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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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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 permafrost 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

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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 μm 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; $MgCl_2 \cdot 6H_20$, 0.4 g; $CaCl_2 \cdot 2H_2O$, 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 °C. 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 inoculation). 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 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. 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 Technology PLC, Belfast, Northern Ireland) as detector. An apochromatic 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 backscattered 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 μm, which acts as the entrance slit (pinhole) of the microspectrometer. The Raman experiments were conducted at diffraction limited conditions of about 320 nm 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 ($r=1$: high correlated data/ identify), 1000 ($r=0$: uncorrelated data) and 2000 ($r=-1$: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 7900*g* for 40 min and 4 °C and washed in 100 mL of distilled water at 4600*g* for 30 min and 4 °C. 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 wolfram (Quorum Q150T S, Gala Instrumente GmbH, Bad Schwalbach, Germany). The samples were examined using a LEO 1530 Scanning Electron Microscope

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