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Planetary and Space Science

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Potential use of highly insoluble carbonates as carbon sources by methanogens in the subsurface of Mars



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ARTICLE INFO

Article history:

Received 17 January 2014

Received in revised form

6 June 2014

Accepted 8 July 2014

Available online 30 July 2014

Keywords:

Methanogens

Mars

Carbonates

Carbon source

ABSTRACT

Methanogens, microorganisms in the domain Archaea, have been studied as life forms that might inhabit the subsurface of Mars. These organisms can use carbon dioxide as a carbon source, a compound that is abundant in the martian atmosphere. But if they exist in the deep subsurface where the carbon dioxide may not penetrate, they would have to rely on another source of carbon. Magnesium carbonate and calcium carbonate have been detected at the martian surface, and there is no reason to believe that they would not be in the subsurface as well. In the research reported here, we asked if these carbonates could possibly serve as carbon sources for four species of methanogens. *Methanothermobacter wolfei*, *Methanobacterium formicicum* and *Methanococcus maripaludis* were able to produce a small amount of methane (approximately 0.4–0.8% headspace gas) when either carbonate was the carbon source available while *Methanosarcina barkeri* only produced significant methane (also 0.4–0.8%) when calcium carbonate was the carbon source. The amounts produced were dependent on methanogenic species, carbonate used and pH. At equilibrium, a small amount of carbon dioxide (approximately 0.05–0.15% headspace gas as well as in liquid media) was generated by these carbonates, and this carbon dioxide was most likely the carbon compound that was being metabolized. Background carbon dioxide from the atmosphere was not sufficient for measureable methane production.

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1. Introduction

Methanogens have been studied as possible life forms on Mars for many years (Chastain and Kral, 2012; Chastain and Kral, 2010a, 2010b; Kendrick and Kral, 2006; Kral et al., 1998, 2004, 2011; Kral and Altheide, 2013; McAllister and Kral, 2006; Moran et al., 2005; Ormond and Kral, 2006; Ulrich et al., 2010). These microorganisms are members of the domain Archaea, and can be found in a variety of locations on Earth including, but not limited to, human and animal intestines, swamp marshes, wetlands, permafrost, hot springs, and hydrothermal vents beneath the oceans (Chapelle et al., 2002; Morozova et al., 2007). All of these environments are depleted of molecular oxygen (O₂), ideal for these anaerobic organisms. Methanogens are chemoautotrophs, so they use an inorganic energy source and do not require sunlight. In fact, the majority of methanogen species need just liquid water, molecular hydrogen (H₂), a carbon source, and a few other nutrients for optimum growth (Staley, 1989). Methanogens obtain their name from the fact that they emit methane (CH₄) gas as a waste product

due to consumption of H₂ and carbon dioxide (CO₂). These organisms play an important role in the global carbon cycle (Dickens, 2003) and are responsible for the bulk of the CH₄ in the Earth's atmosphere today (Pavlov et al., 2000).

All the ingredients required for the existence of methanogens are believed to be present on Mars. H₂, the simplest energy source for a majority of methanogens, might arise from volcanic or hydrothermal activity, or the reaction of anaerobic water and basalt (Boston et al., 1992; Stevens and McKinley, 1995; Chapelle et al., 2002). In the past, liquid water flowed on the surface of Mars (Carr, 1996; Chandler, 2004; Leshin et al., 2013; Meslin et al., 2013). Today, most of the martian water is frozen, however, some of the frozen water beneath the surface may be melted by volcanic activity or geothermal energy (Schorghofer and Aharonson, 2005; Urquhart, 2005; Ulrich et al., 2010). As for the carbon source, methanogens near the surface may use the predominant martian atmospheric gas, CO₂. Fanale et al. (2012) have determined that a substantial amount of the atmospheric CO₂ could be harbored within a few meters of the surface. The terrain of Mars contains approximately 2% or greater atmosphere-exchangeable CO₂, and this absorbed CO₂ is the main repository of atmosphere-exchangeable CO₂ (Fanale and Cannon, 2012).

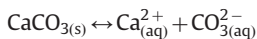
But microbes in the subsurface may not have access to the atmospheric CO₂. These methanogens would need to locate an

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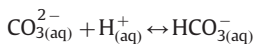
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alternate carbon source. Carbonate mineralization has been found on the martian meteorite ALH84001 (Borg et al., 1999). The carbonate is estimated to have formed in the early history of Mars, during its warmer and wetter days. The properties of these carbonates suggest that they are a product of precipitation in a closed system. The dissolution of carbonates is consistent with an origin from the CO₂ prevalent in the martian atmosphere. Due to a possible greenhouse effect in Mars earlier history, atmospheric CO₂ may have dissolved in oceans and then sequestered as relatively insoluble carbonates in rock (Quinn et al., 2006). Carbonates have also been observed in two other martian meteorites, EETA79001 and Nakhla (Brack and Pillinger, 1998). In addition to the evidence found on martian meteorites, calcium carbonate (CaCO₃) has been detected by the Mars Phoenix Lander (Boynton et al., 2009) and magnesium carbonate (MgCO₃) by the Mars Reconnaissance Orbiter (Ehlmann et al., 2008).

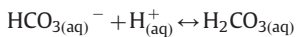
It is important to note that even though they are very often termed insoluble, CaCO₃ and MgCO₃ are slightly soluble in aqueous solutions ($K_{sp}=4.8 \times 10^{-9}$ at 25 °C for CaCO₃ [Patnaik, 2003] and $K_{sp}=10^{-7.8}$ at 25 °C for MgCO₃ [Bénézech et al., 2011]). For their metabolism, methanogens might take advantage of this solubility and utilize the small amount of dissolved CO₂ that is produced from the equilibration between the carbonate and CO₂ present in the water (Altheide et al., 2007; Hastings et al., 1927). The reaction begins with the carbonate partially dissolving into its component ions. Using CaCO₃ as an example,



Some of the CO₃²⁻ combines with H⁺ in the aqueous mixture to form a bicarbonate ion (HCO₃⁻):



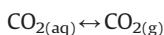
HCO₃⁻ can combine with H⁺ to form carbonic acid (H₂CO₃):



Decomposition of H₂CO₃ produces CO₂ and H₂O:



It is this CO₂ yielded from carbonic acid that the methanogens might be able to metabolize. This aqueous CO₂ in solution would be in equilibrium with gaseous CO₂, for instance, in the headspace of a culture tube:



The research reported here seeks to determine if this relatively small amount of CO₂ derived from carbonates can support CH₄ production by methanogens.

2. Materials and methods

2.1. Organisms and growth media

The methanogens, *Methanothermobacter wolfeii* (OCM 36), *Methanosarcina barkeri* (OCM 38), *Methanobacterium formicicum* (OCM 55) and *Methanococcus maripaludis* (OCM 151), were obtained from the Oregon Collection of Methanogens, Portland State University, OR, and grown in their respective growth media, MM, MS, MSF, and MSH, as described by Kral and Altheide (2013).

2.2. Preparation of media with supplements

Standard methanogenic growth media are typically prepared with a CO₂-saturated buffer in a Coy Anaerobic Chamber (Coy Laboratory Products Inc., Grass Lake Charter Township, MI)

containing approximately 90% CO₂. In the experiments where CO₂ was not added, media were prepared in flasks on the bench top using de-ionized water. Tubes containing these media (10 mL) were purged with argon gas for 7–10 min followed by sealing with butyl rubber stoppers to eliminate as much atmospheric O₂ as possible.

For tubes containing MgCO₃ (CAS #39409-82-0, Alfa Aesar, Ward Hill, MA), CaCO₃ (CAS #471-34-1, Alfa Aesar, Ward Hill, MA), magnesium chloride (MgCl₂; CAS #7791-18-6) or calcium chloride (CaCl₂; No. C-3881, Sigma Chemical Co., St. Louis, MO), these ingredients were added to the individual media as they were being prepared to give a final concentration of 1% wt/vol. All tubes of media (with or without supplements) were sealed with butyl rubber stoppers, crimped with aluminum crimps, and autoclaved at 121 °C at 15 psi for 30 min.

2.3. Carbonate as carbon source experiments.

For each methanogen being tested, tubes were prepared containing MgCO₃ with and without added CO₂ or CaCO₃ with and without added CO₂. In addition, because of the very different pH values of the media, portions of the MM and MS media were pH-adjusted to a final value (value after sealing, autoclaving, and addition of sodium sulfide [Na₂S]) of approximately 7.5 (Fisher Accumet pH Meter, Model 610 with a Fisher Electrode, Calomel Combination Polymer Body, Fisher Scientific, Pittsburgh, PA). The MgCO₃-containing media without CO₂ (unadjusted pH of 8.38 ± 0.08) were adjusted with 12 N hydrochloric acid (HCl; CAS #7647-01-0, EM Science, Darmstadt, Germany) while the CaCO₃-containing media without CO₂ (unadjusted pH of 6.94 ± 0.18) were adjusted with 18 N sodium hydroxide (NaOH; prepared using pellets, CAS #1310-73-2, JT Baker, Phillipburg, NJ).

Methanogenic cells, previously grown in standard growth media, were centrifuged at 6000 rpm (Beckman GP Tabletop Centrifuge, Beckman Coulter, Irving, TX) for 20 min followed by washing the cells with carbonate-containing media without added CO₂. Following a second centrifugation, the cells were re-suspended in the same medium (10 mL). A half-milliliter of each suspension was then added to the appropriate tubes. Prior to inoculation, 0.15 mL of sterile 2.5% Na₂S was added to each tube to eliminate residual O₂ (Boone et al., 1989). All tubes were pressurized with 200 kPa of H₂ and then incubated at temperatures conducive to growth (55 °C for *M. wolfeii*, 37 °C for *M. barkeri* and *M. formicicum*, 25 °C for *M. maripaludis*). Headspace gas samples (1 mL) were removed from individual tubes using sterile 3cc syringes at regular time intervals (typically seven days) and injected into a Varian Micro-GC with a thermal conductivity detector, model CP-4900 (Palo Alto, Ca) for CH₄ analysis. Scotty Analyzed Gases (0.5%, 1.01%, 2.5%, 5.70%, 50.0% CH₄; Supelco, Bellefonte, PA) were used to calibrate the gas chromatograph.

Only highest headspace methane values were included in Fig. 1. As a negative control, media containing no carbonates and no added CO₂ were inoculated with methanogens that had been centrifuged and washed with the same media (no carbonates and no added CO₂). Individual experiments contained three tubes for each medium, and the entire experiment was repeated three times.

2.4. Media with MgCl₂ or CaCl₂

Standard methanogenic growth media were also prepared containing 1 wt%/vol% MgCl₂ or CaCl₂. Cells were washed as previously described, except that they were washed with the appropriate chloride-containing media. Inoculation, incubation, and CH₄ measurements were as described previously. These cultures contained added CO₂ in the headspace. Only highest

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