak from 2 consecutive years separated into non-photoautotrophs (named heterotrophs; **a**) and photoautotrophs (i.e., *Cyanobacteria*; **b**). The cyanobacterial cell type (filamentous or unicellular) is depicted on the graph

Cyanobacteria host methanogens or that the latter have a significant role in the formation of the oxic methane peak in Lake Stechlin.

Despite the lack of *archaeal* sequences in the metagenomic and metatranscriptomic datasets, we specifically searched for genes involved in all methanogenesis pathways using methylamines, acetate, methanol, and H_2/CO_2 as substrates (Fig. 5). All these pathways involve the reduction of methylcoenzyme-M and the release of methane by reductases. This set of genes, known to be present in all methanogens, was entirely absent from the metagenomic and metatranscriptomic datasets of Lake Stechlin. Additional key enzymes were missing from the different pathways, and the only enzymes present were those that are not unique to

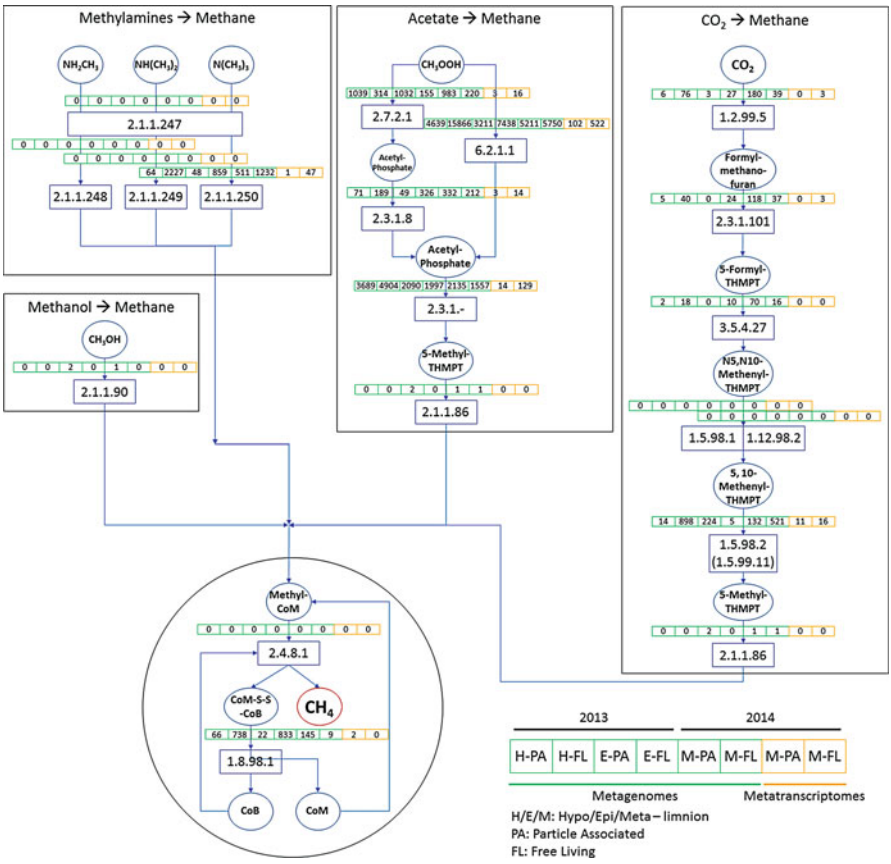

Fig. 5 Read abundance of genes involved in classical methanogenesis in the different metagenomic and metatranscriptomic datasets of Lake Stechlin. The counts are placed on methanogenesis KEGG pathway maps. Values bordered in green and orange represent read counts from metagenomes and metatranscriptomes, respectively. Metagenomic samples in 2013 were collected from the hypolimnion and epilimnion of Lake Stechlin and fraction filtered for **p**article-**a**ssociated and **f**ree-**l**iving bacteria (H-PA/H-FL and E-PA/E-FL). Samples in 2014 were similarly fraction filtered and collected in the metalimnion (at the thermocline) (M-PA/M-FL)

methanogenesis and are involved in multiple pathways. Methylene-THMPT reductase (ec:1.5.98.2 formerly ec:1.5.99.11) is coenzyme-F420 dependent and is usually associated with methanogenesis. Nevertheless, numerous coenzyme F420-dependent enzymes have been found to be abundant in *Actinobacteria* (Selengut and Haft 2010) and other gram-positive bacteria. The methylene-THMPT reductase found abundantly in the Lake Stechlin metagenomic and metatranscriptomic datasets was almost exclusively annotated as of actinobacterial origin. This enzyme is associated with functions other than methanogenesis (Selengut and Haft 2010); hence, its presence in the *Actinobacteria* from the lake does not imply that these organisms contribute directly to the oxic methane peak.

Interestingly, trimethylamine methyltransferase (ec:2.1.1.250), a gene involved in the demethylation of trimethylamine, is highly abundant in the Lake Stechlin datasets. Tang et al. (2016) have shown that mono-, di-, and trimethylamine methyltransferases were present in the genomes of non-methanogenic organisms. The downstream enzyme, coenzyme-M methyltransferase, was however absent, suggesting that no methane was produced via the classical TMA-based methanogenesis pathway. Nevertheless, as discussed below, incubation experiments with TMA led to the formation of methane, suggesting the presence of an alternative non-archaeal pathway.

6 Potential Precursors for Oxic Methane Production

Cyanobacteria have been shown to produce methane in the process of demethylation of methylphosphonates (MPN) while scavenging for phosphorus.

This process does not require anaerobic conditions, and no methanogenesis specific enzymes are needed in addition to the genes of the *phosphonate* (*Phn*) operon (Gomez-Garcia et al. 2011). Lake Stechlin is considered P limited (Allgaier and Grossart 2006); hence, it is likely that cyanobacteria as well as other lake organisms will try to find alternative P sources.

The *Phn* operon is found in many bacteria and has been initially identified in *Escherichia coli*. Nevertheless, the ability of these organisms to emit methane has never been evaluated. In the marine environment, *Thaumarchaeota* are considered as MPN producers (Metcalf et al. 2012) though recent studies have questioned their ability to quantitatively be responsible for the marine oxic methane peak (Repeta et al. 2016). In freshwater, *Actinobacteria* may be a potent MPN source. This phylum is known to produce phosphonates both for P storage and an infochemical (Francis and Martodam 1983; Ju et al. 2015). Additionally, *Actinobacteria* have been shown to have efficient exophosphatases and thus are able to take up P (Srivastava et al. 2015) even in P-limited Lake Stechlin. Since the abundance of *Actinobacteria* may reach up to 60% of the total free-living microbial community (Bižić-Ionescu et al. 2014), they may be quantitatively important phosphonate producers for MPN-based oxic methane production. A remaining question is how do methane producers acquire MPN from *Actinobacteria*, especially under P-limiting condition? We suggest two possibilities: (1) Methane producers and *Actinobacteria* form some form of co-dependence such that methane producers acquire MPN from *Actinobacteria* in

exchange for other essential substrates required by the latter such as vitamins (Garcia et al. 2015); (2) Methane producers can induce *Actinobacteria* to release MPN similarly to what has been shown for the cyanobacterium *Aphanizomenon ovalisporum*, which uses the cylindrospermopsin toxin to induce phosphatase activity in other bacteria (Bar-Yosef et al. 2010).

A second well-known precursor to methane is trimethylamine (TMA) which is a common product of organic matter decomposition. In contrast to MPN, so far, there is no evidence that TMA serves as a precursor to methane in oxic environments; however, genes involved in TMA-based methanogenesis were highly abundant in the metagenomic and metatranscriptomic data from the oxic methane peak in Lake Stechlin (Fig. 5).

Accordingly, we tested the ability of a *Cyanobacteria* culture as well as a natural lake community resuspended in controlled growth medium (BG11) to produce methane using MPN or TMA as precursors and incubated under different light intensities and nutrient concentrations (Fig. 6). Surprisingly, a slight increase in methane concentration was observed in all nutrient-depleted treatments (i.e., -P and -N). Addition of TMA and MPN to both N- and P-depleted treatments resulted in increased production of methane in the nature lake community under high-light condition. The combination of MPN and high light also resulted in increased methane production in the *Cyanobacteria* enrichment culture. No difference, however, was observed between the TMA-treated *Cyanobacteria* enrichment culture and the non-treated N-depleted treatment, both showing a minor increase in methane, more pronounced under high light as well. The final dissolved oxygen concentration was above 80% saturation in all incubation vessels, indicating well-oxygenated conditions in all treatments.

Since methane production was particularly pronounced under high-light condition, these experiments were chosen for microbial community analysis. As a control for the presence of classical methanogens in the experiments, primers targeting the 16S rRNA gene of both *Archaea* and *Bacteria* were used. No *Archaea* sequences were found. The community composition and the relative abundance of bacteria (sequence frequency) varied among the experiments. Interestingly, despite the light availability, the cyanobacterium *Anabaena* (*Dolichospermum*) in the enrichment culture was outcompeted by other organisms as evidenced by its near absence from the sequence data, except when N was available and P was supplied as MPN. Both the TMA and MPN lake community experiments were dominated by unicellular *Cyanobacteria* (Fig. 7) as was also evidenced by the green color of the water.

The ability of cyanobacteria to demethylate MPN to obtain P and release methane as a byproduct has been shown previously (Gomez-Garcia et al. 2011); nevertheless it is unclear whether the cyanobacteria directly used TMA as an N source or the latter was first oxidized by heterotrophic bacteria, releasing part of the nitrogen to the water providing the cyanobacteria with both carbon and nitrogen source. In the absence of axenic cultures, these experiments do not prove the ability of cyanobacteria to produce methane from TMA; however, there seems to be a link between *Cyanobacteria* and methane production in the oxic layer of lakes as has been hypothesized earlier (e.g., Tang et al. 2016). It seems evident that there are multiple potential pathways to produce methane in oxic waters with MPN and TMA

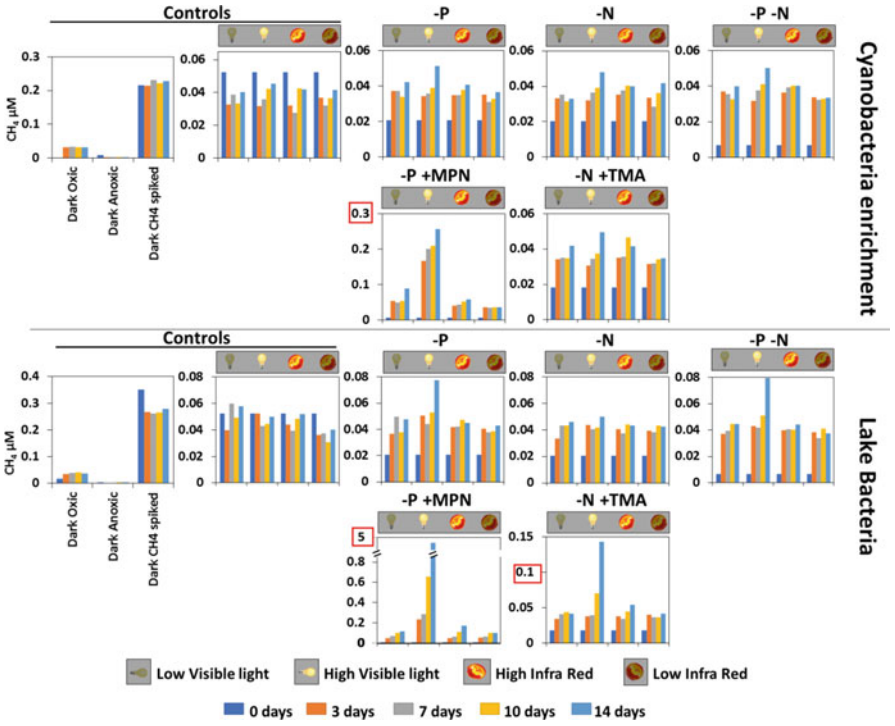

Fig. 6 Changes in methane concentrations over 2 weeks in incubation experiments conducted with a cyanobacterial enrichment culture and natural lake water community. Both experiments were conducted in BG11 medium with or without P and N sources as specified in the panels above. Lake bacteria were concentrated using tangential filtration and resuspended in fresh media to produce the original cell concentration. Low visible and infrared light intensities were ~10 µmol quanta m^{-2} s^{-1}. High visible and infrared lights were ~100 µmol quanta m^{-2} s^{-1}. Methylphosphonate (MPN) and trimethylamine (TMA) were added each at a final concentration of 5 µM

being just two of several possible precursors. Both phosphonate uptake and degradation genes were found in Lake Stechlin metagenomes and metatranscriptomes albeit in low numbers. This suggests that while the mechanism was present in the lake during the time of sampling, it likely was not the most significant one for methane production.

7 Research Needs

The "methane paradox" exists because active methane production in oxic waters is traditionally considered impossible. However, by now it is evident that *in situ* production of methane in oxic waters is a common phenomenon fueled by several potential parallel pathways, some of which are still not well understood. When

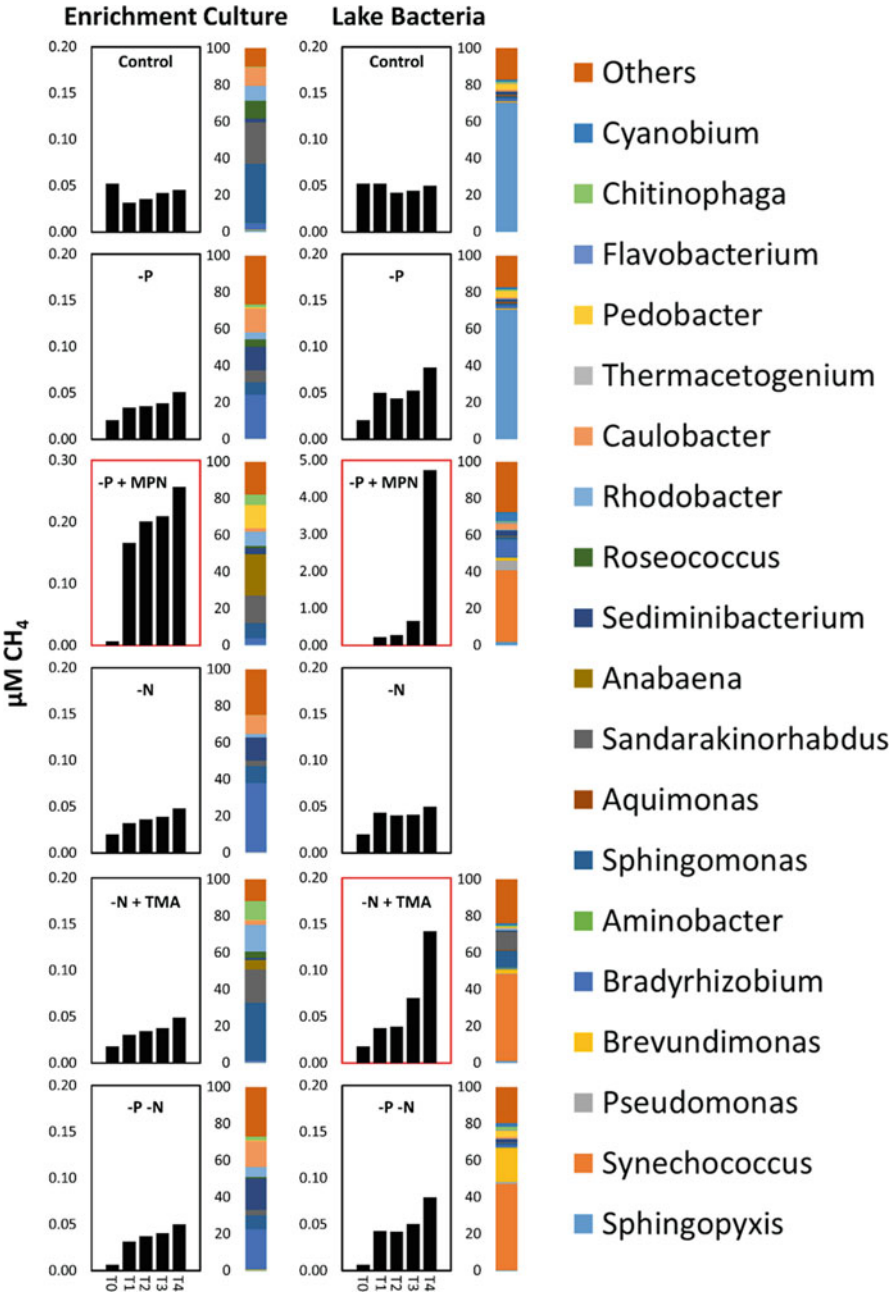

Fig. 7 Microbial community composition of the high-light (100 µmol quanta m^{-2} s^{-1}) incubation experiments presented in Fig. 6

considering Lake Stechlin as a model system, it seems that the contribution of methane from the littoral zone is low, and most of the methane in the pelagic zone and methane emission from the lake are related to methane production in fully oxic waters. Methanogenic *Archaea*, previously regarded as primary candidates for methanogenesis, do not substantially contribute to the localized production of methane in the oxic pelagial of the lake. Hence, it becomes clear that additional, perhaps (partly) novel, biochemical pathways are involved.

A well-established oxic methanogenesis pathway is the demethylation of methylphosphonates by *Cyanobacteria* and other organisms (Fig. 8). Nevertheless, the abundance and source of methylphosphonates in freshwater need to be further evaluated. In marine environments, *Thaumarchaeota* have been shown to produce MPNs, but their contribution to the total oceanic oxic methane production has not been quantified. In freshwater, possible producers of MPNs have not been evaluated to the best of our knowledge. Yu et al. (2013) conducting a genomic survey on available genomic and metagenomics data concluded that the pathway for phosphonate production is encoded in 5.7% of the bacterial genomes available at the time. Nevertheless, as previously demonstrated (Gomez-Garcia et al. 2011; Repeta et al. 2016), degradation of phosphonates does not always result in methane formation. The *Actinobacteria*, a highly abundant phylum in the free-living fraction of many temperate lakes, represent good candidates. These bacteria are known to produce phosphonates, and they also constitute up to 60% of the free-living bacteria

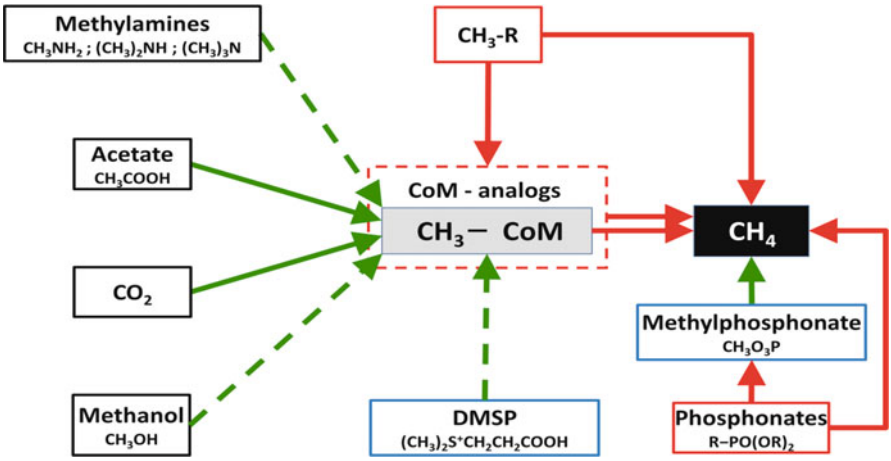

Fig. 8 Classical, known oxic, and hypothetical methanogenesis pathways represented by substrates. Full or dashed green arrows mark **sections** of known methanogenesis pathways for which all or some of the genes were found in genomes of *Bacteria* (i.e., non-*Archaea*), respectively. Compounds marked in blue have been shown as substrates in oxic methanogenesis. Compounds first published here as substrates for oxic methane production are marked with an asterisk (*). Methyl-coenzyme-M (gray box) is entirely absent from genomes of *Bacteria*; however, analogs have been suggested (dashed red line). Substrates and pathways marked in red are hypothetical. (The figure was modified from Tang et al. (2016))

in Lake Stechlin. Additionally, the bacterial sequence most similar to the archaeal methylphosphonate synthase is of actinobacterial origin.

A second link that needs to be explored is the role of phototrophs in oxic methane production. Is the reported co-occurrence of cyanobacterial blooms together with oxic methane peaks a coincidence or are cyanobacteria responsible for the oxic methane production? If so, does methane formation occur in the absence of MPNs as suggested by our experimental TMA incubation data presented in Fig. 6?

Lastly, a survey of all published genomes of *Bacteria* (i.e., not *Archaea*) shows that most of the genes required for classical methanogenesis are present also in *Bacteria* (Fig. 8). Missing links are the presence of coenzyme-M methyltransferase and subsequently the methyl-coenzyme-M reductase (MCR). Accordingly, this prompts us to hypothesize a possible alternative pathway carried out by *Bacteria* (Fig. 8). This would consist of a demethylation pathway, a known or novel C1-carrier (e.g., tetrahydrofolate or tetrahydromethanofuran), a methyl transferase, a methyl-coenzyme-M equivalent (e.g., mercaptopropionate), and a methyl reductase which most likely can directly act on the C1 carrier.

In conclusion, despite recent studies proposing that oxic methane production is the result of organic phosphonate degradation (Yu et al. 2013; Repeta et al. 2016; Wang et al. 2017), our data suggest that more than one mechanism is involved. Whether they occur in parallel or sequentially still has to be determined. Furthermore, most genes necessary for methanogenesis are present in the genomes of *Bacteria*. Alongside with the production of methane from TMA by concentrated lake *Bacteria*, this implicates the involvement of genes different from those of traditional pathways as well as other C1-carrying molecules. The presence of alternative pathways has been suggested for *Cyanobacteria* not containing the Phn genes but which are able to degrade phosphonates (Gomez-Garcia et al. 2011). Thus, the different triggers for oxic methane production, the organisms involved, and the employed biochemical pathways are far from being resolved.

8 Methods

8.1 Environmental Parameters

Oxygen measurements were obtained from ongoing hourly measurements at the Lake Lab facility (www.lake-lab.de) in Lake Stechlin.

8.2 Methane Measurements

Methane was measured using the headspace method (McAullife 1971; Magen et al. 2014). Shortly, water was collected in pre-weighed 60 ml serum bottles and sealed with gas tight rubber stoppers without trapping any gas bubbles. The bottles were placed in ice to inhibit further activity till processed in the lab within 1–2 h of sampling. Subsequently, the full bottles were weighed, a portion of the headspace

was replaced with He, the flushed bottles were weighed again, and the bottle was vigorously shaken for 30 s. A volume of 500 µl was collected from the headspace, and methane concentration was measured using a Shimtazu 1A gas chromatograph.

8.3 DNA and RNA Extraction

On 26 June 2013 and 31 August 2014, water samples from Lake Stechlin (2 L) were fractionated by filters into 5 µm and 0.22 µm fractions, representing particle-associated and free-living microbes, respectively. In 2013, the water was collected from above and below the thermocline for DNA extraction. In 2014, samples were collected within the thermocline for both DNA and RNA extraction. DNA was extracted using the phenol/chloroform method (Nercessian et al. 2005). For RNA extractions (two samples from 2014), acidic phenol (pH 4.0) was used. Following a DNAse treatment (Turbo DNA free, Ambion) the RNA samples were enriched for mRNA following Stewart et al. (2010). Single strand cDNA was synthesized using High Capacity cDNA reverse transcription kit (Applied Biosystems) according to the manufacturer's instructions.

DNA and cDNA samples were sent for Illumina HiSeq 2X150 bp sequencing at Mr. DNA laboratories (Shallowater, Texas, USA).

8.4 Bioinformatic Analysis

Metagenomic and metatranscriptomic raw reads were quality trimmed using Nessoni (Victoria bioinformatics). DNA and RNA sequences were uploaded to MG-RAST (Wilke et al. 2016) for analysis. Additionally, DNA sequences were assembled using SPADES assembler (V. 3.6) (Bankevich et al. 2012). RNA sequences were assembled using Trinity (Grabherr et al. 2011) and annotated following the Trinotate (https://trinotate.github.io/) pipeline.

8.5 Incubation Experiment

For incubation experiments, a *Cyanobacteria* enrichment culture from Lake Stechlin and lake bacteria were used. The cyanobacterial culture was filtered on a 5 µm pore size polycarbonate filter prior to the experiment and resuspended in freshly made BG11 medium (Rippka et al. 1979) depleted of P and N sources. To obtain lake bacteria in natural concentrations but in controlled growth medium, 2 L of lake water were concentrated using a tangential filtration system into 10 ml of water which were then added to 2 L of fresh BG11 medium depleted of N and P. The latter were added to both sets of experiments as described in Fig. 6 to create full medium N-, P-, and N+P-depleted conditions. Anoxic controls to test for methanogenic *Archaea* in the experiment were prepared by flushing the medium for 30 min with N_2 gas. Tests for methane oxidation were conducted with full media and were spiked with 100 µl 1%

methane standard gas (Supelco, Bellefonte PA, USA). All cultures contained 65 ml of medium in 100 ml bottles and were weighed empty and full and during the experiment to obtain an accurate measure of the headspace. Methane samples were collected every 3–4 days for 2 weeks by sampling 1 ml of gas from the headspace and replacing it with 1 ml of air to avoid sub-pressure in the bottle. The incubation bottles were placed in an 18 °C temperature-controlled room and were illuminated as depicted in Fig. 6. Visible light was obtained from two neon bulbs directly applied or channeled through a narrow slit to obtain high and low light, respectively. High and low lights were measured at ~125 and ~10 μmol photons m^{-2} s^{-1}, respectively. Infrared light was obtained using an IR bulb and was placed similarly to the visible light to obtain high and low intensities of ~50 and ~5 μmol photons m^{-2} s^{-1}. The open space incubation was supplemented with ventilation to prevent heating from the high infrared light. Illumination was supplied in a day-night cycle of 14 h light and 10 h dark. At the end of the experiment following the dark period, O_2 was measured using a micro-optode inserted through a needle into the bottles.

Community analysis was conducted for the high-light treatments only as those that produced methane. Water samples were filtered on a 0.22 μm pore size filter at the end of the experiment. DNA was extracted as described above, and the samples were sent for 2 × 250 bp paired-end sequencing on an Illumina MiSeq using the general *Bacteria* and *Archaea* primers 515F and 806R (Caporaso et al. 2011). Microbial community composition was analyzed using the SILVA NGS pipeline (Ionescu et al. 2012).

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Contribution of Methane Formation and Methane Oxidation to Methane Emission from Freshwater Systems

16

Carsten J. Schubert and Bernhard Wehrli

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Abstract

Lakes and reservoirs have been only in the early twenty-first century identified to be main methane emitters to the atmosphere (Bastviken et al., Glob Biogeochem Cycles 18:1–12, 2004; St. Louis et al., Bioscience 50:766–775, 2000). With an estimated yearly amount of 12–29.6 Tg CH_4 for reservoirs (Deemer et al., Bioscience 66:949–964, 2016) and up to 71.6 Tg CH_4 for lakes (Bastviken et al., Science 331:50–50, 2011), they represent up to 10% of total methane emissions and hence have to be taken into account in global budgets. Freshwater systems are emitting more methane than oceans although only covering about 3% of the earth surface since methanogenesis, the building process of methane, is the main organic matter degradation step compared to oceans where sulfate reduction is dominant. Reservoirs in comparison to lakes have two additional methane release mechanisms, which are loss from methane-rich hypolimnion waters at the turbine and then degassing in the river to which the turbined water has been released. A still poorly constrained mechanism occurring in both systems is ebullition, the transfer of methane bubbles directly through the water column

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towards the atmosphere. Whereas in the oceans, mainly archaea often in a consortium with bacteria oxidize the methane in the sediments or water column, in freshwater systems the oxidation process seems to be much more versatile in respect to electron acceptors (oxygen, nitrate, iron, and manganese) as well as to the microorganisms involved. We believe that in the future there will be more discoveries and surprises when investigating freshwater methane oxidation.

1 Introduction

Methane (CH_4) is the smallest hydrocarbon molecule and the most abundant organic compound in the atmosphere. It is a potent greenhouse gas due to its ability to absorb in the infrared, at bandwidths that are not occupied by carbon dioxide. Taking into account its ~10 year residence time in the atmosphere, its concentration, and its specific absorbance wavelength, methane is about 28 times more potent as a greenhouse gas than CO_2 on a mass and 100 year basis (34 times if climate carbon feedbacks are included) (Myhre et al. 2013).

In general, methane sources can be divided into abiotic (20%) and biogenic sources (80%). Coal mining, industrial waste treatment, and combustion processes (e.g., aircraft and automobile exhausts), as well as volcanic emissions, biomass burning during deforestation or for heating purposes, belong to abiotic sources (Yusuf et al. 2012). However, most methane is produced via biological processes in freshwater wetlands, and rice paddies, but also the guts of ruminants and colonies of termites are important contributors (Kirschke et al. 2013).

The total global methane budget is divided into emissions from natural (347 Tg yr^{-1}) and anthropogenic sources (331 Tg yr^{-1}), and we give here the data from bottom-up estimates of emissions in the decade 2000–2009 (Kirschke et al. 2013) (Fig. 1). Aquatic systems including ocean and freshwater systems have become a focus for methane emissions only over the last decades (Bastviken et al. 2004; St. Louis et al. 2000). Although the oceans cover over 70% of the earth surface, the emissions are rather small compared to the huge area they cover. This is mainly because methane is already oxidized to carbon dioxide in the sediments (Boetius et al. 2000) or later in the water column before reaching the water surface where emissions occur (Schubert et al. 2006). The oxidation is mainly performed by a consortium of methane-oxidizing archaea with sulfate-reducing bacteria (Boetius et al. 2000); however, also single archaea and bacteria have been described and recently several new pathways have been proposed (Knittel and Boetius 2009; Milucka et al. 2012; Wegener et al. 2015). Compared to those rather minor emissions from oceans, freshwater systems including rivers, natural wetlands, lakes, and reservoirs have been identified as large contributors to the global methane emissions (Bastviken et al. 2004; Deemer et al. 2016). Here, in contrast to marine systems where methane is mainly oxidized by anaerobic processes mediated by archaea, methane in freshwater systems is mainly oxidized by aerobic methanotrophic bacteria (Milucka et al. 2015; Oswald et al. 2016b).

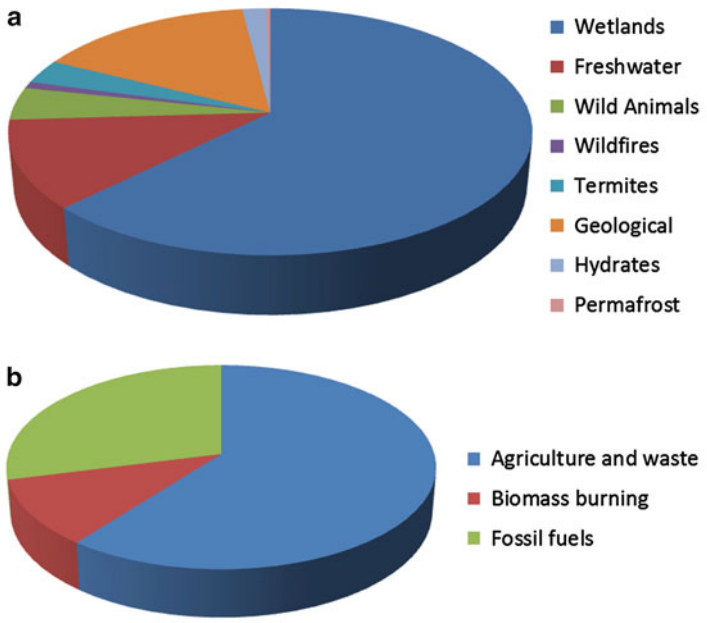

Fig. 1 Net emissions of methane stemming from natural (**a**) and anthropogenic sources (**b**). (Numbers from Kirschke et al. 2013)

The chapter first discusses very briefly the process of methanogenesis, then we assess the current knowledge of methane emissions from different freshwater systems such as wetlands, lakes, reservoirs, and rivers. We continue with a rather detailed overview of methane oxidation processes in inland waters. Finally, we end with a brief evaluation of knowledge gaps and recommendations for further research.

1.1 Methanogenesis in Aquatic Systems

The global budget of atmospheric CH_4, which is on the order of 700 Tg CH_4 per year, is mainly the result of environmental microbial processes, such as archaeal methanogenesis in wetlands, rice fields, ruminant, and termite digestive systems and of microbial methane oxidation under anoxic and oxic conditions (Conrad 2009). In all above-mentioned environments, methane is produced as the end product of organic matter degradation under anaerobic, i.e., oxygen free, conditions. The methanogenic degradation of organic matter is accomplished by a complex microbial community (Conrad 1989; Conrad and Frenzel 2002).

Until recently, the main agreement was that all methane that is found in surface waters whether it would be in the marine or freshwater realm stems from the underlying sediments. This was explained with the fact that methane produced by

methanogenesis will only be produced under anoxic conditions (Boone et al. 1993), since methanogens are obligate anaerobes (Borrego et al. 1999; Chistoserdova et al. 1998) and will not survive and produce methane under aerobic conditions. This dogma was recently challenged in marine as well as in freshwater systems (Grossart et al. 2011; Karl et al. 2008). Several authors have pointed out that methane concentrations in near-surface waters throughout much of the global ocean are supersaturated with respect to the atmosphere, implying local methanogenesis and a net flux from the ocean to the atmosphere (Lamontagne et al. 1973; Scranton and Farrington 1977; Tilbrook and Karl 1995).

However, since those waters are normally saturated or even supersaturated with oxygen with respect to the atmosphere, this phenomenon is also called the "oceanic methane paradox" (Karl et al. 2008). Karl et al. (2008) explained the existence of methane in the surface as a by-product during methylphosphonate decomposition in phosphate-stressed waters and methane production may be enhanced by the activity of nitrogen-fixing microorganisms.

In lakes, in contrast, neither the addition of inorganic phosphate nor methylated compounds affected methane production and so pelagic methanogenesis seemed not to depend on phosphate or methylated substrates like in the marine realm (Grossart et al. 2011). In Lake Stechlin, a co-occurrence of high numbers of cyanobacteria, algae, and attached Archaea within the epilimnion may enable a direct transfer of H_2 or acetate from the autotrophs to the methanogenic Archaea to support methane production in oxygenated water (Grossart et al. 2011). Rather acetoclastic than hydrogenic methanogenesis linked to in situ, algal DOC production potentially plays a central role in supporting water column CH_4 production in oxic freshwaters (Bogard et al. 2014). Nevertheless, other investigations show that it is sufficient to have methane transported from the littoral zone (where methane is mainly produced in the sediments and not in the water column) to explain methane concentrations in the surface water of the pelagic zone (Encinas Fernández et al. 2016).

1.2 Freshwater Emissions

1.2.1 Lakes and Wetlands

Wetlands are globally the largest emitters of methane from natural sources and are estimated to vary between 92 and 232 Tg CH_4 yr^{-1} (Wuebbles and Hayhoe 2002). More recent global modeling studies centered around emission values of 180–190 Tg CH_4 yr^{-1} but varied 40% about this mean (Bridgham et al. 2013). They occur in different climate zones but are mainly found in tropical, subtropical, northern high latitudes and arctic regions. A recent analysis confirmed general controls on wetland methane emissions from soil temperature, water table, and vegetation, but also show that these relationships are modified depending on wetland type (bog, fen, or swamp) and region (subarctic to temperate) (Turetsky et al. 2014). Mean methane fluxes between ecosystem types (bogs, rich fens, and poor fens) are relatively similar 93–96 mg m^2 d^{-1}, whereas swamps only emit less than half the amount 41 mg m^2 d^{-1} (Turetsky et al. 2014). By region, subarctic and temporal

regions emit more methane with 112 and 109 mg m^2 d^{-1}, respectively, whereas boreal and subtropical regions emit less methane (73 and 48 mg m^2 d^{-1}, respectively) (Turetsky et al. 2014). More than half of global wetland emissions are coming from tropical areas (Bloom et al. 2010).

Global CH_4 emissions from natural lakes have been estimated by Bastviken et al. (2004). Organic matter in lakes kept at natural state or with only minor influence by humans undergoes aerobic degradation and hence will be emitted mainly as CO_2. However, when eutrophication by intensive agriculture or sewage effluents leads to high organic matter production, lakes might turn anoxic and will now, due to anaerobic degradation, release methane formed by methanogenesis. In these systems, three different pathways for methane emission are described: (1) ebullition, i.e., emission through bubbles, (2) diffusive flux, which mainly depends on surface methane concentrations and wind speed over surface waters, and (3) storage, i.e., methane released during lake turnover (Fig. 2). On average, these three pathways, respectively, contribute 62%, 31%, and 7%, to total emissions. Bastviken et al. (2004) also stated that methane emission per lake is mainly related to lake area, which implies relatively constant flux per unit area. They further stated that key variables for predicting areal emission include concentrations of total phosphorus, dissolved organic matter, and methane, as well as the anoxic volume fraction determining storage and water depth being important for ebullition.

One difficulty in making predictions of emissions of greenhouse gases (GHG) from lakes is to estimate the global surface area of lakes. Over the last years several estimates have been made using various approaches (Lehner and Döll 2004) first set out to estimate global lake size distribution via maps and GIS analysis and estimated 2.4 million km^2 of surface waters >1 km^2. Downing et al. (2006) applied a Pareto distribution to the results of Lehner and Döll (2004) under the assumption of a canonical distribution in lake size to estimate the smallest lakes (< 1 km^2) and found

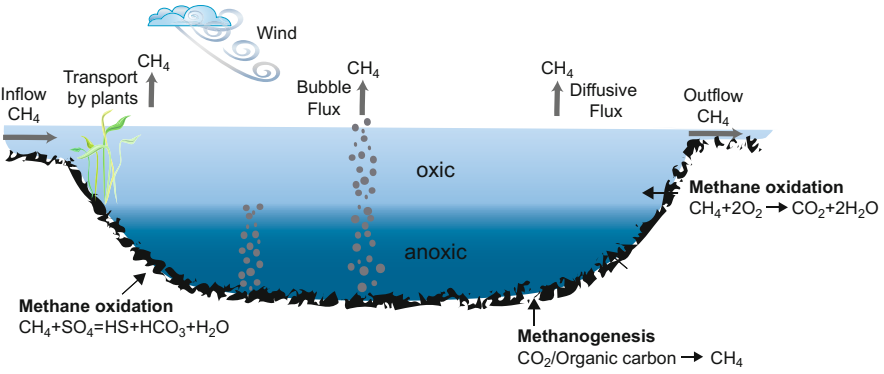

Fig. 2 Methane production, oxidation, and emission pathways in a natural lake (after Bastviken et al. 2004). Methane is produced via methanogenesis in the sediments or in the water column. Subsequently, it is then anaerobically (with sulfate, nitrate, iron, or manganese oxides) or aerobically (with oxygen) oxidized. Emissions take place via water surface diffusion, bubbling formation (ebullition), direct plant transport in the littoral zone, or via outflow by rivers

~304 million lakes that occupy 4.2 million km^2 of the continental terrestrial area, dominated by millions of water bodies smaller than 1 km^2. Downing et al. (2006) found an additional 0.4 million km^2 of land surface covered by impoundments and farm ponds and thus estimated that >3% of the continents were covered by water. The newest estimate comes from high-resolution satellite imagery that produced a GLObal WAter BOdies database (GLOWABO), comprising all lakes greater than 0.002 km^2 (Verpoorter et al. 2014). GLOWABO contains geographic and morphometric information for ~117 million lakes with a combined surface area of about 5×10^6 km^2, which is 3.7% of the Earth's nonglaciated land area.

Using different regression equations for surface CH_4 concentrations, ebullition, diffusive flux, and storage, together with data on lake area and lake numbers (Kalff 2002), methane emissions from lakes on a global basis were estimated (Bastviken et al. 2004). When compared to global nonanthropogenic emission values from Wuebbles and Hayhoe (2002), the estimated lake emissions of 8–48 Tg CH_4 yr^{-1} represent 6–16% of global nonanthropogenic or 2–10% of total emissions. A more recent estimate of the emission of lakes including ebullition, diffusive, and storage flux of approximately 100 lakes added up to an emission of 71.6 Tg yr^{-1} (Bastviken et al. 2011). While those numbers are already 67% higher than previous ones (Bastviken et al. 2004), it was again pointed out that ebullition is still underestimated since it is not captured representatively due to short-term measurements.

An area, which is especially vulnerable to environmental change, is the Arctic realm with estimated annual temperature increase up to 9 °C (Collins et al. 2013). Hence, whereas the total CH_4 emissions north of 50°N are today estimated to be 16.5 ± 9.5 Tg yr^{-1}, they might increase to 21.3 ± 11.9 Tg yr^{-1} when +20 days of ice-free lakes and ponds will occur under a warmer climate (Wik et al. 2016). An earlier estimate from (Walter et al. 2006) based on measurements of only 16 lakes revealed emissions from all lakes north of 45°N to be 67% higher (24.2 ± 10.5 Tg yr^{-1}). Other regions will also be influenced more than the Arctic by rising temperatures and higher degradation rates of organic material, and reduced oxygen concentrations in the water column due to stronger stratification will most likely lead to higher methane emission in the future.

1.2.2 Reservoirs

Since the beginning of the twenty-first century, freshwater environments, including lakes and reservoirs for hydropower production, attract attention not only for CO_2 but also for CH_4 emissions (Bastviken et al. 2004; St. Louis et al. 2000; Tremblay et al. 2005).

The construction and operation of over one million dams globally (Lehner et al. 2011) has provided a variety of services important to a growing human population (e.g., hydropower, flood control, navigation, and water supply), but has also significantly altered water, nutrient, and ecosystem dynamics and fluxes in river networks (Deemer et al. 2016), as well as carbon dynamics (Aufdenkampe et al. 2011). Over the next decades a new wave of dam construction will expand the number of large dams significantly and increase the fraction of global river water that is passing through reservoirs from 50% today to about 90% in 2030 (Van Cappellen and

Maavara 2016; Zarfl et al. 2015). By damming rivers, huge areas are flooded and the water is stored for energy production via turbines. Normally, forests, peatlands, or grasslands bind CO_2 via photosynthesis and store organic carbon in plant material. However, if these areas are flooded during water level build-up in the reservoir, they will become a source of greenhouse gases due to aerobic and anaerobic decomposition of the submerged organic material (Deshmukh et al. 2014; Guérin et al. 2008). For example, in the boreal region of Canada, flooded peatlands represent a worst-case scenario since they store large amounts of organic carbon, which will decompose rather effectively when flooded and release CO_2 and CH_4 over a long period. Here, an experimental flooding of a boreal forest wetland caused the wetland to change from being a small, natural carbon sink, with respect to the atmosphere, of -6.6 g of C m^{-2} yr^{-1} to a large source of $+130$ g of C m^{-2} yr^{-1} (Kelly et al. 1997). As stated by the authors, this change was caused by the decaying vegetation, which eliminated the photosynthetic CO_2 sink and stimulated the microbial production of CO_2 and CH_4 from decomposition of plant tissues and peat.

Reservoirs are different from natural systems (i.e., lakes) in several ways (Fig. 3). First, when they are built, huge areas are flooded and the organic material that is now covered by water will be degraded and form CO_2 and CH_4 (e.g., Guérin et al. 2008). Dam operation often causes fluctuations in water level that can, in turn, via the decrease in hydrostatic pressure, enhance CH_4 bubbling (e.g., ebullition) rates at least over the short (Harrison et al. 2017; Maeck et al. 2013). The high catchment area-to-surface area ratio and close proximity to human activities (Thornton et al. 1990)

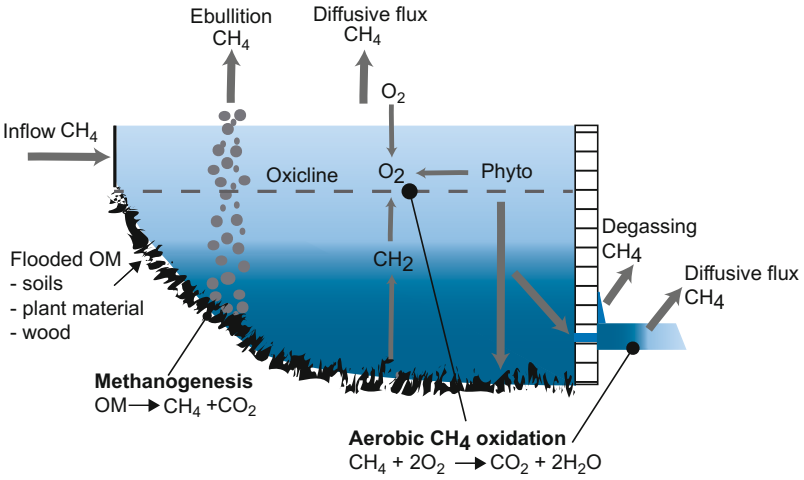

Fig. 3 Methane production, oxidation, and emission pathways in a reservoir (after Guerin 2006). Methane is produced via methanogenesis in the sediments or in the water column similar to lakes. Beside the emission pathways that are found in lakes, i.e., bubble formation (ebullition) and surface water diffusion, additionally pathways are direct degassing at the turbines or subsequently in the river fed by the turbinated water. This emission pathway is especially important since the water that is used for energy production is normally enriched in methane since it is derived from methane rich water of the hypolimnion

characteristic of many reservoirs are likely to increase the delivery of organic matter and nutrients from land to water (relative to natural lakes), potentially stimulating additional production and decomposition (Deemer et al. 2016; West et al. 2012, 2016). And finally, releasing water for energy production leads to high CH_4 emissions since this deep water is normally CH_4 enriched (Abril et al. 2005; Diem et al. 2012; Kemenes et al. 2011; Deshmukh et al. 2016).

It makes a difference to the emission rate whether an area has only been flooded recently or was flooded decades ago. Age was suggested as the most important variable beside latitude and DOC input in determining the time course of carbon emissions in reservoirs (Barros et al. 2011). In younger reservoirs (less than ~15 years), the main source of carbon is flooded biomass (Teodoru et al. 2011). This study showed that (1) total CO_2 emission to the atmosphere was highest in the first year after flooding, and that surface fluxes were spatially heterogeneous. (2) Spatial heterogeneity was not random, but was linked to the preflood landscapes: reservoir areas overlying former peatbogs and mature forests had the highest average emissions, whereas areas overlying former nonforest and burned soils had the lowest emissions. (3) Total reservoir emissions appeared to decline exponentially in the next 2 years and so did the degree of spatial heterogeneity in surface fluxes, suggesting a progressive weakening of the link to the preflood landscapes, and a homogenization of reservoir processes (Chanudet et al. 2011).

Altogether, a newly formed reservoir will emit methane at a much higher rate since fresh organic material is rapidly degraded. In contrast, reservoirs that are already decades old show lower methane emissions since older, i.e., more refractory organic material is harder to degrade but few studies have actually measured this decrease over time directly (Abril et al. 2005; Chanudet et al. 2011; Teodoru et al. 2011). In a tropical reservoir (Petit Saut hydroelectric reservoir, Sinnamary River, French Guiana), methane and carbon dioxide emissions were quantified for 10 years after impounding (Abril et al. 2005). Total carbon emissions were 0.37 ± 0.01 Mt yr^{-1} C (CO_2 emissions, 0.30 ± 0.02; CH_4 emissions, 0.07 ± 0.01) the first 3 years after impounding (1994–1996) and then decreased to 0.12 ± 0.01 Mt yr^{-1} C (CO_2, 0.10 ± 0.01; CH_4, 0.016 ± 0.006) since 2000. On average over the 10 years, 61% of the CO_2 emissions occurred by diffusion from the reservoir surface, 31% from the estuary, 7% by degassing at the outlet of the dam, and a negligible fraction by bubbling. CH_4 diffusion and bubbling from the reservoir surface were predominant (40% and 44%, respectively) only the first year after impounding (Abril et al. 2005). However, there are differences between reservoir emissions and even reservoirs that overall act as carbon sinks since carbon dioxide uptake is higher that methane emissions exist (Chanudet et al. 2011).

Beside the age of a reservoir, CH_4 emissions are triggered by temperature as was shown for lakes (Bastviken et al. 2004) and ponds (DelSontro et al. 2016a; Yvon-Durocher et al. 2014) and hence can express a relationship with latitude. In a compilation, methane fluxes from temperate reservoirs varied from 1 to 260 mg m^{-2} d^{-1}, while tropical reservoirs with their pronounced productivity showed even higher fluxes of 2–3800 mg m^{-2} d^{-1} (St. Louis et al. 2000). On average, tropical reservoirs were thought to emit CH_4 at rates about an order of

magnitude higher than temperate reservoirs (20 mg m^{-2} d^{-1} CH_4 vs 300 mg m^{-2} d^{-1} CH_4; (St. Louis et al. 2000)). The relevance of latitude for GHG emissions has been questioned recently by a study of a far greater number of reservoirs showing that the amount of nutrients and hence production of algal material is more important for the extent of GHG emissions (Deemer et al. 2016). In addition, high latitude (Barros et al. 2011) and high elevation reservoirs (Diem et al. 2012) emit GHG at lower rates than their warmer counterparts, particularly in the case of CH_4.

Hydroelectric reservoirs alone emit CO_2 and CH_4 of about 16% of those from all human-made reservoirs and 4% of all total (natural plus human-made) carbon emissions by fresh waters (Barros et al. 2011). They estimate that globally hydroelectric reservoirs emit about 48 Tg C as CO_2 per year and 3 Tg C as CH_4 per year from the reservoir surface. The increase of GHG emitted from reservoirs in the future will depend greatly on where geographically the new reservoirs are built. For example, if the surface area of hydroelectric reservoirs increases by 5000 km^2, the carbon emissions (as CO_2 equivalents) could either increase by 1–2 Tg C yr^{-1} when build in northern temperate and boreal regions or up to 4 Tg of C yr^{-1} when build in tropical areas (Barros et al. 2011).

CH_4 ebullition has only been measured in detail over the last 10–15 years. Conventional methods based on inverted funnels or floating chambers often fail to capture the highly intermittent and localized bubble streams. In recent years, the application of the eddy-covariance technique (Eugster et al. 2011; Schubert et al. 2012) improved the temporal resolution to detect ebullition events, and the echosounding technique allowed for better spatial coverage and mechanistic analysis (DelSontro et al. 2015). Originally, ebullition was thought to be only important in tropical systems due to the consistently warm temperatures and abundance of organic matter (Tremblay et al. 2005) – ideal conditions for methanogenesis and supersaturated sediments that induce bubble formation. Relatively recent work, however, illustrated that ebullition can contribute a significant portion to total CH_4 emissions from temperate systems (Beaulieu et al. 2014; DelSontro et al. 2010, 2011; Maeck et al. 2013). In this respect, it is also interesting that analyzing methane concentrations in sediments where methane bubbles form was until recently a big challenge due to overpressure and gas ebullition. Hence, methane concentrations were normally underestimated. A new sampling method solves this problem and allows for precise measurements of methane concentrations in sediments even under oversaturated conditions (Tyroller et al. 2016).

First estimates of general GHG emissions were estimated taking an area of 0.9×10^6 km^2 for temperate and 0.6×10^6 km^2 for tropical reservoirs into account, leading to a global flux of 70 Tg CH_4 (i.e., 2.3 Pg CO_2 equivalents) (St. Louis et al. 2000).

In 2016, Deemer et al. (2016) came up with a global reservoir emission of 0.8 (0.5–1.2) Pg CO_2 equivalents per year (12–29.6 Tg CH_4), with the majority of this forcing due to CH_4 (beside CO_2 and N_2O). The big difference between the former studies of St. Louis (2000) and Barros et al. (2011) is that Deemer et al. (2016) also included ebullition as far as possible into account. Although this is difficult to measure because of spatial and temporal changes but one of the main pathways of

methane emissions in reservoirs (Wehrli 2011), this is a big step forward. The reason that numbers are still lower, although ebullition is taken into account, lies in the extended data set used by Deemer et al. (2016). Using those numbers in comparison to global emission shows that the CO_2 equivalent emission (CO_2, CH_4, N_2O) of reservoirs add up to 1.5% of all anthropogenic emissions (Ciais et al. 2013).

1.2.3 Rivers

Although the role of rivers as methane emitters has been neglected so far, the available studies indicated that most fluvial systems are supersaturated with CH_4. The contribution of river systems to global emissions is estimated to be 26.8 Tg CH_4 yr^{-1}, which would be equivalent to ~15–40% of wetland and lake effluxes (Stanley et al. 2016). A recent study demonstrated that fluvial methane fluxes are comparable to those from tropical aquatic systems (320 mg CH_4 m^{-2} d^{-1}) and that temperature-driven sediment methane ebullition and flow-dependent contribution by hydraulic exchange with adjacent wetlands and small side-bays is important (McGinnis et al. 2016). In this study, about 80% of the total emissions were due to methane bubbles and hence ebullition made an even higher contribution to emission than in lakes and reservoirs.

In another study, first-order streams exhibited the greatest variability in fluxes of CO_2 and CH_4, and hence, up-scaled basin estimates of stream gas emissions suggested that streams may contribute significantly to catchment-wide CH_4 emissions (Crawford et al. 2014). A follow-up study showed that streams emit roughly the same mass of CO_2 as lakes at a regional scale and that stream methane emissions are an important component of the regional greenhouse gas balance (Crawford et al. 2014). Other researcher presented evidence for ebullition fueled by methane formation in river sediments (Wilkinson et al. 2015). On a landscape scale, the fluvial CH_4 emissions quantified in a study represented 41% of the regional aquatic (lakes, reservoirs, and rivers) CH_4 emissions, despite the relatively small riverine surface (4.3% of the total aquatic surface) indicating a disproportionately large role of the fluvial networks in boreal lowlands for CH_4 emissions (Campeau and del Giorgio 2014).

1.2.4 Combined Emissions and Emissions Under a Changing Climate

It can be seen from these few examples that GHG emission estimates from natural freshwater systems are not at all straightforward and still huge uncertainties exist.

Taking the three systems (natural lakes, reservoirs, and river) together results in global methane emissions of 134 Tg yr^{-1} (Bastviken et al. 2011; Deemer et al. 2016; Stanley et al. 2016). These figures are similar to emissions from natural wetlands (100 Tg yr^{-1}), the largest natural contributor, and much higher than estimated oceanic (4 Tg yr^{-1}) emissions (Wuebbles and Hayhoe 2002). Although these estimates might be on the high side, they clearly show that emissions from freshwaters have to be included in global methane budgets.

Recent work has revolved around how CH_4 emissions from natural systems will react to environmental changes, such as eutrophication and climate warming. For example, Yvon-Durocher et al. have shown that CH_4 emissions should increase with increasing temperature from the microbial- to ecosystem-scale (Yvon-Durocher et al.

2014), albeit mesocosm studies show that temperature impacts may not be so straightforward and that other factors, such as nutrient input and macrophyte abundance, modulate emissions at the ecosystem-scale (Davidson et al. 2015). Little work thus far has been done on how ebullitive CH_4 emissions respond to environmental change, despite the fact that ebullition can dominate total emissions in some systems. In the most sensitive and carbon-rich Northern regions, however, significant relationships have been found between relative environmental variables and ebullition. For example, Wik et al. have recently shown that ebullitive emissions from 3 sub-Arctic lakes could be explained by heat input via solar radiation (Wik et al. 2014). DelSontro et al. found in Boreal lakes and ponds that a temperature relationship with ebullition was not significant until the trophic state or nutrient level (via total phosphorus) was taken into account (DelSontro et al. 2016b). Thus, ebullitive CH_4 emissions were more temperature sensitive in more productive system.

Concerning rivers, the major predictors of ambient gas concentrations and exchange are water temperature, velocity, and DOC, and total GHG emissions ($C–CO_2$ equivalent) from a boreal river network were estimated to increase between 13% and 68% under plausible scenarios of climate change over the next 50 years (Campeau and del Giorgio 2014). This study showed also that the predicted increases in fluvial GHG emissions are mostly driven by a steep increase in the contribution of CH_4 (from 36% to over 50% of total CO_2-equivalents).

1.3 Methane Oxidation

Methane oxidation can occur with and without oxygen, i.e., aerobically and anaerobically, and is mediated by bacteria or archaea. A first series of studies about aerobic methane oxidation in lakes and its dependence on oxygen and nitrate was published in the 1970s (Rudd et al. 1974, 1976; Rudd and Hamilton 1975). In the marine environment, anaerobic oxidation of methane (AOM) was suggested to occur in Cariaco Trench and in Santa Barbara basin sediments in 1976 (Barnes and Goldberg 1976; Reeburgh 1976). In these papers, the reaction using sulfate to oxidize methane and a complementary, not competitive, metabolisms between sulfate reducing bacteria and methanogens was suggested. After these first reports, it took over 20 years before the active organisms were detected. A first study by Hinrichs et al. (1999) showed that biomarkers of archaea and sulfate reducing bacteria were isotopically depleted. A second paper visualized for the first time a consortium of archaea and sulfate reducing bacteria by using FISH (fluorescence in situ hybridization); biomarker and stable isotope measurements supported the methane oxidizing consortium (Boetius et al. 2000). It is interesting that in all sediments of the first studies, i.e., from Eel River Basin, Hydrate Ridge, and the Black Sea, those aggregates of archaea and SRB (AOM consortia) were highly abundant, representing >90% of the total microbial community (Boetius et al. 2000; Michaelis et al. 2002; Orphan et al. 2001). At this time, the hypothesis was that methane could be used as a substrate source in the cooperation of archaea and SRB (Hoehler et al.

1994). The dependence of the communities on both substrates, methane and sulfate, also defines a specific zone where those consortia can be retrieved called the "sulfate-methane transition zone" (SMTZ) which is found in all anoxic sediments where methane diffuses from below and sulfate from above (Reeburgh 2007). Those zones including active AOM have been described in sediments of the Pacific Ocean (Boetius et al. 2000), in the Gulf of Mexico (Orphan et al. 2001), in the Arctic Ocean (Niemann et al. 2006), and in the water column of the Black Sea (Schubert et al. 2006). In the AOM process, alkalinity is produced which drives carbonate precipitation. Therefore, carbonate crusts or even chimney-like structures were revealed at many AOM sites (Aloisi et al. 2002; Michaelis et al. 2002). Furthermore, AOM coupled to nitrate reduction can also be performed by the novel archaeal clade ANME-2d as recently described (Haroon et al. 2013). Evidence for AOM proceeding concurrently with iron or manganese reduction also exists for marine settings (Beal et al. 2009; Riedinger et al. 2014; Slomp et al. 2013; Wankel et al. 2012), but the involved microorganisms have not yet been identified. An informative review of AOM, the involved organisms, and processes can be found in Knittel and Boetius (2009).

In 2012 Milucka et al. (2012) proposed zero-valent sulfur compounds (S0) are formed during AOM through a new pathway for dissimilatory sulfate reduction performed by the methanotrophic archaea. They concluded that AOM might not be an obligate syntrophic process but may be carried out by the ANME alone, i.e., without SRB.

Newest findings show that consortial growth of thermophilic ANME-1 archaea and HotSeep-1 bacteria is probably based on direct interspecies electron transfer (DIET) mediated by intercellular wiring made up of pili-like structures and outer membrane multihaem cytochromes (Wegener et al. 2015). With the detection of large multihaem cytochromes in the genomes of methanotrophic archaea and the demonstration of redox-dependent staining of the matrix between cells in consortia, further evidence for syntrophic coupling through direct electron transfer was provided (McGlynn et al. 2015).

In lakes, methane is generally produced in anoxic sediments by methanogenic archaea. In fully mixed lakes, where oxygen is present throughout the water column and even penetrates into the upper sediment layers, methane is efficiently eliminated through aerobic oxidation (Bastviken et al. 2002). However, in permanently stratified (meromictic) and frequently also in seasonally stratified lakes (mono- or dimictic), an anoxic hypolimnion can be formed below the oxycline, where CH_4 can potentially accumulate to high concentrations (Blees et al. 2014; Lehmann et al. 2015; Schubert et al. 2010b). Here instead of AOM other, mainly oxidative, processes prevail.

Conventional sulfate-coupled AOM is an efficient pathway for CH_4 oxidation in oceans, but although there is some biogeochemical and microbiological indication of AOM in freshwater systems (Durisch-Kaiser et al. 2011; Eller et al. 2005), it has not been shown to play a significant role in anoxic hypolimnia of lakes. This is most likely due to relatively low sulfate concentrations (μM range) in freshwater environments compared to the mM concentrations in the oceans. Instead, methane

oxidation (MO) mediated by aerobic methane-oxidizing bacteria (MOB) belonging to the alpha- or gamma-subdivision of the Proteobacteria is considered the principal pathway for methane removal in lakes (e.g., Hanson and Hanson 1996; King 1992, #19797). The division of alpha-MOB (type II) and gamma-MOB (type I and type X) is based on functional differences with regards to carbon assimilation and the ability to fix nitrogen (Hanson and Hanson 1996). Genes encoding for soluble- (sMMO) or particulate methane monooxygenase (pMMO), the principle enzyme involved in MO, are expressed by both alpha- and gamma-MOB (Semrau et al. 2010).

Maximum MO rates and MOB abundances usually occur at the oxic/anoxic interface within sediments or the water column, where gradients of both O_2 and CH_4 intersect (Rudd et al. 1976). However, MO in the absence of detectable O_2 coinciding with populations of predominantly aerobic MOB below the oxycline has been reported for several stratified lakes (Biderre-Petit et al. 2011; Blees et al. 2014; Brand et al. 2016; Schubert et al. 2010b). In shallow lakes where light reaches below the oxycline, aerobic MO may be coupled to in situ photosynthetically production of oxygen (Brand et al. 2016; Milucka et al. 2015; Oswald et al. 2015). This has been shown, for instance, in Lago di Cadagno where diatoms producing oxygen co-occur with MOB (Milucka et al. 2015). Recently, more and more studies report on the possible involvement of electron acceptors alternative to oxygen and sulfate in methane oxidation below the oxycline of stratified lakes, despite the fact that the microbial community seems to be composed predominantly of aerobic methanotrophs in these lacustrine settings (Oswald et al. 2016a).

For example, MO (by facultative aerobic MOB) may be coupled to denitrification under oxygen limitation (Kits et al. 2015a). Methylomirabilis oxyfera (phylum NC 10), for example, has been shown to couple nitrite reduction to NO with methane oxidation, where O_2 is produced intracellularly by NO dismutation and used to oxidize methane aerobically (Ettwig et al. 2010). This process is likely also relevant in natural anoxic waters, as has been suggested for lake sediments (Deutzmann et al. 2014). Similarly, both iron and manganese oxides are important electron acceptors in terrestrial and aquatic settings, and geochemical evidence suggests that MO in lakes may also be linked to iron reduction (Norði et al. 2013; Sivan et al. 2011), or the cycling of both metals (Crowe et al. 2011).

Here we will use one particular example to show how the potential process of AOM in a lake in Spain was investigated (Oswald et al. 2016a) in order to illustrate how different analyses can be combined to investigate AOM in lakes. In order to study methane oxidation below the oxic-anoxic transition zone in detail, we need special monitoring and sampling devices. In our lab we have a custom-made profiling in situ analyzer (PIA) equipped with a multiparameter probe and various other sensors. Using this device it is possible to monitor conductivity, turbidity, temperature, depth (pressure) and pH, photosynthetically active radiation (PAR), chlorophyll a, and dissolved oxygen. The two micro-optodes attached to the multi-parameter probe allow for the detection of dissolved oxygen concentrations of 125 and 20 nM, respectively (Kirf et al. 2014). Highly sensitive O_2 sensors are absolutely necessary if one would like to investigate processes in the sub- to anoxic zone of aquatic systems. Twelve syringes (60 ml each) are attached to the profiling device

that could be triggered in situ from the boat to sample the water layer of interest. Additionally water was pumped to the surface with a peristatic pump via gas tight tubing attached to the PIA.

Water was then distributed into vials with the appropriate preservative. Zinc acetate was used to fix total sulfide ($H_2S + HS^-$). Samples for the determination of dissolved (filtered through a < 0.45 µm cellulose acetate filter) and total metal species were added directly to 65% HNO_3. Similarly, HCl was used to acidify dissolved (<0.45 µm, cellulose acetate filter) and total fractions of Fe(II)/(III) for photometric determination. Aliquots for nitrate (NO_3^-), nitrite (NO_2^-), ammonium (NH_4^+), phosphate (PO_4^{3-}), sulfate (SO_4^{2-}), dissolved inorganic carbon (DIC), and dissolved organic carbon (DOC) were filtered (<0.22 µm, cellulose acetate filter). For methane analysis, serum bottles (120 ml) were filled anoxically by allowing the water to overflow at least two volumes and adding Cu(I)Cl to stop microbial activity before closing the bottles (without headspace or bubbles) with butyl stoppers and aluminum crimp seals.

1.3.1 Methane Concentrations and Stable Carbon Isotopes

Dissolved methane concentrations were analyzed with a gas chromatograph equipped with a Carboxen 1010 column (30 m × 0.53 mm) and a flame ionization detector (FID). Solubility constants were used to calculate the original amount of CH_4 in the water phase (Wiesenburg and Guinasso 1979). To analyze the $^{13}C/^{12}C$ isotopic ratios of the headspace methane a trace gas unit (T/GAS PRECON, Micromass UK Ldt) connected to an isotope ratio mass spectrometer (IRMS; GV Instruments, Isoprime) was used and results are expressed in the conventional $\delta^{13}C$-notation, normalized to the Vienna Pee Dee Belemnite (VPDB) reference standard.

1.3.2 Biological and Geochemical Parameters

Primary production via photosynthesis took place in surface waters with maximum activity around 8.5 m, corresponding to a peak in chlorophyll a (Fig. 4). Below the sunlit zone, organic matter respiration was evident from the incremental depletion of available electron acceptors more or less following the canonical redox cascade. The O_2 concentration profile outlined a zone of net O_2 consumption between 13.5 and 14 m (defined here as the oxycline; dashed line in Fig. 4). Below the oxycline, the decrease in NO_3^- and a concurrent NO_2^- maximum (14–15 m; Fig. 5), provided evidence for an active zone of denitrification. Finally, an increase in dissolved/reduced Mn (below 12 m) and reduced Fe (below 16 m) indicated ongoing metal reduction in the hypolimnion (Fig. 5). As indicated by the concomitant decrease of SO_4^{2-} and the increase in H_2S towards the sediment, sulfate reduction might have occurred below 17 m in the water column (Fig. 5).

1.3.3 Methane Production/Methanogenic Zone

Maximum water column CH_4 concentrations were found nearest to the sediment (Fig. 4), suggesting its production within the sediments and diffusion into the water column. The $\delta^{13}C$–CH_4 in the deepest methane sample was about −50‰, which fell between the range of ratios indicative for biogenic (<−60‰) and thermogenic

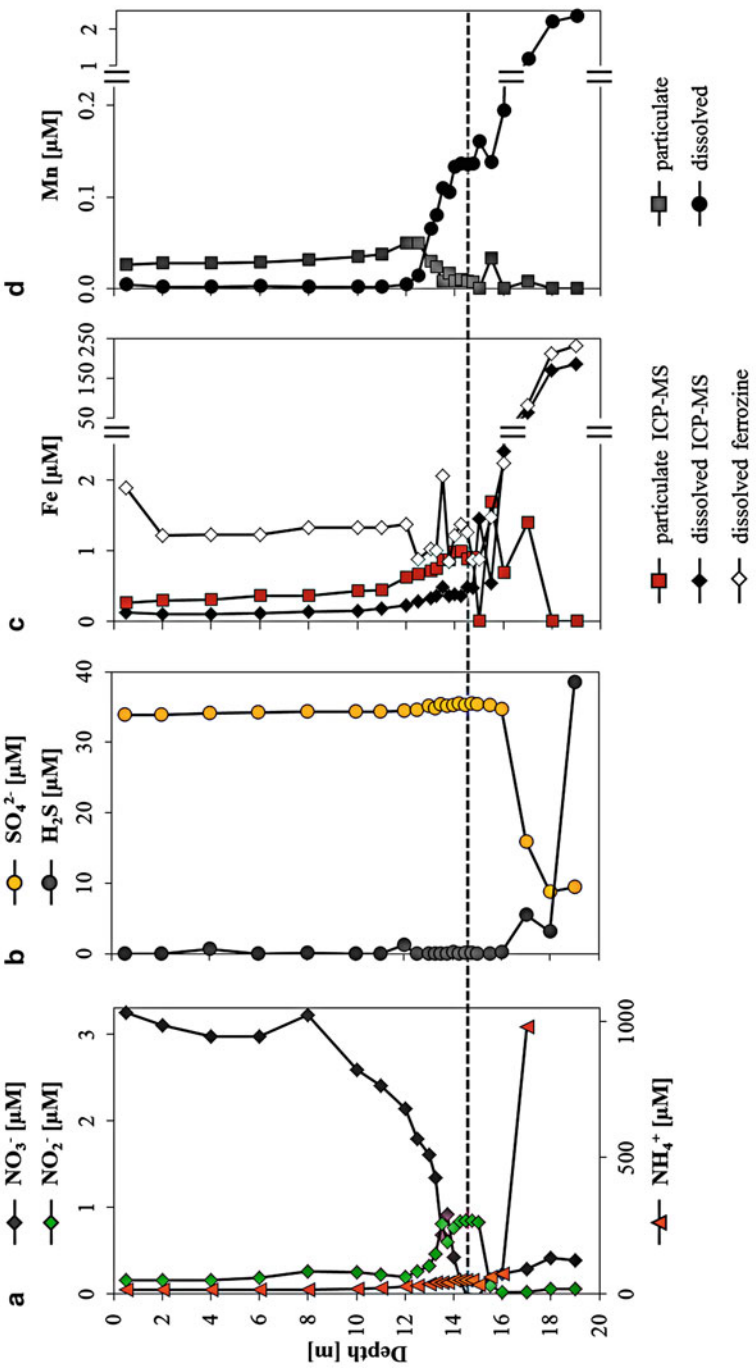

Fig. 4 Physicochemical parameters, methane concentrations, and stable carbon isotope ratios in Lake La Cruz (Oswald et al. 2016a). (**a**) Profiles of oxygen and temperature with a detailed view from 13 to 15 m depth, where the oxygen concentration was measured with a trace optode; (**b**) profiles of photosynthetically active radiation (note the logarithmic scale) and chlorophyll a concentrations; (**c**) depth profiles of methane concentrations and the corresponding δ13C–CH_4. The dashed line denotes the location of the oxycline

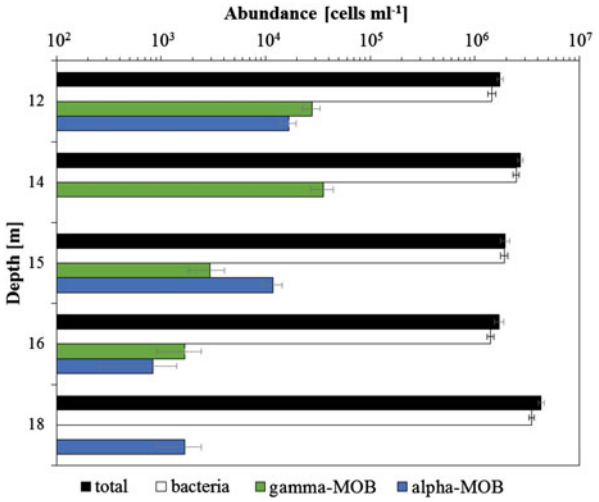

Fig. 5 Water column distribution of dissolved ions and metal species (Oswald et al. 2016a). Depth profiles of (**a**) nitrate, nitrite, and ammonium concentrations; (**b**) sulfate and total sulfide concentrations; (**c**) dissolved and particulate iron concentrations quantified by ICP-MS along with dissolved Fe(II) determined with the ferrozine assay; and (**d**) dissolved and particulate manganese as determined by ICP-MS. The dashed line represents the depth of the oxycline. Note the break in the x-axis in (**c**) and (**d**)

origin (>−50‰) (Whiticar 1999); however, the isotopic discrimination factor between CH_4 and DIC ($\Delta\delta^{13}C_{CH4\text{-}DIC}$ = 51‰) supported a biotic (i.e., methanogenesis in the sediment) rather than a thermogenic source (Whiticar 1999).

1.3.4 Methane Diffusion Zone

A sharp decrease in methane concentrations was measured, yet the $\delta^{13}C$–CH_4 was more or less invariant (~50‰) between the sediment surface and 16 m (Fig. 4). This constant signal indicated the absence of active MO in this zone since methanotrophs would preferentially incorporate the light carbon isotope, ^{12}C, leaving the residual pool of CH_4 enriched in ^{13}C. This finding was supported by minor MO rates in incubated samples of this zone.

1.3.5 Methane Oxidation Zone

A subtle convex CH_4 profile was visible between 16 and 12 m coinciding with a shift to notably higher $\delta^{13}C$–CH_4 values (−48‰ at 16 m to −19‰ at 12 m; Fig. 4), which, along with measured MO rates at depths throughout this zone, confirmed ongoing microbial methane consumption (Oswald et al. 2016a). The observed profiles suggested that reaction must have occurred below 14 m, and although the calculated fractionation factor of −1.005 was relatively low compared to typical values reported in the literature, it was not inconsistent with aerobic MO as the dominant methane removal mechanism (Templeton et al. 2006).

1.3.6 Oxic Zone

In the oxic zone, methane concentrations were very low ($\leq$ 300 nM) compared to the deep water, indicating that most methane was consumed before reaching the epilimnion. These concentrations were likely too low to sustain significant MO there. Indeed, the MO potential above the oxycline was an order of magnitude lower than below. The oxygenated region of the water column was likely unfavorable even for aerobic methanotrophs, not only because of methane limitation, but likely also due to the inhibitory effects of high O_2 concentrations (Rudd and Hamilton 1975) and light intensity (Dumestre et al. 1999; Murase et al. 2005).

In Lake La Cruz the methane turnover potential was similar under oxic and anoxic conditions showing potential methane oxidation rates (1.5–2.6 μM d^{-1}) similar in magnitude to estimates for other stratified lakes (e.g., (Blees et al. 2014; Lidstrom and Somers 1984). In shallow stratified lakes, highest methane turnover is commonly observed right at the oxycline (Panganiban et al. 1979; Sundh et al. 2005), where aerobic MOB have access to both oxygen and methane.

Using the trace oxygen probe, it was possible to detect sub-micro-oxic conditions below 13.6 m and complete anoxia below 14.6 m. Active MO in the apparent absence of O_2 (15, 16 and 18 m) was supported by the $\delta^{13}C$–CH_4 profiles exhibiting C isotopic fractionation of residual CH_4 from and above 17 m (Fig. 4). Below 14 m the only known electron acceptor available in high enough concentrations to account for measured MO potential was sulfate (35 μM). However, sulfate-coupled AOM seemed to be very unlikely, as there was no visible consumption of SO_4^{2-} or production of H_2S within the zone of methane oxidation, and fluxes of both species were extremely low. Additionally, the authors found no targeted groups of known AOM-performing ANME or AAA of the investigated depths, supporting previous evidence that these organisms are rare in lacustrine water columns (Eller et al. 2005; Oswald et al. 2016b). Altogether, this suggested that classical AOM was not occurring in Lake La Cruz.

Hence, while true obligate anaerobic methanotrophs seemed to be absent, aerobic alpha- and gamma-proteobacterial MOB were detected at all investigated depths (12, 14, 15, 16, and 18 m; Fig. 6). The observed decrease in aerobic MOB cell abundances (2.5% at 12 m to 0.04% at 18 m) correlated with depth and oxygen availability suggesting a pivotal role of O_2 in methane oxidation, as also observed in other lakes (Oswald et al. 2016b; Schubert et al. 2010a; Zigah et al. 2015). At 18 and 19 m 16S rRNA gene sequences belonging to the NC 10 phylum were detected. To some extent, members of the NC10 phylum might explain the occurrence of methane oxidation in the anoxic waters of Lake La Cruz in the absence of AOM-mediating archaea. However, as potential oxidation rates at 18 m were extremely low and given that neither NO_3^- nor NO_2^- were available at these depths under natural conditions throughout most of the year, these microorganisms were not considered to contribute substantially to methane consumption in the lake (Oswald et al. 2016a).

1.3.7 Oxygen and Light

Aerobic MOB in anoxic waters might be supported by a cryptic or transient supply of O_2 below the oxycline. While oxygen might have been introduced into the

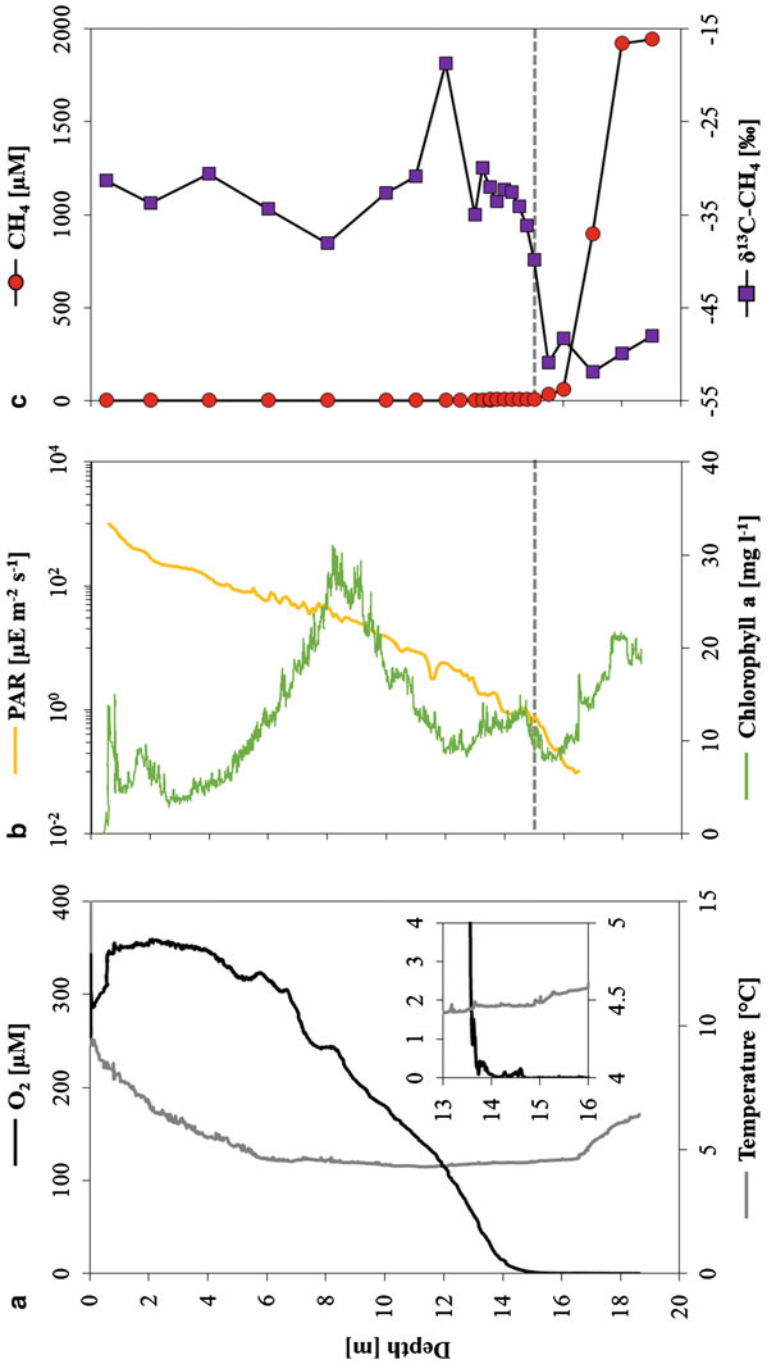

Fig. 6 In situ cell abundances of different microbial groups (Oswald et al. 2016a). Total cell numbers determined by DAPI cell counting. Cell abundances of bacteria (probes EUB338 I-III), gamma-MOB (probe mix Mgamma84 + 705) and alpha-MOB (probe Ma450) were assessed using CARD-FISH. Note the logarithmic scale. Error bars denote the standard error of the mean between counted fields of view

hypolimnion of Lake La Cruz through sublacustrine springs after sporadic rainfalls, cryptic in situ oxygen production by low light photosynthesis might have supported aerobic MO throughout the year. Such photosynthesis-linked MO had been observed in other shallow lake systems with deep light penetration (Brand et al. 2016; Milucka et al. 2015; Oswald et al. 2015) and seemed to be a plausible explanation for aerobic MO in the anoxic parts of Lake La Cruz. Laboratory and field evidence had suggested that the lower PAR threshold for oxygenic photosynthesis in freshwater is 0.09 μE m^{-2} s^{-1} (Gibson 1985) to 0.34 μE m^{-2} s^{-1} (Brand et al. 2016), respectively. Thus, light would have been sufficient to support photosynthesis well below the oxycline (0.1 μE m^{-2} s^{-1} at 16.5 m; Fig. 4). Indeed, peaks in the chlorophyll a profiles indicated the presence of phototrophic organisms down to 18 m, and more importantly, light had stimulated experimental methane consumption for all tested depths (Fig. 7). Nevertheless, stimulation of MO was much less prominent in Lake La Cruz compared to previous studies (Milucka et al. 2015; Oswald et al. 2015) and appeared to slow down after ~12 h. In other shallow lakes, e.g., light promoted continuous linear oxidation (Milucka et al. 2015) and increased the MO potential by up to an order of magnitude (Oswald et al. 2015). Low nitrate

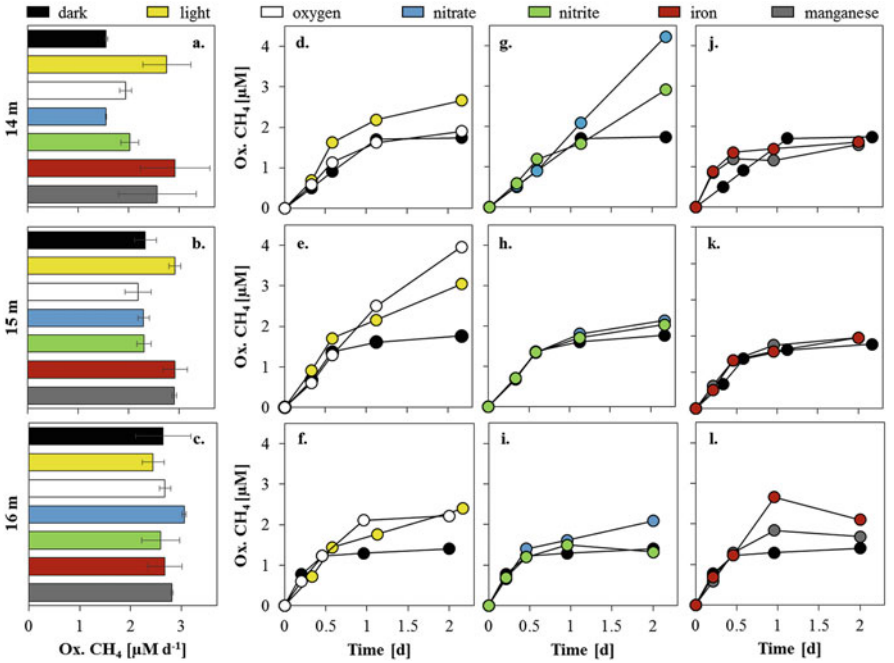

Fig. 7 Methane oxidation potential at and below the oxycline (Oswald et al. 2016a). Methane oxidation rates (**a**–**c**) and corresponding oxidation time series are displayed for 14 m (top panel), 15 m (center panel) and 16 m (bottom panel). (**d**–**f**) Time series of oxidized methane under dark and light conditions; (**g**–**i**) with additions of nitrate and nitrite; and (**j**–**l**) iron and manganese. The dark control series is displayed for reference in all panels. Methane oxidation rates in (**a**–**c**) are calculated based on the initial ~12 h linear segments of the time series in (**d**–**l**)

and phosphate concentrations (average of 40 nM) throughout the water column might have limited primary production in Lake La Cruz. In any case, light-driven methane oxidation alone could not fully explain all of the methane removal at and below the oxycline of Lake La Cruz.

Although in situ concentrations of other potential electron acceptors (NO_3^-, NO_2^-, Fe(III), and Mn(IV)) were proved to be to too low to sustain the observed rates, their addition promoted MO to some degree at all depths (under dark conditions; Fig. 7). Although initial rates with supplemented nitrate or nitrite were only slightly higher (~2 μM d^{-1}) than in the untreated dark control (1.5 μM d^{-1}), both additions resulted in constant oxidation of methane throughout the incubation period with water from 14 m (i.e., the depth of the oxycline; Fig. 7). This steady turnover resulted in total oxidized CH_4, which was about 3 (4.2 μM) and 2 (2.9 μM) times higher with supplemented nitrate and nitrite, respectively, compared to the dark control experiments (1.7 μM). Stimulation of MO was much less pronounced at 15 and 16 m and bulk methane turnover was only slightly higher (max. 2.1 μM at 15 m with added nitrate) than in the control.

Simultaneous denitrification (NO_3^- and NO_2^- supported) occurred in all incubations; however, NO_x reduction rates and $^{30}N_2$ production rates were considerably below (4–700 times lower) what would have been expected if it were directly linked to methane turnover. NO_x reduction might have been partly balanced by nitrification, explaining the comparatively low net nitrate/nitrite reduction rates (Oswald et al. 2016a). Ongoing production by nitrifying bacteria, at least at the oxycline where O_2 and NH_4^+ co-occur, could have been a constant source of both NO_3^- and NO_2^-. This "hidden" nitrite/nitrate regeneration might have resulted in constant MO without the corresponding apparent NO_x consumption. It was difficult to make a clear case for a link between MO and denitrification, at least in the deeper waters. Nevertheless, the possibility that canonical denitrification and NO_x-dependent MO co-occur could not be excluded.

In the study of Oswald et al. (2016a) we did not specifically test for nitrite-reducing *Methylomirabilis oxyfera* (Ettwig et al. 2010) or nitrate-reducing ANME-2d (Haroon et al. 2013; Raghoebarsing et al. 2006) via CARD-FISH. However, as 16S rRNA gene sequences related to the NC 10 phylum were only retrieved below the zone of high methane oxidation potential, where NO_x was lacking (18 and 19 m) and other representatives of ANME (−1 and −2) and AAA were absent, it was unlikely that either group contributed to methane turnover in Lake La Cruz. However, microbiological/molecular (i.e., CARD-FISH, sequencing) evidence for the presence of aerobic gamma-MOB at anoxic depths was found. It had been shown recently that some of these bacteria can couple methane oxidation to nitrate/nitrite reduction under oxygen limitation, although trace amounts of oxygen are probably still required for the initial oxidation of methane to methanol (Kits et al. 2015a, b). This metabolic switch would provide gamma-MOB with a competitive advantage in an environment with fluctuating O_2 conditions (i.e., transient submicromolar O_2 concentrations) and could explain why zones of aerobic MO and nitrate-/nitrite-dependent methane oxidation appear to overlap in the environment (Deutzmann et al. 2014). A facultative aerobic MO mechanism could also explain why MO was

simultaneously stimulated by O_2, NO_x, and light herein. Altogether, we were only able to speculate about any direct coupling of MO and denitrification. Indirect stimulation of MOB to oxidize methane by nitrate and nitrite must also be considered. Most MOB either fix nitrogen (Davis et al. 1964) or derive it from an inorganic source to build up their biomass (Bodelier and Laanbroek 2004; Rudd et al. 1976). The addition of an inorganic nitrogen source may simply have remedied N limitation, thus enhancing N uptake and growth of aerobic MOB. In this case the electron acceptor involved in the oxidation of methane would remain unknown.

It was speculated that Mn(IV) and Fe(III) could have served as the electron acceptors for MO although the organisms performing this reaction have yet to be identified (Crowe et al. 2011; Norði et al. 2013; Sivan et al. 2011). Since aerobic gamma-MOB are, with regards to their metabolic requirements, more versatile than previously believed (Kits et al. 2015a, b), it is conceivable that both Mn(IV) and Fe (III) could serve as viable electron acceptors in the respiratory chain, in addition to O_2 and nitrate or nitrite (Kits et al. 2015a, b).

While the addition of birnessite and ferrihydrite did appear to increase MO rates and methane turnover at some depths in the study of Oswald et al. (2016a), a clear link between observed MO and concurrent metal reduction could not be established. In experiments with birnessite, dissolved (reduced) Mn concentrations, which would indicate Mn oxide reduction, did not increase over time. In experiments with ferrihydrite, it was also not clear whether iron reduction really occurred. In the 14 and 15 m incubations, the Fe(III) concentrations did not show any decreasing trend, whereas at 16 m, Fe(III) decreased by 5% (or 2.4 μM), yet the observed concentration change was insufficient (by a factor of 5) to explain observed methane consumption. It is important to note, however, that produced Fe(II) can be re-oxidized by a variety of abiotic (O_2 and MnO_2) and biotic oxidative processes (i.e., Fe-oxidizing bacteria utilizing O_2 or NO_3^-) (Melton et al. 2014). Whereas both O_2 and MnO_2 were scarce at these depths, the activity of phototrophic and nitrate-dependent Fe-oxidizers could have continuously recycled reduced Fe in deeper waters of the lake (Bruun et al. 2010; Emerson 2009; Sobolev and Roden 2002). Both phototrophic (Walter et al. 2014) and nitrate-dependent Fe-oxidizers (Walter 2011) were found in Lake La Cruz, yet phototrophic recycling of Fe was not possible in our dark experiments. Similarly, to NO_x, the addition of Mn(IV) or Fe(III) might also have indirectly stimulated MO, especially at 14 m. It was seen possible that MnO_2 in the incubations oxidized in situ NH_4^+ (e.g., Luther et al. 1997), supplying NO_3^- as an oxidant for MO or as an inorganic N source for MOB. Iron is an important trace nutrient for some methanotrophs (Glass and Orphan 2012; Semrau et al. 2010), and iron addition thus could also enhanced their activity (without being exploited as electron acceptor).

Hence, methane emissions from Lake La Cruz were effectively mitigated by methane oxidation both in oxic and anoxic waters. Under both oxic and micro-oxic conditions, aerobic MOB utilized oxygen as the oxidant. Under anoxic conditions, aerobic methane turnover was most likely supported through the coupling with oxygenic photosynthesis. In addition, there was circumstantial evidence that electron acceptors besides oxygen, especially NO_3^- and NO_2^-, stimulated methane

consumption at the oxycline. Potential direct links between MO and the reduction of other further alternative electron acceptors and at other depths remained inconclusive and require further investigation. Aerobic MOB alone appeared to be responsible for methane removal in the La Cruz water column, though their actual activity remains to be quantified (i.e., with nanoSIMS). Although the presence of yet unknown anaerobic methanotrophs could not be completely ruled out, the data provided putative evidence for nonarchaeal methane oxidation under anoxic conditions in an aquatic environment. Future research in Lake La Cruz should focus on further characterizing the methanotrophic community and activity, with particular focus on aerobic MOB that are also known to oxidize methane with nitrate and nitrite (e.g., *Methylomonas denitrificans* and *Methylomicrobium album*) (Oswald et al. 2016a). These compounds seemed to play an essential role in the methane biogeochemistry of the lake.

Most recently, Oswald et al. have described new players on the stage of methane oxidation (Oswald et al. 2017), i.e., the putative family Crenotrichaceae. Belonging to an important subgroup of gamma-proteobacterial MOB, but contrary to "classical" MOB these gamma-MOB are multicellular and filamentous. Only two of these bacteria *Crenothrix polyspora* and *Clonothrix fusca* are so far described in the literature and both were retrieved from groundwater (Stoecker et al. 2006; Vigliotta et al. 2007). Crenothrix, a filamentous gamma-proteobacteria, was discovered by Ferdinand Crohn and has been known as contaminant of drinking water supplies since 1870 (Cohn 1870). However, despite its worldwide importance as a problem organism in drinking water supply (Bumb and Schweisfurth 1981), Crenothrix polyspora remained phylogenetically and physiologically uncharacterized. In 2006, Stoecker et al. were able to show that Crenothrix polyspora encodes a phylogenetically very unusual particulate methane monooxygenase whose expression is strongly increased in the presence of methane (Stoecker et al. 2006).

Sporadically, environmental occurrence of Crenothrix was reported in literature based on retrieved 16S rRNA or pmoA sequences (Dörr et al. 2010; Drewniak et al. 2012), but its role in methane cycling has remained unclear. In 2017 it was shown for the first time that Crenothrix is involved in methane oxidation in natural systems, i.e., in two lakes in Switzerland (Oswald et al. 2017). Here, Crenothrix is the main methane oxidizer in Lake Rotsee showing higher methane oxidation rates then the small classical round shape gamma MOB. In Lake Zug the contribution of Crenothrix to total methane oxidation was smaller but still significant. Detection of these bacteria in the water column also proved its capacity to thrive as plankton.

2 Research Needs

2.1 Methane Emissions

Estimating GHG fluxes from natural and anthropogenic freshwater systems is still not perfect and needs further developments, such as daylong continuous measurements year round. Here it makes sense to use laser dependent or mass spectrometric

methods (Schubert et al. 2012) to be able to quantify diffusive flux, ebullition, and storage flux during lake turnover. Having those measurements form numerous systems that are different in various aspects such as nutrient status, size, extension of littoral zones, DOM concentrations would make estimates of GHG emissions more precise. Additionally, precise methane emission measurements in rivers and wetlands are very rare, and their temporal and geographical coverage needs to increase to allow for reliable global estimates.

2.2 Methane Oxidation

Methane oxidation in marine environments is relatively well understood and numerous rate measurements made it possible to come up with global estimates. However, the final word about how the mechanisms of methane oxidation in the marine realm work at the consortium level is not spoken: recent studies propose a direct electron transfer (McGlynn et al. 2015; Wegener et al. 2015). Before, Milucka et al. (2012) showed that methanotrophic archaea produce zero-valent sulfur compounds (S0) via a new pathway for dissimilatory sulfate reduction. In this case, AOM might not be an obligate syntrophic process but may be carried out by the ANME alone. It is important to understand why sometimes consortia are forming although they do not seem to be necessary for AOM. Additionally, the different electron acceptors like nitrate and Fe and Mn oxides that are active in marine settings have further to be tested for their relevance in lacustrine environments. Finally, the discovery of the water-supply bacterium Crenothrix as a planktonic agent of MO in natural systems (Oswald et al. 2017) shows that other unknown organisms might be involved in methane oxidation. Although our knowledge in the last years has grown, there are still lots of surprising discoveries to be made.

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Diversity and Taxonomy of Aliphatic Hydrocarbon Producers

17

Serina L. Robinson and Lawrence P. Wackett

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Abstract

Microbially sourced alkanes and alkenes derived from fatty acids are important in nature and in society with potential as bio-based fuels and other industrial, medical, and consumer products. While the production of hydrocarbons by bacteria was first reported in the literature over half a century ago, most biosynthetic gene clusters and biochemical pathways have only been uncovered within the past decade. A deepened understanding of the genes and enzymes for fatty acid-derived hydrocarbon production has spurred genome mining efforts to determine the diversity of hydrocarbon-producing bacteria. In this chapter, we focus on prokaryotic pathways for the biosynthesis of medium- and long-chain alkanes and alkenes that have fatty acid precursors. Emphasis is placed on the taxonomy of hydrocarbon-producing organisms and the physiological and ecological role of these compounds. Hydrocarbons produced by bacteria have diverse cellular functions, including modulating membrane fluidity in response to environmental stressors. In microbial communities, hydrocarbons drive interspecies interactions and global biogeochemical cycles. Future research needs include harnessing biochemical knowledge to engineer known pathways and using genomics to better inform the discovery of novel hydrocarbon-based natural products.

1 Introduction

Hydrocarbons, compounds containing only carbon and hydrogen, are abundant wherever they have been looked for in the universe and may represent the major forms of carbon in interstellar space (Snow and McCall 2006; Alata et al. 2015). In our solar system, the outer planets and their satellites are known to contain seas composed of hydrocarbons (Dermott and Sagan 1995). On Earth, petroleum and natural gas hydrocarbons are prized in modern society, and so their formation, properties, and reactions have been heavily studied (Olah and Molnar 2003). Chemical studies have revealed a seemingly infinite variety of hydrocarbon structures. Naturally occurring aliphatic hydrocarbon chains vary greatly in length and branching levels further modified by the petroleum refining process. Cyclization of aliphatic chains adds another level of structural diversity. As hydrocarbon deposits age and proceed to greater thermodynamic stability, aromatic rings form and fuse resulting in a complex mixture.

Given the abundance of hydrocarbons, it is not surprising that microorganisms show extensive capabilities to catabolize and synthesize these molecules. The aerobic catabolism of aliphatic and aromatic hydrocarbons has been known for more than 60 years, with details of anaerobic catabolism emerging a few decades later (Zobell 1946; Gibson 1982). These microbes were often discovered in regions of natural hydrocarbon seepage or in locations impacted by hydrocarbon pollution from human activities. With respect to hydrocarbon biosynthesis, bacterial methane (Stephenson and Stickland 1933) and carotenoid (Thomas 1950) production was known in the first half of the twentieth century, and the biosynthesis of longer-chain

aliphatic hydrocarbons was reported in the late 1960s (Tornabene et al. 1967; Han and Calvin 1969). With the exception of methanogenesis, microbial hydrocarbon biosynthesis was generally not viewed to contribute to global hydrocarbon flux. This view has changed and will be discussed later in this chapter.

A burst of new inquiry into hydrocarbon biosynthesis occurred in the beginning of the twenty-first century. This wave was triggered by high oil prices and economic interests in generating energy-dense and easily extracted biofuels with better combustion properties than ethanol. During that time, investigators discovered several new biosynthetic pathways, expanding both the number and diversity of microorganisms known to produce aliphatic hydrocarbons. The advent of the genomic era provided a new platform for data-driven discovery of hydrocarbon biosynthetic genes in microorganisms. Bacterial hydrocarbon biosynthesis is no longer considered an anomaly. Indeed, certain phyla, such as the cyanobacteria, appear to universally produce hydrocarbons (Coates et al. 2014).

This review will focus on hydrocarbon biosynthesis by bacteria. The major classes of hydrocarbons covered here are medium- to long-chain length alkanes and alkenes derived from fatty acids (Fig. 1). While most are linear chains, branched fatty acids can serve as precursors to generate branched hydrocarbons. The alkenes, known as olefins, have an internal or terminal double bond, depending on the

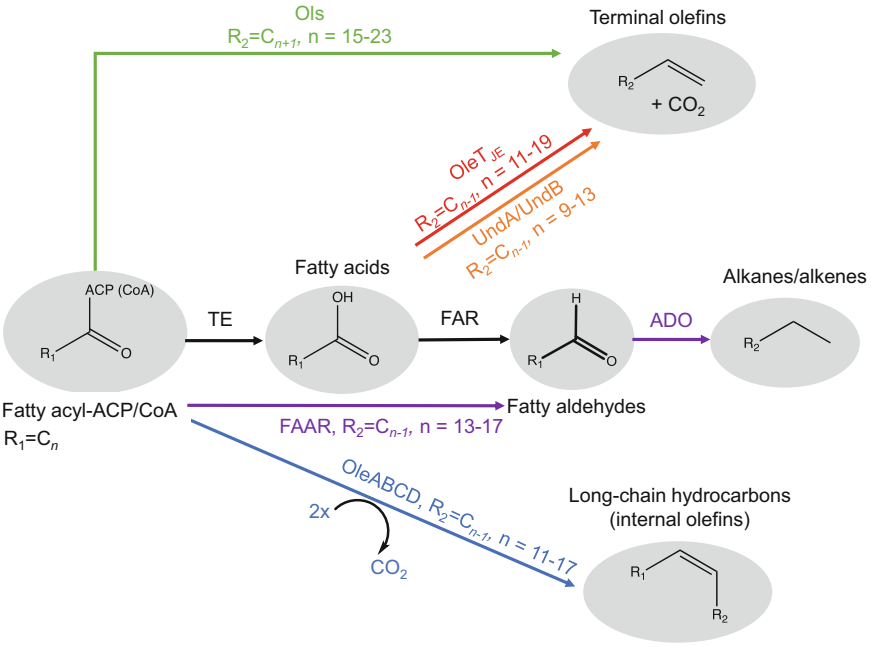

Fig. 1 Simplified prokaryotic pathways to fatty acid-derived hydrocarbons. "R" groups may have variable chain lengths and unsaturation levels depending on fatty acid precursors. Enzymes are abbreviated as described in the text with the exception of the generic thioesterase (TE) and fatty acid reductase (FAR) enzymes not covered in this review

mechanism of biosynthesis. The direct mechanisms of hydrocarbon generation include decarboxylation of fatty acids, deformylation of fatty aldehydes, and condensation of fatty acyl groups to produce long-chain internal olefins. Plants and animals are known to biosynthesize hydrocarbons for protection, communication, and insulation (Jacob 1978; Aarts et al. 1995), but these are outside the scope of the review. *Archaea* also produce interesting hydrocarbon moieties including fatty acid chains with cyclobutane rings known as ladderane lipids (Damste et al. 2002), but these are not strictly hydrocarbons and so will not be covered here. In this chapter, the mechanisms and taxonomic distribution of bacteria that use those mechanisms are described and are summarized in Table 1. The sections that follow will cover known interactions between aliphatic hydrocarbons, the organisms that produce them, and their environments.

2 Pathways and Enzymes

2.1 FAAR/ADO Enzymes and Organisms

Hydrocarbons produced by the cyanobacterium *Synechococcus* were identified nearly half a century ago (Winters et al. 1969), but the genes required for biosynthesis of these compounds were not known until 40 years later (Schirmer et al. 2010). In a landmark study, Schirmer et al. demonstrated that two enzymes, fatty acyl-ACP reductase (FAAR) and aldehyde-deformylating oxygenase (ADO), act sequentially to transform fatty acyl groups to medium-length alkanes (Fig. 1). The ADO enzyme catalyzes a novel carbon-carbon bond cleavage reaction with an aldehyde substrate to yield a hydrocarbon and formate as products (Schirmer et al. 2010). This pathway has been intensively investigated due to its novel reaction mechanism and potential for generating diesel-length alkanes from recombinant hosts. The active site of ADO resembles soluble methane monooxygenase, and the enzyme was found to catalyze an adventitious oxygenation of shorter-chain-length ($C_{9\text{-}10}$) aldehydes, presumably proceeding via a free-radical mechanism (Aukema et al. 2013). Evidence of the free-radical intermediate along the reaction pathway was later confirmed through detection of radicals during single-turnover experiments (Rajakovich et al. 2015). A model organometallic complex suggests that the reaction pathway of binuclear iron oxygenating enzymes can partition depending upon the way that the incipient substrate radical is quenched, either by oxygen rebound, as in oxygenases, or by a hydrogen donor, as with ADO (Shokri and Que 2015). There have been numerous efforts to express the ADO pathway heterologously in yeast for optimal production of diesel-length alkanes via fermentation; however, titers from this pathway still remain discouragingly low (22 μg/grams dry weight) (Foo et al. 2017).

The FAAR/ADO pathway is ubiquitous across all subdivisions of the cyanobacteria (Fig. 2) and was present in 122 of 139 strains that were analyzed in a comparative study by Coates et al. (2014). Phylogenomic analyses suggest FAAR/ADO is the ancestral hydrocarbon pathway in cyanobacteria, supported by its widespread distribution in species that span different morphologies and lifestyles

Table 1 Prokaryotic pathways for aliphatic hydrocarbon biosynthesis from fatty acid precursors covered in this review

Enzyme	Function	Precursor(s)	Product(s)	Select organisms	Reference(s)
OleT	Fatty acid decarboxylase (cytochrome P450 family)	Medium-to-long-chain fatty acids (C_{12}–C_{20})	Terminal olefins, CO_2	*Jeotgalicoccus* sp., *Corynebacterium efficiens*, *Kocuria rhizophila*, *Methylobacterium populi*, *Bacillus subtilis*	Rude et al. (2011), Belcher et al. (2014)
OleABCD	OleA: fatty acyl condensase OleD: keto reductase OleC: beta-lactone synthetase OleB: beta-lactone decarboxylase	Two long-chain fatty acyl-ACPs (CoAs) each (C_{12}–C_{18})	Long-chain internal olefins (C_{23}–C_{33})	*Micrococcus luteus*, *Chloroflexus aurantiacus*, *Kocuria rhizophila*, *Brevibacterium fuscum*, *Xanthomonas campestris*, *Shewanella oneidensis*, *Planctomyces maris*, *Geobacter bemidjiensis*, *Arthrobacter aurescens*	Frias et al. (2009), Sukovich et al. (2010a, b), Christenson et al. (2017a, b, c)
Ols	Polyketide synthase	Long-chain fatty acyl-ACPs (CoAs) (C_{16}–C_{24})	Long-chain terminal olefins, CO_2	*Synechococcus*, *Moorea*, *Cyanothece*, *Leptolyngbya*, *Geminocystis*, *Prochloron*, *Stanieria*, *Pleurocapsa*, *Xenococcus*	Mendez-Perez et al. (2011), Coates et al. (2014)
FAAR	Fatty acyl-ACP reductase	Medium-to-long-chain fatty acyl-ACPs (CoAs) (C_{12}–C_{20})	Fatty aldehydes	Widespread in all organisms, i.e., plants, eukaryotic algae, bacteria, humans	Schirmer et al. (2010), Coates et al. (2014)
ADO	Aldehyde-deformylating oxygenase	Medium-to-long-chain fatty aldehydes (C_{12}–C_{20})	Diesel-length alkanes	*Acaryochloris*, *Anabaena*, *Synechococcus*, *Prochlorococcus*, *Trichodesmium*, *Oscillatoria*, *Planktothrix*, *Gloeocapsa*, *Microcoleus*, *Nostoc*	Schirmer et al. (2010), Coates et al. (2014)

(*continued*)

Table 1 (continued)

Enzyme	Function	Precursor(s)	Product(s)	Select organisms	Reference(s)
UndA	Fatty acid oxidase/ decarboxylase	Medium-chain fatty acids (C_{10}–C_{14})	Terminal olefins, CO_2	*Pseudomonas*, *Burkholderia*, *Acinetobacter*, *Myxococcus*, *Nocardia*, *Leptospira*, *Turneriella*	Rui et al. (2014)
UndB	Fatty acid oxidase/ decarboxylase	Medium-chain fatty acids (C_{10}–C_{14})	Terminal olefins, CO_2	*Pseudomonas mendocina, Acinetobacter baumannii*	Rui et al. (2015)

Abbreviations: *ACP* acyl-carrier protein

(Coates et al. 2014). The FAAR enzyme is ubiquitous in all domains of life including plants, eukaryotic algae, and even humans, but ADO has not been detected in any other organisms outside of the cyanobacterial lineage.

2.2 Ols Pathway and Organisms

Cyanobacteria that lack the FAAR/ADO pathway produce terminal alkenes (Fig. 1) with an odd number of carbon atoms ($C_{n\ +\ 1}$) via a polyketide synthase (PKS) pathway, known as olefin synthase or Ols (Mendez-Perez et al. 2011). The Ols pathway was first discovered through a homology search of the biosynthetic gene cluster for curacin A, an anticancer natural product that has a terminal olefin tail (Chang et al. 2004). The Ols open reading frame shared ~45% sequence identity with the *curM* module, which is responsible for forming the terminal olefin in curacin A (Chang et al. 2004; Mendez-Perez et al. 2011). The N-terminal domain of the PKS was found to contain two additional modules: a fatty acid acyl-ACP ligase and an acyl-carrier protein (Mendez-Perez et al. 2011). Ols module architecture was perfectly conserved between verified PKS gene clusters in 17 different cyanobacteria (Coates et al. 2014). Comparative analysis across a subset of 32 cyanobacterial strains showed that cyanobacteria with the Ols pathway had an average 2.5-fold higher content of hydrocarbons than FAAR/ADO ($0.173 \pm 0.032\%$ vs. $0.070 \pm 0.008\%$) (Coates et al. 2014).

Despite its higher yield, the Ols pathway is significantly less prevalent than FAAR/ADO among cyanobacterial species (Coates et al. 2014). With a few notable exceptions, Ols is confined to a monophyletic clade consisting of *Pleurocapsales*, *Oscillatoriales*, and *Chroococcales* (Fig. 2) (Coates et al. 2014). Interestingly, *Leptolyngbya* sp. PCC 6406 and a set of filamentous cyanobacteria belonging to the genus *Moorea* contain the Ols pathway but do not cluster with other Ols-containing organisms. The presence of the Ols pathway in *Moorea producens*, formerly *Lyngbya majuscula*, and all other *Moorea* species analyzed to date is particularly interesting, since *M. producens* is the natural

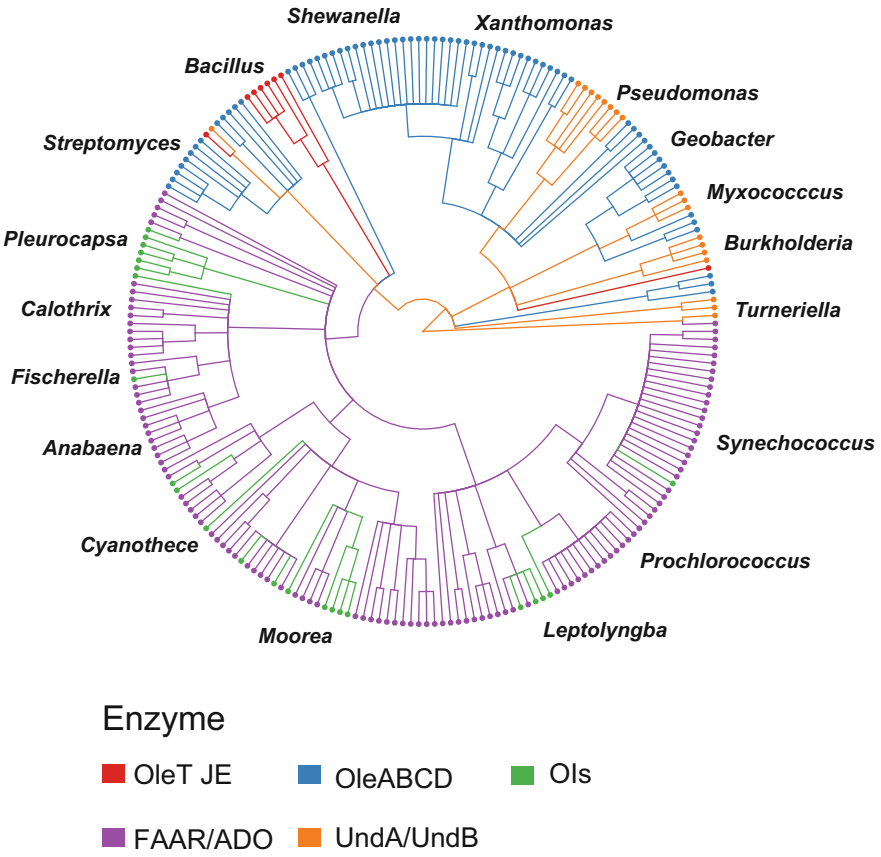

Fig. 2 Unrooted phylogenetic tree of prokaryotic aliphatic hydrocarbon producers. Leaves and branches are colored by hydrocarbon biosynthesis pathway. All taxa included in the tree were collected from literature sources in which organisms were experimentally verified to produce hydrocarbons. Pathway presence or absence is limited by what has been experimentally investigated and likely does not reflect true pathway abundances in nature. FAAR/ADO classification requires the presence of both enzymes for a pathway to be considered complete. UndA/UndB classification refers to any organism that possesses either UndA, UndB, or both pathways. Phylogeny was generated using phyloT and visualized using iTOL (Letunic and Bork 2016) based on full NCBI taxonomy. Tree branch lengths are uniformly scaled to each NCBI taxonomic level and do not necessarily reflect evolutionary distances

producer of curacin A (Chang et al. 2004). Despite high homology between CurM and Ols, genomic analysis indicated that *M. producens* has gene clusters encoding both biosynthetic pathways. The Ols gene clusters in *Moorea* and *Leptolyngbya* have significantly lower G + C content than the rest of the genomes, suggesting the Ols cluster may have been acquired by horizontal gene transfer (Coates et al. 2014). The most striking finding from the study by Coates et al. was that either the FAAR/ADO or Ols pathway was always present, but mutually exclusive, in the 139 cyanobacteria

analyzed. The obligate occurrence of one or the other pathway, but not both, indicates a strong selective pressure to maintain hydrocarbon pathways in cyanobacterial genomes (Coates et al. 2014).

2.3 $OleT_{JE}$ Enzymes and Organisms

The $OleT_{JE}$ pathway (Fig. 1) was first discovered in *Jeotgalicoccus* sp. ATCC 8456 (Rude et al. 2011), a halophilic organism isolated from the Korean fermented seafood (Yoon et al. 2003). $OleT_{JE}$ converts medium- and long-chain fatty acids (C_{12}–C_{20}) into terminal alkenes via oxidative decarboxylation. The general mechanism of action for the $OleT_{JE}$ pathway is consistent with characterized cytochrome P450 enzymes in which a ferryl-oxo porphyrin radical abstracts a hydrogen atom to initiate decarboxylation (Rude et al. 2011; Belcher et al. 2014; Grant et al. 2015). Interestingly, $OleT_{JE}$ uses hydrogen peroxide rather than oxygen as a reactant and an electron source (Rude et al. 2011).

$OleT_{JE}$ belongs to a family of peroxygenases known as CYP152 within the cytochrome P450 superfamily (Rude et al. 2011). The closest characterized enzymes are fatty acid hydroxylases which catalyze α- and β-hydroxylation of long-chain fatty acids (Matsunaga et al. 1997, 1999). Despite strong phylogenetic evidence for inclusion in CYP152, Belcher and colleagues noted that $OleT_{JE}$ was highly divergent (<40% sequence identity) from all but one sequence in the same clade, suggesting a remarkable functional diversity of enzymes in this family (Belcher et al. 2014).

To investigate the functionality of close homologs, Rude et al. purified putative $OleT_{JE}$ enzymes from six species and found that only four of the homologs were able to decarboxylate fatty acids to terminal alkenes (Rude et al. 2011). The apparent lack of correlation between sequence identity and alkene production highlights the difficulty in using bioinformatic methods to predict the distribution of the $OleT_{JE}$ pathway in bacteria. Indeed, $OleT_{JE}$ is one of the least understood pathways with respect to its taxonomic distribution. Despite a plethora of mechanistic and metabolic engineering studies on $OleT_{JE}$ (Grant et al. 2016; Hsieh and Makris 2016; Matthews et al. 2017), none to date have examined the diversity of organisms with the pathway beyond the five which were identified by Rude et al. (2011). Interestingly, the $OleT_{JE}$ pathway remains the only hydrocarbon pathway present in the genomes of low-G + C content, gram-positive *Firmicutes* (Fig. 2). Four of these five organisms containing $OleT_{JE}$ are *Firmicutes*, while only one, *Corynebacterium efficiens*, belongs to the *Actinobacteria* and has high G + C content. Further analysis of the $OleT_{JE}$ pathway distribution is required but challenging due to the widespread prevalence of homologous non-$OleT_{JE}$ cytochrome P450 enzymes in bacterial genomes.

2.4 OleABCD Pathway and Organisms

The production of long-chain hydrocarbons by *Micrococcus luteus* was originally discovered in the late 1960s, but the biosynthetic genes were not identified until 40

years later (Tornabene et al. 1967; Albro and Dittmer 1969; Friedman and DaCosta 2008; Beller et al. 2011). These early studies established the OleABCD pathway as the major mechanism for long-chain (C_{23}–C_{31}) internal olefin production (Fig. 1) in bacteria (Beller et al. 2011). The functions of the four genes in the *oleABCD* cluster are now well established. The OleA enzyme performs a head-to-head Claisen condensation of two fatty acyl-CoA substrates to form a β-keto acid, while OleD transforms the β-keto acid to a β-hydroxy acid via an NAD(P)H-dependent reduction (Bonnett et al. 2011; Frias et al. 2011; Goblirsch et al. 2016). A report by Kancharla et al. (2016) suggested that OleC catalyzed the final step in the pathway, converting the β-hydroxy acid intermediate to the final *cis*-olefin product, leaving no apparent role for OleB. Recently, the reaction catalyzed by OleC was revised to one in which β-hydroxy acids are converted to β-lactones instead of olefins. The *cis*-olefin product observed in previous studies with purified OleC was found to be artifact from thermal degradation of the β-lactone moiety in the gas chromatograph inlet (Christenson et al. 2017b). This new insight revealed that OleB functions to catalyze the final step in the OleABCD pathway by decarboxylating the β-lactone to the *cis*-olefinic hydrocarbon product (Christenson et al. 2017c). This led to OleC being characterized as the first known β-lactone synthetase enzyme. The interest in this discovery is heightened by the fact that non-hydrocarbon β-lactone natural products are important antibiotic, anticancer, and anti-obesity medicines and there appears to be some overlap between the enzymes involved in hydrocarbon and natural product biosynthetic pathways (Christenson et al. 2017b).

OleABCD gene clusters were identified in over 70 diverse bacteria, comprising about 5.2% of bacterial genomes (Sukovich et al. 2010a). More recent bioinformatic analysis detected homologs in >300 microorganisms, mainly environmental aquatic and soil organisms in the phyla *Actinobacteria* and *Gammaproteobacteria*, and some were identified in the *Chloroflexi*, *Verrucomicrobia*, *Planctomycetes*, and *Elusimicrobia* (unpublished data). No known human pathogens contain the OleABCD gene clusters.

Variations in the architecture of *oleABCD* gene clusters carry a strong phylogenetic signal. For example, long-chain hydrocarbon producers within the *Actinobacteria* contain only three *ole* genes, with *oleB* and *oleC* genes fused into one open reading frame. The OleBC fusion was shown to be fully active and capable of producing olefins in *Kocuria rhizophila* and *Micrococcus luteus* (Sukovich et al. 2010a; Christenson et al. 2017b). Prediction of the precise structure of the final olefin products produced by different microorganisms is not clear-cut based on *oleABCD* sequence signatures. Biochemical studies have demonstrated considerable variation in hydrocarbon chain length and degrees of unsaturation, even among members of the same species. A study that characterized ten different species of *Arthrobacter* found that six species produced head-to-head alkenes, while four did not (Frias et al. 2009). This unexpected variation among closely related species also suggested that production of hydrocarbons in *Arthrobacter* sp. is not essential or may be latent. By contrast, species in many genera including *Micrococcus*, *Shewanella*, and *Xanthomonas* universally produce head-to-head hydrocarbons, suggesting a more conserved role for hydrocarbons in these strains (Sukovich et al. 2010a).

2.5 UndA/UndB Enzymes and Organisms

UndA is a non-heme iron oxidase and the most recently identified enzyme for medium-chain hydrocarbon biosynthesis in bacteria (Fig. 1) (Rui et al. 2014). Originally characterized in four *Pseudomonas* species, UndA converts medium-chain fatty acids (C_{10}–C_{14}) into terminal olefins by oxidative decarboxylation proposed to proceed via a radical intermediate (Rui et al. 2014). Studies on UndA suggested a mechanism of β-hydrogen abstraction from free fatty acid substrates, not activated CoA or ACP-modified derivatives (Rui et al. 2014). The sequential binding mechanism of UndA is similar to many other non-heme iron enzymes including naphthalene dioxygenase and superoxide reductase (Karlsson et al. 2003; Katona et al. 2007).

Rui et al. identified UndA homologs in over 1500 published genomes, suggesting this pathway may be more prevalent in sequenced bacterial genomes than the genes encoding OleABCD or $OleT_{JE}$ pathways (Rui et al. 2014). However, this hypothesis requires further investigation due to challenges in inferring function from homology alone. Rui and co-workers found no homologs for Ols or $OleT_{JE}$ pathways in *Pseudomonas* sp., nor did they detect any evidence of the UndA pathways in *Shewanella* (Rui et al. 2014). The mutual exclusivity of these genes suggests there are multiple cellular routes to hydrocarbons, but the majority of known organisms likely only utilize one class of hydrocarbon pathway.

Rui et al. used transposon mutagenesis to demonstrate that UndA was both necessary and sufficient for 1-undecene production in several species of *Pseudomonas* (Rui et al. 2014). Interestingly, the investigators noted that 1-undecene titers varied by several orders of magnitude between closely related *Pseudomonas* (Rui et al. 2014). They hypothesized the existence of another route for hydrocarbon biosynthesis and later proved their hypothesis by characterizing UndB, a second fatty acid oxidase/decarboxylase belonging to the Und family (Rui et al. 2015). Feeding studies confirmed that the enzyme accepts substrates in free fatty acid form and proceeds via a similar mechanism to UndA (Rui et al. 2015).

Comparative analysis between UndA/UndB pathways suggested UndB genes were found in similar taxa but were far less prevalent than UndA (Rui et al. 2015). Notably, while some strains of *Pseudomonas* (namely, *P. aeruginosa* and *P. putida)* did not encode UndB homologs, numerous strains, including *Acinetobacter baumannii* and *Pseudomonas mendocina,* had verified expression and activity of both UndA and UndB (Rui et al. 2015). UndA/UndB pathways are highly conserved in three main genera: *Burkholderia*, *Pseudomonas*, and *Myxococcus* (Fig. 2). Interestingly, these genera all belong to different phyla and do not form a monophyletic clade (unpublished data). The evolutionary pressures that led to the UndA/UndB pathway arising multiple times and, in some cases, maintaining both the UndA and UndB gene copies despite functional redundancy remains to be explored.

2.6 CYP4G and Eukaryotic Hydrocarbon Pathways

Eukaryotic pathways for hydrocarbon biosynthesis are not covered in this review, but it is worth mentioning parallels between the prokaryotic and eukaryotic

pathways. CYP4G, a class of insect-specific cytochrome P450 enzymes, and the ADO enzymes in bacteria both catalyze the production of long-chain terminal alkanes from aldehydes (Qiu et al. 2012). The CYP4G enzyme is distinct from ADO in that it releases CO_2 rather than formate and consumes NADPH (Qiu et al. 2012). Bacteria also use the cytochrome P450 enzyme $OleT_{JE}$ to make 1-alkenes through a β-hydroxylation mechanism, but $OleT_{JE}$ differs significantly from CYP4G in both its sequence and mechanism of action. The CYP4G pathway is universally conserved among insects but is not known to be present in any other kingdoms outside *Insecta*. This observation leads Qiu et al. (2012) to speculate that acquisition of the CYP4G pathway for hydrocarbon biosynthesis was required to prevent desiccation and that may have been a key trait that allowed insects to colonize land. The evolutionary history of cytochrome P450 enzymes for hydrocarbon biosynthesis remains poorly understood.

3 Physiology

3.1 Growth

Recent investigations on the role of hydrocarbons in cyanobacteria demonstrated that hydrocarbon-deficient mutants had significant growth and division defects (Lea-Smith et al. 2016). Mutants were significantly larger than wild-type cells and also exhibited reduced membrane curvature (Lea-Smith et al. 2016). Although hydrocarbons accumulated in the thylakoid membrane, the organelles where photosynthetic reactions occur, the rates of photosynthesis were not significantly different (Lea-Smith et al. 2016). Overall Lea-Smith and colleagues demonstrated that hydrocarbons were essential for normal growth, division, and membrane fluidity (Lea-Smith et al. 2016). These results were generated from a singular study, indicating the need for further research to test the effects of hydrocarbon biosynthesis on cell biology and morphology.

3.2 Temperature Stress

It is now widely accepted that bacteria modulate fatty acid composition to adjust membrane fluidity in response to temperature fluctuations. However, little is known about the mechanism of action of aliphatic hydrocarbons during temperature stress. A seminal study of hydrocarbons in polar marine bacteria identified a novel long-chain olefin, hentriacontanonene (n-$C_{31:9}$) produced in large quantities (>200 μg g^{-1} cells dry weight) by 7 of 19 strains isolated from Antarctic sea ice cores (Nichols et al. 1995). This same alkene compound was later identified as a product from the *oleABCD* pathway from *Shewanella oneidensis* (Sukovich et al. 2010b). *S. oneidensis* produced increasing titers of hydrocarbons when it was subjected to a temperature downshift to 4 °C (Sukovich et al. 2010b). Furthermore, hydrocarbon-deficient *S. oneidensis* mutants exhibited a longer lag phase than wild type during growth under cold-shock conditions (Sukovich et al. 2010b). It was hypothesized,

but has still not been rigorously shown, that pathways producing olefins rapidly act to modulate membrane fluidity properties and allow for increased flexibility during large shifts in temperature.

Studies of temperature stress were also conducted in cyanobacterial systems. In *Synechococcus* sp. strain PCC 7002 containing the Ols pathway, production of 1,14-nonadecadiene ($C_{19:2}$) was found to be inversely correlated with temperature (Mendez-Perez et al. 2014). A fatty acid desaturase gene that introduced the internal double bond in $C_{19:2}$ was shown to be essential for cyanobacterial growth at 22 °C, further supporting the role of hydrocarbons in temperature adaptation (Mendez-Perez et al. 2014). Berla et al. engineered an alkane-deficient *Synechocystis* mutant by replacing the ADO pathway with a kanamycin resistance cassette (Berla et al. 2015). As expected, they observed a reduction in growth of the hydrocarbon-deficient mutant at 25 °C and 20 °C compared to wild type. The investigators also measured redox kinetics at low temperatures and found an increased reliance in the mutant on cyclic electron flow (CEF), likely in order to maintain redox poise and reductant partitioning. Taken together, these results suggest hydrocarbons in thylakoid membranes may play a critical role in regulating redox balance under temperature stress (Berla et al. 2015).

4 Ecology

4.1 Volatile Organic Compounds

In addition to membrane components, hydrocarbons also can contribute to cell signaling and defense. Bacteria produce a diverse array of hydrocarbon-derived volatile organic compounds (VOCs) to communicate with other bacteria, fungi, and plants (Schmidt et al. 2016; Bailly et al. 2017). The VOC 1-undecene has received considerable attention for its effective inhibition of oomycetes, including the potato pathogen *Phytophthora infestans* (Hunziker et al. 2015). The investigators isolated 32 bacterial strains from the rhizosphere and phyllosphere of field-grown potatoes and found the VOC profiles of four rhizosphere *Pseudomonas* strains to be uniquely dominated by 1-undecene. Application of 1-undecene directly to *P. infestans* resulted in impaired mycelial growth, sporangia formation, and zoospore release, suggesting it may be an effective biocontrol agent for potato crop protection (Hunziker et al. 2015). The production of 1-undecene by *Pseudomonas* was confirmed in a meta-analysis of 31 VOC studies. Bos et al. identified 1-undecene as a unique biomarker capable of differentiating *Pseudomonas aeruginosa* from five other common human pathogens in sepsis (Bos et al. 2013). 1-Undecene has also been detected in the VOC profiles of species in the genera *Burkholderia*, *Bacillus*, and *Serratia* (Blom et al. 2011). Interestingly, these genera are all known to possess homologs of the UndA/UndB pathway (Rui et al. 2014, 2015), which raises the

hypothesis that the UndA/UndB pathway may have evolved to produce VOCs to mediate intra- and interspecies signaling and defense.

4.2 Biogeochemical Cycles

Hydrocarbons are prevalent in marine systems, even in pristine regions of the ocean far removed from human sources or natural seeps (Schwarzenbach et al. 1978; Gschwend et al. 1980). Concentrations ranging from 2 to 130 pg/mL of saturated and unsaturated hydrocarbons in unpolluted ocean regions are frequently detected, but their source is unknown (Schwarzenbach et al. 1978; Gschwend et al. 1980). Lea-Smith et al. explored this phenomenon by quantifying the contribution of cyanobacterial hydrocarbons to the marine hydrocarbon cycle (Lea-Smith et al. 2015). The investigators focused on the two most dominant cyanobacterial genera in marine systems: *Prochlorococcus* and *Synechococcus*, both containing the FAAR/ADO pathway. Lea-Smith et al. measured hydrocarbon production per cell and scaled these amounts to reflect global species abundances estimated by Flombaum et al. (2013). They reported cyanobacterial hydrocarbon production on the order of ~308–771 million tons per annum, an amount rivaling the oil production of Saudi Arabia (U.S. Energy Information 2017). According to these findings, the global pool of hydrocarbons produced by cyanobacteria is orders of magnitude higher than anthropogenic and abiotic sources. To explain the apparent imbalance between this number and relatively low quantities of measurable hydrocarbons in the ocean, the investigators proposed a tight coupling between hydrocarbon-producing cyanobacteria and hydrocarbon-degrading heterotrophic marine bacteria. They hypothesized that cross-feeding interactions between the hydrocarbon degraders and cyanobacteria form a beneficial partnership in which cyanobacteria provide alkanes for consumption by the hydrocarbon degraders in exchange for CO_2 to power photosynthesis. While the existence of this "short-term hydrocarbon cycle" still remains to be rigorously proven, the global importance of these compounds in marine biogeochemical cycling is clear.

5 Research Needs and Conclusions

Major breakthroughs in the past decade have helped draw the genetic and biochemical blueprints for microbial hydrocarbon production. These studies have laid the foundation to creatively build upon and broaden the applications of these pathways for consumer, medical, and energy needs. In addition to optimizing and engineering of existing pathways, the endless diversity of biology ensures that numerous hydrocarbon-derived products and pathways still await characterization. In this review, we highlight current research needs to help fill knowledge

gaps for existing pathways and promote discovery of novel cellular routes to hydrocarbon natural products.

5.1 Investigate Hydrocarbon Pathways in Understudied Organisms

Progress in elucidating fungal and other eukaryotic hydrocarbon biosynthetic pathways lags behind knowledge about bacterial pathways. For example, Shaw et al. (2015) demonstrated a novel PKS pathway that produced odd-carbon chain length terminal olefins in the endophytic fungal isolate *Nigrograna mackinnonii*. None of the 17 PKSs encoded in the *N. mackinnonii* genome contained a sulfotransferase domain characteristic of CurM and Ols clusters, suggesting an alternate mechanism of displacement than the Ols pathway, which was confirmed by ^{13}C-acetate labeling (Shaw et al. 2015). A Patagonian fungal endophyte *Gliocladium roseum* (NRRL 50072) was also found to produce straight- and branched-chain alkanes and alkenes via an unknown pathway (Strobel et al. 2008). Further biochemical knowledge is also required for known pathways such as the CER1 protein for long-chain hydrocarbon biosynthesis in plants (Aarts et al. 1995). Although CER1 was discovered in 1995, few studies have been conducted since then, leaving many open questions about its mechanism (Bernard et al. 2012). These examples likely only represent the "tip of the iceberg" for the diversity of hydrocarbon-producing pathways in fungi, plants, and other understudied domains on the tree of life.

5.2 Engineer Custom Chain Length Hydrocarbons and Derivatives

The discovery of the Ols pathway ignited great interest in genetic engineering of PKS modules to produce custom oleochemicals (Mendez-Perez et al. 2011). Despite significant efforts to express Ols in *Escherichia coli*, no olefin production was achieved, likely due to alternate codon usage and challenges associated with expressing a large, multi-domain complex proteins (Mendez-Perez et al. 2012). To the best of our knowledge, expression of the Ols pathway in other host organisms has not been demonstrated. A major discovery in the Ols protein complex was the existence of a unique active-site flap. This flap affects substrate selectivity as was determined through X-ray crystal structures obtained for the sulfotransferase domains in the Ols and CurM modules (McCarthy et al. 2012). Soon afterward, Whircher et al. (2013) solved crystal structures for two cyanobacterial PKS docking domains which were identified as intermolecular recognition elements that could be amenable to genetic engineering. Advances in structural knowledge have now revealed that PKS active sites are not as modular as previously believed and in fact communicate with and reposition downstream modules (Whicher et al. 2014). Highly variable linker regions previously thought to be unimportant "junk" are now known to be critical for communication between modules (Whicher et al. 2014).

Blueprints for PKS modules continue to be rewritten as a 2017 publication demonstrated that processing enzymes migrate with the ketosynthase that is downstream, rather than directly upstream of the assembly line (Zhang et al. 2017). This dramatic reshuffling of the textbook understanding of PKS module order attests to both the grand challenges and tantalizing potential of harnessing these systems for custom hydrocarbon-derived products.

Although the "plug-and-play" architecture of PKS domains makes them attractive engineering targets, the OleABCD pathway also presents intriguing opportunities for rational design. Sukovich et al. demonstrated that the OleA protein dictated the pattern of hydrocarbon products formed, presumably because the OleBCD complex has a reasonably broad substrate specificity (Sukovich et al. 2010a; Christenson et al. 2017a). The "gatekeeping" ability of OleA was tested in vivo by introducing different *oleA* genes into a heterologous host and showing that swapping OleA altered the chain length of hydrocarbon products (Sukovich et al. 2010a). Engineering OleA to modulate long-chain hydrocarbon products via the OleABCD pathway warrants further inquiry.

5.3 Characterize the Diversity of Natural Products with Hydrocarbon Moieties

Nature produces a vast array of secondary metabolites, including hydrocarbons and their derivatives. The rate of discovery of microbial natural products is staggering; over 32,000 compounds have been identified for the past two decades (Pye 2017). However, due to limited resources, the clinical potential of these products remains poorly characterized. An exception to the rule, curacin A, has achieved relative fame for its anti-tubulin activity and potent cytotoxicity against breast cancer cell lines (Verdier-Pinard et al. 1998; Chang et al. 2004). Verdier-Pinard et al. showed that the $C_{9\text{-}10}$ olefinic bond was critical for interactions with tubulin, suggesting a functional role for the olefin side chain in inhibiting microtubule assembly (Verdier-Pinard et al. 1998). Only a handful of other compounds with the terminal alkene moiety have been identified to date, for example, in jamaicamide, mupirocin, and pederin (Hopwood 2009). Given the therapeutic potential of these alkene-derived natural products, further research is needed on the source and mechanism of action of the terminal olefin in natural products to advance drug discovery efforts.

5.4 Discover Culture Conditions to Trigger Cryptic Gene Cluster *Expression*

Expression of natural products, including hydrocarbons, is highly dependent on culture conditions (Blom et al. 2011; Okada et al. 2017). Many "cryptic" biosynthetic gene clusters appear to be inactive until triggered by an environmental signal such as low levels of antibiotics or other small molecules (Seyedsayamdost 2014). Antibiotics are known to function in nature not only as growth inhibitors but also as

signal molecules to elicit transcriptional responses (Romero et al. 2011). Therefore, studies using antibiotics to elicit production of cryptic hydrocarbon products are of interest and may help to improve production. Leveraging ecological knowledge to co-culture different strains may also serve to upregulate latent hydrocarbon production pathways. Co-culture of different bacterial and fungal strains has been shown to promote production of an array of secondary metabolites that differ from growth in axenic cultures (Schmidt et al. 2016). Modulating growth conditions through alterations in nutrient levels, pH, and especially lower temperatures could also be effective in advancing discovery of novel hydrocarbon-derived natural products.

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Alkane Biosynthesis in Bacteria

18

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Abstract

Biofuels are a commercial reality with ethanol comprising approximately 10% of the US retail fuel market, and biodiesels contributing a little under 5% to the EU retail fuel market. These biofuels are derived from the fermentation of sugars by yeast (ethanol) and from the chemical modification of animal fats and plant oils (biodiesel). However, these biofuel molecules are chemically distinct from the petroleum fuels that they are blended with. Petroleum-based fuels are predominantly composed of alkane and alkene hydrocarbons. These differences impact on fuel properties and infrastructure compatibility resulting in a "blend wall" that – without significant infrastructure realignment and associated costs – limits the use of biofuels. For this reason, there is great interest in biosynthetic routes for alkane and alkene production. Here we will review the known biological routes to alkane/alkene biosynthesis with a focus on bacterial alkane and alkene biosynthetic pathways. Specifically, we will review pathways for which the underlying genetic components have been identified. We will also investigate the development of engineered metabolic pathways that permit the production of alkanes and alkenes that are not naturally synthesized in bacteria (heterologous production) but are suitable for industrial commercial application. Finally, we will highlight some of the challenges facing this research area as it moves from proof-of-principle studies toward industrialization.

1 Introduction

Biogenic production of alkane and alkene hydrocarbons has received much attention in recent years. This is largely driven by the growth of the biofuels sector in response to climate change legislation and fuel security concerns. Current retail biofuels fall into two categories: alcohols (primarily ethanol) that are blended with petrol, and both fatty acid ethyl esters (FAEEs) and fatty acid methyl esters (FAMEs) that are blended with diesel to form fatty acid-derived biodiesel. Ethanol is produced predominantly by yeast fermentation and is mixed with petrol (gasoline). Biodiesels, derived from animal fats or plant oils, are mixed with diesel. These first-generation biofuels, most notably ethanol, have demonstrated a rapid increase in their penetration of the fuel market. In the USA, for example, ethanol content in petrol rose from little over 1% in 2001 to nearly 10% within a decade. Six years later, however, ethanol remains at approximately 10% of the market. The failure to penetrate the fuel market further is in part due to the challenges that these biofuel molecules present to the fuel sector. They are not wholly compatible with our petroleum-focused infrastructure. Ethanol, for example, is hygroscopic, meaning that it has a high tendency

to absorb water from the air. The presence of water makes it corrosive to transport infrastructure and reduces the heat of fuel combustion. Biodiesel is also moderately hygroscopic and, if not blended correctly, can form waxes within fuels at cool temperatures. Both of these biofuels therefore have limitations imposed on the ratio of biofuel to petroleum – the so-called blend wall. This is typically in the region of 10–15%. It is to circumvent this blend wall that there has been a renewed interest in alkane biogenesis.

Alkane and alkene hydrocarbon molecules are chemically and structurally identical to the molecules found within petroleum-based fuels (Fig. 1). As such there is no theoretical or practical limitation on their inclusion within current transport infrastructure. Within the last decade, the underlying genetic components of alkane biogenesis have been discovered in diverse species from many taxa. This knowledge is in turn permitting a greater understanding of the biochemical processes involved and spurring the search for further discoveries. Coupled to this "discovery-focused" effort, there are significant advances in engineering natural biosynthetic pathways to produce alkanes and alkenes in microbial hosts, such as *Escherichia coli* and *Saccharomyces cerevisiae*. The aim of this research is to improve yields and to direct carbon flux toward infrastructure-compatible molecules – the so-called drop-in biofuels. This chapter will review current knowledge of the underlying genetic and biochemical basis for alkane/alkene biogenesis in bacteria and will describe efforts to manipulate these biological systems to develop metabolic pathways that can produce alkane and alkene hydrocarbons tailored to the specific requirements of

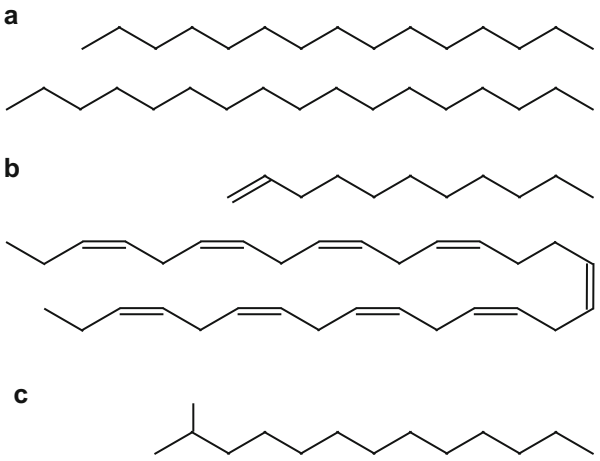

Fig. 1 Examples of alkane and alkene molecules that can be synthesized by microbes. (**a**) Pentadecane (*top*) and heptadecane (*bottom*) are produced by cyanobacteria (Schirmer et al. 2010). (**b**) Alkenes include 1-undecene (*top*) produced by *Pseudomonas* sp. (Rui et al. 2014) and the highly unsaturated very-long-chain alkene, 3,6,9,12,15,19,22,25,28-hentriacontanonaene (*bottom*) produced by *Shewanella* sp. (Sukovich et al. 2010). (**c**) Branched alkanes bearing a methyl group (e.g., methyl tridecene illustrated) can be produced by the creation of synthetic metabolic pathways (Howard et al. 2013)

our transport infrastructure. The biosynthesis of other hydrocarbon molecules, for example isoprenoids, will not be considered here, nor will we review the extensive literature manipulating and enhancing fatty acid biosynthesis. For reviews on these topics, see Schrader and Bohlmann (2015) and Mehrer et al. (2016), respectively.

2 Natural Alkane Biosynthetic Pathways

Biogenic sources of alkane have been known for some considerable time (Albro and Dittmer 1969a, b), and alkane biosynthesis can be observed across the biological domains. Examples include alkanes as components in waxes of plants (Bernard et al. 2012) and animals (Cheesbrough and Kolattukudy 1988), in storage reserves and membrane components of microalgae (Dennis and Kolattukudy 1992; Grossi et al. 2000; Sorigue et al. 2016), and in pheromones, defense compounds and cuticular waxes of insects (Howard 1982; Howard et al. 1982; Reed et al. 1994; Tillman et al. 1999; Qiu et al. 2012). Alkanes are also known to be synthesized in fungi (Griffin et al. 2010; Gianoulis et al. 2012; Spakowicz and Strobel 2015) and in many bacterial species (McInnes et al. 1980; Beller et al. 2010; Schirmer et al. 2010; Sukovich et al. 2010b; Rude et al. 2011) though their physiological roles are less well understood. In eukaryotic systems, the genetic components have been identified in plant cuticular wax biosynthesis and in fruit fly wax biosynthesis. In plants, very-long-chain (VLC) alkanes are an integral component of the waxy cuticle, comprising up to 70% of the cuticle in the model species *Arabidopsis thaliana*. VLC alkanes have a carbon chain length (C_n) of between C_{20} and C_{36}. In *Arabidopsis*, the proteins ECERIFERUM1 (CER1) and ECERIFERUM3 (CER3) and endoplasmic reticulum-associated cytochrome b5 isoforms CYTB5s interact to enable the conversion of fatty acids to VLC alkanes (Bourdenx et al. 2011; Bernard et al. 2012). This proposed conversion is via a fatty acyl-CoA intermediate, with the subsequent release of CO. Clear evidence for the role of CER1/CER3 and CYTB5 has been demonstrated by the reconstitution of this pathway in the yeast, *S. cerevisiae*, and the resulting production of VLC alkanes (Bernard et al. 2012). Within the fruit fly *Drosophila melanogaster*, a P450 enzyme of the CYP4G family oxidatively produces alkanes from fatty aldehydes (Qiu et al. 2012). The enzyme reaction allows the production of the alkane heptadecane from the fatty aldehyde octadecanal, with the release of CO_2. The biochemical and genetic mechanisms underlying the production of alkanes and alkenes in other eukaryotic systems remain to be elucidated. In this section, we will review the remainder of the alkane and alkene biosynthetic pathways for which the genetic components have been identified (Fig. 2). All of these biogenic routes are found within bacteria. They include the cyanobacterial biosynthesis of alkanes and alkenes (Sect. 2.1) catalyzed by an acyl-[acyl carrier protein] (ACP) reductase and an aldehyde-deformylating oxygenase, the production of VLC alkanes (Sect. 2.2) via the *oleABCD* gene cluster, and the production of mid-chain length terminal alkenes (2.3) that can be accomplished via three independent mechanisms.

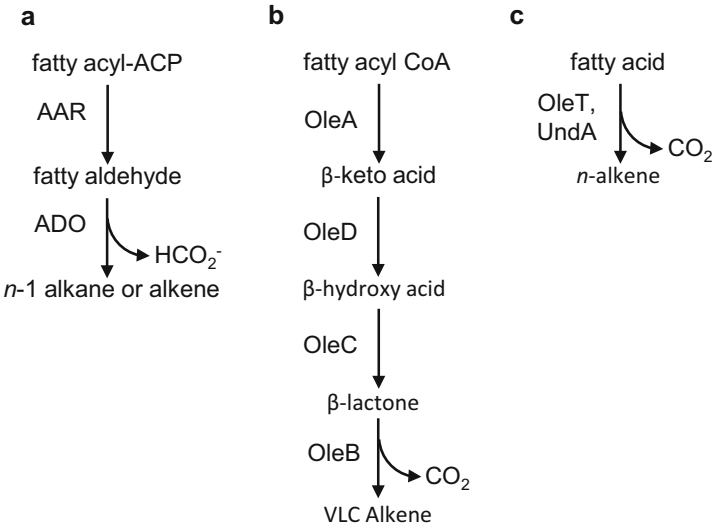

Fig. 2 Overview of bacterial alkane and alkene biogenesis. (**a**) Cyanobacterial conversion of fatty acyl-ACP molecules to C_{n-1} alkanes and alkenes via acyl-ACP reductase (AAR) and aldehyde-deformylating oxygenase (ADO) (Schirmer et al. 2010). (**b**) Biosynthesis of VLC alkenes by the *oleABCD* gene cluster enzymes OleA, OleD, OleC and OleB (Beller et al. 2010; Sukovich et al. 2010; Sugihara et al. 2010; Christenson et al. 2017b). (**c**) Biosynthesis of mid-chain length alkenes directly from fatty acids accomplished using either OleT (Rude et al. 2011) or UndA (Rui et al. 2014)

2.1 Cyanobacterial Biosynthesis of Alkanes and Alkenes

The discovery and elucidation of a two-step alkane biosynthetic pathway in cyanobacteria stimulated a renewed interest in alkane biosynthesis (Schirmer et al. 2010). The cyanobacterial alkane biosynthetic pathway comprises a two-step reaction involving, firstly, the reduction of intermediates of fatty acid biosynthesis (specifically the growing fatty acyl-ACP chain) to a fatty aldehyde and, secondly, the deformylation of the fatty aldehyde to an C_{n-1} alkane or alkene (Das et al. 2011; Warui et al. 2011). The genes responsible for this reaction were discovered through a subtractive genomics approach (Schirmer et al. 2010). Cyanobacteria benefit from reproducible reports of alkane biosynthesis (Winters et al. 1969) and the availability of whole genome sequences. An assessment of 11 cyanobacterial species for their ability to make alkanes in culture identified 10 that could synthesize alkanes and one that could not. Subtractive analysis of the available genomes identified 17 genes common to all species that produced alkanes but absent in the one species that did not. While it was possible to assign biological functions to ten of these genes, seven candidates remained without clear biological roles. Of these seven, two of these genes were hypothesized as likely candidates, and as a result they were cloned from *Synechococcus elongatus* PCC7942. These were orf1594 and orf1593. They were expressed in *E. coli*, and when combined, the result was the biosynthesis of

heptadecane (C_{17}) and pentadecane (C_{15}) (Fig. 1a). Further investigations of homologs of orf1594 and orf1593 in other cyanobacteria identified this to be the common genetic basis for alkane biosynthesis across all cyanobacteria investigated within this study (Schirmer et al. 2010).

The two genes, orf1594 and orf1593, encode an acyl-ACP reductase (AAR) and an aldehyde-deformylating oxygenase (ADO), respectively (Fig. 2b) (Schirmer et al. 2010; Das et al. 2011; Eser et al. 2011). Both AAR and ADO are soluble enzymes, and the reactions proceed in the cytosol, in contrast to the *Arabidopsis* CER1/CER3/CYTB5s and *Drosophila* CYP4G proteins which are membrane associated. The ADO enzyme is responsible for generating alkanes from fatty aldehydes, though it can also generate $C_{n\text{-}1}$ fatty aldehydes and alcohols (Aukema et al. 2013). Initially, it was suggested that the decarbonylation of fatty aldehyde produces carbon monoxide as a by-product (Schirmer et al. 2010). Further investigations with *Nostoc punctiforme* ADO heterologously expressed in *E. coli* have indicated that the by-product was in fact formate (Warui et al. 2011). The cyanobacterial ADO is a member of the non-heme dinuclear iron oxygenase family of enzymes. This family of enzymes uses molecular oxygen and a reducing system, typically ferredoxin, ferredoxin oxidoreductase, and NADPH, to reduce the enzyme at the start of each reaction. Initially, studies of ADO from *Prochlorococcus marinus* MIT9313, heterologously expressed and purified from *E. coli*, indicated that the reaction mechanism also produces formate and alkane under anaerobic conditions (Das et al. 2011). Other experiments on *N. punctiforme*, *Synechococcus* sp. RS9917 and *Synechococcus* sp. PCC6803 also exhibited alkane biosynthesis in anaerobic conditions (Eser et al. 2011). It was therefore thought that the enzyme may convert fatty aldehydes to alkane in the absence of molecular oxygen. If ADO operates in the absence of oxygen, it was hypothesized that water provides the oxygen required for the formation of formate (Eser et al. 2011). Radiolabelling studies with ^{18}O-water suggested that a hydrolytic event was solely responsible for the conversion of fatty aldehyde to its corresponding *n*-1alkane and formate (Eser et al. 2011). A reducing system is still required even under anaerobic conditions, and reaction rates were further improved using a nonphysiological reducing system, 5-methylphenazinium methylsulfate (PMS):NADPH as opposed to the ferredoxin system (Eser et al. 2011). In direct contradiction to these investigations, an equivalent study with ADO from *N. punctiforme* suggested an absolute dependence on molecular oxygen (Li et al. 2011). Later studies have confirmed this view, with labelled $^{18}O_2$ and ^{18}O-water demonstrating a strict dependence of ADO on molecular oxygen with *P. marinus* and *N. punctiforme* orthologs (Li et al. 2012). The discrepancy with the early work was attributed to oxygen contamination in a supposedly anoxic condition during enzymatic assays. To reflect the formation of formate and use of oxygen, the original nomenclature for the enzyme aldehyde decarbonylase has been changed to aldehyde-deformylating oxygenase.

Catalytic turnover of ADO is incredibly slow in vitro with single turnover rates measured in hours (Das et al. 2011; Eser et al. 2011; Warui et al. 2011). Solubility of the substrate may play a part in the slow turnover rates, as even at low concentrations, long substrates such as octadecanal form micelles. Low rates are typical for

enzymes that catalyze reactions involving insoluble substrates. Shorter-chain length substrates, such as heptanal, are also suitable substrates (Eser et al. 2011). There are also improvements in rates when the reaction is reconstituted in the presence of the endogenous cyanobacterial reducing system (Zhang et al. 2013). Reaction rates in these circumstances can be measured at 0.4 min^{-1} against octadecanal. The low activity and stability of the ADO present a significant challenge for its deployment in metabolic engineering, though it has undoubtedly proven a popular choice of enzyme for alkane biosynthesis.

2.2 Very-Long-Chain (VLC) Alkene Biosynthesis

The presence of VLC alkenes has been reported in many Gram-positive and Gram-negative bacteria (Sukovich et al. 2010a), including in *Sarcina lutea* (Albro and Dittmer 1969a, b), *Micrococcus luteus* (Beller et al. 2010), *Arthrobacter aurescens* (Frias et al. 2009), *Stenotrophomonas maltophilia* (Bonnett et al. 2011), and *Shewanella* species (Sugihara et al. 2010; Sukovich et al. 2010b). These alkenes typically fall into the range C_{25} to C_{33} and can be highly unsaturated molecules. The highly unsaturated alkene identified in *Shewanella* is shown in Fig. 1b. The biosynthesis of these long-chain hydrocarbons is proposed to be important in maintaining membrane integrity, particularly in marine environments and cold conditions (Nichols et al. 1995; Sukovich et al. 2010b). This function is not restricted to bacterial membranes, with similar physiological function proposed in some unicellular marine eukaryotes (Grossi et al. 2000). The genes responsible have been termed *ole* genes (the name derived from *ole*fin synthesis), and biosynthesis of alkenes requires the presence of three or four genes, *oleA*, *oleB*, *oleC*, and *oleD* (Sukovich et al. 2010a; Christenson et al. 2017a). A combination of biochemical deduction and genome analysis was responsible for the identification of three *ole* genes in *M. luteus* (Beller et al. 2010). In this instance, research benefited from well-documented alkene biosynthesis in the closely related *S. lutea* species (Albro and Dittmer 1969a, b). The pathway in *S. lutea* was hypothesized to involve an initial decarboxylation of a fatty acyl-CoA substrate, followed by a "head-to-head" condensation reaction. The availability of the genome from the closely related *M. luteus* allowed searches for homologs of enzymes involved in condensing reactions in fatty acid biosynthesis to identify three candidate genes (Beller et al. 2010). Of these, one corresponded closely to the β-ketoacyl-ACP synthase II (KASII) gene *fabF* and another corresponded to the β-ketoacyl-ACP synthase III (KASIII) gene *fabH*. These two genes were also present in a six-gene cluster encoding several other enzymes of fatty acid biosynthesis, strongly suggesting their role was in the synthesis of fatty acids rather than alkenes. The third gene, Mlut_13230, was also homologous to *fabH*, but the sequence similarity diverged to a greater extent than the other candidate genes. Heterologous expression of these candidate genes in *E. coli* did not result in the production of alkenes. Careful examination of the metabolite profile of *E. coli* expressing Mlut_13230 however did reveal the appearance of monoketones. This is consistent with the proposed mechanism of alkene biosynthesis. As a result,

further analysis of the *M. luteus* genome revealed that Mlut_13230 is present as part of a three-gene cluster, comprising Mlut_13230, Mlut_13240, and Mlut_13250. It was shown that expression of all three of the genes in this gene cluster in *E. coli* results in the biosynthesis of VLC alkenes (Beller et al. 2010).

In two similar studies, VLC alkenes were identified in *Shewanella oneidensis* (Sukovich et al. 2010b) and *Shewanella* sp. strain osh08 (Sugihara et al. 2010). The principal alkene produced was a highly unsaturated molecule, containing nine C=C double bonds. NMR and GC/MS analysis revealed it to be 3,6,9,12,15,19,22,25,28-hentriacontanonaene, shown in Fig. 1b. Genetic evidence indicates that the production of this alkene in *Shewanella* requires the biosynthesis of polyunsaturated fatty acids (PUFAs). Strains in which PUFA biosynthesis is inhibited through the removal of key PUFA biosynthetic genes are unable to synthesize alkenes (Sugihara et al. 2010; Sukovich et al. 2010b). Unlike in *M. luteus*, however, *Shewanella* alkene biosynthesis requires four genetic components. One of the proteins identified in *S. oneidensis*, gi 24373309, has a 31% homology at the amino acid level to Mlut_13230. Another, identified in *S. oneidensis* as gi 24373312, shows 31% homology to Mlut_13250, while the two remaining proteins both have homology to Mlut_13240 from *M. luteus*. In fact, on closer examination it is evident that Mlut_13240 is a fusion protein of these proteins (Beller et al. 2010; Sukovich et al. 2010b). Genomic analysis indicates that the occurrence of this type of "head-to-head" condensation is widespread in bacteria, and different genomic arrangements have been described (Sukovich et al. 2010a). It is now recognized that the *oleABCD* gene cluster encodes the following enzymes: *oleA* encodes a thiolase, *oleB* encodes an alpha/beta hydrolase, *oleC* encodes an AMP-dependent ligase/synthase, and *oleD* encodes short-chain dehydrogenase/reductase. None of the *ole* genes has yet been identified in the Archaea or Eukaryota (Sukovich et al. 2010a).

The biosynthesis of these VLC alkenes commences with the reaction catalyzed by the OleA thiolase (Fig. 2b). In this reaction, the non-decarboxylative Claisen condensation of two fatty acyl-CoA molecules, such as tetradecanoyl-CoA (C_{14}), results in the production of a C_{27} β-keto acid, 2-myristoylmyristic acid (Frias et al. 2011). The next step, catalyzed by OleD, an NADPH-dependent 2-alkyl-3-ketoalkanoic acid reductase, results in the production of a hydroxyl alkanoic acid (Bonnett et al. 2011). Finally, OleC has been shown to generate a thermally labile β-lactone which can spontaneously and nonbiologically decarboxylate to an alkene. However, it is currently believed that this final reaction is catalyzed in vivo by OleB – a β-lactone decarboxylase (Kancharla et al. 2016; Christenson et al. 2017b). Recent evidence indicates that OleB, OleC, and OleD enzymes assemble into a large, multiprotein complex (Christenson et al. 2017a). It is proposed that such a configuration retains the highly reactive beta-lactone intermediate produced by the OleC-catalyzed reaction from unwanted reactions – such multiprotein complexes are observed across the biological domains (for examples, see Singleton et al. (2014)). Genetic tools available in *S. oneidensis* permit the removal of the endogenous *oleA* gene and its replacement with homologs from other species. When *oleA* is replaced with the equivalent gene from *S. maltophilia*, there is an alteration in the alkene profile. In the wild-type strain, 3,6,9,12,15,19,22,25,28-hentriacontanonaene is the sole product,

whereas in the modified strain, an abundance of more saturated monoketones is produced (Sukovich et al. 2010b). Likewise, swapping in *oleA* genes from a range of diverse bacteria alters the hydrocarbon profile in such a way that it resembles the hydrocarbon profile from the donor *oleA*, rather than the host *S. oneidensis* (Sukovich et al. 2010a).

2.3 Biosynthesis of Mid-Chain Length Alkenes

The third example of hydrocarbon biogenesis in bacteria is the production of mid-chain length terminal alkenes, and there are three distinct genetic examples of how this may be achieved. These include their production via cytochrome P450-catalyzed reactions in *Jeotgalicoccus* sp. (Rude et al. 2011), via a non-heme iron oxidase in *Pseudomonas aeruginosa* (Rui et al. 2014), and via a modular polyketide synthase (PKS) in *Synechococcus* sp. (Mendez-Perez et al. 2011, 2014). These reactions produce mid-chain length 1-alkenes far shorter than those described in Sect. 2.2, with chain lengths typically in the region of C_{10} (for the non-heme iron oxidase) and C_{18} to C_{20} (for the P450 and PKS reactions). Mid-chain length terminal alkenes are of interest not just for their fuel properties but also because they can be readily derivatized; they are important precursor molecules for commodity chemicals such as plastics, lubricants, and detergents.

A reverse genetics approach was used to isolate the genes responsible for medium-chain length (C_{18} to C_{20}) alkene biosynthesis in *Jeotgalicoccus* sp. (Rude et al. 2011). *Jeotgalicoccus* species are low GC Gram-positive *Firmicutes*. Analysis of micrococci reported to produce alkenes revealed several genes that produced very-long-chain alkenes (as discussed in Sect. 2.2) and one that produced medium-chain length alkenes. This ability to produce medium-chain length alkenes rather than VLC alkenes was identified in a *Jeotgalicoccus* species, and subsequent investigation revealed this was a common feature in many *Jeotgalicoccus* species. Both feeding assays – in which media were supplemented with fatty acids – and the ability of crude cell lysates to convert fatty acids to the corresponding $C_{n\text{-}1}$ alkenes suggested direct activity on fatty acids (Rude et al. 2011). Importantly, the ability to assay alkene production in this manner permits protein purification and fractionation experiments. As a result, fractions of partially purified proteins with the ability to convert fatty acids to alkenes were identified that contained two candidate proteins. One of these was identified as orf880, and heterologous expression of orf880 in *E. coli* resulted in the production of pentadecene and 1,10-heptadecadiene. The gene was termed *oleT*, and it encodes an enzyme that is a cytochrome P450 fatty acid peroxygenase (Rude et al. 2011), specifically a member of the cyp152 subfamily of P450s. They are found in a diverse range of bacteria, including many that catalyze the hydroxylation of fatty acids. Members of this family are active using H_2O_2, rather than O_2, NADPH, or other redox partners. $OleT_{JE}$ has been shown to catalyze the formation of $C_{n\text{-}1}$ alkenes through the H_2O_2-dependent decarboxylation of C_{12}, C_{14}, C_{16}, C_{18}, and C_{20} saturated fatty acids (Belcher et al. 2014). Other P450s known

with similar activity include a P450 from *Rhodotorula minuta* that converts isovalerate to isobutene (Fukuda et al. 1994).

Another pathway for the biosynthesis of mid-chain length alkenes is found in *P. aeruginosa* (Rui et al. 2014). Discovery of the gene responsible for the biosynthesis of the 11-carbon semi-volatile 1-undecane began with feeding labelled putative substrates [12-^{13}C]dodecanoic acid and [1-^{13}C]dodecanoic acid to *P. aeruginosa* cultures. The result was the biogenic production of [12-^{13}C]undecene and [U-^{12}C11] undecene. This confirmed that the substrates are fatty acids and that the terminal carboxylic acid moiety is removed in the reaction (Rui et al. 2014). A high-throughput screen of approximately 6000 fosmid-containing *E. coli* cultures was employed and identified a single gene for 1-undecene production, termed *undA*. UndA is a small protein of 261 amino acids. Biochemical analysis reveals that Fe^{2+} is essential for its function in vitro. Moreover, while the dominant function in vivo appears to be the production of 1-undecene, it is also capable of acting on fatty acids in the range of C_{10} to C_{14}. The reaction is proposed to proceed via the sequential binding of the carboxylate moiety of the fatty acid to ferrous iron within UndA, followed by binding of O_2 to form a Fe(III) superoxide complex. Electron transfer results in the production of 1-undecene, CO_2, and H_2O and the reduction of an unstable Fe(IV) = O species. Importantly, unlike AAR/ADO and the *oleABCD* pathway, both $OleT_{JE}$ and UndA work directly on fatty acid substrates to produce the corresponding $C_{n\text{-}1}$ alkene in a single-enzyme-catalyzed reaction.

Finally, 1-alkene production by a modular synthase-encoding gene from *Synechococcus* sp. PCC 7002 has been reported (Mendez-Perez et al. 2011, 2014). *Synechococcus* sp. 7002 has been demonstrated to produce $C_{19:1}$ and $C_{19:2}$ alkenes, but interestingly, the largest fatty acid detected from *Synechococcus* sp. 7002 is a C_{18} fatty acid. This suggests that the mechanism is not analogous to the various $C_{n\text{-}1}$ mechanisms for generating mid-chain length terminal alkenes or the cyanobacterial ADO mechanism. Rather, an elongation-decarboxylation mechanism is proposed (Mendez-Perez et al. 2011, 2014). The *ols* gene (*ol*efin *s*ynthesis) was identified as a putative candidate, and Δ*ols* strains did not contain alkenes. The Ols system comprises a single, multi-domain protein and is found in only a small number of cyanobacteria (Xie et al. 2017). The biochemical steps are yet to be fully elucidated.

3 Synthetic Alkane Biosynthetic Pathways

The identification of genetic components responsible for alkane biosynthesis across the biosphere is the crucial first step in the production of petroleum-replica hydrocarbons by microbial fermentation. However, the demands the fuel industry places on the chemical and structural diversity for alkanes are not the same as the evolutionary pressure that has led to the biochemistry capable of biosynthesizing alkanes (Rude and Schirmer 2009; Jimenez-Diaz et al. 2017). Natural biosynthetic routes predominantly produce very-long-chain alkanes (e.g., *A. thaliana*, *M. luteus*), terminal alkenes (*oleT*), or a narrow range of linear alkanes (dominated by C_{17} and C_{15} in cyanobacteria). For alkane biosynthesis to be of commercial relevance, as well as

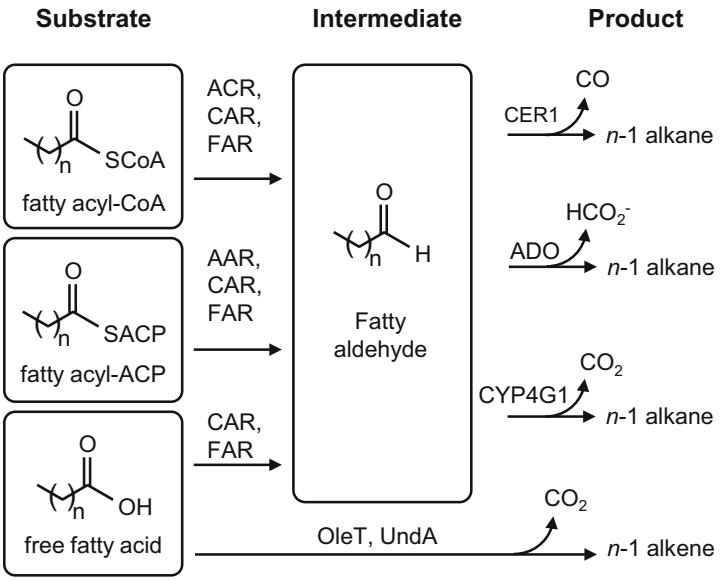

Fig. 3 Various substrates have been used as a starting point for alkane and alkene biosynthesis in genetically engineered pathways. These include fatty acyl-CoAs, fatty acyl-ACPs, and fatty acids. The enzymes that can exploit these substrates are a fatty acyl-CoA reductase (ACR) from *Acinetobacter* sp. (Yan et al. 2016), the carboxylic acid reductase (CAR) from *Mycobacterium marinum* (Akhtar et al. 2013), the fatty acid reductase (FAR) complex from *Photorhabdus luminescens* (Howard et al. 2013), OleT from *Jeotgalicoccus* sp. (Rude et al. 2011), and UndA from *Pseudomonas* sp. (Rui et al. 2014). With the exception of OleT and UndA, the other reactions generate a fatty aldehyde that can then be converted to an *n*-1 alkane or alkene using an aldehyde reductase from *Arabidopsis thaliana* (CER1) (Choi and Lee 2013), cyanobacterial aldehyde-deformylating oxygenase (ADO) (e.g., Akhtar et al. 2013; Howard et al. 2013), or CYP4G1 from *Drosophila melanogaster* (Qiu et al. 2012). Different permutations of these various steps have been successfully assembled, primarily in *E. coli* or *S. cerevisiae*

biological interest, novel pathways must be developed that permit the production of the range and diversity of chemicals blended in fuel (Fig. 3).

3.1 Altering Starting Substrates

The alkane profile of organisms is greatly influenced by the first step in the alkane biosynthetic pathway. For example, the cyanobacterial enzyme AAR reveals a preference for C_{18} over C_{16} fatty acyl-ACPs (Schirmer et al. 2010; Howard et al. 2013), while the nature of the OleA present in *Shewanella* greatly affects the resulting alkane profile (Sukovich et al. 2010a). The ability to manipulate the output of alkane biosynthetic pathways will therefore be impacted by the ability to control which acyl chains enter the biosynthetic pathway. As a result, many groups have successfully identified novel routes for providing fatty aldehydes for alkane biosynthesis.

In one study, the fatty acid reductase (FAR) complex from *Photorhabdus luminescens*, used to provide fatty aldehyde for bacterial luminescence, replaced the activity of AAR in an engineered *E. coli* (Howard et al. 2013). The *P. luminescens* FAR complex (encoded by *luxCDE*) exploits fatty acids, fatty acyl-CoAs, and fatty acyl-ACP molecules to generate fatty aldehydes (Meighen 1998), thereby widening the pool of substrates available to feed into alkane biosynthesis. When coupled with the activity of ADO, there was a change in the abundance and composition of the alkane profile that more closely reflected the composition of the fatty acid profile than did the reconstituted AAR/ADO pathway (Howard et al. 2013). In two further examples, the carboxylic acid reductase (CAR), a large, single-chain polypeptide from *Mycobacterium marinum* (Akhtar et al. 2013) and a fatty acyl-CoA reductase from *Acinetobacter* sp. M-1 (Yan et al. 2016) were coupled with ADO to enable the used of free fatty acid pools, rather than fatty acyl-ACP, as starting substrate. By using fatty acids rather than fatty acyl-ACP, the nature of the alkanes produced can be altered, and any future metabolic engineering strategies can benefit from the extensive literature on improving fatty acid yields in bacteria.

Another study exploited the plant alkane biosynthetic system, rather than ADO, but also permitted access to the fatty acid pool in *E. coli* (Choi and Lee 2013). In this instance, the authors increased the natural ability of *E. coli* cells to produce fatty acyl-CoAs from fatty acids, via fatty acyl-CoA synthetase (FadD), and coupled this activity with a fatty acyl-CoA reductase (ACR) activity from *Clostridium acetobutylicum* to reduce fatty acyl-CoAs to fatty aldehydes. The final conversion of these aldehydes to alkanes was completed following addition of an *E. coli* codon-optimized *Arabidopsis CER1* gene. This report is important in two respects. Firstly, previous attempts to heterologously express an active CER1 protein had been unsuccessful (Bourdenx et al. 2011; Bernard et al. 2012). Results indicated that CER1 and CER3 are mandatory for activity and that they rely on an interaction with their partner CYTB5s. By providing modifications upstream in *E. coli* that provide the required fatty aldehyde, the authors have been able to demonstrate that the difficulties are, at least in part, metabolic in nature; without CER3 – a putative acyl-CoA reductase – there is no substrate for CER1 to act on. Secondly, this analysis supports the assertion that CER1 catalyzes the second stage of alkane biosynthesis and is therefore informative as to the metabolic routes for alkane biosynthesis in planta.

3.2 Manipulating Chain Length

The chain length of alkanes and alkenes is one of the most crucial contributors to the properties of the fuel itself. Chain lengths in the region of C_5 to C_{10} are the dominant chain length distribution found in petrol (gasoline) fuel blends, whereas C_{12} to C_{18} chain lengths are found in jet fuels and diesel. The ability to alter the chain length is therefore of great interest and has been demonstrated by several groups independently.

The first group to demonstrate the ability to manipulate the alkane output was a team of undergraduate students from the University of Washington, competing in the International Genetically Engineered Machines (iGEM) competition in 2011 (Harger et al. 2013). They achieved this through a combination of genetic and media manipulations. To do this, the two-step cyanobacterial AAR/ADO pathway was expressed in *E. coli*. An additional modification was made through the addition of the gene encoding KASIII from *Bacillus subtilis* (*fabHB*). The KASIII enzyme from *B. subtilis*, unlike its *E. coli* counterpart, accepts a wider range of primer molecules into fatty acid biosynthesis. In addition to this genetic manipulation, they also grew their *E. coli* in the presence of the three-carbon molecule propanoate. The presence of propanoate in *E. coli* growth media increases the pool of propanoyl-CoA molecules which are available for incorporation into fatty acid biosynthesis via the introduced KASIII. This ensured that – at least for some cycles – a three-carbon compound was incorporated into the fatty acid elongation cycle as well as the normal two-carbon acetyl-CoA. The resultant fatty acids are therefore one carbon longer than the typical fatty acids. Following the C_{n-1} rule for cyanobacterial AAR/ADO alkane biosynthesis, the combined effect of these genetic and media manipulations is the production of even- as well as odd-chain length alkanes.

Entirely genetically encoded manipulations of alkane output have also been successful. For example, very-short-chain alkanes (e.g., propane) have been achieved in *E. coli* (Choi and Lee 2013; Kallio et al. 2014; Sheppard et al. 2016; Zhang et al. 2016). These manipulations are invariably made possible because of the switch from an acyl-ACP-dependent system to one that can exploit a manipulated fatty acid pool. In each instance, alkane production in *E. coli*, whether via the FadD/ACR/CER1 manipulations of Choi and Lee (2013) or via CAR (Akhtar et al. 2013; Kallio et al. 2014), FAR (Howard et al. 2013), or ACR (Yan et al. 2016), was redirected to shorter-chain alkanes by the inclusion of various thioesterases.

3.3 Production of Branched Alkanes

Branched-chain alkanes (Fig. 1c) are crucial for fuel performance, preventing stacking (gelling) of fuel at cold temperatures or altitude. To date there are no elucidated natural pathways that produce mid-chain length branched alkanes suitable for retail fuels. To demonstrate that alkane biosynthesis can be directed toward such branched molecules, *E. coli* expressing the FAR/ADO pathway was analyzed for the ability to incorporate exogenous branched fatty acids into alkanes. The results indicated that branched molecules could be used by this pathway (Howard et al. 2013). Establishing these capabilities at the genetic level in *E. coli* is complicated by the fact that *E. coli* cannot naturally synthesize branched fatty acids (Choi et al. 2000; Smirnova and Reynolds 2001) though other bacteria, notably *B. subtilis*, do produce branched fatty acids (Oku and Kaneda 1998). The difficulty in establishing branched fatty acid biosynthesis in *E. coli* is twofold. In vitro assembly of *E. coli* fatty acid biosynthesis indicates firstly that biosynthesis of branched fatty acids relies on the addition of appropriate branched primer molecules and secondly that it relies

on the addition of an alternative KASIII enzyme (Choi et al. 2000). To make branched fatty acids in *E. coli* therefore the KASIII gene from *B. subtilis* needs to be heterologously expressed, and suitable activity to generate the branched primer molecules not naturally present in *E. coli*, is also required. For this to occur a further four genes are required that code for the multienzyme branched-chain keto-dehydrogenase complex. Introducing all five genes leads to the production of branched fatty acids in *E. coli*, and the further addition of the FAR/ADO pathway results in the appearance of branched alkanes (Howard et al. 2013). The biosynthesis of short-chain branched alkanes has also been established (Sheppard et al. 2016). Taken together with the results discussed in 3.2, it is apparent that it is entirely possible to synthesize a range of linear and branched alkanes and alkenes of the chain lengths appropriate for petroleum-replacement biofuels that can be blended depending upon the required fuel properties.

4 Research Needs

Further advances in the metabolic engineering of microbes for alkane biosynthesis have naturally shifted toward increasing yields and efficiencies of the biosynthetic processes. These may be considered in four broad areas: choice of organism and growth conditions, pathway engineering, enzyme engineering, and the removal of the alkane product. In addition, further discovery of the genetics underlying alkane biosynthesis across all living systems will improve our understanding of the evolutionary pressures and biochemical diversity of alkane biosynthesis, with a resulting impact inspiring novel engineering strategies.

4.1 Choice of Chassis

While it is possible to relatively rapidly develop a proof-of-principle strain that produces a product of interest, it is far more challenging to develop a strain that meets commercial targets and fits within a biorefinery concept (Runguphan and Keasling 2014). The choice of cell factory is critical in the assessment of the industrial production of chemicals, and recently there has been a consolidation and focus on a few industrial cell factories (Rumbold et al. 2009; Vickers et al. 2010; Hong and Nielsen 2012). The proven ability of cell factories such as *E. coli*, *Corynebacterium glutamicum*, *Aspergillus niger*, *Pichia stipitis*, and *S. cerevisiae*, to perform robustly within industrial processes highlights their potential as industrial cell factories that may replace the current production of chemicals from crude oil in a sustainable way. The large-scale industrial production of cellulosic ethanol using the yeast *S. cerevisiae* ("Project Liberty": a joint venture between POET and DSM in the USA) shows the economic feasibility of using this cell factory for production of a relatively cheap, high-volume commodity and is a success story related to the production of biofuels.

The AAR/ADO pathway has been shown to operate in a wide range of microbes beyond *E. coli*, including non-AAR/ADO cyanobacterial species (Yoshino et al. 2015) and the chemoautotrophic bacterium *Cupriavidus necator* (Crepin et al. 2016). The latter is already grown as a commercial concern for production of bioplastics. Alkane biosynthesis has also been demonstrated in eukaryotic microbes such as *S. cerevisiae* (Bernard et al. 2012; Buijs et al. 2015; Kang et al. 2017) and in the filamentous fungus *Aspergillus carbonarius* (Sinha et al. 2017). It is also important to note however that highly significant gains were made in the production of semisynthetic artemisinin not only from metabolic engineering strategies but through optimization of fermentation and extraction conditions (Westfall et al. 2012; Paddon et al. 2013). It is therefore a key challenge to metabolic engineers to consider not only the optimization of the genetic components (e.g., choice of chassis and pathway engineering) but to include the optimization of environmental (i.e., fermentation) conditions at the same time (Mukhopadhyay et al. 2008).

4.2 Pathway Engineering

Exploiting fatty acid biosynthesis for fuel production is advantageous, as many species have high carbon flux into these energy-rich chemicals. Large increases in yields have been achieved in fatty acid and fatty acid-derived chemicals (reviewed in Mehrer et al. 2016), and similar methods can be applied to increasing alkane biosynthesis efficiencies. There are early examples of this being applied successfully to microbial production of alkanes. Greater alkane titers have been achieved by manipulating the supply of substrates to the pathway and removal of competitive reactions (Cao et al. 2016; Song et al. 2016). In addition to these specific genetic manipulations, advances in computer-guided metabolic engineering strategies will greatly assist these efforts (Patel et al. 2016).

4.3 Enzyme Engineering

One means of addressing the need to improve system performance is through improvements to the performance of the enzymes responsible for catalyzing the conversion from substrate (typically fatty acids) through to alkanes and alkenes. This is particularly important given the slow catalytic turnover of many of the enzymes. There are already examples of successful catalytic manipulations of both the ADO and OleT systems through their fusion to alternative reducing systems or proteins capable of removing inhibiting compounds. As a peroxygenase, $OleT_{JE}$ uses H_2O_2 as its redox partner. However, excess reactive oxygen species can cause cellular damage and result in apoptosis. The cell therefore elicits many responses leading to rapid detoxification of reactive oxygen species and the removal of a driver of $OleT_{JE}$ alkene biosynthesis. To circumvent this, studies have shown that $OleT_{JE}$ can perform H_2O_2-independent catalysis in vitro using either a flavodoxin/flavodoxin reductase system or a P450 RhFRED reductase domain from *Rhodococcus* sp. (Liu et al.

2014). Furthermore, an OleT-RhFRED fusion has been shown to perform the same enzymatic activity but supported by NADPH and oxygen in an engineered fatty acid-overproducing strain of *E. coli* (Liu et al. 2014). An alternative strategy has been to fuse $OleT_{JE}$ with an alditol oxidase (AldO) from *Streptomyces coelicolor* (Matthews et al. 2017). The addition of AldO fused to $OleT_{JE}$ enables local generation of H_2O_2 from polyols (e.g., glycerol, sorbitol, and xylitol) and an increased conversion of tetradecanoic acid to alkenes compared to direct addition of H_2O_2. For ADO, H_2O_2 has been shown to reversibly inhibit its catalytic activity. In order to circumvent this, the creation of a fusion protein consisting of ADO fused to a catalase capable of removing local H_2O_2 led to a dramatic fivefold improvement in catalytic turnover in vivo (Andre et al. 2013). In each instance these protein engineering efforts demonstrate that catalytic activity can be maintained and indeed improved when the proteins are assembled into larger structures providing optimism that different strategies for improving alkane production through protein manipulations can be achieved.

4.4 Removal of Product

As with any metabolic engineering strategy, as pathways improve and titers of target molecules increase, it is likely that toxicity of the product will become a problem. This is an underexplored area that will need to be developed to maximize outputs of petroleum-replica hydrocarbons. Manipulating efflux pumps to increase yield of target chemicals has been successful (Dunlop et al. 2011; Lennen et al. 2012; Kato et al. 2015). One of the key challenges identified in this research, however, is the need to increase efflux pump efficiency and specificity, rather than simply increasing the number of pumps. The latter scenario can result in physiological damage to membrane integrity with deleterious effects on cell survival.

4.5 Gene Discovery

Alkane biosynthesis is known in many living organisms, yet only a small number of the biochemical pathways and the underlying genetic components are so far known. Elucidation of new pathways, for example, in fungi (Gianoulis et al. 2012), mammalian waxes (Cheesbrough and Kolattukudy 1988), algae (Dennis and Kolattukudy 1992), or bacteria, will enhance our understanding of the biochemistry and enzymology involved in alkane biosynthesis. This knowledge will broaden the choices available to bioengineers for developing suitable bioprocesses and facilitate greater forward engineering of pathways for commercial alkane biosynthesis.

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Oil and Hydrocarbon-Producing Bacteria

19

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Abstract

Bacteria are recognized as a sustainable source of renewable feedstocks for production of biofuels and other chemicals. Triacylglycerols and wax esters, with potential applications similar to the ones derived from plants, can be produced by several groups of bacteria using inexpensive carbon sources, such as organic residues from industry or municipal sources. Also, aliphatic hydrocarbons, which are the main components of gasoline, diesel, and jet fuels, can be produced by some bacteria directly from sunlight and CO_2 or by other groups using renewable organic sources.

This chapter highlights the advantages and biotechnological applications of bacterial oil and hydrocarbon (O&H) production, in particular for the biofuel industry, gives an overview of the bacterial groups having this capacity, and finally outlines major research needs in the field.

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1 Introduction

Bio-based sustainable production of fuels and other chemicals has been attracting increasing interest, greatly stimulated by the need to reduce dependence on non-renewable petroleum products.

In the last years, bacteria have gain status as potential candidates to supply renewable oleochemical substitutes for petrochemicals and for agricultural oils and animal fats. Some bacteria are able to produce fatty acids (FA) and neutral lipids, such as triacylglycerols (TAG) and wax esters (WE), with similar properties to those derived from plants and animals. In addition, they present some important advantages, namely do not require fertile land and only little space is needed for their cultivation, are easy to cultivate and can be grown with a wide range of inexpensive carbon sources, such as organic residues from industry or municipal sources.

Bacterial TAG are suitable feedstocks for different types of applications. One possibility is in the production of edible oil and fats. It was shown that *Rhodococcus opacus* PD630 cultivated on octadecane produced TAG with a fatty acid profile similar to those from vegetable sources (Alvarez et al. 1996; Alvarez and Steinbüchel 2002). Moreover, engineered strains of *R. opacus* PD630 grown on gluconate were able to produce TAG similar to those present in cocoa butter oil (Wältermann et al. 2000). Bacterial TAG can also be applied in therapeutic and pharmaceutical industries as drug carriers, namely, in the preparation of vehicles for different medical compounds such as demulcents, emollients, and laxatives (Alvarez and Steinbüchel 2002). There is still a lack of information regarding the economic feasibility and health security for nutritional purposes. Nevertheless, the use of bacterial oils for edible purpose is likely hindered by social acceptance and thus is mainly restricted to animal feeding.

Additionally, bacterial lipids can be used in the production of valuable products such as cleaning and cosmetic products, detergents, paints, resins, and lubricants, among others (Wältermann et al. 2000; Alvarez and Steinbüchel 2002; Alvarez 2010). Not only bacterial TAG but also TAG-synthesizing enzymes can be important for industrial purposes. Several hydrocarbonoclastic bacteria have wax ester synthase/diacylglycerol acyltransferase (WS/DGAT) (Kalscheuer and Steinbüchel 2003; Kalscheuer et al. 2007; Alvarez et al. 2008). This is a novel bifunctional enzyme family, not found until now in other organisms, that mediates the last acylation reaction in TAG and WE synthesis. An interesting feature of this enzyme is the low substrate specificity, allowing the use of acyl groups with different compositions, forming TAG with a wide range of fatty acids. Therefore, this ability makes this enzyme a promising alternative for the production of different types of fine chemicals and/or oleochemicals (Stöveken et al. 2009).

The most prominent bacterial TAG application is for biofuel production, namely, biodiesel, which is currently undergoing a very active phase of research. Several efforts aiming to optimize bacterial TAG-based biofuel productions were already achieved by using inexpensive wastes and by-products as carbon sources (Gouda et al. 2008; Wei et al. 2015a; Kumar et al. 2015), by the development of fermentation processes at high scale (Voss and Steinbüchel 2001) and by the construction of

engineered strains with improved TAG ability biosynthesis (Kalscheuer et al. 2006; Xiong et al. 2012).

Similar to TAG, bacterial WE can also be applied to different biotechnological and industrial fields. The most significant applications are in the manufacturing of cosmetics, additives, candles, lubricants, polishes, surfactants, coatings, and ultimately in the biofuel industries (Ishige et al. 2003; Alvarez 2010; Westfall and Gardner 2011).

Besides oils, also aliphatic hydrocarbons, which are the main components of gasoline, diesel, and jet fuels, can be produced by some bacteria. Many cyanobacteria are capable of synthesizing intracellular alka(e)nes directly from sunlight and CO_2, whereas chemotrophic bacteria can produce intracellular or extracellular hydrocarbons from renewable carbon sources (Wang and Lu 2013; Fu et al. 2015). Bio-alka(e)nes have the potential to be used as advanced biofuels, presenting similar performance as the petroleum-based fuels and superior properties relatively to other biofuels (e.g., the energy content of these compounds is 30% higher than ethanol) (Wang and Lu 2013). Additional advantages over the petroleum are the fact that they do not contain any sulfur, sulfates, and polycyclic aromatic hydrocarbons; after burning, no SO_2, CO, and particulate matters are released, and the released CO_2 can be recycled by plants via photosynthesis so that air pollution such as smog can be greatly reduced. Their hydrophobic property and compatibility with existing liquid fuel infrastructure are also advantageous characteristics that facilitate their use as "drop-in" biofuels (Wang and Lu 2013; Jiménez-Díaz et al. 2017).

2 Biosynthesis and Accumulation of Neutral Lipids

The process of neutral lipid (triacylglycerols (TAG) and wax esters (WE)) production and accumulation is a wide spread and well-described phenomenon in eukaryotes. However, in prokaryotes it has only been reported in members of the *Actinomycetales* and in some marine and other Gram-negative bacteria.

TAG are reserve lipids composed by glycerol esterified with fatty acids. Generally, the chemical composition, properties, and amount of TAG in bacterial cells are determined by the type of fatty acids, carbon chain length, and the degree of fatty acid saturation and are highly influenced by the carbon source used and cultivation conditions. TAG levels increase substantially when bacterial cells reach stationary phase of growth and/or when bacteria are cultivated in nitrogen-limiting conditions, being fatty acid production channeled from phospholipid synthesis to TAG biosynthesis (Packter and Olukoshi 1995; Wältermann et al. 2005).

The biosynthesis of bacterial TAG from unrelated carbon sources (e.g., gluconate/glucose/organic acids) and related carbon sources (e.g., hexadecane) involves different metabolic pathways. When cells are grown on unrelated carbon sources, the substrate is converted to acetyl-CoA by oxidative decarboxylation of pyruvate. Acetyl-coA serves as precursor for fatty acid biosynthesis. On the other hand, during cultivation with alkanes, the cells oxidize hydrocarbons to originate fatty acids for TAG biosynthesis (Alvarez et al. 1997a; Alvarez 2003; Manilla-Pérez et al. 2011).

WE are a class of neutral lipid compounds mainly composed by oxoesters of long-chain fatty alcohols and long-chain fatty acids (Jetter and Kunst 2008). The physical properties of WE are mainly determined by the carbon chain length of fatty acyl and fatty alcohol moieties, by the degree of saturation, the level of ramification, and the presence of unusual chemical groups (Rontani et al. 1999; Patel et al. 2001; Uthoff et al. 2005). Bacterial WE are functionally similar to the other bacterial reserve compounds (Alvarez and Steinbüchel 2002). Like TAG, bacterial WE are mainly synthesized under stressful cultivation conditions, such as limited nitrogen concentrations combined with an excess of carbon source, and also under high hydrostatic conditions (Wältermann and Steinbüchel 2005; Grossi et al. 2010; Rontani 2010). The majority of WE-producing strains uses hydrocarbons as carbon and energy sources (Alvarez et al. 2000; Ishige et al. 2002; Manilla-Pérez et al. 2011); however some bacteria also produce WE from other types of substrates, namely, acetate and sugars (Alvarez 2010; Kalscheuer 2010).

2.1 The *Actinomycetales*

Biosynthesis and accumulation of TAG is thought to be common to bacteria belonging to the actinomycete group, such as *Rhodococcus*, *Mycobacterium, Streptomyces*, *Nocardia*, *Dietzia*, or *Gordonia* (Alvarez and Steinbüchel 2002) (Table 1). The genus *Rhodococcus* has been widely studied due to its ability to synthesize high amounts of TAG from different carbon sources during cultivation under nitrogen starvation conditions (Alvarez et al. 1997a; Alvarez and Steinbüchel 2002; Alvarez 2003; Silva et al. 2010; Castro et al. 2016). Within this genus, the highest TAG levels were reported in *R. opacus* PD630, i.e., 18–87% of cellular dry weight (cdw) (Table 1), when grown on defined carbohydrate and noncarbohydrate carbon sources (Alvarez et al. 1996). Members of this genus are also capable of biotransformation and degradation of different environmental pollutants (Martinkova et al. 2009; Kuyukina and Ivshina 2010). The potential for storage lipid production was demonstrated using a wide range of complex and low-cost wastes, namely, lubricant-based wastewaters, lignocellulosic biomass, fishery waste, light oil from pyrolysis, and dairy wastewater (Da Silva et al. 2016 Wei et al. 2015a, b; Palmer and Brigham 2016; Kumar et al. 2015). In these cases, lipid production ranged from 14% to 29% cdw.

In *Mycobacterium tuberculosis*, TAG were identified as crucial compounds associated with pathogenesis, whereas in *Streptomyces lividans* TAG are important in secondary metabolite production, particularly antibiotics (Table 1; Barksdale and Kim 1977; Olukoshi and Packter 1994). Moreover, *Streptomyces coelicolor* is explored regarding fatty acid (suitable for green chemistry applications) synthesis, due to its ability to use agricultural lignocellulosic residues (Dulermo et al. 2016).

The filamentous bacterium *Microthrix parvicella*, found in activated sludge systems of wastewater treatment plants, was also reported to be able to accumulate lipids. Storage lipid bodies were detected during in situ studies after addition of labeled oleic acid (Nielsen et al. 2002).

Table 1 Synthesis of lipid storage compounds in bacteria of the *Actinomycetales* group. (*cdw* cellular dry weight, *n.r.* not reported)

Bacterium	Storage compound	Carbon source	Content	Reference
Dietzia maris WR-3	WE-like compounds	n-Hexadecane	10.5 g L^{-1} medium	Nakano et al. (2011)
	TAG	n-Hexadecane	19.2% (cdw)[a]	Alvarez (2003)
Gordonia sp. KTR9	TAG	Ethanol	28 umol g^{-1} (cdw)[b]	Eberly et al. (2013)
Gordonia sp. DG	TAG	Orange waste	57.8 mg L^{-1} medium[b]	Gouda et al. (2008)
Mycobacterium tuberculosis	TAG	Glycerol	n.r.	Barksdale and Kim (1977)
Nocardia corallina	TAG	Valerate	23.9% (cdw)[a]	Alvarez et al. (1997a)
Rhodococcus opacus PD630	TAG	Gluconate	76% (cdw)[a]	Alvarez et al. (1996)
		Olive oil	87% (cdw)[a]	
		Fructose	18% (cdw)[a]	
		Acetate	31% (cdw)[a]	
		Propionate	40% (cdw)[a]	
		Pentadecane	39% (cdw)[a]	
		n-Hexadecane	38% (cdw)[a]	
		Heptadecane	28% (cdw)[a]	
		Octadecane	39% (cdw)[a]	
		Citrate	37% (cdw)[a]	Alvarez et al. (1997a)
		Succinate	22% (cdw)[a]	
		Propionate	18% (cdw)[a]	
		Valerate	38% (cdw)[a]	
		Phenyldecane	38% (cdw)[a]	
		Agro-industrial wastes	45.3 g L-1 medium[b]	Gouda et al. (2008)
		Glucose	53.9% (cdw)[a]	Kurosawa et al. (2010)
		Lignocellulosic autohydrolysates	24.8–28.6% (cdw)[a]	Wei et al. (2015a)
		Vanillic acid	14.6% (cdw)[a]	Kosa and Ragauskas (2012)
		Dairy wastewater	2 g L^{-1} medium[a]	Kumar et al. (2015)
		Light oil	25.8% (cdw)[a]	Wei et al. (2015b)
	TAG; WE	Phenyldecane	n.r.	Alvarez et al. (2002)

(*continued*)

Table 1 (continued)

Bacterium	Storage compound	Carbon source	Content	Reference
Rhodococcus opacus B4	TAG	Glucose	23.0% (cdw)[b]	Castro et al. (2016)
		Acetate	25.2% (cdw)[b]	
		Hexadecane	18.6% (cdw)[b]	
Rhodococcus opacus DSM 1069	TAG	Light oil	22% (cdw)[a]	Wei et al. (2015b)
		Glucose	17.9% (glucose);	Kosa and Ragauskas (2012)
		Vanillic acid	16.8% (4-HBA);	
		4-Hydroxybutyl acrylate (4-HBA)	6.7% (vanillic acid) (cdw)[a]	
Rhodococcus ruber	TAG	Glucose; valerate; n-hexadecane	19.0%; 12.2%; 26.0% (cdw)[a]	Alvarez et al. (1997a)
Rhodococcus aetherivorans IAR1	TAG	Acetate; toluene	24% (acetate; toluene) (cdw)[a]	Hori et al. (2009)
Rhodococcus sp. 602	TAG	Gluconate; benzoate	71.2% (gluconate);	Silva et al. (2010)
			64.9% (benzoate) (cdw)[a]	
Rhodococcus jostii RHA1	TAG	Glucose; gluconate; acetate	n.r.	Hernandez et al. (2008)
	WE	Hexadecane; hexadecanol	n.r.	
Rhodococcus sp. A5	TAG	Glucose	11.0% (glucose); 32.1% (n-hexadecane) (cdw)[a]	Bequer Urbano et al. (2013)
		n-Hexadecane		
Rhodococcus corynebacterioides DSM 20151	TAG	Glucose	9.2% (glucose) 17.9% (n-hexadecane) (cdw)[a]	Bequer Urbano et al. (2013)
		n-Hexadecane		
Streptomyces lividans	TAG	Glucose	125 mg L^{-1} [b]	Olukoshi and Packter (1994)
Pseudomonas aeruginosa 44T1	TAG	Olive oil	38% (cdw)[a]	de Andrés et al. (1991)

[a]Total amounts of cellular fatty acids
[b]Total amounts of storage compound

More recently, several genetic engineering studies have been performed to increase TAG yields as well as to confer novel substrate degradation abilities to members of actinomycete group. There are already several reports describing effective TAG production from engineered *Rhodococcus* strains using glycerol, arabinose, xylose, and levoglucosan for production of renewable biofuels (Kurosawa and Sinskey 2013, 2015a, b; Xiong et al. 2012, 2016a, b). Comba and co-workers (2014) introduced into *E. coli* a TAG production metabolic pathway from *Streptomyces*

coelicolor. This engineered strain was able to accumulate a TAG content of 4.85% cdw, using glucose as carbon source.

2.2 Marine Bacteria and Other Gram-Negative Bacteria

The production of storage lipid compounds has been described in Gram-negative bacteria, belonging to the genus *Acinetobacter*, and also in some groups of marine bacteria belonging to *Marinobacter*, *Thalassolituus*, and *Alcanivorax* genera (Scott and Finnerty 1976; Rontani et al. 1999; Manilla-Pérez et al. 2011) – see Table 2.

Most of *Acinetobacter* species accumulates preferentially WE than TAG using different carbon sources (Ishige et al. 2002; Kalscheuer et al. 2003; Santala et al. 2011a). Maximum WE content was detected in *Acinetobacter* sp. strain H01-N (17% cdw) and *Acinetobacter* sp. strain 211 (25% cdw) grown in *n*-hexadecane and olive oil, respectively (Fixter et al. 1986; Alvarez et al. 1997b). On the other hand, the genus *Alcanivorax* is able to accumulate TAG or WE, depending on the carbon source used. For example, accumulation of TAG as main neutral lipids was found in *Alcanivorax borkumensis* SK2 when grown with acetate and pyruvate (Kalscheuer et al. 2007), while this organism accumulated mainly WE when cultivated in hexadecane (Manilla-Pérez et al. 2010, 2011). Members of the genus *Marinobacter*, widely known as hydrocarbon-degrading bacteria and found only in petroleum-contaminated waters, produce exclusively WE as storage lipids. *Marinobacter squalenivorans* and *Marinobacter hydrocarbonoclasticus* produced isoprenoid WE during the degradation of squalene, phytol, and 6,10,14-trimethylpentadecan-2-one (Rontani et al. 1999, 2003). More recently, it was found that *Marinobacter hydrocarbonoclasticus* SP17 produced high levels of WE (0.47 mg mg^{-1} protein) when grown in the form of biofilm at the hexadecane-water interface (Klein et al. 2008).

In recent years, *Acinetobacter baylyi* ADP1 was genetically modified by heterologous expression of *E. coli* genes, which promoted an increase in gluconate/glucose consumption with concomitantly high WE biosynthesis (Kannisto et al. 2014). Gene deletion performed on this bacterium by Santala et al. (2011b) induced a 5.6-fold higher TAG yield.

Genes from *A. baylyi* ADP1 and *Zymomonas mobilis* were successfully cloned in *Escherichia coli*, producing considerable amounts of fatty acid ethyl esters (FAEE), using glucose and oleic acid as carbon sources. FAEE are a promising alternative to other biofuels and were considered the first microbial biodiesel, commonly known as microdiesel (Kalscheuer et al. 2006).

3 Biosynthesis of Alka(e)nes

Hydrocarbons, such as alkanes or alkenes, can be produced by a wide range of microorganisms. Within the domain Bacteria, many marine cyanobacteria are capable of synthesizing intracellular alka(e)nes directly from sunlight and CO_2, and several chemotrophic bacteria can produce intracellular or extracellular

Table 2 Synthesis of lipid storage compounds in marine and other Gram negative bacteria. (*cdw* cellular dry weight, *n.r.* not reported)

Bacterium	Storage compound	Carbon source	Content	Reference
Acinetobacter baylyi ADP1	TAG; WE	Gluconate	1.4% TAG; 6.9% WEs (cdw)[a]	Kalscheuer and Steinbüchel (2003)
	TAG	n-Hexadecane	3–4% (cdw)[a]	Reiser and Somerville (1997)
	TAG; WE	Glucose	4.0 mg L^{-1} medium (TAG)[b]	Santala et al. (2011b)
			5.2 mg L^{-1} medium (WE)[b]	Santala et al. (2011a)
Acinetobacter sp. strain H01-N	WE	n-Hexadecane	17% (cdw)[a]	Ishige et al. (2002)
				Makula et al. (1975)
	WE	n-Hexadecanol	1.9% (cdw)[a]	Singer et al. (1985)
Acinetobacter sp. strain 211	TAG	Olive oil	25% (cdw)[a]	Alvarez et al. (1997b)
Alcanivorax borkumensis SK2	TAG	Pyruvate	23% (cdw)[b]	Kalscheuer et al. (2007);
	WE	n-Hexadecane	9.2% (cdw)[b]	Manilla-Pérez et al. (2011)
Alcanivorax jadensis T9	TAG	Pyruvate	7% (cdw)[b]	Bredemeier et al. (2003);
	TAG; WE	n-Hexadecane	8.6% TAG; 13.4% WE (cdw)[b]	Manilla-Pérez et al. (2011)
Marinobacter hydrocarbonoclasticus SP17	WE	Pyruvate;	30.6% (cdw)[b]	Klein et al. (2008);
		n-hexadecane	2.4% (cdw)[b]	Manilla-Pérez et al. (2011) and Holtzapple and Schmidt-Dannert (2007)
Marinobacter sp. strain CAB	WE	Phytol; 6,10,14-trimethylpentadecan-2-one	n.r.	Rontani et al. (1999)
Marinobacter squalenivorans	WE	Squalene	n.r.	Rontani et al. (2003)

[a]Total amounts of cellular fatty acids
[b]Total amounts of storage compound

hydrocarbons from renewable carbon sources (Wang and Lu 2013; Fu et al. 2015). According to their chain length, alkanes and alkenes are categorized as short or medium chain from C3 to C12 and long chain when containing more than 13 carbon atoms. The carbon chains can be linear or branched. The hydrocarbon content and profile varies significantly among the different microorganisms (Ladygina et al. 2006; Fu et al. 2015).

The mechanisms of alka(e)ne biosynthesis are linked to fatty acid metabolism. Up to now, five different microbial pathways that convert free fatty acids or fatty acid derivatives into alka(e)nes have been identified. Synthetic biology strategies have been recently applied for optimum bio-alka(e)ne production (Wang and Lu 2013).

3.1 Cyanobacteria

Cyanobacteria are a diverse group of photosynthetic bacteria, from which several species have been reported to produce intracellular hydrocarbons, e.g., *Anabaena* (*Nostoc*) sp. PCC 7120 and *Nostoc punctiforme* PCC 73102 (more detailed information in the reviews from Ladygina et al. 2006; Wang and Lu 2013; Fu et al. 2015; Jiménez-Díaz et al. 2017). The ability of these microorganisms to synthesize alka(e)nes directly from sunlight and CO_2 is highly advantageous for industrial applications, as well as for environmental protection, and is a sustainable way of producing biofuels.

Coates et al. (2014) studied the hydrocarbon composition of 32 strains of cyanobacteria, selected from a wide phylogenetic distribution, and concluded that hydrocarbon production is a universal trait among cyanobacteria. However, the biological function of these compounds in cyanobacteria is still not understood. Some possible roles for bio-alka(e)ne production have been suggested, namely, chemical signaling, adaptation to certain conditions (e.g., desiccation), enhanced buoyancy, or membrane fluidity/stability (Jiménez-Díaz et al. 2017).

Cyanobacteria synthesize long-chain alka(e)nes using two different pathways: the "elongation-decarboxylation" pathway for alkane biosynthesis and the α-olefin synthase pathway (OLS or PKS pathway) for medium-chain α-olefin (1-alkene) biosynthesis (Coates et al. 2014). While the first is widely distributed among cyanobacteria, the second is present in only a small number of species, e.g., *Synechococcus* sp. PCC 7002, and the coexistence of these pathways was never reported (Coates et al. 2014; Yoshino et al. 2015).

Within this phylum, the presence of a variety of linear and branched alka(e)nes in the carbon range of C15–C19 has been reported, but generally heptadecane (C17 alkane) is most abundant, representing 68–98% of the total hydrocarbons produced (Ladygina et al. 2006; Jiménez-Díaz et al. 2017). Cyanobacteria possess the unique ability to produce branched C18 hydrocarbons (7- and 8-methylheptadecanes) in a ratio of 1:1.

In general, the native production of alka(e)nes range from 0.02% to 0.13% cdw (Fu et al. 2015). Nevertheless, hydrocarbon yields higher than 0.10% cdw were reported in nine cyanobacterial strains by Liu et al. (2013), with the maximum value

of 0.18% cdw in *Nostoc spongiaeforme* FACHB 130 and up to 0.26% cdw in *Pleurocapsa* sp. PCC 7516 (Coates et al. 2014).

Heterologous expression of cyanobacterial genes involved in the biosynthesis of alkanes has been performed in *E. coli* and in *Synechococcus* sp. PCC 7002, which does not synthesize alkanes, leading to the production of C15 and C17 alkanes. In *E. coli*, alkane titers higher than 300 mg L^{-1} were obtained, with more than 80% of the alkanes found outside the cells (Schirmer et al. 2010), and yields up to 0.50% cdw were reported in *Synechococcus* sp. PCC 7002 (Reppas and Ridley 2010). Overexpression of alkane biosynthetic genes in *Synechocystis* sp. PCC 6803 enhanced 8.3 times the bio-alka(e)nes yields when compared with the wild-type strain (Wang et al. 2013). The simultaneous production of alkanes and α-olefins was also promoted by molecular engineering in *Synechococcus* sp. NKBG15041c, which is a native producer of 1-alkenes. The engineered strain produced heptadecane by expressing the alkane biosynthesis genes from *Synechococcus elongatus* PCC 7942, but maximum yields obtained were only around 0.0004% cdw (Yoshino et al. 2015). The use of engineered cyanobacteria, or *E. coli* modified with cyanobacterial genes, for alka(e)ne biofuel production was patented (Reppas and Ridley 2010; Schirmer et al. 2014).

3.2 Chemotrophic Bacteria

The capacity to synthesize hydrocarbons is present in several chemotrophic bacteria. Detailed review of the different hydrocarbon-producing bacterial species can be found in Ladygina et al. (2006) and Fu et al. (2015). Briefly, intracellular production of alka(e)nes has been reported in the following microorganisms:

(i) Aerobic Gram-positive bacteria (e.g., *Micrococcus* and *Sarcina* genera) – synthesize saturated and unsaturated C14–C34 hydrocarbons
(ii) Facultative anaerobic Gram-negative bacteria of the genus *Vibrio* – produce up to 80% *n*-heptadecane
(iii) Anaerobic phototrophic bacteria from the genera *Rhodopseudomonas*, *Rhodospirillum*, *Rhodomicrobium*, and *Chlorobium* – synthesize pristane and phytane (isoprenoid hydrocarbons)
(iv) *Desulfovibrio desulfuricans*, a Gram-negative sulfate-reducing bacterium, produce alkanes in the range of C11–C35, from which *n*-C25–C35 are generally most abundant and may account for 80% of the total hydrocarbons synthesized
(v) Anaerobic Gram-positive bacteria of the genus *Clostridium* (e.g., *C. pasteurianum*) – produce mainly C11–C35 hydrocarbons, with the predominance of C18–C27 *n*-alkanes

For all these bacteria, intracellular hydrocarbon content generally ranges from 0.005% to 2.69% cdw (Ladygina et al. 2006; Fu et al. 2015; Jiménez-Díaz et al. 2017).

Bacteria from the genera *Desulfovibrio* and *Clostridium* can also produce extracellular long-chain hydrocarbons, although the chain length of these is

generally lower than the intracellular hydrocarbons (C16–C18 and C19–C21 in *D. desulfuricans* and *C. pasteurianum*, respectively) (Ladygina et al. 2006). The process for extracellular production of C14–C25 hydrocarbons of normal and iso-structure by *Desulfovibrio desulfuricans* was patented (Belyaeva et al. 1995). The synthesis of extracellular C21–C33 alkanes was also reported in *Pseudomonas fluorescens*, grown in defined medium with glucose (Ladygina et al. 2006).

The role of bacterial hydrocarbons in the metabolism remains unclear. Due to the low yields and titers, intracellular hydrocarbons are not expected to act as carbon and energy sources and are probably involved in cell wall composition or influence the properties of cytoplasmic membranes. The biosynthesis of extracellular hydrocarbons may be associated with the formation of capsules, with protective functions, or involved in cell aggregation (Ladygina et al. 2006).

The biosynthetic pathways of α-olefins in chemotrophic bacteria are different from the one reported for cyanobacteria (OLS pathway). Bacteria from the genus *Jeotgalicoccus* can synthesize terminal olefins, such as 18-methyl-1-nonadecene and 17-methyl-1-nonadecene, by a fatty acid decarboxylase $OleT_{JE}$ (a cytochrome P450 enzyme that was isolated from *Jeotgalicoccus* sp. ATCC 8456). Biosynthesis of long-chain olefins occurs in some bacteria (e.g., in *Micrococcus luteus* ATCC 4698 and *Sarcina lutea* ATCC 533) by head-to-head condensation of fatty acids (Wang and Lu 2013; Beller et al. 2015), a pathway that seems to be specific for bacteria, since it was never identified in Archaea or Eukarya (Sukovich et al. 2010).

In vivo expression of *Jeotgalicoccus* sp. ATCC 8456 genes in different *E. coli* strains resulted in a maximum titer of total hydrocarbons of 98 mg L^{-1}, corresponding to 0.3% cdw (Liu et al. 2014). The combined expression in *E. coli* of cyanobacterial genes and other alternative genes was successfully applied for the production of alkanes, leading to the synthesis of C9–C14 linear and branched alkanes with a yield of 5.81% cdw (Choi and Lee 2013). The expression of a *Bacillus subtilis* gene in addition to the cyanobacterial genes of *S. elongatus* contributed to modify the alkane product profile in *E. coli* (Harger et al. 2013). Detailed information on the use of metabolic engineering for improving bio-alka(e)ne synthesis was recently published by Fu et al. (2015), Kang and Nielsen (2017), and Jiménez-Díaz et al. (2017).

4 Research Needs

Although an intensive research on fundamental and applied aspects of bacterial oil and hydrocarbon (O&H) production has been made in the last decade, scientific research and applications on this topic are still in its infancy. A significant body of knowledge was gathered on the identification of bacterial genera or species capable of synthesizing neutral lipids or alka(e)nes, as well as on the necessary culturing conditions and carbon sources. However, up to now, the amount of hydrocarbons produced by native or engineered bacteria is low (generally less than 10% of cdw). In most cases, the accumulation of alka(e)nes, as well as neutral lipids, occurs intracellularly, which makes extraction and purification obligatory increasing process costs. Additionally, extraction procedures are not optimized. Exploring the potential of native bacteria

capable of exporting O&H, and construction of genetically improved strains with the ability to export O&H, will contribute for the development of these processes.

Several genes and metabolic pathways were studied and identified in different lipid- and alka(e)ne-producing bacteria. Moreover, some metabolic engineering studies were performed in order to increase the yields and titers. With the increasing availability of more powerful molecular biology and omics technologies, it will be possible to get the whole picture concerning the regulatory basis and interactions between different metabolic pathways. In this way, it is expected to improve neutral lipid/alka(e)ne biosynthesis in native and modified bacteria, as well as to rationally design oil-based compounds with suitable properties for biofuels and other industrial applications.

Cultivation of O&H-producing bacteria can be performed in bioreactors, which facilitates the industrial large production of biofuels. However, few attempts were made on the scale-up of the process. Commercialization and entry into the market of bacterial O&H is still not feasible, and thus the development of scalable and cost-effective biotechnological process based on O&H bacterial synthesis is one of the most challenging research needs.

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