# METHANOGENS FROM SIBERIAN PERMAFROST
# AS MODELS FOR LIFE ON MARS

## Response to simulated martian conditions and biosignature characterization

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# Methanogens from Siberian permafrost as models for life on Mars

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*"Science is much more than a body of knowledge. It is a way of thinking. A way of skeptically interrogating the universe with a fine understanding of human fallibility."*

*CARL SAGAN (1934-1996)*

**PREFACE**

This project focuses on the response of methanogenic archaea from Siberian permafrost-affected soils to simulated Mars conditions and on the characterization of their biosignatures by means of confocal Raman microspectroscopy.

Fieldwork was not necessary for the completion of this study since the samples were collected during the expedition Lena Delta 2002 (Samoylov Island, Siberia, Russia). Laboratory work was performed at the Alfred Wegener Institute for Polar and Marine Research, Research Unit Potsdam and the GFZ German Research Centre for Geosciences, Potsdam. The Raman measurements were mainly carried out at the Robert Koch Institute, Berlin. Initial Raman measurements and the preparation of regolith pellets for the BIOMEX experiment were performed in the German Aerospace Agency DLR, Berlin.

The following dissertation is written in English and presented as a cumulative Ph.D. thesis to the University of Potsdam (Faculty of Mathematics and Natural Sciences). It is divided in three core sections. Section I consists of an introductory review about the particular research field including the scientific background, the description of the study area and the strains used, the objectives of the study and the brief overview of the manuscripts. Section II represents the main body of the thesis and is composed of three manuscripts with first authorship. Finally, section III presents the synthesis of this investigation including a review of the most important findings, a general conclusion, methodological remarks and future perspectives.

**TABLE OF CONTENTS**

## LIST OF ABREVIATIONS

| | |
|---|---|
| a.u. | arbitrary units |
| BIOMEX | Biology and Mars Experiment (ESA space exposure experiment) |
| $CH_4$ | methane |
| CO | carbon monoxide |
| $CO_2$ | carbon dioxide |
| CRM | confocal Raman microspectroscopy |
| DLR | Deutsches Zentrum für Luft- und Raumfahrt (German Aerospace Agency) |
| DNA | deoxyribonucleic acid |
| DSM | Deutsche Sammlung von Mikroorganismen (German collection of microorganisms) |
| ESA | European Space Agency |
| ExoMars | Exobiology on Mars mission (by ESA in collaboration with the Russian Federal Space Agency) |
| ISS | International Space Station |
| JSC Mars-1 | martian regolith simulant |
| MCR | methyl coenzyme-M reductase |
| mcrA | gene encoding methyl coenzyme-M reductase |
| NASA | National Aeronautics and Space Administration |
| $NaClO_4$ | sodium percholate |
| $MgCl_2O_8$ | magnesium perchlorate |
| PCR | polymerase chain reaction |
| P-MRS | phosphatic Mars regolith simulant |
| qPCR | quatitative polymerase chain reaction (also known as real-time PCR) |
| RLS | Raman laser spectrometer |
| RNA | ribonucleic acid |
| rRNA | ribosomic RNA |
| S/V | surface-to-volume ratio |
| SMA | Siberian methanogenic archaea |
| S-MRS | sulphatic Mars regolith simulant |
| T | temperature |

**SUMMARY**

Mars is one of the best candidates among planetary bodies for supporting life. The presence of water in the form of ice and atmospheric vapour together with the availability of biogenic elements and energy are indicators of the possibility of hosting life as we know it. The occurrence of permanently frozen ground – permafrost, is a common phenomenon on Mars and it shows multiple morphological analogies with terrestrial permafrost. Despite the extreme inhospitable conditions, highly diverse microbial communities inhabit terrestrial permafrost in large numbers. Among these are methanogenic archaea, which are anaerobic chemotrophic microorganisms that meet many of the metabolic and physiological requirements for survival on the martian subsurface. Moreover, methanogens from Siberian permafrost are extremely resistant against different types of physiological stresses as well as simulated martian thermo-physical and subsurface conditions, making them promising model organisms for potential life on Mars.

The main aims of this investigation are to assess the survival of methanogenic archaea under Mars conditions, focusing on methanogens from Siberian permafrost, and to characterize their biosignatures by means of Raman spectroscopy, a powerful technology for microbial identification that will be used in the ExoMars mission. For this purpose, methanogens from Siberian permafrost and non-permafrost habitats were subjected to simulated martian desiccation by exposure to an ultra-low subfreezing temperature (-80ºC) and to Mars regolith (S-MRS and P-MRS) and atmospheric analogues. They were also exposed to different concentrations of perchlorate, a strong oxidant found in martian soils. Moreover, the biosignatures of methanogens were characterized at the single-cell level using confocal Raman microspectroscopy (CRM).

The results showed survival and methane production in all methanogenic strains under simulated martian desiccation. After exposure to subfreezing temperatures, Siberian permafrost strains had a faster metabolic recovery, whereas the membranes of non-permafrost methanogens remained intact to a greater extent. The strain *Methanosarcina soligelidi* SMA-21 from Siberian permafrost showed significantly higher methane production rates than all other strains after the exposure to martian soil and atmospheric analogues, and all strains survived the presence of perchlorate at the concentration on Mars. Furthermore, CRM analyses revealed remarkable differences in the overall chemical composition of permafrost and non-permafrost strains of methanogens, regardless of their phylogenetic relationship. The convergence of the chemical composition in non-sister permafrost strains may be the consequence of adaptations to the environment, and could explain their greater resistance compared to the non-permafrost strains. As part of this study, Raman spectroscopy was evaluated as an analytical technique for remote detection of methanogens embedded in a mineral matrix.

This thesis contributes to the understanding of the survival limits of methanogenic archaea under simulated martian conditions to further assess the hypothetical existence of life similar to methanogens on the martian subsurface. In addition, the overall chemical composition of methanogens was characterized for the first time by means of confocal Raman microspectroscopy, with potential implications for astrobiological research.

## ZUSAMMENFASSUNG

Der Mars ist unter allen Planeten derjenige, der aufgrund verschiedener Faktoren am wahrscheinlichsten Leben ermöglichen kann. Das Vorhandensein von Wasser in Form von Eis und atmosphärischem Dampf zusammen mit der Verfügbarkeit biogener Elemente sowie Energie sind Indikatoren für die Möglichkeit, Leben, wie wir es kennen, zu beherbergen. Das Auftreten von dauerhaft gefrorenen Böden, oder auch Permafrost, ist ein verbreitetes Phänomen auf dem Mars. Dabei zeigen sich vielfältige morphologische Analogien zum terrestrischen Permafrost. Permafrostgebiete auf der Erde, welche trotz extremer, Bedingungen durch eine große Zahl und Vielfalt mikrobieller Gemeinschaften besiedelt sind, sind hinsichtlich möglicher Habitate auf dem Mars die vielversprechendste Analogie. Die meisten methanogenen Archaeen sind anaerobe, chemolithotrophe Mikroorganismen, die auf der Marsoberfläche viele der metabolischen und physiologischen Erfordernisse zum Überleben vorfinden. Methanogene Archaeen aus dem sibirischen Permafrost sind zudem extrem resistent gegenüber unterschiedlichen Formen von physiologischem Stress sowie simulierten thermo-physikalischen Marsbedingungen.

Die Hauptziele dieser Untersuchung bestehen darin, das Überleben der methanogenen Archaeen unter Marsbedingungen zu beurteilen, wobei der Fokus auf methanogenen Archaeen aus dem sibirischen Permafrost liegt, sowie deren Biosignaturen mit Hilfe der Raman-Spektroskopie zu charakterisieren, einer starken Technologie zur mikrobiellen Identifikation, welche bei der ExoMars-Mission zum Einsatz kommen wird. Zu diesem Zweck wurden methanogene Archaeen aus dem sibirischen Permafrost sowie aus Nicht-Permafrost-Habitaten in Simulationen Marsbedingungen ausgesetzt, wie Austrocknung durch Langzeitversuche bei ultraniedrigen Temperaturen unter dem Gefrierpunkt (-80ºC), Mars-analogen Mineralien (S-MRS und P-MRS) sowie einer Marsatmosphäre. Weiterhin wurden die Kulturen verschiedenen Konzentrationen von Magnesiumperchlorat, einem starken Oxidant, der im Marsboden nachgewiesen wurde, ausgesetzt. Ferner wurden die Biosignaturen einzelner Zellen der methanogenen Archaeen mit Hilfe der konfokalen Raman-Mikrospektroskopie (CRM) charakterisiert.

Die Ergebnisse zeigten für alle untersuchten methanogenen Stämme Überleben und Methanbildung, nachdem diese simulierten Austrocknungsbedingungen ausgesetzt worden waren. Nach Versuchen mit Temperaturen unter dem Gefrierpunkt zeigten die Stämme aus dem sibirischen Permafrost eine schnellere Wiederaufnahme der Stoffwechseltätigkeit, wohingegen bei den Referenzorganismen aus Nicht-Permafrost-Habitaten die Zell-membranen im größeren Ausmaß intakt blieben. Der Stamm *Methanosarcina soligelidi* SMA-21 aus dem sibirischen Permafrost zeigte nach dem Belastungstest mit Marsboden und Mars-analoger Atmosphäre signifikant höhere Methanbildungsraten. Zudem überlebten alle untersuchten Stämme die Zugabe von Magnesiumperchlorat in der entsprechenden Konzentration, die auf dem Mars vorkommt. Weiterhin konnten durch die Raman-Spektroskopie beachtliche Unterschiede in der chemischen Zusammensetzung zwischen methanogenen Archaeen aus Permafrost- und Nicht-Permafrost-Habitaten, trotz ihrer phylogenetischen Verwandtschaft, ermittelt werden. Die Konvergenz der chemischen Zusammensetzung der Permafrost-Stämme könnte das Resultat ihrer Anpassung an die Umgebung sein, was auch die Unterschiede hinsichtlich ihrer Resistenz verglichen mit Nicht-

Permafrost-Stämmen erklären könnte. Als Teil dieser Studie wurde die Raman-Spektroskopie als Analyse-Technik zur Ferndetektion von methanogenen Archaeen, welche in eine Mineral-Matrix eingebettet sind, evaluiert.

Diese Dissertation trägt zu einem besseren Verständnis hinsichtlich der Grenzen für ein Überleben von methanogenen Archaeen unter simulierten Marsbedingungen bei und damit zu einer Beurteilung der Hypothese, ob es ähnliches Leben unter der Marsoberfläche geben könnte. Darüber hinaus wurde erstmalig die chemische Zusammensetzung von methanogenen Archaeen mit Hilfe der Raman-Mikrospektroskopie charakterisiert. Dieser Technologie kommt eine wesentliche Bedeutung für weitere Forschungstätigkeit in der Astrobiologie zu.

## I. INTRODUCTION

### 1.1 Habitability of Mars

Mars has been the centre of attention within the field of Astrobiology since the 1960's. Constant efforts made by space agencies to explore this planet and the possibility to host life have outnumbered the missions to any other celestial object, making Mars the best studied planetary body besides Earth. The habitability of Mars has been and still is one the most debated topics associated to the Red Planet. Habitability is the potential of an environment (past or present) to support the origin of life or its continued existence (Jakosky et al., 2007; Westall et al., 2013). The NASA Astrobiology Roadmap included among the absolute requirements for life the presence, persistence, and chemical activity of liquid water, conditions essential for the assembly of complex organic molecules and the presence of thermodynamic disequilibria as energy sources to sustain metabolism (NASA, 2007). Atmospheric conditions on Mars today do not favour the presence of stable liquid water on the surface (Kminek et al., 2010) because the global average temperature is 220 K (-53°C), varying from 145 K (-128°C) during polar night to 300 K (27°C) at the equator during midday at the closest point in orbit to Sun (NASA, 2004). There is evidence that water exists at the surface today in the form of ice at the polar caps and vapour in the atmosphere (Jakosky and Phillips, 2001). In addition, the Mars Odyssey orbiter reported an increasing gradient of water equivalent hydrogen (interpreted as ground ice) towards the martian poles (Fig. 1) (Boynton et al., 2002; Feldman et al., 2004). Despite temperatures regularly rise up to the melting point, the low vapour pressure of water in the atmosphere would cause the ice to sublimate rather than melt, and water appears either solid or gaseous. However, liquid water has existed on the martian surface and subsurface at various times in its history and there are multiple indicators in the geomorphology (such as networks of valleys and eroded ancient impact craters, Fig. 2) together with geochemical and mineralogical proofs (like the presence of carbonates and hydrated minerals found on martian meteorites, Jakosky, et al. 2007; Grotzinger 2014). In fact, new research has proven the existence of liquid water on Mars as recently as 200,000 years ago in a mid-latitude crater based on geomorphological attributes (Johnsson et al., 2014). Early Mars was warmer and wetter than it is today as a result of greenhouse warming by atmospheric gases. Carbon dioxide is thought to have existed in an amount that, if present in the atmosphere, could have caused substantial warming. Another plausible greenhouse gas is methane ($CH_4$), whose source could potentially have been early life or geological processes such as serpentinization (Krasnopolsky et al., 2004; Oze and Sharma, 2005). The presence of methane on Mars has been long debated.

It was first detected in some regions of the planet by the Mars Express orbiter and the Fourier-Transform Spectrometer at the Canada-France Hawaii telescope (Formisano et al., 2004; Lefèvre and Forget, 2009; Mumma et al., 2009). Shortly afterwards, Zahnle et al. (2011) defended the lack of compelling evidence of the measurements and recent data from the Tunable Laser Spectrometer on Curiosity rover reported no detection of atmospheric methane (Webster et al., 2013). A recent contribution to this discussion proved that wind-mediated erosion processes of ordinary quartz crystals can sequester methane to produce activated quartz grains, which would explain the fast destruction of methane observed on

Mars (Jensen et al., 2014). In addition to the contribution of greenhouse gases to the increase of the martian temperatures, changes in tilt also cause alterations in the summertime heating of the polar water-ice deposits. Therefore, ice temperatures might rise enough to allow thin, unfrozen water films to form, even at sub-freezing temperatures. Temperatures might rise even up to the melting point (Jakosky et al., 2003) and heating of ice deposited at lower latitudes might melt rather than sublimate, thus releasing short-term localized liquid water (Christensen et al., 2003)
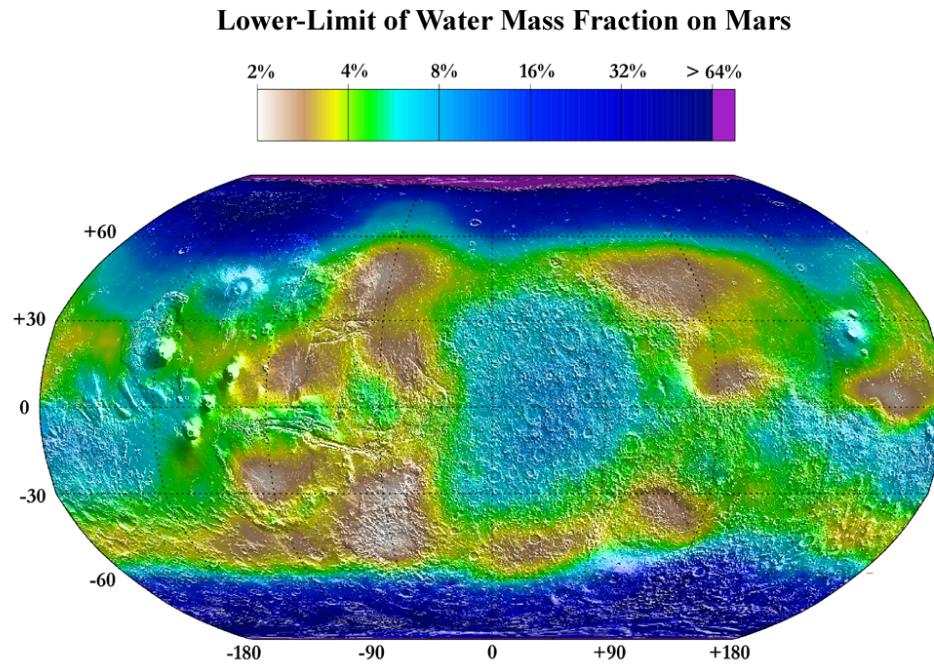


**Figure 1.** Water mass map from neutron spectrometer. This map shows the estimated lower limit of the water content of the upper meter of Martian soil. The estimates are derived from the hydrogen abundance measured by the neutron spectrometer component of the gamma ray spectrometer suite on NASA's Mars Odyssey spacecraft. Image credit: NASA/JPL/Los Alamos National Laboratory.

Regarding the biogenic elements and the energy to support life, the essential elements necessary for life (C, H, O, N) are present in the atmosphere in the form of $H_2O$, $CO_2$ and $N_2$. Although the abundance of $N_2$ is low (2.5%), evidence based on the enhanced ratio of $^{15}N/^{14}N$ suggests that this element must have been abundant in the past (McElroy et al., 1976). Its presence is possible in the form of nitrates or nitrogen bearing minerals within the martian crust (Jakosky et al., 2007). Other elements important for terrestrial life, such as S, P, Fe, Ca, etc. have been detected with in situ observations from landed spacecraft as well as analysis of martian meteorites (McSween, 1994). The energy available includes the combination of energy from volcanism that might have driven hydrothermal systems, and chemical weathering reactions. It has been estimated that the availability of energy to support the origin and maintenance of life on Mars over its entire history is comparable to the one available on early Earth in its first 50-100 Myr (Jakosky et al., 2007).

The damaging effect of ultraviolet and ionising radiation on cellular structures is one of the main limiting factors on the survival of life in the martian surface. Ultraviolet radiation can be screened by the top layer of regolith of the martian soils and it is therefore rapidly attenuated in the subsurface (Moores et al., 2007). However, Mars lacks a magnetic field and

thus penetrating ionizing radiations are at a much higher dose than on the Earth (Kminek and Bada, 2006; Pavlov et al., 2012). The habitability of impact craters, deep and near subsurface of Mars has been thoroughly discussed by Cockell (2014). He has also argued the possibility that even if the martian subsurface meets the requirements for habitability, it might be devoid of life, appearing as an uninhabited habitable environment (Cockell et al., 2012).
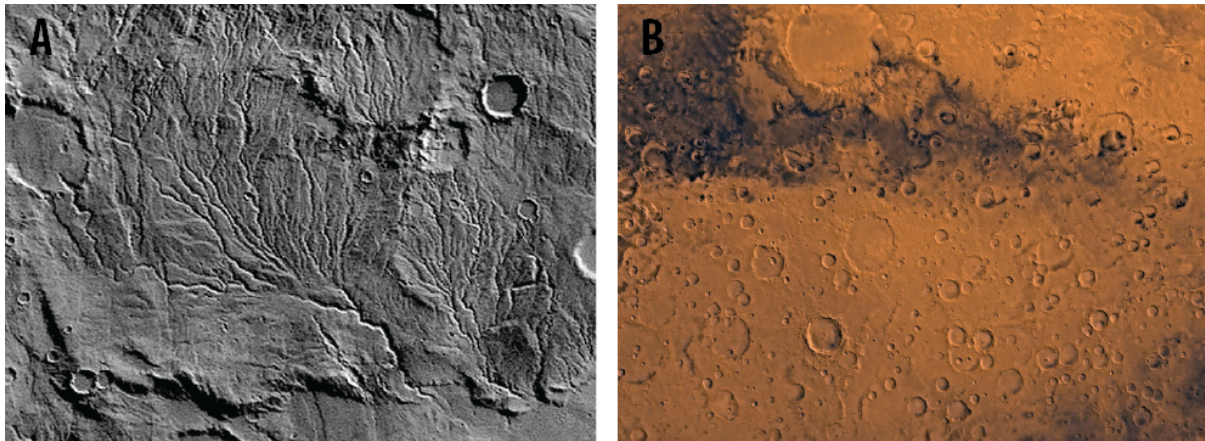


**Figure 2.** Viking Orbiter images showing some of the geomorphological indicators for the evidence of liquid water on Mars. A) Finely dissected valley network within Martian ancient highlands terrain near 42ºS, 92ºW B) Heavily cratered surface on the martian highlands in the Sinus Sabeus region of Mars (note the heavy degradation). Image credits to NASA/USGS.

## 1.2 Terrestrial permafrost regions as analogues of habitable martian zones

Permafrost is the region of the cryosphere defined as permanently frozen ground and it represents a common phenomenon on Mars. It appears in different forms, primarily in polygonal frost-cracking forms that are ubiquitous in high latitudes and cover geomorphological features of a varied origin (Kuzmin, 2005). Periglacial landforms across Mars have been the focus of numerous investigations because of the morphological analogies to terrestrial permafrost (Fig. 3) such as polygonal patterned ground, scalloped depressions and pingo-like features (Burr et al., 2009; Ulrich et al., 2011; Ulrich et al., 2012). In both martian hemispheres, pole-ward from 40º stable ground ice can be found in the subsurface. Above the ground ice, a thin dry layer of regolith (loose, heterogeneous material) of variable thickness (from a few centimetres to one meter) protects the ice by reducing its sublimation (Fig. 4) and attenuating the temperature fluctuations (Mitrofanov, 2005; Schorghofer and Aharonson, 2006). The Mars Express orbiter indicated that the frozen sediments in the polar caps extend to depths of at least 1.8 km in the north and 3.7 km in the south (Picardi et al., 2005).
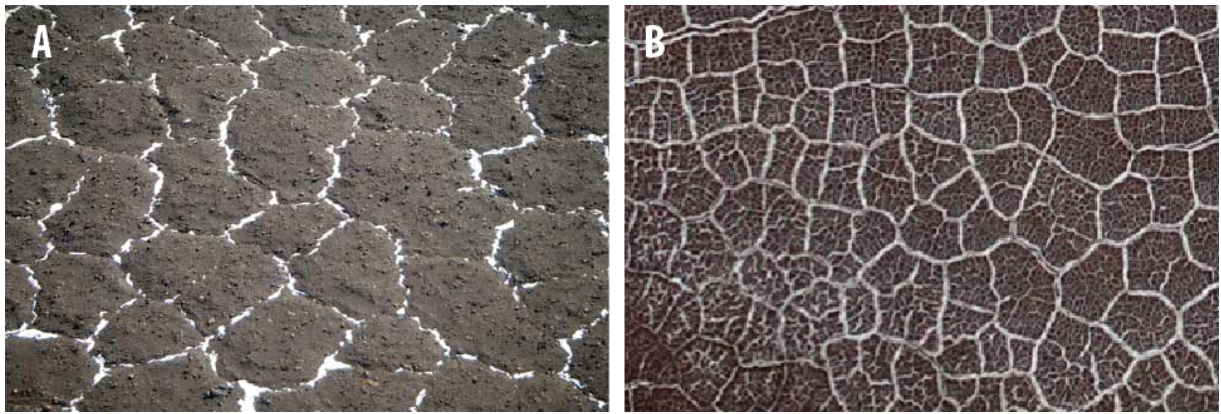
**Figure 3:** Geomorphological similarities between permafrost landscapes on Earth (A) and Mars (B). A) Sublimation-type polygons in a permafrost landscape in the Antarctic Dry Valleys. Source: Marchant and Head (2007) B) Polygons in the Northern Hemisphere of Mars. Image credit: NASA/JPL/University of Arizona.

On Earth permafrost predominantly occurs in the Northern Hemisphere and covers more than 25% of the land surface (Zhang et al., 1999) and significant parts of the coastal sea shelves (Romanovskii et al., 2005). It is considered the oldest most inhabited part of the cryosphere and it reaches depths of several thousand meters. A wide variety of microorganisms have colonised it in high numbers (up to dozen millions of cells per gram of soil), living adsorbed on organic or mineral particles (Demidov and Gilichinski, 2009). They have been isolated from "extreme" (for us humans) habitats from extremely low temperatures in the Arctic and the Antarctic (-17ºC on the northernmost latitude: 80º in Canada (Steven et al., 2007) and -27ºC in the southernmost latitude: 78º in Dry Valleys (Gilichinsky et al., 2007)), down to 400m depth in the Mackenzie Delta (Gilichinsky, 2002) and up to 4700m elevation in the Tibetan Plateau (Zhang et al., 2007). In addition, cell replication and metabolic activity of permafrost bacteria has been reported to occur at extremely low temperatures, between -6ºC and -20ºC (Carpenter et al., 2000; Rivkina et al., 2000; Jakosky et al., 2003; Junge et al., 2004; Wagner et al., 2007) and down to -28ºC and -35ºC (Rivkina et al., 2005; Panikov and Sizova, 2007). Moreover, microorganisms inhabiting permafrost are one of the few living organisms that can be preserved over a geologically significant time and viable microorganisms have been isolated from sediments dating back from a few thousand to 2-3Myr in the Arctic to 5-8 Myr in Antarctica (Gilichinsky et al., 2007). These findings indicate that the subzero temperatures of the cryosphere are not a limiting but rather a stabilizing factor, allowing a substantial decrease of the biochemical and metabolic activities of the cells that therefore survive significantly longer than in other habitats (Gilichinsky et al., 1995). Nevertheless, permafrost on Earth and on Mars differ in age, being of a few million years on Earth (Sher, 1974) and a few billion years on Mars (Tokano, 2005). The disparity in timescales may have an impact on the possibility of preserving life on Mars, since the number and biodiversity of microorganisms is inversely proportional to permafrost age. Terrestrial permafrost therefore should be studied as an approximate analogue to Mars. The preservation of viable microorganisms in permafrost soils is to a great extent explained by the occurrence of unfrozen water films within cryopegs, ground in which freezing is prevented by freezing-point depression due to the dissolved-solids content of the pore water (Gilichinsky et al., 2005). These films allow the protection of viable cells from mechanical destruction by ice crystal formation and allow the diffusion of nutrients and metabolic by-

products. For instance, cryopegs found in Siberian permafrost show a high salt content (170-300 g L$^{-1}$) and remain liquid at the in situ temperature of -10ºC. The uptake of [$^{14}$C] Glucose by the biomass in these unfrozen water films takes place down to -15ºC, indicating an active microbial metabolism (Gilichinsky et al., 2003).
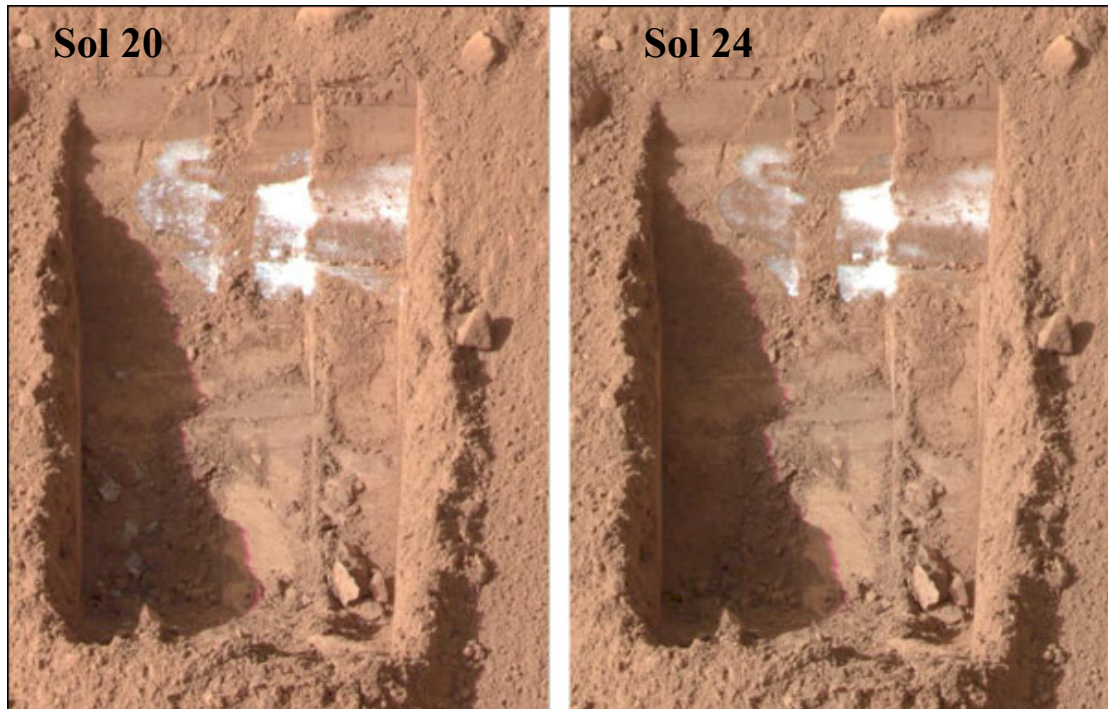


**Figure 4:** Images from the Phoenix Mars Lander showing small clumps of material found in a trench dug by the craft have disappeared over four martian days, suggesting they have vaporized between Sols 20 and 24 (June 15 and 18, 2008. Image credit: NASA/JPL-Caltech/University of Arizona/Texas A&M University.

Microorganisms from cryopegs are not only cold tolerant but also salt tolerant, indicating that the two parameters may be associated. Experimental studies show that in the presence of 25% NaCl halophiles present a higher survival at low temperatures (-20ºC to -80ºC) than non-halophiles (Mancinelli et al., 2004). The Gamma Ray Spectrometer onboard the Mars Odyssey orbiter detected chlorine in a rather high concentration in the upper 10-20 cm of some regions of Mars (Keller et al., 2006) and the presence of perchlorate salts (primarily $MgCl_2O_8$ and $NaClO_4$) were shown at the Phoenix landing site (Hecht et al., 2009; Stoker et al., 2010), which suggests the potential presence of unfrozen water in the martian subsurface.

In conclusion, Mars is a planetary body of cryogenic nature where liquid water may occur in the near-surface and subsurface in the presence of high solute content, probably as supercooled brines within permafrost, where, similarly to terrestrial analogues, microorganisms adapted to subzero temperatures and high salinity, among other parameters, might survive. Thus, the microbiology of permafrost represents a plausible prototype for microbial life on Mars, be it as an "oasis" for extant life, or as the last refuge of an extinct biota (Mancinelli et al., 2004).

**1.3 Methanogenic archaea from Siberian permafrost as models for martian life**

Methanogenic archaea (methanogens) are exclusive to the Euryarchaeota kingdom of the Archaea domain (Woese et al., 1990) and they form a polyphyletic group that comprises six orders (Ferry, 2010). They are strictly anaerobic chemotrophic microorganisms that produce methane as a metabolic end product through at least one of the three main metabolic pathways: 1. hydrogenotrophic (oxydize $H_2$ and reduce $CO_2$), 2. methylotrophic (use methyl compounds, e.g. methanol) and 3. acetoclastic (use acetate). The most energetically favourable reaction is the hydrogenotrophic pathway, which, by natural pressure of selection, it is also the most common (Garcia et al., 2000). Methanogens are the only organisms known to produce abundant methane as main end product of their metabolism and methanogenesis is their exclusive energy conservation approach. It is estimated that microbial processes contribute 85% of annual global methane formation on Earth (Thauer et al., 2008). Methanogenic archaea are found widespread in anoxic ($O_2$-free) or microaerophilic (2-10% $O_2$) habitats in nature, including wetlands, aquatic sediments (ponds, marshes, swamps, rice soils, lakes and oceans), the digestive tract of mammals (especially the rumen of herbivores), heartwood of trees, sewage digesters, decomposing algal mats etc. They are also abundant in a wide range of "extreme" environments from permafrost and cold climates to hot springs, submarine 'black smokers', hypersaline environments and habitats with high/ low pH (Ferry, 1993; Garcia et al., 2000). Given their metabolic features and their ecological versatility, methanogens have been proposed numerous times as models for martian life (Kral et al., 2004a; Kral et al., 2011) and even as the potential source of methane on Mars (Moran et al., 2005; Wagner, 2008; Price, 2010)

Terrestrial methane production is believed to mainly occur in wetlands, especially in cold regions. Approximately 75% of the biosphere is subjected to permanently low temperatures and thus, psychrophiles and cold-adapted microorganisms are widespread in nature (Cavicchioli 2006). Cold-adapted methanogens are ubiquitous in natural wetlands, freshwater sediment, landfills, permafrost and the basal layer of glaciers, among others, being equipped with cold-adaptive mechanisms that involve modifications of the cell components to enable an adequate performance of the cellular processes (Dong and Chen, 2012). It has been estimated that 25% of the methane from natural sources on Earth is released from permafrost (Fung et al., 1991). Recent findings show that methanogenic communities in permafrost environments are composed by representatives of the major phyla and the total biomass is comparable to temperate soil ecosystems (Wagner et al., 2005). In permafrost environments, in addition to the cold temperatures, methanogens experience seasonal freeze-thaw cycles in the upper active layer, with temperatures fluctuating between -45ºC and 25ºC in the Arctic tundra (Wagner et al., 2005), desiccation, starvation and the effects of long-lasting background radiation over geological time scales (Wagner and Liebner, 2010). For instance, methanogenic archaea isolated from the active layer of a permafrost-affected soil in the Lena Delta (Siberia, Russia) have shown a remarkable resistance against desiccation, starvation, osmotic stress and low temperatures when compared to other methanogens from non-permafrost environments (Morozova and Wagner, 2007; Wagner et al., 2013). They also survive the exposure to high doses of monochromatic and polychromatic UV and ionizing radiation (Morozova, et al., in preparation) in levels comparable to those of *Deinococcus*

*radiodurans* (Brooks and Murray, 1981). Moreover, Siberian permafrost strains are able to survive simulated martian thermo-physical conditions, in contrast to other psychrophilic methanogens from non-permafrost habitats (Fig.5, Morozova, et al. 2007) such as *Methanogenium frigidum* (Franzmann et al., 1997) from Ace Lake, Antarctica, and they show metabolic activity under simulated martian subsurface analogue conditions (Schirmack et al., 2013). These facts suggest that methanogenic archaea from Siberian permafrost are ideal candidates among methanogens proposed to date as models for potential life on Mars.
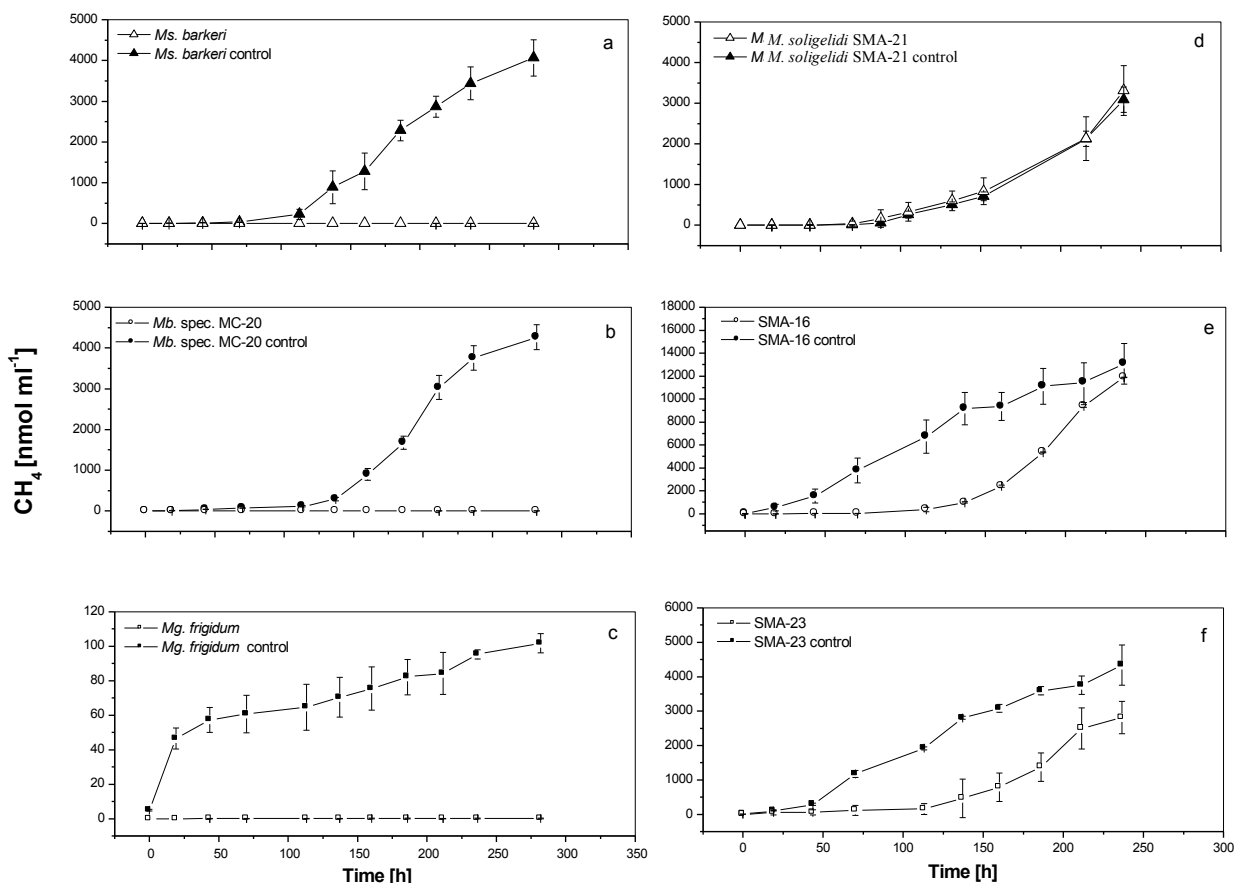


**Figure 5:** Methane production of methanogens from non-permafrost habitats (a) *Methanosarcina barkeri*, (b) *Methanobacterium* spec. MC-20 and (c) *Methanogenium frigidum*, and methanogens isolated from Siberian permafrost (d) *Methanosarcina soligelidi* SMA-21, (a) SMA-16, (f) SMA-23 before and after exposure to simulated martian thermo-physical conditions. Source: Morozova et al. (2007)

## 1.4 Raman spectroscopy for biosignature description and relevance in planetary exploration

Raman spectroscopy is an analytical technique based on inelastic scattering of laser excitation light able to reveal information on the vibrational and rotational modes within a system. The Raman spectrum of a biological sample shows the information about the overall chemical composition of the cells and provides a "spectral fingerprint" that allows the chemical characterization and identification of biological systems on a molecular level. For organic molecules, the Raman shifts relate primarily to vibrational modes and so are determined by the molecular structure, including functional groups and bond types (Naumann et al., 1991; Maquelin et al., 2002). Raman spectroscopy has proven to be a powerful molecular structural technique by generating multidimensional data. It presents several advantages for biological investigations such as minimal sample preparation, the possibility

16

of a non-invasive analysis and the study of aqueous samples, given the low Raman intensity for water (Petry et al., 2003; Harz et al., 2009). However, the main disadvantage of Raman is the low scattering efficiency, since Raman spectra measured in the visible or near infrared regions are often interfered with a more intense fluorescence emission (Gremlich and Yan, 2001). Raman spectroscopy therefore allows a rapid detection of biological and microbial signatures with a high sensitivity and selectivity and it has proven a relevant potential for microbial identification in recent years (Popp, 2007; Harz et al., 2009)

As one of the variants, confocal Raman microspectroscopy (CRM) combines the Raman setup with a light microscope equipped with a numerical aperture objective and high magnification (Fig. 6.A). CRM achieves a great spatial resolution in the range of 1μm, enabling the study of individual cells. This setup allows the diffraction-limited investigation of the microbial samples according to their chemical nature (Krause et al., 2008; Hermelink et al., 2009). Furthermore, CRM has proven to be an accurate chemotaxonomical tool and a considerable amount of microorganisms have been successfully characterized to the species and even strain levels (Fig. 6.B, Harz et al., 2005; Rösch et al., 2005; Hermelink et al., 2009; Hermelink et al., 2011). Therefore, this technique shows a great potential for the fast diagnosis of microbial infections as well as the identification of pathogens in the food industry. Additionally, Raman spectroscopy has proven to be an effective tool for the in situ investigation of microbial communities inhabiting extreme environments, such as the McMurdo Dry Valleys in Antarctica (Edwards et al., 1997; 2012) and natural halite the hyper-arid Atacama Desert (Vítek et al., 2010; 2012), both relevant places for Astrobiology.

In recent years, scientists have become interested in the applications of Raman spectroscopy for the remote detection of biosignatures in the context of planetary exploration (Ellery and Wynn-Williams, 2003; Jorge-Villar and Edwards, 2006; Tarcea et al., 2008). The ExoMars rover (Fig. 6.C) (Vago et al., 2006) is an ESA mission due for launch in 2018 that will incorporate a Raman Laser Spectrometer (RLS) to analyse samples of the martian subsurface collected by the probe's 2-m drill. The RLS will use green excitation light at a wavelength of 532 nm, a spectral resolution of 6-8 $cm^{-1}$, and the spectrometer unit offers a spectral range of 200-3800 $cm^{-1}$, covering both mineralogical and biological Raman regions (Rull et al., 2010; Edwards et al., 2012; 2013). Within this framework, the BIOMEX space experiment (de Vera et al., 2012) was designed to evaluate the stability of biomolecules under space and Mars-like conditions. The methanogenic archaeon *Methanosarcina soligelidi*, will be one of the extremophiles onboard this mission, that will be launched on the space exposure facility EXPOSE R-2 to the International Space Station (ISS) this summer 2014. The creation of a Raman biosignature database of terrestrial extremophilic organisms to interpret future data from the ExoMars mission is one of the goals of BIOMEX. Given the high involvement of the present investigation within the BIOMEX mission, the publication in *Planetary and Space Science* describing this project (where the candidate is a co-author) is included in the appendix.
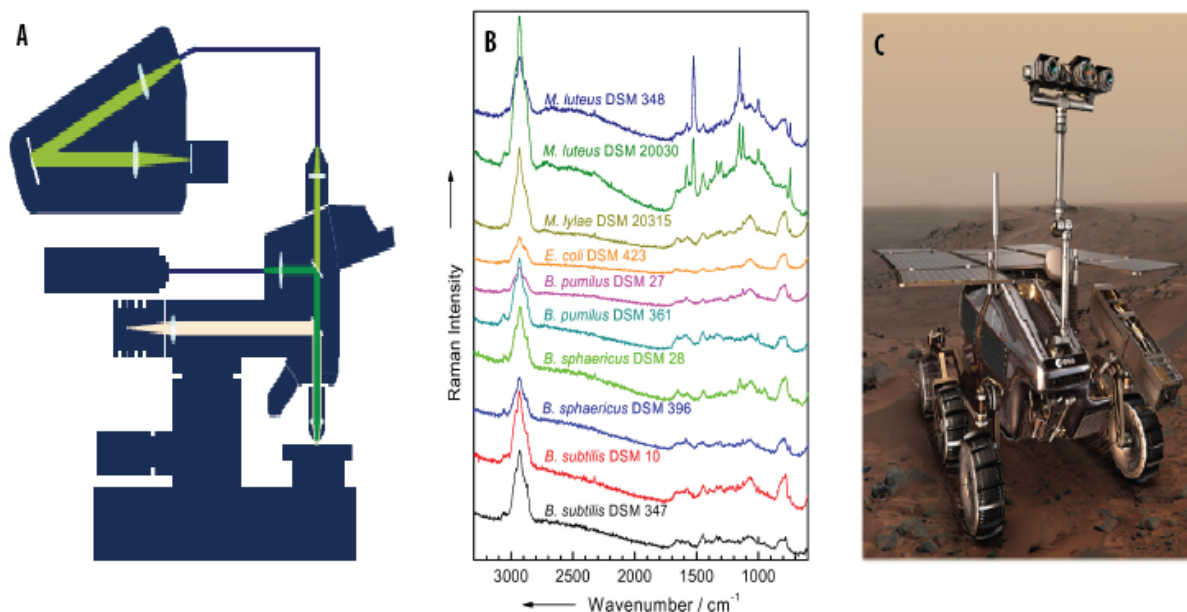
**Figure 6**: A) Diagram of a confocal Raman microspectrometer composed by a confocal microscope coupled to a laser source. Image courtesy of the Robert Koch Institute. B) Micro-Raman spectra of single bacterial cells recorded with 532nm excitation. Source: Popp (2007) C) Digital illustration of ExoMars rover. Image credits: ESA.

## 1.5 Study area

Samoylov Island (N 72°22, E 126°28, Fig. 7) is located in the active and geologically youngest area (~8500 years) of the Lena Delta, in the far north of eastern Siberia, Russia. The Lena Delta has a total area of 32.000 km$^2$, being one of the world's largest deltas (Schneider et al., 2009). It is located at the Laptev Sea coast between the Taimyr Peninsula and the New Siberian Islands, showing a characteristic arctic continental climate with a low mean annual air temperature of -14.7°C ($T_{min}$ = -48°C, $T_{max}$ = 18°C) and a low mean annual precipitation of 190 mm (WWIS, 2004). The delta is within the zone of continuous permafrost and therefore soils and sediments are permanently frozen, with the exception of 20-50 cm in the upper layer, forming the so-called active layer that thaws during the summer months.

Soil samples were collected from the active layer during the expedition to the Lena Delta in 2002, in the framework of the Russian-German cooperation 'System Laptev Sea 2000'. Two soil profiles with a diverse genesis and properties that represent the most characteristic geomorphic units of the island were sampled. One of the profiles was extracted from the depression of a low-centred ice-wedge polygon (N 72°22.2', E 126°28.5') with a diameter of about 20 m in the eastern part of the island. The polygon depression was dominated by *Typic Historthels*, according to the US Soil Taxonomy (Soil Survey Staff, 1998) and characterized by a water level near the soil surface and a predominantly anaerobic accumulation of organic matter. The second profile was located at a flood plain in the northern part of the island, where annual floods lead to a continuous accumulation of fluvial sediments. The predominant substrate is sandy and silty fluvial material. The prevalent soil type of the flood plain was a *Typic Aquorthel*, according to the US Soil Taxonomy (Soil Survey Staff, 1998). Soil samples were filled in gastight plastic jars (Nalgene) and

transported to Germany in frozen condition.
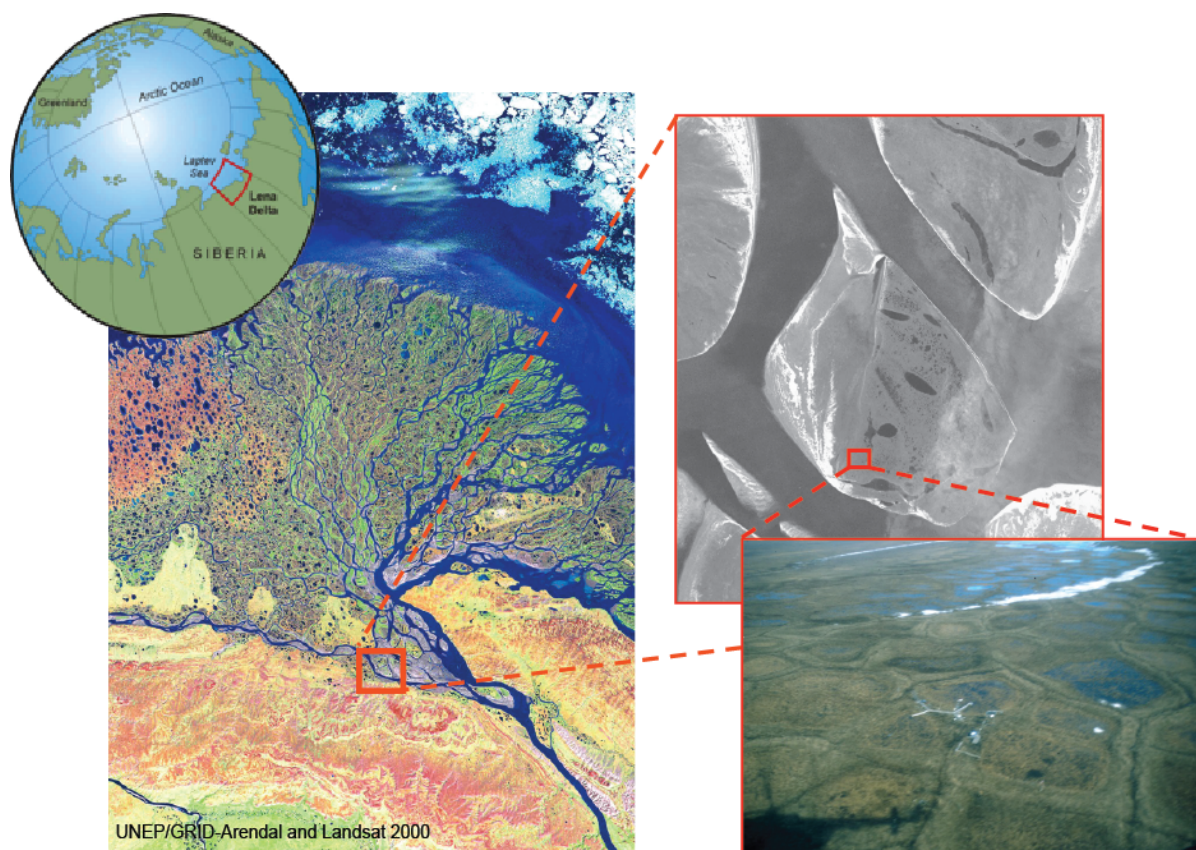


**Figure 7:** Geographical location and geomorphological landscape of Samoylov island, in the Lena Delta, Siberia, Russia (N 72°22.2', E 126°28.5'). Image credits: Landsat 2000 and Dirk Wagner.

## 1.6 Methanogenic archaea used in this study

This project focuses on the investigation of five strains of methanogenic archaea: three were isolated from the active layer of Siberian permafrost-affected soils in the Lena Delta (*Methanosarcina soligelidi* SMA-21, *Candidatus Methanosarcina* SMA-17 and *Candidatus Methanobacterium* SMA-27) and two belong to mesophilic non-permafrost environments (*Methanosarcina mazei* and *M. barkeri*). For a better understanding of this study, the main phylogenetic, morphological and physiological features are described as follows:

**Siberian permafrost-associated strains:**

- *Methanosarcina soligelidi* DSM 26065[T], strain SMA-21. According to the 16S rRNA gene sequences, it shows a 99.9% sequence homology with *M. mazei* and a 99.8% homology with SMA-17. Cells appear as cocci, ~1μm in diameter and often form aggregates (Fig 8.A). It grows on $H_2/CO_2$ (80:20, v/v), methanol (20 mM) and acetate (20 mM) and the optimal growth temperature is 28ºC (Wagner et al., 2013).

- SMA-17. It shows a 99.8 % identity on the 16S rRNA gene sequences with *M. mazei*. Cells appears as ~1μm-diameter cocci and may form cell aggregates, although not as often as *M. soligelidi* (Fig 8.B). It grows on $H_2/CO_2$ and the optimal growth temperature was observed at 28ºC (Malaszkiewicz, 2009).

- SMA-27. Its closest relative is *Methanobacterium congolense* (Cuzin et al., 2001) showing a 96.4 % homology in the 16S rRNA gene sequence. SMA-27 cells are elongated rods, ~3-4 μm long (Fig 8.C). It grows on $H_2/CO_2$ and the optimal growth temperature was observed at 28ºC (Malaszkiewicz, 2009).

**Reference strains from mesophilic environments:**

- *Methanosarcina mazei* DSM 2053 (Mah, 1980) was isolated from a mesophilic sewage sludge plant in California, USA. It appears as irregular cocci, ~1μm in diameter that often form macrocysts (Fig 8.D). It is capable of growing on $H_2/CO_2$, acetate, all methylamines, and methanol. The optimal growth temperature is 37ºC.

- *Methanosarcina barkeri* DSM 8687 (Maestrojuan et al., 1992), isolated from a peat bog in northern Germany. They are irregular cocci, ~1μm in diameter and form aggregates (Fig 8.E). It is extremely oxygen-sensitive and grows on $H_2/CO_2$, methanol, methylamines, and acetate. The optimal growth temperature is 30ºC.
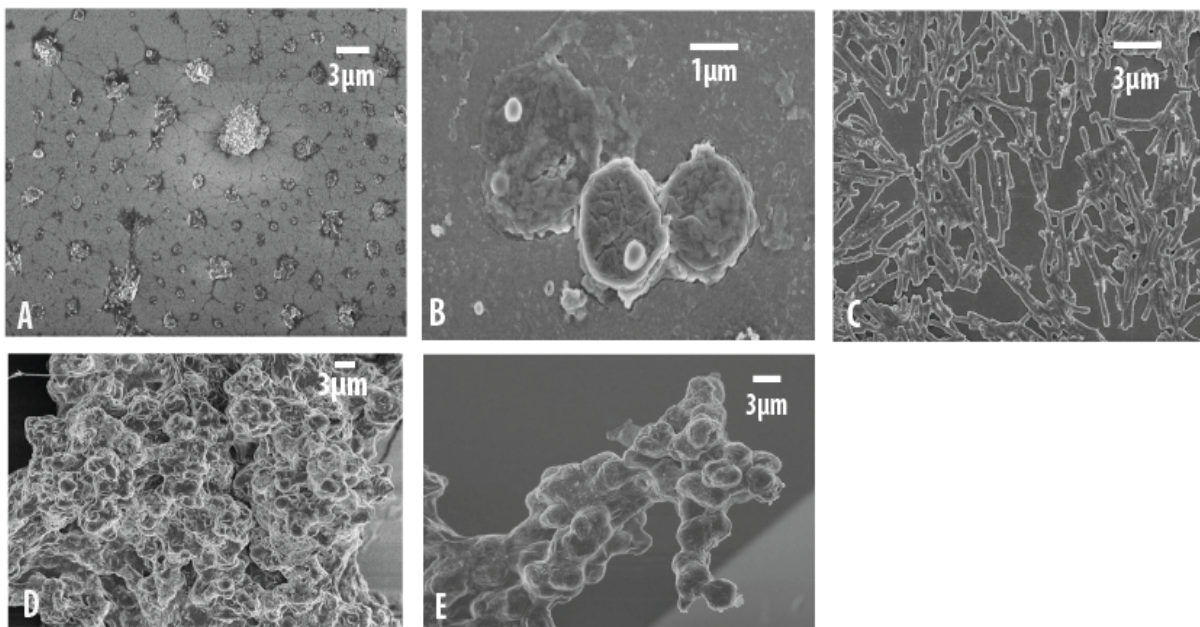


**Figure 8:** Scanning electron microscopy images of the studied strains in culture conditions A. *Methanosarcina soligelidi* SMA-21, B. SMA-17, C. SMA-27, D. *M. mazei* and E. *M. barkeri*. Courtesy of the Robert Koch Institute.

**1.7 Aims and objectives**

The main goals of this investigation are 1) to assess the survival of methanogenic archaea under Mars conditions including temperature, soil and atmospheric composition and the presence of oxidants, with special emphasis on Siberian permafrost strains, and 2) to characterize for the first time the biosignatures of methanogenic archaea by means of confocal Raman microspectroscopy in order to evaluate the potential of this technique for life detection in the context of planetary exploration. In order to achieve these goals, the following questions should be answered:

*1) Are methanogenic archaea from Siberian permafrost environments optimal model organisms for potential life on the martian subsurface?*

Methanogenic archaea have been proposed as candidates for life on Mars given their metabolic characteristics and the resistance shown under certain simulated martian conditions. Previous studies in our research group put in evidence significant differences in the survival of Siberian permafrost strains compared to non-permafrost strains when exposed to physiological stress including simulated martian thermo-physical conditions. As part of the present investigation, we aim to gain a deeper insight into the resistance of Siberian permafrost and non-permafrost strains by exposing them to martian parameters that have never been tested before with methanogens. These include long-term freezing, desiccation by exposure to regolith simulants together with a simulated atmospheric composition, and the presence of perchlorate, recently reported in the martian geology.

*2) Is Raman spectroscopy a suitable tool for the in situ detection of methanogenic archaea in the context of planetary exploration?*

Raman spectroscopy is currently one of the most promising tools for remote life detection and the ExoMars mission will include a Raman laser spectrometer among its instrumentation in order to study the martian mineralogy, perhaps encountering biosignatures. In this thesis, a detailed study on the biosignatures of the methanogenic archaeon *Methanosarcina soligelidi* during the different growth phases was performed using confocal Raman microspectroscopy. The data obtained in this investigation help to evaluate the potential of Raman spectroscopy for planetary exploration.

*3) What do the Raman biosignatures of methanogenic archaea tell us about their chemical composition?*

Through the biosignature characterization of methanogenic archaea from Siberian permafrost and non-permafrost environments by confocal Raman microspectroscopy, this study aims to improve our understanding about the overall chemical composition of methanogens, the individual Raman "fingerprints" for each strain as well as help interpret the previously reported differences in survival between permafrost and non-permafrost strains.

**1.8 Overview of the publications and manuscripts**

**"Response of methanogenic archaea from Siberian permafrost and non-permafrost environments to simulated martian desiccation and the presence of perchlorate"**

**Authors:** Paloma Serrano[1,2], Mashal Alawi[2] and Dirk Wagner[2]

**Aim:** The objective of this study was to analyse the survival and metabolic activity of methanogens from Siberian permafrost and non-permafrost environments after their exposure to simulated martian desiccation and the presence of perchlorate in order to gain insights into their robustness as candidates for potential life on Mars and validate whether methanogens from Siberian permafrost soils show a differential survival compared to non-permafrost strains.

**Summary:** To attain this goal, Siberian permafrost strains and methanogens from non-permafrost environments were subjected to simulated martian desiccation by long-term exposure to 1. ultra- low subfreezing temperatures 2. Mars regolith analogues together with a simulated martian atmosphere. The survival and methane production rates after desiccation were analysed by quantitative PCR and gas chromatography respectively. Additionally, liquid cultures of the same strains were exposed to different concentrations of magnesium perchlorate, a strong oxidant detected on martian soils, while monitoring the methane production. The permafrost strains showed the highest recovery of the metabolic activity after long-term freezing, although the non-permafrost strains revealed a higher number of cells with intact cell membranes (assumed living cells) in these conditions. All strains survived in large numbers the exposure to simulated martian soil and atmospheric composition and significantly higher methane production rates were found for *Methanosarcina soligelidi* from Siberian permafrost after regrowth. A new method to isolate DNA from intact cells from the martian regoliths was proposed and evaluated as part of the study. The presence of magnesium perchlorate at the concentration reported on Mars (2.4mM) had no significant effects on the methanogenic activity of the studied strains and at increasing salt exposures, permafrost strains experienced metabolic shutdown at a lower concentration than non-permafrost strains. Overall, this study suggests that there is no evidence to dismiss methanogenic archaea as candidates for potential life on Mars, and Siberian permafrost strains might pose some advantages to be considered more appropriate model organisms.

**Contribution of the co-authors:** *Paloma Serrano* proposed and developed the objectives of this investigation, carried out the experimental work, analysed the results and wrote the manuscript. *Mashal Alawi* developed and optimized the DNA isolation method from the martian regolith and participated in the interpretation of the results. *Dirk Wagner* contributed to design the experiments, to interpret the results and provided valuable discussion.

**Manuscript II** (in press, accepted in 2013 in *Planetary and Space Science*)

**"Single-cell analysis of the methaonogenic archaeon *Methanosarcina soligelidi* from Siberian permafrost by means of confocal Raman microspectroscoy for astrobiological research."**

**Authors:** Paloma Serrano[1,2], Dirk Wagner[2], Ute Böttger[3], Jean-Pierre de Vera[3], Peter Lasch[4], Antje Hermelink[4].

**Aim:** This study aims to characterize the biosignatures of the methanogenic archaeon *Methanosarcina soligelidi* during the growth phases using confocal Raman microspectroscopy and to evaluate the advantages and limitations of this analytical approach in the context of remote detection of biosignatures and planetary exploration missions.

**Summary:** The Raman signatures of single cells of *M. soligelidi* SMA-21 were described in the exponential, stationary and senescent phases of growth, highlighting a high heterogeneity within one phase of growth and diversity in the chemical composition of the cells, which correlated with changes in the cell morphology during the growth. The analysis of the spectral data grouped into growth-phase-specific clusters, showing that the most noticeable differences in the chemical composition of cells occur at the senescent phase. Alongside the cell spectra, extracellular microbial products such as lipid droplets and calcium carbonate were detected and their presence confirmed by scanning electron microscopy. However, the in situ detection of biosignatures of methanogenic archaea in a mineral substrate faces technical challenges that must be overcome for a successful use in remote detection. Among them figure the small Raman scattering of the biological material compared to minerals (in the case of non-pigmented cells), the unexpected heterogeneity effects, and the specificity linked to single-cell analysis, in terms of spatial resolution and alignment between the laser and the target.

**Contribution of the co-authors:** *Paloma Serrano* defined the objectives of this investigation, carried out the Raman measurements, performed the data analysis and wrote the manuscript. *Dirk Wagner* helped to develop the objectives and contributed with useful comments. *Ute Böttger* contributed to the interpretation of the results. *Jean-Pierre de Vera* established the objectives related to the background of the investigation (the BIOMEX project) and provided useful comments. *Peter Lasch* helped to develop the discussion. *Antje Hermelink* supervised the Raman measurements, data analysis and contributed to the interpretation of the results.

**Manuscript III** (submitted for publication to *Applied and Environmental Microbiology*)

**"Convergence of the chemical composition in methanogenic archaea from a Siberian permafrost-affected soil: investigation by confocal Raman microspectroscopy"**

**Authors:** Paloma Serrano[1,2], Antje Hermelink[4], Peter Lasch[4], Jean-Pierre de Vera[3], Nicole König[4], Oliver Burckhardt[2] and Dirk Wagner[2]

**Aim:** The aim of this study is to gain a deeper insight into the Raman signatures of methanogenic archaea from Siberian permafrost and non-permafrost habitats in order to identify similarities and differences in their overall chemical composition that might explain the differential survival rates reported in previous studies.

**Summary:** This investigation presents the biosignature characterization of five strains of methanogens from Siberian permafrost and non-permafrost environments in stationary phase of growth by means of confocal Raman microspectroscopy. The cluster analysis of the spectra reveals two groups: the strains from Siberian permafrost and those from non-permafrost environments, evidencing a high homology in the overall chemical composition of the cells within the same group and remarkable differences between permafrost and non-permafrost strains. These differences were primarily focused on the aliphatic chain composition of lipids, specific aromatic amino acids, ribonucleotides, proteins, carbohydrates and polysaccharides. Furthermore, a phylogenetic reconstruction of the studied strains based on the functional gene *mcrA* was performed, showing a diverse evolutionary origin within the strains originating from Siberian permafrost. The convergence of the chemical composition in methanogens from Siberian permafrost habitats regardless of their phylogenetic relationship suggests a complex adaptive process to the environmental conditions that resulted in a similar chemistry and this fact might be the reason underlying their previously reported resistant nature.

**Contribution of the co-authors:** *Paloma Serrano* formulated and developed the structure and objectives of this investigation, carried out the Raman measurements, performed the data analysis and wrote the manuscript. *Antje Hermelink* supervised the Raman measurements, data analysis and contributed to the interpretation of the results. *Peter Lasch* helped to develop the discussion. *Jean-Pierre de Vera* provided useful comments. *Nicole König* assisted with the Raman measurements. *Oliver Burckhardt* contributed to the isolation, amplification and sequencing of the functional gene *mcrA*. *Dirk Wagner* formulated the hypothesis and contributed to the interpretation of the results and valuable discussion.

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# II. MANUSCRIPTS

**Manuscript I** - (In preparation for submission to *Astrobiology*)

**Response of methanogenic archaea from Siberian permafrost and non-permafrost environments to simulated martian desiccation and the presence of perchlorate**

Paloma Serrano[1, 2], Mashal Alawi[2] and Dirk Wagner[2]

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**Abstract**

Methanogenic archaea are widespread anaerobic chemotrophic microorganisms whose metabolism is in principle compatible with the conditions on early and recent Mars. Some strains from Siberian permafrost-associated environments have shown a particular resistance against desiccation, radiation, low temperatures and martian thermo-physical conditions. In this investigation we analysed the response of Siberian permafrost strains (*Methanosarcina soligelidi* SMA-21, SMA-17 and SMA-27) and related strains from non-permafrost environments (*M. mazei, M. barkeri*) to desiccation by exposure to ultra-low temperatures (-80ºC), and to the combination of a Mars regolith simulant (S-MRS, P-MRS) together with a simulated martian atmosphere. The survival and metabolic recovery after the stress were analysed by quantitative PCR of the DNA from cells with intact membranes and by gas chromatography, monitoring the methane production, respectively. Moreover, the methanogens were subjected to increasing concentrations of perchlorate, in order to find out their threshold for metabolic shutdown. We found that in the desiccation setups all strains survived in high numbers and permafrost strains increased their methane production rates by 1000% after exposure to ultra-low temperatures. *M. soligelidi* SMA-21 showed an extremely active metabolism in the presence of regolith analogues and after desiccation for up to 16 days in S-MRS and P-MRS under a simulated martian atmosphere. The non-permafrost strains showed a moderate metabolic activity, but resisted the deep-freezing conditions in higher cell numbers than the permafrost strains. The perchlorate concentration reported at the Phoenix landing site (2.4 mM) did not reduce the survival of any strain, although the permafrost strains were more sensitive to high concentrations of perchlorate. The differences in the methanogenic activity and survival were discussed and a new method to extract DNA from intact cells embedded in the Mars regolith simulants S-MRS and P-MRS was presented and evaluated. We conclude that methanogenic archaea from permafrost and non-permafrost environments show no evidence to be dismissed as candidates for potential life in the martian subsurface.

**Introduction**

Mars has long been proposed as a potential location for life within the Solar system. The presence of liquid water is, however, crucial for the origin, evolution and preservation of life as we know it. However, despite the evidence of ancient aqueous environments on the

surface and subsurface of Mars that could have been potentially inhabited (Grotzinger, 2014), the present atmospheric conditions do not favour the existence of liquid water on the martian surface (Kminek et al., 2010). Today most of the water on Mars probably exists as ground ice in the subsurface, which is supported by the findings of the Gamma Ray Spectrometer onboard NASA's Mars Odyssey spacecraft, that detected an increasing gradient of water-equivalent hydrogen towards the martian poles (Boynton et al., 2002; Feldman et al., 2004). Therefore, if microbial life emerged on the aqueous environments of early Mars, it either adapted to the dramatically different conditions found in the present, survived in well-protected relicts, which remain unknown (Horneck, 2000) or otherwise became extinct. The potential habitability of Mars' polar and high-latitude regions was proposed and assessed by Jakosky et al. (2003) and Stoker et al. (2010) based on the possible periodic presence of liquid water as orbital dynamics influence the regional climate and the fact that terrestrial microorganisms are able to grow at subfreezing temperatures. Ulrich et al. (2012) discussed the potential habitability of periglacial landscapes in martian mid-latitudes by relating terrestrial analogues to permafrost landforms on Mars. Subsurface permafrost environments on Mars represent potential habitable regions where extant life could have survived and additionally, terrestrial permafrost is considered the most promising analogue to a potential martian habitat (Gilichinsky, 2002b) among the extreme habitats on Earth (Rothschild and Mancinelli, 2001). Microbial communities in terrestrial permafrost and cold-associated environments often appear in high density and consist of specialized psychrophilic (cold-adapted) microorganisms. Most of them present a chemolithoautotrophic metabolism, meaning that they can use carbon dioxide, inorganic compounds and external chemical compounds as the carbon, reducing equivalents and energy sources respectively. Moreover, these microorganisms can be active with extremely low amounts of liquid water (Gilichinsky et al., 2007).

Methanogenic archaea pose a number of metabolic features that might be favourable for their survival in the martian subsurface. On the one hand, they are strictly anaerobic chemolithotrophs that use hydrogen and carbon dioxide as energy and carbon sources. On Earth, they are found widespread in anaerobic habitats and they appear in high numbers in extreme environments ranging from permafrost and cold climates to hot temperatures, high salinity and high/low pH (Ferry, 1993). On the other hand, methanogenic archaea produce methane as a metabolic end product. The presence of methane on Mars remains controversial. Despite the gas was reported in some areas by the Mars Express Orbiter and the Fourier Transform Spectrometer at the Canada-France Hawaii Telescope (Formisano et al., 2004; Lefèvre and Forget, 2009; Mumma et al., 2009), the lack of compelling evidence was later discussed by Zahnle et al. (2011). Moreover, even if the presence of methane was confirmed, it could either be produced biotically (by microorganism such as methanogens) or abiotically, as a product of geological processes such as serpentinization (Krasnopolsky et al., 2004). In addition to this ongoing discussion, it was recently proven that the wind-mediated erosion process of ordinary quartz crystals can sequester methane to produce activated quartz grains, fact that would explain the observed fast destruction of methane on Mars (Jensen et al., 2014).

Methanogenic archaea from Siberian permafrost meet many of the pre-conditions for survival in the martian subsurface, showing a remarkable resistance against desiccation, osmotic stress, low temperatures, starvation and thermo-physical martian conditions,

compared to non-permafrost strains (Morozova et al., 2007; Morozova and Wagner, 2007). They are also subject of astrobiological interest for future planetary exploration missions involving life detection (Serrano et al., 2013). However, their survival possibilities in martian conditions remain uncertain and must be further assessed to confirm whether they are promising model organisms for potential life on Mars, given an adequate environment.

Some of he main threats that cell integrity would face under martian conditions are the desiccation due extremely low temperatures, the dehydration of the martian soils and atmosphere, and the presence of oxidants such as perchlorate. The Mars Exploration Rover Opportunity reported surface temperature averages of -53°C (-64°F), varying from -128°C (-199°F) during polar night to 27°C (80°F) at equator during midday at closest point in orbit to Sun (NASA, 2004). On Earth, survival and metabolic activity of permafrost bacteria has been reported down to -20°C (Rivkina et al., 2000).

Schuerger et al. (2012) discussed the biotoxicity of multiple Mars soils and concluded that they are not likely to be overtly biotoxic to terrestrial microorganisms. Previous studies on methanogenic archaea show not only survival, but active methanogenesis under desiccating conditions in presence of the Mars soil simulant JSC Mars-1 (Kral et al., 2011). The Mars regolith analogues used in these experiment contain terrestrial minerals and rocks that are structurally and chemically similar to those in martian meteorites, with a particular focus on the composition of early (phylosicatic, P-MRS) and late (sulphatic, S-MRS) Mars (Böttger et al., 2012). They were previously tested on methanogenic archaea with positive results for survival (Wagner, unpublished data) but never in combination with a martian atmospheric composition. The DNA isolation from these regolith analogues has been optimized by using a high phosphate solution that contained ethanol (Direito et al., 2012). Ethanol, however, would interfere with the analysis of surviving cells and an adapted protocol has become an emergent need. Finally, the presence of perchlorate on Mars has potential implications for the occurrence of liquid water by lowering the freezing point in solutions, although it may be detrimental for life due to its strong oxidant nature (Stoker et al., 2010). A concentration of 2.4 mM of perchlorate ($ClO_4^-$) on average was reported at the Phoenix lander site and the most abundant ion found was $Mg^{2+}$ (3.3mM on average) (Hecht et al., 2009). On Earth, the increasing presence of (per)chlorate in the environment is associated to human activities (mainly to agriculture and the paper industry) and its ecotoxicity has been evaluated on aquatic organisms (van Wijk and Hutchinson, 1995).

The aim of this study is to analyse the survival and metabolic activity of three methanogenic archaea from a Siberian permafrost-affected soil and two close relatives from non-permafrost habitats when exposed to single (ultra-low temperatures) and multiple (regolith and atmospheric composition) simulated martian desiccating parameters as well as to several concentrations of magnesium perchlorate to further progress in the complex assessment of the prospective survival and performance of methanogens as candidates for potential life on the martian subsurface.

**Materials and Methods**
*Methanogenic strains*

Three strains from permafrost-affected soils and two strains from non-permafrost habitats were used for this study. The permafrost strains, *Methanosarcina soligelidi* SMA-21, (Wagner et al., 2013), SMA-17 and SMA-27 were isolated from the active layer of

permafrost-affected soils in the Lena Delta, Siberia (Russia). *M. soligelidi* SMA-21 (DSM 26065[T]) and SMA-17 appear as ~1µm-diameter cocci, sometimes forming cell aggregates. They both show a 99.9 % homology on the 16S rRNA sequence with *Methanosarcina mazei* (Mah, 1980). SMA-27 cells are large rods, ~3-4 µm long. According to the 16S rRNA molecule, it presents a 96.4 % homology with *Methanobacterium congolense* (Cuzin et al., 2001). The non-permafrost methanogenic strains in this study are *M. barkeri* DSM 8687, isolated from a peat bog in northern Germany (Maestrojuan et al., 1992), and *M. mazei* DSM 2053, which originates from a mesophilic sewage sludge plant in California, US. They are found in multiple environments and appear as irregular cocci, ~1µm in diameter that often form aggregates. Cultures of both strains were purchased from the German Culture Collection of Microorganisms and Cells (DSMZ, Braunschweig, Germany).

MW medium (Serrano et al., 2013) was used to grow *M. soligelidi* SMA-21, SMA-17, M. mazei and M. barkeri. SMA-27 was grown CS medium, a modification of MW containing (L[-1]): $NH_4Cl$, 0.3 G, $MgCl_2$ x $6H_2O$, 0.4 G, $CaCl_2$ x $2H_2O$, 0.16 G, NaCl, 1.0 G, KCl, 0.5 G, $K_2HPO_4$ 0.25 G, Na $HCO_3$, 2.7 G, Na-Acetate, 0.25 G, $Na_2S_2O_4$, 0.1 G, $Na_2S$, 0.25 G; trace element solution (Imhoff-Stuckle and Pfennig, 1983), 1mL and vitamin solution (Bryant et al., 1971), 1mL.

*Exposure to ultra-low subfreezing temperatures*

A temperature of -80°C was selected as a hypothetical constant (or invariable for long terms) subfreezing temperature present in numerous regions of the martian subsurface at variable depths, latitudes and times of the year. Pure cultures were grown at 28°C in sealed bottles containing 50 mL medium, flushed and pressurized to one atmosphere with $H_2/CO_2$ (80:20, v/v). Once they reached the exponential growth phase (~12% $CH_4$, approx. three weeks), all cultures but the controls were frozen to -80°C and kept at constant temperature (+/- 2ºC) for 63, 152, 209, 254 and 315 days. The cultures were thawed in triplicates for 30 min at 4°C followed by 3h at 24°C. Intact cell numbers and metabolic activity after exposure were studied by DNA quantification and by monitoring the methane production after re-incubation in fresh medium (one atmosphere $H_2/CO_2$ (80:20, v/v) and 28°C) respectively. Three replicates were used.

*Exposure to martian soil and atmospheric analogues*

Two different Mars regolith simulants were provided by the Museum für Naturkunde Berlin and consisted of mineral and rock mixtures structurally and chemically similar to those found in martian meteorites and reported by orbited and rovers. The two regoliths contain a predominant phylosillicatic (P-MRS) and a sulphatic (S-MRS) composition characteristic of different Mars geological epochs: the Noachian and Hesperian were represented by hydrothermal alteration or weathering of crustal rocks and secondary mineralization, simulated in P-MRS, and were followed by the Amazonian, where the prevailing cold and dry oxidising conditions lead to the formation of anhydrous iron oxides, present in S-MRS. A more detailed description can be found in Böttger et al. (2012).

1 L of pure culture of each strain was grown for 4 weeks with an initial pressure of one atmosphere $H_2/CO_2$ (80:20, v/v). The cultures were centrifuged in two rounds at 6000 g for 50 min and 4°C and the volume concentrated to 5 mL. 100 µL of the concentrated culture were deposited in sterile 7-mm-diameter pellets of the Mars regolith simulants S-MRS and P-

MRS and desiccated in sealed bottles containing the desiccant 'köstrolith' (Chemiewerk Bad Köstritz, Germany), flushed and pressurized to one atmosphere with a Mars gas mix containing the same elements and proportions as recently reported by the rover Curiosity (Mahaffy et al., 2013). The pellets were left to desiccation for 8 and 16 days, and three replicates per time were used to study intact cell numbers and metabolic activity after exposure. As controls, three replicates of inoculated pellets were transferred into sealed bottles with medium and $H_2/CO_2$ (80:20, v/v) in regular culture conditions at the start of the experiment.

*Exposure to magnesium perchlorate*

10 mL of pure culture of the five strains were grown for four days in sealed bottles, previously flushed and pressurized to one atmosphere with $H_2/CO_2$ (80:20, v/v). Commercial magnesium perchlorate ($MgCl_2O_8$) (Sigma Aldrich, St. Louis, MO) was then added to a final concentration of 2.4, 25, 100 and 500 mM, preparing three replicates to test each condition. A control without perchlorate was included and the methane production of the cultures was measured during the following 50 days.

*DNA extraction and quantification*

After exposure to subfreezing temperatures, 1 mL of the thawed cultures was concentrated by centrifugation and treated with 20 µM propidium monoazide (PMA) (Biotium, Hayward, CA). PMA binds the DNA from cells with non-intact membranes, allowing the quantification of DNA from intact cells exclusively in downstream applications. The DNA extraction was performed using UltraClean Microbial DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA).

Regarding the samples exposed to the martian regolith simulants, a quantifiable DNA recovery from these analogue soils was possible for the first time. Each of the inoculated pellets was transferred to an Eppendorf tube and mechanically disassembled in 500 µL of an RNA solution (10 µG mL$^{-1}$). This mix was subjected to gentle agitation in a horizontal vortex for 20 min and centrifuged at 500 g for 2 min and 4°C, the supernatant containing the cells was collected and kept at 4°C. The remaining regolith was washed twice with phosphate buffer ($KH_2PO_4$, $Na_2HPO_4$ 0.2M, pH 8; based on Ogram et al. (1987)) following the steps above and the supernatant was collected and treated with PMA (20 µM) to finally centrifuge the solution at 10000 g for 15 min and 4°C. The supernatant was discarded and the pellet used for DNA extraction with UltraClean Microbial DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA).

The DNA quantification was performed by real-time PCR, also known as quantitave PCR (qPCR), in the cycler CFX Connect (Bio-Rad) using the universal archaeal primer pair from 16S rRNA (5'- 3') Arch751-F CCg ACg gTg AgR gRY gAA and UA1406R Acg ggC ggT gWg TRC AA and the polymerase iTaq™ Universal SYBR® Green Super Mix. The amplification process consisted in 45 cycles of 95°C, 0:20 min (denaturation); 80°C, 0:03 min (annealing); 60°C, 1:30 min (extension). The qPCR was carried out in undiluted conditions for the samples subjected to freezing and using 1:5 dilutions for the DNA extracted from the martian regolith, since it contains certain inhibitors of the amplification process and optimal results were achieved by diluting the sample.

*Methane production analysis*

Methane production was monitored by gas chromatography during the re-incubation phase. The gas chromatographer Agilent 7890A (Agilent Technologies, Santa Clara, CA) was equipped with an HP-PLOT capillary column (Ø 0.53 mm, 30 m in length) and a flame ionization detector. The temperature in the injector was 45°C, whereas the detector reached 250°C. Helium was used as carrier gas. For statistical analysis of the methane production rates, the R project (R Core Team, 2014, Vienna, Austria) was used to perform two-tailed t-tests between samples exposed to S-MRS and P-MRS regolith analogues. An additional analysis of variance (ANOVA) was run per time period and regolith simulant and when $p < 0.001$ a Tukey post-hoc test was performed to find out the strain/s with significantly different parameters.

*Scanning electron microscopy (SEM)*

The images illustrating the cell morphology and aggregation were taken in collaboration with the Robert Koch Institute, Berlin. 50 mL of cell culture were centrifuged at 7900 g for 40 min and 4°C followed by a washing step in 100 mL of distilled water at 4600 g for 30 min and 4°C. Dilutions 1:10 and 1:100 were done for a better observation of the individual cells. One microliter of the suspension was vacuum-dried onto a SiN chip and 5 nm of wolfram (Quorum O150T S, Gala Instrumente GmbH, Bad Schwalbach, Germany) were used for sputter-coating the sample. The sample examination was carried out using a LEO 1530 Scanning Electron Microscope (Carl Zeiss SMT AG, Oberkochen) operated at 3 kV in SE mode (Dalle et al., 2010).
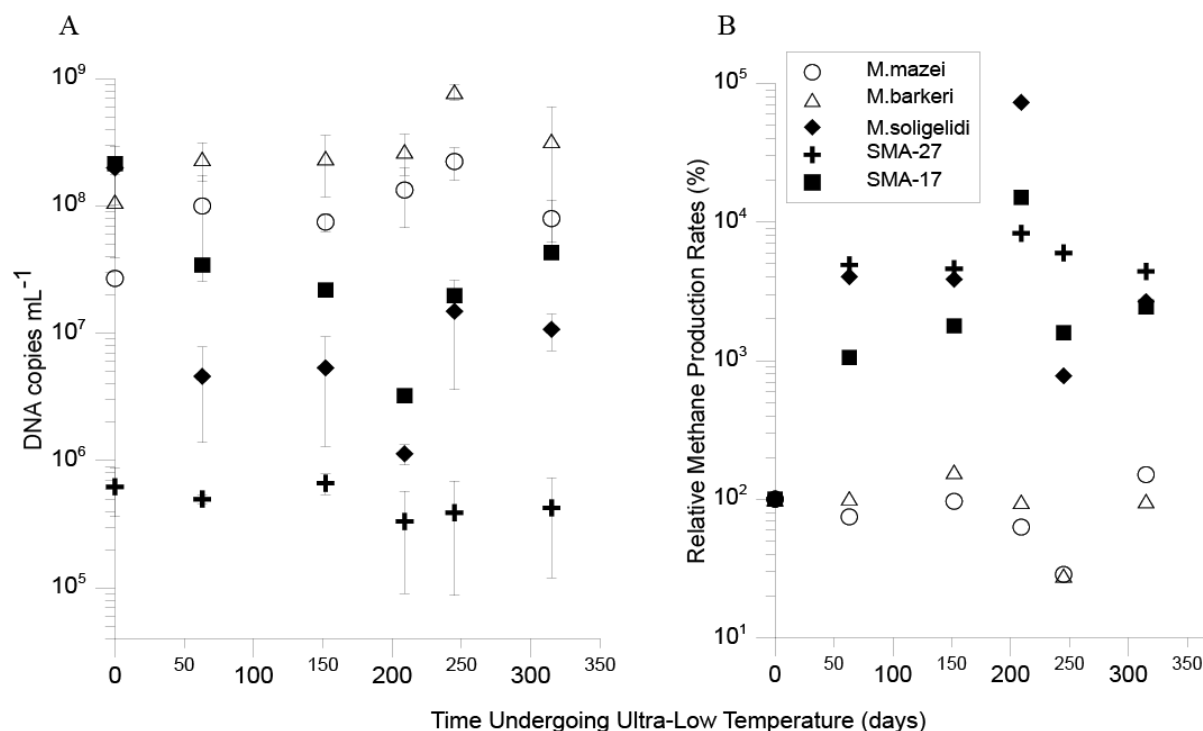
**Results**

*Survival and methanogenic activity after long-term desiccation by exposure to ultra-low subfreezing temperature (-80°C)*

The survival was analysed as DNA copies from cells with intact membranes after the exposure to -80°C during different periods of time. Initial cell numbers in non-frozen control cultures were around $10^8$ cells mL$^{-1}$ for *M. barkeri, M. soligelidi* SMA-21 and SMA-17, $10^7$ cells mL$^{-1}$ for *M. mazei* and $10^5$ cells mL$^{-1}$ in the case of SMA-27 (Fig 1A). The permafrost strains *M. soligelidi* SMA-21 and SMA-17 experienced a significant initial reduction in the DNA copy numbers from living cells after undergoing subfreezing conditions, decreasing three and two orders of magnitude respectively. After the initial drop, they kept constant values until the end of the experiment, except for an unexpected drop that takes place after 209 days of exposure, followed by a recovery. The third permafrost strain of the study, SMA-27, and the non-permafrost strains *M. mazei* and *M. barkeri* kept relatively constant values over the 315 days. The latter showed a significant increase after 245 days of exposure, followed by a drop to the previous DNA values.

The relative methane production rates indicate the methane production per living cell and time unit in regard to the methane production rates in unfrozen conditions (Fig 1B). According to this parameter, the permafrost and the non-permafrost strains presented a different behaviour after the exposure to subfreezing temperatures for different time intervals (Fig. 1B). The permafrost strains, *M. soligelidi* SMA-21, SMA-17 and SMA-27 showed a prominent increase in the methane production rates, reaching levels 10 times and above higher than the non-frozen controls as a response to the cold shock. The activity remained

constant, except for a sudden increase in *M. soligelidi SMA-21* and SMA-17 at 209 days frozen, reflecting the decrease in the DNA copy numbers at that time interval. In contrast, the non-permafrost strains *M. mazei* and *M. barkeri* showed similar methane productions rates than their respective non-frozen controls after the exposure to subfreezing temperatures. A punctual decrease in the activity appeared after 245 days frozen, which correlated with the unexpected increase in the DNA copies.

Data fluctuations in individual events that recovered their stable level in the following time interval (both at the DNA levels and the methane production rates) were assumed as technical artefacts on those punctual samples.



**Figure 1.** Survival and metabolic recovery after desiccation by exposure to ultra-low subfreezing temperatures (-80ºC) up to 315 days. A) DNA copies per millilitre from surviving cells over time. B) Methane production rates relative to the methane levels of the non-frozen controls.

*Survival and methanogenic activity after desiccation by exposure to Mars regolith simulants and simulated martian atmospheric composition.*

DNA was extracted in quantifiable amounts for the first time from S-MRS and P-MRS. Table 1 shows the DNA copy numbers of the concentrated culture deposited per pellet (100 µL) and the DNA amount extracted with our protocol from S-MRS and P-MRS pellets once the culture was adsorbed at the start of the experiments. The DNA recovery from both regolith analogues was only partial, but the DNA levels in both cases were well above the detection limits for quantification by qPCR. SMA-27 experienced a moderate decrease of one order of magnitude for both Mars mineral analogues. The *Methanosarcina* strains (including SMA-17, whose closest relative is *M. soligelidi* SMA-21) showed a decrease of one or two orders of magnitude in the case of P-MRS and their recovery rates dropped four orders of magnitude for S-MRS.

| Methanogenic strain | Control (no regolith) (DNA copies per 100µL) | P-MRS (DNA copies per pellet) | S-MRS (DNA copies per pellet) |
|---|---|---|---|
| SMA-27 | $8.45 \times 10^5$ | $2.11 \times 10^4$ | $1.02 \times 10^4$ |
| *M. soligelidi* SMA-21 | $5.30 \times 10^8$ | $4.53 \times 10^7$ | $2.26 \times 10^4$ |
| SMA-17 | $6.20 \times 10^8$ | $1.04 \times 10^7$ | $1.79 \times 10^4$ |
| *M.mazei* | $1.28 \times 10^8$ | $1.54 \times 10^6$ | $1.06 \times 10^4$ |
| *M.barkeri* | $6.70 \times 10^7$ | $4.16 \times 10^6$ | $8.74 \times 10^3$ |

**Table 1.** Efficiency of the new method for isolation and quantitification of DNA from surviving cells using RNA and phosphate buffer. The colums shows the DNA copy numbers from living cells of each strain present in 100 µL of concentrated culture (control, no regolith) vs DNA copy numbers from living cells recovered from a pellet of P-MRS and S-MRS regolith analogue with an initial inoculum of 100 µL of concentrated culture (P-MRS, S-MRS).

After exposure to the analogue P-MRS (early Mars, phyllosilicatic regolith simulant) and a simulated martian atmosphere for 8 and 16 days, the non-permafrost strains and SMA-27 presented DNA copy numbers within the same order of magnitude than without being subjected to desiccation (Fig 2A). The permafrost strains *M. soligelidi* SMA-21 and SMA-17, however, showed similar DNA levels after 8 days of desiccation compared to the start of the experiment (without desiccation), and these levels experienced a significant reduction of one order of magnitude after 16 days to exposure to martian regoliths. The methane production rates remained constant throughout the experiment for non-permafrost strains, whereas permafrost strains behaved differently (Fig 2B). SMA-17 experienced a progressive decrease in the methanogenic activity, and after 16 days of desiccation the recovery was extremely slow. The methane production rates of SMA-27 increased within the same order of magnitude after 8 days of desiccation followed by a drop to the initial levels after 16 days in Mars desiccating conditions. The methanogenic activity of *M. soligelidi* SMA-21 remained relatively constant after 8 days of desiccation and increased after 16 days of exposure. *M. soligelidi* SMA-21 showed statistical evidence for significantly higher methane production rates both without desiccation (ANOVA ($F_{4,10}= 5.645$ p=0.0149) and post-hoc test significant for *M. soligelidi* SMA-21 - SMA-27 and *M. soligelidi* SMA-21 - *M. barkeri* (p < 0.001)) and after 16 days of desiccation (ANOVA ($F_{4,10}= 79.07$ p= $3.25 \times 10^{-5}$) and post-hoc test significant for *M. soligelidi* SMA-21 (p < 0.001) with no other significances recovered).

The exposure to the analogue S-MRS (late Mars, sulphatic regolith simulant) presented fluctuating values for the DNA copy numbers corresponding to cells surviving desiccation (Fig 2C). Only the strain SMA-27 showed relatively constant DNA levels over the 16 days of desiccation. After 8 days of desiccation, *M. mazei* presented levels comparable to the non-desiccated controls, whereas *M. barkeri* and *M. soligelidi* SMA-21 experienced a remarkable increase and SMA-17 showed a substantial decrease. In terms of the methane production rates (Fig 2D), the non-permafrost strains and SMA-27 showed constant rates after 8 days of desiccation, followed by a moderate decrease after 16 days. SMA-17 presented approximately constant values over the 16 days of desiccation and *M. soligelidi* SMA-21 showed statistical evidence for significantly higher methane production rates both without

desiccation (ANOVA ($F_{4,10}$= 29.88 p= $3.3 \times 10^{-5}$) and post-hoc test significant for *M. soligelidi* SMA-21 (p < 0.001) with no other significances recovered) and after 16 days of desiccation (ANOVA ($F_{4,10}$= 22.03 p= $6.03 \times 10^{-5}$) and post-hoc test significant for *M. soligelidi* SMA-21 (p < 0.001) with no other significances recovered). However, the methane production rates of *M. soligelidi* SMA-21 dropped after 8 days of desiccation. Additionally, the exposure of *M. soligelidi* SMA-21 to S-MRS followed by the re-incubation in medium and $H_2/CO_2$ (80:20, v/v) led in most cases to the progressive development of a shiny black film attached to the inner wall of the bottle, in the region in contact with the medium (not shown). Only the strain SMA-17 showed statistically significant differences between exposure to S-MRS and P-MRS in the methane production rates.

Punctual data fluctuations during the desiccation experiment that increased in the following time interval were assumed as technical artefacts on the experimental setups.

*Shutdown of methanogenesis under exposure to magnesium perchlorate in liquid medium*

The shutdown of the methanogenic pathway in the presence of magnesium perchlorate ($MgCl_2O_8$) was studied by subjecting pure cultures of the methanogenic strains to increasing concentrations (2.4, 25, 100 and 500 mM), followed by monitoring the methane production (Fig. 3). The non-permafrost strains *M. mazei* and *M. barkeri* showed the same activity as the controls when exposed to 2.4 and 25 mM $MgCl_2O_8$. A significant decrease in the activity was evidenced for 100 mM $MgCl_2O_8$ and the complete shutdown of the metabolic activity took place at 500 mM. The permafrost strains SMA-17 and *M. soligelidi* SMA-21 also presented methane production rates similar to the controls for 2.4 and 25 mM $MgCl_2O_8$, although the methanogenic shutdown occurred at 100Mm $MgCl_2O_8$. The results for SMA-27 were not conclusive due to the low methane production rates that characterise this strain even in control conditions. They indicated no significant differences for perchlorate concentrations up to 100 mM. In addition, at 500 mM $MgCl_2O_8$, the methanogenic activity is low but existent, displaying values one order of magnitude higher than those for the other strains when exposed to 500 mM $MgCl_2O_8$, despite the moderate basal rates characteristic of SMA-27.

Exposure to P-MRS
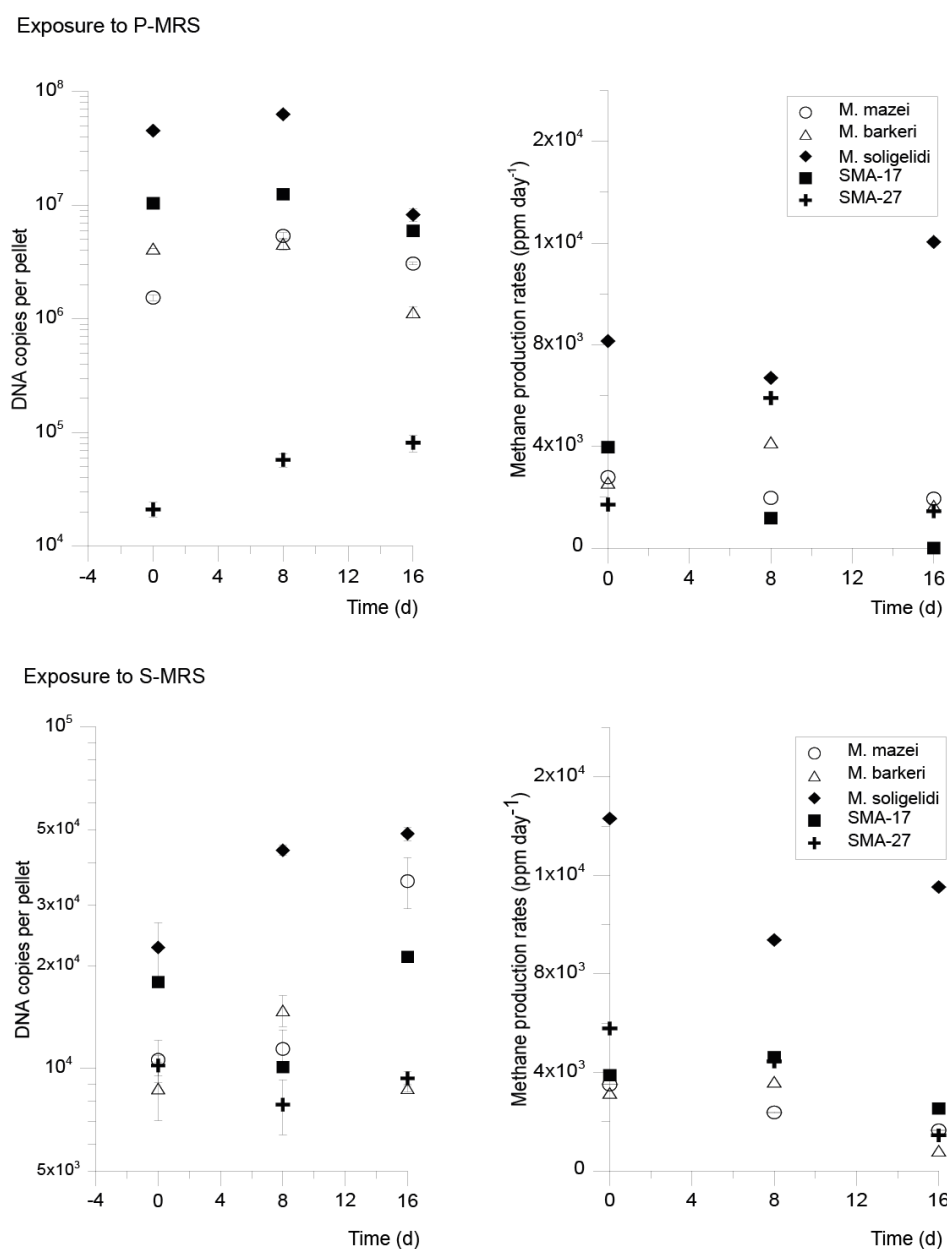


Exposure to S-MRS



**Figure 2.** Survival and metabolic recovery after desiccation by exposure to one Mars regolith analogue (P-MRS or S-MRS) and a simulated martian atmosphere composition. A) DNA copies extracted from P-MRS pellets B) Methane production rates after the exposure to P-MRS C) DNA copies extracted from S-MRS pellets D) B) Methane production rates after the exposure to S-MRS.

*Cell morphology and aggregation*

Figure 4 shows the SEM images of the five strains of this study. SMA-27 cells (Fig. 4A) appear as elongated rod-shaped cells and they seem to group to a certain extent. The *Methanosarcina* species (Fig 4B-E) are cocci of the same size (~1µm) and, at least in culture conditions, the non-permafrost strains *M. mazei* and *M. barkeri* form large aggregates, whereas *M. soligelidi* SMA-21 and SMA-17 often appear as single cells or small sarcina-like clusters.
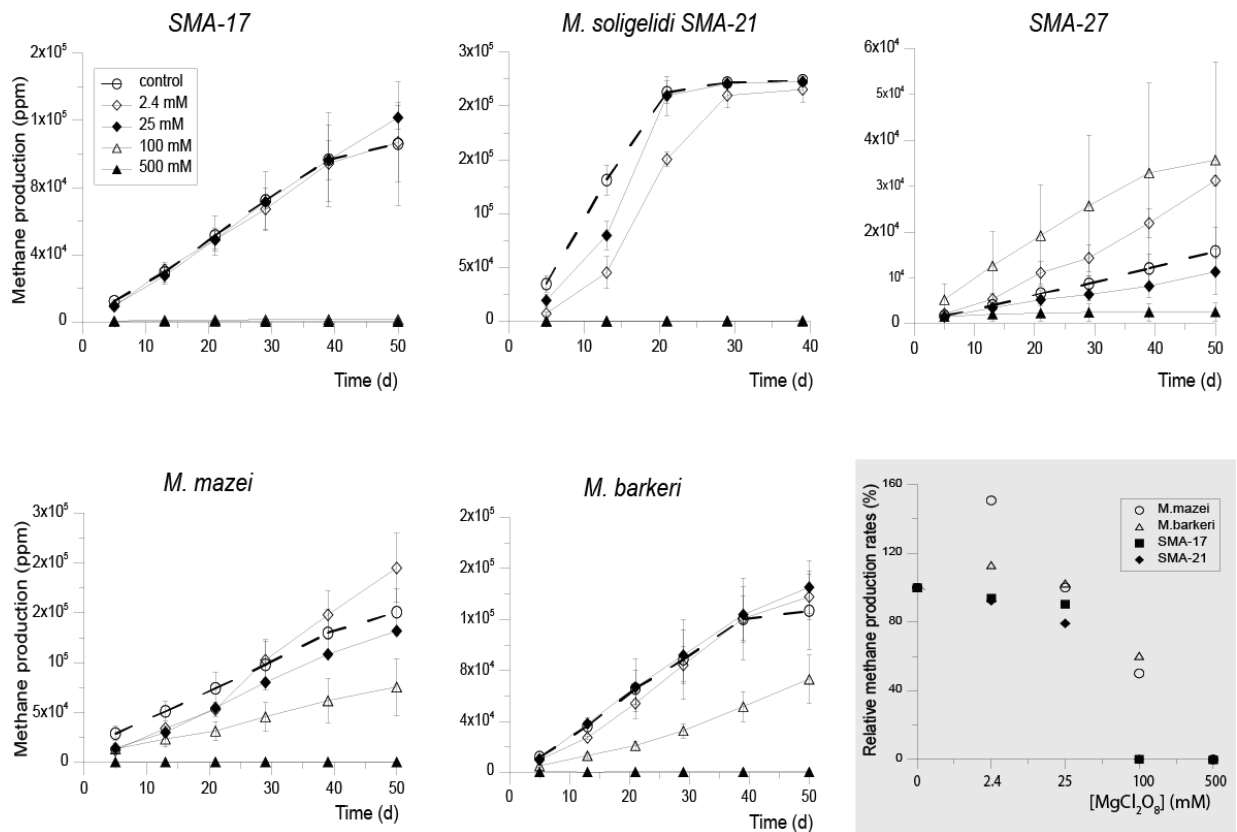
**Figure 3.** Methane production under different concentrations of magnesium perchlorate (2.4, 25, 100 and 500mM). The bottom right chart displays the relative methanogenic rates of the four Methanosarcina strains at different concentrations of perchlorate.
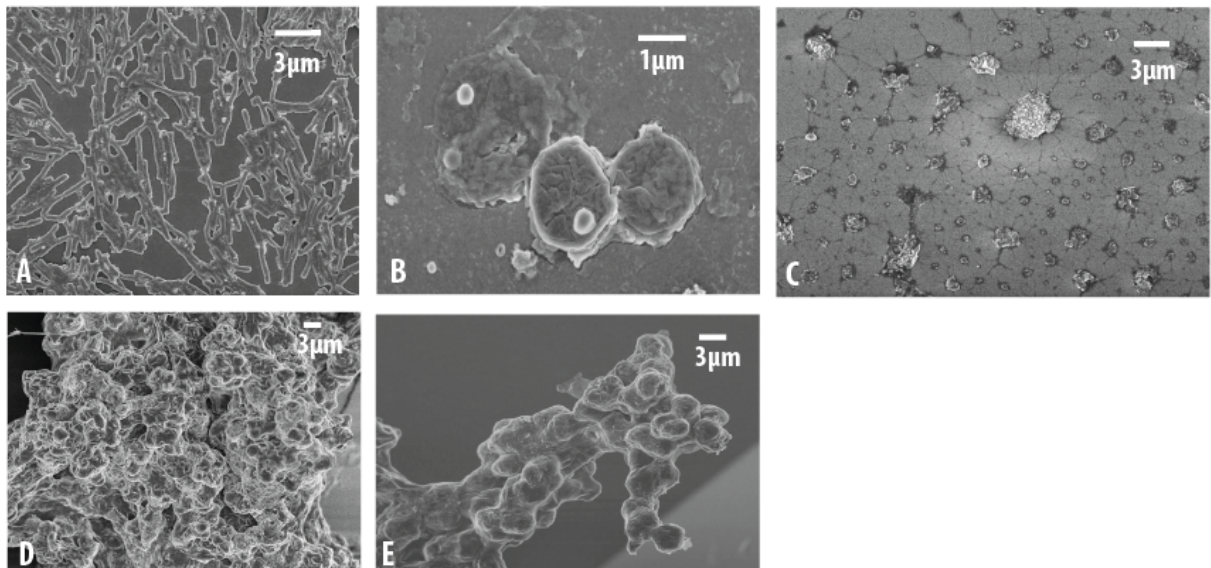


**Figure 4.** Scanning electron microscopy images of the studied strains in culture conditions A. SMA-27, B. SMA-17, C. *Methanosarcina soligelidi* SMA-21, D. *M.mazei* and E. *M. barkeri*. Courtesy of the Robert Koch Institute.

**Discussion**

Microorganisms have survived in permafrost environments at subzero temperatures for hundreds of thousands to millions of years (Gilichinsky, 2002a). For instance, in the Siberian Arctic, the soils are permanently frozen with the exception of the thin top layer, denominated active layer, that experiences seasonal freezing-and-thawing cycles with temperatures ranging from -45°C to 25°C. In this study, we found that the permafrost strains, adapted to extreme cold and this fluctuating environment, showed relative methane production rates ten times (and above) higher after their exposure to -80°C for up to 315 days, than in control conditions (Fig 1B), whereas their non-permafrost relatives just presented a similar methanogenic activity than in control conditions. This fact suggests that permafrost strains are able to cope better with extreme cold, even at temperatures well below the ones reported in their natural habitat. Cold-adapted archaea naturally have a variety of adaptations to deal with the difficulties that come along with exposure to cold, like intracellular ice formation, membrane fluidity, genetic expression, reduced enzyme activity and protein function, and altered transportation of nutrients and waste products (D'Amico et al., 2006). The adaptations aim to preserve the structural integrity, cover the necessities for metabolism, and regulate it in response to environmental changes (Cavicchioli et al., 2000). Previous studies indicated that the permafrost and non-permafrost strains in this study have a different chemical composition in terms of aliphatic chain composition of lipids, aromatic amino acids and carbohydrates among others (Serrano, unpublished data), which may be related to cold adaptive mechanisms and therefore a differential post-freezing recovery in the two groups. The freezing process might have activated a cold-shock or heat-shock response, that consists on the transient over- expression of cold-shock proteins (Csps) and heat-shock proteins (Hsps) respectively, which mediate cellular processes such as transcription, translation, protein folding and the regulation of membrane fluidity (Phadtare, 2004). As a result, a higher metabolic activity was recorded after the exposure to cold, a feature that theoretically may be hugely beneficial in Mars due to the short-term periods of liquid water (Christensen et al., 2003). However, non-permafrost methanogenic strains together with the permafrost strain SMA-27 kept their membranes intact to a greater extent than the permafrost strains (Fig 1A). SMA-27 presented an extremely slow growth, which makes it not comparable to the rest of the permafrost strains in this study, *M. soligelidi* SMA-21 and SMA-17. The freeze-thawing process represents a double stress for the cells: thermal and osmotic stress act simultaneously as the cells cool down (Muldrew and McGann, 1990). The cooling rate is the main factor conditioning cell viability (Dumont et al., 2003), although its influence is not relevant for this study since all the strains were identically frozen and defrosted. Another important factor for cryopreservation is the cell size, which defines the surface-to-volume (S/V) ratio and directly determines the transfer surface for intracellular heat and mass (Dumont et al., 2004). In this context, the differences evidenced in the membrane integrity may find plausible explanations in the cell morphology, the phenomenon of cell aggregation and the presence of antifreeze proteins (AFPs). Figure 4 shows the SEM images of the five strains of this study, evidencing large aggregate formations for the non-permafrost strains, whereas the permafrost strains *M. soligelidi* SMA-21 and SMA-17 (cocci) occasionally group and SMA-27 appear as rods that often stick together. According to the cell morphology, the cylindrical shape of SMA-27 cells allows a substantially larger S/V ratio compared to round cocci and thus a smoother freezing process is expected. On the other hand, the phenomenon

of cell aggregation shown by the non-permafrost strains has proven to be a microbial survival strategy that leads to favourable forms of life, since they live as a synergistic microconsortium (Wingender et al., 1999). In addition, biofilm cells tolerate higher concentrations of many biocides (Flemming and Wingender, 2001) and the formation of biofilms may protect the organisms from ice-crystal damage (Thomas and Dieckmann, 2002). Nevertheless, it must be considered that the morphology might have changed while being exposed to subfreezing temperatures, since cold-adapted archaea have previously shown to form multicellular aggregates embedded in EPS after exposure to subfreezing conditions (Reid et al., 2006). AFPs are able to bind to ice crystals through a large complementary surface, creating thermal hysteresis and allowing the organisms to survive at lower temperatures (Jia and Davies, 2002), and in this case, they might help the cells to withstand the exposure to ultra-low subfreezing temperatures.

With regards to the second kind of simulated martian desiccation by exposure to Mars regolith analogues and atmospheric composition, this study presents a relevant innovation from a technical perspective, since we were able to extract quantifiable amounts of DNA from surviving cells from two broadly used Mars regolith simulants for the first time. P-MRS and S-MRS  regolith analogues are broadly used in Mars simulation experiments (e.g. BIOMEX space experiment (de Vera et al., 2012)). For instance, Baque et al. (2013) were able to extract the DNA from dried cells mixed with S-MRS and subjected to desiccation, although it remains unknown whether the DNA belonged exclusively to viable cells or originated from lysed cells. The proposed method involves a step prior to the DNA isolation, where the sample is treated with RNA (10 $\mu G\ mL^{-1}$) and phosphate buffer (0.2 M, pH 8) as competitors for the DNA binding sites within the regolith, given their stereochemical and electrostatic similarities with the DNA molecule. Nevertheless, the efficiency of the DNA recovery is not complete and it is three orders of magnitude higher for P-MRS than for S-MRS in the case of *Methanosarcina* species. The lower DNA recovery yields from S-MRS might be due to the presence of anhydrous iron oxides on the late-Mars regolith analogue. The DNA molecule has two binding sites for iron (III) (Netto et al., 1991), forming chelation complexes that might prevent the correct DNA isolation and subsequent amplification.

Concerning the fluctuations in the DNA copy numbers from surviving cells, sometimes showing higher copy numbers in desiccating conditions than unexposed (Fig. 2A, 2C), the authors propose two possible explanations. The first one is related to the minor differences in the adsorption of the inoculum deposited in each pellet, e.g. pellets adsorb the inoculum to a different extent according to minimal variations in their local morphology and thickness.  The second factor that could explain the fluctuations could be a differential efficiency of the PMA treatment due to dissimilarities in the turbidity of the samples, in the sense of different numbers of regolith particles in the solution. A different turbidity would lead to the differential light penetration in the final step of the PMA treatment and therefore would result in different binding ratios of the accessible DNA to the PMA that would ultimately reflect on the DNA quantification.

The methane production rates after the exposure to simulated martian regoliths and atmosphere declined in general after 16 days of desiccation (Fig 2B, 2D). *M. soligelidi* SMA-21 shows statistically significant values for a higher methane production rate compared to the rest of the strains in the presence of both S-MRS and P-MRS,  for the controls and after 16 days of desiccation. A considerable drop after 8 days of desiccation was also observed in

both Mars regolith analogues (not correlated with a decrease in the cell survival), suggesting that desiccation has a drastic initial effect on the metabolic recovery of *M.soligelidi* (SMA-21). However, it showed a strong resilience: after 16 days of desiccation, the methane production rates were higher than at 8 days in both regolith analogues, and in the case of exposure to P-MRS, higher even than the initial levels without desiccation. The rest of the strains showed relatively constant levels after 8 days of desiccation (with a higher variability on P-MRS), followed by a general drop in the levels, after 16 days. *M. soligelidi* SMA-21 is therefore the strain of this study with the best metabolic adaptation to both Mars regolith simulants, which might be related to the modification of some enzymes following the exposure to the stress. In addition, the black film that developed in the presence of S-MRS suggests the precipitation of the iron present in the 'late Mars' regolith (likely as iron sulphide) driven by the microbial activity. This film was only observed in the presence of *M. soligelidi* SMA-21 though, possibly due to its high metabolic rate.

Only SMA-17 showed significant differences in the methanogenic activity between S-MRS and P-MRS, which might be explained by technical irregularities, since the P-MRS samples were accidentally in contact with oxygen, so this difference is not conclusive.

The perchlorate experiment illustrates a first approach of the survival threshold of methanogens in the presence of magnesium perchlorate (Fig. 3), evidencing that the non-permafrost strains are more resistant and experience a complete metabolic shutdown in medium containing up to 500 mM $MgCl_2O_8$ *vs* the permafrost strains *M. soligelidi* SMA-21 and SMA-17 that stop their metabolic activity at 100 mM $MgCl_2O_8$, of the conditions hereby tested. We hypothesise that the reasons underlying the differences in the resistance to perchlorate rely on the higher likelihood of *M. mazei* and *M. barkeri* to form aggregates that protect the cells against adverse conditions, which are likely related to the reported differences in the chemical composition between permafrost and non-permafrost strains (Serrano, unpublished data), as already discussed. The average concentration of perchlorate reported at the Phoenix landing site was 2.4 mM (Hecht et al., 2009) and at this concentration, all strains showed methane production rates similar to control conditions (only *M. mazei* showed significantly higher rates). Despite perchlorate is a strong oxidant, in solution it is highly soluble and remains chemically stable (Urbansky, 1998), while in a solid state in combination with minerals perchlorate is expected to be more reactive. On Earth, the occurrence of natural perchlorate was long ago described in the Atacama desert, Chile (Ericksen, 1981), and recently reported in the McMurdo Dry Valleys in Antarctica (Jackson et al., 2012). Both systems are inhabited by a variety of microbial communities (Brambilla et al., 2001; Parro et al., 2011), fact that suggests that life forms have adapted and are able to withstand the detrimental effects of perchlorate. SMA-27 presents an extremely slow growth (Fig. 3) and the results obtained in this test evidenced cell survival and active methane production, although due to the low growth rates, the methanogenic activity cannot be compared with the rest of the strains. It is worth to mention that at the concentration of 100 mM $MgCl_2O_8$, it reaches the highest levels of methane production and at 500 mM methane production occurs and its levels are above those of the *Methanosarcina* strains, suggesting that it did not yet experience a complete metabolic shutdown. The findings in this study are consistent with those reported by Goodhart and Kral (2010), who exposed several methanogens to perchlorate concentrations up to 1% (45 mM) and registered methane production in similar levels to the controls.

In conclusion, this study shows that methanogenic archaea are metabolically active and able to survive in high numbers after the long-term exposure to stable subfreezing temperatures that may occur in the martian subsurface, the combination of a martian regolith simulant with the chemical composition of early/late Mars together with a simulated atmospheric composition, and the presence of perchlorate in culture medium at the concentration reported in the Phoenix landing site. The Siberian permafrost strains pose some advantages for a more active methanogenesis after undergoing ultra-low subfreezing temperatures and resist the presence of perchlorate at reported martian concentrations. *M. soligelidi* SMA-21 shows an outstanding methanogenic activity after exposed to simulated martian regolith analogues and atmosphere for up to 16 days, together with the highest survival, based on DNA from intact cells. These facts make *M. soligelidi* SMA-21 the most successful methanogen reported to date for potential survival in Mars conditions. On the other hand, non-permafrost strains show a higher proportion of cells with intact membranes when subjected to long-term freezing, and also experience metabolic shutdown at a higher concentration of perchlorate than the permafrost strains, presumably due to the fact that they often form large aggregates, which may be relevant for cell survival. Furthermore, a new method to isolate and quantify DNA from cells with intact membranes embedded in martian regolith was presented. The authors conclude that the findings of this investigation support previous studies on methanogenic archaea under Mars-like conditions, proving that methanogens are good model organisms for potential life forms on the martian subsurface.

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**Single-cell analysis of the methanogenic archaeon *Methanosarcina soligelidi* from Siberian permafrost by means of confocal Raman microspectrocopy for astrobiological research**

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**Keywords**

**Abstract**

Methanogenic archaea from Siberian permafrost are suitable model organisms that meet many of the preconditions for survival on the martian subsurface. These microorganisms have proven to be highly resistant when exposed to diverse stress factors such as desiccation, radiation and other thermo-physical martian conditions. In addition, the metabolic requirements of methanogenic archaea are in principle compatible with the environmental conditions of the Red Planet. The ExoMars mission will deploy a rover carrying a Raman spectrometer among the analytical instruments in order to search for signatures of life and to investigate the martian geochemistry. Raman spectroscopy is known as a powerful nondestructive optical technique for biosignature detection that requires only little sample preparation. In this study, we describe the use of confocal Raman microspectroscopy (CRM) as a rapid and sensitive technique for characterization of the methanogenic archaeon *Methanosarcina soligelidi* SMA-21 at the single cell level. These studies involved acquisition of Raman spectra from individual cells isolated from microbial cultures at different stages of growth. Spectral analyses indicated a high degree of heterogeneity between cells of individual cultures and also demonstrated the existence of growth-phase specific Raman patterns. For example, besides common Raman patterns of microbial cells, CRM additionally revealed the presence of lipid vesicles and $CaCO_3$ particles in microbial preparations of *M. soligelidi* SMA-21, a finding that could be confirmed by electron microscopy. The results of this study suggest that heterogeneity and diversity of microorganisms have to be considered when using Raman-based technologies in future space exploration missions.

## 1. Introduction

Methanogenic archaea are anaerobic microorganisms that produce methane gas as a metabolic byproduct. Several novel strains were recently isolated from Siberian permafrost in the Lena Delta (Russia). They present a remarkable resistance against desiccation, osmotic stress, low temperatures and starvation when compared with methanogenic archaea from non-permafrost environments (Morozova and Wagner, 2007). In addition, these methanogens from Siberian perma- frost are able to survive simulated thermo-physical martian conditions (Morozova et al., 2007) as well as high doses of UV-C and ionizing radiation (Wagner, personal communication), making them model organisms for potential past or present life on the martian subsurface.

Furthermore, the Mars Express Orbiter and the Fourier Transform Spectrometer at the Canada–France–Hawaii Telescope reported the presence of methane in some areas of the Red Planet (Formisano et al., 2004; Lefèvre and Forget, 2009; Mumma et al., 2009), although this subject remains controversial and a more recent study defended the lack of compelling evidence for methane on Mars (Zahnle et al., 2011). If methane were present, its origin could be either biotic, as a product of the methanogenic activity, or abiotic, resulting from geological processes such as serpentinisation etc. (Krasnopolsky et al., 2004).

The ExoMars mission, foreseen to be launched in 2018 by ESA and with a possible collaboration of Roscosmos and NASA, will investigate the martian surface and subsurface searching for possible biosignatures of past or present martian life (European Space Agency, 2012a). It will include a Raman Laser spectrometer (RLS) among other analytical instruments, emphasizing the increasing importance of Raman spectroscopy in biosignature description. Further investigation on biosignature identification of microorganisms by means of Raman has therefore become an emerging challenge for space exploration in the context of life detection. As part of the "Biology and Mars Experiment" (BIOMEX) project, the creation of an international Raman biosignature database of terrestrial extremophilic organisms has been proposed (de Vera et al., 2012).

Raman spectroscopy is a vibrational spectroscopic technique that has demonstrated a relevant potential as a microbial identification technique in recent years (Hermelink et al., 2009; Puppels et al., 1990). It is a powerful molecular structural tool that provides fingerprint-like information about the overall chemical composition of investigated microbial samples (Harz et al., 2009; Naumann et al., 1991). Moreover, it allows a nondestructive investigation and requires only minimal sample preparation. Confocal Raman microspectroscopy (CRM) allows the rapid detection and identification of the microorganism compared to the classical approach of microbial diagnosis based on isolation and culturing as well as modern molecular techniques (Harz et al., 2009). In addition, it allows the detection of biochemicals as possible signature of extinct or fossilized life (Edwards et al., 2012). CRM combines a dispersive Raman spectroscopy setup with a confocal microscope equipped with high numerical aperture objectives, enabling to visualize individual cells. This setup allows the diffraction-limited investigation of the microbial samples in respect of their chemical nature (Harz et al., 2009; Krause et al., 2008).

Diverse microorganisms and their molecular composition have been successfully characterized to the species level using CRM (Hermelink et al., 2009, 2011; Rösch et al., 2005). However, most of them are disease-associated microorganisms. In recent years, Raman studies concerning organisms inhabiting extreme environments that have certain

relevance for astrobiology have become more popular, focusing on the detection of photoprotective pigments and compatible solutes as biomarkers (Jehlicka et al., 2012; Vítek et al., 2010) rather than on the microbial characterization based on the biomolecules.

In this first-ever Raman study on methanogenic archaea, we aim to gain a deeper insight into the biosignatures of these microorganisms by means of CRM. The recently described strain from Siberian permafrost, *Methanosarcina soligelidi* SMA-21, has proven to be particularly resistant by surviving the hardest conditions in all the performed tests and was therefore selected for this study (Wagner et al., 2013). *M. soligelidi* SMA-21 was investigated at four points of the microbial growth (early and late exponential phase, stationary phase and senescent phase) in order to obtain information about its chemical composition as well as to study the phenotypic heterogeneity at the single-cell level.

## 2. Materials and methods

### 2.1. Microbial cultures

The strain used in this study is *M. soligelidi* SMA-21, isolated using serial dilution techniques from permafrost sediment samples from the Lena delta, Siberia (Russia). The isolation procedure is described in detail by Wagner et al., 2013. This archaeon appears as irregular cocci of about 1 mm in diameter. Phylogenetic studies have revealed that the closest relative described to date is *Methanosarcina mazei*, with a 99% homology in the 16 S rRNA sequences (Wagner et al., 2013). Cell aggregation is often observed. For cultivation, sealed bottles containing 50 ml of MW anaerobic medium were used (per liter): $NH_4Cl$, 1.0 g; $MgCl_2d6H_2O$, 0.4 g; $CaCl_2d2H_2O$, 0.1 g; $NH_4Cl$, 0.25 g; KCl, 0.5 g; $KH_2PO_4$, 0.2 g; $NaHCO_3$, 2.7 g; Cysteine, 0.3 g; $Na_2S$, 0.2 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). The cultures were pressurized with $H_2/CO_2$ (80:20v/v, 150 kPa) as substrate and incubated at 28 1C. Cells were harvested from the medium for spectroscopic and microscopic examination at four points in time, according to the $CH_4$ production: early exponential phase (5% $CH_4$), late exponential phase (14% $CH_4$), stationary phase (20% $CH_4$) and senescent phase (20–30% $CH_4$, 5 months after inocula- tion). Note that the growth of permafrost strains is very slow: a culture inoculated with 5 mL and grown without shaking reaches the stationary phase in around 14 days. Fresh and old sterile medium were used as blank and negative controls respectively.

Two hundred milliliters of culture was centrifuged at 7900g for 40 min and 4 1C and washed twice in 200 mL of distilled water at 4600g for 30 min and 4 1C. Seven microliters of the cell suspension was air-dried onto a $CaF_2$ slide, previously diluted 1:10 and 1:100 for a better observation of the single cells.

### 2.2. Confocal Raman microspectroscopy (CRM)

Raman spectra were obtained using a WITec (Ulm, Germany) Model alpha 300R confocal Raman microspectroscope (CRM). The CRM was equipped with an ultra-high throughput spectrometer (UHTS300) and a back-illuminated EMCCD camera (Andor Tech-nology PLC, Belfast, Northern Ireland) as detector. An apochro- matic Nikon (Tokyo, Japan) E Plan (100 /0.95) objective with a working distance of 0.230 mm and an excitation wavelength of 532 nm (frequency doubled Nd-YAG laser; 35 mW laser power) was used for

all measurements presented in this paper. The laser power at the sample was estimated to be $1 mW. Raman back- scattered intensity was collected via the objective, passed the edge filter and was subsequently focused into a multimode optical fiber with a core diameter of 50 mm, which acts as the entrance slit (pinhole) of the microspectrometer. The Raman experiments were conducted at diffraction limited conditions of about 320nm laterally and an integration time of 5 s per spectrum. A grating of 600 lines/mm was used, giving point spacing between 3 and 9 1/cm in the resulting Raman spectra. We performed a minimum of 20 measurements of single cells for each of the four selected points of the microbial growth, with 10 accumulations under full pixel binning and without gaining at the camera.

For hierarchical clustering spectra were first subjected to a cosmic ray removal procedure and then individually exported via an ASCII interface into OPUS 5.5 (Bruker Optik GmbH, Rheinstetten, Germany). Further pre-processing involved a quality test (test for signal noise ratio and pre-selection of the cell-based spectra based on the principal components of the spectrum), the application of a first derivative Savitzky–Golay smoothing/ derivative filter with nine smoothing points and vector normalization. Spectral distances between pairs of individual spectra were obtained on the basis of information in the 796–1854 and 2746–3205 cm$^{-1}$ spectral regions as D-values derived from normalized Pearson's product momentum correlation coefficient (Naumann, 2000). The normalization prevents negative values and permits a value variation between 0 (r1⁄41: high correlated data/ identify), 1000 (r 1⁄4 0: uncorrelated data) and 2000 (r 1⁄4 _ 1:anti- correlated spectra) (Moss et al., 2010). Average linkage was used as the clustering method. For cluster analysis, spectra from the early and late exponential growth phase were merged into the group named "exponential phase".

### 2.3. Scanning electron microscopy (SEM)

For SEM, 50 mL of culture were centrifuged at 7900g for 40 min and 4 1C and washed in 100 mL of distilled water at 4600g for 30 min and 4 1C. One microliter of the cell suspension was vacuum-dried onto a SiN chip, previously diluted 1:10 and 1:100 for a better observation on the single cell level. The sample was sputter-coated with 5 nm of wolfram (Quorum O150T S, Gala Instrumente GmbH, Bad Schwalbach, Germany). The samples were examined using a LEO 1530 Scanning Electron Microscope (Carl Zeiss SMT AG, Oberkochen) operated at 3 kV in SE mode (Dalle et al., 2010).

## 3. Results
### 3.1. Phenotypic heterogeneity

Initially, the prepared microbial samples showed a high spectroscopic diversity, although the particles looked almost the same in the bright field microscope (Fig. 1). Raman spectral heterogeneity was observed among particles from cultures in different phases of growth and within the same stage of growth giving rise to a growth-phase specific Raman pattern. Heterogeneity within one phase of growth is illustrated in Fig. 1, corresponding to particles of a culture undergoing the exponential phase. The band assignment for the different spectra in Fig. 1 points to the existence of several subpopulations: in some of the cells protein is the predominant component, whereas in others either lipids are more abundant. However, the majority of the cells show a combination of the protein-based and the lipid-based spectra,

mixed in different proportions depending on the cell. In other words, an average cell shows a spectrum where all the biomolecules are represented in variable moderate proportions. Note that different subpopulations present a similar morphology.
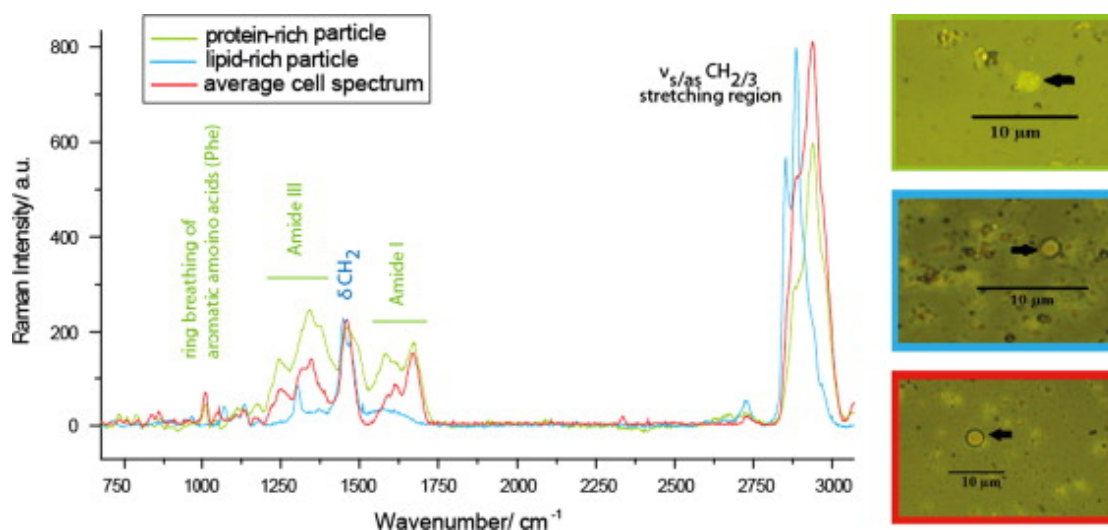


**Fig. 1.** Raman spectra and confocal microscope images showing the chemical heterogeneity of cells from a culture of *M. soligelidi* SMA-21 undergoing the exponential phase of growth. Some particles have a predominant component such as protein or lipid, whereas most cells show a combination of the spectra from different molecules, evidencing several subpopulations in the culture. All particles show a similar shape in the bright field microscopic images, despite the different chemical composition.

### 3.2. Changes in cell composition and morphology over time

The Raman biosignatures of *M. soligelidi* SMA-21 change over time as the archaeal cultures become older, evidencing changes in the composition of the culture colony lifetime, as it undergoes the three classical developmental states of a terrestrial microbial sample. An overview of the spectra corresponding to the four selected time points over the growth of the strain (early and late exponential phase, stationary and senescent phase) can be found in Fig. 2, which depicts the inter-phase diversity reflected in the corresponding Raman signatures. In all phases, the signal with the highest Raman intensity is the $CH_2$ stretching vibration around 2936 $cm^{-1}$. Raman modes of proteins can be found at 1669 $cm^{-1}$ (amide I) and at 1344 $cm^{-1}$ (amide III) and the aromatic amino acids tyrosine and tryptophan depict a band at 1338 $cm^{-1}$ (which also corresponds to signatures of the nucleotides adenosine monophosphate, AMP and guanosine monophosphate, GMP). Phenylalanine and tryptophan show an additional band at 1008 $cm^{-1}$, attributed to the symmetric benzene/pyrrole in- phase and out of phase breathing modes. All the mentioned bands are present and slightly change in bandwith, position and intensity over the growth of M. soligelidi. However, the spectral region evidencing the most relevant differences is located between 950 and 1200 $cm^{-1}$. Within this region, the band at 1008 $cm^{-1}$, assigned to the amino acids tryptophan and phenylalanine, increases over time and reaches the highest level in the stationary phase. In the same way, the characteristic CO symmetric stretching band observed at 1054 $cm^{-1}$ and the band at 713 $cm^{-1}$, presumably corresponding to carbonates (Frost et al., 2003; Buzgar N. and Buzatu, 2009)

show a gradual increment over time reaching its maximum in the stationary phase and maintaining these levels over the senescent phase. This region, together with the band at 1172cm$^{-1}$, is characteristic from the D-ribose and 2-deoxy- D-ribose (Mathlouthi and Seuvre, 1983). This band at 1172 cm$^{-1}$ also corresponds to the amino acid tyrosine (Takeuchi et al., 1989) and it shows its maximum in the late exponential phase.

These changes over time are also evidenced in the cell morphology when observed under the scanning electron microscope (SEM, Fig. 2b). The cell size is constant during the exponential and stationary phase, but the cell surface becomes smoother as the culture ages. In addition, the presence of small vesicles and crystalline structures around the cells is especially remarkable during the stationary phase, as shown in Fig. 2. During the senescent phase the cells become slightly smaller.
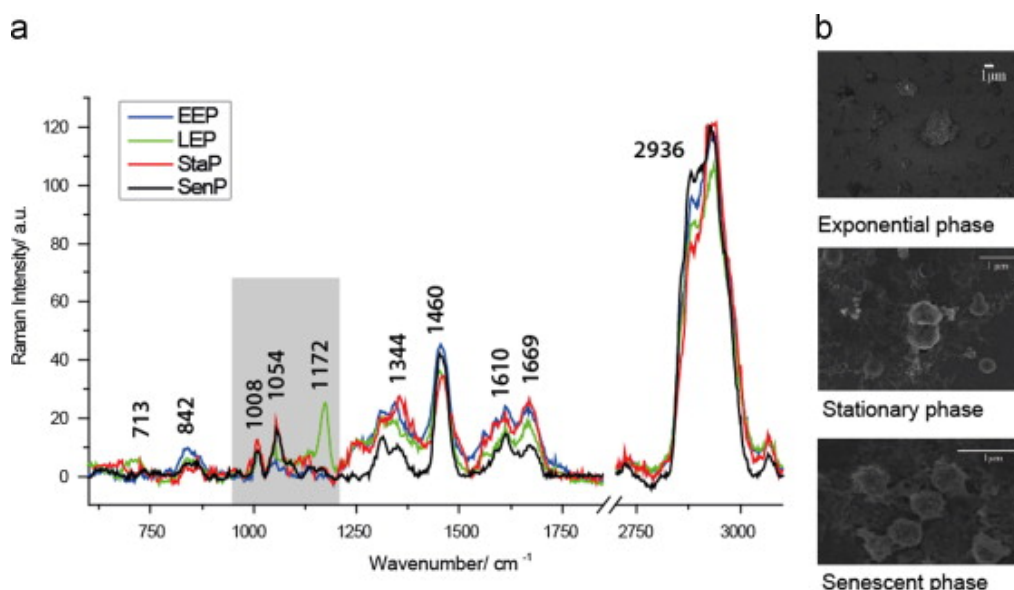


**Fig. 2.** Spectral and morphological diversity of *M. soligelidi* SMA-21 cells from cultures undergoing different phases of growth. (a) Raman spectra of the methanogenic archaea *M. soligelidi* SMA-21. Four timepoints over the microbial growth were selected for this study (early exponential phase – EEP, late exponential phase – LEP, stationary phase – StaP and senescent phase – SenP). Changes in the cell composition over time are evidenced between 950 and 1200 cm$^{-1}$. The protein (1008 cm$^{-1}$) and carbonate (1054 cm$^{-1}$) levels reach their maximum in the stationary phase, while the RNA (1172 cm$^{-1}$) is most abundant in the late exponential phase. (b) SEM images showing the morphological changes that *M. soligelidi* cells experiment over growth. Note the cell aggregate in the center of the top image, corresponding to a culture in exponential phase, and the presence of small smooth vesicles (lipid bodies) and crystalline structures (presumably carbonate) in the cells undergoing the stationary phase. In the senescent phase, the cells show a considerable reduction of their size.

### 3.3. Detection of microbially induced products

During the measurements, some Raman active bodies that were neither cells nor medium were detected. The size thereof was approximately 200–300nm and they show characteristic Raman spectra corresponding in some cases to the mineral calcite (CaCO3), depicting bands at 713, 1089 cm$^{-1}$ and in other cases to pure lipid, with prominent bands in the regions 1063–1125 cm$^{-1}$, 1290, 1437, 1655 and 1753 cm$^{-1}$. None of these spectra were found in fresh/old sterile media. The microscope images confirm that some of these particles have a crystalline structure (CaCO3), whereas others are perfectly round and with a smooth surface, attributed to lipid bodies (Fig. 3).
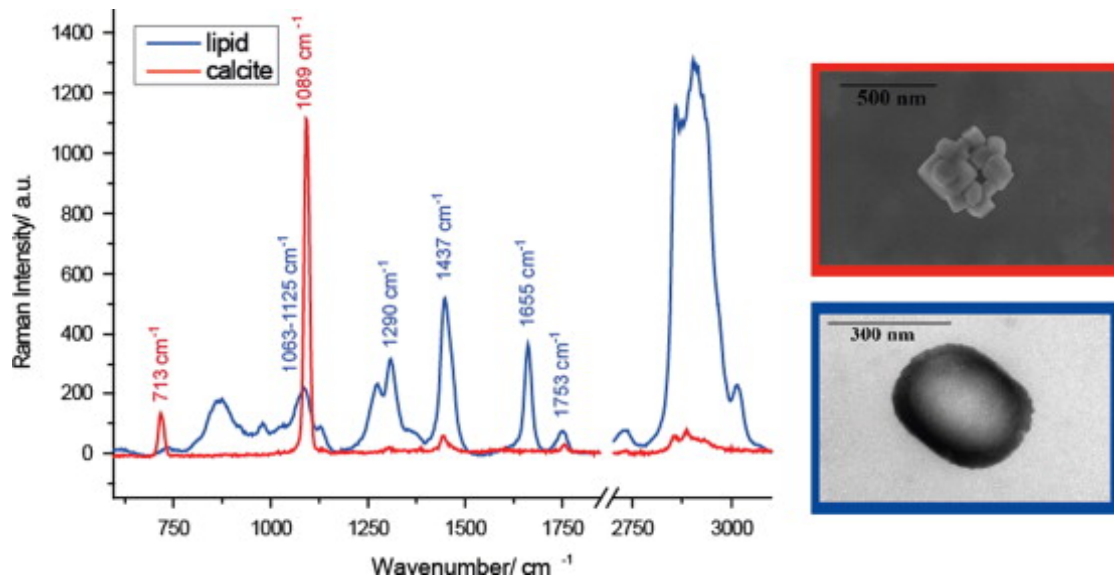
**Fig. 3.** Confocal Raman spectra and SEM images of the microbially-induced *in situ* detected products of *M. soligelidi* SMA-21: calcite (CaCO$_3$) and lipid bodies.

## 3.4. Cluster analysis

The cluster analysis demonstrates Raman spectroscopic differ- ences in the cells of *M. soligelidi* SMA-21 in different stages of growth (Fig. 4). The spectra corresponding to the cells in the same stage of growth cluster together, with the exception of three spectra from the exponential phase. These spectra are more similar to spectra of cells in stationary phase and are separated from other exponential phase spectra by the distance of 88 D- value units (Naumann, 2000). The spectra, and therefore the composition of the cells in the stationary phase, are more similar to spectra from cells in the exponential phase of growth, which are separated by a distance of 112 D-value units within one large cluster. Finally, the total distance between *M. soligelidi* SMA-21cells in senescent phase and the cluster exponential-stationary phase is 182 D-values.
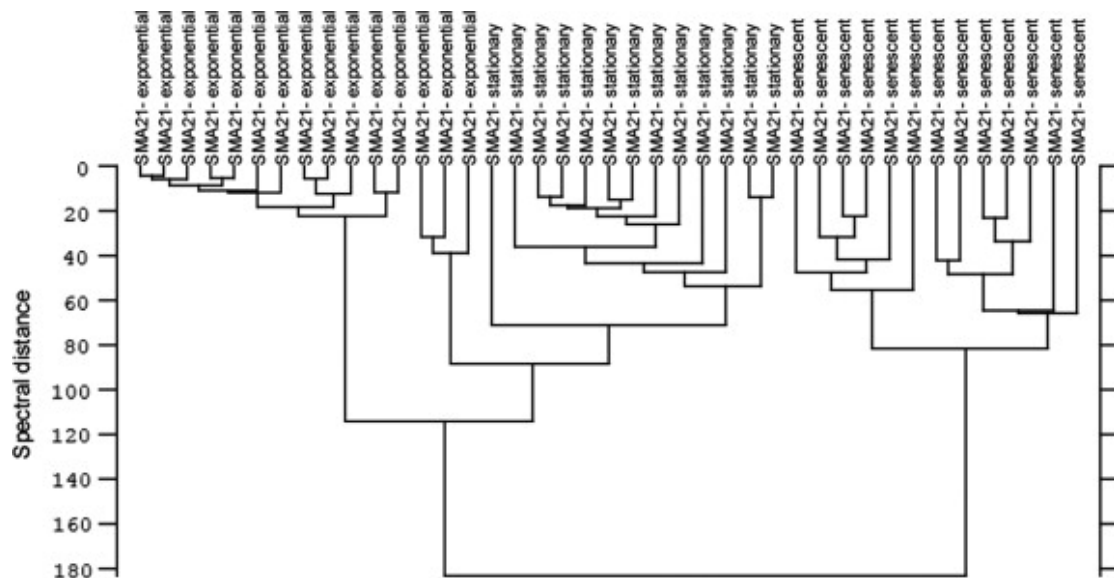
**Fig. 4.** Unsupervised hierarchical cluster analysis of CRM spectra obtained from individual cells of *M. soligelidi* SMA-21 in different growth phases. Spectra from individual growth phases form phase-specific clusters. The largest Raman spectroscopic differences were found between cells in the exponential/stationary phases and cells from the cultures in the senescent growth phase.

## 4. Discussion

In this first CRM study of methanogenic archaea, we show that *M. soligelidi* from Siberian permafrost has unique Raman fingerprints that contain information about the chemical composition of single cells, presenting diversity within the same phase and between different phases of growth.

The diversity of the Raman spectroscopic patterns within a population of SMA-21 cells in different phases of growth (Fig. 2) or even within a certain growth phase (Fig. 1) indicates that there are clear subpopulations that differ in the predominant component of the cell (protein, lipid or the combination of different biomolecules). However, most of the spectra obtained are a mixture of the spectral components of different molecules, since the mentioned components are usually evenly distributed within a cell. Hence, the spectra recorded and the cell composition slightly differs from one cell to another, being the protein and the lipid-based spectra two extreme examples. The phenotypic heterogeneity was also evidenced in spectra of SMA-21 cells from cultures in different stages of growth (Fig. 2). The band at 1172 cm$^{-1}$ was assigned to the ribose and deoxyribose that form the nucleic acids RNA and DNA. The cell content is particularly high during the late exponential phase, suggesting that during this phase the metabolism is very active and the cell division takes place at a high frequency. The high intensity of this peak during the late exponential phase compared to the other phases could be attributed to the RNA molecule, key for the transcription and translation processes that will give rise to the proteins for the new cells. On the other hand, the protein (1008 cm$^{-1}$) and carbonate (1054 and 713 cm$^{-1}$) contents in the cells gradually increase to reach their maximum during the stationary phase, where the mature differentiated cells divide more slowly and accumulate proteins and other products of the metabolism. As mentioned, the spectrum resulting from a cell is the combination of the individual spectra from the biomolecules and other inorganic components present in the sample. As a result, certain peaks can experience a slight shift/broadening due to overlapping and intermolecular interaction when compared to the spectrum of the pure substance. As more complex the chemical composition of the measurement spot becomes as more a band is broadened and harder to assign clearly (Hermann et al., 2011). These findings corroborate the presence of subpopulations with a different chemical composition and confirm the phenotypic heterogeneity within microbial populations found in other microorganisms (Hermelink et al., 2009; Schuster et al., 2000a)

The changes in the cell composition evidenced by CRM seem to correlate with changes in the cell morphology as the cultures age (Fig. 2b), with special emphasis on the appearance and accumulation of round vesicles with a smooth surface and crystalline structures (Fig. 2b, stationary phase). They possibly correspond to the spectra of bodies rich in lipid content found in the Raman measurements and under the SEM and CaCO3 crystals, respectively (Fig. 3). Cells of senescent cultures are smaller compared to cells of other growth phases,

evidencing the lack of nutrients in the medium after 5 months of cultivation without any additional supply of metabolic substrates.

Our results from the CRM measurements and the SEM revealed that carbonates are present in the cell populations over growth and CaCO3 is detected as individual bodies, but neither in fresh nor in old sterile medium. The in situ detection of carbonates suggests that its precipitation is enhanced by the presence of microorganisms. During anaerobic degradation, the methanogenic process removes $CO_2$ from the medium, leading to pH increase and this fact could initiate the precipitation of $CO_2^-$ (Morse, 1983) together with ions available in the medium, for instance giving rise to CaCO3. In addition, the role of methanogenic archaea in carbonate precipitation has already been defined for modern stromatolites (Lundberg et al., 2009) and in a mixed anaerobic microbial consortium (Kenward et al., 2009). This finding is also in agreement with previous CRM studies that have reported the presence of microbial metabolic products, such as poly- β-hydroxybutyric acid in *Legionella* (Hermelink et al., 2011) and granulose in *Clostridium* (Schuster et al., 2000b).

The dendrogram in Fig. 4 illustrates that despite heterogeneity, spectra of individual growth phases cluster together. This finding highlights the accuracy of confocal Raman spectroscopy for bio- signature description on the single cell level and supports the fact that the cell composition changes as the culture matures. The only exception in the cluster is three cells in exponential phase whose spectra and therefore chemical composition are more related to those from cultures in stationary phase. This could be a consequence of the intra-phase heterogeneity and suggests that these cells from an exponential culture seem to be slightly more developed than the average and represent a transition between the exponential and the stationary phase.

In this study we demonstrate that the Raman biosignatures of *M. soligelidi* SMA-21 can be detected and they are diverse within the cell population. As mentioned in Section 1, this archaeon from Siberian permafrost is a good model for potential past or present life on the Red Planet, due to its resistant nature and suitable metabolism. However, given the spectral properties and chemical heterogeneity of these microorganisms, the direct in situ detection in a mineral substrate faces some challenges. For a successful use of CRM for identification purposes, three main problems need to be overcome from the authors' view:

1.      The very small Raman scattering cross section of biological substances compared to minerals. Some Raman studies on other microorganisms interesting for Astrobiology show that it is possible to detect the photoprotective pigment β-carotene in cyanobacteria embedded in a martian regolith analog (Böttger et al., 2012) as well as in cyanobacteria and cryptoen- dolithic lichens from Antarctica in their natural substrates (Edwards et al., 2012; Edwards et al., 1997). The presence of carotenoids was also detected in natural halite from the hyperarid regions of Atacama Desert (Vítek et al., 2012). Other photoprotective pigments and calcium oxalate have been detected in situ within *Acarospora* lichens from the Antarctic and Mediterranean (Holder et al., 2000). Nevertheless, photo- protective pigments are biomolecules that contain resonant structures with an exceptionally intense Raman signature (Schulz et al., 2005). Methanogenic archaea lack these molecules and, although not crucial for microbial detection when they are not embedded in a mineral matrix, the low signal from the biomolecules compared to the mineral particles might involve technical challenges for their in situ detection.

2.      Unexpected heterogeneity effects cannot be covered by a Raman reference database. Raman spectroscopy was already proposed as a potential tool for planetary exploration by Wang et al. (1995), but to date it has not been included in any martian lander mission. ExoMars mission planned for 2018 will be the first rover incorporating a Raman Laser spectrometer (RLS) among its analytical instruments for biosignature detection, among other objectives related to the study of the martian geology (European Space Agency, 2012b). Thus, the creation of a Raman biosignature database of terrestrial extremophiles has become an emerging need in order to interpret future data from Mars, assuming that life there would be of the same nature as the one on Earth. This database must include a wide range of spectra resulting from the phenotypic diversity caused by the environmental and culture conditions as well as the developmental state of the cells. Moreover, it should be considered that the spectral heterogeneity within a certain strain must be lower than the spectral heterogeneity between that strain and other strains with a similar composition, in order to perform a reliable microbial discrimination and create an accurate database.

3.      The single-cell analysis is very specific, requires high spatial resolution and a very accurate laser sample alignment for a successful identification. An aspect to evaluate for the creation of the mentioned Raman signature database would be whether to focus on the average spectral information recorded from large sample volumes rather than only one single cell. This setup would prevent heterogeneity effects due to artifactual measurements and the investigation of events that are rather exclusive than representative of the entire strain/sample. In this case, the sample size measured should be considerably larger in order to overcome the intraspecific heterogeneity.

## 5. Conclusions

In this study, we get a deeper insight into the Raman biosignatures of methanogenic archaea from Siberian permafrost, since they are interesting model organisms for potential life on Mars and Raman spectroscopy is a promising technology for space exploration. We use the highly resistant strain M. soligelidi SMA-21 as our model organism.

The Raman spectra obtained at different stages of the microbial growth evidence the phenotypic heterogeneity within the cell population of M. soligelidi, suggesting the presence of subpopula- tions with a different chemical composition. The changes in composition over time correlate with morphological modifica- tions. Microbially induced products like lipid vesicles and $CaCO_3$ are detected by means of CRM and SEM.

The in situ detection of these microorganisms on mineral substrate might involve technical challenges and the creation of an accurate and standardized Raman signature database is an emerging challenge to interpret Raman data from future space missions.

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**Manuscript III** (submitted for publication to *Applied and Environmental Microbiology*)

**Convergence of the chemical composition in methanogenic archaea from a Siberian permafrost-affected soil: investigation by confocal Raman microspectroscopy.**

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Running title: Convergent chemistry in methanogens from permafrost.

**Abstract**

Methanogenic archaea are the only organisms responsible for the production of biogenic methane. Several new species of methanogenic archaea were recently isolated from a permafrost-affected soil in the Lena delta (Siberia, Russia), showing an exceptional resistance against desiccation, osmotic stress, low temperatures, starvation, UV and ionizing radiation when compared to methanogens from non-permafrost environments. In this study, we attempt to gain a deeper insight into the chemical composition of these methanogens using confocal Raman microspectroscopy (CRM) to explain the origin of their resistant nature. CRM is a powerful tool for microbial identification and provides fingerprint-like information about the chemical composition of the cells. Our results show that the chemical composition of methanogens from permafrost-affected soils presents a high homology and is remarkably different from strains inhabiting non-permafrost environments. In addition, we performed a phylogenetic reconstruction of the studied strains based on the functional gene *mcrA* to demonstrate the different evolutionary relationship of the permafrost strains. We conclude that the permafrost strains show a convergent chemical composition regardless of their genotype and this fact is likely to be the consequence of a complex adaptive process to the Siberian permafrost environment and might be the reason underlying their resistant nature.

**Introduction**

Terrestrial permafrost predominantly occurs in the Northern Hemisphere and covers approximately 24 % of Earth's land surface. It represents a significant natural source of methane, largely of biological origin (1, 2). Methanogenesis, or biological methane production, is a relevant process in the global carbon cycle and represents about 1.6 % of the total carbon that is fixed yearly by photosynthetic organisms. The atmospheric methane concentration has increased more than twofold in the last 200 years (3), contributing to the increase in the Earth's temperature over the last decades. About 85% of the annual global methane formation is mediated by microorganisms (4). Biogenic methane is exclusively produced by methanogenic archaea, strictly anaerobic microorganisms that belong to the phylum *Euryarchaeota* and produce methane as an obligate catabolic end-product (5). Biogenic methane can either be oxidized in biotic and abiotic processes or accumulate in the Earth's atmosphere as a greenhouse gas, where it will slowly oxidize by means of photochemical reactions. It is therefore crucial to study the global distribution of methane and the physiological and ecological roles that the methanogenic archaea play in the global greenhouse effect.

Arctic tundra soils in Siberia are permanently frozen throughout the year with the exception of the thin active layer, subjected to seasonal freeze-thaw cycles with *in situ* temperatures ranging from $-45^{\circ}C$ to $25^{\circ}C$ (6). The microorganisms inhabiting this extreme environment must be adapted to withstand the extremely low temperatures, desiccation and osmotic stress as well as the seasonal fluctuations in temperature and salinity. Several novel strains of methanogenic archaea were recently isolated from the active layer of a permafrost-affected soil in the Lena Delta (Siberia, Russia) and they show remarkable resistance against desiccation, osmotic stress, low temperatures and starvation when compared to methanogenic archaea from non-permafrost environments (7, 8). They also exhibit a high level of resistance to monochromatic and polychromatic UV and ionizing radiation (D. Wagner, unpublished data), which is comparable to that of *Deinococcus radiodurans* (9). In addition, methanogens from Siberian permafrost environments are able to survive simulated Martian thermo-physical conditions (10) and simulated Martian subsurface analog conditions (11), in contrast to other psychrophilic methanogens from non-permafrost habitats such as *Methanogenium frigidum* (12) from Ace Lake, Antarctica, which cannot resist these conditions (10). Among the Siberian permafrost isolates, the genera *Methanosarcina* and *Methanobacterium* are broadly represented. The genus *Methanosarcina* belongs to the order *Methanosarcinales* and family *Methanosarcinaceae*. They are acetotrophic methanogens found in marine and freshwater sediments, anoxic soils, animal-waste lagoons and anaerobic digestors (13). The genus *Methanobacterium* belongs to the order *Methanobacteriales* and the family *Methanobacteriaceae*. *Methanobacterium* species present a hydrogenotrophic metabolism, growing on $H_2+CO_2$ or formate. They are found in various freshwater habitats and soil environments usually growing at pH values near neutrality, but some species are acidophilic or alkaliphilic (5). Microbial phylogeny nowadays is often based on the 16S rRNA molecule, although other important molecular markers for classification are known. For instance, in methanogenic archaea, the functional gene *mcrA* codes for the α subunit of the methyl coenzyme-M reductase (*MCR*), which catalyzes the last step of the methanogenesis, reducing a methyl group bound to coenzyme-M, with the consequent release of methane (14). MCR is thought to be unique to methanogens and since it retains a common function, sequence

comparisons are considered to provide valid phylogenetic data (15). The gene *mcrA* has proven to be an alternative to 16S rRNA in the phylogenetic analysis of methanogen populations (16) and is therefore a valid phylogenetic marker.

The reason why methanogenic archaea from Siberian permafrost environments are more resistant to a broad range of extreme parameters than their relatives from psychrophilic and mesophilic non-permafrost habitats remains unknown. This difference might be accounted for by the distinct chemical composition of the cells. In this study we used confocal Raman microspectroscopy (CRM) to investigate the chemical composition of methanogens from permafrost and non-permafrost habitats. Raman spectroscopy is a vibrational spectroscopic technique that has shown a remarkable potential in microbial identification in recent years (17). It provides fingerprint-like information about the overall chemical composition of the cell and requires a minimal sample preparation, allowing a nondestructive investigation and thus the rapid detection and diagnosis of the microorganism compared to the classical methods for microbial identification based on isolation and culturing (18). It is often difficult to systematically classify closely related strains and even species according to certain genetic markers, since they might be highly homologous or identical. However, the specific Raman spectrum obtained with this vibrational spectroscopy technique enables to discriminate between different phenotypes according to their overall chemical composition. The strains in this study were previously investigated by Fourier-transformed Raman spectroscopy in an attempt to perform a bulk analysis of their chemical composition, though due to the nature of the cells and the presence of metabolic byproducts (19), CRM proved to be the optimal method for our study. CRM combines a dispersive Raman setup with a high-numerical aperture confocal microscope, enabling the study of the chemical structure and composition of individual cells under diffraction-limited conditions (20, 21). Diverse microorganisms and their chemotaxonomic features have been successfully characterized to the species and even strain levels using CRM (22).

In this paper we describe the overall chemical composition of three strains of methanogenic archaea from Siberian permafrost and two strains of methanogens from non-permafrost habitats by means of CRM in an attempt to explain their different resistance to extreme conditions. In addition, we give a phylogenetic overview of the studied strains and their evolutionary relationship based on the functional gene *mcrA*. Finally, we discuss the differences in the chemical nature in relation to the reconstructed phylogeny.

**Materials and Methods**

The strains from Siberian permafrost environments *Methanosarcina soligelidi* SMA-21 (8), SMA-17 and SMA-27 were used for this study. They were isolated from the active layer of permafrost-affected soils in the Lena Delta, Siberia (Russia). *Ms. soligelidi* SMA-21 (DSM 26065$^T$) and SMA-17 appear as irregular cocci, ~1μm in diameter and cell aggregation is often observed. They show 99.9 % homology on the 16S rRNA sequence with *Methanosarcina mazei* (23). SMA-27 cells are elongated rods, ~3-4 μm long.  Their closest relative according to the 16S rRNA molecule is *Methanobacterium congolense (24) (*96.4 % homology). Additionally, two strains from non-permafrost habitats were used as reference strains. *Ms. barkeri* DSM 8687 originates from a peat bog in northern Germany (25) and *Ms. mazei* DSM 2053 was isolated from a mesophilic sewage sludge plant in California, USA. Both strains were obtained from the German Culture Collection of Microorganisms and Cells

(DSMZ, Braunschweig, Germany), appear as irregular cocci, ~1μm in diameter, grow in colonies and are found in diverse environments.

Pure cultures were grown for three weeks until reaching stationary phase of growth at $28^0$C in sealed bottles containing 50 mL of MW medium [(L$^{-1}$): NH$_4$Cl 0.25 G, MgCl$_2$ x 6H$_2$0, 0.4 G, CaCl$_2$ x 2H$_2$O 0.1 G, KCl, 0.5 G, KH$_2$PO$_4$, 0.2 G, Na HCO$_3$, 2.7 G, Cysteine, 0.3 G, Na$_2$S, 0.2 G; trace element solution (26), 10mL; vitamin solution (27), 10mL] and CS medium [(L$^{-1}$): NH$_4$Cl, 0.3 G, MgCl$_2$ x 6H$_2$O, 0.4 G, CaCl$_2$ x 2H$_2$O, 0.16 G, NaCl, 1.0 G, KCl, 0.5 G, K$_2$HPO$_4$ 0.25 G, Na HCO$_3$, 2.7 G, Na-Acetate, 0.25 G, Na$_2$S$_2$O$_4$, 0.1 G, Na$_2$S, 0.25 G; trace element solution (28), 1mL; vitamin solution (27), 1mL] in the case of SMA-27. Both media contain 2 mL resazurin (7-Hydroxy-3H-phenoxazin-3-on-10-oxide). The bottles were flushed and pressurized to one atmosphere with H$_2$/CO$_2$ (80:20 v/v). For sample preparation, 200 mL from four sets of pure cultures in the stationary phase of growth were centrifuged at 7900 g for 40 min and 4°C and washed twice in 200 mL of distilled water at 4600 g for 30 min and 4°C. 7 μL of the cell suspensions were air-dried onto a CaF$_2$ slide, previously diluted 1:10 and 1:100 for a better observation of the single cells.

Raman spectra were captured using a WITec (Ulm, Germany) Model alpha 300R confocal Raman microspectroscope (CRM). The CRM contained an ultra-high throughput spectrometer (UHTS300) and used a back-illuminated EMCCD camera (Andor Technology PLC, Belfast, Northern Ireland) as detector. All the measurements presented in this article were performed with an apochromatic Nikon E Plan (100x/0.95) objective (Tokyo, Japan) and a working distance of 0.230 mm at an excitation wavelength of 532 nm (frequency doubled Nd-YAG laser; 35mW laser power). A minimum of 20 individual cells were measured, each of them with 10 accumulations under full pixel binning and without gaining at the camera. Further technical details about the Raman equipment and measurements were reported in detail in Serrano, et al. (19).

For hierarchical clustering of the CRM spectra, a cosmic ray removal procedure was first performed on the spectra, followed by the individual export of each spectrum via an ASCII interface into OPUS 5.5 (Bruker Optik GmbH, Rheinstetten, Germany). As part of the pre-processing, we carried out a quality test in order to assess the signal-to-noise ratio and a pre-selection of the cell-based spectra that contains the principal components of the spectrum. The first derivative with Savitzky-Golay smoothing/ derivative filter was applied using 9 smoothing points and normalized vectors. Spectral distances between pairs of individual spectra were obtained based on the data from the 796-1854 and 2746-3205 cm$^{-1}$ spectral regions as D-values (29) derived from normalized Pearson's product momentum correlation coefficient. The normalization allows a variation between D-value=0 (r=1: high correlated data/identity), D-value=1000 (r=0: uncorrelated data) and D-value=2000 (r=-1:anti-correlated spectra) and prevents negative values (30). Average linkage was used as the clustering method. For the cluster analysis in figure 3.a, the same method was applied to the average spectra obtained from averaging the individual spectra of each strain shown in Fig.2, including the outlying spectra.

For phylogenetic analysis based on the *mcrA* sequence, the DNA was extracted from pure cultures of the five mentioned strains following the user manual of the UltraClean® DNA purification kit. The *mcrA* gene (31) was amplified with the primers ME1 (forward: gCMATgCARATHggWATgTC) and ME2 (reverse: TCATKgCTAgTTDggRTAgT) and resulted in a 710 base pairs gene product. Sequencing was performed by GATC Biotech

(Constance, Germany). The consensus sequence was obtained using the software CodonCode Aligner (Codoncode Cooperation, MA, USA). The nucleotide sequences from the Siberian permafrost strains were uploaded in GeneBank under the numbers KJ432634 (*mcrA Ms. soligelidi* SMA-21), KJ432635 (*mcrA* SMA-17) and KJ432633 (*mcrA* SMA-27).

A multiple alignment of the five *mcrA* sequences was performed with ClustalW (32) through Geneious pro 5.6.6 (Biomatters Ltd.) and a maximum likelihood tree (1000 bootstraps) was built using the GTR substitution model including the methanogenic archaea *Methanopyrus kandleri* (33) order *Methanopyrales*, (Genbank U57340) as an outgroup. In addition, an integrated alignment of the *mcrA* sequences from permafrost strains was carried out using ARB (34) in order to contrast them with the *mcrA* database containing all *mcrA* sequences described to date.

**Results**

*Raman spectra of permafrost and non-permafrost methanogens*

The Raman spectra of the analyzed strains *Ms. soligelidi* SMA-21, SMA-17 and SMA-27 from Siberian permafrost and *Ms. barkeri* and *Ms. mazei* from non-permafrost habitats are illustrated in Figure 1. The signal with the highest Raman intensity in all spectra was the $CH_2$ stretching vibration around 2936 cm$^{-1}$. The spectra from permafrost strains exhibited a shoulder at 2885 cm$^{-1}$, corresponds to the symmetric $CH_3$ stretching (35), indicating significant differences in the aliphatic chain composition between permafrost and non-permafrost strains. Raman modes of proteins were found at 1669 cm$^{-1}$ (amide I) and at 1243-1275 cm$^{-1}$ (region of amide III). Their intensities are correlated and show slightly lower values for *Ms. soligelidi* SMA-21 and SMA-17. The peak at 1610 cm$^{-1}$ corresponds to the bond C=C found in aromatic amino acids phenylalanine and tyrosine and reached higher intensities in non-permafrost strains, whereas the peak at 1589 cm$^{-1}$ is associated to the ring breathing modes of ribonucleotides guanine and adenine as well as the amino acid tryptophan and was absent in permafrost strains. The intensity of the 1460 cm$^{-1}$ band, attributed to $CH_2$ deformation, was similar in all strains investigated. The peaks at 1344 cm$^{-1}$ and 1338 cm$^{-1}$ were both assigned to the deformation of the group CH in carbohydrates and proteins (36). The peak at 1344 cm$^{-1}$ reached the highest intensity for *Ms. mazei*, the lowest for *Ms. soligelidi* SMA-21 and SMA-17 and intermediate values for SMA-27 and *Ms. barkeri,* whereas the one at 1338 cm$^{-1}$ was unique to the permafrost strains SMA-21 and SMA-17. All the mentioned bands varied slightly in bandwidth, position and intensity for each strain. The peaks in the spectral region located between 1200 and 800 cm$^{-1}$ showed relative higher intensities in permafrost strains than in non-permafrost strains, including the bands located at 1167 cm$^{-1}$ (C-C and C-O ring breathing), 1128 cm$^{-1}$ (characteristic of the C-O-C in the glycosidic link) and 1054 cm$^{-1}$ (C-O and C-C from carbohydrates, and C-C and C-N in proteins) (37). The band at 1008 cm$^{-1}$ was attributed to the symmetric benzene/ pyrrole in-phase and out-of-phase breathing modes of phenylalanine (36). The band at 860 cm$^{-1}$ corresponded to the C-C stretching modes and the C-O-C glycosidic link in polysaccharides (38), and the peak at 835 cm$^{-1}$ was exclusive to the permafrost strains and was attributed to the ring breathing of the amino acid tyrosine and the group O-P-O present in nucleic acids (36).

The cluster analysis based on the Raman spectra showed the similarities and differences in the overall chemical composition of permafrost and non-permafrost strains in

stationary phase, revealing two chemically different clusters illustrated in Figure 2 (individual spectra) and 3.a (average spectra). In general, CRM spectra corresponding to individual cells of the same microbial strain clustered together, with the exception of two spectra from SMA-27 and three spectra from *Ms. soligelidi* SMA-21 (Fig.2). The outlying spectra of SMA-27 were equally distant to the spectra of the SMA-27 cluster and the *Ms. soligelidi* SMA-21/ SMA-17 cluster, separated by the distance of 104.6 and 123.1 D-value units, respectively. Three spectra of *Ms. soligelidi* SMA-21 were separated by 70.8 D-value units from the *Ms. soligelidi* SMA-21/ SMA-17 cluster. Spectra from *Ms. mazei*, *Ms. barkeri* and SMA-17 cells were less heterogeneous and grouped into unique clusters at the strain level.
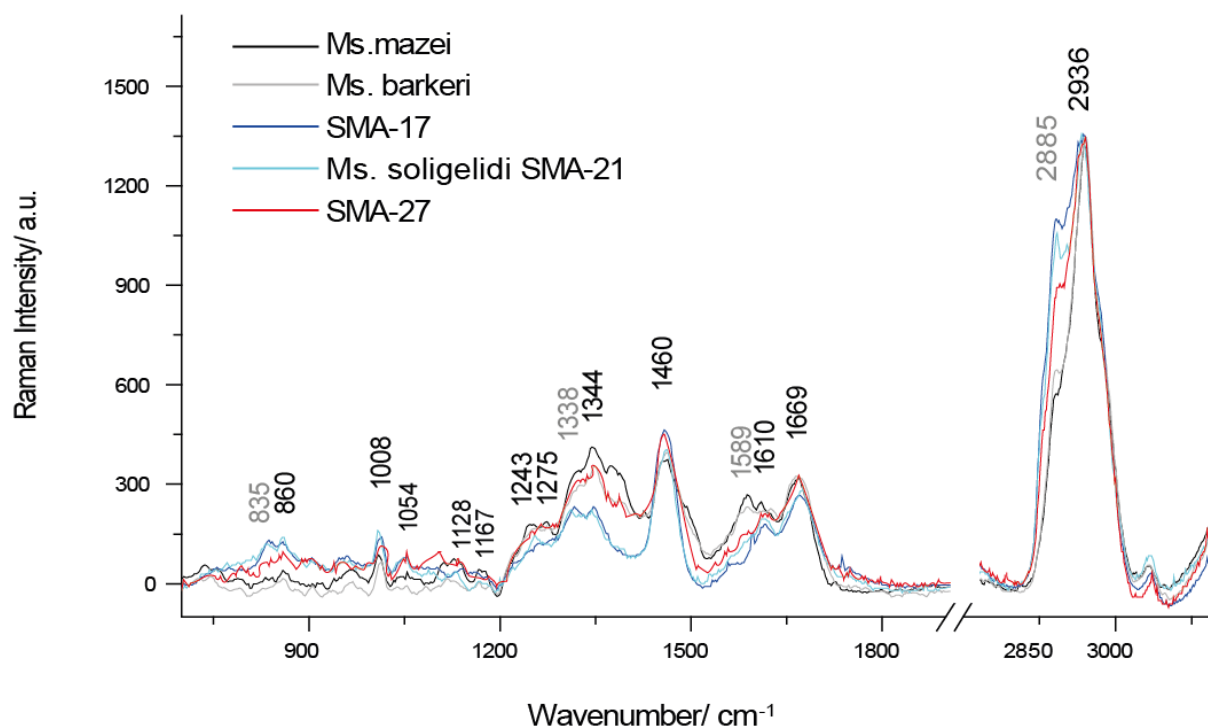


**Figure 1.** Average Raman spectra of the Siberian permafrost strains *Methanosarcina soligelidi* SMA-21, SMA-17 and SMA-27 and the non-permafrost strains *Ms. mazei* and *Ms. barkeri* measured with an excitation wavelenght of 532nm. Note that values corresponding to the band positions common to all strains are shown in black whereas those specific to one or a few strains are presented in grey.

The cluster analysis in figure 3.A shows an overview of the phenotypic resemblance in the chemical composition based on the average spectra of each strain, obtained from averaging the individual spectra, including the outliers (and therefore disregarding the intraspecific variances in the heterogeneity). Strains *Ms. soligelidi* SMA-21 and SMA-17 were most similar, separated by 15.6 D-values. The cluster *Ms. soligelidi* SMA-21/ SMA-17 was closely related to the strain SMA-27, also from Siberian permafrost, distanced by 37.8 D-values. Apart from the permafrost group, the spectra from *Ms. mazei* and *Ms. barkeri* (non-permafrost strains) grouped together, separated by 24.4 D-value units. The total distance between the permafrost and the non-permafrost cluster was 84.4 D-values.
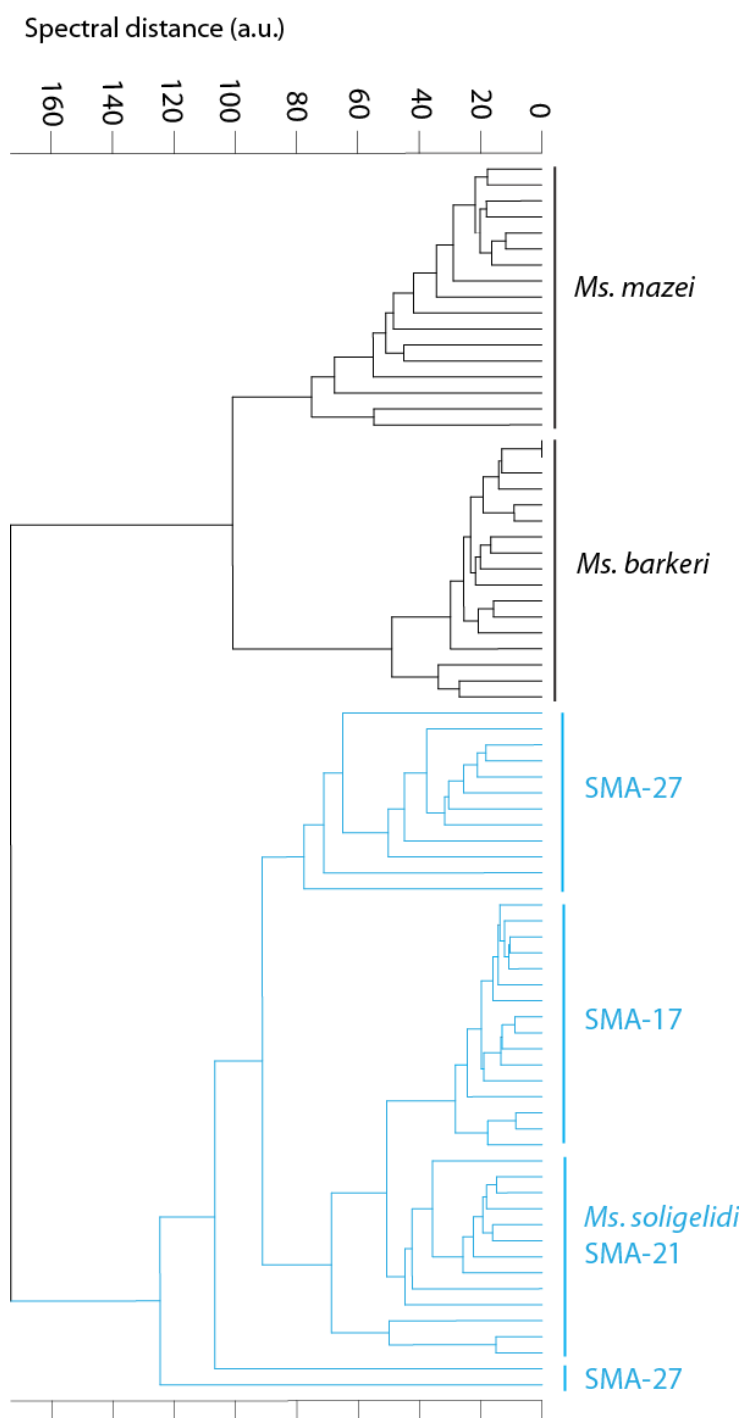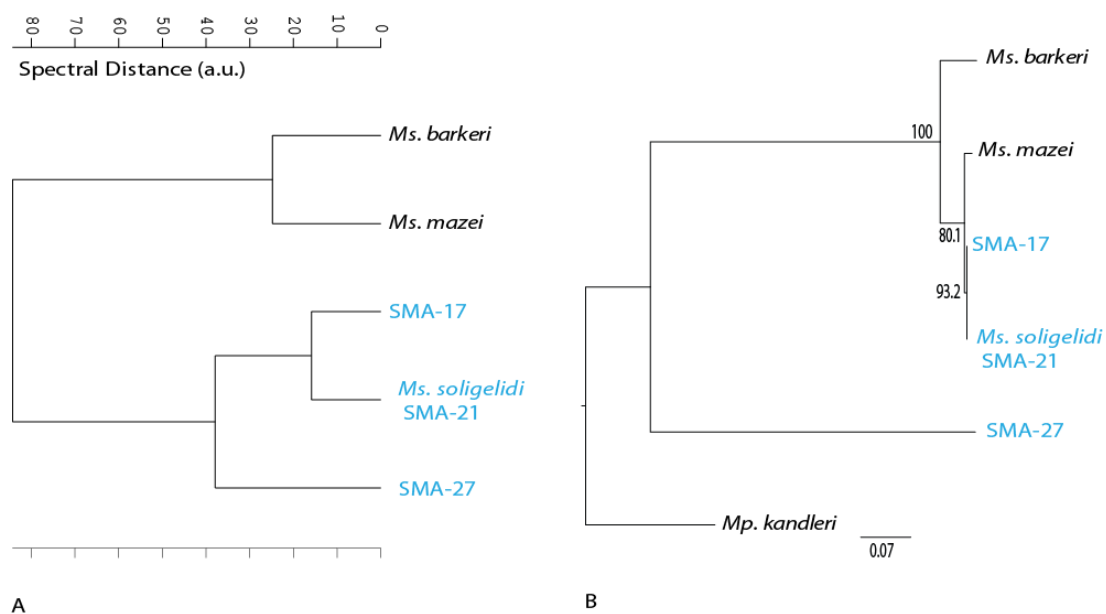
**Figure 2.** Cluster analysis (average linkage method) of Raman spectra from individual cells from permafrost and non-permafrost strains in stationary phase. CRM spectra from *Ms. mazei* and *Ms. barkeri* (non-permafrost strains) form a cluster, which is well separated from the cluster of permafrost strains (SMA-27, *Ms. soligelidi* SMA-21 and SMA-17).

*Phylogenetic relationships of methanogenic archaea*

A maximum likelihood tree (GTR substitution model, 1000 bootstraps) was built for the studied methanogens according to the *mcrA* nucleotide sequence, using *Methanopyrus kandleri* as the outgroup (Fig.3.B). All the *Methanosarcina* species clustered together, with *Ms. soligelidi* SMA-21 and SMA-17 from the Siberian permafrost showing identical *mcrA* sequences. The cluster *Ms. soligelidi* SMA-21/ SMA-17 was closely related to *Ms. mazei*, sharing a 98.5 % identity in their sequences. *Ms. mazei* and *Ms. barkeri* presented a 91.5 % homology. Finally, SMA-27 was the most evolutionary distant strain, sharing only 61% of the *mcrA* nucleotide sequence with the rest of the studied strains. To verify the outcome of

this tree, the sequences corresponding to the permafrost strains were aligned with the *mcrA* database that contains all the nucleotide sequences from the *mcrA* gene described to date (Fig. 4). Once again, *Ms. soligelidi* SMA-21 and SMA-17 grouped together with other members of the genus *Methanosarcina* and directly clustered with a previously described culture of *Ms. mazei (MtnMaz42, Methanosarcina mazei, AY260440, [EBI][1]: Simankova M.V.,Kot)*. On the other hand, SMA-27 showed the highest homology with the genus *Methanobacterium*, being *Mb. formicicum (MtbFor15, Methanobacterium formicicum, EF465108, [EBI][1]:Ma K. Dong X [2])* the closest described relative. The sequence alignment with the *mcrA* database therefore supports the robustness of our phylogenetic reconstruction.



**Figure 3.** Chemical *vs*. phylogenetic relationships of methanogenic archaea from Siberian permafrost *Methanosarcina soligelidi* SMA-21, SMA-17 and SMA-27 (in blue) and the two non-permafrost strains used as reference *Ms. barkeri* and *Ms. mazei* (A) Cluster analysis of the average Raman spectra from permafrost and non-permafrost strains in stationary phase using the average linkage clustering method. (B) Maximum likelihood tree (GTR substitution model, 1000 bootstraps) according to the mcrA nucleotide sequence. *Methanopyrus kandleri* (*Methanopyrales*) was used as the outgroup. The branch support values indicated in the nodes show the robustness of the phylogenetic reconstruction.
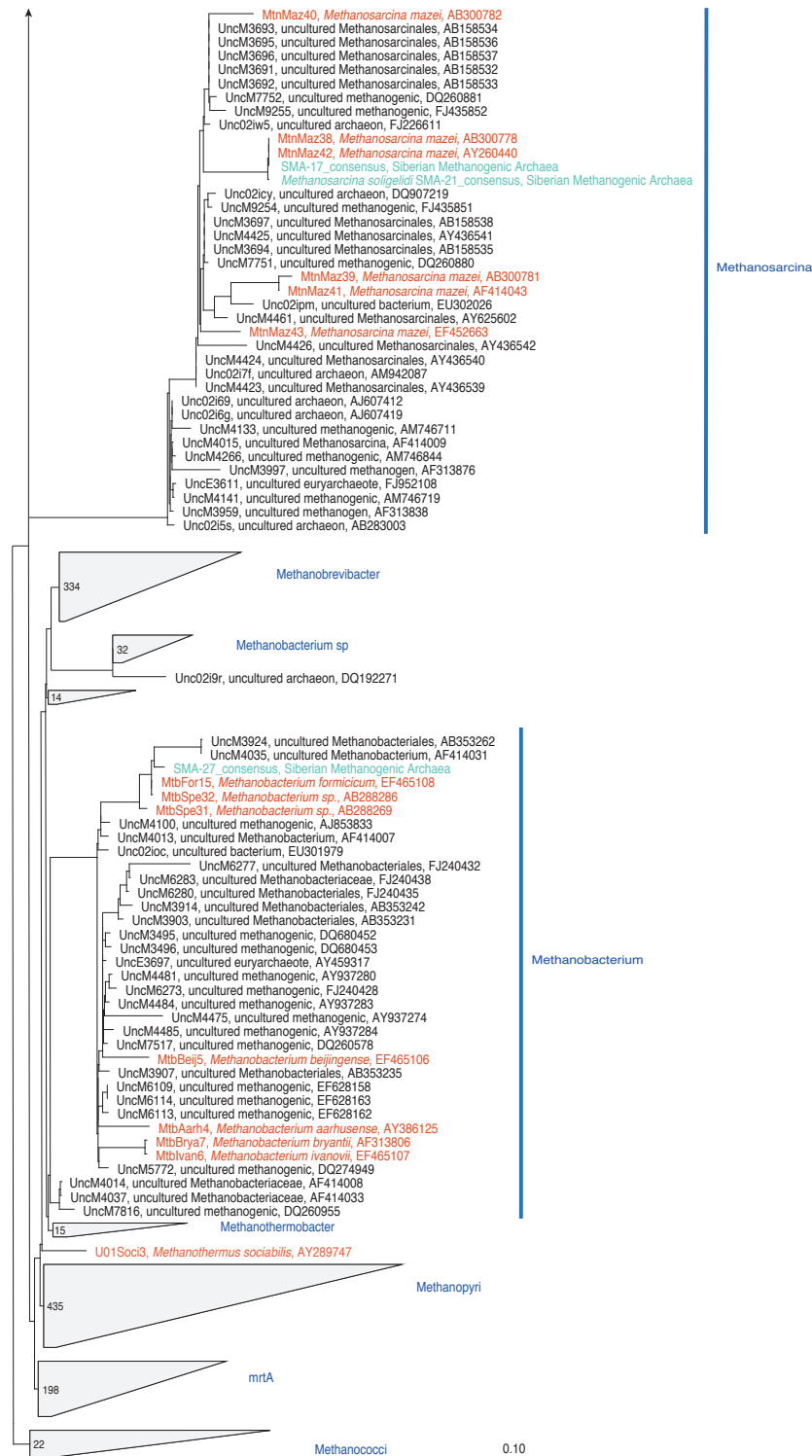
**Figure 4.** Overview of the nucleotide sequence alignement of Siberian permafrost strains (light blue) with the mcrA database containing all the sequences described to date. Reference species are marked in red and several genera of methanogenic archaea are presented in dark blue. *Methanosarcina soligelidi* SMA-21 and SMA-17 are aligned with other members of the genus Methanosarcina, more specifically with *Ms. mazei*. SMA-27 shows a higher homology with members of the genus *Methanobacterium*.

## Discussion

Previous studies have shown that methanogenic archaea from permafrost habitats are more tolerant against different environmental stress factors compared to those from non-permafrost areas (7, 10). In this study, we have shown that on the basis of CRM analysis Siberian permafrost and non-permafrost strains could be classified into two different groups according to their chemical composition. The Siberian permafrost strains *(Ms. soligelidi*

SMA-21, SMA-27 and SMA-17) show a relatively high similarity in their chemistry in spite of the heterogeneity between individual spectra (Fig 2 and 3.A). The cluster analysis in figure 2 shows two branches from SMA-27 and three from *Ms. soligelidi* SMA-21 that do not group together with the cluster of the corresponding strain, suggesting that the SMA-27 and *Ms. soligelidi* SMA-21 populations are more chemically heterogeneous than the other strains. The high phenotypic heterogeneity and diversity of cell populations of *Ms. soligelidi* SMA-21 (39) were also observed in the strains investigated in this study. When comparing the cluster analysis of the individual spectra (Fig. 2) with the average spectra (Fig. 3.a), the scales vary, although they refer to the same data. In addition, the heterogeneity within the SMA-27 population (individual spectra) is larger than the overall distance in the average spectra. The explanation relies on the fact that the average spectra were obtained by averaging the single spectra from each strain, including the outlying spectra, which considerably increase the variance of the corresponding strains (*Ms. soligelidi* SMA-21 and most remarkably SMA-27). The largely different variances within each strain were therefore not proportionally weighed for the cluster analysis of the average spectra, and despite this assumption, the permafrost and the non-permafrost strains cluster in different groups regarding their chemical composition.

On the other hand, the evolutionary relationships among the strains do not correspond in all cases with the topology found for the chemical composition. The molecular information provided by the gene *mcrA* proves that the permafrost strains do not form a monophyletic group (Fig 3.B and 4). The *Methanosarcina* strains (SMA-21 and SMA-17) share identical sequences, whereas SMA-27 presents only 61% of homology in the *mcrA* sequence with the rest of the strains and its 16S rRNA sequence affiliates more closely with the genus *Methanobacterium*. Therefore, SMA-27 is the phylogenetically most different strain in this study. The maximum likelihood analysis based on *mcrA* shows a full bootstrap support for the node that separates *Ms. barkeri* (Fig. 3.B). Although the other two nodes within that group are not completely resolved, it is evidenced that *Ms. mazei* is the most closely related strain to *Ms. soligelidi* SMA-21 and SMA-17. SMA-27 forms a distantly-related sister group, as it is corroborated by the sequence alignment of permafrost strains with the *mcrA* database (Fig. 4). In addition, the non-permafrost strains *Ms. mazei* and *Ms. barkeri* share a remarkable degree of homology in both chemical composition and genetic information. Previous findings based on the 16S rRNA sequence alignment (D. Wagner, unpublished) have proven the same relationship among these strains, confirming the fact that *mcrA* is an accurate phylogenetic marker for methanogenic archaea (16).

The studied permafrost strains share a related chemistry, regardless of their evolutionary origin. In other words, methanogens with different genotypes can exhibit an analogous phenotype in terms of chemical composition. This finding points to the evidence of the complexity of the adaptations to the environmental conditions, suggesting that permafrost strains may have developed common biochemical adaptations to sub-zero temperatures, freeze-thaw cycles, osmotic stress and high levels of background radiation. A plausible phenomenon explaining the convergent chemical composition in permafrost strains despite their different genotype is the horizontal gene transfer (HGT). HGT allows the rapid incorporation of novel functions that provide a selective advantage to the organism and there is proof of HGT in the evolution of some genes coding for enzymes involved in methanogenic pathways (40). However, they only concern certain enzymes in the acetoclastic

pathway and methanogenesis from methylamines and not MCR, common to all methanogenic pathways. All *mcr* operons appear to have evolved from a common ancestor and since MCR plays a key role in the methanogenesis, it is highly conserved and should provide valid phylogenetic information, independent of the 16S rRNA information (15). Despite this fact, other operational genes involved in metabolic pathways not related to methanogenesis may have experienced HGT with the consequent production of molecules/metabolites that might have provided a selective phenotypic advantage to the cells, enabling them to survive in the Siberian permafrost environment and leading to a convergent chemical phenotype of the methanogenic archaea.

CRM allows the discrimination between molecules based on their specific vibrational modes. When investigating the composition of a whole cell, CRM can be used to describe only the Raman-active biomolecules such as molecules containing aromatic rings (phenylalanine, tryptophan, pigments etc.), but this technology does not allow the discrimination of specific biomolecules (e.g. a particular protein or carbohydrate). Both quantitative (band intensities) and qualitative (band position) differences found between spectra of permafrost and non-permafrost strains are illustrated in Figure 1. The underlying structural and compositional differences might be correlated with biochemical adaptations to permafrost conditions and could explain the resistant nature of the permafrost strains when compared to other non-permafrost strains. Some cold-adaptive mechanisms described for archaea involve modifications in the lipid composition, increasing the levels of unsaturation of the fatty acids to maintain the fluidity of the membrane (41). This fact is evidenced in the Raman spectra from Figure 1, as the intensity of the peak at 2936 cm$^{-1}$ in the $CH_2$ stretching region of the spectra indicates that the lipid content is comparable. The peak at 2885 cm$^{-1}$ corresponds to the symmetric $CH_3$ stretching and reveals a noticeable contrast between permafrost and non-permafrost strains, denoting differences in the aliphatic chain composition of the lipids. Other adaptations to cold include modifications in the ribosomes, protein folding and compatible solutes in cellular cytoplasm with a cryoprotective function (41, 42). In that regard, permafrost and non-permafrost strains show qualitative differences in aromatic amino acids and ribonucleotides (1589 cm$^{-1}$), carbohydrates and proteins (1344 cm$^{-1}$ and 1338 cm$^{-1}$) and Tyrosine and phosphate (835 cm$^{-1}$), which likely correspond to some of these adaptive mechanisms. Moreover, the band at 860 cm$^{-1}$ is especially prominent in permafrost strains and was previously assigned to the C-O-C 1,4-glycosidic link present in carbohydrates and polysaccharides (36, 38). This specific band together with the one mentioned at 1338 cm$^{-1}$ suggest the presence of polysaccharide in permafrost strains, which represents an interesting finding since many microorganisms, including archaea, have been reported to produce exopolysaccharides (EPSs) as a strategy to survive adverse conditions (43). EPSs are sugar-based polymers that are secreted by microorganisms to the surrounding environment and in some cases they constitute a substantial component of the extracellular polymers. In addition, they have have been shown to play a protective role against desiccation (44), fact that would bring a suitable adaptation to the permafrost strains, given the arid conditions of the Siberian permafrost environment.

Life has evolved similar adaptations multiple times in extreme environments in an attempt to survive (45) and, for instance, cold-adaptive responses are not unique to the domain Archaea. Sub-zero temperatures and osmotic stress generally affect macromolecule structures and the thermodynamics of chemical reactions, having the same impact on all

microorganisms. Hence, the biochemistry and physiology of psychrophiles (like other groups of extremophiles) demonstrate that the features and adaptations that unite them as a group are stronger than the variation imposed by their phylogeny (46). This study therefore presents proof of concept that distantly related methanogens (*Methanosarcina* and *Methanobacterium)* occurring in the same habitat, have independently developed similarities in chemical composition. The microbial community of permafrost has been often referred to as a "community of survivors" (47) that have found themselves trapped in this environment and have outcompeted those unable to withstand the given extreme conditions through a process of continuous selection that lasted millions of years (48). The Siberian permafrost strains in this study corroborate the convergence of a certain phenotype in response to the surrounding environment, independent of the genotype.

## Acknowledgements

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## III. SYNTHESIS

### 3.1 Discussion

This investigation contributes to the scientific understanding of the survival limits of methanogenic archaea, with a focus on methanogens from Siberian permafrost, in simulated martian conditions and to the characterization of their biosignatures by means of confocal Raman microspectroscopy. Methanogenic archaea have a metabolism that is in principle compatible with the martian environmental conditions reported to date and they occur in a wide variety of anaerobic habitats. Used as models for life on Mars since the late 90's, it has been proven that certain methanogens can survive and retain metabolic activity when exposed to martian conditions such as the presence of the regolith analogue JSC Mars-1 (Kral et al., 2004b) and desiccation at low pressure for extended periods of time (Kral et al., 2011). Although martian conditions are extreme, in many aspects they resemble to the most extreme cold regions of the terrestrial biosphere. More than 80% of the biosphere on Earth is permanently at temperatures below 4ºC, therefore, indigenous microorganisms have been proposed as candidates for life on planetary bodies of cryogenic nature, such as Mars. Given the multiple geomorphological similarities with martian permafrost, terrestrial permafrost is the most promising analogue to potential habitats on Mars (Gilichinsky, 2002). Previous studies have shown that methanogenic archaea isolated from Siberian permafrost environments are more resistant to desiccation, starvation, osmotic stress and low temperatures than non-permafrost methanogens (Morozova and Wagner, 2007) and experiments under simulated Mars conditions indicate survival when exposed to martian thermo-physical (Morozova et al., 2007) as well as subsurface-analogue conditions (Schirmack et al., 2013). To further analyse their survival to yet untested simulated Mars conditions, methanogens from Siberian permafrost and non-permafrost were exposed in this study to ultra-low subfreezing temperatures, the presence of perchlorate, simulated Mars regoliths and Mars atmospheric composition. For a better interpretation of the biosignatures that future space missions might encounter (e.g. ExoMars (Vago et al., 2006), due for launch in 2018) it is necessary to gain deeper insight into the biosignatures of terrestrial organisms is necessary. In this context and as part of the BIOMEX space experiment (de Vera et al., 2012), the present investigation describes the use of biosignatures to characterize the chemical composition of methanogenic archaea by means of confocal Raman microspectroscopy (CRM) and to evaluate the potential of this tool for the remote detection of methanogens. Based on the presented and discussed results in the three chapters that compose this investigation, the following conclusions could be drawn:

❖ *Methanogenic archaea survive in high numbers and retain metabolic activity after the exposure to desiccation under simulated Mars conditions and the presence of perchlorate.*

All strains both from Siberian permafrost and non-permafrost successfully survived desiccating conditions when subjected to 1. long-term exposure for up to 315 days to ultra-low subfreezing temperatures that simulate a hypothetical constant temperature (-80ºC) in some regions of the martian subsurface and 2. medium-term exposure for up to 16 days to

martian soil and atmospheric analogues. The regolith analogues reproduced the mineralogical composition of certain regions on early (P-MRS) and late (S-MRS) Mars, and the simulated martian atmospheric composition contained 95% $CO_2$ and other trace elements (Ar, $N_2$, $O_2$, CO) in the proportions recently reported by Mars rover lander Curiosity (Mahaffy et al., 2013). Cold-exposed strains from Siberian permafrost show a methane production rate at least 10 times higher than their respective non-frozen controls. This increase in the activity after cold shock likely represents an adaptation evolved in the active layer of the permafrost-affected soils, where the thawing only occurs for a few months during summer. In a similar way, liquid water on Mars is scarce and only present for short periods (Christensen et al., 2003; Jakosky et al., 2007), depending on the tilt of the planet's axis (Jakosky et al., 2003) and on the presence of salts such as perchlorate (Keller et al., 2006; Stoker et al., 2010). Non-permafrost strains reported similar methanogenic rates after freezing than in control conditions. Regarding the tolerance to regolith and atmospheric analogues, all the strains showed a high methane production after exposure. One of the Siberian permafrost representatives, *Methanosarcina soligelidi* SMA-21, showed significantly higher methane production rates when exposed to both Mars regolith simulants, P-MRS and S-MRS, and after 16 days of desiccation. However, non-permafrost strains and the permafrost strain SMA-27 were able to preserve intact cells in higher numbers after the exposure to subfreezing temperatures, which could find an explanation in the cell morphology (Dumont et al., 2004) and the phenomenon of cell aggregation, that provides protection to the cells (Flemming and Wingender, 2001). For the first time DNA from surviving cells embedded in the Mars regolith simulants P-MRS and S-MRS was isolated in quantifiable amounts and revealed relatively stable levels over the time of desiccation in the presence of martian regolith and atmospheric analogues. The DNA isolation from the regolith S-MRS (corresponding to late Mars) was more problematic and the efficiency three orders of magnitude lower than for P-MRS, yet the cells were in such high numbers that could be quantified by qPCR. A possible explanation for the different extraction efficiency may be the presence of iron in the late-Mars regolith analogue, which sequestrates the DNA (Netto et al., 1991), and thus hampers the correct isolation process. Moreover, all the methanogenic strains were able to produce methane in levels similar to the controls when exposed to magnesium perchlorate at the concentration reported at the Phoenix landing site (2.4 mM, Hecht, et al. 2009; Stoker, et al. 2010). The exposure to increasing concentrations of perchlorate up to 500 mM showed that the metabolism was inhibited at lower concentrations for permafrost strains (100 mM) than for non-permafrost strains (500 mM). This might be explained by the capability of the latter to form cell aggregates. The different response of permafrost and non-permafrost strains exposed to simulated martian parameters might find an ultimate interpretation in the different chemical composition of these organisms, as described in the last manuscript of this thesis. The main findings of this study suggest that, regardless their origin, methanogens are in principle suitable models for life on the martian subsurface.

❖ *Methanosarcina soligelidi SMA-21 is an optimal model organism for potential life on the martian subsurface.*

The strain from Siberian permafrost *M. soligelidi* SMA-21 has proven to survive in high numbers the long-term exposure to ultra-low subfreezing temperatures (-80ºC) and the presence of martian regolith and atmospheric analogues. Furthermore it showed a methanogenic activity up to 73 times higher after undergoing -80ºC for up to 315 days compared to unfrozen controls. It also presented the highest methane production rates after exposure to early and late Mars regolith analogues in combination with a simulated martian atmosphere for up to 16 days, and it did not register significant changes on the methanogenic rates during the exposure to 2.4 mM magnesium perchlorate ($MgCl_2O_8$) compared to the controls. Previous studies reported the highest resistance against desiccation, starvation, osmotic stress and low temperatures for pure cultures of this strain (Morozova and Wagner, 2007). It also presented the highest survival when exposed to high doses of monochromatic and polychromatic UV and ionizing radiation (Morozova, et al., in preparation), and it was able to resist simulated martian thermo-physical conditions (Morozova et al., 2007). Additionally, it showed metabolic activity under simulated Martian subsurface analogue conditions, including a low atmospheric pressure of 50 kPa (500 mbar, Schirmack, et al. 2013) , and the survival and metabolic activity after > 300 days of desiccation on S-MRS, P-MRS and JSC Mars-1 (Schirmack and Wagner, unpublished data). Other investigations concerning methanogenic archaea as models for life on Mars have focused on *Methanosarcina barkeri, Methanothermobacter wolfeii, Methanococcus maripalidus* and *Methanobacterium formicicum* and have reported methane production in the presence of 1% perchlorate (~ 45 mM, Goodhart and Kral 2010)  and at reduced pressures of 400 and 50 mbar on JSC Mars-1 (Kral et al., 2004b; 2011), all conditions compatible with the survival of *M. soligelidi* SMA-21. To summarize, *M. soligelidi* SMA-21 is a well-described permafrost strain able to withstand a wide range of physico-chemical stresses more effectively than other permafrost and non-permafrost strains. It is so far the best-studied highly resistant methanogenic model for life on Mars and will be used on the BIOMEX space project (de Vera et al., 2012) onboard the EXPOSE-R2 mission, where it will be exposed to simulated Mars conditions (Mars atmosphere, minerals and analogue solar radiation) in space. As part of the project verification test in DLR (German Aerospace Agency) Cologne, samples of *M. soligelidi* SMA-21 embedded in S-MRS and P-MRS analogues were exposed to a Mars gas mixture at reduced pressure conditions of 1000 Pa and polychromatic UV wavelengths 200-400 nm (as expected on Mars) with an intensity of $5 \times 10^5$ kJ m$^{-2}$ simulating a mission duration of 12 months ($T_{max}$= 10ºC; $T_{min}$= -25ºC). Preliminary results show slowly increasing levels in the methane production after the simulation.

❖ *Methanosarcina soligelidi SMA-21 shows high heterogeneity and diversity in the chemical composition of the cells over their growth.*

As part of the first study of methanogenic archaea using confocal Raman microspectrocopy, *M. soligelidi* SMA-21 showed unique Raman fingerprints with a high degree of heterogeneity among cells within the same phase of growth. This finding suggests the presence of subpopulations with a different predominant cell component, which is in

agreement with previous studies that proved that the phenotypic heterogeneity within microbial populations can be detected by CRM (Schuster et al., 2000; Hermelink et al., 2009). Moreover, cells at different stages of growth also showed diversity in the chemical composition, in particular with regards to the protein (1008 cm$^{-1}$), carbonate (1054 cm$^{-1}$) and RNA (1172 cm$^{-1}$) content. Proteins and carbonates reached their maxima in the stationary phase, and RNA presents the highest levels in the late exponential phase. The changes in the chemistry of the cells correlate with modifications of the cell morphology and the appearance of lipid vesicles and carbonate, described as microbially induced products. Phenotypic heterogeneity has proven the differential sensitivity to stress of genetically identical cells and is thought to be fundamental to the persistence and fitness of an organism (Sumner and Avery, 2002). These facts taken together suggest a link between the high heterogeneity and diversity in the chemical composition of the populations of *M. soligelidi* SMA-21 and its endurance and fast recovery after the exposure to simulated Martian conditions observed in this study, as well as their resistance against physiological stress previously reported (Morozova et al., 2007; Morozova and Wagner, 2007; Wagner et al., 2013). However, the degree of heterogeneity and diversity of the other permafrost and non-permafrost strains has not been assessed and therefore this explanation is not conclusive.

❖ *Raman spectroscopy faces some challenges for the remote detection of methanogenic archaea.*

One of the goals of the BIOMEX space experiment is to create a Raman biosignature database of terrestrial extremophiles that will serve to interpret data from future space missions, e.g. ExoMars (Vago et al., 2006), that will incorporate a Raman laser spectrometer (RLS) among the analytical instruments. However, in the course of the biosignature characterization of *M. soligelidi* SMA-21 by CRM, a number of technical and theoretical issues regarding their description on mineral substrates arose. This investigation was carried out using a laser source of 532 nm, which is the same wavelength than the RLS onboard the ExoMars mission will have (Rull et al., 2011). However, the laser spot size planned for ExoMars will be around 50 μm on the target, whereas in this study the laser spot used to cover a single cell and obtain its Raman fingerprint was 1 μm, requiring a high spatial resolution as well as an accurate laser-sample alignment. Furthermore, the Raman scattering of non-pigmented microorganisms like methanogenic archaea is very small compared to minerals, and thus the biosignatures would be difficult to detect as masked by the signal of the mineral. Several microorganisms of interest for Astrobiology have been detected *in situ* in nature (Edwards et al., 1997; 2012; Vítek et al., 2012) and in the lab while embedded in a mineral matrix (Böttger et al., 2012). Nevertheless, the presence of photoprotective pigments, carotenoids in most cases, was crucial for their detection, since these biomolecules present resonant structures with an exceptionally intense Raman signal (Schulz et al., 2005). The Raman characterization of microbial pigments has been studied diffusely in recent years (Holder et al., 2000; Jehlička et al., 2014), although their detection does not provide the same comprehensive information than the Raman fingerprint of the entire cell. Finally, provided that microbial detection was feasible, unexpected effects on the spectral heterogeneity cannot be predicted and included in an accurate Raman database for microbial identification.

❖ *Methanogens from Siberian permafrost show a convergent chemical composition, different from non-permafrost strains.*

Comparing the chemical composition of methanogenic archaea from Siberian permafrost to those from non-permafrost environments, the results of a cluster analysis of their CRM spectra highlighted the existence of two groups according to the similarities in the chemical composition: the strains from Siberian permafrost clustered together and so did those from non-permafrost habitats. Despite their similar chemical composition, permafrost strains showed a diverse phylogenetic origin, based on the homologies of the sequence in the functional gene *mcrA*, which is considered to provide valid phylogenetic data comparable to the 16S rRNA (Reeve, 1992). On the other hand, the non-permafrost strains, though close relatives to some of the permafrost strains and presented differences in their chemistry greater than those between non-phylogenetically related strains from the same permafrost habitat. The main differences between permafrost and non-permafrost strains consisted in the aliphatic chain composition of lipids (2885 cm$^{-1}$), aromatic amino acid and ribonucleotides (1589 cm$^{-1}$), proteins and carbohydrates (1338 cm$^{-1}$), tyrosine and phosphate (835 cm$^{-1}$) and the C-O-C 1,4 glycosidic link characteristic of carbohydrates and polysaccharides (860 cm$^{-1}$) (Neugebauer et al., 2007; Ivleva et al., 2009). These modifications likely correspond to cell adaptations to the environmental features of the Siberian permafrost, namely cold adaptive mechanisms, such as unsaturation of the lipids to maintain the fluidity of the membranes (Cavicchioli et al., 2000; Mangelsdorf et al., 2009) or the presence of extracellular polymeric substance (EPS) mostly composed of polysaccharides and proteins, which have demonstrated a protective role against desiccation (Ophir and Gutnick, 1994). Furthermore, the differences observed in the survival and recovery of the methanogenic activity of the permafrost and non-permafrost strains after the exposure to simulated martian conditions might be explained in part by the different chemical composition. For instance, after the exposure to subfreezing conditions, the permafrost strains show a substantially higher relative methane production rates than the non-permafrost strains, which might be related to the presence of cold-shock proteins, leading to a more active metabolism once the stress has ceased. In addition, the better preservation of cell membranes in non-permafrost strains must be related to the phenomenon of aggregation in these cells, which is ultimately related to the chemical composition. The same argument could provide an explanation to the higher resistance of non-permafrost strains to elevated concentrations of perchlorate. The convergence of the chemical composition in Siberian permafrost strains is a proof of concept that illustrates that organisms can evolve similar adaptations multiple times independently to adapt to the same extreme environment. (Rothschild and Mancinelli, 2001).

## 3.2 Conclusion

The present investigation has found that methanogens from Siberian permafrost are able to survive in high numbers and recover their metabolic activity after the exposure to two types of simulated martian desiccation: exposure to an ultra-low subfreezing temperature (-80ºC), and to Mars regolith (S-MRS/P-MRS) and a martian atmosphere. They also show a normal metabolic activity when exposed to the concentration of perchlorate reported on Mars. The non-permafrost strains, however, present a less prominent methanogenic activity

when exposed to subfreezing temperatures, and higher cell numbers with intact membranes compared to some of the permafrost strains. The non-permafrost strains can also survive the exposure to perchlorate and to Mars regoliths (S-MRS/P-MRS) and atmosphere as well as recover their metabolic activity to the same extent as Siberian permafrost strains. *Methanosarcina soligelidi* SMA-21, from Siberian permafrost, shows an extremely fast metabolic recovery in all the tested simulated martian conditions. These facts together with the previously reported resistance against multiple physiological stresses (starvation, osmotic stress, UV and ionizing radiation, etc.) and simulated martian thermo-physical and subsurface conditions make of *M. soligelidi* SMA-21 one of the best methanogenic models for potential survival in the martian subsurface. In the framework of the BIOMEX project, this work also presents the biosignatures of methanogenic archaea using confocal Raman microspectroscopy (CRM) and might help to interpret future data from space exploration missions, such as the upcoming ExoMars mission. The biosignatures of *M. soligelidi* SMA-21 were characterized during the growth phases at a single-cell level, presenting a high heterogeneity and diversity in the chemical composition of the cells as well as highlighting the occurrence of subpopulations with different predominant biochemical components and microbially induced products, such as lipid vesicles and carbonate. Throughout this study, some technical challenges were foreseen concerning the Raman detection of methanogenic archaea (and other non-pigmented microorganisms) embedded on a mineral substrate, such as the spatial resolution and the low Raman signal from the biosignatures compared to the mineral signal. Finally, the biosignatures of permafrost and non-permafrost strains in the stationary phase of growth were characterized by CRM. A cluster analysis of the spectra revealed that permafrost and non-permafrost strains have a different overall chemical composition, which likely explains their different responses after exposure to simulated Mars conditions. A phylogenetic study based on the functional gene *mcrA* was performed, showing that the methanogenic strains from Siberian permafrost are of a diverse phylogenetic origin, and suggesting that the similarities in their chemical composition are likely the result of an adaptive process to the environment.

Overall, this work contributes to the existing knowledge of the survival and metabolic activity of methanogens to simulated martian conditions, confirming previous investigations and proving that methanogens are good model organisms for potential life forms on the martian subsurface, with special emphasis on *M. soligelidi* SMA-21 from Siberian permafrost. In addition, this is the first time that CRM has been used to characterize the biosignatures of methanogenic archaea. The convergence of the chemical composition found in permafrost strains is a relevant finding that will serve as a base for future studies, and the biosignatures reported in this study have possible practical applications in future planetary exploration missions.

## 3.3 Remarks and future perspectives

A common limitation of any simulation study is the only partial recreation of the simulated conditions, in this case, martian conditions. This work also faced the additional difficulty of the limited knowledge about Mars that we still have in many aspects, such as the lack of data about the temperature on the subsurface at different latitudes and depths or the accurate mineralogical and chemical composition of soils, which remain unknown for most

of the regions. This study focused on the exposure to single (subfreezing temperatures, perchlorate) and double (martian regolith and atmospheric composition) simulated martian parameters and given the successful results regarding survival, future approaches could include the simultaneous simulation of multiple parameters in the same experiment. For instance, future tests could be performed in a Mars simulation chamber, which allows the simultaneous recreation of temperature fluctuations in the martian day and night, radiation exposure (for surface studies), martian atmospheric composition, low pressure (or high pressure for subsurface studies), etc.

Further limitations of this investigation are related to the number and the phylogenetic diversity of the study-organisms. This study included three strains from Siberian permafrost (*Methanosarcina soligelidi* SMA-21, *Candidatus Methanosarcina* SMA-17 and *Candidatus Methanobacterium* SMA-27) and two strains from non-permafrost habitats (both *Methanosarcina* species). The non-permafrost strains were chosen according to their close phylogenetic relationship to *Methanosarcina soligelidi* SMA-21, the best-studied and most resistant permafrost candidate so far. Now that the differences in chemical composition between permafrost and non-permafrost strains have been described, future experimental setups may contemplate to include a more extended study-group of methanogens and to increase the phylogenetic diversity.

With regards to the methodological approaches, a further optimisation of the DNA extraction from the martian regolith S-MRS would be advisable for future studies, perhaps with iron chelating agents that prevent its binding to the DNA molecules. In terms of confocal Raman microspectroscopy as a possible approach for the remote life detection of non-pigmented microorganisms in future planetary exploration missions, the discussed technical difficulties must be overcome (e.g. setting up a careful sample preparation that allows the detection of microbial biosignatures), or perhaps other technologies that require little sample preparation and allow specific biomolecule recognition should be considered.

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**V. APPENDIX**

**Supporting Mars exploration: BIOMEX in Low Earth Orbit and further astrobiological studies on the Moon using Raman and PanCam technology**

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**Abstract**

The Low Earth Orbit (LEO) experiment *Bio*logy and *M*ars *Ex*periment (BIOMEX) is an interdisciplinary and international space research project selected by ESA. The experiment will be accommodated on the space exposure facility EXPOSE-R2 on the International Space Station (ISS) and is foreseen to be launched in 2013. The prime objective of BIOMEX is to measure to what extent biomolecules, such as pigments and cellular components, are resistant to and able to maintain their stability under space and Mars-like conditions. The results of BIOMEX will be relevant for space proven biosignature definition and for building a biosignature data base (*e.g.* the proposed creation of an international Raman library). The library will be highly relevant for future space missions such as the search for life on Mars. The secondary scientific objective is to analyze to what extent terrestrial extremophiles are able to survive in space and to determine which interactions between biological samples and selected minerals (including terrestrial, Moon- and Mars analogs) can be observed under space and Mars-like conditions. In this context, the Moon will be an additional platform for performing similar experiments with negligible magnetic shielding and higher solar and galactic irradiation compared to LEO. Using the Moon as an additional astrobiological exposure platform to complement ongoing astrobiological LEO investigations could thus enhance the chances of detecting organic traces of life on Mars. We present a lunar lander mission with two related objectives: a lunar lander equipped with Raman and PanCam instruments which can analyze the lunar surface and survey an astrobiological exposure platform. This dual use of testing mission technology together with geo- and astrobiological analyses will significantly increase the science return, and support the human preparation objectives. It will provide knowledge about the Moon′s surface itself and, in addition, monitor the stability of life-markers, such as cells, cell components and pigments, in an extraterrestrial environment with much closer radiation properties to the surface of Mars. The combination of a Raman data base of these data together with data from LEO and space simulation experiments, will lead to further progress on the analysis and interpretation of data that we will obtain from future Moon and Mars exploration missions.

## 1. Introduction

The search for life or fossil remnants of life on other planets is a prime goal of future space missions due to its high scientific and philosophical relevance. The possibility that organisms could survive in harsh environments outside the Earth was investigated in previous experiments (Horneck et al., 2010), see also (Table 1). Spaceflight and ground based experiments have shown that especially micro-colonies of bacteria, meristematic black fungi and symbiotic associations of microorganisms such as lichens are able to survive and to be reactivated after space experiments (de la Torre Noetzel et al., 2007, de la Torre et al., 2010, de Vera et al., 2003, de Vera et al., 2004a, de Vera et al., 2004b, de Vera et al., 2007, de Vera et al., 2008, de Vera et al., 2009, de Vera, 2005, Horneck et al., 1994, Kordyum et al., 1983, Olsson-Francis et al., 2009, Onofri et al., 2008, Onofri et al., 2009, Sancho et al., 2007 and Tarasenko et al., 1990). Bacteria strains, such as *Bacillus subtilis* and *Deinococcus radiodurans*, are radiation and vacuum tolerant ( Horneck, 1993, Horneck et al., 1994, Horneck et al., 2001, Moeller et al., 2007a, Moeller et al., 2007b and Moeller et al., 2007c). Gram-negative endophytic bacteria and cyanobacteria survived during a 14 day-shuttle flight and their co-existence with plants exposed to microgravity showed enhanced plant colonizing

activity ( Tarasenko et al., 1990). During the BIOPAN 5 and 6 experiments the lichens *Rhizocarpon geographicum*, *Xanthoria elegans* and *Aspicilia fruticulosa* (renamed as *Circinaria gyrosa*) were analyzed after an exposure time to space conditions of about 10–16 day and parallel ground based tests were performed. The results after flight and simulation experiments lead to the conclusion that the tested symbiotic eukaryotic associations of alga and fungi in the lichen were not seriously damaged and nearly 70%–90% of the tested lichens have survived. They were physiologically active and able to germinate and grow. Some investigations on the mutation rate expressed by photoproducts produced in the DNA have also shown that the fungus was practically not affected by UV radiation but the algal symbiont was more sensitive ( de la Torre Noetzel et al., 2007, de la Torre et al., 2010, de Vera et al., 2003, de Vera et al., 2004a, de Vera et al., 2004b, de Vera et al., 2007, de Vera et al., 2008, de Vera et al., 2009, de Vera et al., 2010b, de Vera, 2005, de Vera and Ott, 2010a and Sancho et al., 2007). Cyanobacteria were also able to survive Low Earth Orbit (LEO) environment and simulated extraterrestrial conditions, as shown by analysis of the akinetes (resting-state cells of cyanobacteria, Olsson-Francis et al., 2009). Vegetative cells of *Chroococcidiopsis* sp. CCMEE 029 survived prolonged desiccation ( Billi, 2009) and a few minutes of exposure to unattenuated martian UV flux ( Cockell et al., 2005). The cells also survived, shielded by 3 mm of sandstone, in real space as well as simulated martian conditions representing 1.5 years exposure in LEO ( Billi et al., 2011). Moreover, these same vegetative cells associated with an epilithic microbial community survived 548 day on the EXPOSE-E facility ( Cockell et al., 2011). Numerous species mentioned here are even able to survive simulated asteroid impacts ( Horneck et al., 2001, Horneck et al., 2008, Meyer et al., 2011 and Stöffler et al., 2007). Mars simulation tests on methanogenic archaea have also shown a remarkable level of survival ( Morozova et al., 2007a and Morozova and Wagner, 2007b). The same has been observed for meristematic black fungi during a ground based experiment in the space simulation facilities at DLR Cologne named EVT (Experiment Verification Test), performed in preparation for the recent and completed EXPOSE experiments ( Onofri et al., 2008). They survived also further exposure to real space conditions on the ISS with a total exposure time of 1.5 years ( Onofri et al., 2012). In other ground-based experiments, we were able to demonstrate that *Paenibacillus* sp. IMBG221 caused bio-corrosion of an anorthosite rock—a Moon analog ( Lytvynenko et al., 2006). This brief overview shows that a wide variety of different microorganisms, including evolved ones, is able to resist and to survive space conditions for certain periods of time.

## 2. Experimental approach of BIOMEX—a step further

BIOMEX is an exposure experiment which will be performed on the exposure facility EXPOSE-R2 attached to the Russian Zveszda module of the International Space Station (ISS). The Launch is foreseen for mid-2013. The planned exposure duration is expected to be in maximum 1.5 years. EXPOSE-R2 is a hardware payload built by Kayser-Threde, where the samples can be placed into sample holders and exposed outside the module (Fig. 1). The concept of this experiment is an improvement on the previously executed experiments in Section 1.
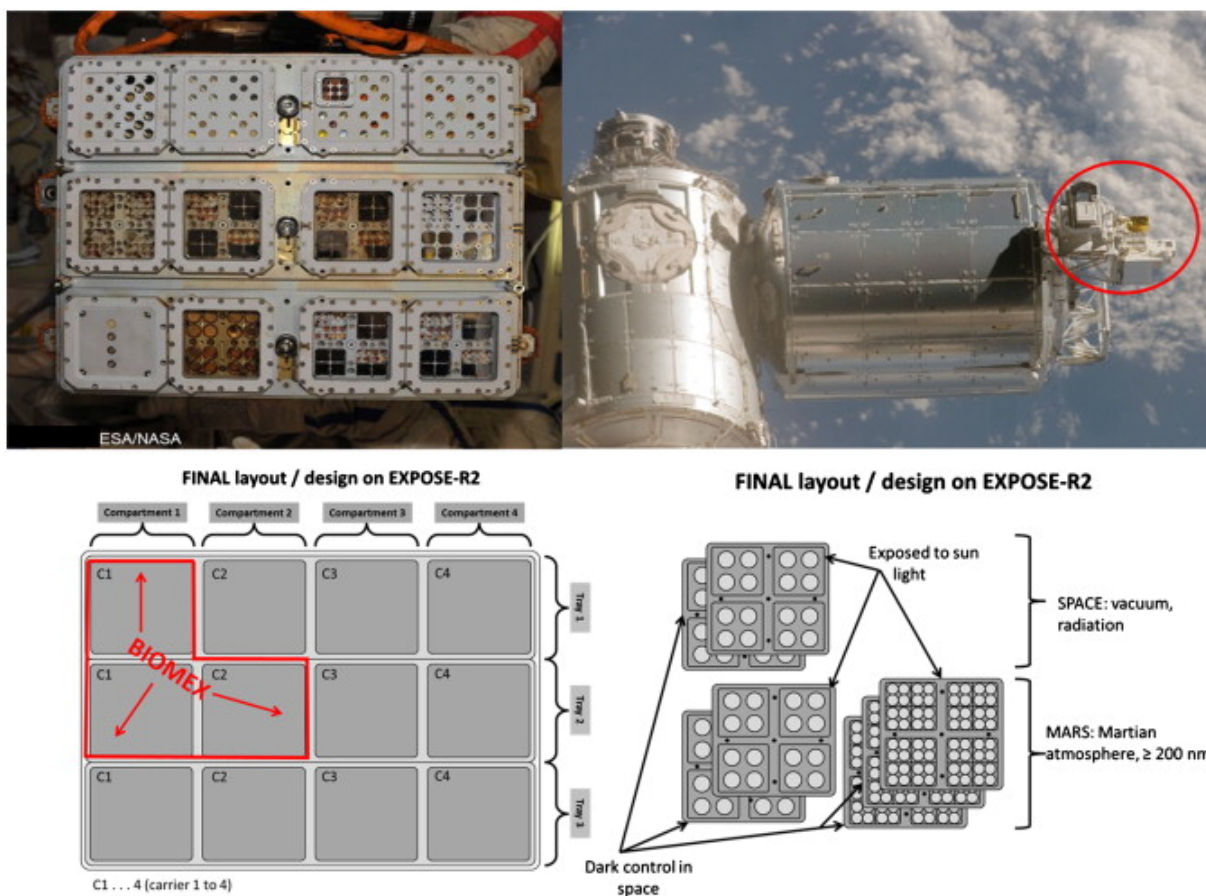
**Table 1**
Survival in Mars and space environment

| Tested organisms | Mars atmosphere | Vacuum | UV/space radiation | Shock experiments |
|---|---|---|---|---|
| **Archaea** | | | | |
| *Methanosarcina* | + | + | +/− | n.d. |
| **Bacteria** | | | | |
| *Bacillus subtilis* | + | + | + | + |
| *Deinococcus radiodurans* | + | + | + | n.d. |
| Endophytic gram⁻ bacteria | + | + | + | n.d. |
| *Cyanobacteria (e.g. Chroococcidiopsis)* | +/− | +/− | +/− | +/− |
| **Lichens** | | | | |
| *Aspicilia fruticulosa (renamed: Circinaria gyrosa)* | + | + | + | n.d. |
| *Rhizocarpon geographicum* | + | + | + | n.d. |
| *Xanthoria elegans* | + | + | + | + |
| **Merismatic black fungi** | | | | |
| *Cryomyces antarcticus* | + | + | + | n.d. |

(+) Positive survival; (−) No survival; (n.d.) Not determined.

## 2.1. First scientific objective of BIOMEX

Its primary objective is to pay more attention to the structure and stability of most of the biological components, especially to the protecting surface coats, membranes, proteins, pigments or secondary metabolite deposits on cell surfaces. In previous exposure experiments, these investigations were incomplete with respect to the identification of space-resistant biosignatures, although the results we might get by such a systematic analysis are of high relevance for the characterization and detection of potential biosignatures of recent/extant microorganisms on future missions to Mars. Another reason for using the BIOMEX experiment is to deepen these investigations and to analyze the effect of space environment by using the biological samples in contact with Lunar Regolith analog rocks, such as anorthosite (Mytrokhyn et al., 2003) and two Mars regolith analog mixtures with the characteristics of phyllosilicatic Mars Regolith soils (P-MRS) and sulfatic Mars Regolith soils (S-MRS) reflecting alteration minerals formed during different environmental conditions of the Noachian and Hesperian/Amazonian, respectively (Böttger et al., 2012). These two Mars analog mineral mixtures have been developed by the Naturkundemuseum Berlin in the framework of the Helmholtz-Alliance "Planetary Evolution and Life". The production of the analogs is based on recent Mars data and publications (Bibring and Langevin, 2005, Bibring and Squyres, 2006, Chevrier and Mathé, 2007 and Poulet and Bibring, 2005).

**Fig. 1.** EXPOSE R (Zvezda module/ top left) and EXPOSE-E on racks of Columbus module (top right, red circle); position of BIOMEX experiment in the EXPOSE-R2 hardware (bottom left) and sample holders (bottom right).

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rock and mineral analogs is an important parameter for optimizing exploration procedures focusing on the detection of mineral composition as a context for biological investigations. Hence space exposure experiments, such as presented by the concept of BIOMEX, might help to avoid possible pitfalls which might occur due to possible overlapping of signals during spectroscopic detection procedures. The influence of interfering mineralogical features or changes in the molecular conformation of biological substances after exposure to the space environment on the ISS may be identified. Such experiments will greatly aid to identify if signatures of extinct or extant life are present on another planet.

As mentioned above, a number of pigments and membrane components, representing bio-relevant substances extracted from cells and embedded in the Mars and Lunar regolith analog mineral pellets are also part of the experiment. This will provide valuable information about the stability of the pigments before and after space exposure and might also help to determine the location of protected bio-signatures in Mars-like soils.

## 2.2. Second scientific objective of BIOMEX

The BIOMEX space experimental platform provides an ideal opportunity to pursue a second objective: the samples used can also test the viability and space resistance of microorganisms in the context of (Litho-)Panspermia (Arrhenius, 1903, Arrhenius, 1908, Arrhenius, 1918, Chyba and Sagan, 1992 and Martins et al., 2008). This concept was tested in space experiments on FOTON/BIOPAN (Sancho et al., 2007 and de la Torre et al., 2010) and more recently on the latest EXPOSE missions on the ISS. Replicates are needed, as well as data during different phases of the sun′s activity for a re-evaluation of the Lithopanspermia theory. In addition to replicates, a new set of microorganisms, representing species of all three main branches of the tree of life have been chosen (Fig. 2), in particular microorganisms which are known to be relevant for Mars (*e.g.* methane producing archaea, cyanobacteria and iron bacteria/ Fig. 3).
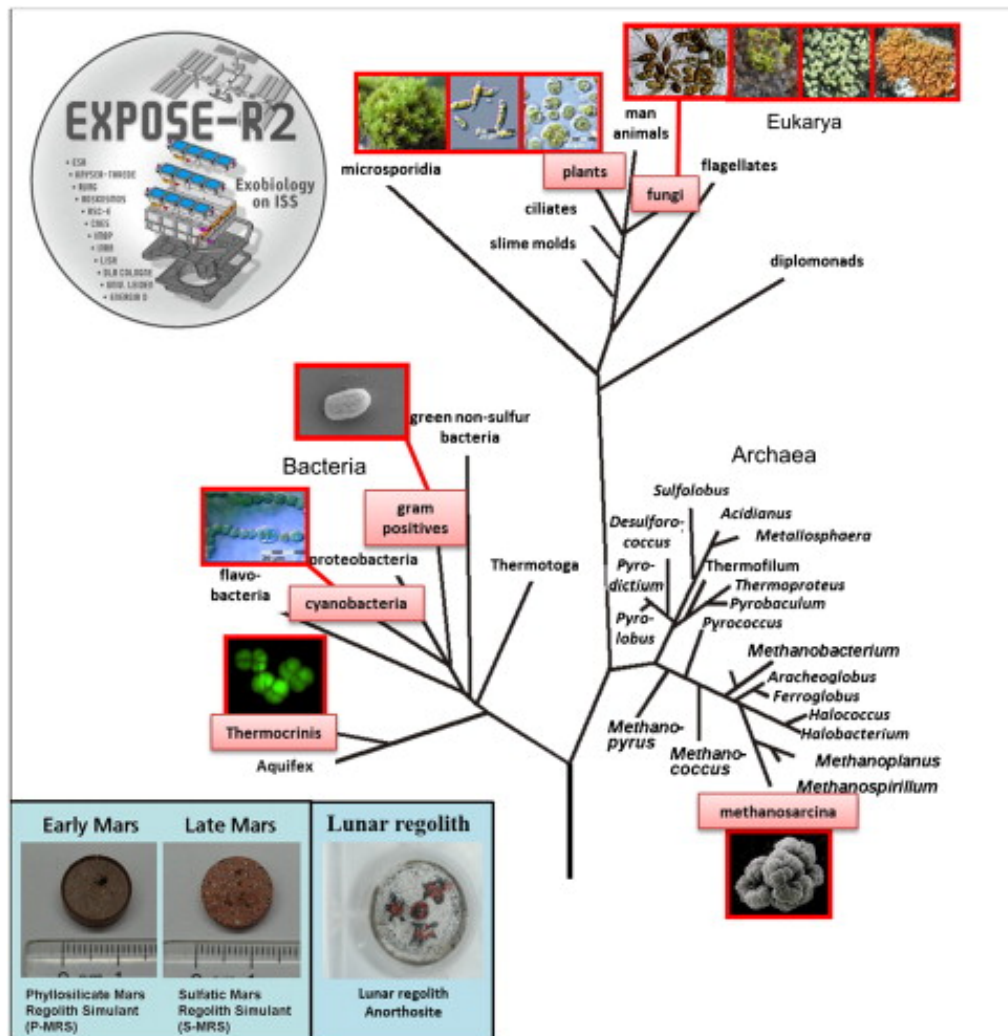
**Fig. 2**. The rRNA analysis-based phylogenetic tree of terrestrial life including the selected organisms for the BIOMEX space experiments onboard the ISS. Bottom left: mineral pellets selected for BIOMEX.

Currently, a number of space simulation experiments, the Experiment Verification Tests (EVTs) and Scientific Verification Tests (SVTs), are being performed and are in continuation at the space simulation facilities at DLR Cologne to aid sample preparation and the selection processes of the best samples to be sent to space. The preliminary results indicate a high survival rate of the investigated organisms and the stability of their pigments. Therefore the final arrangement of the sample distribution in the space hardware will not differ much from the layout presented in Fig. 2 and Fig. 3.
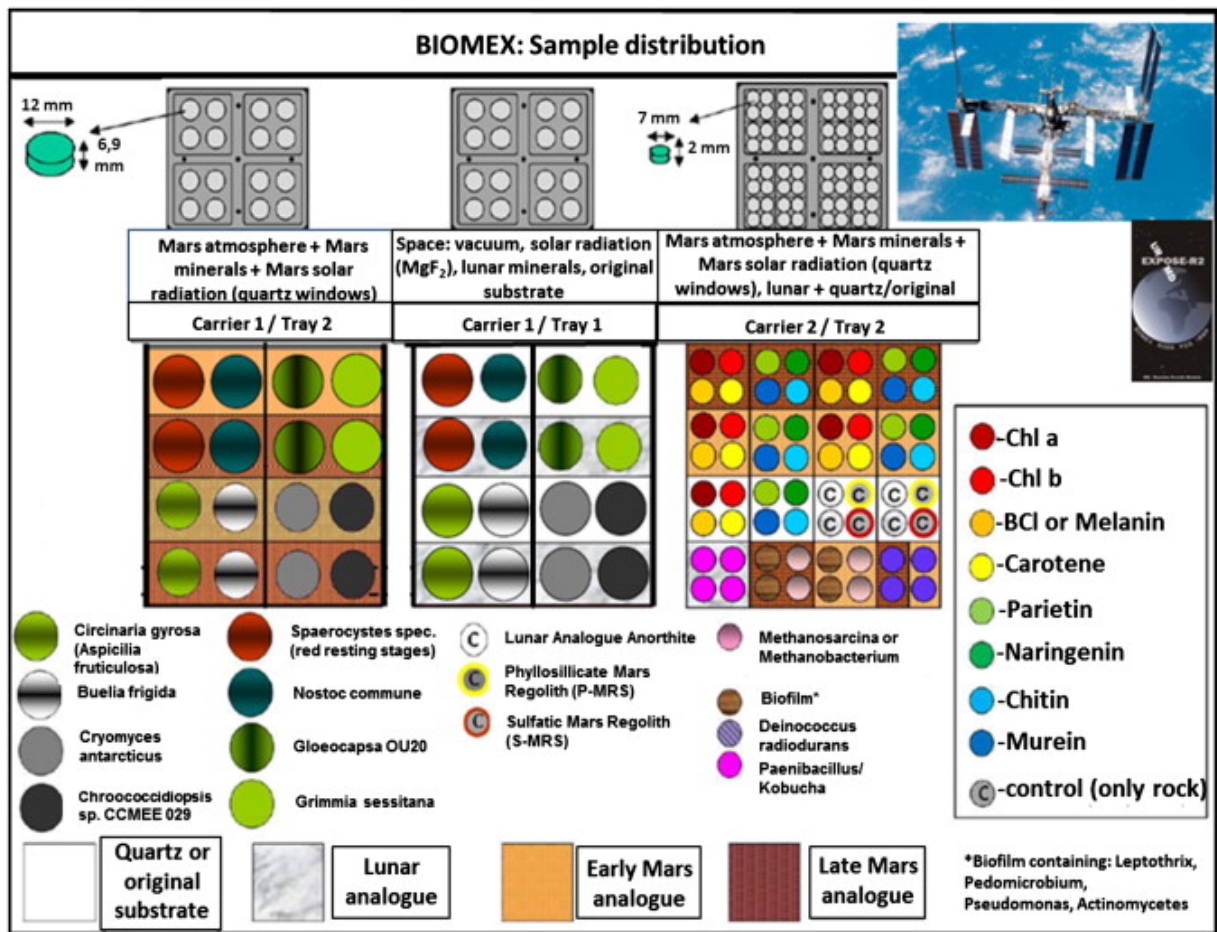
**Fig. 3**. Sample positions in hardware and parameter description.

## 3. The relevance of exposure experiments on the Moon

BIOMEX is designed as a precursor experiment for follow-on geo-biological studies on the Moon and as preparation and support for future Mars exploration missions as described in the original proposal (ILSRA-2009-0834).

In response to ESA's call for letters of interest and proposals for a Lunar Lander, the national and international HGF-Alliance "Planetary Evolution and Life" and its cooperating teams have submitted a proposal, named LOGOS (Lunar-Organisms and Geology Operation Sciences), emphasizing research on space weathering on organisms and rocks.

The LOGOS-Cube is designed as a package which has a closely connected context to the present BIOMEX experiment in LEO. It is intended to serve as an exposure platform on the Moon for organisms, organics and mineral surfaces of different rocks with the same scientific focus as described for the BIOMEX experiment. The LOGOS-Cube will obtain information on the effect of space weathering on organisms and rocks and the degradation or resistance of organics produced by life forms, thus contributing to our understanding of relevant life markers/bio-signatures as a reference to the search for life on other planets. The Moon is a natural vantage platform with an enhanced radiation exposure due to the absence of both, an own magnetic field and an atmosphere. The data expected from the present BIOMEX project on the ISS will facilitate identification of the most suitable samples for lunar orbiter or lander experiments and will provide reference data for future Moon experiments. These data will be extremely useful in support of future missions to Mars to search for biosignatures as evidence

for remnant or fossil and therefore extinct life and for the search of extant life on other Solar System bodies.

## 4. Our proposal: biosignature analysis combining PanCam and Raman in situ measurement devices on one exposure-platform

For a new lander with multifunctional technology tests and additional scientific outcome, our suggestion is to combine existing and space proved exposure platforms and technologies with a Technical Readiness Level (TRL) of about 6–9. We propose to use a small version of the EXPOSE platform, as used on the ISS, with a maximum of 3 carriers in a compartment which could serve as sample holder for geo-biological samples as shown in Fig. 1. One additional sensor carrier will be used to collect temperature and radiation data. This particular exposure device is space proven and its TRL level is almost 9 (Rabbow et al., 2009). In addition, *in situ* measurements in the visible and multispectral wavelength are required. Therefore, instruments such as a multispectral camera, sensitive in the visible (VIS) and near infrared (NIR) range, as well as fluorescence, Raman and IR spectrometers are needed for detection of the spectral characteristics of life forms and biosignatures and for the observation of their behavior during the exposure time (*e.g.* position and possible changes of specific detected peaks in different spectral ranges previously determined as specific bio-relevant finger prints of, for example, unaffected pigments can indicate changes and destruction of the molecular structure during space exposure ( Dartnell et al., 2012))".

## 4.1. Panoramic wide angle multispectral Stereo Camera (PanCam) combined with IR and Raman spectroscopy

One of the proposed instruments which can be helpful to support this task is a panoramic wide-angle multispectral stereo camera (PanCam, Coates et al., and Griffiths et al., 2006) with TRL 6–7 that has been selected as part of the ExoMars rover (Vago and Kminek, 2007 and Cousins et al., 2010). A similar device is also already a part of the model payload of ESA′s Lunar Lander (Carpenter, 2011).

This camera system (1) delivers the geological context of the landing site (cf. Bell et al., 2004 and Smith et al., 1997) and (2) helps to identify the best locations for further study via multi-level analyses (Vago, 2005). Further capabilities of this system include multispectral analysis in the VIS/NIR (400–1000 nm) range that provides compositional information. The final optical characterization is done by a close-up imager inspection of surfaces at mm- or sub mm-scale (such as ExoMars PanCam HRC) or microscopically (Clupi on ExoMars (Josset et al., 2012), MAHLI (Edgett et al., 2005) on MSL). For ExoMars, the PanCam (Fig. 4A and B) consists of two wide angle cameras (WACs), which form a stereo pair and a narrow angle high resolution camera (HRC). Its first and foremost task in any payload is the delivery of geological context information. Geological information that can be gathered from greyscale WAC and HRC images includes outcrop context, structure, morphology, and brightness. Such knowledge can be used to make a preliminary classification of rock type and its weathered state. Additionally, the WACs are equipped with 3 wideband RGB filters and 11 narrow band (30–40 nm) geological filters to provide color and visible and near infrared spectral data, respectively. This provides both mineralogical and compositional information, which, in combination with morphology, structure and context helps for interpretation of the lithology of the landing site. In the context of life detection missions, such as ExoMars, the

utilization of recorded VIS/NIR spectra is especially important to assess astrobiological objectives such as habitability condition like detection of hydrated minerals that might indicate past aqueous activity (Fig. 4A and B). In addition, the same instrument can be utilized for visible and multispectral monitoring of samples on an EXPOSE-like exposure platform.
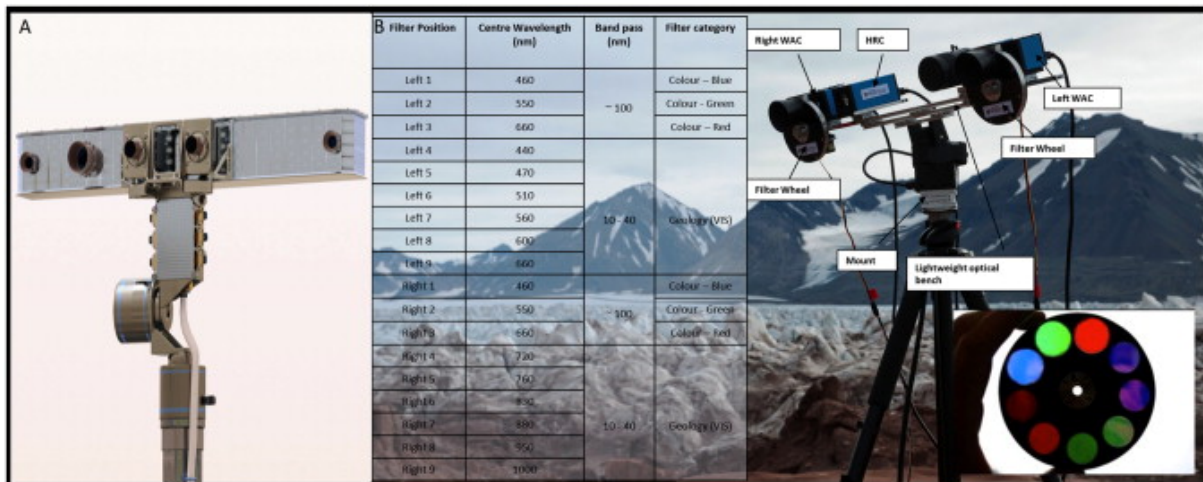


**Fig. 4.** (A) Drawing of the ExoMars PanCam instrument accommodated on a Pan-Tilt Unit on top of the ExoMars rover for multiple use. (B) Field Model of the ExoMars Panoramic Camera, as used during the Arctic Mars Analog Svalbard Expeditions (AMASE), with a range of VIS/NIR filters for multispectral composition analysis.

Multiple spectrometers can be mounted on the mast in addition to PanCam including IR- and Raman-spectrometers; which could be used for (3) *in situ* measurements of the exposed geo-bio-samples (see Fig. 5), as well as geological investigations of the Moon's surface.
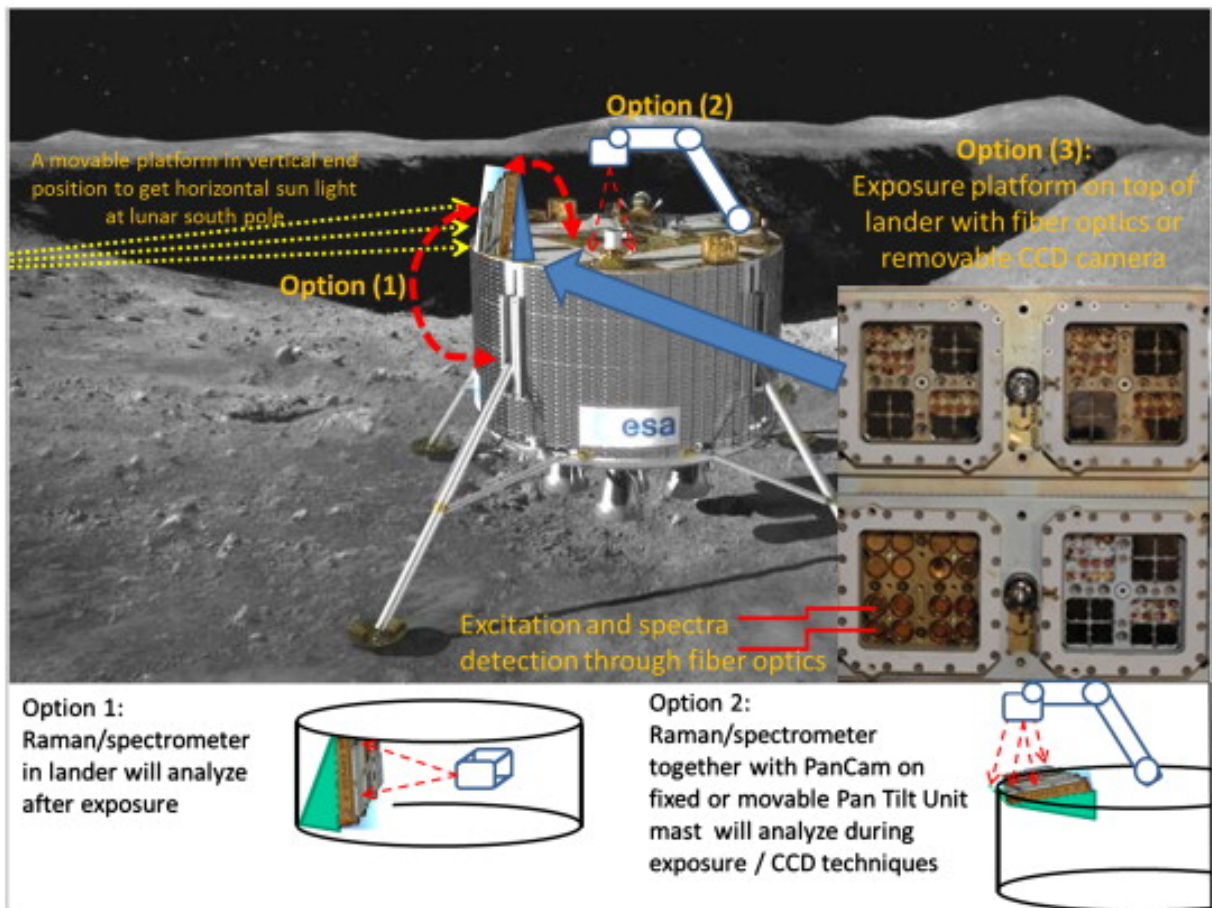
**Fig. 5**. The 3 options of operation scenarios to combine remote and *in situ* measurements.

## 5. Operation scenarios

We have envisaged three different scenarios for operations during the lander mission (Fig. 5):

• Option (1) is a movable, *i.e.* in- and out-side gliding exposure platform which exposes the geo-biological samples to the lunar environment (vacuum, radiation) and upon which the samples can be analyzed by a PanCam (outside) and a Raman/LIBS-spectrometer accommodated inside the lander (TLR 6–9, ISS and ExoMars techniques).

• Option (2) is the use of the PanCam instrument combined with different spectrometers including Raman and IR on top of a tiltable mast (Fig. 4A and Fig. 5) that can analyze the landing site geology as well as monitor changes in the geo-biological samples on the exposure platform during the mission, as previously described (TLR 6–9, ISS and ExoMars techniques).

• Option (3) is an exposure platform (TRL 9) on the top of the lander which has fiber optics in the exposure sample holders or a removable CCD camera with a connection to an internal data logger. But this option needs further investigation and the present TRL is at 5–6.

Our preferred option is (2) because of its multi-functionality and the high expected scientific outcome. Another advantage is the possibility of using a similar but smaller version of the exposure platform, as used on the ISS, because the carriers with the samples are also easily removable. This might be relevant for future lunar manned space missions, where astronauts can remove or exchange the carriers from the lander to transfer the samples back to Earth for further investigations. Additional techniques that would not be possible to be conducted on

the Moon during the lander mission because of technical and mass restrictions can be applied in ground-based laboratories.

## 6. Conclusion

The presented technology is available and the individual components have a high level of TRL. The new combination of existing and already applied techniques in LEO, as well as tested instruments for other space missions comparable to Mars missions, together with new innovative instrumentation, will support the ESA lunar lander objectives to prepare for human lunar exploration and will lead to high and valuable scientific outcome including astro-/exo-biology investigations.

In particular, data from the BIOMEX experiment obtained from spectrometers, such as Raman technology, will serve as reference for future exposure experiments on the Moon surface. By comparing data from LEO with data obtained on the surface of the Moon, we will improve our knowledge regarding the stability of selected biosignature spectra in high radiation environments similar to those expected on the surface of Mars. For such a proposed lunar lander mission we do not have to reinvent the wheel but can use resources, know-how and knowledge already available due to innovative enterprises in LEO and developed for Mars exploration studies (see Fig. 6).
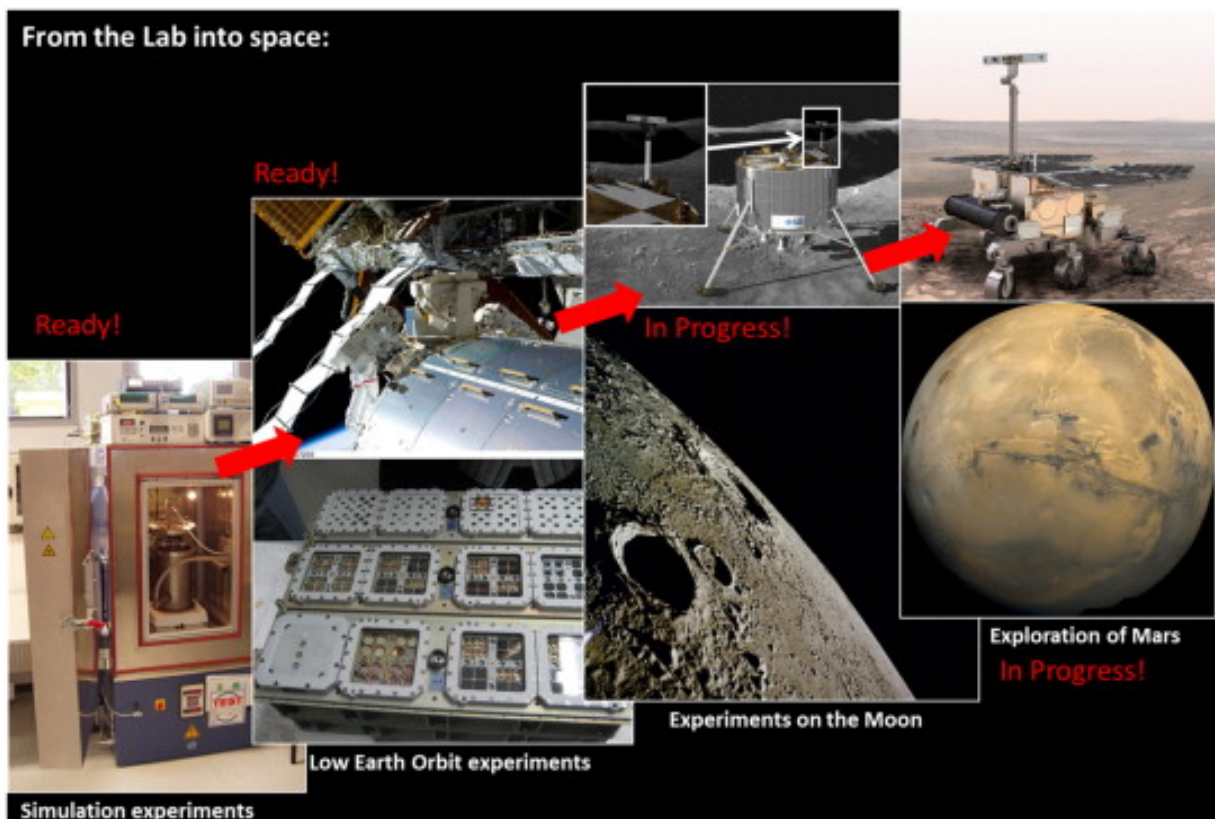


**Fig. 6.** The way to Mars exploration: the necessity and relevance of technology improvement and science tests in simulation facilities, in Low Earth Orbit and on the Moon.

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Hiermit versichere ich an Eides statt, dass die vorliegende Arbeit selbständig und unter Verwendung keiner anderen als der angegebenen Hilfsmittel verfasst wurde.

(Paloma Serrano)                              (Potsdam, Mai 2014)
Unterschrift                                        Ort, Datum