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Stable carbon isotope fractionation by methanogens growing on different Mars regolith analogs

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ABSTRACT

In order to characterize stable carbon ($^{13}C/^{12}C$) isotope fractionation of metabolically produced methane by methanogens in martian settings, *Methanothermobacter wolfeii*, *Methanosarcina barkeri*, and *Methanobacterium formicicum* were cultured on four different Mars regolith analogs – JSC Mars-1, Artificial Mars Simulant, montmorillonite, and Mojave Mars Simulant – and also in their growth supporting media. These chemoautotrophic methanogens utilize CO_2 for their carbon source and H_2 for their energy source. When compared to the carbon isotope signature of methane when grown on their respective growth media, *M. wolfeii* and *M. barkeri* demonstrated variability in carbon isotope fractionation values during methanogenesis on the Mars analogs, while *M. formicicum* showed subtle or negligible difference in carbon isotope fractionation values. Interestingly, *M. wolfeii* and *M. barkeri* have shown relatively consistent enriched values of ^{12}C on montmorillonite, a kind of clay found on Mars, compared to other Mars regolith analogs. In general, *M. barkeri* showed large carbon isotope fractionation compared to *M. wolfeii* and *M. formicicum* during methanognesis on various kinds of analogs. Stable carbon isotope fractionation is one of the techniques used to infer different origins, environments, and pathways of methanogensis. The results obtained in this novel research can provide clues to determine ambiguous sources of methane on Mars.

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1. Introduction

Mars is a cold, arid, and seemingly inhospitable planet. The detection of methane in the martian atmosphere (Formisano et al., 2004; Krasnopolsky et al., 2004; Mumma et al., 2009; Webster et al., 2014) and the probability of the existence of liquid water during the early history of Mars (Christensen et al., 2004; Herkenhoff et al., 2004; Klingelhofer et al., 2004; Rieder et al., 2004; Squyres et al., 2006; Ehlmann et al., 2011) and possibly today (McEwen et al., 2011, 2013) prompts enthusiasm about plausible life forms on Mars. Mumma et al. (2009) have obtained temporal, spatial, and seasonal distribution of methane in the Martian atmosphere. The half-life of methane in a planetary atmosphere is about 300 years (Hunten, 1979), so in order to be detected on Mars, methane would need to be replenished continuously.

Methane on Mars may be ancient or contemporary. If ancient, it may have been stored in clathrate ices and released into the atmosphere from time to time as environmental conditions changed (Chastain and Chevrier, 2007). Various potential sources of methane in the martian atmosphere have been suggested, such as volcanic, meteoritic, cometary, hydrogeochemical, and biogenic sources (Atreya et al., 2007). On Earth, however, about 90–95% of atmospheric methane has a biological origin, either from living organisms or decay of organic matter (Atreya et al., 2007). Hence, one explanation for the finding and non-uniform distribution of methane on Mars could be localized microbial sources, either extinct or extant, such as methanogens. Methanogens are anaerobic chemoautotrophs that mostly consume CO_2 as a carbon source and H_2 as an energy source and produce methane as an end product of metabolism.

Methanogens have been considered models for possible martian life-forms even before the discovery of methane in Mars' atmosphere (Boston et al., 1992; Chapelle et al., 2002; Kral et al., 1998; Weiss et al., 2000). Methanogens are anaerobes, and certain strains can tolerate low pressure, desiccation (Kral et al., 2011), and very cold temperature (Reid et al., 2006), like conditions present on Mars. Additionally, CO_2 is abundant in the Martian atmosphere while molecular hydrogen could be formed by serpentinization on Mars (Oze and Sharma, 2005; Lyons et al., 2005; Schulte et al., 2006). These gases could provide both a carbon and an energy source for methanogens.

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To determine if methanogens could be plausible extinct or extant life on Mars, various experimental techniques need to be utilized. Stable carbon isotope fractionation is one of the important techniques that can suggest potential sources of methane (Allen et al., 2006). Due to difference in mass between isotopes of the same element, partial separation of isotopes occurs during any physical or chemical process. The magnitude of the fractionation depends on the associated isotopic effect of an element in any system. Hence, carbon isotope fractionation values aid in recognition of the surroundings of methanogenesis, such as type and availability of different types of substrates, as these factors have an effect on carbon isotope fractionation of methane (Londry et al., 2008). Life, as we know it, tends to favor the lighter isotope of carbon, carbon-12, because of the lower energy costs during the bond formation in any biochemical reactions. Consequently, bioorganic molecules are rich in carbon-12 but deficient in carbon-13. Due to this, carbon isotopic fractionation may be able to distinguish between biologically and geologically produced methane. Moreover, carbon isotope composition, along with hydrogen isotope composition, may possibly discriminate microbial methane from thermogenic methane, such as methane produced by thermal decay of complex organic matter (Allen et al., 2006; Schoell, 1988).

The carbon isotope fractionation, $\delta^{13}C$, value allows an understanding of different pathways of methanogenesis. Microbial methane usually has $\delta^{13}C(CH_4)$ lower than −60‰, whereas thermogenic methane has $\delta^{13}C(CH_4)$ higher than −50‰ (Cicerone and Oremland, 1988). Moreover, $\delta^{13}C(CH_4)$ between −110‰ and −60‰ is the result of autotrophic methanogenesis, while $\delta^{13}C(CH_4)$ ranging between −65‰ and −50‰ is the result of acetotrophic methanogenesis (Whiticar et al., 1986). Hence, the characterization of the carbon isotope fractionation of methane is crucial to identify the potential sources of methane on Mars.

In this paper, we present the carbon isotope fractionation pattern of methane produced by three different strains of methanogens growing on four different Mars regolith analogs. These methanogens have shown survivability on these analogs (see Section 2) in previous studies (Kral et al., 2004; Chastain and Kral, 2010). In this work, methanogens were provided CO_2, in the form of bicarbonate buffer, and molecular hydrogen in the gaseous form. Molecular hydrogen acts as an electron donor and reduces CO_2 to methane. Micronutrients were provided by the regolith analogs.

The carbon isotope fractionations of methane by methanogens on Earth have been studied for a long time in both natural and carefully controlled laboratory environments. The nature of the stable carbon isotope fractionation of methane by methanogens growing on martian regolith simulants has not been studied so far. Therefore, this novel work attempts to further our understanding of the source of methane on Mars.

2. Materials and methods

2.1. Methanogenic cultures and growth media

Pure cultures of *Methanothermobacter wolfeii* (OCM36), *Methanosarcina barkeri* (OCM38), and *Methanobacterium formicicum* (OCM55) were initially obtained from Oregon Collection of Methanogens, Portland State University, Portland, OR. Each species was grown in its respective growth supporting medium:

(1) MS medium (Boone et al., 1989) for *M. barkeri,* which contains yeast extract, trypticase peptone, mercaptoethanesulfonic acid, potassium phosphate, ammonium chloride, calcium chloride, resazurin as an oxygen indicator, and many trace minerals;
(2) MSF medium for *M. formicicum,* which contains the same composition as MS medium but also includes sodium formate;
(3) MM medium (Xun et al., 1988) for *M. wolfeii,* which contains the same components as MS medium except yeast extract, trypticase peptone, and mercaptoethanesulfonic acid.

These media were prepared in a Coy Laboratories anaerobic chamber (Coy Laboratory Products Inc., Grass Lake Charter Township, MI), which was filled with 90% carbon dioxide and 10% hydrogen. Different growth media were then transferred into anaerobic culture bottles inside the anaerobic chamber as described by Boone et al. (1989). These containers were then sealed with butyl rubber stoppers, removed from the chamber, crimped with an aluminum cap, and autoclaved for sterilization.

In order to eliminate any residual molecular oxygen from the vessels containing the media, a sterile sodium sulfide solution (2.5% wt/vol; 1.5 mL per 100 mL of media) was added to each vessel about an hour prior to inoculation of the methanogens (Boone et al., 1989). The vessels were pressurized with 200 kPa of hydrogen gas and incubated at their respective optimal growth temperatures. *M. wolfeii* grows optimally around 55 °C while the optimal growth temperature for *M. barkeri* and *M. formicicum* is around 37 °C.

2.2. Mars regolith analog substrate preparation

Four different Mars regolith analogs utilized in this experiment were JSC Mars-1 (Allen et al., 1998); Artificial Mars Simulant (AMS) (Fanale et al., 1982), which is a mixture of 45% smectite, 45% basalt, and 10% hematite; montmorillonite (Bentonite, WA: 46E 0438, size $<63\ \mu m$); and Mojave Mars Simulant (MMS; Peters et al., 2008), which mainly consists of basalt. Montmorillonite, a clay mineral, is abundant on Mars (Bishop and Murad, 2004). A total of thirty-six 150 mL serum bottles were used for three different strains of methanogens and four different Mars simulants. For statistical sampling, each experiment was done in triplicate. Four bottles were also prepared for negative controls consisting of regolith simulant without methanogens. Each bottle contained 3 g of the regolith analog. They were left overnight in the anaerobic chamber to deoxygenate. On the following day, 60 mL of bicarbonate buffer were added to each bottle. Bottles were sealed with butyl rubber stoppers, removed from the chamber, secured with aluminum crimps and autoclaved. For positive controls, three bottles of each medium containing 60 mL of MM, MS, and MSF were also prepared. Another three bottles of a negative control consisting of only sterile buffer were also utilized.

2.3. Inoculation of methanogens in growth media and Mars analogs

Actively growing microbial cells were centrifuged at 6000 rpm (Beckman CP centrifuge) for 10 min and washed two times with reduced sterile bicarbonate buffer in sterile plastic centrifuge tubes. McAllister and Kral (2006) determined that these methanogen strains could tolerate up to 1.5 h of exposure to atmospheric oxygen during the washing procedure. Washing of cells with buffer ensures that they do not carry over any residual growth media. The washed cells of each species were then suspended in 15 mL of sterile bicarbonate buffer. Each bottle containing a Mars regolith analog received a 1 mL aliquot of their respective cell suspension (except negative control bottles). All bottles, except buffer-containing bottles, were pressurized with 200 kPa of H_2 and incubated at the organisms' respective optimal growth temperatures.

Headspace gas was analyzed periodically for methane concentration using a Varian CP-4900 Micro-GC. Stable carbon isotopic fractionation of the methane and starting CO_2 was measured by a Cavity Ringdown Spectrometer G2201 (University of Arkansas Stable Isotope Laboratory).

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