



MASTERARBEIT / MASTER'S THESIS

Titel der Masterarbeit / Title of the Master's Thesis

„A cumulative work on different aspects of biological
methanogenesis“

verfasst von / submitted by

Stadlbauer Michael, BSc

angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Master of Science (MSc)

Wien, 2022 / Vienna 2022

Studienkennzahl lt. Studienblatt /
degree programme code as it appears on
the student record sheet:

UA 066 830

Studienrichtung lt. Studienblatt /
degree programme as it appears on
the student record sheet:

Masterstudium Molecular Microbiology, Microbial Ecology
and Immunobiology

Betreut von / Supervisor:

Mag. Mag. Dr. techn. Simon Karl-Maria Rasso Rittmann,
Privatdoz., Bakk.

Mitbetreut von / Co-Supervisor:

Dr. Jennifer Zwicker, Bakk. MSc

Zusammenfassung

In dieser kumulativen Masterarbeit sind zwei Projekte zusammengefasst, an denen im Rahmen des praktischen Teils meines Studiums gearbeitet wurde. Beide Teile, die sich inhaltlich voneinander unterscheiden haben jedoch den Fokus auf den Stoffwechsel anaerober Archaeen gemeinsam, den Methanogenen. Der erste Teil befasst sich mit einem biotechnologischen Ansatz, bei dem ein carboxydotrophes und hydrogenogenes Archaeon in einer künstlichen Ko-Kultur mit ausgewählten hydrogenotrophen Methanogenen kultiviert wurde, um Biomethan zu produzieren. Diese anwendbare Methode zur Biomethanproduktion ist hier als Teil einer schon veröffentlichten Publikation enthalten. Im zweiten Teil der Arbeit geht es um die Entdeckung eines Eisenoxid-Minerals, dem Magnetit, in der methanogenen Zone in marinen Sedimenten. Hier war das Ziel, den notwendigen Schritt der Magnetitbildung, der Eisenreduktion, an die im marinen Sediment vorherrschende, methylotrophe Methanogenese zu koppeln. Hierbei lag der Fokus auf den Methanosarcinales und deren Fähigkeit ihren Metabolismus an einen externen Elektronentransfer zu verknüpfen. Dieser Teil der Arbeit wurde theoretisch und praktisch in einem noch unveröffentlichten Manuskript zusammengefasst und ist ebenfalls hier enthalten.

Abstract

In this cumulative master's thesis two projects are summarized which were part of the practical part of my master study. Both parts, which differ with regards to content, have one important similarity. The metabolism of the strictly anaerobic methanogenic archaeal organisms, the methanogens. In the first chapter a biotechnological approach is summarized in which a carboxydutrophic hydrogenogenic archaeon and hydrogenotrophic methanogens are cultivated in an artificial co-culture with the aim of biomethanation, hence biogas production. This successful and applicable approach is summarized in an already published paper and is part of this thesis. The second chapter deals with the findings of a novel magnetite in the methanogenic zone of marine sediments. The goal was to link iron reduction to methylotrophic methanogenesis, the predominant methanogenic pathway in marine sediments. Methanosarcinales, with their capability to link their metabolism to external electron transfer were selected as representative organisms. This part is summarized theoretically and methodically in a yet unpublished manuscript and is also part of this thesis.

Table of contents

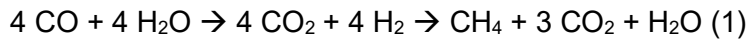
1	Introduction	7
2	Results.....	8
2.1	Chapter 1	8
2.2	Biomethanation of Carbon Monoxide by Hyperthermophilic Artificial Archaeal Co-Cultures	10
2.3	Chapter 2	23
2.4	Iron reduction and possible magnetite precipitation by methylotrophic Methanosarcinales.....	27
3	Discussion	45
4	References	48

1 Introduction

Methane (CH₄) is a colorless, odorless but explosive, and highly effective greenhouse gas, contributing to current global warming (Dean et al., 2018). Despite showing a lower abundance than carbon dioxide (CO₂) in the atmosphere, CH₄ has a higher global warming potential (GWP) (Myhre et al., 2013). Nevertheless, CH₄ is a naturally occurring gas and is used extensively as a fuel and therefore energy resource. As a main constituent of natural gas, which is known as fossil fuel, CH₄ can be divided into the already well-known non-renewable resource, but also more recently as a renewable resource, in the form of biogas, from the 5th generation of the proposed biofuel generations (Porqueras et al., 2012) where in contrast no biomass is destroyed, but generated. This 5th generation is based on the energy metabolism of strictly anaerobic archaea belonging to the order Euryarchaeota (Liu & Whitman, 2008) but also the orders Crenarchaeota, Halobacterota and Thermoplasmatota as recently demonstrated by Lyu et al., (2018) which makes them phylogenetically more diverse than previously assumed. This archaeal energy metabolism is referred to as microbial or biological methanogenesis. Methanogenesis is the last step of the global carbon cycle in which the strictly anaerobic methanogenic archaeal organisms (methanogens) recycle organic matter produced in primary production. Methanogens thrive in a broad range of ecosystems from marine sediments to landfills and also in gastrointestinal tracts of mammals (Liu & Whitman, 2008), as well as within a wide spectrum of temperatures ranging from subzero (Reid et al., 2006) to over 100°C (Kurr et al., 1991). Depending on the aforementioned factors, methanogens are able to utilize different substrates which include mainly: H₂/CO₂, the prevalent form of methanogenesis, where molecular hydrogen (H₂) donates electrons to reduce CO₂, the methyl-group type (methanol, methylamines), where these methylated C₁ molecules are partly oxidized to CO₂ donating electrons to reduce them to CH₄, and the acetate pathway, where this C₂ molecule is also partly oxidized to CO₂ donating electrons to produce CH₄. (Liu & Whitman, 2008; Thauer et al., 2008). A few methanogens, mainly Methanosarcinales (Bott et al., 1986; Rother & Metcalf, 2004) but also *Methanothermobacter* spp. (Daniels et al., 1977) are even able to metabolize carbon monoxide (CO). Furthermore, Methanosarcinales exhibit cytochromes, electron carrier proteins (Kühn et al., 1983), and methanophenazine, hydrophobic phenazine derivatives and cofactors, which additionally play a major role in electron transport (Abken et al., 1998). Interestingly the presence of cytochromes and hyperthermophilic methanogenesis are negatively correlated since no organism in this range exhibits them and they seem to be more correlated to lower temperatures (Thauer et al., 2008).

For this **cumulative master's thesis** two different projects were performed. First, a more biotechnological approach with the establishment of an artificial co-culture for biomethanation (Zipperle et al., 2021) using the hyperthermophilic, carboxydophilic, hydrogenogenic archaeon *Thermococcus onnurineus* (Bae et al., 2006) performing the water-gas shift reaction, and three hyperthermophilic autotrophic hydrogenotrophic Methanocaldococcus, with focus on

Methanocaldococcus villosus (Bellack et al., 2011), according to following equation:



The second project was a basic research approach based on findings of (Lin et al., 2020) where a novel occurrence of authigenic magnetite was discovered in the methanogenic zone in marine sediments. The goal was to link magnetite formation to methylotrophic methanogenesis of certain Methanosarcinales and their capability of external electron transfer using cytochromes (Holmes et al., 2019). *Methanosarcina acetivorans* was used as the representative organism.

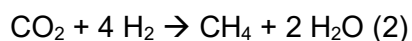
Since in both cases methanogens were involved, following text will focus on the organisms used, and their metabolism regarding energy conservation will be described in more detail.

2 Results

2.1 Chapter 1

***Methanocaldococcus villosus*, hydrogenotrophic methanogenesis without cytochromes:**

M. villosus was first isolated from a hydrothermal system in northern Iceland. Since this archaeon is hyperthermophilic the temperature for growth ranges from 55 to 90°C with an optimum of 80°C. Regarding pH and salinity the optimum is 6.5 and 2.5% (w/v) NaCl respectively (Bellack et al., 2011). Interestingly this organism exhibits polar orientated flagella which are used for active movement and considering size make *M. villosus* the fastest organism on Earth (Herzog & Wirth, 2012). Additionally the flagella are used for cell to cell contact and adherence to surfaces (Bellack et al., 2011). Regarding energy conservation this organism solely thrives on CO₂ reduction via H₂, making it a representative organism for hydrogenotrophic methanogenesis without cytochromes. Summarized this pathway is based on following equation:



in which CO₂ is reduced successively to CH₄ in the extensively described methyl branch (Buckel & Thauer, 2013; Liu & Whitman, 2008; Thauer et al., 2008) (Fig.1).

Here CO₂ is fixed and binds to methanofuran (MFR), which then is further reduced to the formyl level through electrons from the reduced ferredoxin (Fd), which gets the electrons from the original electron donor H₂ (Thauer et al., 2008). The formyl group from MFR is then transferred to tetrahydromethanopterin (H₄MTP) which is dehydrated to methenyl- H₄MTP and furthermore reduced to methylene-, and methyl- H₄MTP. These steps use electrons from F₄₂₀H₂ for reduction. The methyl group is then transferred to the coenzyme M (CoM) which is an exergonic step, and involved in energy conservation by creating an electrochemical sodium

ion potential for ATP synthesis (Thauer et al., 2008). Then CoM is reduced to CH₄ through the methyl coenzyme reductase (Mcr) with electrons from the coenzyme B (CoB). CoB and CoM form a heterodisulfide (CoM-S-S-CoB) which is getting reduced through the heterodisulfide reductase, and thereby regenerating the coenzymes. This is the second exergonic and energy conserving step, in which reducing CoM-S-S-CoB and reducing Fd is coupled (Thauer et al., 2008). The reduced Fd is again used to fix CO₂.

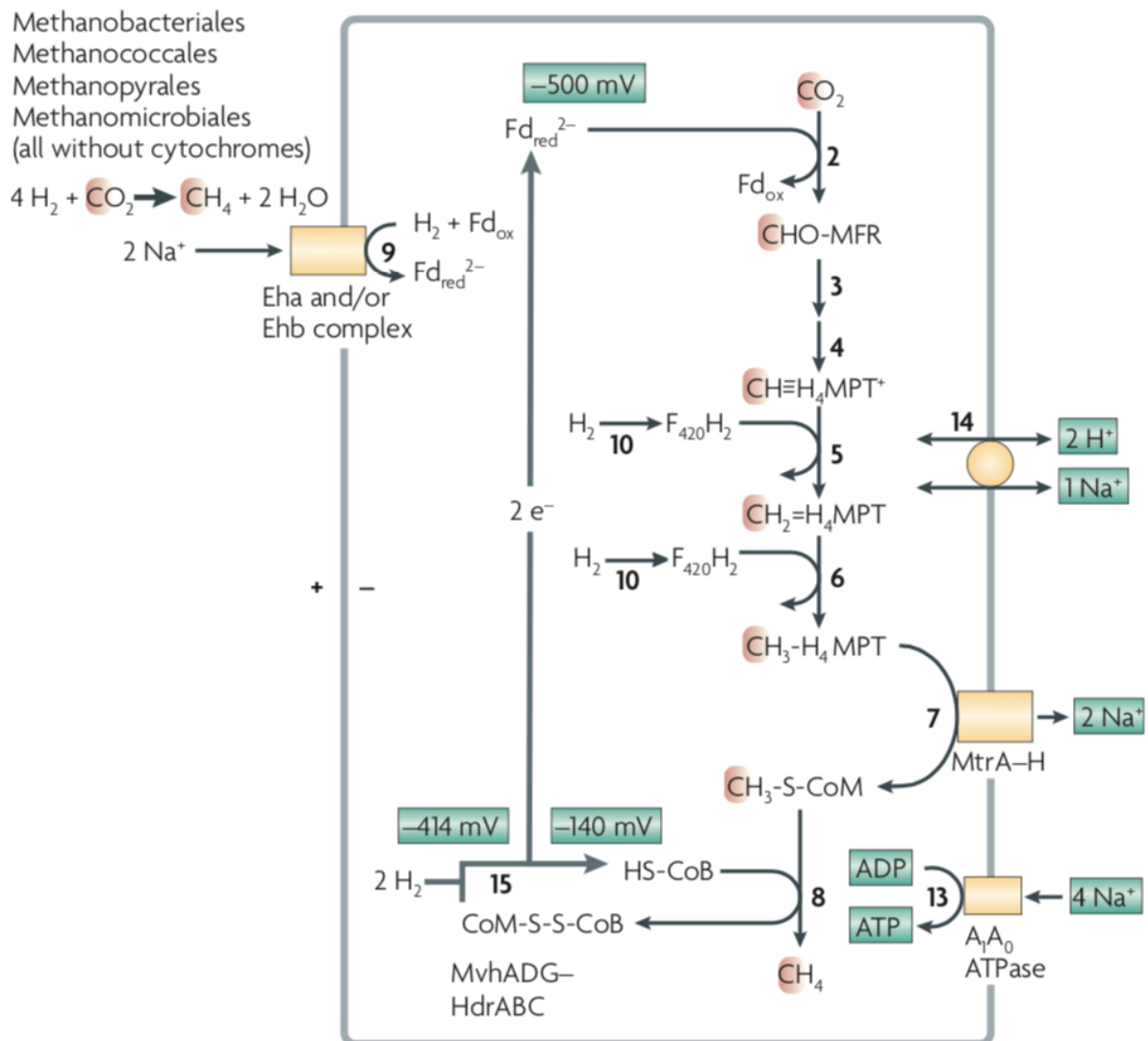


Fig. 1: Methyl branch and CO₂ reduction with H₂ to CH₄. Energy conservation pathway for hydrogenotrophic methanogens without cytochromes. Figure taken from Thauer et al., 2008

Based on this metabolic pathway (Fig.1) combined with the capability of *T. onnurineus* to oxidize CO for ATP synthesis (Rittmann et al., 2015) a successful and applicable approach for biomethanation was established using pure CO or synthetic CO-containing industrial waste gases (Zipperle et al., 2021).

2.2 Biomethanation of Carbon Monoxide by Hyperthermophilic Artificial Archaeal Co-Cultures

Published in MDPI Fermentation

Personal contributions: Investigation

Article

Biomethanation of Carbon Monoxide by Hyperthermophilic Artificial Archaeal Co-Cultures

Aaron Zipperle ^{1,†} , Barbara Reischl ^{1,2,†}, Tilman Schmider ¹, Michael Stadlbauer ¹, Ivan Kushkevych ³ , Christian Pruckner ¹, Monika Vítězová ³  and Simon K.-M. R. Rittmann ^{1,2,*} 

¹ Archaea Physiology & Biotechnology Group, Department of Functional and Evolutionary Ecology, Universität Wien, 1030 Wien, Austria; aaronzipperle@gmail.com (A.Z.); barbara.reischl@univie.ac.at (B.R.); tilman.schmider@univie.ac.at (T.S.); a01005948@unet.univie.ac.at (M.S.); greeese@gmail.com (C.P.)

² Arkeon GmbH, 3430 Tulln an der Donau, Austria

³ Department of Experimental Biology, Faculty of Science, Masaryk University, 60200 Brno, Czech Republic; kushkevych@mail.muni.cz (I.K.); vitezova@sci.muni.cz (M.V.)

* Correspondence: simon.rittmann@univie.ac.at; Tel.: +43-4277-76513; Fax: +43-4277-876513

† These authors contributed equally to this work.

Abstract: Climate neutral and sustainable energy sources will play a key role in future energy production. Biomethanation by gas to gas conversion of flue gases is one option with regard to renewable energy production. Here, we performed the conversion of synthetic carbon monoxide (CO)-containing flue gases to methane (CH₄) by artificial hyperthermophilic archaeal co-cultures, consisting of *Thermococcus onnurineus* and *Methanocaldococcus jannaschii*, *Methanocaldococcus vulcanius*, or *Methanocaldococcus villosus*. Experiments using both chemically defined and complex media were performed in closed batch setups. Up to 10 mol% CH₄ was produced by converting pure CO or synthetic CO-containing industrial waste gases at a high rate using a co-culture of *T. onnurineus* and *M. villosus*. These findings are a proof of principle and advance the fields of Archaea Biotechnology, artificial microbial ecosystem design and engineering, industrial waste-gas recycling, and biomethanation.

Keywords: Archaea Biotechnology; anaerobic microbiology; methanogenesis; biohydrogen; biological gas conversion



Citation: Zipperle, A.; Reischl, B.; Schmider, T.; Stadlbauer, M.; Kushkevych, I.; Pruckner, C.; Vítězová, M.; Rittmann, S.K.-M.R.

Biomethanation of Carbon Monoxide by Hyperthermophilic Artificial Archaeal Co-Cultures. *Fermentation* **2021**, *7*, 276. <https://doi.org/10.3390/fermentation7040276>

Academic Editor: Christian Kennes

Received: 18 October 2021

Accepted: 22 November 2021

Published: 25 November 2021

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

In the European Union, around 70% of primary energy is generated by the combustion of fossil fuels, contributing about 78% (3367 Tg-CO₂ equ.) of the total emitted greenhouse gases [1,2]. A transition to a carbon dioxide (CO₂)-neutral and sustainable energy production system is urgently needed. One of the possibilities is to utilize the power-to-gas process [3–5]. Within this process, biomethanation of CO₂ to methane (CH₄) offers a sustainable opportunity to enable the transition from fossil fuels, as it is an autocatalytic process. Therefore, it is envisioned that biomethanation will become an essential part of future energy production systems, as CH₄ could be produced at a stable pace and stored in vast amounts in the natural gas grid network [6]. Pure cultures of methanogenic archaea (methanogens) [7–12] and enrichment cultures containing methanogens [13–17] can be utilized for in situ or ex situ biomethanation [18–20].

Carbon monoxide (CO)-containing rich waste gases are a by-product of industrial processes such as steelmaking [21]. CO-containing gases can also be obtained by gasification of carbon-rich materials, such as domestic organic waste or lignocellulose conversion to syngas [22]. The fact that biofuel production directly from lignocellulose is still costly and biotechnologically challenging makes microbial gas to gas conversion from CO a promising alternative bioprocess [7,23]. Developments in the transformation of gaseous waste products to energetically valuable compounds emphasize the potential for syngas as a substrate [24–27]. CO has a high potential for donating electrons, making it a favourable

substrate for lithotrophic microorganisms [28,29]. Besides CO, waste and syngas mainly consist of molecular hydrogen (H₂), CO₂, and CH₄. As early as 1990, it was demonstrated that methanogens can metabolize some components from syngas [30].

The direct conversion of pure CO to CH₄ was performed by hydrogenotrophic methanogens and subsequently analysed [31–34]. Furthermore, growth adaption to CO did not change the CH₄ production rates significantly in the case of *Methanothermobacter marburgensis*, and the specific growth rate (μ) of *Methanothermobacter thermoautotrophicus* on pure CO was only a hundredth of the growth rate achieved using H₂:CO₂ as a substrate [31]. This led to the assumption that an artificial co-culture, where CO is converted to CH₄ in a successive bioprocess, would lead to higher efficiency, as microorganisms specifically adapted to the task of converting CO and producing CH₄ can be selected. Studies showed that the independent performance of the water gas shift reaction (WGSR) and biomethanation by two different organisms in the same vessel resulted in a more than 20-fold faster conversion than direct conversion by a single organism [34]. Therefore, we hypothesized that an artificial co-culture of a carboxydutrophic, hydrogenogenic microbe with a hydrogenotrophic, autotrophic organism would drive favourable thermodynamic conditions for the WGSR. These conditions are created by direct removal of the gaseous metabolic end products of the WGSR, that is H₂:CO₂, by the methanogen. The conversions that are successively performed by the two organisms can be summarized with the following equation:



Overall, studies using a co-culture approach showed promising results for biomethane production rates [34,35]. Based on a previous study where a bacterial/archaeal co-culture was utilized [36], we wanted to investigate the potential of artificial archaeal co-cultures consisting of a carboxydutrophic, hydrogenogenic archaeon, and different hydrogenotrophic, autotrophic, and methanogenic archaea for biomethanation. We selected *Thermococcus onnurineus* for performing the catalysis of the WGSR, as it was shown that μ and H₂ productivity on CO was substantially higher compared to other carboxydutrophic and hydrogenogenic microbes [37–40]. To catalyse the second part of the reaction, *Methanocaldococcus jannaschii*, *Methanocaldococcus vulcanius*, and *Methanocaldococcus villosus* were selected, because of their similar cultivation requirements to *T. onnurineus* with respect to temperature, salt concentration, and pH optimum. All four organisms were isolated from deep-sea hydrothermal vents and belong to the Euryarchaeota. They are able to grow in a temperature range of 63 to 86 °C, a salt concentration of 1 to 5%, and a pH of 5.5 to 7.0. *T. onnurineus* is a heterotroph, while the methanogens are chemolithoautotrophs [41–44]. Moreover, hyperthermophilic organisms are more advantageous over mesophiles, since at higher temperatures, a faster conversion of CO by the carboxydutrophic microorganism occurs, and a three times faster removal of H₂ is obtained by the methanogen [34,45]. Therefore, the properties of a hyperthermophilic environment positively affects growth and conversion rates and is, thus, advantageous over mesophilic conditions. Here, we analysed whether *T. onnurineus* together with one of the three methanogens can be grown as a powerful artificial archaeal co-culture to efficiently generate biological CH₄ from synthetic waste gases.

2. Materials and Methods

2.1. Chemicals

CO (99.999 Vol.-%), H₂:CO (60 Vol.-% in CO), H₂:CO₂ (80 Vol.-% in CO₂), and an artificial CO-containing syngas (CO₂ 16.7 Vol.-%, H₂ Vol.-% 16.8%, CH₄ Vol.-%14.7, N₂ Vol.-% 14.5%, and CO 37.3 Vol.-%) were used for closed batch experiments. For gas chromatography (GC), H₂ (99.999 Vol.-%), CO₂ (99.999 Vol.-%), CO (99.999 Vol.-%), H₂/CO₂ (80 Vol.-% in CO₂), H₂/N₂ (4.5 Vol.-% H₂ in N₂), CH₄ (99.995 Vol.-%), and the standard test gas (Messer GmbH, Wien, Austria) (containing 0.01 Vol.-% CH₄, 0.08 Vol.-% CO₂ in N₂) were used in addition to the gases mentioned above. All gases, except the standard

test gas, were purchased from Air Liquide (Air Liquide GmbH, Schwechat, Austria). All other chemicals were of the highest grade available.

2.2. Media

Medium A is a modified version of the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) medium 282. The exact composition of medium A and medium B can be found in Supplementary Materials, Tables S1 and S2. Balch's vitamin solution was used [46]. For *M. marburgensis* and *M. thermautotrophicus*, a phosphate-buffered medium was used [47].

2.3. Strains and Cultivation Conditions

The strains *M. jannaschii* JAL-1, *M. villosus* KIN24-T80, *M. vulcanius* M7, *M. marburgensis* DSM 2133 (Marburg), and *M. thermautotrophicus* DSM 1053 (delta H) were purchased from the DSMZ. *T. onnurineus* NA1 was provided by Prof. Dr. Sung Gyun Kang (Korea Institute of Ocean Science and Technology (KIOST), Ansan, Korea).

Every cultivation was performed in closed batch mode [48]. Experiments were conducted in 120 mL serum bottles (Ochs Glasgeraetebau, Langerwehe, Germany), sealed with a 20 mm butyl rubber stopper and an aluminium crimp cap (Chemglass Life Science LLC, Vineland, NJ, USA). Serum bottles were filled with the corresponding medium and sealed. Anaerobic conditions were created by evacuating and re-pressurizing with H₂:CO₂ (4:1) to 0.5 barg five times. The bottles were autoclaved and stored at 4 °C until further use. Unless otherwise stated, the medium was augmented with the following sterile filtered stock solutions before inoculation: NaHCO₃, L-Cysteine-HCL, and Balch's Vitamin solution [46] (see Supplementary Materials, Tables S1 and S2). Afterwards, the bottles were flushed with H₂:CO₂ (4:1) to 0.5 barg, before the addition of autoclaved Na₂S·9H₂O.

The inoculum, 1 mL (2% v/v) of an actively grown culture, was added anaerobically. The final liquid phase in each serum bottle added up to 50 mL. Depending on the experiment, the headspace gas phase was exchanged with the corresponding gas. Bottles were incubated at 80 °C in either a double-layer shaking incubator at 100 rpm (LABWIT ZWYR-2102C, Labwit Scientific Pty Ltd., Burwood East, Australia) or in a water bath at ~100 lateral shakes per minute (GFL 1083, LAUDA-GFL, Burgwedel, Germany).

2.4. Pure-Culture Closed Batch Experiments

The methanogens *M. villosus*, *M. vulcanius*, and *M. jannaschii* were grown under 2 barg H₂:CO₂ in either medium A or B. A reduced version of them without vitamins, yeast extract, trace elements, or a combination of the three was also tested. The incubation rhythm consisted of 13 and 7 h incubation periods, with 2 h of sampling in between every period. The same rhythm was applied for cultivation of *T. onnurineus*. It was grown in the same medium, but under 1 barg CO. These incubation periods did not apply for *M. marburgensis* and *M. thermautotrophicus*, because of their slow growth. Furthermore, they were grown in a phosphate-buffered *M. marburgensis* medium with either 1.7 barg CO, 1.7 barg H₂:CO (4:1), or 1.7 H₂:CO₂ (4:1).

2.5. Co-Culture Closed Batch Experiments

The co-cultures consisted of *T. onnurineus* and either *M. villosus*, *M. vulcanius*, or *M. jannaschii*. The experiments were conducted according to two general schemes. In the first setup, the experiment started by growing the respective *Methanocaldococcus* strain under 2 barg H₂:CO₂ first. After 13 h in the incubator, *T. onnurineus* was added. The bottles were then pressurized with CO, H₂:CO, or artificial syngas to 1 barg. In the second setup, *T. onnurineus* grew 13 h under pure CO, H₂:CO, or artificial syngas before one of the *Methanocaldococcus* strains was added. From this point onward, both schemes followed the same incubation rhythm, as described in the pure-culture experiments, and ended after a maximum of 80 h of cumulative incubation time. Every experiment was performed in hexuplicates (n = 6) on media A and B, as well as on reduced versions of them.

2.6. Sampling

The routinely performed sampling consisted of removing the cultures from the incubator and measuring the pressure as soon as the bottles cooled down to room temperature (~60 min). To analyse growth, 0.7 mL of the cultures were withdrawn for optical density (OD) measurements ($\lambda = 578$ nm). Lastly, the bottles were flushed and re-pressurized with the corresponding gas.

2.7. Analytical Procedures

OD of the cultures was measured via a spectrophotometer at $\lambda = 578$ nm (Specord 200 Plus, Analytik Jena, Jena, Germany). The headspace gas composition before and after inoculation in pure cultures was determined via the gas headspace pressure difference [18,47,49]. Gas evolution and uptake rates were calculated according to methods described in refs. [47,50,51]. Samples that were withdrawn for gas chromatography (GC) analysis did not undergo the sampling procedure. The headspace gas composition in co-culture experiments was analysed via GC, and the evolution and uptake rates were calculated [52]. Some of the negative controls revealed “air contamination” and were removed from the calculations of the results. To maintain the correct atmosphere, OD was measured after completion of the GC run and as such is only an estimate of the true OD.

3. Results

3.1. Growth Kinetics of Methanogens in Defined Medium

Growth and gas conversion by the methanogens *M. villosus*, *M. vulcanius*, and *M. jannaschii* were analysed in defined versions of media A and B on $H_2:CO_2$ (4:1), by removing complex components. Removal of the trace elements from the media hindered growth. *M. villosus* showed a higher methane evolution rate (MER) than the other two methanogens in medium A and B (Figure 1).

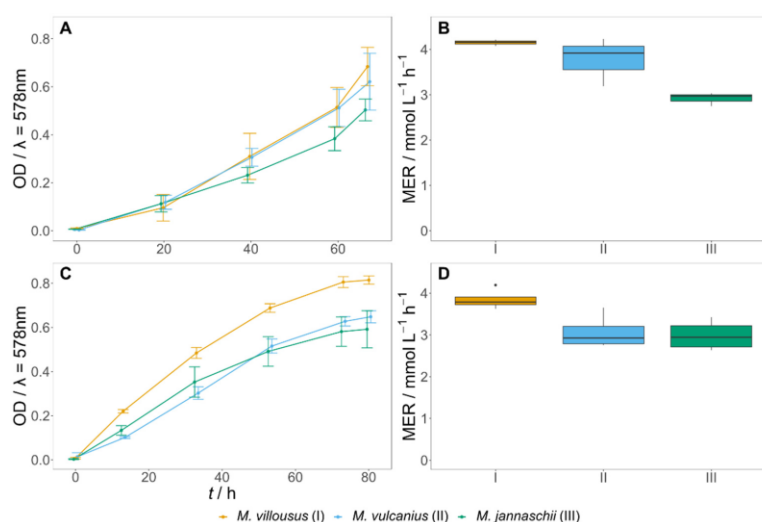


Figure 1. Growth kinetics and MER of *M. villosus*, *M. vulcanius*, and *M. jannaschii*. (A,B) grown in medium A; (C,D) grown in medium B. Both media were without yeast extract and vitamins. Error bars in the line graphs show the standard deviation. All experiments are $N = 1$, $n = 3$. However, $N = 2$, $n = 3$ for *M. villosus* using medium B.

M. marburgensis and *M. thermotrophicus* were grown on CO, H₂:CO (3:1) and H₂:CO₂ (4:1) [53]. The analysis showed that *M. marburgensis* and *M. thermotrophicus*, when grown on H₂:CO₂ (4:1), had a high turnover, and furthermore, experiments on H₂:CO resulted in poor CH₄ conversion. No growth was observed on pure CO (see Supplementary Materials, Figure S1, Table S5). Due to these findings, *M. marburgensis* and *M. thermotrophicus* were excluded from the co-culture experiments.

3.2. *T. onnurineus* Grown on CO

T. onnurineus was grown individually in a pure culture in media A and B. Results indicated that the organism reached higher OD₅₇₈ when grown on medium A as well as achieved a higher gas conversion than in media B. Omission of vitamins from medium B showed no difference regarding the H₂ evolution rate (HER) (see Supplementary Materials, Table S6, Figure S2).

3.3. Artificial Archaeal Co-Culture Engineering

Conversion of CO to CH₄ and CO₂ was performed with a co-culture consisting of *T. onnurineus* together with either *M. villosus*, *M. jannaschii*, or *M. vulcanius*. After pre-growth of the methanogens for 13 h, the cultures were inoculated with *T. onnurineus*. Growth and gas rates were analysed for all three co-cultures in media A and B (Tables 1 and 2, Figure 2). Co-cultures in medium A had relatively similar MERs ranging from 1.4 to 1.6 mmol L⁻¹ h⁻¹, whereas in medium B, the co-culture consisting of *M. villosus* and *T. onnurineus* achieved MERs between 1.6 and 2.0 mmol L⁻¹ h⁻¹. In general, the measured mean gas evolution and uptake rates were either equal or slightly higher in medium B. The higher CO uptake rate (COUR) and CO₂ evolution rate (CER) in medium B indicated that *T. onnurineus* performed better under this condition. It is likely that the H₂ uptake rate (HUR) of the methanogens positively influenced the COUR of *T. onnurineus* by creating favourable thermodynamic conditions [34].

Table 1. Mean gas evolution and uptake rates of the co-cultures cultivated in medium A on 100% CO¹.

Co-Culture	MER/mmol L ⁻¹ h ⁻¹	HUR/mmol L ⁻¹ h ⁻¹	CUR/mmol L ⁻¹ h ⁻¹	COUR/mmol L ⁻¹ h ⁻¹	CER/mmol L ⁻¹ h ⁻¹
<i>M. villosus</i> + <i>T. onnurineus</i>	1.4 ± 0.4	6.4 ± 1.5	1.4 ± 0.4	6.4 ± 1.5	5.0 ± 1.1
<i>M. vulcanius</i> + <i>T. onnurineus</i>	1.6 ± 0.3	7.0 ± 1.3	1.6 ± 0.3	7.0 ± 1.3	5.4 ± 0.9
<i>M. jannaschii</i> + <i>T. onnurineus</i>	1.5 ± 0.4	6.5 ± 1.5	1.5 ± 0.3	6.5 ± 1.5	5.0 ± 1.2

¹ Measurements were taken after a 7 h incubation period. Data collected at indicated timepoints in Figure 2 A. (N = 1, n = 6). Values are shown with standard deviation.

Table 2. Mean gas evolution and uptake rates of the co-cultures cultivated in medium B on 100% CO¹.

Co-Culture	MER/mmol L ⁻¹ h ⁻¹	HUR/mmol L ⁻¹ h ⁻¹	CUR/mmol L ⁻¹ h ⁻¹	COUR/mmol L ⁻¹ h ⁻¹	CER/mmol L ⁻¹ h ⁻¹
<i>M. villosus</i> + <i>T. onnurineus</i>	2.0 ± 0.3	8.3 ± 1.3	2.1 ± 0.3	8.4 ± 1.3	6.3 ± 0.9
<i>M. vulcanius</i> + <i>T. onnurineus</i>	1.6 ± 0.5	10.3 ± 0.2	2.6 ± 0.3	11.3 ± 0.7	8.7 ± 0.5
<i>M. jannaschii</i> + <i>T. onnurineus</i>	1.8 ± 0.4	9.0 ± 1.5	1.9 ± 0.5	9.1 ± 1.6	7.2 ± 1.2

¹ Measurements were taken after a 7 h incubation period. Data collected at indicated timepoints in Figure 2 B. (N = 1, n = 6). Values are shown with standard deviation.

The results suggested that the co-culture with *M. vulcanius* was less efficient in medium B, as it had a higher CO₂ uptake rate (CUR) and HUR, compared to medium A, but not a higher MER. This agrees with the results obtained from pure-culture experiments, where medium B led to a slightly lower MER of *M. vulcanius* compared to the other methanogens.

Comparison of the MERs between the pure and the co-cultures suggested that, on average, a lower MER was measured in the co-cultures (Tables 1 and 2, Figure 1). A likely reason for this was that they were dependent on the conversion of CO to CO₂ and H₂ by *T. onnurineus*. Figure 3 shows that the CH₄ production and growth occurred at the highest capacity, as the H₂ content in most cultures was below 1 mol%, except for one case, suggesting it was fully converted. As such, the rate-limiting step in CH₄ production by the methanogens in the co-culture was the availability of H₂.

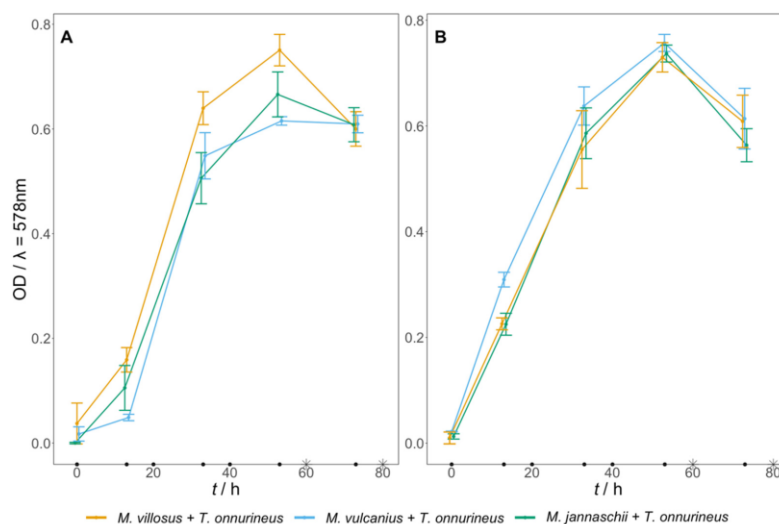


Figure 2. Growth kinetics of either *M. villosus*, *M. jannaschii*, or *M. vulcanius* with *T. onnurineus*. After the first 13 h, the medium was inoculated with *T. onnurineus* and the gas phase was exchanged from $\text{H}_2\text{:CO}_2$ to CO . The error bars show the standard deviation. Closed black circles on the x-axis mark the re-pressurising of the headspace with CO . The asterisks on the x-axis indicate the sampling points for GC analysis. (A) Growth in medium A; (B) growth in medium B. $N = 1$, $n = 6$.

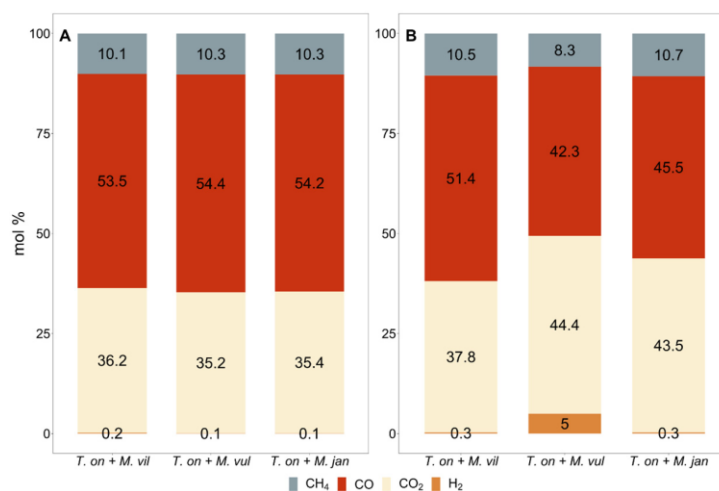


Figure 3. Relative mean molar gas composition in the co-cultures headspace after a 7 h incubation period on CO . Measurements were taken at the timepoints indicated in Figure 2. (A) Co-culture cultivated in medium A; (B) co-culture cultivated in medium B. $N = 1$, $n = 6$.

To circumvent H_2 limitation in the co-culture experiments, two strategies were employed. First, to create a functional culture with a high H_2 production rate in advance of the methanogen being added, an inoculation scheme was tested, where *T. onnurineus* was initially grown 13 h on CO , prior to addition of said methanogen. Second, a different gas

composition, H₂:CO (3:1) instead of pure CO, was used to provide additional H₂ available in the atmosphere. In addition, an experiment with a gas composition resembling that of industrial waste or syngases was performed [21]. The co-cultures were exposed to an artificial syngas (see Section 2.1) that could arise by industrial processes (Table 3). As *T. onnurineus* grown with *M. villosus* in medium B was considered the best co-culture, this pair was used. However, it should be noted that these were closed batch experiments and that the productivities are dependent on the conversion kinetics and on the tested time interval.

Table 3. Mean gas evolution rates of co-cultures in medium B under different gas compositions and inoculation orders ¹.

Co-Culture	Gas	MER/mmol L ⁻¹ h ⁻¹	CER/mmol L ⁻¹ h ⁻¹
<i>M. villosus</i> + <i>T. onnurineus</i>	CO	2.0 ± 0.3	6.3 ± 0.9
<i>T. onnurineus</i> + <i>M. villosus</i>	CO	1.1 ± 0.1	3.7 ± 1.0
<i>M. villosus</i> + <i>T. onnurineus</i>	H ₂ :CO	1.0 ± 0.4	0.0 ± 0.0
<i>T. onnurineus</i> + <i>M. villosus</i>	H ₂ :CO	1.0 ± 0.3	0.0 ± 0.0
<i>M. villosus</i> + <i>T. onnurineus</i>	Art. syngas	1.5 ± 0.5	0.9 ± 0.6
<i>T. onnurineus</i> + <i>M. villosus</i>	Art. syngas	0.9 ± 0.3	NA

¹ Measurements were taken after a 7 h incubation period (N = 1, n = 6). Values are shown with standard deviation.

In experiments with pure CO, the MER was twice as high when *M. villosus* was added first. The average H₂ content turned out to remain below 0.3 mol% (see Supplementary Materials, Table S7). When gassing with H₂:CO, interestingly, both inoculation schemes showed a decreasing performance over time, as revealed by a MER, which was only half of what was achieved when pure CO was used (Table 3). However, the measured CO₂ levels were below 0.1 mol% in both cases (see Supplementary Materials, Table S7). This could have been caused by the presence of H₂ in the H₂:CO, as the additional application of H₂ within the gas negatively affected growth and performance of *T. onnurineus*, which in turn most likely hindered the MER. The reduced CER by *T. onnurineus* may have been caused by the lower partial pressure of CO in the serum bottle headspace.

In the experiment where the artificial syngas mix was used and where *M. villosus* was pre-grown, the MER was 1.5 ± 0.5 mmol L⁻¹ h⁻¹. The average CH₄ mol% increased by about 10% to 24.8 mol%. This increase was similar to what was obtained when using pure CO, although the MER decreased as the experiment progressed. We did not observe a negative impact on the MER during co-culture experiments when elemental sulphur was added.

4. Discussion

This study is a new brick in the emerging research field of Archaea Biotechnology [54] and artificial microbial ecosystem design and engineering [55]. We investigated the conversion of one-carbon substrates (CO, H₂:CO, and CO-rich waste gases) by the artificially designed co-cultures of either *M. villosus*, *M. jannaschii*, or *M. vulcanius* together with *T. onnurineus*. Our results showed fast and reliable gas conversion, with a reduction in pure CO to about 50 mol% and simultaneous production of ~10 mol% CH₄ within 7 h. The most efficient conversion of the artificial syngas was performed by the co-culture *M. villosus* with *T. onnurineus*, inoculated in this order. This culture showed a CO reduction of 7%, starting from 37.3 mol% and an increase in CH₄ by ~10 mol% within 7 h. This proved the ability of the co-cultures to convert a variety of different CO-containing gas compositions with a lower proportion of CO.

A study with a similar experimental setup reported an ~6% CH₄ increase after 22 h [35]. We obtained 10% increase in 7 h. This suggests that the established co-culture (*T. onnurineus*/*M. villosus*) is of higher catalytic power. Nonetheless, most up-to-date published co-cultures were tested in different setups than the one reported in this study, making a direct comparison of evolution and uptake rates rather difficult [26,30,34]. Establishing different co-cultures in a bioreactor setup will, thus, be of great importance to fully understand their growth and production kinetics.

Pure-culture closed batch experiments of the methanogens in a defined medium on H₂:CO₂ showed a higher MER ($4.2 \pm 0.1 \text{ mmol L}^{-1} \text{ h}^{-1}$) than the co-cultures ($2.0 \pm 0.3 \text{ mmol L}^{-1} \text{ h}^{-1}$) (Figure 1, Table 3). Therefore, the potential for achieving a higher MER in co-cultures is possible if the necessary gas supply can be performed, and the inhibitory concentration of CO would not be surpassed [56]. Although the main limitation of the co-culture grown on pure CO was the availability of H₂ for methanogenesis and, hence, the conversions of CO to H₂ and CO₂ by *T. onnurineus* (Figure 3 and Supplementary Materials, Table S7). The addition of H₂ to the gas phase did not provide an increase in the MER. Rather, it led to a limitation of CO₂ availability (Supplementary Materials, Table S7), most likely due to the reduced performance of *T. onnurineus* under the lower CO partial pressure. Consequently, application of the artificial syngas led to an increase in MER, as biomethanation seems to be neither limited by CO₂ nor H₂ availability. Nonetheless, it did not reach the same values as in pure CO (Table 3). This can be explained by the concentration reduction in CO, CO₂, and H₂ in syngas by the other initially present gases, reducing substrate availability.

A change in the cultivation method to a bioreactor setup with higher pressure, agitation, and a constant gassing, resulting in having a higher gas solubility and higher gas transfer rate to the liquid phase, might increase the conversion of CO by *T. onnurineus* and the potential of the co-culture [37,39,48]. However, the ratio of the gas in the liquid phase has to be considered carefully, as high agitation might also lead to an excessive CO availability for the methanogens [34]. As recently more and more genetic tools for archaea become available, an overexpression of the carbon monoxide dehydrogenase could also be a solution to debottleneck CO conversion by *T. onnurineus* [39,57].

Unfortunately, *T. onnurineus* is still dependent on a complex medium containing yeast extract, which limits the potential industrial applicability. However, co-cultivation could be used to gain advantages or improve growth of both microorganisms through improving their syntrophic relationships [34]. Finding a suitable defined medium for *T. onnurineus* or replacing it by a different organism that can catalyze the WGS from CO in minimal conditions is one suggested avenue of research. *Carboxydocella thermotrophica* or *Carboxydocella sporoproducens* would fall into the same pH and temperature conditions as the herein employed methanogens [35].

A direct conversion of CO to CH₄ by a single organism such as *M. marburgensis* or *M. thermotrophicus* is likely not the most suitable biotechnological approach, as the inhibitory concentration of CO for these methanogens is very low [31,34]. The artificially created archaeal co-cultures consisting of one of the hyperthermophilic methanogenic archaea *M. villosus*, *M. jannaschii*, and *M. vulcanius* together with *T. onnurineus*, as performed in this study, are highly efficient and reliable for biomethanation. By adaptation to a minimal medium and by performing targeted bioprocess development, artificial archaeal co-cultures could be of environmental, economic, and industrial value in renewable energy production and storage.

5. Conclusions

This contribution is another step in the rapidly developing research field of Archaea Biotechnology. Furthermore, it is a proof of principle for the design and engineering of artificial microbial co-cultures. Additionally, it marks a cornerstone in the use of artificial archaeal co-cultures for the biomethanation of syngas. This design of artificial archaeal co-cultures represents a novelty in the field of biomethanation, due the unique selection of

organisms and their hyperthermophilic growth conditions. The created co-cultures convert a variety of different CO-containing gases, including syngas, to CH₄. As the WGSR might still be the limiting factor, finding the best organism for conversion of H₂O:CO to H₂:CO₂ will be of future relevance to enhance the kinetics of the artificial co-culture. Apart from the choice of the archaea, the inoculation sequence of the strains and medium development are crucial factors for a successful design. These factors will be important in engineering the next steps for scaling up the bioprocess of artificial archaeal co-cultures. Inferring from our results, hyperthermophilic and anaerobic archaea are of great biotechnological relevance, as they act as highly efficient H₂ and CH₄ cell factories in artificial co-culture.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/fermentation7040276/s1>, Table S1: Chemical composition of medium A, Table S2: Chemical composition of medium B, Table S3: 141 trace element solution modified from DSMZ, Table S4: Holden's trace element solution 2, Table S5: The physiological maximal and mean values of CH₄ production and growth kinetics of *M. marburgensis* and *M. thermotrophicus*, Table S6: HER of *T. onnurineus* after 7 h of incubation, Table S7: Relative mean molar gas composition of the co-culture's headspace after a 7 h incubation period, Figure S1: Growth of *M. marburgensis* and *M. thermotrophicus* in defined medium, Figure S2: Growth of *T. onnurineus* at 1 barg CO.

Author Contributions: Conceptualization, A.Z., T.S. and S.K.-M.R.R.; methodology, B.R., I.K., M.V. and S.K.-M.R.R.; validation, A.Z., B.R. and S.K.-M.R.R.; formal analysis, A.Z.; investigation, A.Z., B.R., T.S., M.S. and C.P.; resources, M.V. and S.K.-M.R.R.; data curation, A.Z. and B.R.; writing—original draft preparation, A.Z., B.R. and S.K.-M.R.R.; writing—review and editing, A.Z., B.R., I.K., M.V. and S.K.-M.R.R.; visualization, A.Z.; supervision, S.K.-M.R.R.; project administration, M.V. and S.K.-M.R.R.; funding acquisition, M.V. and S.K.-M.R.R. All authors have read and agreed to the published version of the manuscript.

Funding: Greatly acknowledged is the Österreichische Forschungsförderungsgesellschaft (FFG) for funding the project BioHyMe (grant no. 853615). The BMBWF is acknowledged for supporting the research with the WTZ project CZ 08/2020. Open access funding by the University of Vienna.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Acknowledgments: S.K.-M.R.R. greatly acknowledges Sung Gyun Kang (Korea Institute of Ocean Science and Technology (KIOST), Ansan, Republic of Korea) for providing *T. onnurineus* NA1. We want to thank Angus Hilt for comments on and proofreading of the manuscript.

Conflicts of Interest: B.R. and S.K.-M.R.R. declare competing interests due to their employment in the Arkeon GmbH. All other authors declare no competing interest.

Abbreviations

CO ₂	Carbon dioxide
CH ₄	Methane
CO	Carbon monoxide
H ₂	Molecular hydrogen
WGSR	Water gas shift reaction
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
OD	Optical density
GC	Gas chromatography
MER	Methane evolution rate
HER	Molecular hydrogen evolution rate
COUR	Carbon monoxide uptake rate
CER	Carbon dioxide evolution rate
HUR	Molecular hydrogen uptake rate
CUR	Carbon dioxide uptake rate

References

- EEA (European Environment Agency). *Primary Energy Consumption by Fuel in the EU-27*; EEA: København, Denmark, 2020; Volume 28.
- EEA (European Environment Agency). *National Emissions Reported to the UNFCCC and to the EU Greenhouse Gas Monitoring Mechanism*; EEA: København, Denmark, 2019; Available online: <https://www.eea.europa.eu/data-and-maps/data/national-emissions-reported-to-the-unfccc-and-to-the-eu-greenhouse-gas-monitoring-mechanism-14> (accessed on 10 November 2021).
- Sterner, M.; Specht, M. Power-to-Gas and Power-to-X—The History and Results of Developing a New Storage Concept. *Energies* **2021**, *14*, 6594. [\[CrossRef\]](#)
- Götz, M.; Lefebvre, J.; Mörs, F.; McDaniel Koch, A.; Graf, F.; Bajohr, S.; Reimert, R.; Kolb, T. Renewable Power-to-Gas: A Technological and Economic Review. *Renew. Energy* **2016**, *85*, 1371–1390. [\[CrossRef\]](#)
- Rönsch, S.; Schneider, J.; Matthieschke, S.; Schlüter, M.; Götz, M.; Lefebvre, J.; Prabhakaran, P.; Bajohr, S. Review on Methanation – From Fundamentals to Current Projects. *Fuel* **2016**, *166*, 276–296. [\[CrossRef\]](#)
- Eyl-Mazzega, M.-A.; Mathieu, C.; Boesgaard, K.; Daniel-Gromke, J.; Denysenko, V.; Liebetrau, J.; Cornot-Gandolphe, S. *Biogas and Bio-Methane in Europe: Lessons from Denmark, Germany and Italy*; Études de l’Ifri; French Institute of International Relations (IFRI): Paris, France, 2019; ISBN 979-10-373-0025-6.
- Rittmann, S.K.-M.; Seifert, A.H.; Bernacchi, S. Kinetics, Multivariate Statistical Modelling, and Physiology of CO₂-Based Biological Methane Production. *Appl. Energy* **2018**, *216*, 751–760. [\[CrossRef\]](#)
- Mauerhofer, L.-M.; Zwirtmayr, S.; Pappenreiter, P.; Bernacchi, S.; Seifert, A.H.; Reischl, B.; Schmider, T.; Taubner, R.-S.; Paulik, C.; Rittmann, S.K.-M.R. Hyperthermophilic Methanogenic Archaea Act as High-Pressure CH₄ Cell Factories. *Commun. Biol.* **2021**, *4*, 289. [\[CrossRef\]](#)
- Mauerhofer, L.-M.; Reischl, B.; Schmider, T.; Schupp, B.; Nagy, K.; Pappenreiter, P.; Zwirtmayr, S.; Schuster, B.; Bernacchi, S.; Seifert, A.H.; et al. Physiology and Methane Productivity of Methanobacterium Thermaggregans. *Appl. Microbiol. Biotechnol.* **2018**, *102*, 7643–7656. [\[CrossRef\]](#) [\[PubMed\]](#)
- Abdel Azim, A.; Pruckner, C.; Kolar, P.; Taubner, R.-S.; Fino, D.; Saracco, G.; Sousa, F.L.; Rittmann, S.K.-M.R. The Physiology of Trace Elements in Biological Methane Production. *Bioresour. Technol.* **2017**, *241*, 775–786. [\[CrossRef\]](#)
- Seifert, A.H.; Rittmann, S.; Herwig, C. Analysis of Process Related Factors to Increase Volumetric Productivity and Quality of Biomethane with Methanothermobacter Marburgensis. *Appl. Energy* **2014**, *132*, 155–162. [\[CrossRef\]](#)
- Seifert, A.H.; Rittmann, S.; Bernacchi, S.; Herwig, C. Method for Assessing the Impact of Emission Gasses on Physiology and Productivity in Biological Methanogenesis. *Bioresour. Technol.* **2013**, *136*, 747–751. [\[CrossRef\]](#)
- Burkhardt, M.; Jordan, I.; Heinrich, S.; Behrens, J.; Ziesche, A.; Busch, G. Long Term and Demand-Oriented Biocatalytic Synthesis of Highly Concentrated Methane in a Trickle Bed Reactor. *Appl. Energy* **2019**, *240*, 818–826. [\[CrossRef\]](#)
- Burkhardt, M.; Koschack, T.; Busch, G. Biocatalytic Methanation of Hydrogen and Carbon Dioxide in an Anaerobic Three-Phase System. *Bioresour. Technol.* **2015**, *178*, 330–333. [\[CrossRef\]](#) [\[PubMed\]](#)
- Jensen, M.B.; Strübing, D.; de Jonge, N.; Nielsen, J.L.; Ottosen, L.D.M.; Koch, K.; Kofoed, M.V.W. Stick or Leave—Pushing Methanogens to Biofilm Formation for Ex Situ Biomethanation. *Bioresour. Technol.* **2019**, *291*, 121784. [\[CrossRef\]](#)
- Nock, W.J.; Serna-Maza, A.; Heaven, S.; Banks, C.J. Evaluation of Microporous Hollow Fibre Membranes for Mass Transfer of H₂ into Anaerobic Digesters for Biomethanization. *J. Chem. Technol. Biotechnol.* **2019**, *94*, 2693–2701. [\[CrossRef\]](#)
- Tao, B.; Alessi, A.M.; Zhang, Y.; Chong, J.P.J.; Heaven, S.; Banks, C.J. Simultaneous Biomethanisation of Endogenous and Imported CO₂ in Organically Loaded Anaerobic Digesters. *Appl. Energy* **2019**, *247*, 670–681. [\[CrossRef\]](#)
- Rittmann, S.K.-M.; Seifert, A.; Herwig, C. Essential Prerequisites for Successful Bioprocess Development of Biological CH₄ Production from CO₂ and H₂. *Crit. Rev. Biotechnol.* **2015**, *35*, 141–151. [\[CrossRef\]](#) [\[PubMed\]](#)
- Rittmann, S.K.-M.R.; Seifert, A.H.; Krajete, A. Biomethanisierung—ein Prozess zur Ermöglichung der Energiewende? *BIOspektrum* **2014**, *20*, 816–817. [\[CrossRef\]](#)
- Rittmann, S.K.-M.R. A Critical Assessment of Microbiological Biogas to Biomethane Upgrading Systems. In *Biogas Science and Technology*; Guebitz, G.M., Bauer, A., Bochmann, G., Gronauer, A., Weiss, S., Eds.; Advances in Biochemical Engineering/Biotechnology; Springer International Publishing: Cham, Switzerland, 2015; pp. 117–135. ISBN 978-3-319-21993-6.
- Van der Stricht, W.; De Maré, C.; Plattner, T.; Fleischanderl, A.; Haselgriber, M.; Nair, P.; Wolf, C. *Sustainable Production of Low Carbon, Renewable Fuels by Fermenting Industrial Process Gasses from the Iron and Steel Industry*; ArcelorMittal, Primetals Technologies, LanzaTech: Gent, Belgium, 2017.
- Safari, F.; Tavasoli, A.; Ataie, A.; Choi, J.-K. Hydrogen and Syngas Production from Gasification of Lignocellulosic Biomass in Supercritical Water Media. *Int. J. Recycl. Org. Waste Agric.* **2015**, *4*, 121–125. [\[CrossRef\]](#)
- Dürre, P.; Eikmanns, B.J. C1-Carbon Sources for Chemical and Fuel Production by Microbial Gas Fermentation. *Curr. Opin. Biotechnol.* **2015**, *35*, 63–72. [\[CrossRef\]](#)
- de Medeiros, E.M.; Noorman, H.; Maciel Filho, R.; Posada, J.A. Multi-Objective Sustainability Optimization of Biomass Residues to Ethanol via Gasification and Syngas Fermentation: Trade-Offs between Profitability, Energy Efficiency, and Carbon Emissions. *Fermentation* **2021**, *7*, 201. [\[CrossRef\]](#)
- Phillips, J.R.; Huhnke, R.L.; Atiyeh, H.K. Syngas Fermentation: A Microbial Conversion Process of Gaseous Substrates to Various Products. *Fermentation* **2017**, *3*, 28. [\[CrossRef\]](#)

26. Westman, S.Y.; Chandolias, K.; Taherzadeh, M.J. Syngas Biomethanation in a Semi-Continuous Reverse Membrane Bioreactor (RMBR). *Fermentation* **2016**, *2*, 8. [\[CrossRef\]](#)
27. Devarapalli, M.; Lewis, R.S.; Atiyeh, H.K. Continuous Ethanol Production from Synthesis Gas by *Clostridium Ragsdalei* in a Trickle-Bed Reactor. *Fermentation* **2017**, *3*, 23. [\[CrossRef\]](#)
28. Rittmann, S.K.-M.; Lee, H.S.; Lim, J.K.; Kim, T.W.; Lee, J.-H.; Kang, S.G. One-Carbon Substrate-Based Biohydrogen Production: Microbes, Mechanism, and Productivity. *Biotechnol. Adv.* **2015**, *33*, 165–177. [\[CrossRef\]](#) [\[PubMed\]](#)
29. Thauer, R.K. Citric-Acid Cycle, 50 Years on: Modifications and an Alternative Pathway in Anaerobic Bacteria. *Eur. J. Biochem.* **1988**, *176*, 497–508. [\[CrossRef\]](#)
30. Klasson, K.; Cowger, J.; Ko, C.; Vega, J.; Clausen, E.; Gaddy, J. Methane Production from Synthesis Gas Using a Mixed Culture Of *R. Rubrum* M. *Barkeri*, and *M. Formicum*. *Appl. Biochem. Biotechnol.* **1990**, *24*, 317–328. [\[CrossRef\]](#)
31. Daniels, L.; Fuchs, G.; Thauer, R.; Zeikus, J. Carbon Monoxide Oxidation by Methanogenic Bacteria. *J. Bacteriol.* **1977**, *132*, 118–126. [\[CrossRef\]](#)
32. O'Brien, J.M.; Wolkin, R.; Moench, T.; Morgan, J.; Zeikus, J. Association of Hydrogen Metabolism with Unitrophic or Mixotrophic Growth of *Methanosarcina Barkeri* on Carbon Monoxide. *J. Bacteriol.* **1984**, *158*, 373–375. [\[CrossRef\]](#)
33. Diender, M.; Pereira, R.; Wessels, H.J.; Stams, A.J.; Sousa, D.Z. Proteomic Analysis of the Hydrogen and Carbon Monoxide Metabolism of *Methanothermobacter Marburgensis*. *Front. Microbiol.* **2016**, *7*, 1049. [\[CrossRef\]](#)
34. Diender, M.; Uhl, P.S.; Bitter, J.H.; Stams, A.J.; Sousa, D.Z. High Rate Biomethanation of Carbon Monoxide-Rich Gases via a Thermophilic Synthetic Coculture. *ACS Sustain. Chem. Eng.* **2018**, *6*, 2169–2176. [\[CrossRef\]](#)
35. Kohlmayer, M.; Robert, H.; Raimund, B.; Wolfgang, M. Simultaneous CO₂ and CO methanation using microbes. *Microbiology* **2018**. [\[CrossRef\]](#)
36. Schmider, T. Ecophysiology and Methane Productivity of Carboxydotrophy-Based Archaeal Co-Cultures. Master's Thesis, University of Vienna, Vienna, Austria, 2018.
37. Kim, M.-S.; Fitriana, H.N.; Kim, T.W.; Kang, S.G.; Jeon, S.G.; Chung, S.H.; Park, G.W.; Na, J.-G. Enhancement of the Hydrogen Productivity in Microbial Water Gas Shift Reaction by *Thermococcus Onnurineus* NA1 Using a Pressurized Bioreactor. *Int. J. Hydrog. Energy* **2017**, *42*, 27593–27599. [\[CrossRef\]](#)
38. Lee, S.H.; Kim, M.-S.; Lee, J.-H.; Kim, T.W.; Bae, S.S.; Lee, S.-M.; Jung, H.C.; Yang, T.-J.; Choi, A.R.; Cho, Y.-J.; et al. Adaptive Engineering of a Hyperthermophilic Archaeon on CO and Discovering the Underlying Mechanism by Multi-Omics Analysis. *Sci. Rep.* **2016**, *6*, 22896. [\[CrossRef\]](#)
39. Kim, M.-S.; Bae, S.S.; Kim, Y.J.; Kim, T.W.; Lim, J.K.; Lee, S.H.; Choi, A.R.; Jeon, J.H.; Lee, J.-H.; Lee, H.S.; et al. CO-Dependent H₂ Production by Genetically Engineered *Thermococcus Onnurineus* NA1. *Appl. Environ. Microbiol.* **2013**, *79*, 2048–2053. [\[CrossRef\]](#) [\[PubMed\]](#)
40. Bae, S.S.; Kim, T.W.; Lee, H.S.; Kwon, K.K.; Kim, Y.J.; Kim, M.-S.; Lee, J.-H.; Kang, S.G. H₂ Production from CO, Formate or Starch Using the Hyperthermophilic Archaeon, *Thermococcus onnurineus*. *Biotechnol. Lett.* **2012**, *34*, 75–79. [\[CrossRef\]](#) [\[PubMed\]](#)
41. Bae, S.-S.; Kim, Y.-J.; Yang, S.-H.; Lim, J.-K.; Jeon, J.-H.; Lee, H.-S.; Kang, S.-G.; Kim, S.; Lee, J. *Thermococcus onnurineus* Sp. Nov., a Hyperthermophilic Archaeon Isolated from a Deep-Sea Hydrothermal Vent Area at the PACMANUS Field. *J. Microbiol. Biotechnol.* **2006**, *16*, 1826.
42. Bellack, A.; Huber, H.; Rachel, R.; Wanner, G.; Wirth, R. *Methanocaldococcus villosus* Sp. Nov., a Heavily Flagellated Archaeon That Adheres to Surfaces and Forms Cell–Cell Contacts. *Int. J. Syst. Evol. Microbiol.* **2011**, *61*, 1239–1245. [\[CrossRef\]](#) [\[PubMed\]](#)
43. Jeanthon, C.; l'Haridon, S.; Reysenbach, A.-L.; Corre, E.; Vernet, M.; Messner, P.; Sleytr, U.; Prieur, D. *Methanococcus vulcanius* Sp. Nov., a Novel Hyperthermophilic Methanogen Isolated from East Pacific Rise, and Identification of *Methanococcus* Sp. DSM 4213Tas *Methanococcus fervens* Sp. Nov. *Int. J. Syst. Evol. Microbiol.* **1999**, *49*, 583–589. [\[CrossRef\]](#)
44. Jones, W.; Leigh, J.; Mayer, F.; Woese, C.; Wolfe, R. *Methanococcus jannaschii* Sp. Nov., an Extremely Thermophilic Methanogen from a Submarine Hydrothermal Vent. *Arch. Microbiol.* **1983**, *136*, 254–261. [\[CrossRef\]](#)
45. Diender, M.; Stams, A.J.; Sousa, D.Z. Pathways and Bioenergetics of Anaerobic Carbon Monoxide Fermentation. *Front. Microbiol.* **2015**, *6*, 1275. [\[CrossRef\]](#)
46. Balch, W.E.; Wolfe, R. New Approach to the Cultivation of Methanogenic Bacteria: 2-Mercaptoethanesulfonic Acid (HS-CoM)-Dependent Growth of *Methanobacterium Ruminantium* in a Pressurized Atmosphere. *Appl. Environ. Microbiol.* **1976**, *32*, 781–791. [\[CrossRef\]](#)
47. Taubner, R.-S.; Rittmann, S.K.-M. Method for Indirect Quantification of CH₄ Production via H₂O Production Using Hydrogenotrophic Methanogens. *Front. Microbiol.* **2016**, *7*, 532. [\[CrossRef\]](#)
48. Rittmann, S.K.-M.; Herwig, C. A Comprehensive and Quantitative Review of Dark Fermentative Biohydrogen Production. *Microb. Cell Factories* **2012**, *11*, 115. [\[CrossRef\]](#) [\[PubMed\]](#)
49. Pappenreiter, P.A.; Zwiirtmayr, S.; Mauerhofer, L.-M.; Rittmann, S.K.-M.R.; Paulik, C. Development of a Simultaneous Bioreactor System for Characterization of Gas Production Kinetics of Methanogenic Archaea at High Pressure. *Eng. Life Sci.* **2019**, *19*, 537–544. [\[CrossRef\]](#) [\[PubMed\]](#)
50. Rittmann, S.K.-M.; Seifert, A.; Herwig, C. Quantitative Analysis of Media Dilution Rate Effects on *Methanothermobacter Marburgensis* Grown in Continuous Culture on H₂ and CO₂. *Biomass Bioenergy* **2012**, *36*, 293–301. [\[CrossRef\]](#)
51. Bernacchi, S.; Rittmann, S.K.-M.; Seifert, A.H.; Krajete, A.; Herwig, C. Experimental Methods for Screening Parameters Influencing the Growth to Product Yield (Y (x/CH₄)) of a Biological Methane Production (BMP) Process Performed with *Methanothermobacter Marburgensis*. *AIMS Bioeng.* **2014**, *1*, 72–86. [\[CrossRef\]](#)
52. Reischl, B.; Ergal, I.; Rittmann, S.K.-M. Biohydrogen Production Characteristics of *Desulfurococcus Amylolyticus* DSM 16532. *Int. J. Hydrog. Energy* **2018**, *43*, 8747–8753. [\[CrossRef\]](#)

-
53. Pruckner, C. Evolutionary and Physiological Aspects of Potentially Carboxydophilic Archaea. Master's Thesis, University of Vienna, Vienna, Austria, 2019.
 54. Pfeifer, K.; Ergal, I.; Koller, M.; Basen, M.; Schuster, B.; Rittmann, S.K.-M.R. Archaea Biotechnology. *Biotechnol. Adv.* **2021**, *47*, 107668. [\[CrossRef\]](#)
 55. Ergal, I.; Bochmann, G.; Fuchs, W.; Rittmann, S.K.-M.R. Design and Engineering of Artificial Microbial Consortia for Biohydrogen Production. *Curr. Opin. Biotechnol.* **2022**, *73*, 74–80. [\[CrossRef\]](#)
 56. Guiot, S.R.; Cimpoaia, R.; Carayon, G. Potential of Wastewater-Treating Anaerobic Granules for Biomethanation of Synthesis Gas. *Environ. Sci. Technol.* **2011**, *45*, 2006–2012. [\[CrossRef\]](#)
 57. Straub, C.T.; Counts, J.A.; Nguyen, D.M.N.; Wu, C.-H.; Zeldes, B.M.; Crosby, J.R.; Conway, J.M.; Otten, J.K.; Lipscomb, G.L.; Schut, G.J.; et al. Biotechnology of Extremely Thermophilic Archaea. *FEMS Microbiol. Rev.* **2018**, *42*, 543–578. [\[CrossRef\]](#)

2.3 Chapter 2

***Methanosarcina acetivorans*, acetoclastic and methylotrophic methanogenesis possessing cytochromes:**

M. acetivorans was first isolated from methanic marine sediments in California. This organism is mesophilic with an optimal temperature range from 35 to 40°C. Regarding pH and salinity the optimum range is between 6.5 to 7 and at least 2.34% (w/v) NaCl. No growth is observed when NaCl is omitted completely. *M. acetivorans* shows no flagellum and is non-motile but like other Methanosarcinales may form aggregates under certain biogeochemical circumstances (Sowers et al., 1984, 1993). This methylotrophic methanogen thrives preferably on acetate but also on other methyl group substrates like methanol and methylamines (Sowers et al., 1984) and even on CO (Rother & Metcalf, 2004). No growth is observed using H₂/CO₂ or formate as substrate (Sowers et al., 1984). Typical for Methanosarcinales, these organisms have cytochromes and methanophenazine, enzymes and phenazine derivatives connected to electron transport (Abken et al., 1998; Kühn et al., 1983). These two important different pathways described here are based on the extensively performed research on previous studies (Costa & Leigh, 2014; Ferry, 2020; Holmes et al., 2019; Q. Li et al., 2006; Liu & Whitman, 2008; Prakash et al., 2019; M. Wang et al., 2011; Welte & Deppenmeier, 2014).

In the acetoclastic pathway first acetate is fixed and subsequently converted to acetyl-CoA through ATP, which is then cleaved and decarbonylated into the methyl and carbonyl remnant (Fig.2). Here the carbonyl group is oxidized to CO₂ by transferring electrons to Fd and the methyl group is transferred to the cofactor tetrahydrosarcinapterin (H₄SPT). Here the metabolite enters the methyl branch, where the methyl group is transferred to the CoM catalyzed by the membrane-bound methyl transferase. Importantly this step is coupled with the establishment of an electrochemical sodium gradient and therefore involved in energy conservation (Fig.3) (Gottschalk & Thauer, 2001). Ultimately CH₄ is produced by the reduction of the methylated coenzyme through the corresponding reductase Mcr with electrons from CoB which then forms the multi-enzyme complex CoM-S-S-CoB. This multi-enzyme complex is then regenerated through the reduced Fd causing an electron transport chain (ETC) where the representative Rnf complex with the c-type cytochrome (MmcA) and methanophenazine (Mph) channel electrons to the heterodisulfide reductase (HdrED) (Fig. 3). Here again this ETC is coupled to a sodium and proton translocation and hence additionally involved in energy conservation (Ferry, 2020; M. Wang et al., 2011).

For the second part of this cumulative master's thesis *Methanosarcina acetivorans* was grown on methanol since acetate is an unlikely substrate due to the fact that *in situ* bacteria outcompete methanogens for this potential electron donor (Reeburgh, 2007).

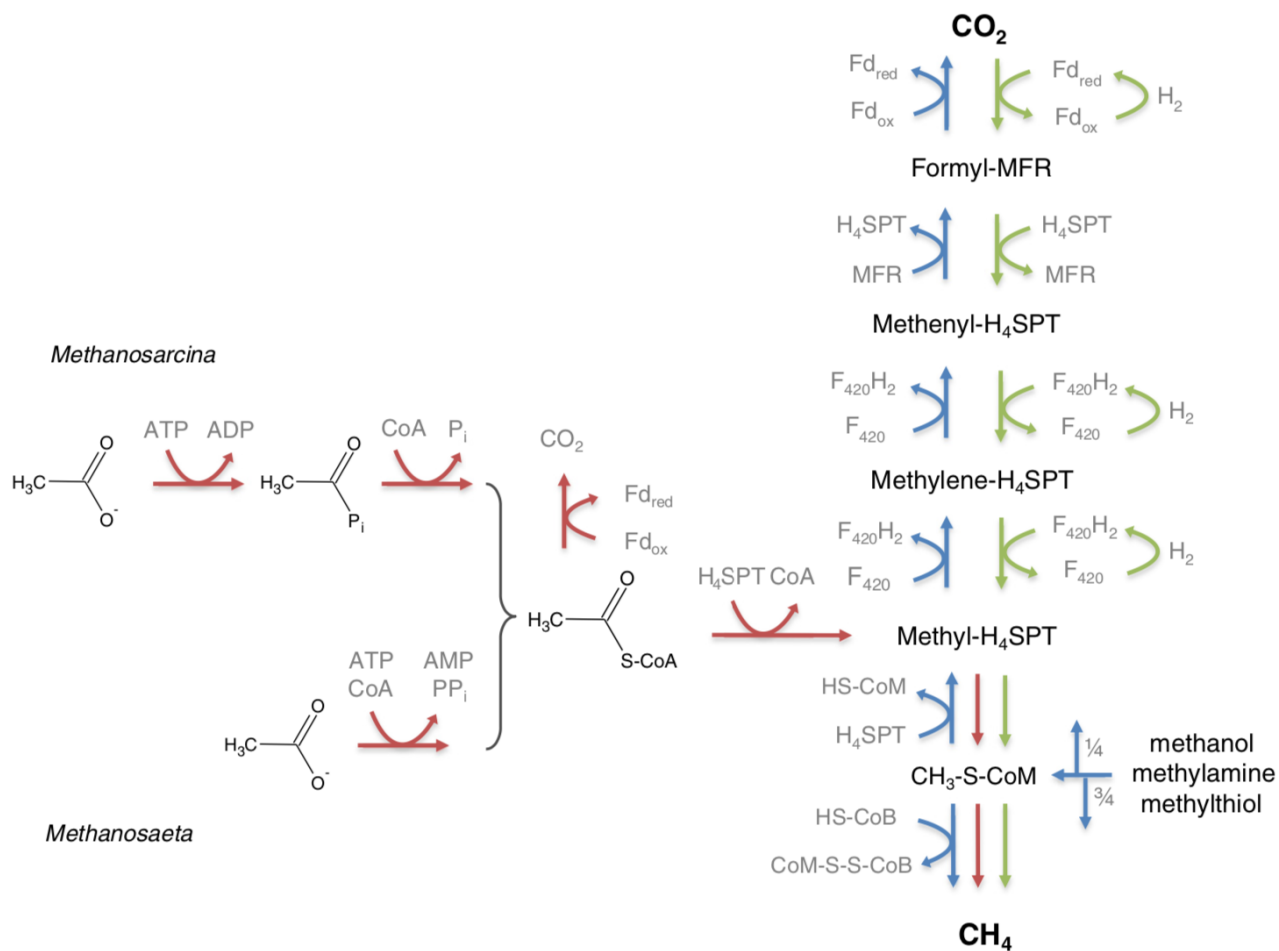


Fig. 2: Pathways of aceticlastic and methylotrophic methanogenesis for *Methanosarcina* and *Methanosaeta*. Red arrows show the aceticlastic pathway whereas the blue arrows show the methylotrophic pathway. Green arrows show the hydrogenotrophic which is described more in detail in the previous section and Fig.1. Figure taken from Welte et al., 2013.

this step endergonic (Gottschalk & Thauer, 2001). The formed $\text{CH}_3\text{-H}_4\text{SPT}$ then is stepwise oxidized to methenyl- H_4SPT reducing the cofactor F_{420} which transfers the electrons to the Fpo complex which in contrast to acetate grown *M. acetivorans* is up-regulated in methanol grown cells (L. Li et al., 2007). The next step is the transfer of the formyl group to MFR in which the corresponding dehydrogenase oxidizes the formed molecule to CO_2 and MFR. Here the electrons are transferred to Fd, hence reducing it (Bertram & Thauer, 1994). In this pathway, the reduced Fd transfers the electrons to the Rnf complex. With the transfer of electrons from the reduced co-factors here again an ETC is established where, additionally to the Rnf and Fpo complex, the MmcA and Mph are involved in channeling electrons to HdrED regenerating CoM-S-S-CoB . Here again the ETC triggers the sodium and proton translocation and therefore energy can be generated (Welte & Deppenmeier, 2014) (Fig.3) (Fig. 4). Naturally linked to this step the remaining 3 molecules of the $\text{CH}_3\text{-S-CoM}$ are reduced to CH_4 .

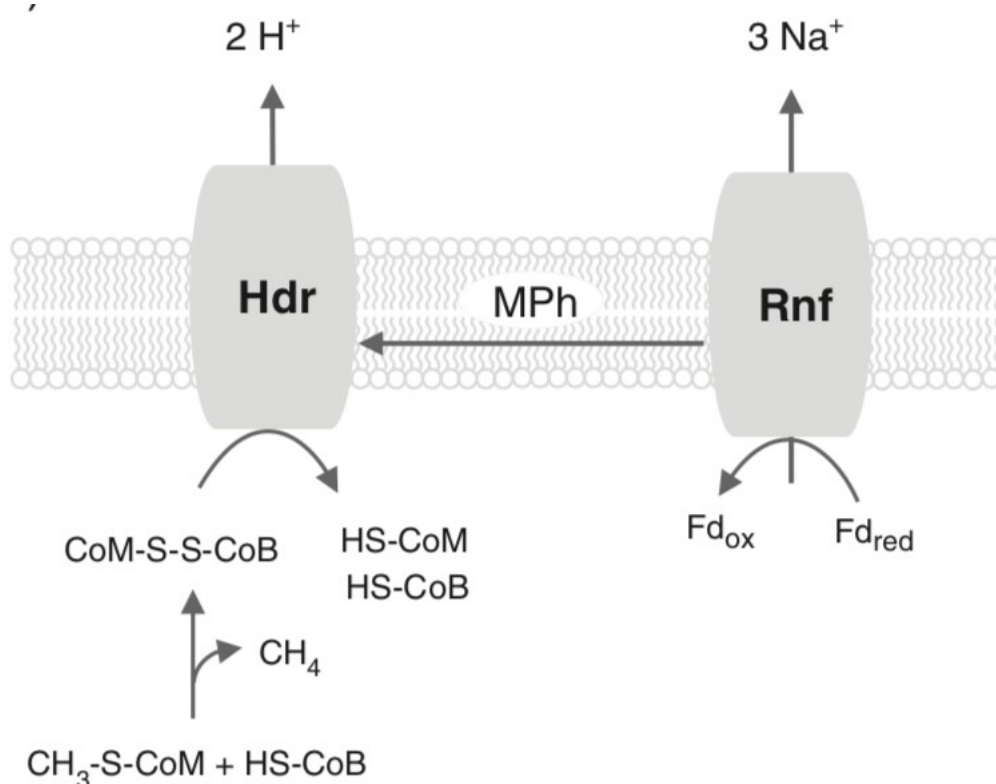


Fig. 4: Sodium and proton translocation coupled with reduced ferredoxin and hence electron transport to Hdr. Figure taken from Welte & Deppenmeier, 2014.

With this detailed knowledge on the energy metabolism of methylotrophic methanogens and the reported possibility of external electron transfer in Methanosarcinales (Bond & Lovley, 2002; Holmes et al., 2019; Prakash et al., 2019; Shang et al., 2020) following unpublished manuscript was composed:

2.4 Iron reduction and possible magnetite precipitation by methylotrophic Methanosarcinales

Unpublished manuscript

Personal contribution: Conceptualization, methodology, formal analysis, investigation, writing

Abstract

Methanogenesis is the last step in the anaerobic part of the global carbon cycle in which organic matter produced by e.g., photosynthesis is recycled. This metabolism is performed by strictly anaerobic microorganisms, methanogenic archaea (methanogens). Despite extensive research on the metabolic potential and substrates utilization that can be used by these organisms, and their biotechnological applicability, their geomicrobiological influence on mineral formation remains largely enigmatic. The current focus on certain methanogens, the Methanosarcinales, on extracellular electron transfer represents a comparatively new area of research. Methanosarcinales show the highest diversity regarding substrate utilization among the characterized methanogens and thrive in a broad spectrum of ecosystems such as marine sediments, whereof e.g., *Methanosarcina acetivorans* was first isolated. In the current study, we examined *M. acetivorans* grown on methanol as substrate with the addition of ferrihydrite as electron acceptor. Microbial iron reduction, and possible magnetite formation by *M. acetivorans* was mimicked in a pure culture approach. We suggest an adapted methylotrophic pathway linked to iron reduction based on our results in which *M. acetivorans* actively reduces Fe(III) to Fe(II). Magnetite formation was observed at a certain degree, but not stable, indicating the complexity of geobiochemical factors for mineral formation. We propose an alternative pathway for energy conservation in methylotrophic Methanosarcinales and highlight its role on the formation of iron minerals.

Introduction

Methanogenesis is the last step of the global carbon cycle, the last step in the anaerobic carbon cycle, and therefore also the last step in the degradation and recycling of organic matter produced by primary production. Methanogenesis is a strictly anaerobic microbial process and is exclusively performed by methanogenic archaea (methanogens) mainly belonging to the Euryarchaeota phylum (Liu & Whitman, 2008). These organisms metabolize a limited variety of substrates to produce methane (CH_4) including molecular hydrogen (H_2) and carbon dioxide (CO_2), with which they mediate the most widespread type of methanogenesis, where H_2 donates electrons to reduce CO_2 ; the methyl-group type methanogenesis, where methylated C_1 compounds are reduced and oxidized; and the acetate type pathway (acetoclastic methanogenesis), where this C_2 compound is likewise reduced and oxidized to produce CH_4 but also CO_2 as final products (Thauer et al., 2008), (Liu & Whitman, 2008). Another distinction of methanogens can be made via the presence of cytochromes, which are electron carrier proteins that are yet only known from the order of Methanosarcinales (Kühn et al., 1983), and furthermore the presence of methanophenazine, a hydrophobic phenazine derivate, which is as well connected to electron transport (Abken et al., 1998). Furthermore, Methanosarcinales show the highest diversity regarding substrate utilization with representative organisms for all main pathways (Liu & Whitman, 2008). Interestingly Methanosarcinales are phylogenetically closely related to a cluster of anaerobic methane oxidizing archaea (ANME-2) (F.-P. Wang et al., 2014) and therefore are linked to the hypothesized possibility of reverse methanogenesis (Hallam et al., 2004) where an external electron acceptor would be mandatory (Timmers et al., 2017). These external electron acceptors are inter alia sulphate (SO_4^{2-}), nitrate (NO_3^-) and metal ions like iron (Fe(III)) (Timmers et al., 2017). ANME are key players in the sulfate-methane transition zone (SMTZ) of marine sediments where they thrive in a syntrophic consortium with sulfate-reducing bacteria (Boetius et al., 2000). These consortia oxidize biogenic and abiogenic CH_4 produced in marine sediments via reduction with sulfate derived from seawater, and are capable for preventing large amounts of CH_4 escaping into the water column and the atmosphere (Jørgensen & Kasten, 2006).

Most recently Lin et al. (2021) reported a novel magnetite nanoparticle mineral from a yet unknown source in sediments associated with methane seepage. These minerals differ in size and characteristic properties compared to the already known magnetite produced by magnetotactic bacteria (Devouard et al., 1998) and dissimilatory iron-reducing bacteria (Li et al., 2009). Magnetite ($\text{Fe}^{2+}(\text{Fe}^{3+})_2\text{O}_4$) is an iron oxide where ferric (Fe(III)) and ferrous (Fe(II)) iron form an octahedral crystal (Cornell & Schwertmann, 2003). Ferric iron, which may be reduced biotically (Lovley, 1991) but also abiotically (Ionescu et al., 2015), incorporates the ferrous iron enabling magnetite formation. The origin and exact formation mechanisms of this newly reported mineral in methanic sediments are still unknown, yet several hypotheses can be considered. The possibility of methanogens performing reverse methanogenesis coupled to iron reduction may be biochemically conceivable (Yan et al., 2018) but is unlikely since until

now no methanogen was discovered performing this reversed reaction. Only a genetically engineered methanogen, *Methanosarcina acetivorans* with the methyl-coenzyme M reductase (Mcr) from a ANME-1 origin was able to oxidize CH₄ with FeCl₃ as electron acceptor (Soo et al., 2016), again highlighting the phylogenetic proximity from ANME to Methanosarcinales (K. U. Hinrichs et al., 1999) but also the limiting step as Mcr does not seem to operate reversed *in situ* (Timmers et al., 2017). CH₄ oxidation was assumed to be limited to the SMTZ and therefore spatially separated from methanic sediments (Claypool & Kaplan, 1974) yet more recent work has shown that these biogeochemical zones are more transient than previously assumed (Treude et al., 2014). Especially the anaerobic oxidation of CH₄ coupled to iron reduction (Fe-AOM) (Beal et al., 2009) is a process that appears to operate below the SMTZ within the methanogenic zone (Aromokeye et al., 2020) and therefore these organisms may play an active role in accelerating magnetite formation. Nevertheless, the majority of CH₄ is oxidized with the coupling of sulfate reduction (K.-U. Hinrichs & Boetius, 2002) and therefore methanogens act as key players in the methanogenic zone. Acetate and H₂ are highly consumed substrates used by both bacteria and methanogens whereby the former outcompete the latter (Reeburgh, 2007) thus making them unlikely electron donors for methanogenesis coupled to iron reduction in marine sediments. Therefore, we hypothesized that rather methylotrophic methanogenesis might be a possible mechanism that is coupled to iron reduction and the formation of magnetite. Moreover, it is quantitatively the dominant methanogenesis pathway in marine sediments (Zhuang et al., 2018). It is well known that certain methanogens, especially Methanosarcinales, are able to reduce iron as external electron acceptor (Bond & Lovley, 2002) (Bodegom et al., 2004). Holmes et al. (2019) demonstrated that *Methanosarcina acetivorans* shows a higher gene expression of a c-type cytochrome when incubated with an external electron acceptor anthraquinone-2,6-disulfonate (AQDS) and methanol as substrate, indicating a possible energy metabolism in a methylotrophic methanogen coupled with an external electron acceptor. Additionally, it was recently discovered that in an attempt to form zerovalent iron, *Methanosarcina barkeri* was able to reduce iron and precipitate magnetite by first growing the organism with H₂/CO₂ but then omitting the electron donor when iron oxides were added, hence decoupling iron reduction from the active metabolism (Shang et al., 2020). Furthermore, it has been demonstrated that *Methanosarcina acetivorans* was able to reduce iron and precipitate magnetite by using acetate as substrate and enhancing external electron transfer with AQDS Prakash et al., (2019). Both routes are therefore conceivable for laboratory conditions but unlikely for magnetite formation in methanic sediments since these substrates are not available for methanogens (Reeburgh, 2007; Schönheit et al., 2004).

Therefore, this study investigates and highlights methylotrophic methanogenesis coupled to iron reduction in an attempt to link magnetite formation to the active metabolism of these organisms. Using *Methanosarcina acetivorans* as the model organism we demonstrate the influence of this newly discovered energy conservation pathway but also the inhibitory factors and challenges in the formation of iron minerals.

Methods

Growth conditions

All experiments were performed under strictly anaerobic closed batch conditions (Taubner & Rittmann, 2016) in sterile 120 mL serum bottles closed with butyl rubber stoppers and sealed with aluminium crimp caps.

Methanosarcina acetivorans (DSM 2834) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). Prior to the experiment the organism was pre-cultured in adapted versions of DSMZ medium 141c by omitting first $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \times 6 \text{H}_2\text{O}$ solution (0.1% w/v), Na-acetate, Trypticase peptone (BD BBL) and Na-resazurin solution (0.1% w/v). In the late exponential phase, the organism was then transferred to the latter medium by omitting L-cysteine and transferred an additional time by omitting L-cysteine and yeast from the medium completely. The last step in the pre-culture setup was transferring *Methanosarcina acetivorans* twice to the final medium containing (per litre) 0.34g KCl, 4g $\text{MgCl}_2 \times 6 \text{H}_2\text{O}$, 0.5g $\text{MgSO}_4 \times 7 \text{H}_2\text{O}$, 0.25g NH_4Cl , 0.14g $\text{CaCl}_2 \times 2 \text{H}_2\text{O}$, 0.14g K_2HPO_4 , 18g NaCl. 10 mL Modified Wolin's mineral solution (Wolin et al., 1964). After sterilization through autoclaving the following sterile prepared solutions were added (per L) 1g NaHCO_3 . 5 mL Methanol. 5 mL Wolin's vitamin solution (Wolin et al., 1964) and a final concentration of 1 mmol L^{-1} Na_2S . Sterile prepared solutions were maintained under anaerobic conditions in a N_2 atmosphere. For the experiment, 9 mmol L^{-1} final concentration sterile prepared ferrihydrite was added to the final medium to an end volume of 50 mL liquid phase and 70 mL gas headspace under a 1 barg N_2/CO_2 (80:20) atmosphere. The pH of the final medium was 6.5 and not further adjusted since it was in the optimal range (Sowers et al., 1984). Media modifications and organism transferring were performed to avoid possible abiotic iron reduction through reducing agents and organics (Sivan et al., 2016). The organism was incubated in the dark in a 35°C water bath while slowly shaking.

Poorly crystalline ferrihydrite was prepared as described formerly in Lovley & Phillips, (1986). Summarized, a FeCl_3 solution was titrated with NaOH to pH 7 while stirring. The slurry then was washed with autoclaved Milli-Q H_2O to remove formed salt and leftover base. The washed ferrihydrite was then resuspended in autoclaved Milli-Q H_2O and anaerobized twice with N_2 for 30 min while shaking.

Experimental setup

To monitor iron reduction and mineral precipitation over time, *Methanosarcina acetivorans* was grown and kept in the experimental bottles over a time period of 18 days. A destructive sampling method was performed where a defined set of bottles, consisting of quadruplicates of the microbiological experiments and a zero control (treated the same but no organism), was sacrificed every 3 days for all analysis procedures. An additional set of bottles which were

treated identically to the microbiological experiments, was established to investigate time and pH on possible magnetite formation. Here after 18 days methanol addition was omitted and the headspace was exchanged from N₂/CO₂ (80:20) to N₂. This row was incubated in the dark at 35°C for additional 14 days without shaking. Additionally, a control group was established to investigate and correlate metabolism to iron reduction. Here after 18 days of incubation ferrihydrite was added and methanol was omitted while maintained under a N₂ atmosphere. Validation and maintaining productivity was reassured via pressure measurements, the addition of methanol (0.15 mL per bottle every 3 days) and flushing all serum bottles again to 1 barg N₂CO₂ (80:20) or N₂, respectively.

Sampling was performed anaerobically in a glove box and consisted of first taking 2 mL of the liquid phase for qPCR measurements, then filtering the formed precipitates from the liquid phase and drying it in the dark to prevent possible photooxidation. The separated liquid phase was furthermore titrated and fixed to pH 1, incubated and also stored in the dark for later analysis.

Analytical methods

After preparation, ferrihydrite was determined by inductively-coupled-plasma mass spectrometry (ICP-MS). Here the slurry was digested with H₂SO₄ to pH 1 to insure Fe(III) solubility, and incubated in the dark for 24h. The solution was then diluted accordingly based on the initial concentration and prepared for measurement.

Iron reduction and therefore the concentration of Fe(II) was determined first through spectrophotometric analysis as described in DIN 38 406: German standard methods for the examination of water, waste water and sludge; cations (group E); determination of iron (E 1). Summarized, the already filtered and fixed anaerobic liquid phase which was titrated to pH 1 with H₂SO₄ was laced with 1,10-Phenanthroline and measured at an absorbance with 510 nm. Standards were used accordingly to the protocol described above.

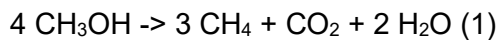
Secondly, total iron, Fe(III), Fe(II) was determined again through mass spectrometry (ICP-MS). The results of both methods should yield identical results, besides possible minor deviations through methodical difference, due to the insolubility of Fe(III) in neutral pH solutions. Fe(III) was expected to be present only in the poorly crystalline ferrihydrite or possibly magnetite which were filtered and separated out of the medium before pH changes were made for Fe(II) determination.

Separated and already dried precipitates were measured via x-ray diffraction (XRD).

Results

Iron reduction and Magnetite formation

Methanosarcina acetivorans is a mesophilic acetotrophic methanogen isolated from marine sediments which is able to metabolize further methyl-group substrates and carbonmonoxide but not H_2/CO_2 (Rother & Metcalf, 2004; Sowers et al., 1984). Here we grew *Methanosarcina acetivorans* on methanol as substrate and ferrihydrite as potential electron acceptor, and in contrast to *Methanosarcina barkeri* which was also considered for the experiment, the former grew without adding L-cysteine as reducing agent. Therefore, *Methanosarcina barkeri* was excluded from further experiments. Methanol uptake and CH_4 production, hence growth and productivity, was monitored via pressure measurements considering the following equation:



Therefore, an increase in pressure was expected. This is shown in Fig.1 indicating no growth inhibition through iron and no further substrate limitation from the medium over the duration of the experiment. The reduction of added ferrihydrite and therefore the production of Fe(II) was identified over the whole experiment run and increased with time in inoculated bottles (Fig. 2a). Furthermore, after ferrihydrite addition on day 1 only a minor amount ($0.47 \pm 0.08 \text{ mmol L}^{-1}$) was reduced abiotically and remained almost identical over time (Fig.2a). Only a short time after ferrihydrite addition, the typical red-brown colored slurry changed to a black precipitate that flocculated (Fig. 2b).

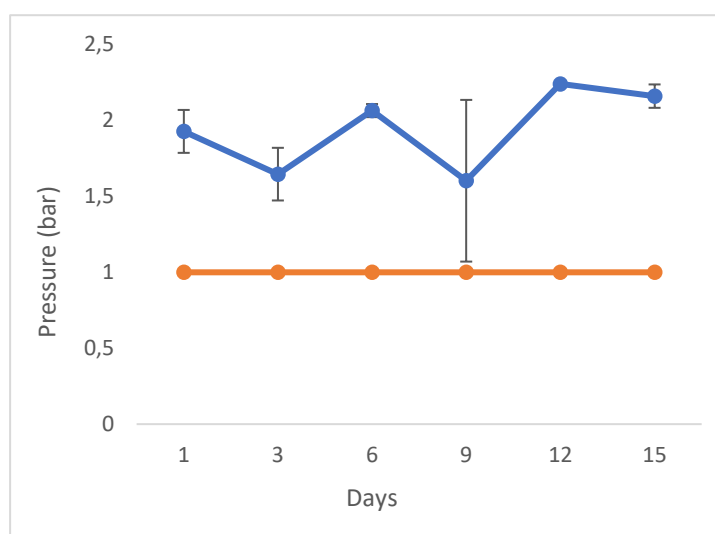


Fig. 1: Productivity measurement via pressure over 15 days. Inoculated system in blue, zero control in orange.

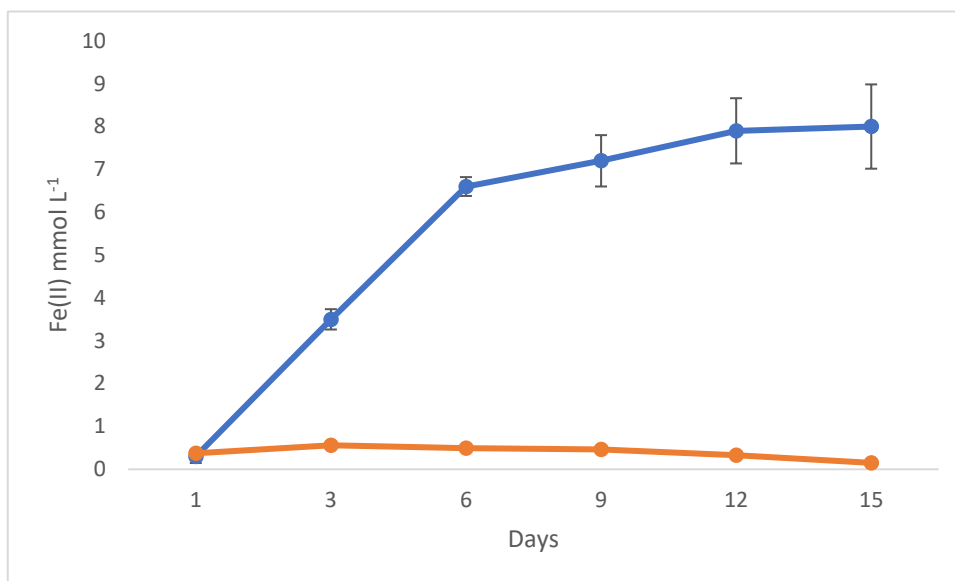


Fig. 2a: Iron reduction/Fe(II) production over 15 days. Inoculated system in blue, zero control in orange.

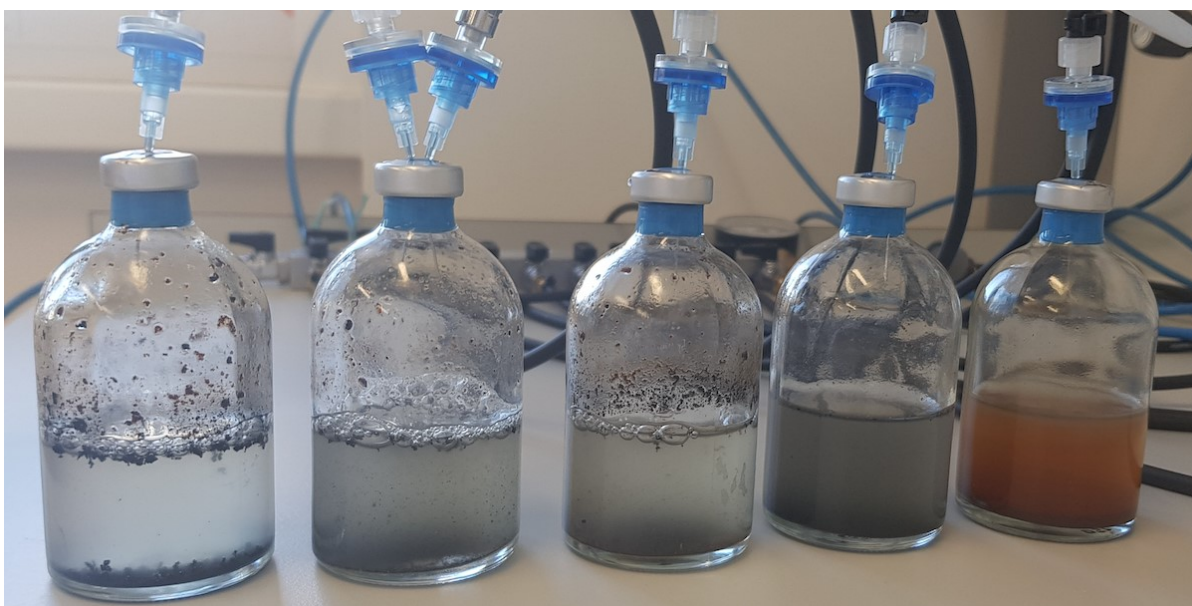


Fig. 2b: Color and structural changes of ferrihydrite. Right zero control, remaining bottles inoculated and augmented ferrihydrite after 15 days of incubation.

These black precipitates were supposed to be magnetite (Prakash et al., 2019; Yamada et al., 2014) but interestingly only in the beginning of the experiment, precisely on the second sampling day, magnetite peaks were identified during XRD measurements (Fig. 3a). These peaks decreased during the third and fourth measurements and no peaks were detectable from the fifth measurement onward. The zero-controls showed similar magnetite peaks as

inoculated bottles indicating magnetite formation in the zero control (Fig. 3b), which contradicts iron reduction values of zero controls (Fig. 2a).

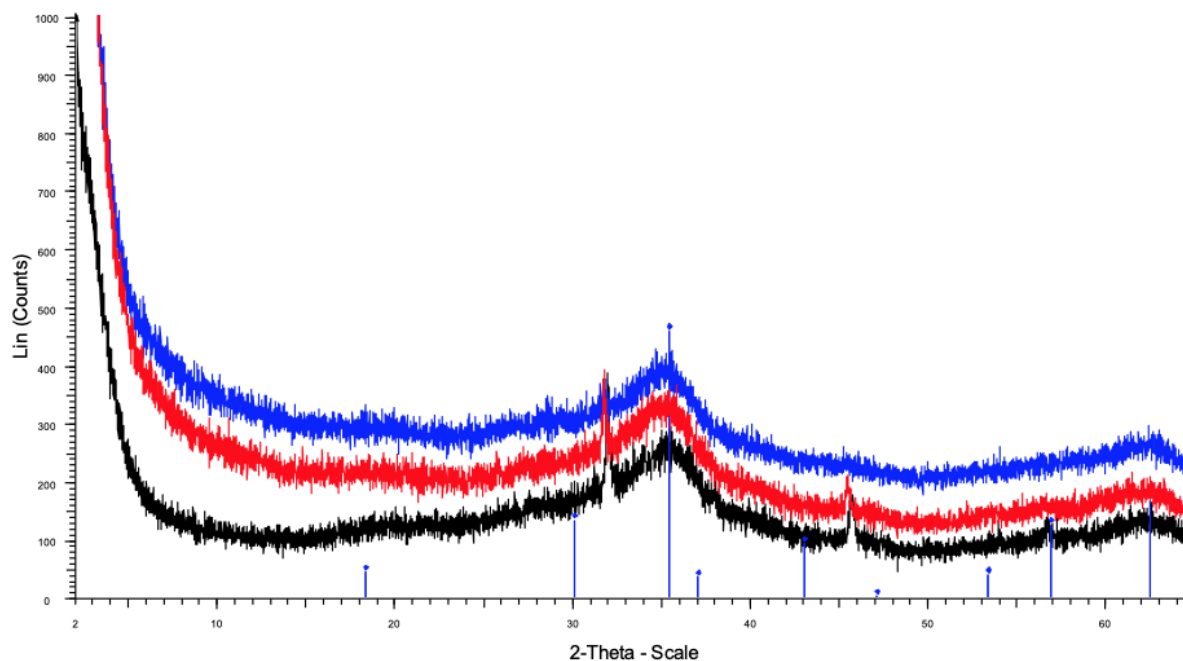


Fig. 3a: XRD spectrum, magnetite peak in inoculated bottles (8,7,6 top down) from the first measurement.

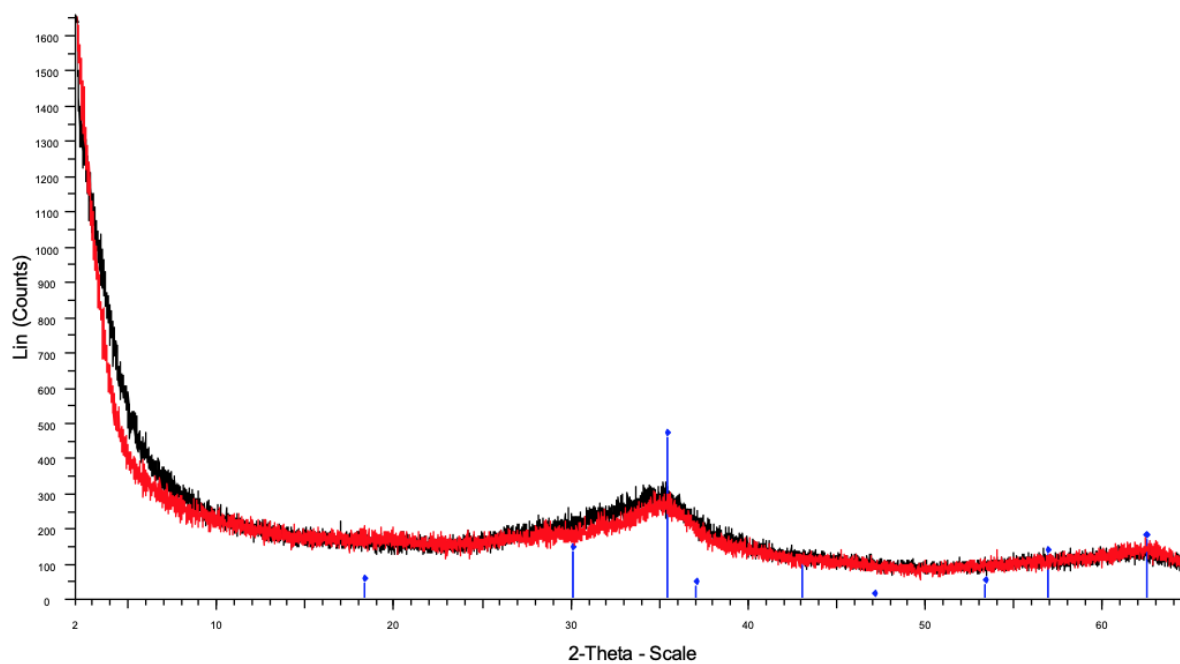


Fig. 3b: XRD spectrum, magnetite peak in inoculated bottle (8, red) compared to zero control (black).

To investigate the role of pH and time on magnetite formation an additional control group was established and after changing the headspace from N₂CO₂ (80:20) to N₂, the pH of the inoculated medium increased from pH 6.5 to pH 7.5 after 18 days of incubation. Here, despite the black colored precipitates no magnetite was identified. Furthermore, to link iron reduction to the metabolism, ferrihydrite was added to an additional control group after 18 days of incubation where methanol was depleted and not further augmented. Here, no magnetite was identified and the ferrihydrite maintained the typical red-brown colored slurry for another 14 days under a N₂ headspace.

Discussion

Previous studies on extracellular electron transfer focused on either using the humic acid analogue AQDS to enhance iron reduction (Bond & Lovley, 2002; Holmes et al., 2019; Prakash et al., 2019; Yamada et al., 2014), decouple iron reduction from the active metabolism (Shang et al., 2020; Sivan et al., 2016), or using acetate, which is unlikely used as a substrate for magnetite formation in the methanogenic zone (Prakash et al., 2019). The impact of iron species on CH₄ production and enumerating the specific gas composition in the headspace was not part of this study since this was described extensively in previous studies (Bodegom et al., 2004; Bond & Lovley, 2002; Prakash et al., 2019). We focused on representing a near natural environment reflected in the chosen media and solutions used during the experiment. Ferrihydrite is a prevalent iron mineral in natural environments and therefore a traceable source (Jambor & Dutrizac, 1998). Thus, *M. acetivorans* was cultured in a defined medium without adding crucial reducing agents, organics, or additional electron shuttles. We were able to link iron reduction to methylotrophic methanogenesis through an already proposed (Holmes et al., 2019) and herewith adapted pathway (Fig. 4). As shown in Fig. 4, methanol is converted to CH₃-CoM using 3 specific enzymes (Bose et al., 2006), and is then partly oxidized to CO₂ while being simultaneously reduced to CH₄, according to equation (1). During CO₂ production, the cofactor F₄₂₀ and the electron transfer protein ferredoxin appear in their reduced form but are re-oxidized through the Fpo complex (F₄₂₀:methanophenazine oxidoreductase) (Yan & Ferry, 2018) and the Rnf complex (M. Wang et al., 2011) respectively, where in both cases a proton/sodium translocation takes place and ATP can be generated. Electron transport takes place either via methanophenazine or directly through Rnf to the membrane multiheme cytochrome A (MmcA) which is most likely the direct intermediate for iron reduction (Holmes et al., 2019). Exact ATP yields with methanol as substrate compared to conventional methanogenesis has yet to be determined but *M. acetivorans* showed enhanced ATP yields when grown on acetate and ferrihydrite (Prakash et al., 2019) and enhanced growth rates when grown on methanol and AQDS as external electron acceptor (Holmes et al., 2019). This knowledge leads to the assumption that iron reduction might be a beneficial metabolic pathway in an otherwise challenging and competitive environment such as marine sediments, therefore opening a new ecological niche for methanogens. Considering previous results and combined

with the results demonstrated here, it can be assumed that methylotrophic methanogens from the Methanosarcinales might indeed be a driving force for magnetite formation in marine sediments.

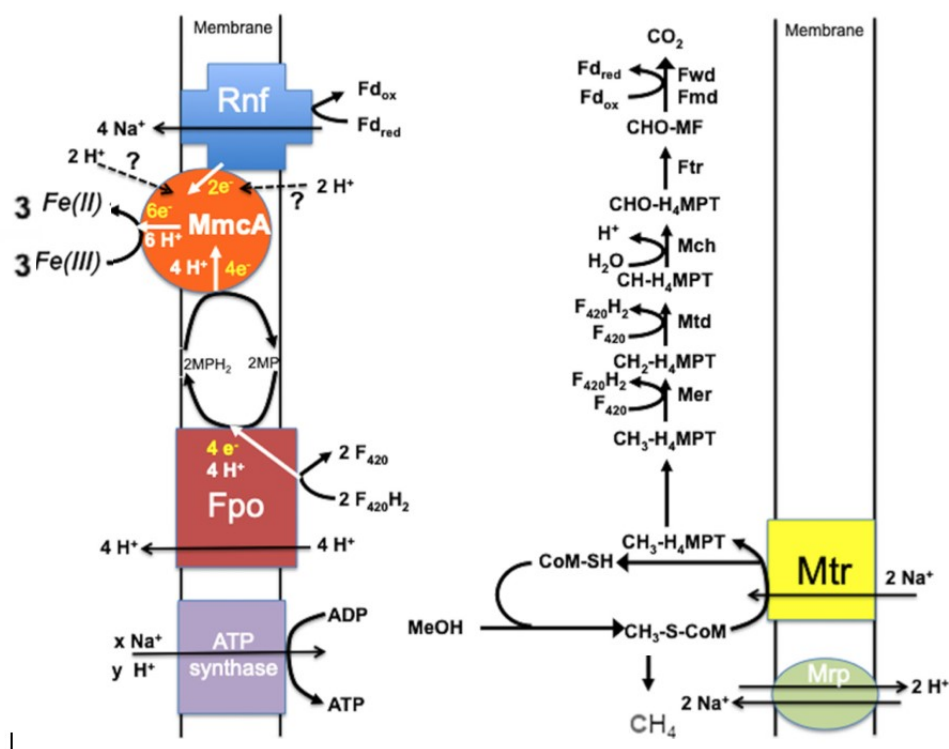


Fig. 4: Adapted and proposed pathway for iron reduction with methanol in *Methanosarcina acetivorans*. (Holmes et al., 2019)

However, in this study only minor magnetite peaks were detected in the precipitates and this might be for several reasons. Hydrogen sulfide and other sulfuric components inhibit magnetite precipitation by enhancing its solubility in natural environments (Roberts, 2015). In our experiments, the concentration of Na_2S was already lowered to 1 mmol L^{-1} but may have been nevertheless a possible inhibitor for magnetite formation. Furthermore, our results showed high Fe(II) production and hence iron reduction might have been too rapid to allow for magnetite formation. This could explain why, up to a certain point, no magnetite peaks were detected in XRD. Methanol was added continuously over the whole experiment run and therefore nearly all of Fe(III) was available for reduction. Further crucial factors for magnetite formation are the ambient pH and redox potential of the parent solution (Roh et al., 2003). The final pH of the medium was 6.5 and not further adjusted by buffering with carbonate, to avoid potential siderite formation (Roh et al., 2003). Therefore, the partial pressure of produced CO_2 might have further lowered the pH to a point where magnetite is no longer stable. By omitting reducing agents like L-cysteine completely, there is the possibility that the redox potential was not

aligned to methanogenesis (Reeburgh, 1983) which is reflected in our unpublished data where *Methanosarcina barkeri* was not able to grow without it. Nevertheless, *Methanosarcina acetivorans* was able to thrive in our defined medium, possibly mediating its own low redox microenvironment (Fetzer & Conrad, 1993). The identified magnetite peak in the zero controls might be due to small abiotically induced reduction via sulfide, which contrasts data from Roberts (2015). Furthermore, no sulfide minerals such as pyrite were identified in XRD. Additionally, zero controls showed only minor iron reduction values and remained almost identical over the whole experiment run making it unlikely to form magnetite.

Our findings suggest that Methanosarcinales possess the capability to shuttle electrons to the outside of the cell (Abken et al., 1998; Kühn et al., 1983). This might have catalyzed iron reduction in conditions resembling marine sediments, which again highlights the incomplete knowledge of the metabolic diversity regarding archaea physiology. Despite yet lacking direct evidence for methanogen-driven magnetite formation, we were able to link these specific organisms to iron reduction with some confidence, thus shedding new light on the impact of methanogenesis on mineral formation. Further data is still required, to overcome experimental challenges by adaptations of required key parameters. Future work can then be conducted to compare the properties of magnetite produced by culture experiments to recently discovered authigenic magnetite in marine sediments. (Lin et al., 2020).

References

- Abken, H. J., Tietze, M., Brodersen, J., Bäumer, S., Beifuss, U., & Deppenmeier, U. (1998). Isolation and characterization of methanophenazine and function of phenazines in membrane-bound electron transport of *Methanosarcina mazei* Gö1. *Journal of Bacteriology*, 180(8), 2027–2032. <https://doi.org/10.1128/JB.180.8.2027-2032.1998>
- Aromokeye, D. A., Kulkarni, A. C., Elvert, M., Wegener, G., Henkel, S., Coffinet, S., Eickhorst, T., Oni, O. E., Richter-Heitmann, T., Schnakenberg, A., Taubner, H., Wunder, L., Yin, X., Zhu, Q., Hinrichs, K.-U., Kasten, S., & Friedrich, M. W. (2020). Rates and Microbial Players of Iron-Driven Anaerobic Oxidation of Methane in Methanic Marine Sediments. *Frontiers in Microbiology*, 10. <https://www.frontiersin.org/article/10.3389/fmicb.2019.03041>
- Beal, E. J., House, C. H., & Orphan, V. J. (2009). Manganese- and iron-dependent marine methane oxidation. *Science (New York, N.Y.)*, 325(5937), 184–187. <https://doi.org/10.1126/science.1169984>
- Bodegom, P. M., Scholten, J. C. M., & Stams, A. J. M. (2004). Direct inhibition of methanogenesis by ferric iron. *FEMS Microbiology Ecology*, 49(2), 261–268. <https://doi.org/10.1016/j.femsec.2004.03.017>
- Boetius, A., Ravensschlag, K., Schubert, C. J., Rickert, D., Widdel, F., Gieseke, A., Amann, R., Jørgensen, B. B., Witte, U., & Pfannkuche, O. (2000). A marine microbial consortium apparently mediating anaerobic oxidation of methane. *Nature*, 407(6804), 623–626. <https://doi.org/10.1038/35036572>
- Bond, D. R., & Lovley, D. R. (2002). Reduction of Fe(III) oxide by methanogens in the presence and absence of extracellular quinones. *Environmental Microbiology*, 4(2), 115–124. <https://doi.org/10.1046/j.1462-2920.2002.00279.x>
- Bose, A., Pritchett, M. A., Rother, M., & Metcalf, W. W. (2006). Differential Regulation of the Three Methanol Methyltransferase Isozymes in *Methanosarcina acetivorans* C2A.

- Journal of Bacteriology*, 188(20), 7274–7283. <https://doi.org/10.1128/JB.00535-06>
- Claypool, G. E., & Kaplan, I. R. (1974). The Origin and Distribution of Methane in Marine Sediments. In I. R. Kaplan (Hrsg.), *Natural Gases in Marine Sediments* (S. 99–139). Springer US. https://doi.org/10.1007/978-1-4684-2757-8_8
- Cornell, R. M., & Schwertmann, U. (2003). *The Iron Oxides: Structure, Properties, Reactions, Occurrences and Uses* (1. Aufl.). Wiley. <https://doi.org/10.1002/3527602097>
- Devouard, B., Posfai, M., Hua, X., Bazylnski, D. A., Frankel, R. B., & Buseck, P. R. (1998). Magnetite from magnetotactic bacteria; size distributions and twinning. *American Mineralogist*, 83(11-12_Part_2), 1387–1398. <https://doi.org/10.2138/am-1998-11-1228>
- Fetzer, S., & Conrad, R. (1993). Effect of redox potential on methanogenesis by *Methanosarcina barkeri*. *Archives of Microbiology*, 160(2), 108–113. <https://doi.org/10.1007/BF00288711>
- Hallam, S. J., Putnam, N., Preston, C. M., Detter, J. C., Rokhsar, D., Richardson, P. M., & DeLong, E. F. (2004). Reverse Methanogenesis: Testing the Hypothesis with Environmental Genomics. *Science*. <https://doi.org/10.1126/science.1100025>
- Hinrichs, K. U., Hayes, J. M., Sylva, S. P., Brewer, P. G., & DeLong, E. F. (1999). Methane-consuming archaeobacteria in marine sediments. *Nature*, 398(6730), 802–805. <https://doi.org/10.1038/19751>
- Hinrichs, K.-U., & Boetius, A. (2002). *The Anaerobic Oxidation of Methane: New Insights in Microbial Ecology and Biogeochemistry*. 457–477.
- Holmes, D. E., Ueki, T., Tang, H.-Y., Zhou, J., Smith, J. A., Chaput, G., & Lovley, D. R. (2019). A Membrane-Bound Cytochrome Enables *Methanosarcina acetivorans* To Conserve Energy from Extracellular Electron Transfer. *MBio*. <https://doi.org/10.1128/mBio.00789-19>
- Ionescu, D., Heim, C., Polerecky, L., Thiel, V., & de Beer, D. (2015). Biotic and abiotic oxidation and reduction of iron at circumneutral pH are inseparable processes under natural

- conditions. *Geomicrobiology*, 32. <https://doi.org/10.1080/01490451.2014.887393>
- Jambor, J. L., & Dutrizac, J. E. (1998). Occurrence and Constitution of Natural and Synthetic Ferrihydrite, a Widespread Iron Oxyhydroxide. *Chemical Reviews*, 98(7), 2549–2586. <https://doi.org/10.1021/cr970105t>
- Jørgensen, B. B., & Kasten, S. (2006). Sulfur Cycling and Methane Oxidation. In H. D. Schulz & M. Zabel (Hrsg.), *Marine Geochemistry* (S. 271–309). Springer. https://doi.org/10.1007/3-540-32144-6_8
- Kühn, W., Fiebig, K., Hippe, H., Mah, R. A., Huser, B. A., & Gottschalk, G. (1983). Distribution of cytochromes in methanogenic bacteria. *FEMS Microbiology Letters*, 20(3), 407–410. <https://doi.org/10.1111/j.1574-6968.1983.tb00157.x>
- Li, Y.-L., Pfiffner, S. M., Dyar, M. D., Vali, H., Konhauser, K., Cole, D. r., Rondinone, A. J., & Phelps, T. J. (2009). Degeneration of biogenic superparamagnetic magnetite. *Geobiology*, 7(1), 25–34. <https://doi.org/10.1111/j.1472-4669.2008.00186.x>
- Lin, Z., Sun, X., Roberts, A. P., Strauss, H., Lu, Y., Yang, X., Gong, J., Li, G., Brunner, B., & Peckmann, J. (2020). A novel authigenic magnetite source for sedimentary magnetization. *Geology*, 49(4), 360–365. <https://doi.org/10.1130/G48069.1>
- Liu, Y., & Whitman, W. B. (2008). Metabolic, phylogenetic, and ecological diversity of the methanogenic archaea. *Annals of the New York Academy of Sciences*, 1125, 171–189. <https://doi.org/10.1196/annals.1419.019>
- Lovley, D. R. (1991). Magnetite Formation During Microbial Dissimilatory Iron Reduction. In R. B. Frankel & R. P. Blakemore (Hrsg.), *Iron Biominerals* (S. 151–166). Springer US. https://doi.org/10.1007/978-1-4615-3810-3_11
- Lovley, D. R., & Phillips, E. J. (1986). Organic matter mineralization with reduction of ferric iron in anaerobic sediments. *Applied and Environmental Microbiology*, 51(4), 683–689. <https://doi.org/10.1128/aem.51.4.683-689.1986>
- Prakash, D., Chauhan, S. S., & Ferry, J. G. (2019). Life on the thermodynamic edge:

- Respiratory growth of an acetotrophic methanogen. *Science Advances*, 5(8), eaaw9059. <https://doi.org/10.1126/sciadv.aaw9059>
- Reeburgh, W. S. (1983). Rates of Biogeochemical Processes in Anoxic Sediments. *Annual Review of Earth and Planetary Sciences*, 11(1), 269–298. <https://doi.org/10.1146/annurev.ea.11.050183.001413>
- Reeburgh, W. S. (2007). Oceanic Methane Biogeochemistry. *Chemical Reviews*, 107(2), 486–513. <https://doi.org/10.1021/cr050362v>
- Roberts, A. P. (2015). Magnetic mineral diagenesis. *Earth-Science Reviews*, 151, 1–47. <https://doi.org/10.1016/j.earscirev.2015.09.010>
- Roh, Y., Zhang, C.-L., Vali, H., Lauf, R. J., Zhou, J., & Phelps, T. J. (2003). BIOGEOCHEMICAL AND ENVIRONMENTAL FACTORS IN Fe BIOMINERALIZATION: MAGNETITE AND SIDERITE FORMATION. *Clays and Clay Minerals*, 51(1), 83–95. <https://doi.org/10.1346/CCMN.2003.510110>
- Rother, M., & Metcalf, W. W. (2004). Anaerobic growth of *Methanosarcina acetivorans* C2A on carbon monoxide: An unusual way of life for a methanogenic archaeon. *Proceedings of the National Academy of Sciences*, 101(48), 16929–16934. <https://doi.org/10.1073/pnas.0407486101>
- Schönheit, P., Kristjánsson, J., & Thauer, R. (2004). Kinetic mechanism for the ability of sulfate reducers to out-compete methanogens for acetate. *Archives of Microbiology*. <https://doi.org/10.1007/BF00407967>
- Shang, H., Daye, M., Sivan, O., Borlina, C. S., Tamura, N., Weiss, B. P., & Bosak, T. (2020). Formation of Zerovalent Iron in Iron-Reducing Cultures of *Methanosarcina barkeri*. *Environmental Science & Technology*, 54(12), 7354–7365. <https://doi.org/10.1021/acs.est.0c01595>
- Sivan, O., Shusta, S. S., & Valentine, D. L. (2016). Methanogens rapidly transition from methane production to iron reduction. *Geobiology*, 14(2), 190–203.

<https://doi.org/10.1111/gbi.12172>

- Soo, V. W. C., McAnulty, M. J., Tripathi, A., Zhu, F., Zhang, L., Hatzakis, E., Smith, P. B., Agrawal, S., Nazem-Bokaei, H., Gopalakrishnan, S., Salis, H. M., Ferry, J. G., Maranas, C. D., Patterson, A. D., & Wood, T. K. (2016). Reversing methanogenesis to capture methane for liquid biofuel precursors. *Microbial Cell Factories*, 15, 11. <https://doi.org/10.1186/s12934-015-0397-z>
- Sowers, K. R., Baron, S. F., & Ferry, J. G. (1984). Methanosarcina acetivorans sp. Nov., an Acetotrophic Methane-Producing Bacterium Isolated from Marine Sediments. *Applied and Environmental Microbiology*, 47(5), 971–978. <https://doi.org/10.1128/aem.47.5.971-978.1984>
- Taubner, R.-S., & Rittmann, S. K.-M. R. (2016). Method for Indirect Quantification of CH₄ Production via H₂O Production Using Hydrogenotrophic Methanogens. *Frontiers in Microbiology*, 7, 532. <https://doi.org/10.3389/fmicb.2016.00532>
- Thauer, R. K., Kaster, A.-K., Seedorf, H., Buckel, W., & Hedderich, R. (2008). Methanogenic archaea: Ecologically relevant differences in energy conservation. *Nature Reviews Microbiology*, 6(8), 579–591. <https://doi.org/10.1038/nrmicro1931>
- Timmers, P. H. A., Welte, C. U., Koehorst, J. J., Plugge, C. M., Jetten, M. S. M., & Stams, A. J. M. (2017). Reverse Methanogenesis and Respiration in Methanotrophic Archaea. *Archaea*, 2017, e1654237. <https://doi.org/10.1155/2017/1654237>
- Treude, T., Krause, S., Maltby, J., Dale, A. W., Coffin, R., & Hamdan, L. J. (2014). Sulfate reduction and methane oxidation activity below the sulfate-methane transition zone in Alaskan Beaufort Sea continental margin sediments: Implications for deep sulfur cycling. *Geochimica et Cosmochimica Acta*, 144, 217–237. <https://doi.org/10.1016/j.gca.2014.08.018>
- Wang, F.-P., Zhang, Y., Chen, Y., He, Y., Qi, J., Hinrichs, K.-U., Zhang, X.-X., Xiao, X., & Boon, N. (2014). Methanotrophic archaea possessing diverging methane-oxidizing and

- electron-transporting pathways. *The ISME Journal*, 8(5), 1069–1078.
<https://doi.org/10.1038/ismej.2013.212>
- Wang, M., Tomb, J.-F., & Ferry, J. G. (2011). Electron transport in acetate-grown *Methanosarcina acetivorans*. *BMC Microbiology*, 11(1), 165.
<https://doi.org/10.1186/1471-2180-11-165>
- Wolin, E. A., Wolfe, R. S., & Wolin, M. J. (1964). Viologen dye inhibition of methane formation by *Methanobacillus omelianskii*. *Journal of Bacteriology*, 87(5), 993–998.
<https://doi.org/10.1128/jb.87.5.993-998.1964>
- Yamada, C., Kato, S., Kimura, S., Ishii, M., & Igarashi, Y. (2014). Reduction of Fe(III) oxides by phylogenetically and physiologically diverse thermophilic methanogens. *FEMS Microbiology Ecology*, 89(3), 637–645. <https://doi.org/10.1111/1574-6941.12365>
- Yan, Z., & Ferry, J. G. (2018). Electron Bifurcation and Confurcation in Methanogenesis and Reverse Methanogenesis. *Frontiers in Microbiology*, 9.
<https://www.frontiersin.org/article/10.3389/fmicb.2018.01322>
- Yan, Z., Joshi, P., Gorski, C. A., & Ferry, J. G. (2018). A biochemical framework for anaerobic oxidation of methane driven by Fe(III)-dependent respiration. *Nature Communications*, 9(1), 1642. <https://doi.org/10.1038/s41467-018-04097-9>
- Zhuang, G., Heuer, V., Lazar, C., Goldhammer, T., Wendt, J., Samarkin, V., Elvert, M., Teske, A., Joye, S., & Hinrichs, K.-U. (2018). Relative importance of methylotrophic methanogenesis in sediments of the Western Mediterranean Sea. *Geochimica et Cosmochimica Acta*, 224. <https://doi.org/10.1016/j.gca.2017.12.024>

3 Discussion

In Zipperle et al., 2021 a biological recycling system was established where toxic waste gases are transformed into biological produced gas usable as a fuel for daily human needs, showing again the massive potential of archaea for biotechnological application (Pfeifer et al., 2021). Furthermore, a system was established where the inhibitory effects of CO on methanogens are circumvented by using another archaeal organism, approaching a more realistic natural environment reflecting the complexity of global biogeochemical cycles in natural ecosystems. Certain methanogens are able to metabolize CO directly (Bott et al., 1986; Daniels et al., 1977; Rother & Metcalf, 2004), but not on a biotechnological relevant scale (Daniels et al., 1977; Diender et al., 2016). This relevance is nevertheless of great importance, since beside the possibility of biogas production through methanogens is given, future research must focus on intensive upscaling to make it economically viable. This transition to alternative and in best case biologically produced fuels (Porqueras et al., 2012) is inevitable since fossil fuels will become depleted in near future (Shafiee & Topal, 2009) and additionally challenge the climate drastically. Another yet limiting factor in our biomethanation process is the complexity of the medium since yeast is still a crucial factor (Zipperle et al., 2021) for the hydrogenogenic but heterotroph organism *Thermococcus onnurineus* (Bae et al., 2006). Yeast extract is a complex mixture of autolyzed cell content which is therefore not entirely characterized and additionally expensive, hence not ideal for industrial application. A reasonable approach is to find a different organism performing the water-gas shift reaction and to further genetically engineer the organism by overexpressing the CO dehydrogenase (Kim et al., 2013) to reach the highest possible yield. The factor temperature may be a controversial topic since hyperthermophilic archaea need high temperatures and therefore energy may be consumed for heating the system. But it is already well established that in running biogas plants the naturally produced heat can be reused through heat exchangers and therefore they run towards self-sustainable systems. Due to generally higher methane evolution rates in hyperthermophilic methanogens and the capability of these organism to withstand high pressure (Mauerhofer et al., 2021) therefore outweigh discussed arguments above and make these hyperthermophilic co-cultures a comprehensive approach for biogas production. These yet limiting factors are therefore no insuperable barriers but feasible challenges for near future research.

Hyperthermophilic methanogens are mainly isolated from hydrothermal vents in marine seafloor environments. They thrive on H₂ and CO₂ through the Wood-Ljungdahl pathway, an ancient carbon fixation pathway (Borrel et al., 2016) which is proposed to be one of the first metabolic pathways in Earth's history with CH₄ as endproduct (Martin & Russell, 2007). With hydrogenotrophic methanogenesis being possibly the most ancient of these metabolic types, the methylotrophic pathway appears to have a partly different evolutionary background (Muñoz-Velasco et al., 2018).

Methylotrophic methanogenesis is the predominant methanogenic pathway in marine sediments (Zhuang et al., 2018). Beside the fact that the transformation from acetate is

responsible for the major global production of CH₄ (Deppenmeier, 2002), in marine sediments, sulfate reducing bacteria outcompete methanogens for this substrate (Schönheit et al., 2004). The same applies for H₂ (Kristjansson et al., 1982), greatly decreasing the potential ecological niche for methanogens vastly. This might be the reason that certain methanogens lost the capability to metabolize H₂ since even methanogens without cytochromes outcompete methanogens with cytochromes for H₂ (Thauer et al., 2008) which might indicate the substrate specification in the methanogenic zone in marine sediments (Zhuang et al., 2018). The methanogenic zone is the layer below the sulfate-methane transition zone (SMTZ) where methanogens dominate in contrast to the dominating anaerobic methane oxidizers (AOM) in the SMTZ (Iversen & Jorgensen, 1985). In these interdependent layers upwardly diffusing CH₄ is oxidized via sulfate reduction by anaerobic methanotrophs (ANME) (Hinrichs & Boetius, 2002) which are often in a syntrophic consortium with sulfate-reducing bacteria (Knittel & Boetius, 2009). This interdependent relationship, as well as their slow growth rates (Girguis et al., 2005), might be one of the reasons why no ANME organism could be isolated and brought to pure culture so far. Phylogenetically ANME organisms can be separated into three clusters (ANME-1, ANME-2, ANME-3) all of which are closely related to methanogens. ANME-1 are closely related to the order of Methanomicrobiales (Hinrichs & Boetius, 2002) and additionally seem to be more adapted to higher temperatures (Biddle et al., 2012) and in contrast ANME-2 and ANME-3 show close relation to the order of Methanosarcinales (Hinrichs & Boetius, 2002) and Methanococcoides (Knittel et al., 2005) respectively, and are more abundant in colder environments. Especially the phylogenetic relation from ANME-2 to the Methanosarcinales and the extensive biochemical research on the potential possibility of these methanogens to perform reverse methanogenesis (Hallam et al., 2004) coupled to an external electron acceptor (Yan et al., 2018) lead us to the assumption that this potential pathway might provide a link to authigenic magnetite formation in marine sediments (Lin et al., 2020). The present study however, comes to the conclusion that this route is unlikely since no methanogen performed reverse methanogenesis in the laboratory environment. Exclusively a engineered *Methanosarcina acetivorans* with the methyl-coenzyme M reductase from a ANME organism was capable to perform this otherwise irreversible step (Scheller et al., 2010) (Soo et al., 2016) indicating the distinct relation but evolutionary specification of the target enzyme. Since the SMTZ and the methanogenic zone are more merged than previously assumed (Claypool & Kaplan, 1974; Treude et al., 2014) the possibility that anaerobic oxidation of methane coupled to iron reduction (Aromokeye et al., 2020) may still be a potential source for magnetite precipitation in methanic sediments.

In our experimental procedure, which is summarized methodically and theoretically in our manuscript, we were able to couple iron reduction to methylotrophic methanogenesis. Besides the lack of significant magnetite formation, the necessary step in this process was achieved with success. In both cases, Fe-AOM and methylotrophic methanogenesis coupled to iron oxides, an external electron acceptor is mandatory for magnetite precipitation. Additionally, both potential organism groups share very similar electron transporting pathways (F.-P. Wang

et al., 2014). Despite clear evidence of methane oxidation coupled to iron reduction in the methanogenic zone (Aromokeye et al., 2020), methylotrophic methanogens remain key players in this ecosystem, making them the more likely candidates in a novel microbial formation pathway of authigenic magnetite in marine sediments (Lin et al., 2020).

For this master's thesis methanogens were the key players in two different scientific approaches. In both cases extensive research and build up methodical procedures lead to the confirmation of established hypotheses. Based on our newly gathered data, further research may expand the application of these organisms for industrial use and for basic science investigating the evolutionary and geologically history of Earth.

4 References

- Abken, H. J., Tietze, M., Brodersen, J., Bäumer, S., Beifuss, U., & Deppenmeier, U. (1998). Isolation and characterization of methanophenazine and function of phenazines in membrane-bound electron transport of *Methanosarcina mazei* Gö1. *Journal of Bacteriology*, 180(8), 2027–2032. <https://doi.org/10.1128/JB.180.8.2027-2032.1998>
- Aromokeye, D. A., Kulkarni, A. C., Elvert, M., Wegener, G., Henkel, S., Coffinet, S., Eickhorst, T., Oni, O. E., Richter-Heitmann, T., Schnakenberg, A., Taubner, H., Wunder, L., Yin, X., Zhu, Q., Hinrichs, K.-U., Kasten, S., & Friedrich, M. W. (2020). Rates and Microbial Players of Iron-Driven Anaerobic Oxidation of Methane in Methanic Marine Sediments. *Frontiers in Microbiology*, 10. <https://www.frontiersin.org/article/10.3389/fmicb.2019.03041>
- Bae, S.-S., Kim, Y.-J., Yang, S.-H., Lim, J.-K., Jeon, J.-H., Lee, H.-S., Kang, S.-G., Kim, S.-J., & Lee, J.-H. (2006). *Thermococcus onnurineus* sp. Nov., a Hyperthermophilic Archaeon Isolated from a Deep-Sea Hydrothermal Vent Area at the PACMANUS Field. *Journal of Microbiology and Biotechnology*, 16(11), 1826–1831.
- Bellack, A., Huber, H., Rachel, R., Wanner, G., & Wirth, R. 2011. (2011). *Methanocaldococcus villosus* sp. Nov., a heavily flagellated archaeon that adheres to surfaces and forms cell–cell contacts. *International Journal of Systematic and Evolutionary Microbiology*, 61(6), 1239–1245. <https://doi.org/10.1099/ij.s.0.023663-0>
- Bertram, P. A., & Thauer, R. K. (1994). Thermodynamics of the Formylmethanofuran Dehydrogenase Reaction in *Methanobacterium thermoautotrophicum*. *European Journal of Biochemistry*, 226(3), 811–818. <https://doi.org/10.1111/j.1432-1033.1994.t01-1-00811.x>
- Biddle, J. F., Cardman, Z., Mendlovitz, H., Albert, D. B., Lloyd, K. G., Boetius, A., & Teske, A. (2012). Anaerobic oxidation of methane at different temperature regimes in Guaymas Basin hydrothermal sediments. *The ISME Journal*, 6(5), 1018–1031.

<https://doi.org/10.1038/ismej.2011.164>

Bond, D. R., & Lovley, D. R. (2002). Reduction of Fe(III) oxide by methanogens in the presence and absence of extracellular quinones. *Environmental Microbiology*, 4(2), 115–124. <https://doi.org/10.1046/j.1462-2920.2002.00279.x>

Borrel, G., Adam, P. S., & Gribaldo, S. (2016). Methanogenesis and the Wood–Ljungdahl Pathway: An Ancient, Versatile, and Fragile Association. *Genome Biology and Evolution*, 8(6), 1706–1711. <https://doi.org/10.1093/gbe/evw114>

Bose, A., Pritchett, M. A., Rother, M., & Metcalf, W. W. (2006). Differential Regulation of the Three Methanol Methyltransferase Isozymes in *Methanosarcina acetivorans* C2A. *Journal of Bacteriology*, 188(20), 7274–7283. <https://doi.org/10.1128/JB.00535-06>

Bott, M., Eikmanns, B., & Thauer, R. K. (1986). Coupling of carbon monoxide oxidation to CO₂ and H₂ with the phosphorylation of ADP in acetate-grown *Methanosarcina barkeri*. *European Journal of Biochemistry*, 159(2), 393–398. <https://doi.org/10.1111/j.1432-1033.1986.tb09881.x>

Buckel, W., & Thauer, R. K. (2013). Energy conservation via electron bifurcating ferredoxin reduction and proton/Na⁺ translocating ferredoxin oxidation. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, 1827(2), 94–113. <https://doi.org/10.1016/j.bbabi.2012.07.002>

Claypool, G. E., & Kaplan, I. R. (1974). The Origin and Distribution of Methane in Marine Sediments. In I. R. Kaplan (Hrsg.), *Natural Gases in Marine Sediments* (S. 99–139). Springer US. https://doi.org/10.1007/978-1-4684-2757-8_8

Costa, K. C., & Leigh, J. A. (2014). Metabolic versatility in methanogens. *Current Opinion in Biotechnology*, 29, 70–75. <https://doi.org/10.1016/j.copbio.2014.02.012>

Daniels, L., Fuchs, G., Thauer, R. K., & Zeikus, J. G. (1977). Carbon Monoxide Oxidation by Methanogenic Bacteria. *Journal of Bacteriology*, 132(1), 118–126. <https://doi.org/10.1128/jb.132.1.118-126.1977>

- Dean, J. F., Middelburg, J. J., Röckmann, T., Aerts, R., Blauw, L. G., Egger, M., Jetten, M. S. M., de Jong, A. E. E., Meisel, O. H., Rasigraf, O., Slomp, C. P., in't Zandt, M. H., & Dolman, A. J. (2018). Methane Feedbacks to the Global Climate System in a Warmer World. *Reviews of Geophysics*, 56(1), 207–250. <https://doi.org/10.1002/2017RG000559>
- Deppenmeier, U. (2002). The unique biochemistry of methanogenesis. In *Progress in Nucleic Acid Research and Molecular Biology* (Bd. 71, S. 223–283). Academic Press. [https://doi.org/10.1016/S0079-6603\(02\)71045-3](https://doi.org/10.1016/S0079-6603(02)71045-3)
- Diender, M., Pereira, R., Wessels, H. J. C. T., Stams, A. J. M., & Sousa, D. Z. (2016). Proteomic Analysis of the Hydrogen and Carbon Monoxide Metabolism of *Methanothermobacter marburgensis*. *Frontiers in Microbiology*, 7. <https://www.frontiersin.org/article/10.3389/fmicb.2016.01049>
- Ferry, J. G. (2020). *Methanosarcina acetivorans*: A Model for Mechanistic Understanding of Aceticlastic and Reverse Methanogenesis. *Frontiers in Microbiology*, 11. <https://www.frontiersin.org/article/10.3389/fmicb.2020.01806>
- Girguis, P. R., Cozen, A. E., & DeLong, E. F. (2005). Growth and Population Dynamics of Anaerobic Methane-Oxidizing Archaea and Sulfate-Reducing Bacteria in a Continuous-Flow Bioreactor. *Applied and Environmental Microbiology*, 71(7), 3725–3733. <https://doi.org/10.1128/AEM.71.7.3725-3733.2005>
- Gottschalk, G., & Thauer, R. K. (2001). The Na⁺-translocating methyltransferase complex from methanogenic archaea. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, 1505(1), 28–36. [https://doi.org/10.1016/S0005-2728\(00\)00274-7](https://doi.org/10.1016/S0005-2728(00)00274-7)
- Hallam, S. J., Putnam, N., Preston, C. M., Detter, J. C., Rokhsar, D., Richardson, P. M., & DeLong, E. F. (2004). Reverse Methanogenesis: Testing the Hypothesis with Environmental Genomics. *Science*. <https://doi.org/10.1126/science.1100025>
- Herzog, B., & Wirth, R. (2012). Swimming behavior of selected species of Archaea. *Applied*

- and *Environmental Microbiology*, 78(6), 1670–1674.
<https://doi.org/10.1128/AEM.06723-11>
- Hinrichs, K.-U., & Boetius, A. (2002). *The Anaerobic Oxidation of Methane: New Insights in Microbial Ecology and Biogeochemistry*. 457–477.
- Holmes, D. E., Ueki, T., Tang, H.-Y., Zhou, J., Smith, J. A., Chaput, G., & Lovley, D. R. (2019). A Membrane-Bound Cytochrome Enables Methanosarcina acetivorans To Conserve Energy from Extracellular Electron Transfer. *MBio*.
<https://doi.org/10.1128/mBio.00789-19>
- Iversen, N., & Jorgensen, B. B. (1985). Anaerobic methane oxidation rates at the sulfate-methane transition in marine sediments from Kattegat and Skagerrak (Denmark)1. *Limnology and Oceanography*, 30(5), 944–955.
<https://doi.org/10.4319/lo.1985.30.5.0944>
- Kim, M.-S., Bae, S. S., Kim, Y. J., Kim, T. W., Lim, J. K., Lee, S. H., Choi, A. R., Jeon, J. H., Lee, J.-H., Lee, H. S., & Kang, S. G. (2013). CO-dependent H₂ production by genetically engineered *Thermococcus onnurineus* NA1. *Applied and Environmental Microbiology*, 79(6), 2048–2053. <https://doi.org/10.1128/AEM.03298-12>
- Knittel, K., & Boetius, A. (2009). Anaerobic oxidation of methane: Progress with an unknown process. *Annual Review of Microbiology*, 63, 311–334.
<https://doi.org/10.1146/annurev.micro.61.080706.093130>
- Knittel, K., Lösekann, T., Boetius, A., Kort, R., & Amann, R. (2005). Diversity and Distribution of Methanotrophic Archaea at Cold Seeps. *Applied and Environmental Microbiology*, 71(1), 467–479. <https://doi.org/10.1128/AEM.71.1.467-479.2005>
- Kristjansson, J. K., Schönheit, P., & Thauer, R. K. (1982). Different K_s values for hydrogen of methanogenic bacteria and sulfate reducing bacteria: An explanation for the apparent inhibition of methanogenesis by sulfate. *Archives of Microbiology*, 131(3), 278–282.
<https://doi.org/10.1007/BF00405893>

- Krzycki, J. A. (2004). Function of genetically encoded pyrrolysine in corrinoid-dependent methylamine methyltransferases. *Current Opinion in Chemical Biology*, 8(5), 484–491. <https://doi.org/10.1016/j.cbpa.2004.08.012>
- Kühn, W., Fiebig, K., Hippe, H., Mah, R. A., Huser, B. A., & Gottschalk, G. (1983). Distribution of cytochromes in methanogenic bacteria. *FEMS Microbiology Letters*, 20(3), 407–410. <https://doi.org/10.1111/j.1574-6968.1983.tb00157.x>
- Kurr, M., Huber, R., König, H., Jannasch, H. W., Fricke, H., Trincone, A., Kristjansson, J. K., & Stetter, K. O. (1991). *Methanopyrus kandleri*, gen. And sp. Nov. Represents a novel group of hyperthermophilic methanogens, growing at 110°C. *Archives of Microbiology*, 156(4), 239–247. <https://doi.org/10.1007/BF00262992>
- Li, L., Li, Q., Rohlin, L., Kim, U., Salmon, K., Rejtar, T., Gunsalus, R. P., Karger, B. L., & Ferry, J. G. (2007). Quantitative proteomic and microarray analysis of the archaeon *Methanosarcina acetivorans* grown with acetate versus methanol. *Journal of Proteome Research*, 6(2), 759–771. <https://doi.org/10.1021/pr060383l>
- Li, Q., Li, L., Rejtar, T., Lessner, D. J., Karger, B. L., & Ferry, J. G. (2006). Electron transport in the pathway of acetate conversion to methane in the marine archaeon *Methanosarcina acetivorans*. *Journal of Bacteriology*, 188(2), 702–710. <https://doi.org/10.1128/JB.188.2.702-710.2006>
- Lin, Z., Sun, X., Roberts, A. P., Strauss, H., Lu, Y., Yang, X., Gong, J., Li, G., Brunner, B., & Peckmann, J. (2020). A novel authigenic magnetite source for sedimentary magnetization. *Geology*, 49(4), 360–365. <https://doi.org/10.1130/G48069.1>
- Liu, Y., & Whitman, W. B. (2008). Metabolic, phylogenetic, and ecological diversity of the methanogenic archaea. *Annals of the New York Academy of Sciences*, 1125, 171–189. <https://doi.org/10.1196/annals.1419.019>
- Lyu, Z., Shao, N., Akinyemi, T., & Whitman, W. B. (2018). Methanogenesis. *Current Biology*, 28(13), R727–R732. <https://doi.org/10.1016/j.cub.2018.05.021>

- Martin, W., & Russell, M. J. (2007). On the origin of biochemistry at an alkaline hydrothermal vent. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 362(1486), 1887–1926. <https://doi.org/10.1098/rstb.2006.1881>
- Mauerhofer, L.-M., Zwirtnayr, S., Pappenreiter, P., Bernacchi, S., Seifert, A. H., Reischl, B., Schmider, T., Taubner, R.-S., Paulik, C., & Rittmann, S. K.-M. R. (2021). Hyperthermophilic methanogenic archaea act as high-pressure CH₄ cell factories. *Communications Biology*, 4(1), 1–12. <https://doi.org/10.1038/s42003-021-01828-5>
- Muñoz-Velasco, I., García-Ferris, C., Hernandez-Morales, R., Lazcano, A., Peretó, J., & Becerra, A. (2018). Methanogenesis on Early Stages of Life: Ancient but Not Primordial. *Origins of Life and Evolution of Biospheres*, 48(4), 407–420. <https://doi.org/10.1007/s11084-018-9570-9>
- Myhre, G., Shindell, D., Bréon, F.-M., Collins, W., Fuglestad, J., Huang, J., Koch, D., Lamarque, J.-F., Lee, D., Mendoza, B., Nakajima, T., Robock, A., Stephens, G., Zhang, H., Aamaas, B., Boucher, O., Dalsøren, S. B., Daniel, J. S., Forster, P., ... Shine, K. (2013). 8 Anthropogenic and Natural Radiative Forcing. 82.
- Pfeifer, K., Ergal, I., Koller, M., Basen, M., Schuster, B., & Rittmann, S. K.-M. R. (2021). Archaea Biotechnology. *Biotechnology Advances*, 47, 107668. <https://doi.org/10.1016/j.biotechadv.2020.107668>
- Porqueras, E. M., Rittmann, S., & Herwig, C. (2012). Biofuels and CO₂ neutrality: An opportunity. *Biofuels*, 3(4), 413–426. <https://doi.org/10.4155/bfs.12.25>
- Prakash, D., Chauhan, S. S., & Ferry, J. G. (2019). Life on the thermodynamic edge: Respiratory growth of an acetotrophic methanogen. *Science Advances*, 5(8), eaaw9059. <https://doi.org/10.1126/sciadv.aaw9059>
- Reeburgh, W. S. (2007). Oceanic Methane Biogeochemistry. *Chemical Reviews*, 107(2), 486–513. <https://doi.org/10.1021/cr050362v>
- Reid, I. N., Sparks, W. B., Lubow, S., McGrath, M., Livio, M., Valenti, J., Sowers, K. R., Shukla,

- H. D., MacAuley, S., Miller, T., Suvanasuthi, R., Belas, R., Colman, A., Robb, F. T., DasSarma, P., Müller, J. A., Coker, J. A., Cavicchioli, R., Chen, F., & DasSarma, S. (2006). Terrestrial models for extraterrestrial life: Methanogens and halophiles at Martian temperatures. *International Journal of Astrobiology*, 5(2), 97.
- Rittmann, S. K.-M. R., Lee, H. S., Lim, J. K., Kim, T. W., Lee, J.-H., & Kang, S. G. (2015). One-carbon substrate-based biohydrogen production: Microbes, mechanism, and productivity. *Biotechnology Advances*, 33(1), 165–177. <https://doi.org/10.1016/j.biotechadv.2014.11.004>
- Rother, M., & Metcalf, W. W. (2004). Anaerobic growth of *Methanosarcina acetivorans* C2A on carbon monoxide: An unusual way of life for a methanogenic archaeon. *Proceedings of the National Academy of Sciences*, 101(48), 16929–16934. <https://doi.org/10.1073/pnas.0407486101>
- Scheller, S., Goenrich, M., Boecher, R., Thauer, R. K., & Jaun, B. (2010). The key nickel enzyme of methanogenesis catalyses the anaerobic oxidation of methane. *Nature*, 465(7298), 606–608. <https://doi.org/10.1038/nature09015>
- Schönheit, P., Kristjánsson, J., & Thauer, R. (2004). Kinetic mechanism for the ability of sulfate reducers to out-compete methanogens for acetate. *Archives of Microbiology*. <https://doi.org/10.1007/BF00407967>
- Shafiee, S., & Topal, E. (2009). When will fossil fuel reserves be diminished? *Energy Policy*, 37(1), 181–189. <https://doi.org/10.1016/j.enpol.2008.08.016>
- Shang, H., Daye, M., Sivan, O., Borlina, C. S., Tamura, N., Weiss, B. P., & Bosak, T. (2020). Formation of Zerovalent Iron in Iron-Reducing Cultures of *Methanosarcina barkeri*. *Environmental Science & Technology*, 54(12), 7354–7365. <https://doi.org/10.1021/acs.est.0c01595>
- Soo, V. W. C., McAnulty, M. J., Tripathi, A., Zhu, F., Zhang, L., Hatzakis, E., Smith, P. B., Agrawal, S., Nazem-Bokaee, H., Gopalakrishnan, S., Salis, H. M., Ferry, J. G.,

- Maranas, C. D., Patterson, A. D., & Wood, T. K. (2016). Reversing methanogenesis to capture methane for liquid biofuel precursors. *Microbial Cell Factories*, 15, 11. <https://doi.org/10.1186/s12934-015-0397-z>
- Sowers, K. R., Baron, S. F., & Ferry, J. G. (1984). *Methanosarcina acetivorans* sp. Nov., an Acetotrophic Methane-Producing Bacterium Isolated from Marine Sediments. *Applied and Environmental Microbiology*, 47(5), 971–978. <https://doi.org/10.1128/aem.47.5.971-978.1984>
- Sowers, K. R., Boone, J. E., & Gunsalus, R. P. (1993). Disaggregation of *Methanosarcina* spp. And Growth as Single Cells at Elevated Osmolarity. *Applied and Environmental Microbiology*, 59(11), 3832–3839. <https://doi.org/10.1128/aem.59.11.3832-3839.1993>
- Thauer, R. K., Kaster, A.-K., Seedorf, H., Buckel, W., & Hedderich, R. (2008). Methanogenic archaea: Ecologically relevant differences in energy conservation. *Nature Reviews Microbiology*, 6(8), 579–591. <https://doi.org/10.1038/nrmicro1931>
- Treude, T., Krause, S., Maltby, J., Dale, A. W., Coffin, R., & Hamdan, L. J. (2014). Sulfate reduction and methane oxidation activity below the sulfate-methane transition zone in Alaskan Beaufort Sea continental margin sediments: Implications for deep sulfur cycling. *Geochimica et Cosmochimica Acta*, 144, 217–237. <https://doi.org/10.1016/j.gca.2014.08.018>
- Wang, F.-P., Zhang, Y., Chen, Y., He, Y., Qi, J., Hinrichs, K.-U., Zhang, X.-X., Xiao, X., & Boon, N. (2014). Methanotrophic archaea possessing diverging methane-oxidizing and electron-transporting pathways. *The ISME Journal*, 8(5), 1069–1078. <https://doi.org/10.1038/ismej.2013.212>
- Wang, M., Tomb, J.-F., & Ferry, J. G. (2011). Electron transport in acetate-grown *Methanosarcina acetivorans*. *BMC Microbiology*, 11, 165. <https://doi.org/10.1186/1471-2180-11-165>
- Welte, C., & Deppenmeier, U. (2014). Bioenergetics and anaerobic respiratory chains of

- aceticlastic methanogens. *Biochimica Et Biophysica Acta*, 1837(7), 1130–1147.
<https://doi.org/10.1016/j.bbabbio.2013.12.002>
- Yan, Z., Joshi, P., Gorski, C. A., & Ferry, J. G. (2018). A biochemical framework for anaerobic oxidation of methane driven by Fe(III)-dependent respiration. *Nature Communications*, 9(1), 1642. <https://doi.org/10.1038/s41467-018-04097-9>
- Zhuang, G., Heuer, V., Lazar, C., Goldhammer, T., Wendt, J., Samarkin, V., Elvert, M., Teske, A., Joye, S., & Hinrichs, K.-U. (2018). Relative importance of methylotrophic methanogenesis in sediments of the Western Mediterranean Sea. *Geochimica et Cosmochimica Acta*, 224. <https://doi.org/10.1016/j.gca.2017.12.024>
- Zipperle, A., Reischl, B., Schmider, T., Stadlbauer, M., Kushkevych, I., Pruckner, C., Vítězová, M., & Rittmann, S. K.-M. R. (2021). Biomethanation of Carbon Monoxide by Hyperthermophilic Artificial Archaeal Co-Cultures. *Fermentation*, 7(4), 276. <https://doi.org/10.3390/fermentation7040276>