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Helmholtz-Zentrum für Polar- und Meeresforschung
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Activity of methanogenic archaea under simulated Mars analog conditions

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Preface

This doctoral thesis was part of the Helmholtz Alliance “Planetary Evolution and Life”. The coordinating Helmholtz Center of this Alliance was the German Aerospace Centre (DLR, Deutsches Zentrum für Luft und Raumfahrt e.V.). The laboratory work was conducted mainly at the Alfred Wegener Institute, Helmholtz Center for Polar and Marine Research, Research Unit Potsdam and partially at the University of Potsdam at the Institute for Biochemistry and Biology. This study is written in English and is presented as a cumulative Ph.D. thesis at the University of Potsdam, Institute for Biochemistry and Biology at the Faculty of Mathematics and Natural Science.

The thesis is composed of an introduction and four main chapters (2-5), followed by a synthesis and conclusion. In the first chapter, an introduction of the research topic, a short description of the two study sites, the main objectives of this work and an overview of the publications is given. The main chapters consist of four manuscripts of which three are first-authorship publications (chapter 3, 4 and 5) whereas one is a co-authorship publication (chapter 2). Chapter 4 has been accepted for publication in an international journal and the chapters 2, 3 and 5 have been published in international journals already. A synthesis of the four research articles is given in chapter 6, including the outline of the basic findings as well as the major conclusions and future perspectives.

Table of contents

Summary

Assumed comparable environmental conditions of early Mars and early Earth in 3.7 Ga ago – at a time when first fossil records of life on Earth could be found – suggest the possibility of life emerging on both planets in parallel. As conditions changed, the hypothetical life on Mars either became extinct or was able to adapt and might still exist in biological niches. The controversial discussed detection of methane on Mars led to the assumption, that it must have a recent origin – either abiotic through active volcanism or chemical processes, or through biogenic production. Spatial and seasonal variations in the detected methane concentrations and correlations between the presence of water vapor and geological features such as subsurface hydrogen, which are occurring together with locally increased detected concentrations of methane, gave fuel to the hypothesis of a possible biological source of the methane on Mars.

Therefore the phylogenetically old methanogenic archaea, which have evolved under early Earth conditions, are often used as model-organisms in astrobiological studies to investigate the potential of life to exist in possible extraterrestrial habitats on our neighboring planet. In this thesis methanogenic archaea originating from two extreme environments on Earth were investigated to test their ability to be active under simulated Mars analog conditions. These extreme environments – the Siberian permafrost-affected soil and the chemoautotrophically based terrestrial ecosystem of Movile cave, Romania – are regarded as analogs for possible Martian (subsurface) habitats. Two novel species of methanogenic archaea isolated from these environments were described within the frame of this thesis.

It could be shown that concentrations up to 1 wt% of Mars regolith analogs added to the growth media had a positive influence on the methane production rates of the tested methanogenic archaea, whereas higher concentrations resulted in decreasing rates. Nevertheless it was possible for the organisms to metabolize when incubated on water-saturated soil matrixes made of Mars

regolith analogs without any additional nutrients. Long-term desiccation resistance of more than 400 days was proven with reincubation and indirect counting of viable cells through a combined treatment with propidium monoazide (to inactivate DNA of destroyed cells) and quantitative PCR. Phyllosilicate rich regolith analogs seem to be the best soil mixtures for the tested methanogenic archaea to be active under Mars analog conditions. Furthermore, in a simulation chamber experiment the activity of the permafrost methanogen strain *Methanosarcina soligelidi* SMA-21 under Mars subsurface analog conditions could be proven. Through real-time wavelength modulation spectroscopy measurements the increase in the methane concentration at temperatures down to -5 °C could be detected.

The results presented in this thesis contribute to the understanding of the activity potential of methanogenic archaea under Mars analog conditions and therefore provide insights to the possible habitability of present-day Mars (near) subsurface environments. Thus, it contributes also to the data interpretation of future life detection missions on that planet. For example the ExoMars mission of the European Space Agency (ESA) and Roscosmos which is planned to be launched in 2018 and is aiming to drill in the Martian subsurface.

Zusammenfassung

Die Vermutung vergleichbarer Umweltbedingungen des frühen Mars und der frühen Erde vor 3,7 Mrd. Jahren – der Zeitpunkt, zu dem die ersten fossilen Spuren des Lebens auf der Erde gefunden werden konnten – weisen auf die Möglichkeit hin, dass das Leben auf beiden Planeten parallel entstanden sein könnte. Als die Bedingungen auf dem Mars schlechter wurden, ist das hypothetische Leben dort entweder ausgestorben, oder es war in der Lage sich anzupassen und könnte noch heute in biologischen Nischen auf dem Planeten existieren. Die kontrovers diskutierte Detektion von Methan auf dem Mars führte zu der Annahme, dass dieses einen rezenten Ursprung haben muss – entweder abiotisch durch aktiven Vulkanismus oder chemische Prozesse oder aber durch biogene Produktion. Räumliche und saisonale Schwankungen der durch Fernerkundung gemessenen Methankonzentrationen, sowie die Korrelation zwischen dem Auftreten von Wasserdampf und im Untergrund detektiertem Wasserstoff zusammen mit lokal erhöhten Konzentrationen von Methan, befürworten die Hypothese einer biologischen Methanquelle auf dem Mars.

Daher werden methanogene Archaeen, welche sich unter den Bedingungen der frühen Erde entwickelt haben, oft als Modellorganismen in astrobiologischen Studien zu potentiellem Leben auf unserem benachbarten Planeten Mars verwendet. In dieser Dissertation wurden methanogene Archaeen aus zwei extremen Habitaten auf der Erde auf ihre Fähigkeiten hin untersucht, unter simulierten Mars-analogen Bedingungen aktiven Metabolismus zu zeigen. Die beiden extremen Habitate – der *active layer* des sibirischen Permafrosts und das auf Chemoautotrophie basierende terrestrische Ökosystem der Movile Höhle in Rumänien – gelten als Analoga für mögliche Habitate auf dem Mars. Im Rahmen dieser Arbeit wurden zwei neue Arten von methanogenen Archaeen beschrieben, die aus den beiden genannten extremen Habitaten isoliert worden sind.

In dieser Studie konnte gezeigt werden, dass Mars-Regolith-Analoga, in Konzentrationen bis zu 1 Gew.-% zum Wachstumsmedium hinzugefügt, einen

positiven Einfluss auf die Methanbildungsraten der getesteten Archaeen hatten, während höhere zugefügte Konzentrationen sinkende Raten verursachten. Dennoch war es den Organismen möglich, auf wassergesättigten künstlichen Böden aus Mars-Regolith-Analoga Methan zu produzieren, auch ohne Zugabe jeglicher weiterer Nährstoffe. Die Resistenz gegenüber Langzeit-Austrocknung von mehr als 400 Tagen wurde mittels Reinkubation sowie indirekter Zellzahlbestimmung von lebensfähigen Zellen nachgewiesen. Dies erfolgte durch eine Kombinationsbehandlung mit Propidium-Monoazide (zur Inaktivierung der DNA aus Zellen mit zerstörter Membran) und quantitativer PCR. Regolith-Analoga mit einem hohen Anteil an Phyllosilikaten schienen die besten Bodenmischungen für die metabolische Aktivität der getesteten methanogenen Archaeen unter Marsanalogen Bedingungen zu liefern. Des Weiteren konnte mittels einer Simulationskammer für den Permafrost-Stamm *Methanosarcina soligelidi* SMA-21 Methanbildung unter Bedingungen analog zum Marsuntergrund nachgewiesen werden. Durch Wellenlängen-Modulations-Spektroskopie konnte die Zunahme der Methankonzentration bei Temperaturen bis zu -5 °C gemessen werden.

Die in dieser Arbeit vorgestellten Ergebnisse erweitern das Verständnis des Potenzials der methanogenen Archaeen, unter Mars-analogen Bedingungen aktiv sein zu können und vermitteln Einblicke in die mögliche Habitabilität vom heutigen Marsuntergrund. Außerdem tragen sie zur Interpretation der Daten von zukünftigen Marsmissionen zur Erkundung von möglichem Leben auf dem Planeten bei. Ein Beispiel hierfür könnte die ExoMars-Mission der Europäischen Weltraumorganisation (ESA) und Roskosmos sein, welche im Jahr 2018 gestartet werden soll und bestrebt ist, in den Marsuntergrund zu bohren.

1. Introduction

1.1. Environmental conditions of past and present Mars

The present-day Mars is a dry planet and considered to be hostile to life we know from Earth. The temperatures at the Martian surface are varying between -138 to +30 °C (Jones *et al*., 2011) depending on the altitude, season and latitude, where the highest temperatures are only reached during the summer months in more equatorial regions. The atmospheric pressure over ground is as low as 6 mbar and the major component of the atmosphere is carbon dioxide (CO_2) to about 95 %. The rest comprises of nitrogen (N_2) 2.7 %, argon (Ar) 1.6 %, oxygen (O_2) 0.13 % and carbon monoxide (CO) 0.08 %. Minor components detected are water (H_2O) 210 ppm, nitrogen oxide (NO) 100 ppm, neon (Ne) 2.5 ppm, hydrogen-deuterium-oxygen (HDO) 0.85 ppm, krypton (Kr) 0.3 ppm and xenon (Xe) 0.08 ppm (NASA Mars Fact Sheet, 2014). Due to its thin atmosphere and to a lacking magnetic field the surface of Mars is exposed to high doses of UV and gamma radiation.

By observations of the Martian surface and geological features it was shown that Mars was warmer and wetter about 3.7 Ga ago (Solomon *et al*., 2005). At that time local water bodies might have been present at the surface as indicated by sedimentary rocks and waterborne features of the present-day surface (McKay and Davis, 1991; McKay *et al*., 1992; Squyers *et al*., 2004). In the period where the environmental conditions of Mars and Earth were almost similar, evolution of life already started on Earth. Detections of prokaryotic microfossils in Archean rocks suggest that the earliest forms of life on Earth date to 3.5-3.8 Ga ago (Ohtomo *et al*., 2014; Schopf, 1993). The microfossils also suggest that these earliest microbial mats possibly were in direct contact with the atmosphere of early Earth and therefore exposed to high doses of UV radiation. So these communities of organisms probably survived under an UV regime exposure at least as high as found on Mars (Cockall *et al*., 2000).

Hence it is possible that life might also have emerged on Mars as well at the same time when it first evolved on Earth. The climate change on Mars possibly resulted to the loss of the magnetic field. In succession the atmospheric gases became depleted due to solar wind stripping, the temperature and pressure at the surface decreased and left Mars with the cold and dry environmental conditions known today. When conditions changed the possible life on Mars either became extinct and only fossil records of it might be found today. Or life was able to adapt to the drastic change of conditions and might as well exist in niches on or below the surface on the planet.

In general the presence of liquid water is a major limiting factor for life as we know it from Earth. Although, the present-day Mars is known to be a dry planet, water might become available periodically at or below the surface. Changing diurnal and seasonal temperatures in addition to pressure conditions result in liquid water in terms of water vapor, interfacial water or in cryobrines (Möhlmann, 2010a, 2010b; Möhlmann and Thomson, 2011). Furthermore, liquid-like adsorption water in potential subsurface habitats could support life (Möhlmann, 2005). The temperature and pressure regime as well as the high UV doses on the Martian surface are unfavorable for life. Therefore the subsurface or near-subsurface might be a more likely possible habitat for any putative organisms. The subsurface offers shielding against radiation, has more stable thermo-physical conditions and a possibly higher availability of liquid water (Jones *et al*., 2011).

Besides liquid water, a source of carbon and energy are also prerequisites for any active life we know from Earth. Hypothetical Martian subsurface ecosystems could be based on lithoautotrophy since carbon dioxide as the mayor gas component of the atmosphere is present as a possible source of carbon. A thinkable source of energy could be gaseous hydrogen. Although it is found on Mars only in trace amounts (Oze and Sharma, 2005) it can presumably be formed through photochemical processes in the atmosphere (Bar-Nun and Dimitrov, 2006). On Earth molecular hydrogen has been reported to be produced through alteration processes of basaltic crust in stable ancient Precambrian cratons, which serve as good Earth analogs for a single-plate planet like Mars (Sherwood

Lollar *et al.*, 2007). In the Martian subsurface, direct interaction with liquid interfacial water may also be a source of hydrogen due to the release of protons.

1.2. Detection of Methane on Mars

It was reported that spectroscopic observations from the ESA Mars Express spacecraft (Formisano *et al*., 2004; Geminale *et al*., 2011) and ground based spectra analysis from Earth (Krasnopolsky *et al*., 2004; Mumma *et al*., 2009) have found trace amounts of methane in the Martian atmosphere. Methane on Mars is believed to have a relatively short lifetime of approximately 340 years due to direct photolysis by solar UV radiation and homogenous oxidation by OH and O (1D) (Gough *et al*., 2010; Krasnopolsky *et al*., 2004). Therefore its leads to the assumption, that the observed methane must have a recent origin on the planet. The spectroscopic detections of methane on Mars have been discussed critically (Zahnle *et al*., 2011) and the measurements performed with the Tunable Laser Spectrometer (TLS) on board of the Curiosity Rover indicated no definitive detection of methane on Mars. The TLS measured a value of only 0.55 ± 1.46 ppbv, in the Gale Crater region (Webster *et al*., 2013). This is at least six times lower in comparison to the predicted global atmospheric average value on Mars of about 10-15 ppbv methane (Formisano *et al*., 2004; Geminale *et al*., 2011). Further measurements with the TLS conducted in the course of a Martian year revealed temporary higher concentrations of up to 7.2 ± 2.1 ppbv implying episodically release of methane from an unidentified source (Webster *et al*., 2015).

Possible sources of methane on Mars could be abiotic through active volcanism, which has not yet been observed on present-day Mars (Krasnopolsky, 2006), or formation via Fischer-Tropsch reactions associated with serpentinization (Michalski *et al*., 2013; Oze and Sharma, 2005; Vance *et al*., 2007). A different possible source for methane would be biogenic production. The observed spatial and temporal variation of methane in the Martian atmosphere (Formisano *et al*., 2004; Geminale *et al*., 2008, 2011; Mumma *et al*., 2009) and its correlation with the presence of water vapor and subsurface hydrogen are

supporting the biogenic production theory (see Figure 1.1). On Earth the aerobic methane production through plants is known (Bruhn *et al*., 2012), but the mayor source of any biogenic methane is the anaerobic methanogenesis performed by methanogenic archaea. Therefore they are regarded as model-organisms for possible life on Mars.

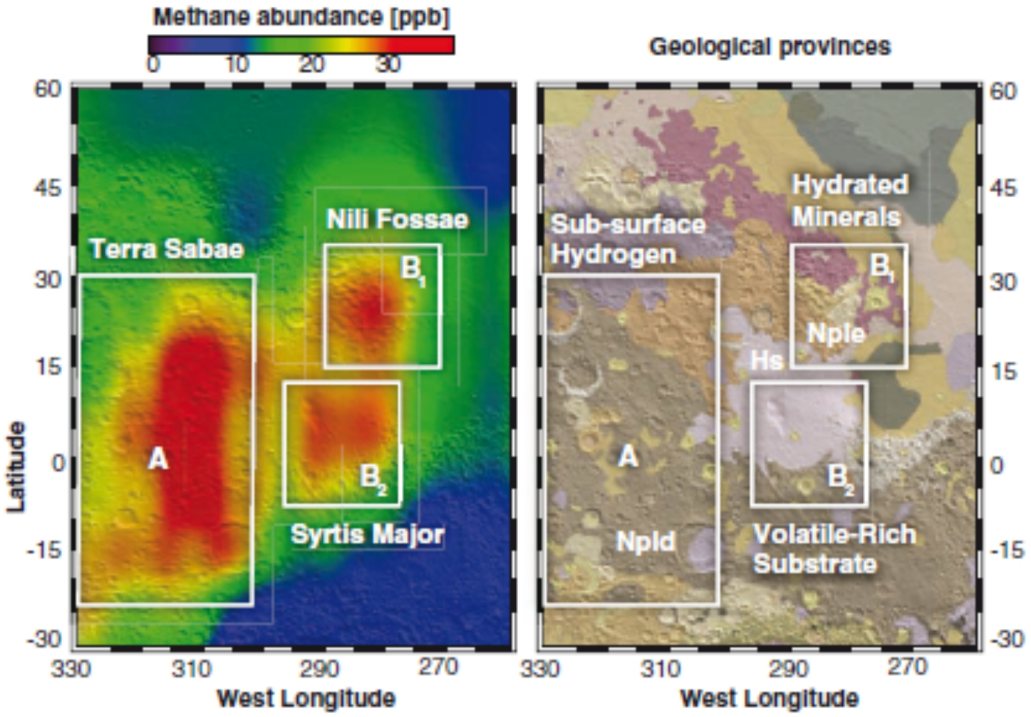

Figure 1.2: left: Regions in the northern hemisphere of Mars with indicated strong release of methane during the summer months; right: correlation with special geological features such as sub-surface hydrogen or hydrated minerals. (Mumma *et al*., 2009).

1.3. Methanogenic archaea

Belonging to the phylum Euryarchaeota in the domain of Archaea (Woese *et al*., 1990), methanogenic archaea are characterized by their ability to produce methane under anaerobic conditions. Methanogenic archaea have a highly specialized metabolism with unique enzymatic pathways and a set of various coenzymes and cofactors allowing them to be capable of lithoautotrophic growth (Whitman *et al*., 1992). They can use hydrogen and carbon dioxide as sole

sources of energy and carbon and can also use a limited number of organic compounds like acetate, formate, methanol, some secondary alcohols and methylamines to produce methane (Zinder, 1993).

Methanogenic archaea are ubiquitous in anaerobic environments like lake and marine sediments, sewage deposits, swamps, rice paddy soils as well as the digestive system of animals (Bryant and Boone, 1987; Chan *et al*, 2005; Grosskopf *et al*., 1998; Jarvis *et al*., 2000; Kendall *et al*., 2006; Zellner *et al*., 1989). Additionally methanogenic archaea can be found in extreme habitats characterized by high temperatures and/or high salinity and pH like hot deep sea vents, hot springs, saltern ponds (Garcia *et al*., 2000; Jeanthon *et al*., 1999; Stetter *et al*., 1990) but also cold habitats like permafrost and permafrost-affected soils or other permanently cold environments (Barbier *et al*., 2012; Bischoff *et al*., 2012; Franzmann *et al*., 1997; Ganzert *et al*., 2007; Kobabe *et al*., 2004; Rivkina, *et al*., 1998; Wagner *et al*., 2013; Zhang *et al*, 2008) as well as groundwater ecosystems (Schirmack *et al*., 2014b).

Methanogenic archaea have evolved under early Earth conditions (e.g. no oxygen, no or little sources of organic substrates) at a time when the environmental conditions of Mars and Earth might have been similar (Carr 1989, 1996; Durham *et al*., 1989; McKay and Davis, 1991; McKay *et al*., 1992). Due to their ability of lithoautotrophic growth under anoxic conditions and their tolerance to low temperatures as well as their capability to survive under the extreme conditions of terrestrial permafrost for millions of years (Rivkina *et al*., 2004) they are regarded as suitable model-organisms for possible life on Mars. They are argued to life in subsurface permafrost layers and metabolize on carbon dioxide and hydrogen from the Martian atmosphere (Formisano *et al*., 2004, Krasnopolsky *et al*, 2004), or might exist in other niches on Mars where liquid water and a source of energy is available.

1.4. Description of the study sites

Two different extreme habitats were chosen to isolate strains of methanogenic archaea. Both can be considered as an analog for potential extraterrestrial habitats on Mars due to their special properties.

Figure 1.2: Location of the Lena Delta (red circle) embedded in the zone model of Permafrost distribution in the northern hemisphere (modified from Brown *et al*., 1997).

The first habitat is the permafrost-affected soil – the so called active layer of permafrost – located on Samoylov island in the Lena Delta, north-east Siberia as

indicated by Figure 1.2 (72° 22' N 126° 28' E). This active layer is the top soil horizon of 20 to 50 cm depth which thaws during the short summer period and freezes back again in the winter months. According to the taxonomic guidelines of the USA Soil survey Staff (1999) this soil is characterized as *Psammentic Aquorthel*. The climate of the Lena Delta belonging to the zone of continuous permafrost is described as arctic continental with a low annual mean air temperature of -14.7 °C (Tmin = -48 °C, Tmax = 18 °C) and a low mean annual precipitation of 190 mm. As permafrost features can be observed on Mars as well, terrestrial permafrost – which covers about 25 % of the continental landmass on Earth (Zhang *et al*., 1999) – can be regarded as an analog for extraterrestrial habitats on our neighboring planet (Ulrich *et al*., 2012). The soil sample used for the isolation of the here described methanogenic archaeon *Methanosarcina soligelidi* SMA-21 was collected from the surface in a depth of 0-5 cm in July 2002 and transported under frozen conditions to Potsdam, Germany.

Figure 1.3: Location map of the Movile cave, taken from: The Movile Cave Project: (http://www.lefo.ro/iwlearn/movile/maps.htm)

The second habitat is the Movile cave located near the city Mangalia in southeast Romania. A location map is shown in Figure 1.3. The cave is a

groundwater system in a karstic region characterized by many thermal springs. It is developed in limestone and consists of an upper dry part and a lower submerged part with several air bells as shown in Figure 1.4. As this cave is almost completely isolated from the surface (Sarbu *et al*., 1996), it is described by constant physical and chemical features. The mean temperature of the atmosphere is 20 °C and around 22–24 °C in the water. Due to the presence of mesothermal water and a lack of openings the temperature does not match the mean annual temperature from the surface of 10–12 °C. The atmospheric composition of the air bells is 7–10 % O2, 2–3.5 % CO2 and 1–2 % CH4 and the groundwater of the subsurface lake in the submerged part of the cave is rich in hydrogen sulfide (up to 10 mg l-1), methane (up to 6 mg l-1) and ammonium (up to 6 mg l-1) and is anoxic from a depth of 5 cm (Sarbu and Kane, 1995; Sarbu *et al*., 1996). Life in this cave has been separated for more than 5 million years from the surface and is based exclusively on chemosynthesis (Falniowski *et al*., 2008; Sarbu *et al*., 1996). The microbial primary production is the base for the cave ecosystem containing of divers invertebrates of which 33 are endemic (Sarbu, 2000). It is the first known terrestrial habitat based on chemosynthesis and therefore can be regarded as an analog for extraterrestrial subsurface habitats for example on Mars. The sample used for the isolation of the here described methanogenic archaeon *Methanobacterium movilense* MC-20 was collected from the anoxic, grey-colored sediment of the subsurface lake in summer 2002.

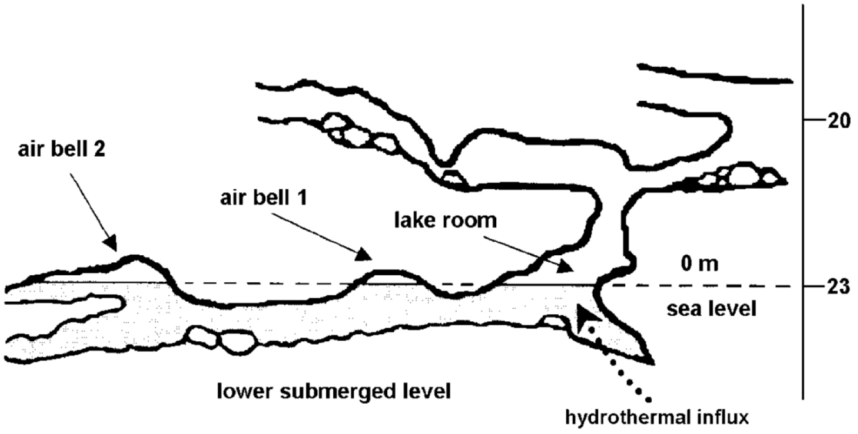

Figure 1.4: Schematic display of the Movile cave, from Sarbu and Popa (1992).

1.5. Aims and approaches

Previous studies have shown the high survival potential of methanogenic archaea – in particular organisms isolated from Siberian Permafrost – under various stress conditions like they can be found on present-day Mars as well as a three weeks lasting exposure to a diurnal profile of simulated Mars surface conditions. The aim of the study presented here is now to assess the potential of methanogenic archaea to have an active metabolism under simulated Mars analog conditions and not only survive the tested parameters. Therefore special interest was given to these key aspects:

- The influence of different Mars regolith analogs on the activity of the methanogenic archaea and the possibility of active metabolism on a water-saturated Mars regolith analog soil matrix with and without additional nutrients.
- The long-term desiccation resistance of the tested strains and any influences thereon through the Mars regolith analogs.
- The differences in the reaction on the tested parameters between the methanogens from permafrost and non-permafrost environments.
- To establish a simulation chamber to be able to test methanogenic archaea under simulated Mars thermo-physical conditions and additionally measure their metabolic activity at the same time.

This study also provides new insights to answer astrobiological questions for the possibilities of life in extraterrestrial habitats with focus on Mars. The two extreme environments, where two of the here tested strains were isolated from and described in frame of this thesis, serve both as analogs for possible habitats on the red planet.

1.6. Overview of the Publications

In the following chapters (2–5) the four published respectively submitted manuscripts are presented, which represent the results of this work.

1st Publication (co-authorship; International Journal of Systematic and Evolutionary Microbiology (2013), 63, 2986–2991; DOI 10.1099/ijs.0.046565-0)

***Methanosarcina soligelidi* sp. nov., a desiccation and freeze-thaw-resistant methanogenic archaeon from a Siberian permafrost-affected soil**

Authors: Dirk Wagner[1,2], Janosch Schirmack[1‡], Lars Ganzert[3‡], Daria Morozova[2] and Kai Mangelsdorf[4]

Aims: In this study a novel methanogenic archaeon strain, isolated from the active layer of permafrost in north-east Siberia on Samoylov Island, was investigated phenotypically and phylogenetically to describe its physiological and biochemical properties and taxonomic affiliation.

Summary: A methanogenic archaeon, strain SMA-21^T, was isolated from a permafrost-affected soil by serial dilution in liquid medium. The cells were non-motile, stained Gram-negative and grew as irregular cocci with a diameter of 1.3–2.5 mm. Optimal growth was observed at 28 °C, pH 7.8 and 0.02 M NaCl. The strain grew on H_2/CO_2, methanol and acetate, but not on formate, ethanol, 2-butanol, 2-propanol, monomethylamine, dimethylamine, trimethylamine or dimethyl sulfide. Major membrane lipids of strain SMA-21^T were archaeol phosphatidylglycerol, archaeol phosphatidylethanolamine and the corresponding hydroxyarchaeol compounds. The G+C content of the genomic DNA was 40.9 mol%. The 16S rRNA gene sequence was closely related to those of *Methanosarcina mazei* DSM 2053^T (similarity 99.9 %) and *Methanosarcina horonobensis* HB-1^T (similarity 98.7 %). On basis of the level of DNA–DNA hybridization (22.1 %) between strain SMA-21^T and *Methanosarcina mazei* DSM 2053^T as well as of phenotypic and genotypic differences, strain SMA-21^T was

assigned to a novel species of the genus *Methanosarcina*, for which the name *Methanosarcina soligelidi* sp. nov. is proposed. The type strain is SMA-21^T (=DSM 20065^T =JCM 18468^T).

Personal contribution: *Myself* carried out all physiological laboratory tests and preparations to the sequencing of the 16S rRNA sequences, the G+C content determination and the DNA-DNA hybridization. Furthermore I contributed to the data interpretation.

2nd Publication (first author; International Journal of Systematic and Evolutionary Microbiology (2014), 64, 522–527; DOI 10.1099/ijs.0.057224-0)

***Methanobacterium movilense* sp. nov., a hydrogenotrophic, secondaryalcohol-utilizing methanogen from the anoxic sediment of a subsurface lake**

Authors: Janosch Schirmack[1‡], Kai Mangelsdorf[4], Lars Ganzert[3Ŧ], Wolfgang Sand[5], Alexandra Hillebrand-Voiculescu[6] and Dirk Wagner[1,2]

Aims: Equivalent to the aims of the 1st Publication. The methanogenic archaeon strain was isolated from the anoxic sediment of the subsurface lake in Movile cave, Mangalia, Romania.

Summary: A novel strain of methanogenic archaea, designated MC-20^T, was isolated from the anoxic sediment of a subsurface lake in Movile Cave, Mangalia, Romania. Cells were non-motile, Gram-negative rods 3.5–4.0 mm in length and 0.6–0.7 mm in width, and occurred either singly or in short chains. Strain MC-20^T was able to utilize H_2/CO_2, formate, 2-propanol and 2-butanol as substrate, but not acetate, methanol, ethanol, dimethyl sulfide, monomethylamine, dimethylamine or trimethylamine. Neither trypticase peptone nor yeast extract was required for growth. The major membrane lipids of strain MC-20^T were archaeol phosphatidylethanolamine and diglycosyl archaeol, while archaeol phosphatidylinositol and glycosyl archaeol were present only in minor amounts. Optimal growth was observed at 33 °C, pH 7.4 and 0.08 M NaCl. Based on phylogenetic analysis of 16S rRNA gene sequences, strain MC-20^T was closely

affiliated with *Methanobacterium oryzae* FPi^T (similarity 97.1 %) and *Methanobacterium lacus* 17A1^T (97.0 %). The G+C content of the genomic DNA was 33.0 mol%. Based on phenotypic and genotypic differences, strain MC-20^T was assigned to a novel species of the genus *Methanobacterium* for which the name *Methanobacterium movilense* sp. nov. is proposed. The type strain is MC-20^T (=DSM 26032^T =JCM 18470^T).

Contribution of co-authors: *Dirk Wagner* wrote and edited the manuscript and was involved in the data interpretation and planning of experimental setup. *Wolfgang Sand* and *Alexandra Hillebrand-Voiculescu* provided the samples from Movile cave used for the isolation and wrote the relevant parts about Movile cave. *Kai Mangelsdorf* provided the phospholipid analysis and *Lars Ganzert* provided the phylogenetical data analysis.

3rd Publication (first author; accepted for publication in Frontiers of Microbiology, section: Extreme Microbiology)

Influence of Martian Regolith Analogs on the activity and growth of methanogenic archaea, with special regard to long-term desiccation

Authors: Janosch Schirmack[1‡], Mashal Alawi[2] and Dirk Wagner[2]

Aims: This study was made to determine the effects of three different Mars regolith analogs on the metabolic activity of selected methanogenic archaea strains: *Methanosarcina soligelidi*, *Methanosarcina mazei* and *Methanobacterium movilense*. Additionally the capability of the methanogens to metabolize in a water saturated soil matrix made of the Mars regolith analogs was investigated and their survival potential after exposure to long-term desiccation was identified.

Summary: Methanogenic archaea have been studied as model organisms for possible life on Mars for several reasons: they can grow autotrophically with hydrogen and carbon dioxide as energy and carbon source, they are anaerobes and have evolved at a time when conditions on early Mars and early Earth are believed to be similar. As, at present days, Mars is dry and cold and water might

be available only in time intervals, any organisms would have to cope with desiccation. Here we present the results of three experiments investigating the influence of three different Martian regolith analogs (JSC-Mars 1A, P-MRA and S-MRA) on the activity and growth of three methanogenic strains under culture conditions as well as long-term desiccation. Concentrations below 1 wt% of regolith in the media resulted in most cases in an increase of the methane production rates, whereas concentration above 1 wt% mostly decreased the rates and therefore prolonged the lag phase. Further experiments showed that methanogenic archaea are capable to grow and produce methane when incubated on a water-saturated sedimentary matrix of regolith without any addition of nutrients. The survival of methanogens under these conditions was analyzed by conducting a long-term desiccation experiment over 400 days in presence of regolith analogs and pure media. Survival of the methanogens was determined via reincubation on fresh medium and quantitative PCR following a propidium monoazide treatment. All three strains survived the desiccation period and regrow on fresh medium.

Contribution of co-authors: *Mashal Alawi* and *Dirk Wagner* contributed to the writing and editing of the manuscript and to the interpretation of the data. *Dirk Wagner* was involved in the planning of the experimental setup.

4th Publication (first author; Planetary and Space Science 98 (2014) 198–204; DOI 10.1016/j.pss.2013.08.019)

Laser spectroscopic real time measurements of methanogenic activity under simulated Martian subsurface analog conditions

Authors: Janosch Schirmack[1‡], Michael Böhm[7], Chris Brauer[7], Hans-Gerd Löhmannsröben[7], Jean-Pierre de Vera[8], Diedrich Möhlmann[8] and Dirk Wagner[2]

Aims: A simulation chamber was constructed to test for metabolic activity of methanogenic archaea under Mars analog conditions. To measure the increase of methane directly during the ongoing simulation process, the method of wavelength modulation spectroscopy was chosen.

Summary: On Earth, chemolithoautotrophic and anaerobic microorganisms such as methanogenic archaea are regarded as model organisms for possible subsurface life on Mars. For this reason, the methanogenic strain *Methanosarcina soligelidi* (formerly called *Methanosarcina spec.* SMA-21), isolated from permafrost-affected soil in northeast Siberia, has been tested under Martian thermo-physical conditions. In previous studies under simulated Martian conditions, high survival rates of these microorganisms were observed. In our study we present a method to measure methane production as a first attempt to study metabolic activity of methanogenic archaea during simulated conditions approaching conditions of Mars-like environments. To determine methanogenic activity, a measurement technique which is capable to measure the produced methane concentration with high precision and with high temporal resolution is needed. Although there are several methods to detect methane, only a few fulfill all the needed requirements to work within simulated extraterrestrial environments. We have chosen laser spectroscopy, which is a non-destructive technique that measures the methane concentration without sample taking and also can be run continuously. In our simulation, we detected methane production at temperatures down to -5°C, which would be found on Mars either temporarily in the shallow subsurface or continually in the deep subsurface. The pressure of 50 kPa which we used in our experiments, corresponds to the expected pressure in the Martian near subsurface. Our new device proved to be fully functional and the results indicate that the possible existence of methanogenic archaea in Martian subsurface habitats cannot be ruled out.

Contributions of co-authors: *Michael Böhm* and *Chris Bauer* were responsible for the wavelength modulation spectroscopy and the performance of the simulation chamber. *Michael Böhm* wrote his relevant parts of the method section. *Hans-Gerd Löhmannsröben*, *Jean-Pierre de Vera*, *Diedrich Möhlmann* and *Dirk Wagner* carried out the initial planning of the simulation system. *Dirk Wagner* was involved in the interpretation of the data and in the editing of the manuscript.

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2. Publication I

Published in: International Journal of Systematic and Evolutionary Microbiology (2013), 63, 2986–2991; DOI 10.1099/ijs.0.046565-0)

Methanosarcina soligelidi sp. nov., a desiccation and freeze-thaw-resistant methanogenic archaeon from a Siberian permafrost-affected soil

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Running title: *Methanosarcina soligelidi* sp. nov.

Keywords: *Methanosarcina*, archaea, permafrost-affected soil

New taxa – Archaea

The GenBank accession number of the 16S rRNA gene sequence of *Methanosarcina soligelidi* SMA-21T is JF812255.

Abstract

A methanogenic archaeon, strain SMA-21^T, was isolated from a permafrost-affected soil by serial dilution in liquid medium. The cells were non-motile, stained Gram-negative and grew as irregular cocci with a diameter of 1.3-2.5 µm. Optimal growth was observed at 28°C, pH 7.8 and 0.02 M NaCl. The strain grew on H_2/CO_2, methanol and acetate, but not on formate, ethanol, 2-butanol, 2-propanol, monomethyamine, dimethylamine, trimethylamine and dimethyl sulfide. Major membrane lipids of SMA-21^T were archaeol phosphatidylglycerol, archaeol phoshatidylethanolamine and the corresponding hydroxyarchaeol compounds. The G+C content of the genomic DNA was 40.9 mol%. The 16S rRNA gene sequence was closely to that of *Methanosarcina mazei* (similarity 99.9%) and *Methanosarcina horonobensis* (similarity 98.7%). On basis of the level of DNA-DNA hybridization (22.1%) between strain SMA-21^T and *Methanosarcina mazei* DSM 2053^T as well as of phenotypic and genotypic differences, strain SMA-21^T was assigned to a novel species of the genus *Methanosarcina*, for which the name *Methanosarcina soligelidi* sp. nov. is proposed. The type strain is SMA-21^T (=DSM 26065^T=JCM 18468^T).

Permafrost and the associated soils cover more than 25% of the land surface (Zhang *et al*. 1999). These ecosystems play an essential role for the Earth's

climate due to the large amount of organic carbon preserved in the soils and sediments (Tarnocai *et al.* 2009) and because of the rapidly warming of permafrost in response to global climate change (Romanovsky *et al*., 2010). Methanogenic archaea are the object of particular interest in permafrost studies, because of their key role in the Arctic methane cycle and their significance for the global methane budget (Graham *et al*., 2012). Recent studies have shown that methanogens from permafrost environments are extremely stress tolerant and can be still metabolic active at subzero temperatures (Morozova and Wagner, 2007; Wagner *et al*., 2007).

Although the abundance and diversity of methanogens in permafrost environments is similar to that of communities of comparable temperate soil ecosystems (Wagner *et al*., 2005), there are only few new strains that have been isolated and described from permafrost habitats. *Methanobacterium arcticum* was isolated from Holocene permafrost deposits (Shcherbakova *et al*., 2011) and *Methanobacterium veterum* was obtained from ancient permafrost, with an age of 3 million years after long-term anaerobic cultivation (Krivushin *et al*., 2010). Another study described new strains of methanogens from different low-temperature environments including strain MT, enriched from an arctic tundra soil, which represents a new ecotype of *Methanosarcina mazei* (Simankova *et al*., 2003). Recently, three different methanogenic archaea closely related to *Methanosarcina mazei* and *Methanobacterium* spec. have been described from permafrost deposits of different ages (Rivkina *et al*., 2007).

In this study, we describe the characteristics of a novel methanogenic archaeon, strain SMA-21^T from a permafrost environment in Northeast Siberia.

The described strain SMA-21^T was isolated from a permafrost-affected soil located on the northern tip of the island Samoylov (N 72°22, E 126°28), Lena Delta, Siberia. According to the US Soil Taxonomy the soil was characterized as *Psammentic Aquorthel* (Soil Survey Staff, 1999). The soil sample was collected in July 2002 from the soil surface (0-5 cm soil depth) and transported in frozen conditions to Potsdam, Germany.

Enrichment of methanogenic archaea was started by adding 2 g fresh soil material into a serum bottle (125 ml) containing 50 ml anaerobic medium of the following composition (l^{-1}): NH_4Cl, 1.0 g; $MgCl_2$ x 6 H_2O, 1.0 g; $CaCl_2$ x 2 H_2O, 0.4 g; KH_2PO_4 x 2 H_2O, 0.8 g; $NaHCO_3$, 12 g; Na_2S x 3 H_2O, 0.5 g; trace element solution (Balch *et al*., 1979), 10 ml; vitamin solution (Bryant *et al*., 1971) 10 ml; and 2 ml resazurin (7-Hydroxy-3H-phenoxazin-3-on-10-oxide) indicator solution. The bottles were sealed with black rubber stoppers and pressurized with H_2/CO_2 (80:20 v/v, 150 kPa) as substrate. Cultures were incubated in the dark at 28°C. After methane production was observed in the headspace, 5 ml of the culture was anaerobically transferred into a new bottle of sterile medium, which was supplemented with the antibiotics erythromycin and phosphomycin (each 50 µl ml^{-1} medium) to suppress growth of non-methanogenic microorganisms (Hilpert *et al*., 1981). This procedure was repeated until a pure culture was obtained. All further incubations including the purity check was done without any antibiotics. Purity of the strain was confirmed by light microscope examination, the absence of growth in rich medium containing (l^{-1}) glucose 4 g, yeast extract 2 g and peptone 2 g, and DGGE analyses of DNA extracts obtained from the culture. The strain was maintained by three month transfer into liquid medium. After re-growth at 28°C the culture was stored at 5°C. All preparation steps were done under strictly anaerobic conditions.

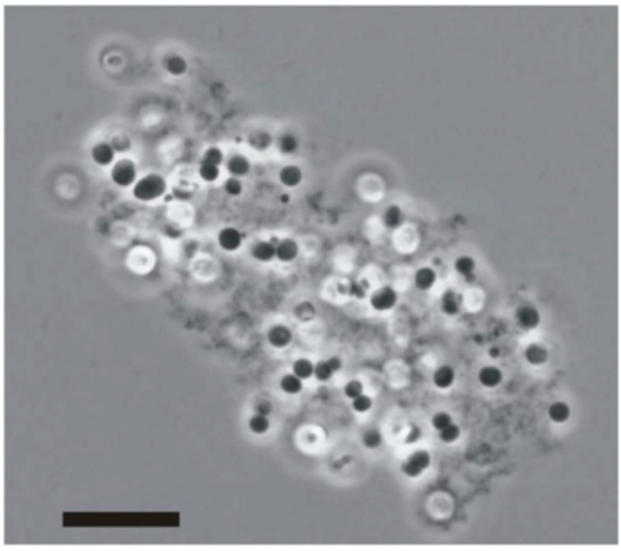

Fig. 1. Phase contrast micrograph of cells of strain SMA-21^T (bar = 10 µm).

Phase contrast microscopy (Axioskop 2, Zeiss) was performed in the exponential growth phase. Cells were irregular cocci, around 1.3-2.5 µm in diameter (Fig. 1) and non-motile. Cells stained Gram-negative, but the reaction was sometimes variable. Lysis of the cells was observed in a 0.01 % (w/v) SDS solution.

Growth and substrate utilization were determined by culturing strain SMA-21^T in medium described above and growth rate was estimated by measuring the concentration of methane in the gas phase (Powell, 1983). The methane concentration was measured by gas chromatography as described previously (Koch *et al*., 2009). All growth tests were performed in triplicates at 28°C. The effect of temperature on growth was tested with H_2/CO_2 (80:20 v/v) as substrate at 0, 5, 10, 16, 22, 28, 33, 38, 44, 54 and 64°C. Growth of strain SMA-21^T was observed at 0-54°C, with optimum growth at 28°C (Fig. 2a). The pH of the growth medium was adjusted to pH 4.1-9.9 with 1 M HCl and 1 M NaOH, respectively. Growth was observed between pH 4.8 and 9.9, with optimum growth at pH 7.8 (Fig. 2b). The salinity range was determined in medium with 0.02-0.6 M NaCl. Optimum growth was measured at 0.02 M, and growth was observed at salt concentrations of up to 0.6 M (Fig. 2c). The substrate specifity of strain SMA-21^T was determined by addition of the following carbon sources to the growth medium: H_2/CO_2 (80:20 v/v, 150 kPa), sodium formate (80 mM), sodium acetate (40 mM), methanol (20 mM), ethanol (20 mM), 2-propanol (10 mM), 2-butanol (20 mM), monomethylamine (20 mM), dimethylamine (20 mM), trimethylamine (20 mM) and dimethyl sulfide (20 mM). With exception of growth test with hydrogen, all other cultures were flushed with N_2/CO_2 (80:20 v/v, 100 kPa). Cultures were incubated at 28°C for 10 weeks, and growth was subsequently monitored through methane formation in the headspace and visual analysis of increasing turbidity. Growth was observed with H_2/CO_2 and methanol. Very slow growth was observed on acetate, but not on formate, ethanol, 2-butanol, 2-propanol, monomethylamine, dimethylamine, trimethylamine and dimethyl sulfide (Tab. 1). The generation time with H_2/CO_2 at 28°C was 5.1 ± 0.3 d.

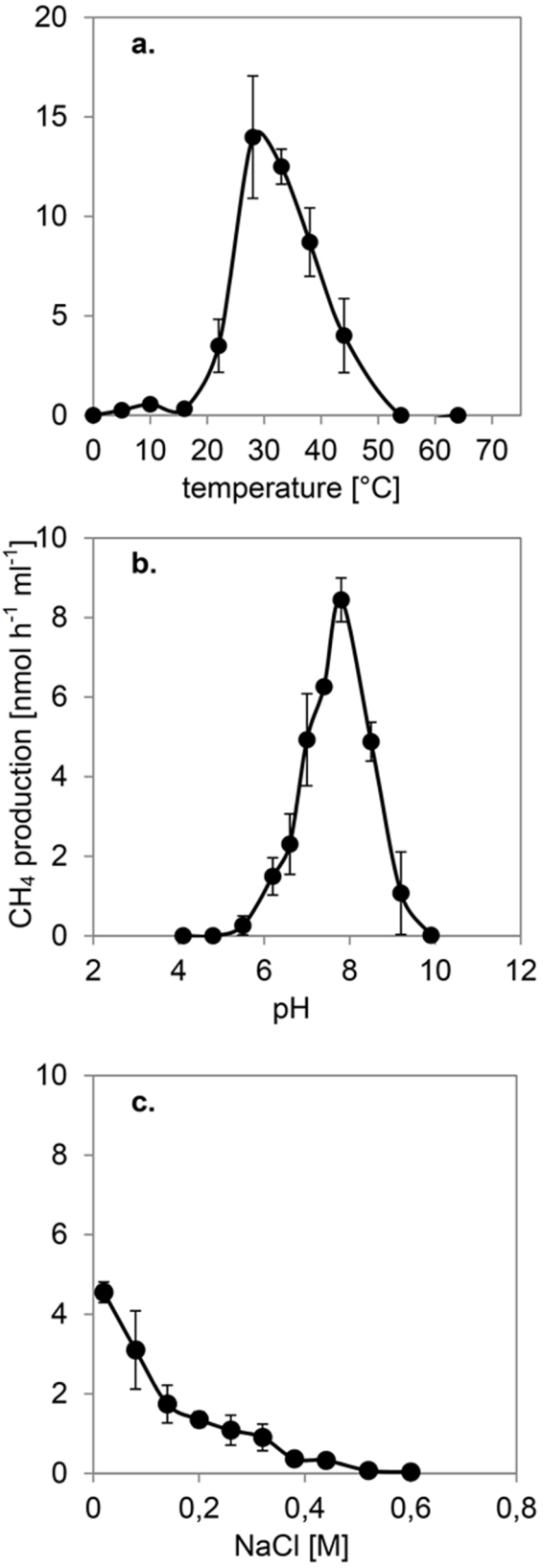

Fig. 2. Physiological characteristics of strain SMA-21^T; methane production in dependence of temperature (a.), pH (b.) and salt concentration (c.).

Intact membrane lipids were examined for strain SMA-21^T and its closest relative *Methanosarcina mazei* (similarity 99.9%) using a method described in Zink and Mangelsdorf (2004). The intact lipids were detected with a high performance liquid chromatography electrospray interface mass spectrometry (HPLC-ESI-MS) system. Furthermore, ether cleavage experiments were conducted to determine the lipid side chain inventory following a method described in Gattinger *et al*. (2003). Compounds were detected using a gas chromatography mass spectrometry (GC-MS) system. Both archaea possess the same set of membrane diether lipids detected in the HPLC-ESI-MS negative ion mode. The major lipids were archaeol phosphatidylglycerol (ArPG; $[M-H]^-$ at m/z = 805), hydroxyarchaeol phosphatidylglycerol (Hydroxy-ArPG; $[M-H]^-$ at m/z = 821), archaeol phosphatidylethanolamine (ArPE; $[M-H]^-$ at m/z = 774) and hydroxyarchaeol phosphatidylethanolamine (Hydroxy-ArPE; $[M-H]^-$ at m/z = 790; Tab. 1). GC-MS measurement on the side chain inventory supports the results obtained from the intact lipid analysis. The major compound in the GC-MS run is phytane being the ether cleavage product of archaeol. Tetraether lipids or biphytanes, respectively, were not detected. Archaeol phospholipids were also the main phospholipids of *Methanococcoides burtonii* isolated from the Ace Lake, Antarctica (Nichols *et al*., 2004).

Environmental stress tolerance was tested as previously described (Morozova and Wagner, 2007). Briefly, oxygen sensitivity and desiccation was tested by adding cell suspensions of 10^8 cells ml^{-1} onto microscope cover slips, which were exposed under aerobic conditions or completely dried. After certain time periods, cells were anaerobically re-suspended and methane production was checked. Freeze-thaw resistance was tested by freezing cultures of 10^8 cells ml^{-1} immediately in medium at -78.5 °C. After 24 h the frozen cells were thawed at room temperature, freeze again at -78.5 °C or methane production was measured. In addition, cells were slowly frozen (0.2 °C min-1) to -20 °C. Initial methane production rates were measured before freezing and compared with those obtained after thawing for samples held at -20 °C for a period of 1–2 years. The results indicated a high survival potential of strain SMA-21^T against air

exposure (up to 72 h), desiccation (up to 25 days), freeze-thaw cycles down to -78.5°C and long-term freezing (up to 2 years at -20°C).

Table 1. Characteristics of strain SMA-21^T and related species of the genus *Methanosarcina*. Strains: 1, *Methanosarcina* ***soligelidi*** sp. nov. SMA-21^T (data from this study); 2, *Methanosarcina mazei* DSM 2053^T (Mah, 1980; Maestrojuán *et al*., 1992); 3, *Methanosarcina horonobensis* HB-1^T (Shimizu *et al*., 2011) and *Methanosarcina siciliae* T4/M^T (Ni & Boone, 1991). +, positive; -, negative; ArPG = archaeol phosphatidylglycerol; Hydroxy-ArPG = hydroxyarchaeol phosphatidylglycerol; ArPE = archaeol phosphatidyl-ethanolamine; Hydroxy-ArPE = hydroxyarchaeol phosphatidylethanolamine; ND, not determined.

Characteristic	**1**	**2**	**3**	**4**
Cell shape	irregular cocci	irregular cocci	irregular cocci	irregular cocci
Cell dimension (µm)	1.3-2.5	1.0-3.0	1.4-2.9	1.5-3.0
Gram stain	-	-	-	-
Temperature range for growth (°C)	0-54	23-50	20-42	25-45
Optimum temperature (°C)	28	30-40	37	40
pH range	4.8-9.9	6.1-8.0	6.0-7.8	6.0-7.7
Optimum pH	7.8	7.0	7.0-7.3	6.5-6.8
Tolerance of NaCl (M)	0.02-0.6	0.1-1.0	0-0.35	0->1.7
Optimum NaCl for growth	0.02	0.1-0.3	0.1	0.4-0.6
Utilization of:				
H_2/CO_2	+	+§	-	-
methanol	+	+	+	+
acetate	+	+	+	-
dimethyl sulfide	-	-§	+	+
monomethylamine	-	-§	ND	ND
dimethylamine	-	+§	ND	ND
trimethylamine	-	+	+	ND
G + C content (mol%)	40.9	42.0	41.4	42-43
ArPG	+	+§	ND	ND
Hydroxy-ArPG	+	+§	ND	ND
ArPE	+	+§	ND	ND
Hydroxy-ArPE	+	+§	ND	ND

§data obtained in this study

DNA was isolated using a Microbial DNA Isolation Kit (MoBio Laboratories) according to the manufacturer's protocol. For 16S rRNA gene amplification, general archaeal primers were used: ArUn4F (TCYGGTTGATCCTGCCRG) and Arc1492R (GGCTACCTTGTTACGACTT). Sequencing by GATC Biotech (Konstanz, Germany) resulted in a 1337 bp gene product. Alignments were performed with all known isolates of the genus *Methanosarcina*. Sequences were obtained from GenBank and aligned using the integrated SINA alignment tool from the ARB-SILVA website (Pruesse *et al*., 2007). Sequences were checked manually using the ARB program (Ludwig *et al*., 2004) and evolutionary distances were calculated based on neighbour-joining. Construction of a phylogenetic tree was done by using the neighbour-joining method (Saitou & Nei, 1987; Fig. 3) and a termini filter that is implemented in the ARB program. To evaluate the tree topologies, a bootstrap analysis with 1000 replications was performed. For strain SMA-21^T, highest 16S rRNA gene sequence similarities were found to the type strains of *Methanosarcina mazei* DSM 2053^T (99.9%), *Methanosarcina horonobensis* HB-1^T (98.7%) and *Methanosarcina siciliae* T4/M^T (97.9%). Determination of G+C content of DNA was done by HPLC according to the method of Mesbah *et al.* (1989). The G+C content of strain SMA-21^T was 40.9 mol%. DNA-DNA hybridization was carried out as described by De Ley *et al.* (1970) under consideration of the modifications described by Huss *et al.* (1983) using a model Cary 100 Bio UV/VIS spectrophotometer equipped with a Peltier-thermostatted 6x6 multicell changer and a temperature controller with *in-situ* temperature probe (Varian). Although high 16S rRNA gene sequence similarity was observed between the strain SMA-21^T and *Methanosarcina mazei*, the results of DNA-DNA hybridization indicated only 22% genomic relatedness (two replications). According to the recommendation of Wayne *et al*. (1987) and based on phylogenetic and physiological characteristics according to the minimal standards for the description of new taxa of prokaryotic strains (Tindall *et al*., 2010), a novel species of the genus *Methanosarcina* is proposed, named *Methanosarcina soligelidi* sp. nov..

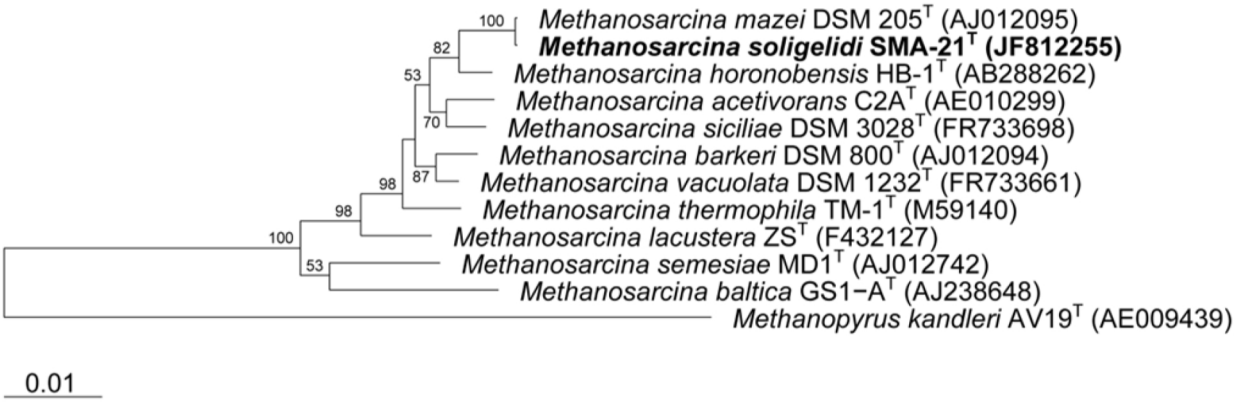

Fig. 3. Phylogenetic tree of the 16S rRNA genes of the novel strain SMA-21^T within the genus *Methanosarcina* (with *Methanopyrus kandleri* AV19^T as outgroup). The tree was constructed by the neighbor-joining algorithm, but all branches were also found in maximum-likelihood (Fitch, 1971) and maximum-parsimony trees (Felsenstein, 1981). Numbers at nodes indicate bootstrap percentages (Felsenstein, 1985) based on a neighbour-joining analysis of 1000 replications; only values ≥ 50% are shown. Bar, 0.01 substitutions per nucleotide position.

Description of *Methanosarcina soligelidi* sp. nov.

Methanosarcina soligelidi (so.li. ge'li.di. L. n. solum, soil; L. adj. gelidus –a –um, icy cold, very cold; N.L. gen. n. soligelidi, of a icy cold soil, referring to the isolation of the type strain from permafrost-affected soil).

Cells are strictly anaerobic, Gram-negative staining, irregular cocci that are 1.3-2.5 µm in diameter. Cells grow on H_2/CO_2, methanol and acetate, but not on formate, ethanol, 2-butanol, 2-propanol, monomethylamine, dimethylamine, trimethylamine and dimethyl sulfide. Optimal growth and growth range were 28°C (0-54°C), pH 7.8 (pH 4.8-9.9) and in 0.02 M NaCl (0.02-0.6 M). The DNA G + C content of the type strain is 40.9 mol%. Major membrane lipids of SMA-21^T were archaeol phosphatidylglycerol, archaeol phoshatidylethanolamine and the corresponding hydroxyarchaeol compounds.

The type strain SMA-21^T (=DSM 26065^T = JCM 18468) was isolated from a permafrost-affected soil on Samoylov Island, Lena Delta, Siberia.

Acknowledgements

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3. Publication II

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Methanobacterium movilense sp. nov., a hydrogenotrophic, secondary alcohol-utilizing methanogen from the anoxic sediment of a subsurface lake

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Running title: *Methanobacterium movilense* sp. nov.

Keywords: *Methanobacterium*, cave ecosystem, archaea

New taxa – Archaea

The GenBank accession number of the 16S rRNA gene sequence of *Methanobacterium movilense* MC-20^T is JF812256.

Abstract

A novel strain of methanogenic archaea, designated strain MC-20^T, was isolated from the anoxic sediment of a subsurface lake in Movile Cave, Mangalia, Romania. Cells were non-motile, Gram-negative rods of 3.5-4.0 µm in length and 0.6-0.7 µm in width, and occurred either singly or in short chains. Strain MC-20^T was able to utilize H_2/CO_2, formate, 2-propanol and 2-butanol as substrate, but not acetate, methanol, ethanol, dimethyl sulphide, monomethylamine, dimethylamine and trimethylamine. Trypticase peptone nor yeast extract was required for growth. The major membrane lipids of strain MC-20^T are archaeol phosphatidylethanolamine and diglycosyl archaeol while archaeol phosphatidylinositol and glycosyl archaeol are present only in minor amounts. Optimal growth was observed at 33°C, pH 7.4 and 0.08 M NaCl. Based on phylogenetic analysis of the 16S rRNA gene sequence, strain MC-20^T closely affiliates with *Methanobacterium oryzae* (similarity 97.1%) and *Methanobacterium lacus* (similarity 97.0%). The G+C content of the genomic DNA was 33.0 mol%. Based on phenotypic and genotypic differences, strain MC-

20^T was assigned to a novel species of the genus *Methanobacterium* for which the name *Methanobacterium movilense* sp. nov. is proposed. Type strain is MC-20^T (= DSM 26032 = JCM 18470).

The Movile Cave is a groundwater system located close to the city of Mangalia, few kilometres away from the Black Sea coast, Romania. This karstic region is characterized by numerous thermal springs (Sarbu & Popa, 1992). The cave is developed in sarmatian limestone on two levels, one of which being dry while the other is submerged and forms a siphon. Where the ceiling of the flooded gallery raises above the level of the water, sealed spaces, called *air bells*, form. Proven to be almost completely isolated from the surface (Sarbu *et al.*, 1996), one of the striking features of this system is the constancy of its physical and chemical parameters. Thus, the cave is known for its unique subsurface ecosystem with groundwater rich in hydrogen sulfide (up to 10 mg l^{-1}), methane (up to 6 mg l^{-1}) and ammonium (up to 6 mg l^{-1}) and anoxic beginning with the depth of 5 cm, and an atmospheric composition in the air bells of 7-10 % O_2, 2-3.5 % CO_2 and 1-2 % CH_4 (Sarbu & Kane, 1995; Sarbu *et al*., 1996). The pH of the water is 7.4 while its temperature is between 22° and 24°C. In contrast with the majority of the caves from the temperate zone, where the inner temperature equals the mean of the annual external temperatures, namely around +10° to +12°C, in Movile Cave, due to the presence of the mesothermal water and to the lack of openings, the temperature of the atmosphere reaches values as high as 20°C in the dry passages and slightly higher in the air bells. Life in the cave has been separated from the outside for the past 5.5 million years and it is exclusively based on chemosynthesis such as sulphur oxidation (Sarbu *et al*., 1996; Falniowski *et al.*, 2008). This microbial primary production acts as food base for invertebrates, such as leeches, spiders, scorpions and insects. Altogether 48 new species have been described of which 33 are endemic (Sarbu, 2000).

Recently, the general diversity of bacteria and archaea was analysed by 16S rRNA gene sequence and functional gene analyses, showing a large diversity of bacteria and archaea in the cave (Chen *et al*., 2009). More detailed investigations on microbial diversity was done for the group of methanotrophic bacteria by stable

isotope probing, which identify a diverse range of methanotrophs belonging to *Alphaproteobacteria* and *Gammaproteobacteria* (Hutchens *et al*., 2004). Although the microbial communities of the Movile Cave have been of interest since the ecosystem was discovered in 1986, to our knowledge only one new strain LV43 of *Thiobacillus thioparus* was isolated so far (Vlasceanu *et al*., 1997). This strain is abundant in the microbial mats and characterized by ribulose-1,5-biphosphate carboxylase activity (Sarbu *et al*., 1994).

The genus *Methanobacterium* (WoRMS 2013) comprises numerous species with validly published names from different environments (e.g. *Methanobacterium oryzae*, rice field soil, Joulian *et al*., 2000; *Methanobacterium aarhusense*, marine sediment, Shlimon *et al*., 2004; *Methanobacterium veterum*, Siberian permafrost, Krivushin *et al.*, 2010; *Methanobacterium lacus*, lake sediment, Borrel *et al*., 2012), but non from a groundwater ecosystem like the Movile Cave. Some of the *Methanobacterium* strains are able to use secondary alcohols as a substrate such as *M. palustre* (Zellner *et al*., 1988) and *M. bryantii* (Balch *et al*., 1979).

In this study, we describe the characteristics of a novel methanogenic archaeon, strain MC-20^T, which was enriched and isolated from anoxic sediment of the subsurface lake in the Movile Cave, Mangalia, Romania.

The described strain MC-20^T was isolated from anoxic, grey-coloured sediment samples obtained in summer 2002 from the subsurface lake of Movile Cave, south east Romania near the city of Mangalia.

Enrichment of the strain was performed with 2 g fresh sediment added to 50 ml of sterile anoxic medium of the following composition (l^{-1}): K_2HPO_4, 0.15 g; KH_2PO_4, 0.3 g; NaCl, 0.6 g; $(NH_4)_2SO_4$, 0.3 g; $MgSO_4$ x 7 H_2O, 0.12g; $CaCl_2$ x 2 H_2O, 0.08 g; $NaHCO_3$, 40 g; Na_2S x 3 H_2O, 0.5 g; Wolfe's trace element solution (composition l^{-1}: MgSO4 x 7 H_2O, 30 mg; Nitrilotriacetic acid, 15 mg; $MnSO_4$ x 4 H_2O, 5 mg; $FeSO_4$ x 7 H_2O, 1 mg; $CoCl_2$ x 6 H_2O, 1 mg; $ZnSO_4$ x 7 H_2O, 1 mg; H_3BO_3, 0.1 mg; $NaMoO_4$ x 2 H_2O, 1 mg; $CuSO_4$ x 5 H_2O, 0.1 mg), 10 ml; Wolfe's vitamin solution (composition l^{-1}: Pyridoxine hydrochloride, 0.1 mg; Calcium D-(+)-pantothenate, 0.05 mg; Lipoic acid, 0.05 mg; Nicotinic acid, 0.05 mg; p-

Aminobenzoic acid, 0.05 mg; Thiamine-HCl, 0.05 mg; Biotin, 0.02 mg; Folic acid, 0.02 mg; Vitamin B12, 0.001 mg), 10 ml; and 2 ml Resazurin indicator solution. The pH was adjusted to 7.2. The bottles were sealed with butyl rubber stopper and secured with an aluminium crimp collar. After flushing the headspace with H_2/CO_2 (80:20 v/v, 100 kPa) the bottles were pressurized with N_2/CO_2 (80:20 v/v, 200 kPa) and incubated at 28°C in the dark. After methane production was observed in the headspace, 5 ml of the culture were transferred to a new bottle with 50 ml of sterile anoxic medium, which was additionally supplemented with the antibiotics phosphomycin and erythromycin (each 50 µl ml^{-1} medium) to suppress growth of non-methanogenic microorganisms (Hilpert *et al*., 1981). This procedure was repeated until a pure culture was obtained. All further incubations, including the purity check, were done without any antibiotics. Purity of the strain was confirmed by light microscope examination, the absence of growth in rich medium containing (l^{-1}) glucose 4 g, yeast extract 2 g and peptone 2 g, and DGGE analyses of DNA extracts obtained from the culture. The strain was maintained by three months transfer into liquid medium. After re-growth at 28°C the culture was stored at 5°C. All preparation steps were done under strictly anaerobic conditions.

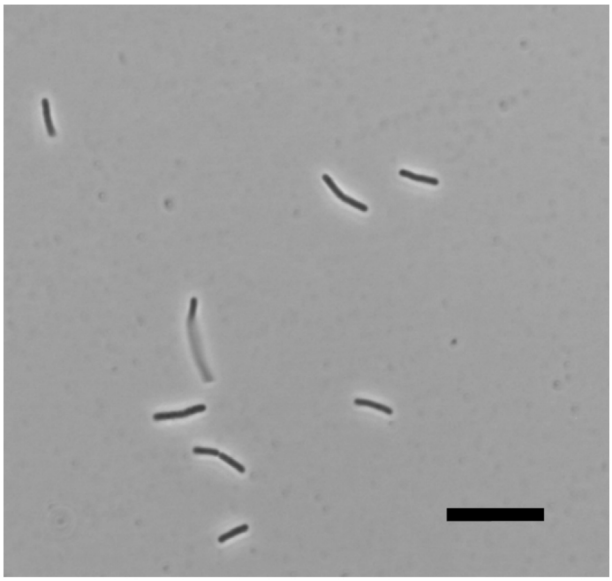

Fig. 1. Light micrograph of strain MC-20T cells (bar = 10 µm).

A light microscope (Axioskop 2, Zeiss) was used to perform phase-contrast microscopy of cells in the exponential growth phase. The results showed that cells of strain MC-20^T were short rods of 3.5-4.0 µm in length and 0.6-0.7 µm in width (Fig. 1). Cells were non-motile and stained Gram-negative. Lysis of the cells was not observed in an SDS solution of up to 1 % (w/v).

Growth and substrate utilization were determined by culturing strain MC-20^T in the medium described above and growth rate was estimated by measuring the concentration of methane in the gas phase (Powell, 1983). The methane concentration was measured by gas chromatography as described previously (Morozova & Wagner, 2007). All growth tests were performed in triplicates at 28°C. The effect of temperature on growth was tested using H_2/CO_2 (80:20 v/v) as substrate at 0, 5, 10, 16, 22, 28, 33, 38, 44, 54 and 64 °C in incubators with the respective temperatures. Growth of strain MC-20^T was observed at 0-44°C with optimum growth at 33°C (Fig. 2a). The pH range for growth was adjusted to pH 4.1-9.9 with 1 M HCl and 1 M NaOH, respectively. Growth was observed between pH 6.2 and 9.9 with optimum growth at pH 7.4 (Fig. 2b). The salinity range was determined in medium with 0.02-0.6 M NaCl. Optimum growth was measured at 0.08 M and salt concentrations of up to 0.3 M were tolerated (Fig. 2c). The substrate spectrum of strain MC-20^T was determined by addition of the following carbon sources to the growth medium: H_2/CO_2 (80:20 v/v, 150 kPa), sodium formate (80 mM), sodium acetate (40 mM), methanol (20 mM), ethanol (20 mM), 2-propanol (10 mM), 2-butanol (20 mM), dimethyl sulfide (20 mM), monomethylamine (20 mM), dimethylamine (20 mM) and trimethylamine (20 mM). Cultures were incubated at 28°C for 10 weeks and growth was subsequently monitored through gas chromatography measurements of methane in the headspace and by visual analysis of increasing turbidity. Growth was observed with H_2/CO_2, formate, 2-propanol and 2-butanol, but not with acetate, methanol, ethanol, dimethyl sulfide, monomethylamine, dimethylamine and trimethylamine (Tab. 1). The generation time with H_2/CO_2 at 28°C was 3.3 ± 0.2 d.

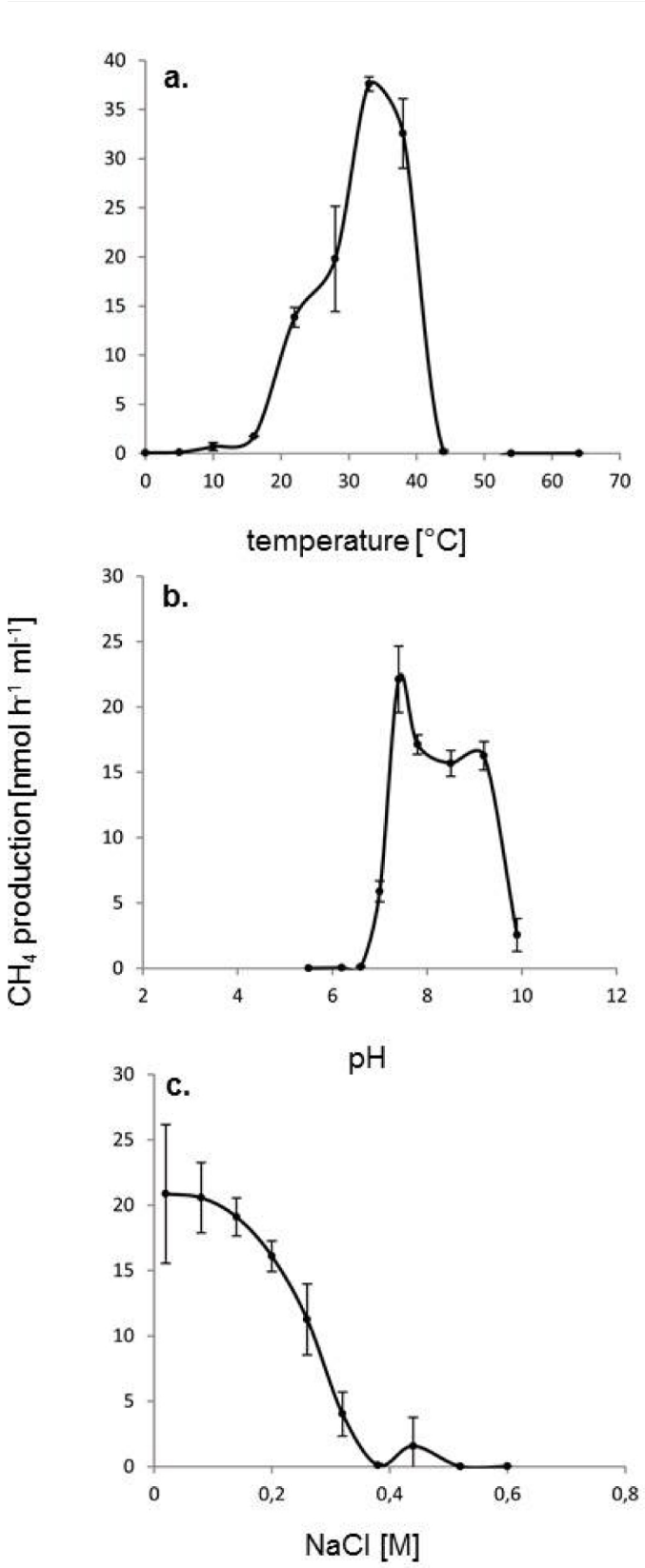

Fig. 2. Physiological characteristics of strain MC-20^T; methane production in dependence of temperature (a.), pH (b.) and salt concentration (c.).

Intact membrane lipids were examined for strain MC-20^T and its closest relatives *Methanobacterium lacus* and *Methanobacterium oryzae* (similarity 97.0 % and 97.1 %, respectively) using a method described by Zink & Mangelsdorf (2004). The intact lipids were detected with a HPLC electrospray interface MS (HPLC-ESI-MS) system. Strain MC-20^T contains a set of membrane diether lipids, detected in the HPLC-ESI MS negative and positive ion mode. The major lipids were archaeol phosphatidylethanolamine (at m/z=774 $[M-H]^-$ or m/z=776 $[M+H]^+$) and diglycosyl archaeol (at m/z=975 $[M-H]^-$ or m/z=999 $[M+Na]^+$) as well as to a smaller extent, archaeol phosphatidylinositol (at m/z=893 [M-H]- or m/z=895 $[M+H]^+$) and glycosyl archaeol (at m/z=837 $[M+Na]^+$; Table 1). The two closest relatives *Methanobacterium lacus* and *Methanobacterium oryzae* also reveal these membrane lipids. However, both species contain some additional tetraether membrane lipids (GDGT; glycerol diphytanyl glycerol tetraether) in small amounts (Table 1), which were not present in strain MC-20^T. *Methanobacterium lacus* additionally exhibits glycosyl-GDGT (m/z=1486 $[M+Na]^+$), diglycosyl-GDGT (m/z=1648 [M+Na]+), glycosyl-phosphatidyl-GDGT (m/z=1544 $[M+H]^+$), diglycosyl-phosphatidyl-GDGT (m/z=1728 $[M+Na]^+$) or glycosyl-phosphatidyl-GDGT-hexose (m/z=1728 $[M+Na]^+$) and diglycosyl-phosphatidyl-GDGT-hexose (m/z=1890 $[M+H]^+$). *Methanobacterium oryzae* additionally only contains glycosyl-phosphatidyl-GDGT and diglycosyl phosphatidyl-GDGT-hexose. Furthermore, *Methanobacterium lacus* reveals in relatively high amounts, a yet unknown diether lipid which is tentatively identified as diglycosyl-di-O-alkyl-glycerol (m/z=1208 $[M+Na]^+$). The mixture of mainly diethers (forming bilayers) with minor amounts of tetraethers (usually forming monolayers) point to a partly “riveted” membrane bilayer in *Methanobacterium lacus* and *Methanobacterium oryzae* as known also from other methanogens (de Rosa *et al*., 1994).

Table 1. Characteristics of strain MC-20^T and related species of the genus *Methanobacterium*.4 Strains: 1, *Methanobacterium movilense* sp. nov. MC-20^T (data from this study); 2, *Methanobacterium oryzae* FPi^T (Joulian *et al*., 2000); 3, *Methanobacterium lacus* 17A1^T (Borrel *et al*., 2012) and 4. *Methanobacterium beijingense* 8-2^T (Ma *et al*., 2005). +, positive; -, negative,

ArPE = archaeol phosphatidylethanolamine; Ar-PI = archaeol phosphatidylinositol; Ar-Gly = glycosyl archaeol; Ar-diGly = diglycosyl archaeol; UK2 = diglycosyl-di-O-alkyl-glycerol; Gly-GDGT = glycosyl-GDGT; diGly- GDGT = diglycosyl-GDGT; GlyP-GDGT = glycosyl-phosphatidyl-GDGT; diGlyPGDGT = diglycosyl-phosphatidyl-GDGT or glycosyl-phosphatidyl-GDGT-hexose; diGlyP-GDGT-Gly = diglycosyl-phosphatidyl-GDGT-hexose; † = detected; †† = medium; ††† = abundant; ND, not determined.

Characteristic	**1**	**2**	**3**	**4**
Source	subsurface lake sediment	rice field soil	lake sediment	anaerobic digester
Cell dimension (µm)	0.6-0.7 x 3.5-4.0	0.3-0.4 x 3-10	0.2-0.4 x 2-15	0.4-0.5 x 3-5
Gram stain	-	ND	-	-
Temp. range (°C)	0-44	20-42	14-41	25-50
Optimum temp. (°C)	33	40	30	37
pH range	6.2-9.9	6.0-8.5	5-8.5	6.5-8.0
Optimum pH	7.4	7.0	6.5	7.2
Tolerance of NaCl (M)	0.02-0.6	0-0.4	0-0.4	0-0.5
Optimum NaCl for growth	0.08	0.08	0.1	ND
Utilization of:				
H_2/CO_2	+	+	+	+
methanol	-	-[§]	(+H_2) +	-
ethanol	-	-[§]	-[§]	-
2-propanol	+	-	-	-
2-butanol	+	-	-	-
acetate	-	-[§]	-	-
formate	+	+	-	+
dimethyl sulfide	-	-[§]	-[§]	-[§]
monomethylamine	-	-[§]	-[§]	-[§]
dimethylamine	-	-[§]	-[§]	-[§]
trimethylamine	-	-[§]	-	-
G + C content (mol%)	33.0	31.0	37.0	38.9
ArPE	†	††[§]	†[§]	ND
Ar-PI	†††	††[§]	†††[§]	ND
Ar-Gly	†	††[§]	†[§]	ND
AR-diGly	†††	†††[§]	†[§]	ND
UK2	-	†††[§]	-[§]	ND
Gly-GDGT	-	†[§]	-[§]	ND
diGly-GDGT	-	†[§]	-[§]	ND
GlyP-GDGT	-	†[§]	†[§]	ND
diGlyP-GDGT	-	†[§]	-[§]	ND
diGlyP-GDGT-Gly	-	†[§]	†[§]	ND
generation time (d) at 28°C	3.3 ± 0.2	ND	0.9	ND

[§]data obtained in this study

For phylogenetic analysis, cells of strain MC-20^T were centrifuged and DNA was extracted using the UltraClean^{TM} Soil DNA Kit (MO BIO Laboratories, USA) according to the manufacturer's protocol. Amplification of the 16S rRNA gene was carried out with primers ArUn4F (5'-TCYGGTTGATCCTGCCRG-3'; Hershberger *et al*., 1996) and Arc1492R (5'-GGCTACCTTGTTACGACTT-3'; Delong, 1992). PCR fragments were purified using the QIAquick PCR Purification Kit (Qiagen, Germany) and sequenced by GATC Biotech AG (Konstanz, Germany). Sequencing resulted in a 1347 bp gene product for MC-20^T that was automatically aligned with closely related sequences obtained from GenBank using the integrated SINA aligner (Pruesse *et al*., 2007) from the ARB-SILVA website (www.arb-silva.de/aligner) and imported into ARB (Ludwig *et al*., 2004). After manual refinement of the alignment, evolutionary distances were calculated and a phylogenetic tree (Fig. 3) was constructed using the neighbour-joining method (Saitou & Nei, 1987) with a termini filter that is implemented in the ARB program. To evaluate the tree topologies, a bootstrap analysis with 1000 replications was performed. Strain MC-20^T showed the highest 16S rRNA gene sequence similarity with the type strains of *Methanobacterium oryzae* (97.1%) and *Methanobacterium lacus* (97.0%). DNA–DNA hybridization experiments were not carried out as the sequence similarities to the nearest relatives were clearly below 98.7 %, which was proposed by Stackebrandt & Ebers (2006). The genomic DNA G+C content of the novel strain was determined through HPLC (Tamaoka & Komagata, 1984) and calculated from the ratio of deoxyguanosine and thymidine according to the method of Mesbah *et al*. (1989). The G+C content of MC-20^T was 33.0 mol%.

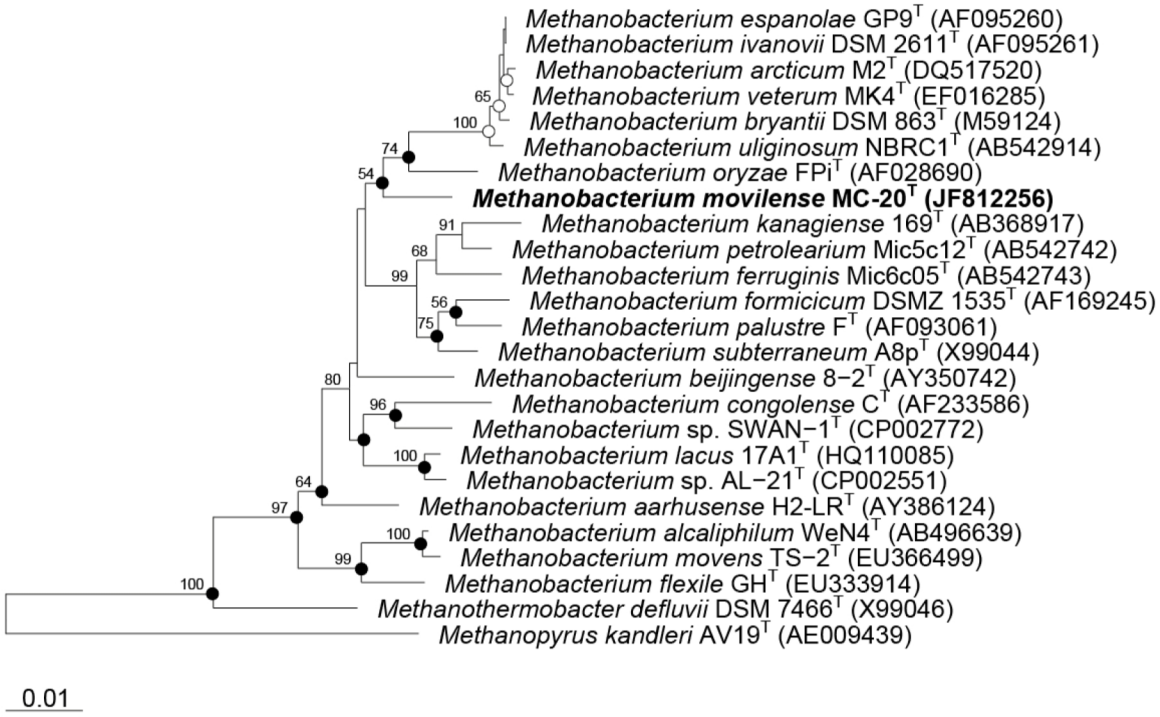

Fig. 3. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic position of strain MC-20^T within the genus *Methanobacterium* (and *Methanopyrus kandleri* AV19^T as outgroup). Branches marked with open circles were also found in maximum-parsimony trees (Felsenstein, 1981) whereas branches with black circles were also found in both maximum-parsimony and maximum-likelihood trees (Fitch, 1971). Numbers at nodes indicate bootstrap percentages (Felsenstein, 1985) based on a neighbor-joining analysis of 1000 replications; only values ≥50% are shown. Bar, 0.01 substitutions per nucleotide position.

Based on the phylogenetic and physiological characteristics according to the minimal standards for the description of new taxa of prokaryotic strains (Tindall *et al*., 2010), a novel species of the genus *Methanobacterium* is proposed, named *Methanobacterium movilense* sp. nov.

Description of *Methanobacterium movilense* sp. nov.

Methanobacterium movilense (mo.vil.en'se. N.L.. neut. adj., *movilense* of Movile, as a reference to the Movile Cave, the source of the isolated strain). Cells are strictly anaerobic, Gram-negative, non-motile rods with 0.6-0.7 µm in width and 3.5-4.0 µm in length, that occur either as single cells or short chains. Cells

grow on H_2/CO_2, formate, 2-butanol and 2-propanol, but not on acetate, methanol, monomethylamine, dimethylamine, trimethylamine and dimethyl sulphide. The strain grows optimal at 33°C (0-44°C), pH 7.4 (pH 6.2-9.9) and in 0.08 M NaCl (0.02-0.6 M). The G+C content of the genomic DNA of the type strain is 33.0 mol%. The major cell membrane lipids of MC-20^T are archaeol phosphatidylethanolamine as well as diglycosyl archaeol and in smaller amounts archaeol phosphatidylinositol and glycosyl archaeol. The generation time of the strain is 3.3 ± 0.2 d at 28°C with hydrogen as substrate. The type strain is MC-20^T (= DSM 26032 = JCM 18470) isolated from sediment of the subsurface lake from Movile Cave, near the city of Mangalia, Romania.

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Influence of Martian regolith analogs on the activity and growth of methanogenic archaea with special regard to long-term desiccation

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Running title: Long-term desiccation resistance of methanogens

ABSTRACT

Methanogenic archaea have been studied as model organisms for possible life on Mars for several reasons: they can grow lithoautotrophically by using hydrogen and carbon dioxide as energy and carbon sources, respectively; they are anaerobes; and they evolved at a time when conditions on early Earth are believed to have looked similar to those of early Mars. As Mars is currently dry and cold and as water might be available only at certain time intervals, any organism living on this planet would need to cope with desiccation. On Earth there are several regions with low water availability as well, e.g. permafrost environments, desert soils and salt pans. Here, we present the results of a set of experiments investigating the influence of different Martian regolith analogs on the metabolic activity and growth of three methanogenic strains exposed to culture conditions as well as long-term desiccation. In most cases, concentrations below 1 wt% of regolith in the media resulted in an increase of methane production rates, whereas higher concentrations decreased the rates, thus prolonging the lag phase. Further experiments showed that methanogenic archaea are capable of producing methane when incubated on a water-saturated sedimentary matrix of regolith lacking nutrients. Survival of methanogens under these conditions was analyzed with a 400 day desiccation experiment in the presence of regolith analogs. All tested strains of methanogens survived the desiccation period as it was determined through reincubation on fresh medium and via qPCR following propidium monoazide treatment to identify viable cells. The survival of long-term desiccation and the ability of active metabolism on water-saturated MRAs strengthens the possibility of methanogenic archaea or physiologically similar organisms to exist in environmental niches on Mars. The best results were achieved in presence of a phyllosilicate, which provides insights of possible positive effects in habitats on Earth as well.

Keywords: Methanogenic archaea, long-term desiccation, Martian regolith analogs, quantitative PCR, propidium monoazide, Mars

INTRODUCTION

The present day Mars is considered hostile to life as we know it on Earth. However, at the time when life first evolved on our planet, the environmental conditions might have been similar to those on early Mars (Carr 1989, 1996; Durham *et al.*, 1989; McKay and Davis, 1991; McKay *et al.*, 1992). Therefore, it is possible that life might have simultaneously evolved on both planets. The detection of methane in the Martian atmosphere has been interpreted as a sign of possible biologic activity, amongst other interpretations (Formisano *et al.*, 2004; Geminale *et al.*, 2011; Krasnopolsky *et al.*, 2004; Mumma *et al.*, 2009); however, the latest measurements performed by a tunable laser spectrometer onboard the rover *Curiosity* indicated that the average methane concentration on Mars (at least in the Gale crater region) is approximately 6 times lower than what was originally estimated (Webster *et al.*, 2013). Nevertheless, temporarily higher concentrations of methane could be observed with measurements conducted over a complete Martian year (Webster *et al.*, 2015).

On Earth, the only biogenic source of methane is methanogenesis, and thus, methanogenic archaea are regarded as model organisms for possible life on Mars (Boston *et al.*, 1992; Jakosky *et al.*, 2003; Morozova *et al.*, 2007; Weiss *et al.*, 2000). Methanogenic archaea have evolved under early Earth conditions, and they are anaerobes that are capable of growing chemolithoautotrophically with hydrogen and carbon dioxide as sole energy and carbon sources, respectively. Although water might be available on the Martian surface-near subsurface (Möhlmann, 2010a, 2010b; Möhlmann and Thomson, 2011), any possible life on Mars has to be able to withstand seasonal desiccation because Mars is considered a dry planet. Previous studies (Morozova *et al.*, 2007) have shown the survival potential of methanogenic archaea – especially strains isolated from permafrost-affected soils such as *Methanosarcina soligelidi* SMA-21 (Wagner *et al.*, 2013) – when exposed to

simulated diurnal variations of Mars analog thermo-physical surface conditions, such as temperatures between -80 and +20°C, changing water activity between a_w 0 and 1, and a pressure of 6 mbar. Methanogenic archaea from permafrost environments also showed high resistance to freezing at -80 °C, high salt concentrations up to 6 M NaCl (Morozova and Wagner, 2007) and methane production under simulated Mars subsurface conditions at a temperature of -5 °C and pressure of 50 kPa (Schirmack *et al.*, 2014a).

Because soil properties and the composition of the sedimentary matrix have a strong influence on the microbial activity and distribution on Earth (e.g., Görres *et al.*, 2013; Rosa *et al.*, 2014), the soil properties are most likely also a very important factor for the habitability of Mars. Therefore we investigated the influence of three different types of Mars regolith analogs (MRAs) on the growth and metabolic activity of three methanogenic strains from permafrost and non-permafrost environments. The regolith mixtures represent differently altered Martian soils, including sulfate-rich deposits and phyllosilicates, and have been designed according to soil types that can be found on Mars (Poulet *et al.*, 2005; Chevrier and Mathé, 2007). The underlying hypothesis is that the properties of the regolith mixtures, due to their mineral composition, may affect the activity of methanogens. Other studies on methanogenic archaea from non-permafrost environments have shown inhibitory effects of Mars regolith analogs on methane production (Kral and Altheide, 2013).

Therefore, the aims of this study are to determine i) the survival potential of methanogenic archaea from permafrost and non-permafrost environments under long-term desiccation (400 days) and ii) the impact of components of different Mars regolith analogs (MRA) at increasing concentrations, with/without nutrient supplements, on the activity and growth of the methanogenic archaea. Survival was estimated via reincubation of the organisms in fresh medium and determination of the number of viable cells via propidium monoazide treatment followed by quantitative PCR. The results of this study contribute to the understanding of factors influencing the survival rate of methanogens under extreme environmental conditions and to the understanding how methanogens were successful over the

time from early Earth up to now, since the last common ancestor of all archaea might have been a methanogen (Gribaldo and Borchier-Armanet, 2006).

MATERIALS AND METHODS

Organisms and Growth media

Three strains of methanogenic archaea were used in these experiments: i) *Methanosarcina soligelidi* SMA-21 isolated from the active layer of permafrost in the Lena Delta, Siberia (Wagner *et al.*, 2013); ii) *Methanosarcina mazei* DSM 2053^T (obtained from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures-DSMZ) isolated from a sewage sludge plant (Mah, 1980; Maestrojuán *et al.*, 1992), which is the phylogenetically closest strain to *M. soligelidi* SMA-21; iii) *Methanobacterium movilense* MC-20 (Schirmack *et al.*, 2014b) isolated from the anoxic sediment of a subsurface thermal groundwater lake in the Movile Cave, Romania.

Two different anaerobic growth media were used to cultivate the organisms. *Methanosarcina soligelidi* SMA-21 and *Methanosarcina mazei* were incubated on MW medium (described in Schirmack *et al.*, 2014a), and *Methanobacterium movilense* MC-20 was incubated on MB medium (described in Schirmack *et al.*, 2014b). All strains were incubated in sealed, 125-ml serum bottles containing 50 ml of medium, and the headspace was filled with a gas mixture of 100 kPa H_2/CO_2 (80:20 v/v) and 200 kPa overpressurization with N_2/CO_2 (80:20 v/v). All incubations were at 28 °C and in the dark but without shaking.

During the course of the experiments, a 300-µl sample was taken from the headspace at time intervals to check for methane production by gas chromatography (GC) using the GC 6890 from Agilent Technologies equipped with a capillary column Plot Q (length 15 m, diameter 530 µm) and a flame ionization detector (FID). Cell numbers were estimated through counting in a Thoma chamber with a Zeiss Axioscop 2 microscope (Carl Zeiss, Germany).

Martian regolith analogs (MRA)

Three different types of MRAs were used in this study. The first, JSC Mars-1A, was obtained from Orbital Technologies Corporation (Madison, WI, USA). JSC Mars-1A is a palagonitic tephra (volcanic ash altered at low temperatures) that was mined from a cinder quarry and sieved to the < 1 mm fraction. The elemental composition is reported in Table 1 and 2.

The second and third MRAs, phylosilicatic MRA and sulfatic MRA (P- and S-MRA, respectively), were provided by the Museum für Naturkunde in Berlin and were produced by mixing terrestrial igneous rocks, phyllosilicates, carbonates, sulfates and iron oxides obtained from KRANTZ (www.krantz-online.de). The minerals and rocks were chosen to be structurally and chemically similar to those identified in Martian meteorites (McSween, 1994) and on the surface of Mars (Poulet *et al.*, 2005; Bibring *et al.*, 2005; Chevrier and Mathé, 2007; Bishop *et al.*, 2008; Morris *et al.*, 2010). The components were mixed in relative proportions to obtain a mafic to ultramafic bulk chemical composition (Table 1 and 2). The two different mineral and rock mixtures reflected the current knowledge of environmental changes on Mars: weathering or hydrothermal alteration of crustal rocks and the perception of secondary minerals during part of the Noachian and Hesperian epoch followed by the prevailing cold and dry oxidizing condition, with the formation of anhydrous iron oxides. The preparation of the two different mixtures account for the orbital observations that the phyllosilicate deposits are generally not occurring together with the sulfate deposits (Poulet *et al.*, 2005).

Table 1 Mineralogical composition of JSC Mars-1A, P-MRA and S-MRA: Composition as weight percent (wt%) of the mixture. Data for JSC Mars-1A were obtained from Morris et al. (1993); dfor P-MRA and S-MRA were obtained from Dr. Jörg Fritz, Museum für Naturkunde Berlin, Germany.

Mineral phase	JSC Mars-1A (wt%)	P-MRA (wt%)	S-MRA (wt%)
Plagioclase Feldspar	64	-	-
(Ferric oxides)			
Olivine	12	-	-
Magnetite	11	-	-
Pyroxene and/or Glass	9	-	-
Fe_2O_3	-	5	-
Montmorillonite	-	45	-
Chamosite	-	20	-
Kaolinite	-	5	-
Siderite	-	5	-
Hydromagnesite	-	5	-
Quartz	-	10	3
Gabbro	-	3	31
Dunite	-	2	16
Hematite	5	-	17
Goethite	-	-	3
Gypsum	-	-	30

Both mineral mixtures contain igneous rocks composed mainly of pyroxene, plagioclase (gabbro) and olivine (dunite). In addition to quartz, the anhydrous iron oxide hematite (α-Fe_2O_3), the only iron oxide that is thermodynamically stable under the present day Martian conditions (Gooding, 1978), was added to both mixtures. P-MRA resembles igneous rocks altered by pH-neutral hydrous fluids to clays of the smectite group, including montmorillonite, chamosite (Poulet *et al.*, 2005) and the clay mineral kaolinite (Mustard *et al.*, 2009). Siderite and hydromagnesite were included to account for carbonates that formed either by precipitation or interaction between a primitive CO_2-rich atmosphere/hydrosphere and basaltic subsurface rocks (Chevrier and Mathé, 2007; Morris *et al.*, 2010). S-MRA serves as an analogue for a more acidic environment with sulfate deposits, and in addition to igneous rocks and anhydrous iron oxides, it includes goethite and gypsum. The materials were crushed to obtain a grain-size distribution for mechanically fragmented regolith, and

to reduce nugget effects, only fragments < 1 mm were used in the mineral mixtures. After mixing the different components, the size distributions of the mixtures were determined by sieving.

Table 2 Major element composition of JSC Mars-1A, P-MRA and S-MRA: Determined through x-ray fluorescence measurements. Data for JSC Mars-1A obtained from Orbital Technologies Corporation, Madison, WI, USA; data for P- and S-MRA obtained from Dr. Jörg Fritz, Museum für Naturkunde Berlin, Germany. For chemical composition the normal convention for data presentation uses oxide formulae from assumed oxidation states for each element and oxygen is calculated by stoichiometry (e.g. silicon is analyzed as an element but presented as SiO_2). These are representations of the chemistry and do not represent actual phases or minerals in each simulant. ND = not determined.

Major element composition	**JSC Mars-1A (wt%)**	**P-MRA (wt%)**	**S-MRA (wt%)**
Silicon Dioxide (SiO_2)	34.5-44	43.6	30.6-31.8
Titanium Dioxide (TiO_2)	3-4	0.36-0.45	0.05-0.98
Aluminum Oxide (Al_2O_3)	18.5-23.5	11.2-11.9	5.6-9.2
Ferric Oxide (Fe_2O_3)	9-12	19.6-20.3	14.9-19.9
Iron Oxide (FeO)	2.5-3.5	-	-
Magnesium Oxide (MgO)	2.5-3.5	4.48-4.52	10.3-10.9
Calcium Oxide (CaO)	5-6	4.67-4.74	17.8-18.4
Sodium Oxide (Na_2O)	2-2.5	0.29-0.32	1.04-1.09
Potassium Oxide (K_2O)	0.5-0.6	1.04-1.07	0.13-0.86
Manganese Oxide (MnO)	0.2-0.3	0.16-0.17	0.31-0.41
Diphosphorus Pentoxide (P_2O_5)	0.7-0.9	0.55-0.56	0.05-0.42
Sulfur trioxide (SO_3)	-	<0.1-0.2	2.7-9.1
Loss of ignition (LOI)	ND	11.8-12.4	5.4-6.4

For all cultivation experiments with MRAs described here, the required amount of each MRA was weighed in serum bottles (125 ml and 25 ml). The bottles were then sealed with a butyl rubber stopper (thickness 12 mm) and an aluminum crimp, and anaerobic conditions were created by degassing (water-jet vacuum pump) and flushing with N_2/CO_2 (80:20 v/v) at 200 kPa. After autoclaving (121 °C for 25

minutes), sterile medium or buffer solution prepared as described previously were added to the bottles.

Influence of MRAs on the activity of methanogenic archaea (1st experiment)

Microbial cells were grown to a cell density of 10^8 cells ml^{-1}, which is the late exponential phase, and 5 ml of the culture was transferred to 125-ml serum bottles containing 50 ml of fresh anaerobe medium mixed with the specific amount of MRA (0.0 wt%, 0.5 wt%, 1.0 wt%, 2.5 wt% or 5.0 wt%). The starting cell concentration in the experimental serum bottles was approximately 5 x 10^7 cells ml^{-1}. The change in pH for samples containing 1.0 and 5.0 wt% MRA was measured separately, and all incubations and methane measurements were carried out as previously described.

Growth of methanogenic archaea in water-saturated MRA soils (2nd experiment)

To test for activity and growth of methanogenic archaea on MRA model soils, the strains were incubated on buffer-saturated MRAs containing $NaHCO_3$ (4 g l^{-1}), $Na_2S \bullet 3H_2O$ (0.3 g l^{-1}) and resazurin (1 g l^{-1}) as a redox indicator. The serum bottles used for this experiment had a volume of 25 ml. Due to the different densities and interstice volumes of the soil material, the total volume of buffer that was needed to achieve saturation differed for each MRA. Five grams of material and 3.1 ml of buffer were used for JSC Mars-1A, 8 g of material and 1.5 ml of buffer were used for S-MRA, and 5 g of material and 2.9 ml buffer were used for P-MRA. Examples of the test-bottles containing the three buffer-saturated MRAs are shown in **Figure 1**.

Cells were grown to a density on the order of 10^8 cells ml^{-1}, which is late exponential phase. To wash the cells, 50 ml of each growth culture medium was added to sealed screw-cap centrifuge tubes (Nalgene, VWR International, Germany; two parallel tubes were used for each methanogenic strain) and centrifuged at 4200

× g for 45 min. The supernatant was discarded, and the pellets were resuspended in buffer solution; this step was repeated twice. After the last centrifugation step, the cell pellets were resuspended either with buffer solution or with fresh medium (each 20 ml). One milliliter of each cell suspension was used as inoculum for each test serum bottle containing MRAs. Bottles with 4 ml of fresh medium and 1 ml of cell inoculum (resuspended in medium or buffer) were used as the positive controls, and the negative controls consisted of 4 ml of buffer with 1 ml of inoculum (cells resuspended in buffer). The resulting cell concentrations at the beginning of the experiment were approximately 4 x 10^7 cells g^{-1} for all JSC Mars-1A and P-MRS samples, 5 x 10^7 g^{-1} for the S-MRS samples, and 1.5 x 10^8 ml^{-1} for the positive and negative control samples. Additional blank controls containing MRAs mixed with buffer or medium without cells were prepared to check for abiotic methane production. The bottles were incubated, and the methane production was measured as previously described.

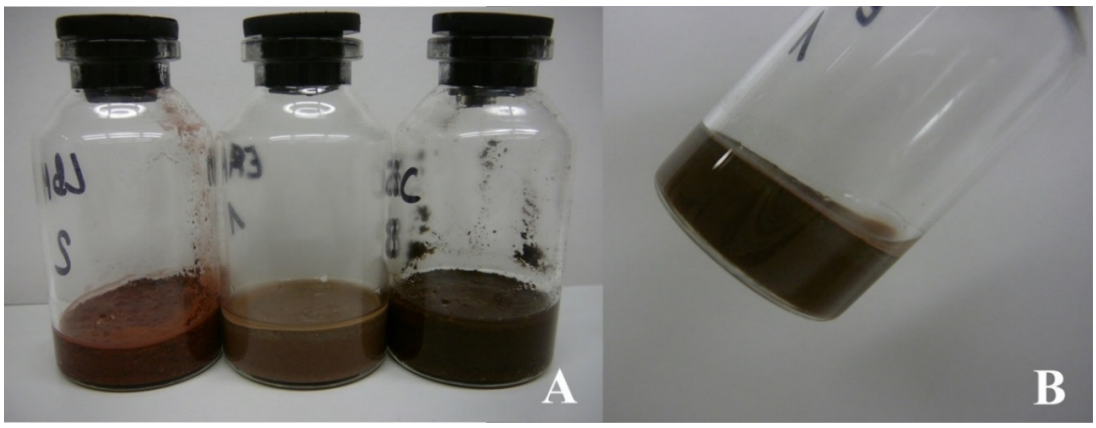

FIGURE 1 Water-saturated MRA in 25 ml serum bottles:
25 ml serum bottles were filled with the different Martian regolith analogs (MRA) to achieve an equal level of filling. Water was added until the water film line reached the surface of the MRA after 24 h of settling. A) From left to right: S-MRA, P-MRA and JSC Mars-1A. B) Bottle with P-MRA held angular to display the water film at the glass margin.

Tolerance OF methanogenic archaea to desiccation in THE presence of MRAs (3rd experiment)

In the 3rd experiment, the effect of MRAs on the survival of desiccated methanogenic archaea was analyzed. Cells were grown as previously described but with 1 wt% of regolith added to the growth medium. No regolith was added to the control samples (desiccation on normal growth medium).

The strains were grown to a cell density of approximately 10^8 cells ml^{-1} for all *Methanosarcina soligelidi* samples, 10^7 cells ml^{-1} for all *Methanosarcina mazei* samples, and 10^9 cells ml^{-1} for all *Methanobacterium movilense* samples. All cells were grown to the exponential or late exponential growth phase and were then harvested together with the Martian regolith analog particles by centrifugation. Two 50-ml serum bottles of the growth media for each strain and sample condition (medium only, JSC Mars-1A, P-MRA and S-MRA) were then transferred to centrifuge tubes (Nalgene, VWR International, Germany), sealed with a screw cap and centrifuged at 4200 × g for 45 minutes at 4°C. After centrifugation, the tubes were placed in an anaerobic chamber, the supernatant was carefully discarded, and the cells as well as the cell-regolith pellets were resuspended in 1 ml (medium only), 4 ml (P-MRA and S-MRA) and 5 ml (JSC Mars-1A) of fresh medium. The cell suspensions were transferred to sterile 500-µl reaction tubes (Eppendorf, Germany) in aliquots of 20 µl (medium only), 80 µl (P-MRA and S-MRA) and 100 µl (JSC Mars-1A) (these differences in volume were due to the different efficiency of the pipetting of the regolith-containing mixtures), which resulted in approximate total starting cell concentrations in the reaction tubes of 2 x 10^9 (*M. mazei*), 2 x 10^{10} (*M. soligelidi*) and 2 x 10^{11} (*M. movilense*). The reaction tubes were then transferred to an anaerobic cylinder outside of the chamber and opened under a constant gas flow of N_2/CO_2 (80:20 v/v). The cylinder was subsequently sealed and flushed several times with H_2/CO_2 (80:20 v/v) through a valve system with sterile filters (0.2 µm), and the gas pressure inside the cylinder was adjusted to 1 bar overpressure to ensure anaerobic

conditions. The cylinder was placed in the dark at room temperature (approximately 22 °C), and 50 g of KÖSTROLITH® (CWK, Chemiewerk Bad Köstritz GmbH, Bad Köstritz, Germany) was placed on the bottom of the cylinder to serve as a drying agent to desiccate the samples. Prior to use, the cylinder and drying agent were sterilized by UV irradiation for 1 hour.

Depending on the sample type, no liquid phase was visible after 2 to 7 days of desiccation. At time intervals of 100, 200, 300 and 400 days, the samples were removed from the anaerobic container, and sampling was performed under a sterile gas flow of N_2/CO_2 (80:20 v/v). The reaction tubes were immediately closed before they were removed and directly transferred inside the anaerobic chamber.

To test the survival and activity of the desiccated cells, the samples were resuspended in fresh medium (200 µl) and left for approximately 6 hours in the anaerobic chamber to allow the regolith to completely dissolve (samples from time step days 300 and 400 were left overnight). The resuspended samples were then mixed with 2 ml of fresh medium in a syringe and inoculated into sterile anaerobic, 5-ml serum bottles. After inoculation, the bottles were incubated, and methane production was measured as described earlier. The time intervals for the measurements ranged from 7 days (samples from time step 100) to 3 weeks (samples from time step day 200 and above), and incubation and measuring continued for up to 80 days after inoculation. All reincubation tests were performed in triplicate.

To estimate the number of cells with an intact cell membrane after the desiccation period, the samples were resuspended in a 1:1 mixture of diethyl dicarbonate water (DEPC) and fresh medium (200 µl in total). A volume of 0.5 µl of propidium monoazide (PMA, Biotium, Hayward CA, USA) was added to the reaction tubes to a final concentration of 50 µM. After addition of PMA, which irreversibly binds to DNA of cells with damaged membranes and inactivates it for further processing (Taskin *et al.*, 2011), the tubes were incubated for 5 minutes on a shaker inside an anaerobic chamber in the dark. The tubes were then placed on ice and

irradiated with a 400 W halogen floodlight from a distance of 20 cm. During the 5 minutes of irradiation, the tubes were frequently shaken and rotated, and after irradiation, the DNA of the desiccated samples was extracted using an UltraClean® Microbial DNA isolation kit according to the manufacturer´s instructions (MO BIO Laboratories, Inc., CA, USA). To increase the amount of eluted DNA, the last step was modified to two elutions with 25 µl of buffer each. Additionally, the elution buffer was warmed to 60 °C before elution. The eluted DNA solution was kept frozen at -20°C until further processing, and isolated DNA from all samples was prepared in triplicate.

Validation of propidium monoazide treatment for methanogenic strains

To ensure that only DNA from intact cells was quantified, the propidium monoazide method in combination with quantitative PCR was tested separately. The three strains were grown as described previously. In two parallel approaches, cells were harvested by centrifugation (8800 × g for 60 minutes) from 20 ml of each culture, and the cell pellets were resuspended in 5 ml of each medium, with one part of the samples treated with 70 % isopropanol for 40 minutes to destroy the cell membranes, and the other part left untreated. After the isopropanol treatment, the samples were washed twice with fresh medium, centrifuged (10000 × g for 30 and 15 minutes) and resuspended again in 5 ml of fresh medium. One milliliter of the treated and untreated samples was processed with propidium monoazide, as described above, or left unprocessed, respectively. The DNA was extracted from all samples, and quantitative PCR was performed to determine the gene copy numbers and hence the number of cells with intact membranes.

Quantitative PCR

To estimate the number of viable cells after desiccation, the desiccated cell samples were treated with propidium monoazide as described previously. After

isolation, the DNA was amplified by quantitative PCR (Rotor Gene Q Qiagen, Germany) using the methanogen-specific functional gene primer pair mlas-F and mcrA-r (Steinberg and Regan, 2008, 2009), which targets the alpha subunit of methyl-coenzyme M reductase (*mcrA*). Based on the data currently deposited in the NCBI database, we assumed that each genome had a single copy of the *mcrA* gene; therefore, the gene copy numbers corresponded to the cell numbers.

The reaction mixture used for gene amplification included the following: 12.5 µl of SYBR green, 0.5 µl of each primer, 6.5 µl of DEPC water, and 5.0 µl of diluted template DNA (1:30). The PCR cycles were as follows: start, 95°C for 10 min; step 1, 95°C for 30 seconds; step 2, 55°C for 30 seconds; step 3, 72°C for 45 seconds; step 4, and 80°C for 3 seconds. Steps 1 to 4 were repeated 40 times. To acquire fluorescence data, the samples were melted from 50 to 95°C, with 5-second holding intervals, and the fluorescence data was acquired. The quantification of DNA was conducted using *Methanosarcina barkeri* as a standard at dilutions from 1.7 x 10^8 to 1.7 x 10^4 copies ml^{-1}.

RESULTS

Influence of different MRAs on the activity of methanogens (1st experiment)

To determine the effect of the different MRAs on the metabolic activity of the archaea strains, we determined the methane production rates based on the linear increase in the methane concentrations measured after 8-10 days of incubation (**Figure 2**).

For all tested strains, MRA concentrations above 1.0 wt% resulted in decreased methane production rates. The methane production rate of *Methanosarcina soligelidi* was reduced from 2.6 ± 0.9 nmol CH_4 $h^{-1}ml^{-1}$ without regolith to 0.7 ± 0.4 on 5 wt% JSC Mars-1A, 0.1 ± 0.1 on 5 wt% P-MRA and 1.9 ± 0.1 on 5 wt% S-MRA. The rates

of *Methanosarcina mazei* were reduced from 4.7 ± 0.4 nmol CH_4 $h^{-1}ml^{-1}$ on medium to 2.2 ± 0.1 (JSC Mars-1A), 0.6 ± 0.1 (P-MRA) and 1.1 ± 0.1 (S-MRA) when incubated with 5 wt% of the regoliths. The methane production rates of *Menthanobacterium movilense* were reduced rom 3.9 ± 0.6 nmol CH_4 $h^{-1}ml^{-1}$ to less than 0 (JSC Mars-1A), 0.1 ± 0.1 (P-MRA) and 3.5 ± 0.4 (S-MRA) when incubated on 5 wt% regolith; however, the latter was a negligible change compared to incubation on medium without MRAs.

Instead, at lower concentrations (0.5 and 1.0 wt%), MRAs had a positive effect on *M. soligelidi* and *M. movilense* and increased their methane production rates. The rates of *M. soligelidi* increased from 2.6 ± 0.9 nmol CH_4 $h^{-1}ml^{-1}$ without regolith to 5.8 ± 2.2 (JSC Mars-1A), 6.0 ± 0.3 (P-MRA) and 4.1 ± 1.3 (S-MRA) with 1 wt% regolith. For *M. movilense,* the rates were from 3.9 ± 0.6 nmol CH_4 $h^{-1}ml^{-1}$ on medium to 5.7 ± 0.9 (JSC Mars-1A), 5.4 ± 1.9 (P-MRA) and 4.2 ± 1.7 (S-MRA) on 1 wt% regolith. The methane production rates of *M. mazei* were reduced in the presence of regoliths in all experiments.

It has to be mentioned that incubation times longer than 40 days resulted in final concentrations of approximately 20 % methane, which equaled the stoichiometric maximum concentration produced by the organisms when incubated under normal growth conditions. However, this methane concentration is usually achieved after fewer than 3 weeks of incubation. The only exception to this observation was *Methanobacterium movilense*, which produced up to 10 % methane until day 50 when incubated in the presence of any concentration of MRA.

The changes in pH due to the addition of MRA to the growth media were negligible. In general, the addition of JSC Mars-1A and P-MRA resulted in a slightly more basic pH, whereas the addition of S-MRA resulted in a more acidic pH.

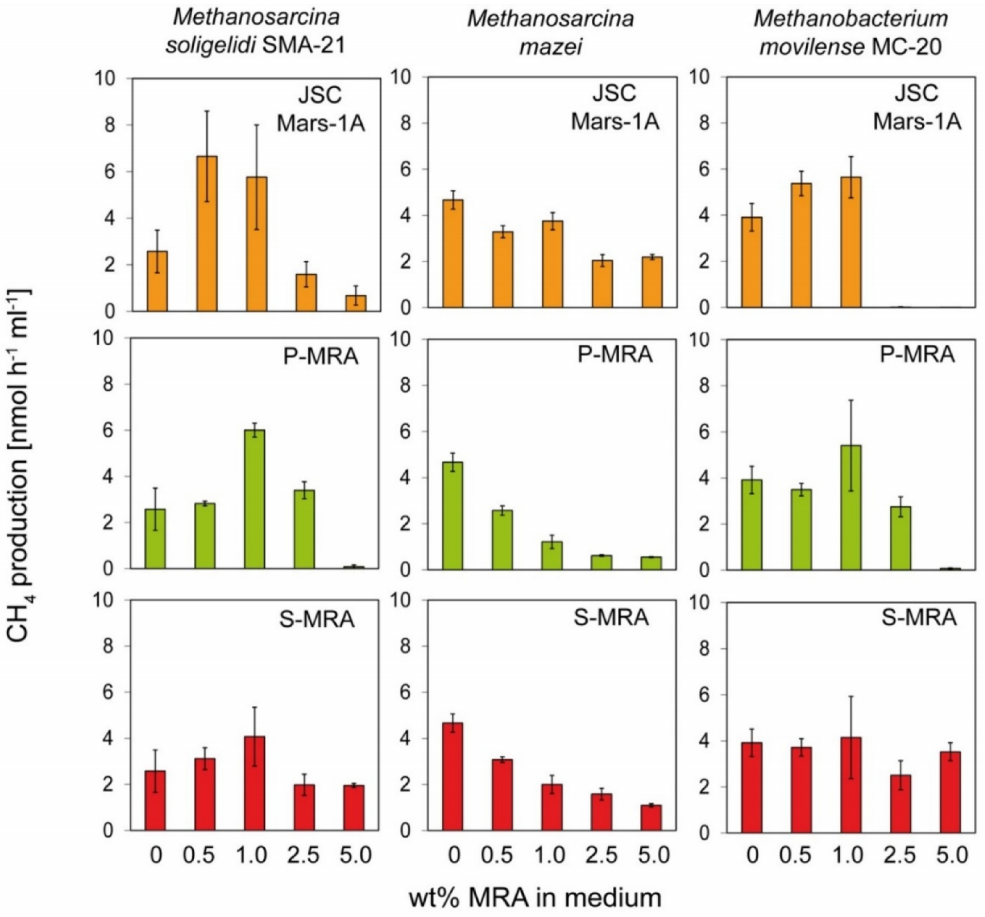

FIGURE 2 Experiment 1, methane production rates of the methanogen strains incubated with increasing concentrations of MRA:
The three methanogen strains where incubated with increasing concentrations of the three Martian regolith analogs (MRA) added to the normal growth medium. The methane production rate was calculated from the increase of methane in the headspace. Error bars indicate standard deviation, n=3.

Growth of methanogens in water-saturated MRAs (2nd experiment)

Methane production was measured by GC for up to 80 days. All positive controls showed continuous methane production, while the negative controls showed no methane production. The additional blank controls (MRA with medium or buffer) showed little methane production in some replicates, e.g., in S-MRA with buffer

solution, where the concentration did not exceed 180 ppm after more than 80 days of incubation. All other tested MRAs reached approximately 30 ppm as a maximum value. To verify that this observed methane release was not due to biotic production through contamination, the blank control bottles were flushed again with fresh gas, and no further increase in methane could be measured.

The increases in the methane concentration during the incubation time for all combinations of methanogenic archaea, MRAs, growth media and buffer solutions are shown in **Figures 3A-F**. In general, all methanogenic strains were able to produce methane on at least one of the tested MRAs when incubated with both growth medium and buffer solution, although this production was lower on buffer than on growth medium. As shown in **Figures 3A** and **3b**, *M. soligelidi* produced more than 20 % methane on P-MRA and approximately 5 % methane on S-MRA when incubated in medium, while it produced 0.3 % methane on P-MRA when incubated in buffer. However, methane production did not exceed the concentration of the blank controls on S-MRA, and no methane was produced on JSC Mars-1A.

Methanosarcina mazei (**Figure 3C**) showed methane production of 8 % only on S-MRA when incubated with medium, and it was able to produce methane on all three tested MRAs when incubated with buffer (**Figure 3D**). The final concentrations, 1.2 %, 0.9 % and 0.4 % methane (P-MRA, S-MRA and JSC Mars-1A, respectively), were higher than that of the blank control.

Methanobacterium movilense produced more than 25 % methane when incubated on P-MRA with medium and 9.4 % methane when incubated on S-MRA with medium (**Figure 3E**). Incubation with buffer resulted in a concentration of more the 20 % on P-MRA and of 1.7 % on S-MRA. *M. movilense* did not produce methane on JSC Mars-1A.

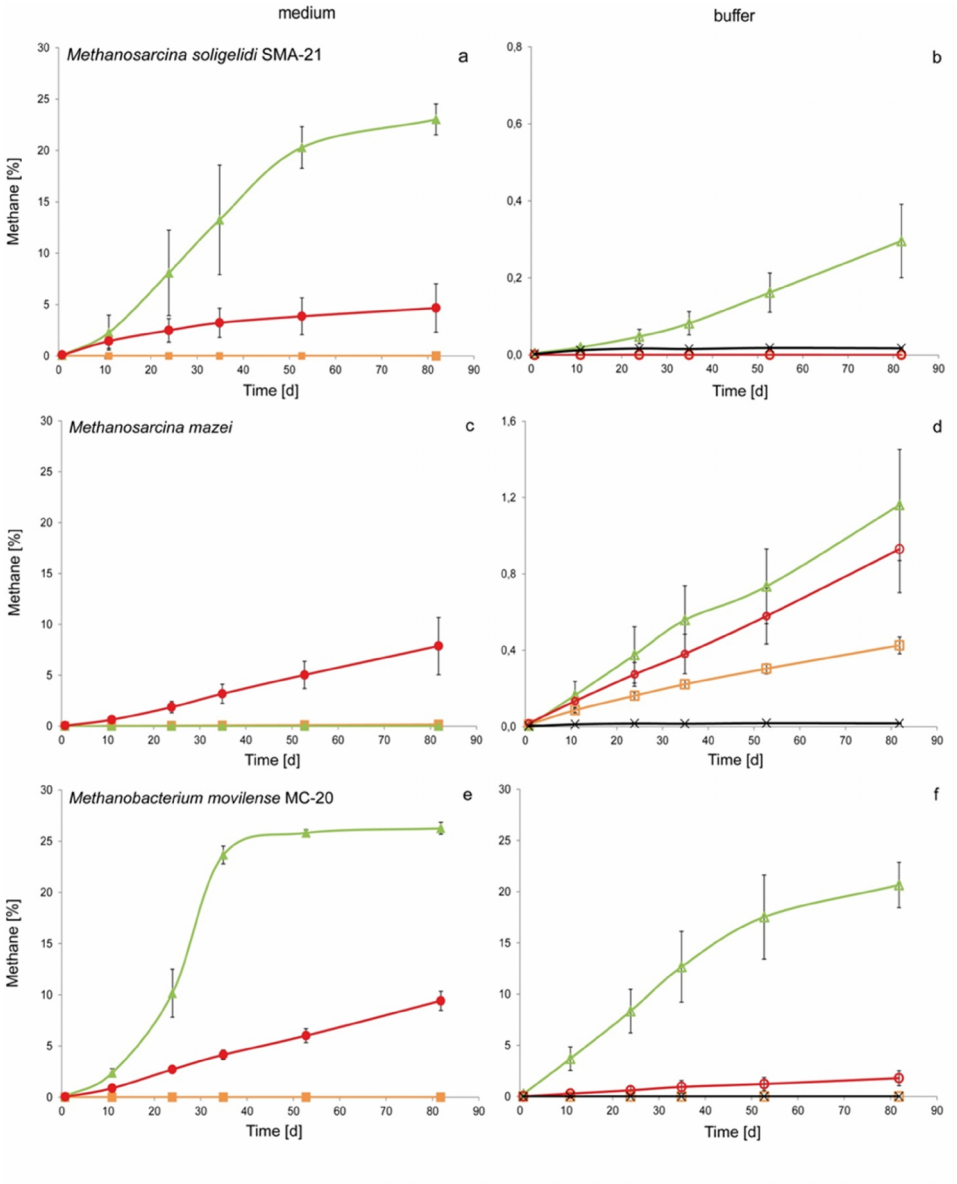

FIGURE 3 Experiment 2, methane production of the three methanogen strains over time when incubated on medium or buffer saturated MRA: 3A, 3C and 3E show the increase of methane over time for (A) *Methanosarcina soligelidi*, (C) *Methanosarcina mazei* and (E) *Methanobacterium movilense* when incubated with standard growth medium on the three Martian regolith analogs (MRA). Green line with closed triangles: P-MRA; red line with closed circles: S-MRA; orange line with closed squares: JSC Mars-1A. 3B, 3D and 3F show the increase of methane over time for (B) *Methanosarcina soligelidi*, (D) *Methanosarcina mazei* and (F) *Methanobacterium movilense* when incubated with buffer solution on the three MRA. Green line with open triangles: P-MRA; red line with open circles: S-MRA; orange line with open squares: JSC Mars-1A;

black line with crosses: highest production of blank controls without cells. All error bars indicate standard deviation, n=3.

Growth of methanogens after desiccation on MRAs (3rd experiment)

Reincubation of the desiccated cell samples showed that methane production could be measured even after 400 days of desiccation, and all strains were able to survive the complete desiccation period under at least three of the four tested conditions. **Table 3** shows the results of methane production after incubation for 80 days. For a better comparison, the produced methane concentrations were rated at levels 0 to 3 on analog to heat map charts. Level 0 indicated no detected methane or that the measured concentration was below 15 ppm; level 1 indicated a methane concentration above 15 but below 100 ppm; level 2 indicated methane concentrations between 100 and 10,000 ppm; and level 3 exceeded 10,000 ppm (1 %). The most important factor for identifying actual methane production was a constant increase in the methane concentration over time, even for the samples marked “1”, whose final concentration of methane did not exceed 100 ppm.

Reincubation of the desiccated samples showed the most constant results for *M. mazei*. In this case, the highest numbers of the tested triplicates were producing methane at least on levels 1 and 2. The highest measured methane production after 400 days of desiccation was detected for *M. movilense* when desiccated on P-MRA, while the weakest results were observed for the time point day 100, on which none of the *M. soligelidi* or *M. movilense* samples showed any methane production. For further verification of methane production, the samples of the last two time points (day 300 and 400) were flushed with a fresh gas mixture (N_2/CO_2, 80:20 v/v) after the first series of measurements (80 days) and incubated again because the headspace pressure in the serum bottles might have dropped due to repeated sampling. Within a few weeks, most samples showed the same level of methane production that was measured at the beginning of the experiments; however, some of the samples did not produce methane. This was the case for some level 1 one

level 1 production of *M. movilense* on S-MRA at time point day 400. In contrast, two of the level 1 productions at day 300 for *M. mazei* (on JSC Mars-1A and S-MRA, respectively) turned out to be level 3 and level 2 productions when incubated after flushing of the headspace.

Table 3 Rated methane production after the specific time steps of desiccation measured for up to 80 days of reincubation:
0 = less than 20 ppm, 1 = between 20 and 100 ppm, 2 = between 100 and 10.000 ppm, 3 = more than 10.000 ppm. 10.000 ppm equals 1% methane. All tests were performed in triplicates; three values are reported for each tested condition per strain and desiccation period. The numbers in each block are sorted decreasingly from the highest to the lowest rating. A dark yellow background indicates three positive results, decreasing coloration of the background marks less positive results.

		Time points of desiccation [d]					
		0	**50**	**100**	**200**	**300**	**400**
M. soligelidi	**medium**	3/3/1	2/1/1	0/0/0	1/0/0	1/0/0	1/1/0
	JSC	3/3/3	3/1/0	0/0/0	2/0/0	1/1/0	2/0/0
	P-MRA	3/3/3	0/0/0	0/0/0	1/1/0	1/1/0	1/0/0
	S-MRA	3/3/3	1/0/0	0/0/0	2/1/0	1/0/0	0/0/0
M. movilense	**medium**	3/3/3	3/3/1	0/0/0	1/0/0	0/0/0	2/0/0
	JSC	3/3/2	3/3/3	0/0/0	0/0/0	3/1/0	0/0/0
	P-MRA	3/3/3	3/3/1	0/0/0	1/1/0	3/3/3	3/0/0
	S-MRA	3/3/3	3/3/3	0/0/0	1/0/0	3/1/0	2/1/0
M. mazei	**medium**	3/3/3	3/3/3	0/0/0	3/3/0	1/1/0	2/2/0
	JSC	3/3/3	3/3/3	3/3/3	3/3/2	1/1/1	2/2/0
	P-MRA	3/3/3	3/3/0	0/0/0	2/0/0	1/1/0	2/0/0
	S-MRA	3/3/3	3/1/0	3/0/0	2/1/0	2/1/1	2/2/0

< 20 ppm | 20-100 ppm | 100-10,000 ppm | > 10,000ppm

Validation and application of propidium monoazide treatment in combination with qPCR

When combined with propidium monoazide (PMA) treatment, qPCR is a valid method to estimate the number of cells (with intact membranes) based on the DNA copy numbers (Taskin *et al.*, 2011). A clear difference was observed in the copy number estimation for the samples treated with isopropanol, depending on whether PMA was added before the DNA isolation. At best, 0.2 % of the copy numbers of the samples not treated with PMA could be found in the PMA samples. For the samples not treated with isopropanol, a difference in the detected copy numbers could also be observed, and treatment with PMA before DNA isolation resulted in reduced copy numbers. At a minimum only approximately 10 % of the copy numbers of the untreated sample could be found in the PMA sample. This was the case for *M. soligelidi*, and the other two strains had approximately 70 % (*M. movilense*) and 30 % (*M. mazei*) of the copy numbers of the untreated samples.

The calculated gene copy numbers per milliliter of culture medium during the desiccation period are shown in **Figure 4**. Although there were variations in the estimated cell concentrations, in most cases, the gene copy numbers did not significantly change, as it was seen with a student's t-test analysis for most of the tested conditions. Moreover, the variations were in the range of the standard deviation. A high concentration of intact cells for all three methanogen strains at all four conditions and even after 400 days of desiccation was detected (**Figure 4**).

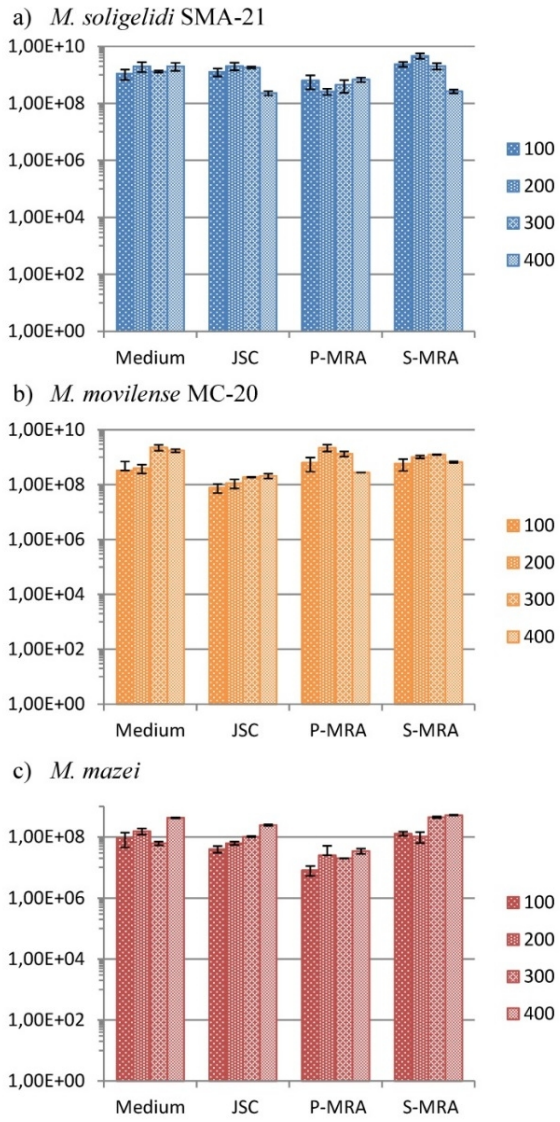

FIGURE 4 Gene copy numbers (mcrA) per ml of culture medium during the desiccation period:
(primers mlas-f and mcrA-r). A) *Methanosarcina soligelidi*, B) *Methanobacterium movilense*, C) *Methanosarcina mazei*. All tests performed at least in triplicates, error bars indicate standard deviation. The reaction efficiencies for all amplification runs were 84 ± 3 %, R^2-values were 0.9973 ± 0.031.

DISCUSSION

Due to their ability for chemolithoautotrophic and anaerobic growth and their evolutionary origin in a time when global environmental conditions on Mars and Earth were supposedly similar (Carr, 1989, 1996; Durham *et al.*, 1989; McKay and Davis, 1991; McKay *et al.*, 1992), methanogenic archaea are considered ideal model organisms for studying possible life on Mars (Boston *et al.*, 1992, Jakosky *et al.*, 2003; Kral *et al.*, 2004; Krasnopolsky *et al.*, 2004; Morozova *et al.*, 2007; Morozova and Wagner, 2007; Schirmack *et al.*, 2014a). In this study, we investigated the effect of different Mars regolith analogs (MRAs) on the metabolic activity and desiccation resistance of methanogenic archaea. Our results prove that the tested methanogenic species have a long-term desiccation resistance (of more than 400 days) and are able to produce methane when incubated on a buffer solution and with MRAs alone.

The methane production rates of the strains *Methanosarcina soligelidi* and *Methanobacterium movilense* increased in the presence of MRAs up to a concentration of 1 wt%. It was noted that each species was differentially affected by the addition of the regoliths. A possible explanation for these differences may be related to the different habitats in which the strains were originally isolated and therefore their specifically adapted physiology. *M. movilense,* for example, inhabits H_2S-rich groundwater (Sarbu *et al.*, 1996), which could explain its higher tolerance to the sulfur-rich S-MRA. In general, the addition of regolith had, up to a certain level, a positive effect on methane production, likely by providing important trace elements such as nickel, cobalt and zinc, which are necessary for the metabolism of the organisms. Additionally, cells attached to regolith particles might have benefited from a shielding effect against environmental influences (Wagner *et al.*, 1999). These positive effects might have become less important with increasing concentrations of MRA in the growth media, and thus, the activity of the methanogens may have been reduced due to inhibitory effects of the mineral mixtures, such as increasing sulfur

concentrations. A comparable observation was made by Kral and Altheide (2013), who showed that the activity of methanogens was decreased in the presence of different Mars analog minerals such as the commonly used JSC Mars-1.

In the second experiment using buffer-saturated Mars regolith analogs, all tested methanogenic strains were able to produce methane in the presence of at least one regolith without any additional nutrients. However, the highest methane production was achieved for all strains after incubation on P-MRA. The production of methane alone might not be proof for actual growth, but in the case of *M. movilense,* which reached a final methane concentration of more than 20 % when incubated on P-MRA, it can be assumed that growth related to high metabolic activity took place. This is in accordance with the study of Kral *et al.* (2004), which showed growth of *Methanothermobacter wolfeii* under comparable conditions on JSC-Mars 1, which was quite similar to the JSC Mars-1A tested here. Nevertheless, a buffer solution and a source of energy and carbon (H_2/CO_2 provided in the headspace) alone are not sufficient to support methanogenic activity, as no methane production could be observed in the control samples containing buffer and cells alone. If *M. movilense* has grown when incubated on P-MRS, the used mineral mixture (Table 2) could be a possible source of phosphorous. Nitrogen is present as molecular nitrogen in the headspace, which can be used by at least some strains of methanogenic archaea such as *Methanosarcina barkeri* (Murray and Zinder, 1984; Leigh, 2000) and *Methanobacterium bryantii* (Belay et al., 1985; Leigh, 2000), which belong to the same genus as *M. movilense*. So, in theory, *M. movilense* might be able to grow diazotrophically; however, this would of course need further verification.

It is remarkable that all of the tested strains were able to sustain the different conditions during the third experiment with up to 400 days of long-term desiccation. For the desiccation test, the quantified gene copy numbers of samples grown on medium only did not change significantly over the course of the experiment. Due to the propidium monoazide (DNA intercalating dye) treatment before DNA isolation and qPCR, damaged cells or free DNA were excluded from the quantification of the *mcrA* genes due to the formation of PMA-DNA complexes. This effect was shown,

for example, by the study of Taskin *et al.* (2011), which tested this method on *Escherichia coli*. The results of the propidium monoazide validation experiment also demonstrate the effectiveness of this method for methanogenic archaea. For the control samples treated with isopropanol to destroy cell membranes, almost no DNA could be quantified when processed with propidium monoazide prior to DNA extraction. The lower copy numbers of the samples processed with propidium monoazide compared to the unprocessed samples showed cells with damaged membranes, where PMA could penetrate, in every culture. However, it is known that cell wall integrity also depends on the growth phase of the culture (Pagán and Mackey, 2000). Moreover, a portion of the intact cells might also have been destroyed during the handling of the samples before the PMA was inactivated by light, and therefore, they were not detected by qPCR. It is notable that the cells maintained their cell wall integrity when desiccated on medium. There was no indication of a positive effect of the added MRAs on cell wall integrity in any of the qPCR experiments, whereas a slightly negative trend was observed in some cases. Therefore, the desiccation resistance of the tested organisms can not be only related to shielding effects of the regolith particles. Other possible reasons could be the secretion of extracellular polysaccharides (EPS) that act as a protective layer, as was shown for *Methanosarcina barkeri* in the study of Anderson *et al*. (2012). In that study, EPS increased the resistance of the strain against desiccation as well as against other environmental stresses, such as oxygen exposure (for 7 days) and high temperature (up to 100 °C).

Due to the application of PMA treatment followed by qPCR, it is possible that a large part of the cells was still intact and viable and therefore survived the desiccation period, even if the methane production that was detected after reincubation of the desiccated samples was comparatively low, which might be dependent on a prolonged lag phase. It is also possible that a portion of the organisms were in a dormant state and therefore not active or just active at a much reduced rate (Hoehler and Jørgensen, 2013). However, in the case of *M. movilense* desiccated for 400 days on P-MRA, the highest production of methane was detected after rehydration. The reason why some of the samples at early time points (e.g., at time step day 100,

Table 3) showed no methane production, but samples at later stages did, cannot definitively be answered. A possible explanation may be the biological variability of the desiccation resistance and activity of the cells. It is also possible that, although the preparations were properly mixed, the samples were not entirely homogeneous. In addition, two of the samples on the starting date showed only little methane production, whereas all other samples reached several percentages of methane production.

Considering all of the results of the experiments, a phyllosilicate-rich soil environment seems to provide the best mineral mixture for methanogenic activity and survival under Mars analog conditions. The major difference between the mineral composition of P-MRA and those of JSC Mars-1A and S-MRA is its high content of phyllosilicate montmorillonite (clay mineral), which is known for its water-binding capacity and expansiveness when exposed to water. This characteristic may be one reason for the resistance of the cells to long-term desiccation in the presence of this mineral. Thus, these cells might have a sufficient source of water present during the desiccating conditions – at least for the later time period when compared to the other MRAs with less clay mineral content. Furthermore, montmorillonite also increases the ion-exchang capacity of P-MRA, which might be a major factor for the increased activity of methanogens on this MRA. Interestingly, montmorillonite has also been discussed as a positive factor influencing the formation of primitive lipid cells or cell precursors as well as RNA binding and therefore is hypothetically involved in the origin of life (Hanczyc *et al.*, 2003).

CONCLUSION

In the scope of the habitability of Mars, it is important for organisms to find all nutrients necessary for growth as well as sources of energy and carbon. Our experiments have shown that, besides being provided hydrogen and carbon dioxide, which are present in the Martian environment, the mineral mixtures of the Martian regolith analogs contain all relevant nutrients to enable metabolic activity of

methanogenic archaea. Albeit survival in a diurnal variation of simulated Martian surface analog conditions for three weeks was proven for *M. soligelidi* (Morozova *et al.*, 2007), the surface-near or deeper subsurface would be more likely habitats due to the relatively stable thermo-physical conditions and better protection from radiation (Jones *et al.*, 2011) as well as better access to liquid water and energy (Vance *et al.*, 2007; Ulrich *et al.*, 2012; Michalski *et al.*, 2013). *M. soligelidi* has also shown its potential for active metabolism under Mars subsurface analog conditions (Schirmack *et al.*, 2014a). In the previous study by Morozova *et al.* (2007), *M. soligelidi* exhibited an explicitly higher survival rate after 3 weeks of exposure to simulated Martian surface analog thermo-physical conditions compared to *M. movilense*. With regard to the results from the present study, it seems that *M. movilense* might be better adapted to cope with the single stress factor of desiccation in the presence of Martian regolith analogs. *M. movilense* was isolated from the Movile cave, which is the first terrestrial ecosystem based on chemosynthesis (Sarbu *et. al.*, 1996) and therefore can be regarded as an analog for extraterrestrial subsurface habitats. The findings of this study may be valuable for future life detection missions, for example ExoMars, which is planned for 2018 and will sample the Martian subsurface (Baglioni *et al.*, 2013).

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5. Publication IV

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Laser spectroscopic real time measurements of methanogenic activity under simulated Martian subsurface analogue conditions

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Abstract

On Earth, chemolithoautothrophic and anaerobic microorganisms such as methanogenic archaea are regarded as model organisms for possible subsurface life on Mars. For this reason, the methanogenic strain *Methanosarcina soligelidi* (formerly called *Methanosarcina spec.* SMA-21), isolated from permafrost-affected soil in northeast Siberia, has been tested under Martian thermo-physical conditions. In previous studies under simulated Martian conditions, high survival rates of these microorganisms were observed. In our study we present a method to measure methane production as a first attempt to study metabolic activity of methanogenic archaea during simulated conditions approaching conditions of Mars-like environments. To determine methanogenic activity, a measurement technique which is capable to measure the produced methane concentration with high precision and with high temporal resolution is needed. Although there are several methods to detect methane, only a few fulfill all the needed requirements to work within simulated extraterrestrial environments. We have chosen laser spectroscopy, which is a non-destructive technique that measures the methane concentration without sample taking and also can be run continuously. In our simulation, we detected methane production at temperatures down to -5°C, which would be found on Mars either temporarily in the shallow subsurface or continually in the deep subsurface. The pressure of 50 kPa which we used in our experiments, corresponds to the expected pressure in the Martian near subsurface. Our new device proved to be fully functional and the results indicate that the possible existence of methanogenic archaea in Martian subsurface habitats cannot be ruled out.

Keywords: Mars; Methanogens; Methane; Sub-zero temperature (Celsius); Wavelength Modulation Spectroscopy (Laser Spectroscopy)

1. Introduction

Although Mars is considered to be the most Earth-like planet of our solar system, its present conditions are characterized as extreme cold and dry. However, there is evidence that 3.8 Ga ago the environmental conditions of early Mars and early Earth have been very similar (Carr 1989, 1996; Durham *et al*., 1989; McKay and Davis, 1991; McKay *et al*., 1992). Considering this, there is the given possibility of life emerging on Mars during the same time as life first appeared on Earth. After its hypothetical initial evolution, life on Mars either might have become extinct resulting in only fossil records being found today, or it might have adapted to the drastically changing conditions and still may be present in some ecological niches.

Spectroscopic observations from the ESA Mars Express spacecraft (Formisano *et al*., 2004; Geminale *et al*., 2011) and ground based spectra analysis from Earth (Krasnopolsky *et al*., 2004; Mumma *et al*., 2009) have detected trace amounts of methane in the Martian atmosphere. Direct photolysis by solar UV radiation and homogenous oxidation by OH and O (^{1}D) are believed to cause a relatively short lifetime of methane on Mars of approximately 340 years (Gough *et al*., 2010; Krasnopolsky *et al*., 2004). This leads to the assumption, that the observed methane must have a recent origin on the planet. At the time of writing latest measurements performed with the Tunable Laser Spectrometer on board of the Curiosity Rover indicated no definitive detection of methane on Mars, with a measured value of only 0.55 ±1.46 ppbv, at least for Gale Crater region (Webster *et al*., 2013). This stands in contrast to the predicted global average value of about 10-15 ppbv methane (Formisano *et al*., 2004; Geminale *et al*., 2011). Nevertheless sources of methane on Mars could be abiotic either via active volcanism, which has not yet been observed on Mars (Krasnopolsky, 2006), or formation via Fischer-Tropsch reactions associated with serpentinization (Michalski *et al*., 2013; Oze and Sharma, 2005; Vance *et al*., 2007). Another possible source for methane would be biogenic production. Although the spectroscopic detection of methane on Mars has been discussed critically (Zahnle *et al*., 2011), the observed spatial and temporal variation

of methane in the Martian atmosphere (Formisano *et al*., 2004; Geminale *et al*., 2008, 2011) and its correlation with the presence of water vapor are supporting the biogenic production theory. On earth aerobic methane production by plants is also known (Bruhn *et al*., 2012), but the main biological source of terrestrial methane is anaerobic methanogenesis (methane production by methanogenic archaea e.g. Barbier *et al*., 2012; Bischoff *et al*., 2012). For this reason several scientific publications have previously dealt with methanogenic archaea as model organisms for possible life on Mars (Boston *et al*., 1992, Jakosky *et al*., 2003, Kral *et al*., 2004, 2011; Krasnopolsky *et al*., 2004; Morozova *et al*., 2007; Morozova and Wagner 2007; Ulrich *et al*., 2012; Weiss *et al*., 2000).

Methanogenic archaea from Siberian permafrost environments have shown extraordinary tolerance against different environmental stresses. However, previous tests were not able to reveal if the microorganisms could be metabolically active in a Mars-like environment or merely were able to survive by entering a dormant state. The methanogenic strain we are using in our recent experiments is *Methanosarcina soligelidi* (formerly called *Methanosarcina* spec. SMA-21), which has been isolated from the active layer of a permafrost-affected soil in northeast Siberia (Wagner *et al*., 2013). In direct comparison to reference strains from non-permafrost environments *M. soligelidi* has shown high resistances against long term freezing for up to two years at -20°C, high salt concentrations up to 6 M NaCl and starvation (Morozova and Wagner 2007), as well as high doses of UV and ionizing radiation (Wagner, D.; unpublished data). Along with two methanogenic strains isolated from the Lena Delta, *M. soligelidi* survived three weeks of simulated Martian thermo-physical conditions (Morozova *et al*., 2007). This was done with a diurnal profile in a Mars-like atmosphere (95.3 % CO_2, 0.6 kPa) with temperature fluctuations from +20 to -75°C and varying humidity with a_w-values between 0.1 and 0.9 corresponding to the Mars average equivalent water vapor pressure of 0.1 Pa. Three in parallel tested reference strains from non-permafrost environments did not show any activity after the simulation period, whereas *M. soligelidi* had almost the same activity than before.

The aim of this study is to present a method to measure the methane production of methanogenic archaea under defined thermo-physical conditions in real time without affecting the simulation conditions through removal of gas samples as it would be necessary for gas chromatography. We developed a system in which we combine laser spectroscopic methane detection with a simulation chamber. In this first study we are focusing on simulated Mars analogue subsurface conditions regarding temperature and pressure like would be found in a potential deep biosphere.

2. Methods

2.1. Wavelength modulation spectroscopy

With laser spectroscopy one can very specifically measure the absorption of methane in the gas phase. With careful selection of the absorption line, cross sensitivities to other gases can be ruled out. Here, we choose the strong absorption line at 1653.45 nm (6047.95 cm^{-1}), to avoid cross sensitivity to water or carbon dioxide (HITRAN database: Rothman *et al*., 2009), as shown in Fig. 1. Although there are stronger absorption lines in the mid infrared, the near infrared spectral region is better addressable because of readily available laser-based telecommunication equipment. For instance, the distributed feedback laser, which was used in this work, is from the telecom provider Anritsu (GB6B5004BDP; Japan). This fiber coupled laser emits cw-light with several mW of optical power, which is tunable across the entire methane line. A highly sensitive InGaAs-photodiode is used (Thorlabs PDA10CS-EC; Newton, New Jersey, USA) to detect the light. Such a near infrared system is less expensive than mid infrared ones, which use e.g. quantum cascade lasers. A near infrared system, like the one presented here, is also better when building up several such devices in parallel. Such a parallel detection scheme seems to be necessary for a systematic analysis of methane production under various conditions.

Due to laser power fluctuations, simple absorption spectroscopy is not sensitive enough and thus a more sophisticated method is needed. Here we use wavelength modulation spectroscopy (Demtröder, 2007). The current of the laser diode is controlled to vary the wavelength of the emitted light (Thorlabs ITC 502; Newton, New Jersey, USA). With lock-in detection technique (lock-in amplifier from Stanford Research System SR830 DSP; Sunnyvale, California, USA), strong rejection of laser fluctuations, background light and noise is achieved.

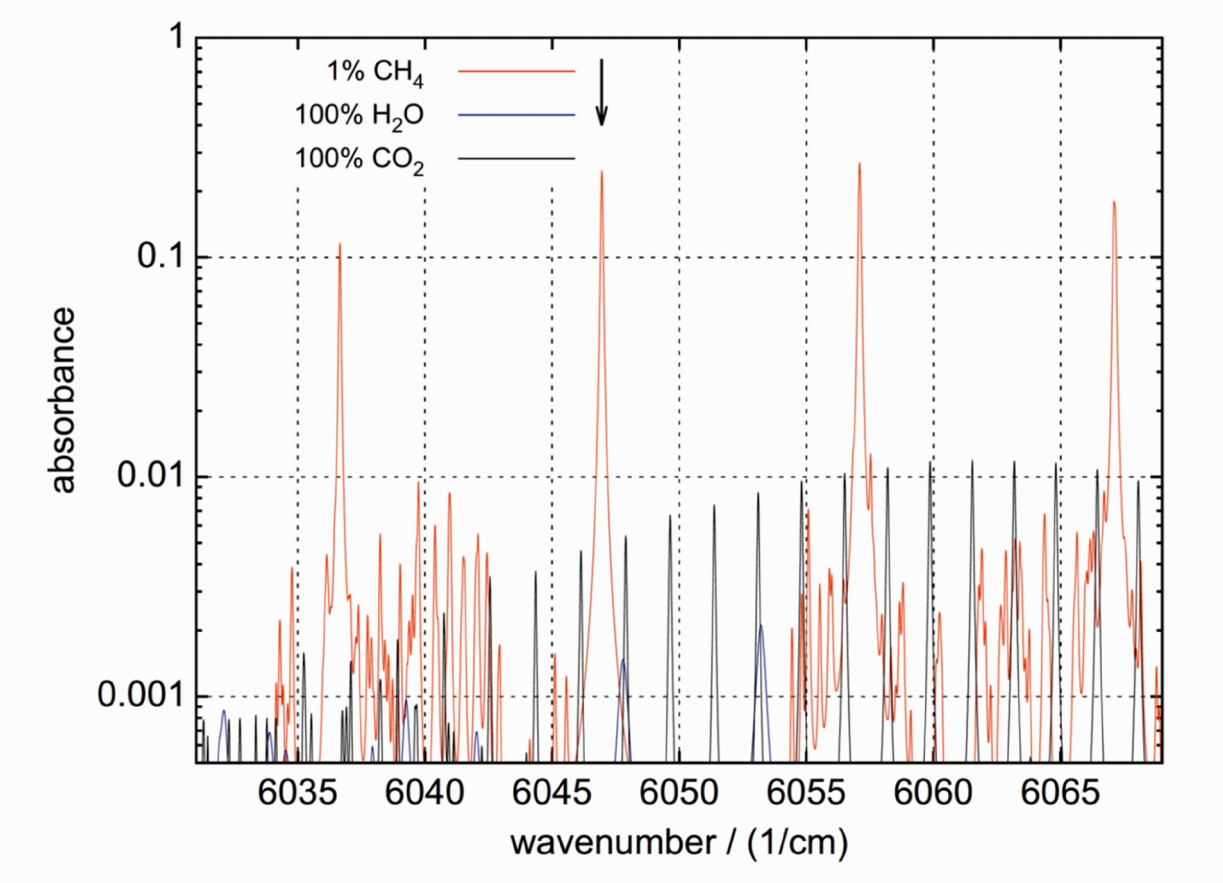

Fig. 1. Comparison of the absorption of 1% CH_4 (red curve), 100% CO_2 (black curve) and 100% H_2O (blue curve) for 1 meter optical path at 50 kPa and 296 K according to the Hitran database. The marked line was chosen to detect methane, because it is strong and avoids cross sensitivities.

The experimental setup is shown schematically in Fig. 2. For a given center wavelength, the laser output wavelength is varied sinusoidal. This modulated light passes the measuring chamber and is analyzed by the lock-in amplifier after detection. The lock-in signal is measured as a function of center wavelength. The received data were recorded every 6 minutes, and the concentration was determined by fitting to the data, as described in more detail below (see chapter 2.2.).

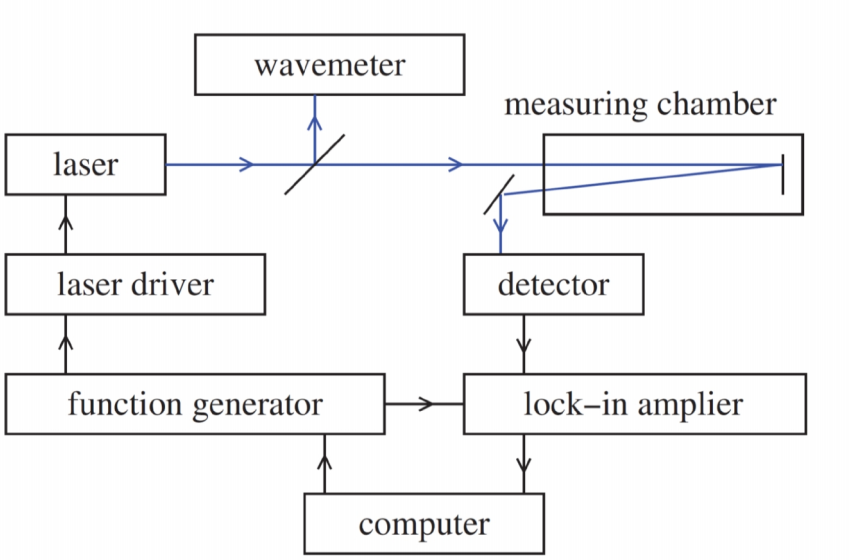

Fig. 2. Schematic diagram of the used wavelength modulation spectroscopic technique: A function generator is used to modulate the laser wavelength via a laser driver, while the actual wavelength is monitored with a wavemeter. The light is, after passing through the measuring chamber, detected with an InGaAs-photodiode. The signal from the detector is then analyzed with lock-in technique. The lock-in signal is finally recorded as a function of the optical center wavelength.

The production of methane takes place in the liquid phase, but the detection is performed in the gas phase. Therefore, the methane must out-gas from the water, which is a slow process. To increase the out-gassing rate, our growth chamber has a high surface to volume ratio (see chapter 2.3.). For an effective measurement a large optical path length is needed. The presented device has an optical path length of 1.7 m with a gas volume of 590 ml and is shown in Fig. 3 a. A better method for

increasing the beam path length to gas volume ratio would be given by an approach presented by Cubillas *et al*. (2009) for measuring methane. However, due to the complexity of this approach it was not investigated here.

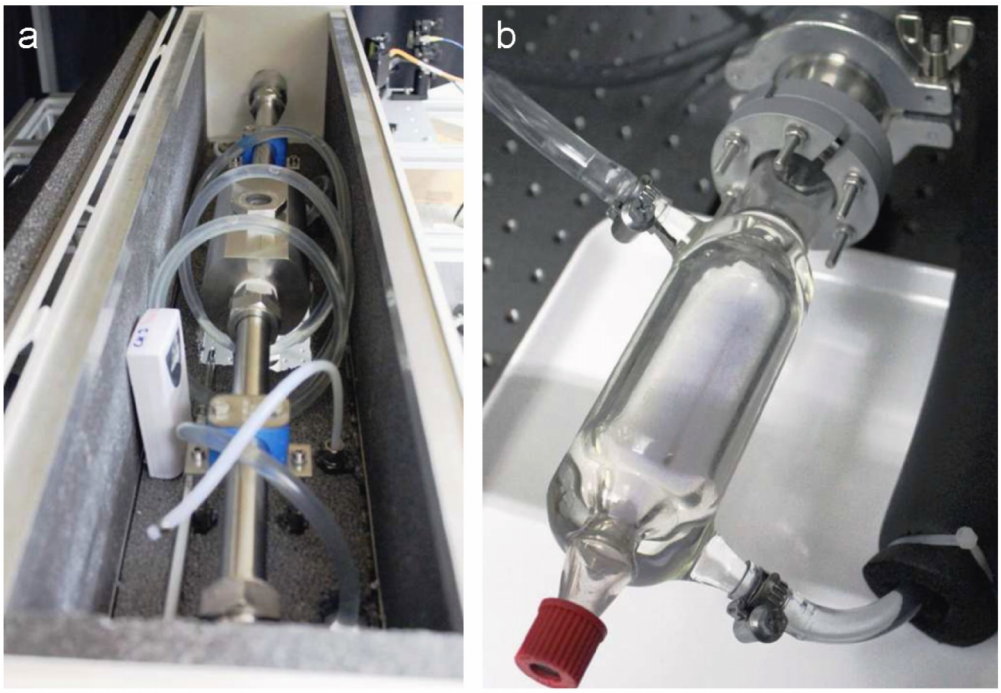

Fig. 3. (a) Photograph of the measuring chamber with the optical path length of 1.7 m. It is placed inside a thermos box and tempered with a water flow. (b) Photograph of the growing chamber. The inner 50 ml cell is connected to the measuring chamber to allow gas and pressure exchange. It is surrounded by an outer oil flow cell, which keeps the temperature constant to the set value.

2.2. Calibration

Although there are methods for quantitative calibration-free and reference-free wavelength modulation spectroscopy (Zakrevskyy *et al*., 2012), we calibrated the whole setup using a reference gas (10,000 ppm methane in N_2).Calibration was performed for different pressures and different dilutions, which is shown in Fig. 4. The received data were fitted assuming a Gaussian shaped spectral line. As a measure for the concentration the area retrieved from the fit is used. Since this kind

of fitting is prone to systematic errors, we used 210 calibration points with known concentration, pressure and temperature of the gas resulting in a nonlinear calibration curve (Fig. 4). For each concentration and pressure three measurements were performed to check reproducibility. A fit to the calibrated data is used to derive a conversion function for the measured data with unknown concentration. Additionally, before and after a measured simulation sequence, we cross checked the validity of the calibration with a single point measurement using the reference gas. This procedure ensures, that even in the case of a residual systematic error in the absolute calibration an increase of the methane concentration is not observed due to a wrong calibration.

To calibrate the wavelength, a wavelength meter WA1100 (Burleigh; Victor, New York, USA) was used achieving a resolution on the order of several pm.

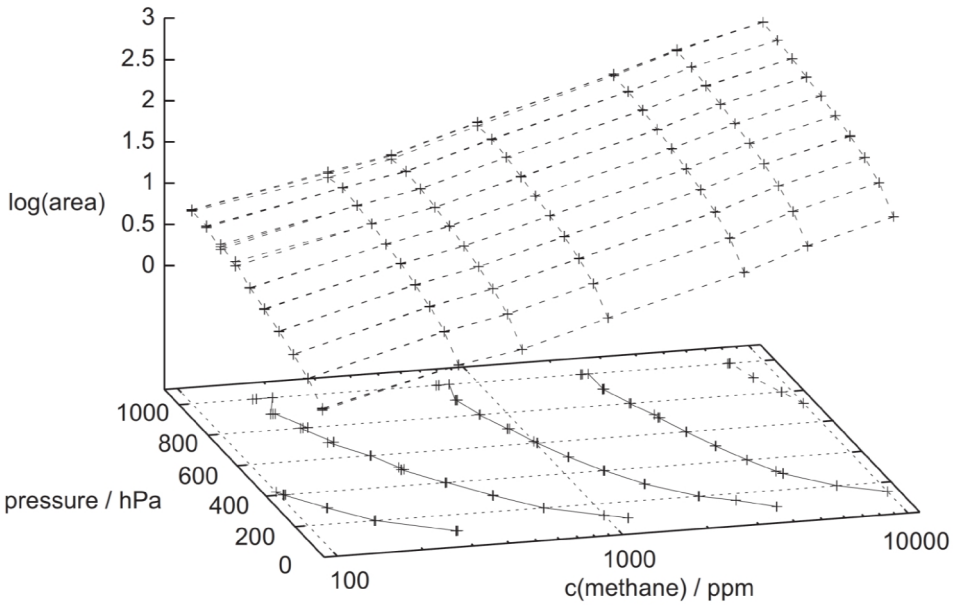

Fig. 4. The measured area (a. u.) of the spectral line is shown for different concentrations of methane and different pressures. This calibration data is used to derive the unknown concentration during a simulation sequence, while measuring the area of the spectral peaks in the same way.

2.3. Two chamber simulation system

Since the methane production should be investigated for different temperatures and pressures, a growing chamber (made of glass), which is surrounded by a controllable temperature flow of oil (Lauda Pro Line RP845; Lauda-Königshofen, Germany) was used, as shown in Fig. 3 b. The pressure was set and recorded with a pressure sensor (Vacuubrand DVR5; Wertheim, Germany). The growing chamber was in gas flow contact with the measuring chamber (Fig. 3 a). The measuring chamber was thermally isolated from the lab conditions and was kept at a constant temperature of 22°C (Lauda Ecoline RE 306; Lauda-Königshofen, Germany) for easy spectroscopic calibration of the methane concentration. The scheme of the two chamber setup is shown in Fig. 5.

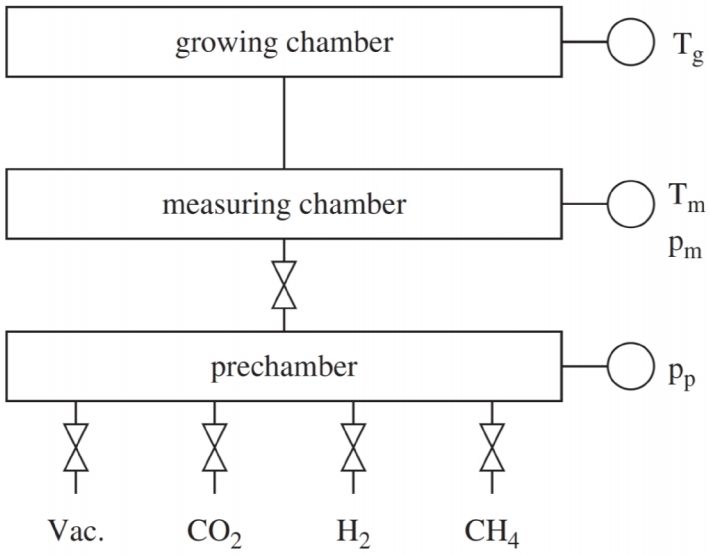

Fig. 5. Schematic diagram of the gas flow between the different simulation system chambers. The temperature T_m of the measuring chamber is held constant at 22°C due to stable measuring conditions. The temperature of the growing chamber T_g is varying over time (as shown in Fig. 5 and 6). The gas mixture (H_2/CO_2, 80:20% v/v) is prepared in the prechamber, p_p and then transferred to the measuring chamber, p_m. The initial pressure of the measuring chamber is adjusted to 50 kPa, the pressure of the growing chamber is according to that. Methane gas (CH_4) was filled in from an external source only during calibration measurements.

2.4. Organism and growth media

The strain *Methanosarcina soligelidi* was isolated from the active layer of permafrost-affected soil on Samoylov Island in the delta of the river Lena in northeast Siberia (72°22′N, 126°28′E; Wagner *et al*., under revision). The cells of *M. soligelidi* were incubated under strict anaerobic conditions in 125 ml serum bottles sealed with a butyl rubber stopper of 12 mm thickness. The medium used for growth had the following composition (l^{-1}): NaCl, 1.0 g; KCl, 0.5 g; MgCl•6 H_2O, 0.4 g; NH_4Cl, 0.25 g; CaCl•2H_2O, 0.1 g; KH_2PO_4, 0.3; $NaHCO_3$, 2.7 g; Na_2S•3H_2O, 0.3 g; cysteine hydrochloride, 0.3 g; trace element solution (Wolin *et al*., 1963), 10 ml; vitamin solution (Bryant *et al*., 1971) 10 ml; and 2 ml resazurin indicator solution. The bottles were flushed with H_2/CO_2 (80:20 v/v, 100 kPa), which served as a substrate and were pressurized with N_2/CO_2 (80:20 v/v, 200 kPa) to ensure anaerobic conditions. The culture was incubated at 28°C in the dark. Growth was monitored by analyzing gas samples with gas chromatography (Agilent GC 6890, with a Flame Ionization Detector and equipped with a Plot Q column, diameter: 530 µm, length: 15 m; Agilent Technologies, Germany).

2.5. Preparation of cells of Methanosarcina soligelidi

After growth in the exponential phase, the cells were harvested through centrifugation (Sigma 6K15-Z3; Sigma-Aldrich, Germany) for 45 minutes with 4200 x g at 4°C, resolved in a fresh sterile medium and transferred to a new serum bottle according to the previous description (see chapter 2.4.). All preparation steps were performed under strict anaerobic conditions. At the start of each experimental run in the simulation chamber, the cell density was adjusted between 1.0 x 10^8 and 5.0 x 10^8 cells ml^{-1}. Cell numbers were determined by Thoma cell counts with a Zeiss Axioscop 2 microscope (Carl Zeiss, Germany).

2.6. Preparing the initial simulation conditions

The removable growing chamber was autoclaved and assembled to the measuring chamber (see chapter 2.3.). The entire system was evacuated for at least 8 h and the gas mixture of H_2/CO_2 (80:20 v/v) was prepared in the pre-chamber before filling the measuring chamber. The pressure of 50 kPa was adjusted after two flushing steps with the gas mixture. 16.5 ml of the prepared methanogenic archaea cell suspension (see chapter 2.5.) was injected through a septum using a syringe under sterile conditions. The temperature was automatically varied as a function of time and at the given pressure of 50 kPa the liquid phase stabilized over the measuring period.

3. Results

Our developed simulation chamber and measuring system (described in chapters 2.1. to 2.3.) was set up and is operating reliably. In both presented experimental simulation sequences (Fig. 6 and 7) an increase of the methane concentration inside the measuring chamber was detected as a function of time and for different temperatures. Within one week of simulation the initial pressure of 50 kPa decreased to approximately 48 kPa. This effect did not occour in tests without organisms, so the biogenic methane formation ($4 \times H_2 + CO_2 \rightarrow CH_4 + 2 \times H_2O$) might explain this observation. After an initial temperature step of 20°C (Fig. 6) and 10°C (Fig. 7) for 24 hours, the temperature of the growing chamber was decreased to 0°C (Fig. 6) and -5°C (Fig. 7), with an intermediate step at 10°C (Fig. 6) and 0°C (Fig. 7), respectively. Each temperature decrease was conducted continuously within 6 hours and the intermediate temperature steps lasted 12 hours. Decreasing temperatures resulted directly in decreasing methane accumulation rates, as it can be seen in the stepwise flattening of the methane concentration curves at each temperature step. However, an increase in the methane concentration over time was still observed down to -5°C. The methanogenic cell suspension inside of the growing chamber remained liquid at a temperature of -5°C. In Table 1, the calculated methane

production rates for each temperature step of both experiments (Fig. 6 and 7) are shown. Although the cell samples for both experiments were treated similarly, a variation in the rates for 10°C and 0°C occurred. Both experiments were performed separately with separately grown microbial cultures. Hence, slightly different initial cell densities, or cells harvested in not absolutely the exact same phase of growth could be an explanation for the observed differences. One should also mention that the measured data show a small variation of the methane concentration with the periodicity of 24 h. From separate measurements we pinned down the origin of this effect in the imperfect complete thermal isolation of the measuring chamber from the environment. A delay between the temperature variation of the growing cell and the methane growth rate is also visible. This effect can be derived from the slow diffusion and outgassing of the methane. All these effects unfortunately limit the methane rate determination for these measurements. Nevertheless, a significant change of the methane accumulation rate for the different temperature regimes was observed.

Table 1. Methane production rates calculated from the increase in the methane concentrations at different temperature intervalls of the two presented experimental simulation sequences shown in Fig. 5 and 6.

Temperature [C°]	**CH_4 production rate [nmol h^{-1} ml^{-1}]**	
	Test 1 (Fig. 5)	Test 2 (Fig. 6)
20.0	17	n.d.
10.0	4	19
0.0	0.4	12
-5.0	n.d.	1

n.d. = not determined in the specific test

4. Discussion

Previous experiments have shown that the archaeal strain *Methanosarcina soligelidi* is able to survive simulated Martian thermo-physical surface conditions (Morozova *et al*., 2007). However, during these experiments it was only possible to measure the methane production and survival rates of the cells before and after the simulation sequence. Our results show, that the constructed simulation system is fully functional and methane production could be observed under the tested conditions.

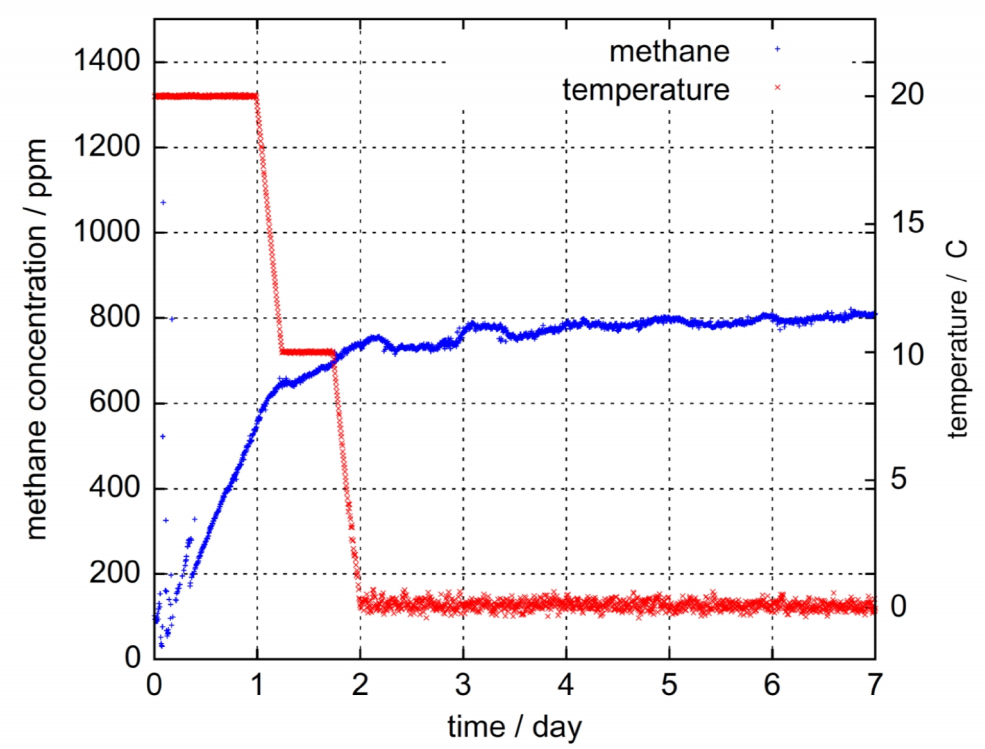

Fig. 6. Methane concentration (blue curve) as a function of time shown stepwise by cooling down from 20°C to 0°C (red curve) within 24 hours with a intermediate step at 10°C for 12 hours. The initial pressure was 50 kPa (H_2/CO_2 80:20% v/v).

The first experiments show a methanogenic activity of *M. soligelidi* at temperatures down to -5°C; lower temperatures were not tested so far. Methane production by a pure culture at temperatures below 0°C was only reported previously from *Methanogenium frigidum* (Franzmann *et al*., 1997). This strain has been isolated from Ace Lake in Antarctica and was used as one of the reference organisms in the previous study done by Morozova *et al*. (2007). No methane production of *M. frigidum* was observed after three weeks exposure to Martian thermo-physical surface conditions shown during the experiment of Morozova *et al*. (2007), whereas the strain *M. soligelidi* showed nearly the same methane production rate after the simulation compared to the rate measured before the experiment. Incubation experiments done by Wagner *et al*. (2007) with Holocene permafrost deposits obtained from Samoylov Island in the Lena Delta, northeast Siberia (the same investigation area *M. soligelidi* was isolated from) have shown methanogenic activity at temperatures down to -6°C. The methane production rates reported in that study, with incubation with hydrogen as a supplemented energy source, resulted in rates of 0.78 ± 0.31 nmol CH_4 $h^{-1}g^{-1}$ for -3°C and 0.14 nmol CH_4 $h^{-1}g^{-1}$ for -6°C, respectively and match the calculated rates for decreasing temperatures presented here (see Table 1). Other tests performed with soil samples obtained from permafrost of the Kolyma lowland from northeast Eurasian Arctic tundra, reported methanogenesis of the native microbial community even at temperatures down to -16.5°C (Rivkina *et al*., 2004). However, the presented observation of methanogenic activity of a pure culture at -5°C was not shown before.

The surface of Mars is an extremely cold place with seasonal and diurnal temperature fluctuations reaching from -138°C to +30°C (Jones *et al*., 2011). The lowest temperature of -5°C tested in this study would only be found on Mars in temporarily variation on the surface, or the first meter below the surface, in mostly equatorial or mid-latitudes. An example for such a periglacial landscape is Utopia Planitia (Ulrich *et al*., 2012). In persistent timeframes -5°C would merely occur in a depth of several kilometers (Jones *et al*., 2011). However, *M. soligelidi* was found to at least survive down to temperatures of -80°C, even repeatedly and reversibly within the three weeks Martian simulation experiment (Morozova *et al*., 2007; Morozova

and Wagner, 2007). Additionally, active microbial metabolism, albeit only close to the maintenance level, was reported from an Antarctic ice-lake even at -13°C (Murray et. al., 2012). If it comes to terms of stable conditions, the low and highly alternating temperature on Mars seems to be a critical factor. Nevertheless, our experimental temperature is consistent with the temperature maxima in diurnal and seasonal fluctuations in the shallow subsurface and also with the assumed stable temperatures in the deep subsurface of Mars.

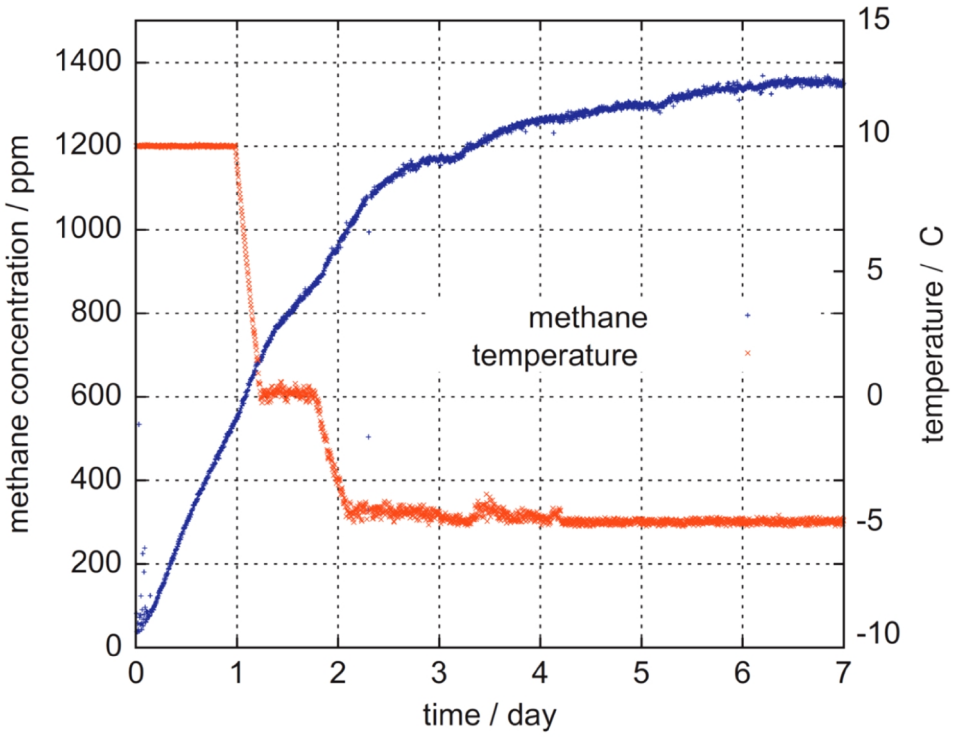

Fig. 7. Methane concentration (blue curve) as a function of time shown stepwise by cooling down from 10°C to -5°C (red curve) within 24 hours with a intermediate step at 0°C for 12 hours. The initial pressure was 50 kPa (H_2/CO_2 80:20% v/v).

The overall atmospheric pressure on Mars is as low as 0.06 kPa. The pressure of 50 kPa used in our experiments equals the hydrostatic pressure which is believed to be found on Mars at depth of approximately 10 to 20 m below the surface, according to Kral *et al*. (2011) and to the PT-diagrams for a potential Martian biosphere

presented by Jones *et al*. (2011). Even lower pressures would not be a problem for methanogenic archaea. In the simulation experiment of Morozova *et al*. (2007) *M. soligelidi* survived the overall Mars like low pressure of 0.06 kPa for three weeks and was almost unaffected. Also, Kral *et al*. (2011) have shown that several archaea strains were able to produce methane at pressures down to 0.5 kPa. Such a pressure is expected in the shallow first few meters of the subsurface of Mars. In deep subsurface environments, the pressure would of course be higher than that in the shallow subsurface, reaching about 10,000 kPa at 1 km depth (Jones *et al*., 2011). On Earth, living methanogens have been isolated from deep subsurface habitats (Kotelnikova *et al*., 1998; Lever *et al*., 2013; Shimizu *et al*., 2011), where the pressure is understandably higher compared to Mars. In addition, the activity of the marine methanogen *Methanococcus jannaschii* has been proved at a pressure of up to 75,000 kPa (Miller *et al*., 1988). Therefore, the pressure at either the shallow or in the deep subsurface of Mars would not be a problem for the existence of methanogenic archaea and thus the pressure chosen for this simulation study is suited over this observed pressure regime.

Liquid water was present during the whole simulation sequence of our experiments and thus was easily accessible for the archaeal cells. However, in general the presence of liquid water is a major limiting factor for life as we know it from Earth which includes methanogens. Although, the present day Mars is known to be a dry planet, water might become available periodically at or below the surface. Changing diurnal and seasonal temperatures in addition to pressure conditions result in liquid water in terms of water vapor, interfacial water or in cryobrines (Möhlmann, 2010a, 2010b; Möhlmann and Thomson, 2011). Furthermore, liquid-like adsorption water in potential subsurface habitats could support life (Möhlmann, 2005). In a previous study, *M. soligelidi* has shown high desiccation tolerance (Morozova and Wagner, 2007). During the three week long Martian thermo-physical simulation experiment done by Morozova *et al*. (2007), the water activity was varying between a_w 0.1 and 0.9 due to diurnal water vapor pressure fluctuations, which had no influence on the viability of *M. soligelidi* tested before and after the exposure. Other methanogenic strains, especially *Methanosarcina barkeri*, have also been

shown to survive long term desiccation for more than 300 days (Kral *et al*., 2011). Hence, even solely periodically available liquid water does not rule out the possible existence of methanogens in the Martian subsurface and the permanent liquid phase during the presented simulation experiments is not necessarily a contradiction to Martian conditions.

As chemolithotrophic microorganisms, methanogenic archaea are able to grow with carbon dioxide and molecular hydrogen as the only carbon and energy sources. Therefore, we used a gas mixture of H_2/CO_2 (80:20% v/v) within the simulation, which serves as a good stoichiometric ratio for hydrogenotrophic methanogenesis ($4\ H_2 + CO_2 \rightarrow CH_4 + 2\ H_2O$). Carbon dioxide is commonly distributed in the Martian atmosphere at about 95%, but hydrogen could be detected only in trace amounts (Oze and Sharma, 2005). On Earth, molecular hydrogen is not available in such high concentrations as it is not on Mars, but it has been reported to be dissolved in groundwater at mM concentrations for subsurface and deep subsurface microbial ecosystems (Sherwood Lollar *et al*., 2007). Not all of these habitats were characterized by hydrogen produced from geothermal activity, whose occurrence on Mars is uncertain (Krasnopolsky, 2006), but it was produced through alteration processes of basaltic crust in stable ancient Precambrian cratons, which serve as good Earth analogues for a single-plate planet like Mars (Sherwood Lollar *et al*., 2007). In the Martian subsurface, direct interaction with liquid interfacial water may also be a source of hydrogen due to the release of protons. Furthermore, it has been shown on Earth that with interspecies hydrogen transfer (e.g. Conrad and Babbel, 1989; Ischii *et al*., 2005), electrons (as hydrogen or formate) which are generated in one microorganism species, can be transferred to another. This might also be a possibility for methanogens in a potential Martian ecosystem to obtain hydrogen as an energy source in a hydrogen limited environment. In addition, methanogens on Earth are also able to substitute hydrogen with carbon monoxide (O'Brien *et al*., 1984), which has been detected in the Martian atmosphere in varying concentrations reaching from 400 to more than 2500 ppm (Sindoni *et al*., 2011). Accordingly, high concentrations of molecular hydrogen as substrate for methanogenesis are consistent with Martian subsurface simulation conditions.

5. Conclusion

We have shown that the developed simulation and measuring system is fully functional and ready to operate with Mars (subsurface) analogue conditions. At temperatures down to -5°C metabolic activity of the pure archaeon strain *Methanosarcina soligelidi* could be detected, which has not been reported for a single methanogenic strain before. This result and the findings of previous reported studies have shown that methanogenic archaea (especially those from permafrost environments) can be regarded as ideal model organisms for possible life on Mars. Although, they are able to survive the harsh thermo-physical conditions found on the Martian surface (Morozova et. al., 2007) and also high doses of UV and ionizing radiation (comparable to *Deinococcus radiodurans*; Wagner, D.; unpublished data) as they would occur on the surface of Mars, the subsurface seems to be a more suitable habitat due to more stable temperature conditions and the higher probability of liquid water. The conditions of potential Martian (deep) subsurface habitats were initially tested in our experiments. Further tests will be needed to check the potential of our model organism *Methanosarcina soligelidi* for methanogenic activity under simulated Martian shallow subsurface conditions. These can be conducted with presence of Mars analogue soil simulates, under colder temperature, lower pressure and humidity, also over diurnal variations which are representative for other possible habitats on Mars.

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6. Synthesis and Conclusion

6.1. Synthesis

The overall scope of this thesis was to achieve a more in-depth understanding of the potential for survival and activity of methanogenic archaea under simulated Mars analog conditions. The driving hypothesis for this study was, that methane detected in the atmosphere of Mars might have a biogenic origin (Formisano *et al*. 2004; Krasnopolsky *et al*., 2004; Krasnopolsky, 2006). Earlier studies carried out in our research group proved a high survival potential of methanogenic archaea isolated from the active layer of permafrost under various stress conditions compared to methanogens from non-permafrost affected environments (Morozova and Wagner, 2007). Some of these experiments were also simulations of the Martian thermo-physical surface conditions in a diurnal profile lasting for three weeks (Morozova *et al*., 2007).

Therefore, the aim of this thesis was to investigate whether methanogenic archaea were not only able to survive but also remain active under Mars analog conditions. One of the model-organisms used in the previous as well as the current research is the permafrost methanogen strain *Methanosarcina soligelidi* SMA-21 (formerly described as *Methanosarcina* spec. SMA-21). Its species description is included in this thesis as Publication I. As methanogenic archaea strains from non-permafrost environments were used *Methanosarcina mazei*, which is phylogenetically closely related to *M. soligelidi*, and *Methanobacterium movilense* MC-20 belonging to a different genus of methanogens. The latter was isolated from the subsurface lake in Movile cave (Mangalia, Romania) and its species description is also included in this thesis as Publication II. The organisms were exposed to different types of Mars regolith analogs (MRA) also in water saturated artificial soil matrixes and in a long-term desiccation experiment in Publication III and their activity in terms of methane production and regrowth capacity was determined. Additionally the activity of *M. soligelidi* was tested directly under simulated Mars analog subsurface thermo-physical conditions in Publication IV. The research presented in this thesis may also provide new insights for future life detection missions on the planet Mars. For example the

ExoMars rover mission of the European Space Agency and Roscosmos, which is planned to launch in 2018 and will probably be drilling in the subsurface of Mars in a depth of up to 2 meters (Baglioni *et al*., 2013).

Considering that physiological features including the potential to withstand and thrive under a variety of stress conditions largely relate to the adaptation to a specific environment, it is important to search for novel methanogenic archaea in diverse habitats. Therefore the descriptions of two strains of methanogenic archaea isolated from two different extreme habitats are included in this thesis.

The first strain *Methanosarcina soligelidi* was isolated from the active layer of permafrost affected soil in north-east Siberia on Samoylov Island. As permafrost features can be observed on Mars as well, terrestrial permafrost is a suitable analog for possible habitats on Mars (Ulrich *et al*., 2012). In comparison to non-permafrost strains it proved high survival potential under various stress conditions such as salinity of 6 M NaCl, starvation, freezing temperature of -78.5 °C and high doses of UV and ionizing radiation (Morozova and Wagner, 2007; Wagner D., unpublished data) as well as a diurnal profile of Martian surface thermo-physical conditions (Morozova *et al*., 2007). In the frame of this thesis it could be shown that it is also able to metabolize in a water saturated MRA matrix and to resist long-term desiccation.

The second archaea strain, *Methanobacterium movilense* was isolated from the anoxic sediment of the subsurface thermal lake of Movile cave (Mangalia, Romania). The Movile cave is a terrestrial cave ecosystem isolated from the surrounding environment and based on chemoautotrophy (Sarbu *et al*., 1996). Therefore it is regarded as an analog for possible extraterrestrial subsurface habitats e.g. on Mars. The strain *M. movilense* has shown the highest methane production rate incubated on the water saturated MRA matrix and also produced the highest methane concentration at reincubation after the long-term desiccation in presence of MRAs.

The three Mars regolith analogs (MRA) used in this study to determine their effect on the activity of the tested methanogenic organisms represent different regolith types known to be present on Mars (Bishop *et al*., 2008; Gooding 1978,

McSween 1994, Morris *et al*. 2010, Poulet *et al*. 2005): JSC Mars-1A (mined palagonitic tephra), P-MRA (a mixture of minerals with mayor part of phyllosilicates) and S-MRA (mixture of minerals with mayor part of sulfate deposits and anhydrous iron oxides). It was shown that MRAs added to the growth media at concentrations up to 1 wt% resulted mostly in an increase of the methane production rates whereas higher concentrations had inhibitory effects and decreased the rates and therefore prolonged the lag phase of the cell growth. The reason for the increase or decrease of activity is not fully understood. Possible explanations for the positive effects could be shielding effects through the regolith particles probably through enhanced biofilm formation (Wagner *et al*., 1999). Or the regolith mixtures complemented important cofactors and trace elements such as nickel, cobalt and zinc for the methanogenic metabolism. The positive effects for P-MRA could also possibly be explained by the capability of the phyllosilicate montmorillonite (a clay mineral), being the mayor part of P-MRA (up to 45 wt%), to bind and retain water and expand once wet. It also enhances the cation exchange capability (CEC) of the mineral mixture, which may be beneficial to the metabolism of the methanogens. At concentrations above the level of 1 wt% the inhibitory effects might have exceeded the beneficial effects and therefore the activity of the methanogenic archaea was decreased. Inhibitory effects might be trough the increase of sulfur components or other toxic minerals. In the study of Kral and Altheide (2013) similar inhibitory effects of different MRAs on methanogenic archaea were observed.

The incubation on the water saturated MRA-matrix revealed that all tested species of methanogenic archaea were able to produce methane when incubated on MRAs, at least in one of the tested conditions. That was not observed in the control samples containing only buffer solution and cells, where no production could be detected. In general, the produced methane concentrations at incubations on MRAs were rather small varying between 4,000 and 12,000 ppm (0.4 and 1.2 %) after 80 days of incubation for both *Methanosarcina* strains, but it was significantly higher than the blank controls containing only analogs and water (maximum concentration of 180 ppm methane). Only *Methanobacterium movilense* reached a final concentration of 200,000 ppm (20 %) methane incubated on P-MRA.

As Mars is a dry planet with water available only sporadically near the surface, all organisms that might potentially exist would have to cope with prolonged times of desiccation. Therefore the long-term desiccation resistance of the methanogenic archaea strains was investigated. They were desiccated for 400 days either in presence of one of the MRA or without. The *mcrA* gene copy numbers determined through quantitative PCR after treatment with propidium monoazide to exclude any source of external DNA (Taskin *et al*., 2011) showed a high surviving potential for all three methanogens tested: the quantified gene copy numbers did not change significantly over the course of the desiccation experiment. Moreover, the reincubation of the desiccated cell samples in fresh growth medium proved that methane production and growth after 400 days of desiccation could be restored. Although the produced concentrations of methane were very little varying mostly between 20 and 200 ppm within the first 80 days of reincubation, one of the tested triplicates for *Methanobacterium movilense* desiccated on P-MRA reached a final concentration of about 200,000 ppm (20 %) methane, which can be regarded as a definite growth of that organism. The in general reduced activity after rehydration in comparison to the high copy numbers determined with qPCR indicate that the cells are viable and thus survived the long-term desiccation, but are possibly in a kind of dormant like state.

To determine whether the strain *Methanosarcina soligelidi* can not only survive (Morozova *et al*., 2007), but also maintain active metabolism under simulated Mars analog thermo-physical conditions, a simulation system was designed which made it possible to measure the increase of methane directly during the course of the simulation. Because of its advantage to perform the measurements without the need to collect a gas sample, wavelength modulation spectroscopy was used as the detection method (Demtröder, 2007). It offered also high time resolution of the measurements with intervals of less than 5 minutes. In the newly developed simulation chamber the gas mixture, atmospheric pressure and temperature could be adjusted according to the specific requirements. In a first set of experiments with the simulation of Mars analog subsurface conditions - meaning -5 °C and 50 kPa as expected to be found on Mars in a depth of 20 m and below (Jones *et al*., 2011; Kral *et al*., 2011) – could be shown that *M. soligelidi* was able to produce methane even at subzero

temperature. Methanogenic activity at subzero temperatures was known for incubations of permafrost soil samples (Rivkina *et al*., 2004; Wagner *et al*., 2007), but until now the methanogen strain *Methanogenium frigidum* was the only single archaeon known to be active at temperatures below 0 °C (Franzmann *et al*., 1997).

As shown in this thesis the survival of long-term desiccation up to 400 days is possible for methanogenic archaea, although the activity after rehydration is decreased in form of a prolonged lag phase. A possible reason might be a dormant like cell state of the organisms due to the desiccation stress. In a desiccated state methanogenic archaea are also able to withstand several other stress factors better compared to a not-desiccated state (Anderson *et al*., 2012). The here observed effect of the prolonged lag phase of 80 days is in line with the results presented by Kral and Altheide (2013), who performed comparable desiccation tests with different methanogen strains (*Methanobacterium formicium, Methanobacterium wolfeii, Methanosarcina barkeri* and *Methanococcus maripaludis)* in presence of regolith analogs and under low pressure of 0.6 kPa for up to 120 days. After rehydration they incubated the methanogens up to 21 weeks to reach the highest final methane concentration of around 25 %. Moreover, *M. formicium* and *M. wolfeii* did show methane production after desiccation only when desiccated without regolith analogs, whereas *M. barkeri* did also produce methane when desiccated on regolith analog JSC Mars-1 as well as montmorillonite, jarosite and basalt. The strain *M. maripaludis* did not survive the desiccation in any of the tested conditions.

Besides the prerequisite of (temporarily) available liquid water, the necessary energy and carbon sources for methanogenic archaea can be provided in form of carbon dioxide and hydrogen, as they are chemoautotrophic organisms. Carbon dioxide is the major gas component in the Martian atmosphere but hydrogen is only available in trace amounts comparable to conditions on Earth (Oze and Sharma, 2005). A possibility is that hydrogen could originate from alteration processes in basaltic crusts similar to Earth (Sherwood Lollar *et al*., 2007) or through geothermal sources. Additionally methanogenic archaea on Earth are also capable to substitute missing hydrogen through carbon monoxide

as their source of energy (O'Brien *et al*., 1984) which has been detected in the Martian atmosphere (Sindoni *et al*., 2011). The subsurface of Mars might be the more likely potential habitat for methanogenic archaea compared to the surface, because of more stable thermo-physical conditions and the higher availability of liquid water (Jones *et al*., 2011).

The findings of this thesis prove the capability of methanogenic archaea to remain active and grow on Martian regolith analogs if water is available. Presence of additional nutrients is beneficial to the methane production but not necessary. It is possible for methanogens to sustain active metabolism under simulated Mars subsurface analog thermo-physical conditions, as it was shown for *M. soligelidi*. Survival of long-term desiccation, like possible organisms might be exposed to in a hypothetic Martian habitat, could be proven and the methanogenic archaea are able to regain full activity after rehydration, like it was the case for *M. movilense*. The phyllosilicate rich regolith P-MRA seems to be the best regolith analog for the here tested methanogen species to be active in Mars analog conditions. It was the only regolith all three strains could produce methane when incubated with water and MRA only. And it was *M. movilense* desiccated on P-MRA that produced 200,000 ppm (20 %) methane at reincubation after the 400 days of long-term desiccation. Although previous research proved significant higher survival rates of *M. soligelidi* compared to *M. movilense* under Mars analog conditions (Morozova *et al*., 2007), the results of this thesis show that the permafrost-strain *M. soligelidi* did not have the highest activity of the investigated strains under the here tested conditions, besides the low temperature of -5 °C. The strain from Movile cave *M. movilense* proved higher activity values incubated on water-saturated MRAs and after long-term desiccation. Therefore it might be that *M. movilense* is better adapted to the here tested conditions compared to *M. soligelidi* and thus showed the highest activity.

6.2. Conclusion and future perspectives

This thesis provided significant insights to the understanding of the potential survival and activity of methanogenic archaea under simulated Mars conditions.

The main conclusions are:

- Methanogenic archaea are able to produce methane when incubated in an artificial soil matrix of water saturated Mars regolith analogs. They can be active even if no additional nutrients are provided and solely using hydrogen and carbon dioxide as energy and carbon source. A buffer solution alone is not sufficient for methane production.
- Methanogenic archaea can survive long-term desiccation of 400 days in presence of Mars regolith analogs. They can produce methane and grow again when rehydrated after the desiccation period, although the lag phase is significantly prolonged.
- The methanogenic archaeon *Methanosarcina soligelidi* retains metabolic activity under simulated Mars subsurface analog thermo-physical conditions and methane production was measured at a temperature of down to -5°C and a pressure of 50 kPa.
- A phyllosilicate rich soil seems to provide the best support for methanogenic archaea to be active under Mars analog conditions – at least for the here tested three organisms.
- The unique physiological adaptions to their specific environment make it important to search and isolate methanogenic archaea from diverse extreme habitats. They have the ability to survive and be active under conditions a potential Martian habitat would most likely have as it could be shown for *Methanosarcina soligelidi* and *Methanobacterium movilense*.

The results of this study indicate that methanogenic archaea – especially isolated from extreme environments – represent well model organisms for potential life on Mars. They can survive and even have an active metabolism under conditions that simulate the environment of the Martian subsurface. It was shown that long-term desiccation, Mars regolith analogs and subzero

temperatures are stress conditions that methanogens can tolerate and be metabolically active when exposed to.

Further studies on methanogenic archaea under Mars analog conditions are continuing, mainly to assess the potential for active metabolism of methanogenic archaea under combined stress factors such as Mars regolith analogs and subzero temperatures and also low water availability. These studies are expected to show the capability of methanogens or physiologically similar organisms to exist in potential habitats on Mars.

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Selbständigkeitserklärung

Ich versichere an Eides statt, dass die bei der Universität Potsdam eingereichte vorliegende Dissertation selbständig von mir und nur unter Verwendung der angegebenen Hilfsmittel erstellt wurde.

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Janosch Schirmack

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