GROWTH OF METHANOGENS ON DIFFERENT MARS REGOLITH ANALOGUES AND STABLE CARBON ISOTOPE FRACTIONATION DURING METHANOGENESIS Navita Sinha[1], T. A. Kral[1,2] [1]Arkansas Center for Space and Planetary Sciences, University of Arkansas, Fayetteville, Arkansas, 72701, USA, [nxs017l@uark.edu], [2]Dept. of Biological Sciences, SCEN 632, University of Arkansas, Fayetteville, Arkansas, 72701, USA.

Introduction: The detection of methane in the Martian atmosphere (1-4) and the probability of the existence of liquid water during the early history of Mars (5-10) prompted enthusiasm about plausible life forms on Mars. The half-life of methane in a planetary atmosphere is about 300 years (11), so in order to be detected on Mars, methane would need to be replenished continuously.

Several potential sources of methane in the martian atmosphere have been suggested, such as volcanic, meteoritic, cometary, hydrogeochemical, and biogenic (12). On Earth, however, about 90 to 95% of atmospheric methane has a biological origin, either from living organisms or decay of organic matter (12). 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 have been considered models for possible martian life-forms even before the discovery of methane in Mars' atmosphere (13-16). Methanogens are anaerobes, and certain strains can tolerate low pressure, desiccation (17), and very cold temperature (18), like conditions present on Mars.

Methanogenic archaea 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. Stable carbon isotope fractionation is one of the important techniques that can distinguish various potential sources of methane (19).

Here, we present the carbon isotope fractionation pattern of methane produced by three different strains of methanogens, *Methanothermobacter wolfeii*, *Methanosarcina barkeri*, and *Methanobacterium formicicum*, growing on four different Mars regolith analogues. Methanogens were provided CO_2, in the form of bicarbonate buffer, and molecular hydrogen in the gaseous form.

Methods: The anaerobic stock cultures in growth-supporting media such as MM, MS, and MSF for *M. wolfeii*, *M. barkeri*, and *M. formicicum,* respectively, were prepared using protocols described previously (20). They were transferred into their respective new growth media every two weeks.

Four different Mars regolith analogues utilized in this experiment were JSC Mars-1 (21); JSC Mars-2 (22), which is a mixture of 45% smectite, 45% basalt, and 10% hematite; montmorillonite (WA: 46 E 0438, size <63μm); and Mojave Mars Simulant (MMS) (23). Montmorillonite, a clay mineral, is abundant on Mars (24). A total of thirty-six 150 mL serum bottles were used for three different strains of methanogens and four different Mars simulants. Samples were in triplicate. Each bottle contained 3g 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, 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.

About an hour prior to inoculation, 1.5 mL of sterile Na_2S were added to each sample bottle to remove any remaining molecular oxygen. Actively growing microbial cells were centrifuged at 6000 rpm for 10 min and washed two times with reduced sterile bicarbonate buffer (25). 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 Mars regolith analogues received a 1 mL aliquot of their respective cell suspension. All bottles were pressurized with 200 kPa of H_2 and incubated at their respective optimum growth temperatures.

Headspace gas was analyzed periodically for methane concentration and stable carbon isotopic fractionation using a Varian CP-4900 Micro-GC, and a Cavity Ringdown Spectrometer G2201-1 isotopic CO_2/CH_4 (University of Arkansas Stable Isotope Laboratory).

Results: The carbon isotope fractionation, $\delta^{13}C$, was calculated using the following equation:

$$\boldsymbol{\delta}^{13}\mathbf{C}_{Sample} = \left\{ \frac{\left(\frac{^{13}\mathbf{C}}{^{12}\mathbf{C}}\right)_{Sample}}{\left(\frac{^{13}\mathbf{C}}{^{12}\mathbf{C}}\right)_{Reference}} \mathbf{-1} \right\} \mathbf{*1000}$$

The reference isotopic standard for $\delta^{13}C$ is PDB (Pee Dee Belemnite). The δ is measured in terms of parts per thousand, or "per mil," and is expressed as ‰.

M. wolfeii, *M. barkeri*, and *M. formicicum* demonstrated substantial headspace methane concentration on JSC Mars-1 and montmorillonite, as well as in their respective media controls over two months of study, but relatively less methane in the headspace gas samples when they were grown on JSC Mars-2 and MMS (Figs. 1A, 1C, and 1E).

M. wolfeii, *M. barkeri*, and *M. formicicum* revealed different magnitudes of carbon isotope fractionation on different substrates. The methane produced by *M. wolfeii* and *M. barkeri* demonstrated relatively depleted ^{13}C when they were cultured on JSC Mars-1, JSC Mars-2, montmorillonite, and MMS, compared to their respective growth media controls (Figs. 1B and 1D). In biogenic methanogenesis, carbon fractionation is due to preferential uptake of lighter isotopes (^{12}C) because of the lower energy costs. Progressive enrichment of ^{12}C can be seen in Figs. 1B and1D, in which the slopes of lines are slowly decreasing. *M. formicicum,* when grown in medium and on four dif-

ferent Mars analogues, demonstrated little or no difference in its carbon isotopic fractionation values. (Fig. 1F).

Discussion and Conclusions: The carbon isotope fractionation in biological processes is an example of "kinetic isotope fractionation" (26). Several factors are responsible for the extent of fractionation of carbon, such as species of methanogen, growth rate of methanogen, substrate concentration, isotope composition of substrates, availability of substrates, growth stage of the species, environmental factors, isotopic effects of enzymes involved in biosynthetic pathways, and use of different metabolic pathways by methanogens (26-32). The shuttling of carbon during methanogenesis involves complex biosynthetic networks. The difference in the rate of fixation of carbon in the metabolic pathways of methanogens could be responsible for the difference in the magnitude of carbon isotope fractionation.

On clay, such as montmorillonite, *M. wolfeii*, and *M. barkeri* showed relatively depleted ^{13}C compared to other Mars regolith analogues. *M. barkeri* had shown continuously depleted ^{13}C during methanognesis on various analogues, which suggests that these organisms are very selective of using ^{12}C in media, as well as on Mars analogues.

Overall, the characterization of carbon isotope fractionation content during methanogenesis on different kinds of Mars analogues represents a step forward toward understanding the ambiguous sources of methane on Mars.

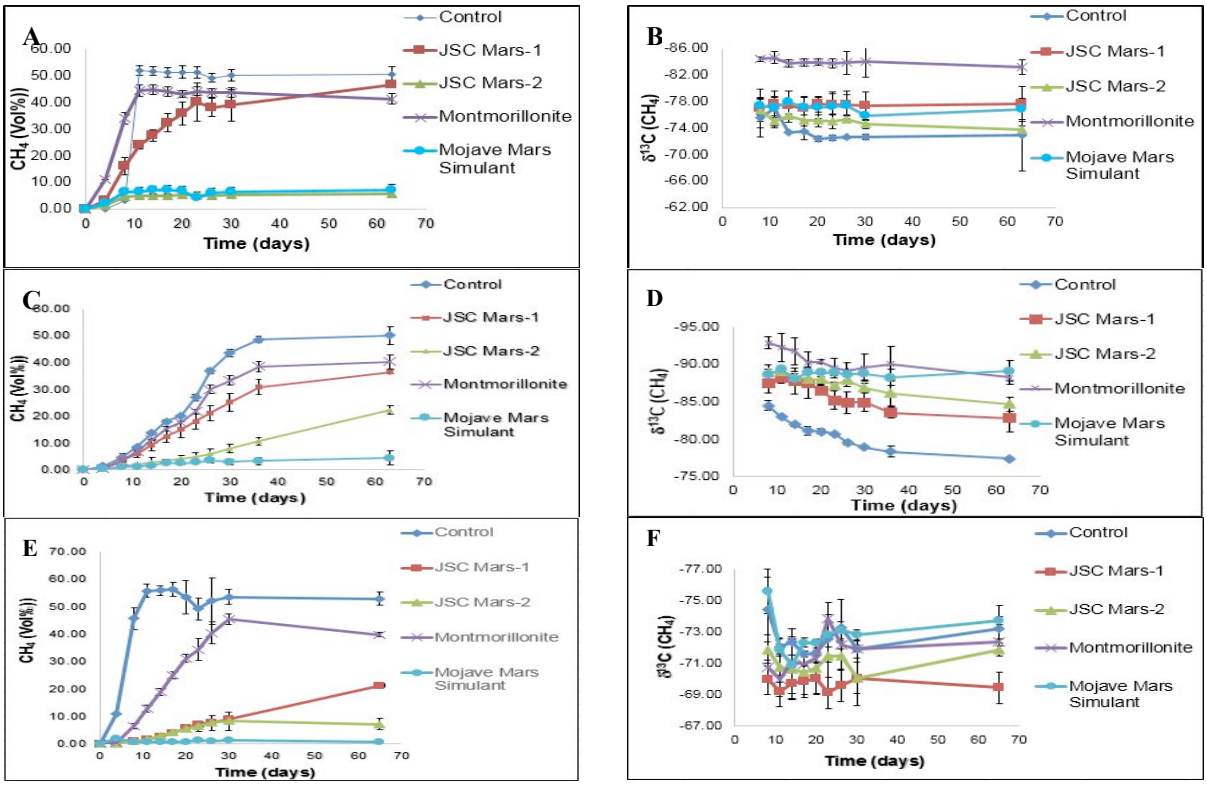

Figure 1: Methane concentration as a function of time in the headspace gas sample during methanogenesis on four different Mars regolith analogues and in their respective growth media for three different methanogen species: (A) *M. wolfeii,* (C) *M. barkeri*, and (E) *M. formicicum.* Carbon isotope fractionation values of methane (in per mil) produced during methanogenesis on four different Mars regolith analogues and in their respective growth media for three different methanogen species: (B) *M. wolfeii,* (D) *M. barkeri*, and (F) *M. formicicum*. Each value is the average of three measurements. Error bars represents +/- one standard deviation.

References: [1] Webster, C. R., et al. (2014) *Sciencexpress*, pg 1-7. [2] Formisano, V., et al. (2004) *Science*, 306, 1758-1761. [3] Krasnopolsky, V. A., et al. (2004) *Icarus,* 172, 537-547. [4] Mumma, M., et al. (2009) *Science,* 323, 1041-1045. [5] Christensen, P. R. et al. (2004) *Science*, 306, 1733-1739. [6] Herkenhoff, K.E., et al. (2004) *Science*, 305, 824-826. [7] Klingelhofer, G., et al. (2004) *Science*, 306, 1740-1745. [8] Rieder, R., et al. (2004) *Science*, 306, 1746-1749. [9] Squyres, S.W., et al. (2006) *Science*, 313, 1403-1407. [10] Ehlmann, B. L., (2011) *Nature*, 479. [11] Hunten, D. M. (1979) *J. Mol. Evol.*, 14(2), 71-78. [12] Atreya, S. K., et al. (2007) *Planetary and Space Science*, 55, 358-369. [13] Boston, P. J., et al. (1992) *Icarus*, 95, 300-308. [14] Chapelle, F. H., et al., (2002) *Nature*, 415 (6869), 312-315. [15] Kral, T. A. et al. (1998) *Origins Life Evol. Biosphere*, 28, 311-319. [16] Weiss, B. P., et al. (2000) Proc. Natl. Acad. Sci. 97, 1395-1399. [17] Kral, T. A. et al. (2011) *Planetary and Space Science*, 59, 264-270. [18] Reid, I. N., et al. (2006) International Journal of Astrobiology, 5, 89-97. [19] Allen, M., et al. (2006) EOS, 87, 433-448. [20] Sowers, K.R. (1995) *Archaea: Alaboratory manual: Methanogens* pp 89-95. [21] Allen, C. C., et al. (1998) *LPSC XXVIII*, 1797. [22] Fanale, F. P., et al. (1982) *Journal of Geophysical Research,* 87, 10215-10225. [23] Peters, G. H., et al. (2008) *Icarus,* 197, 470–479. [24] Bishop, J. L. and Murad, E. (2004) *Journal of Raman Spectroscopy*, 35, 480-486. [25] McAllister, S. A. and Kral, T. A. (2006) *Astrobiology*, 6, 819-823. [26] Whiticar, M. J. (1999) *Chemical Geology*, 161, 291-314. [27] Conrad, R. (2005) *Organic Geochemistry*, 36, 739-752. [28] Conrad, R., et al. (2011) *Biogeosciences*, 8, 795-814. [29] Londry, K. L. and Des Marais, D. J., (2003) *Applied and Environmental Microbiology*, 69, 2942-2949. [30] Londry, K. L., (2008) *Organic Geochemistry*, 39, 608-621. [31] Penning, H., et al. (2005) *Global Change Biology*, 11, 2103-2113. [32] Valentine, D. L., et al. (2004) *Geochemica et Cosmochimica Acta*, 68, 1571-1590.

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