MESOPHILE METHANOGEN SURVIVAL UNDER FREEZE/THAW CYCLES. R. L. Mickol[1], T. A. Kral[1,2] and S. K. Laird[2]. [1]Arkansas Center for Space and Planetary Sciences, 202 Old Museum Building, University of Arkansas, Fayetteville, Arkansas, 72701, USA, [rmickol@uark.edu], [2]Dept. of Biological Sciences, SCEN 632, University of Arkansas, Fayetteville, Arkansas, 72701, USA.

Introduction: Various conditions on Mars deem the planet uninhabitable, such as the lack of atmosphere and cold temperatures, which limits the availability of liquid water on the surface. Methanogens produce methane from H_2 and CO_2 and are ideal candidates for life on Mars due to several characteristics: they are anaerobic, do not require organic nutrients and are non-photosynthetic. Methane has previously been discovered in the Martian atmosphere by a variety of sources, including both space-based and ground-based instruments [1-6].

One of the conditions that any extant life on Mars would face is exposure to the variation in temperature between 0°C and -80°C typical of the surface and near-subsurface. This study subjected two strains of methanogen to varying freeze/thaw cycles between 55°C and -80°C to test the survivability of these species. The species used include *Methanobacterium formicicum* and *Methanothermobacter wolfeii.*

Methods: Two types of methanogen growth media (MSF, MM) were prepared according to Kendrick and Kral [7]. Two separate sets were prepared and subjected to varying freeze/thaw cycles at temperatures of 55°C, 37°C, 24°C, 4°C, -15°C, and -80°C. Transfer sets were also prepared as described below.

Set 1. Ten grams of sand (silica) were added to each of five test tubes. An additional five test tubes contained 5 g sand plus 5 g gravel. Ten milliliters of MSF methanogen growth medium were added to each of the ten test tubes. A sterile solution of 2.5% sodium sulfide was added in the amount of ~125 µL to each test tube following sterilization via autoclave. Each test tube was inoculated with 0.3 mL of MSF medium containing *M. formicicum*. After inoculation, each tube was pressurized with 200 kPa H_2 gas.

After 139 days, a transfer set (Set 1-3) was prepared following the same method as above. The set was inoculated on Day 140 with 0.3 mL from tube #3 from the original set (sand-gravel subset) or 0.3 mL from tube #5 (sand subset).

Set 2. A second set was prepared similarly to Set 1, except that each of 10 test tubes contained only 5 g sand, with five tubes containing 10 mL MSF medium, and five tubes containing 10 mL MM methanogen growth medium. One test tube for each medium type was not inoculated. The MSF tubes were inoculated with 0.5 mL of MSF medium containing *M. formicicum.* The MM test tubes were inoculated with 0.5 mL of MM medium containing *M. wolfeii*. After inoculation, each tube was pressurized with 200 kPa H_2 gas.

After 104 days, a transfer set (Set 2-3) was prepared following the same method as above. On Day 105, the five transfer tubes with MM medium were each inoculated with 0.3 mL from tube #3 from the original Set 2. The five tubes with MSF medium were each inoculated with 0.3 mL from tube #5 from original Set 2.

After 179 days, a second transfer set (2-4) was prepared as above. Each tube in the transfer set was inoculated with 0.5 mL from the corresponding tube in Set 2-3 (for example, tube #1 in Set 2-4 was inoculated from tube #1 in Set 2-3).

Results: *Set 1.* Methane production was monitored over 281 days for two subsets (10 g sand (Fig. 1); 5 g sand + 5 g gravel). Both subsets display survival of methanogens (*M. formicicum*) subjected to freeze/thaw cycles over 281 days. Although the sand-gravel subset initially produced greater amounts of methane (~24% headspace compared to ~12% headspace), exposure to room temperature failed to achieve the same amount of rebound as in the sand subset. For both the sand and sand-gravel sets, only one replicate (out of five) retained metabolizing methanogens at the end of the 281 days.

Transfer Set 1-3. Methane production was monitored over 141 days for the sand transfer set and 105 days for the sand-gravel transfer set. The sand-gravel transfer set failed to produce any significant amount of methane following incubation at 37°C. The sand transfer set achieved greater methane concentration than the initial concentration for the original set (Fig. 1). At Day 281, four out of five sand transfer set replicates contained actively-metabolizing methanogens.

Set 2. Methane production was monitored over 267 days for two subsets (MM medium and MSF medium (Fig. 2)). Similar to Set 1, both subsets display survival following exposure to freeze-thaw cycles, with only one replicate (out of 4) containing actively metabolizing methanogens at the end of 267 days.

Transfer Set 2-3. Two transfer sets were inoculated on Day 105 from the original Set 2 and monitored for 162 days. After 162 days, four out of five replicates in the MM subset contain actively-metabolizing methanogens, while only two replicates in the MSF subset (Fig. 2) display active metabolism.

Transfer Set 2-4. Two transfer sets were inoculated on Day 179 and monitored for 88 days. In the MSF

subset, one tube failed to produce methane following an initial incubation period and was removed from the experiment. After 88 days, all five MM tubes and the four remaining MSF tubes (Fig. 2) contained actively metabolizing methanogens.

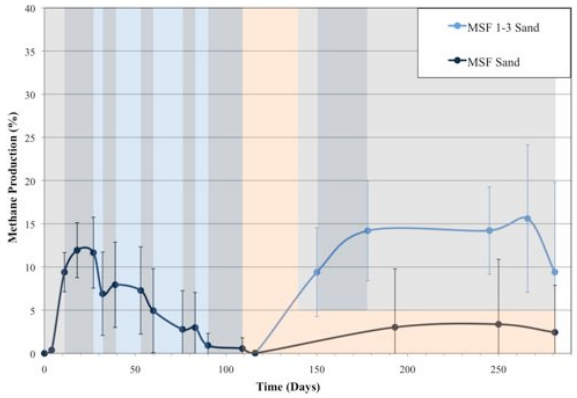

Figure 1. Set 1, MSF. Methane production (% headspace) over 281 days for two sets containing *M. formicicum* in 10 mL MSF medium and 10 g sand. Original tubes were kept at 25°C from Day 109 to Day 281. Transfer tubes were kept at 37°C from Day 178 to Day 281. Colored columns represent the various temperatures to which the tubes were subjected (gray = 37°C, orange = 25°C, dark blue = 4°C, light blue = -15°C). Error bars indicate one standard deviation.

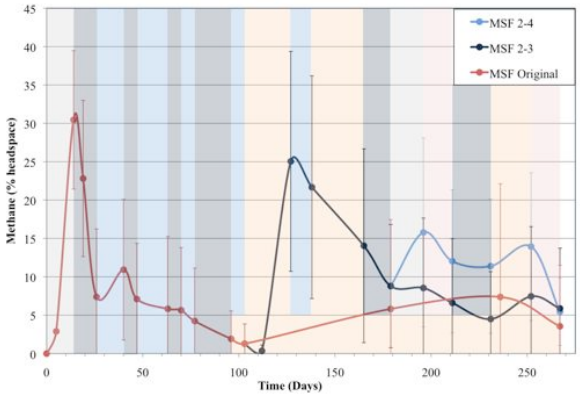

Figure 2. Set 2, MSF. Methane production (% headspace) over 267 days for each of three sets containing *M. formicicum* in 10 mL MSF medium and 5 g sand. Original tubes were kept at 25°C from Day 96 to Day 267. Colored columns represent the various temperatures to which the tubes were subjected (gray = 37°C, orange = 25°C, dark blue = 4°C, light blue = -15°C, red = -80°C). Error bars indicate one standard deviation.

Discussion and Conclusions: An interesting aspect of these experiments is the increase in methane abundance during some exposures to 4°C and -15°C (Figs. 1, 2). The solubility of methane in pure water increases with decreasing temperature, but only reaches about 40 mg/kg (40 ppm) at 0°C [8]. This value is insignificant under these experimental conditions where, at most, only 0.4 mg methane would be dissolved in 10 mL solution. Thus, the increases in methane concentration during exposure to 4°C and -15°C are not attributable to methane solubility.

Methane adsorption experiments using the Martian soil simulant JSC-Mars-1 have demonstrated that the methane release seen on Mars could be the result of seasonal methane adsorption and desorption within the top layers of regolith [9]. The number of CH_4 molecules adsorped decreases exponentially with increasing temperature, approaching zero molecules adsorbed near 145 K (-128°C). While it is possible that increases in methane concentration at 4°C and -15°C could be due to desorption of methane molecules from the sand, further study is necessary for confirmation.

Sets 1, 1-3. Both the sand and sand-gravel subsets in Set 1 displayed similar methane production over the first 100 days of the experiment. In the original sand subset, one test tube greatly rebounded following two months at room temperature, reaching methane concentrations similar to initial maxima (Fig. 1). It is possible that the lack of methane production by the sand-gravel transfer set is due to the fact that most cells within the sand-gravel subset perished prior to the transfer to new media. The role of porosity will be further explored considering differences in survivability between the sand and sand-gravel subsets.

Sets 2, 2-3, 2-4. Both subsets within Set 2 displayed similar methane production over the entire duration of the 267 day experiment. Both subsets within the original Set 2 only retained one replicate with actively metabolizing methanogens with the MSF replicate displaying greater methane production than the MM replicate at the end of 267 days.

Future experiments will test two additional species, *Methanococcus maripaludis* and *Methanosarcina barkeri* for their ability to withstand freeze/thaw cycles. In addition, temperature cycles between 4°C and -80°C over 24 hours will be attempted to better mimic Martian conditions.

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