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

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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 wolfeii, 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.

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 (O_2), 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 (H_2), a carbon source, and a few other nutrients for optimum growth (Staley, 1989). Methanogens obtain their name from the fact that they emit methane (CH_4) gas as a waste product due to consumption of H_2 and carbon dioxide (CO_2). These organisms play an important role in the global carbon cycle (Dickens, 2003) and are responsible for the bulk of the CH_4 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. H_2, 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, CO_2. Fanale et al. (2012) have determined that a substantial amount of the atmospheric CO_2 could be harbored within a few meters of the surface. The terrain of Mars contains approximately 2% or greater atmosphere-exchangeable CO_2, and this absorbed CO_2 is the main repository of atmosphere-exchangeable CO_2 (Fanale and Cannon, 2012).

But microbes in the subsurface may not have access to the atmospheric CO_2. These methanogens would need to locate an

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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 CO_2 prevalent in the martian atmosphere. Due to a possible greenhouse effect in Mars earlier history, atmospheric CO_2 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 ($CaCO_3$) has been detected by the Mars Phoenix Lander (Boynton et al., 2009) and magnesium carbonate ($MgCO_3$) by the Mars Reconnaissance Orbiter (Ehlmann et al., 2008).

It is important to note that even though they are very often termed insoluble, $CaCO_3$ and $MgCO_3$ are slightly soluble in aqueous solutions ($K_{sp}=4.8\times10^{-9}$ at 25 °C for $CaCO_3$ [Patnaik, 2003] and $K_{sp}=10^{-7.8}$ at 25 °C for $MgCO_3$ [Bénézeth et al., 2011]). For their metabolism, methanogens might take advantage of this solubility and utilize the small amount of dissolved CO_2 that is produced from the equilibration between the carbonate and CO_2 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 $CaCO_3$ as an example,

$$CaCO_{3(s)} \leftrightarrow Ca^{2+}_{(aq)} + CO^{2-}_{3(aq)}$$

Some of the CO_3^{2-} combines with H^+ in the aqueous mixture to form a bicarbonate ion (HCO_3^-):

$$CO^{2-}_{3(aq)} + H^{+}_{(aq)} \leftrightarrow HCO^{-}_{3(aq)}$$

HCO_3^- can combine with H^+ to form carbonic acid (H_2CO_3):

$$HCO_{3(aq)}{}^{-} + H^{+}_{(aq)} \leftrightarrow H_2CO_{3(aq)}$$

Decomposition of H_2CO_3 produces CO_2 and H_2O:

$$H_2CO_{3(aq)} \leftrightarrow CO_{2(aq)} + H_2O \quad (1)$$

It is this CO_2 yielded from carbonic acid that the methanogens might be able to metabolize. This aqueous CO_2 in solution would be in equilibrium with gaseous CO_2, for instance, in the headspace of a culture tube:

$$CO_{2(aq)} \leftrightarrow CO_{2(g)}$$

The research reported here seeks to determine if this relatively small amount of CO_2 derived from carbonates can support CH_4 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 CO_2-saturated buffer in a Coy Anaerobic Chamber (Coy Laboratory Products Inc., Grass Lake Charter Township, MI) containing approximately 90% CO_2. In the experiments where CO_2 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 O_2 as possible.

For tubes containing $MgCO_3$ (CAS #39409-82-0, Alfa Aesar, Ward Hill, MA), $CaCO_3$ (CAS #471-34-1, Alfa Aesar, Ward Hill, MA), magnesium chloride ($MgCl_2$; CAS #7791-18-6) or calcium chloride ($CaCl_2$; 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 $MgCO_3$ with and without added CO_2 or $CaCO_3$ with and without added CO_2. 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 [Na_2S]) of approximately 7.5 (Fisher Accumet pH Meter, Model 610 with a Fisher Electrode, Calomel Combination Polymer Body, Fisher Scientific, Pittsburgh, PA). The $MgCO_3$-containing media without CO_2 (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 $CaCO_3$-containing media without CO_2 (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 CO_2. 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% Na_2S was added to each tube to eliminate residual O_2 (Boone et al., 1989). All tubes were pressurized with 200 kPa of H_2 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 CH_4 analysis. Scotty Analyzed Gases (0.5%, 1.01%, 2.5%, 5.70%, 50.0% CH_4; 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 CO_2 were inoculated with methanogens that had been centrifuged and washed with the same media (no carbonates and no added CO_2). Individual experiments contained three tubes for each medium, and the entire experiment was repeated three times.

2.4. Media with $MgCl_2$ or $CaCl_2$

Standard methanogenic growth media were also prepared containing 1 wt%/vol% $MgCl_2$ or $CaCl_2$. Cells were washed as previously described, except that they were washed with the appropriate chloride-containing media. Inoculation, incubation, and CH_4 measurements were as described previously. These cultures contained added CO_2 in the headspace. Only highest