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Survival of Methanogens Desiccated on Mars Soil Simulants: Implications for Life on Mars

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Survival of Methanogens Desiccated on Mars Soil Simulants: Implications for Life on Mars

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Abstract

Because of Mars's chemical features, it can be studied in the search for life and is a significant figure in the study of astrobiology (McKay, 2010). Dr. Kral's laboratory studies methanogens, microorganisms in the domain Archaea, as possible life forms on Mars. Since some methanogens can withstand harsh environmental conditions and emit methane as a waste product, the growth of these microorganisms can be used as an indicator of potential life in the Mars subsurface (Kral et al., 1998). In this research, four methanogens, *Methanothermobacter wolfeii*, *Methanobacterium formicicum*, *Methanosarcina barkeri*, and *Methanococcus maripaludi* were desiccated in the presence of Mars soil simulants for two different time periods to determine their survivability under these conditions. The purpose of desiccating the organisms in an anaerobic chamber was to replicate an extremely dry subsurface Mars environment that could have minimal resources for specific amounts of time (Kendrick & Kral, 2006). The Martian soil simulants used were JSC Mars-1, montmorillonite, basalt, jarosite, and nontronite. A gas chromatograph was used to derive methane concentrations to determine which environment and allocated time were successful for methanogen growth.

Introduction

Earth and Mars have similar chemical characteristics and geological compositions that make Mars a potential habitat for life. Despite no present evidence of life, the ongoing understanding of Mars leads researchers to search for possibilities. Because of this, there are several investigations and missions to explore Mars's geological terrain (Mangold et al., 2016). However, significant differences in Mars's atmosphere create drastic climate differences that imply life would not be as obvious as life on Earth (Mangold et al., 2016). By analyzing different environmental components of Mars that could sustain life, experiments can focus on viable life forms that could withstand the extremities that prevail on Mars (Carr, 2006).

Mars Composition

Variations in size, obliquity, and atmosphere can all affect a planet's climatic conditions and habitability (Carr, 2006). Mars has a 3389.5 km radius compared to Earth's 6378 km, a 24-hour and 39.6 minute day, and a 687-day year (Carr, 2006). Mars's rotational axis has a tilt of 25 degrees with a higher eccentricity and changing obliquity which means Mars has intense seasons with large changes in the climate and atmospheric pressure (Carr, 2006). Despite the variations that come with Mars's atmosphere, Mars has a lower atmospheric pressure than Earth (Leovy, 2001). Not only is the atmospheric pressure at the surface of Mars about 0.6% of Earth's, but ultraviolet radiation exposure is exponentially higher (McKay, 2010). Since Mars is a dry desert planet, Martian temperatures are highly impacted by strong winds and dust storms. (Leovy, 2001) The suspended dust can absorb the ultraviolet radiation that can increase the Martian daily average temperature (Leovy, 2001). The thin atmospheric density along with the radiation absorption create a wide range of surface temperatures that lack a large heat capacity and vary

based on seasonal and latitude changes (Carr, 2006). The atmosphere allows Mars to experience temperatures similar to Earth in the daytime at certain locations, but then have surface temperatures averaging down to -60 degrees C when the sun goes down (McKay, 2010). The abundance of CO_2 in the atmosphere and condensed in the poles can also contribute to the extreme seasonal temperatures (Mangold et al., 2016). However, the deeper into Mars's soil, the less the temperatures fluctuate which is significant because the temperatures become freezing at the surface (Carr, 2006).

Water on Mars

Since Mars's atmospheric pressure is between 0.6-2.0% of Earth's, depending on altitude, and the low temperatures, there is an issue with liquid water availability (e.g. Levin, 1972 and Nazari-Sharabian et al., 2020). Currently, there is minimal water in the Martian CO_2-filled atmosphere, and water ice that was detected at the poles, but the evolution of Mars stems from the evidence of erosion (Carr, 1996). The geological presence of craters, channels, and valleys on the Martian terrain suggests the possibility of past water sources such as oceans and lakes (Carr, 2006). In the present, the primary water source for life would be frozen in the subsurface that melts due to volcanic interactions (Kral et al., 2002). However, since liquid water is essential to life as known on Earth, any potential life form in the Martian subsurface may have to withstand long, dry periods with little to no water intake (Kendrick & Kral, 2006).

Martian Soil

Mars's crust consists of an array of elements within its soil and rocky composition (McSween et al., 2009). Mars's crust is ~50 km thick and covered in craters, volcanic surfaces,

and igneous rocks. (McSween et al., 2009). Mars's surface composition has been measured by several Mars missions such as the Viking Landers, but the Mars Phoenix Lander spacecraft specifically used soil analysis instruments (e.g. Hecht et al., 2009; Smith et al., 2008). The Mars Phoenix Lander used ion-selective electrodes to measure soil concentration and discovered the presence of perchlorate salts which was then confirmed by the Curiosity rover (e.g. Hecht et al., 2009; Kral et al., 2016). The Curiosity Rover also confirmed clay materials to be present on the Noachian terrain which is an indicator of past aqueous environments (e.g. Gil-Lozano et al., 2020; Craig et al., 2017). Viking and Pathfinder landers have observed magnesium sulfate, iron, and basalt concentrations in the Martian environment (e.g. Madden et al., 2004; McSween et al., 2003).

Soil Simulants

JSC Mars-1 is a simulant made from weathered volcanic ash from Pu'u Nene in Hawaii that consists of a mixture of ash particles with the presence of magnetite and olivine (Allen et al., 1998). Jarosite, which is composed of ferric sulfate, has been identified at the Meridiani Planum landing site (Madden et al., 2004). Jarosite is found in acidic drainage sites near volcanic rocks and sulfur-rich fluid volcanic vents on Earth (Madden et al., 2004). Montmorillonite is a smectite clay-based simulant that is rich in iron and contains all the relevant nutrients for methanogen survival (Schirmack et al., 2015). Nontronite is another clay-based simulant that is Fe- smectite (Craig et al., 2017). Both nontronite and montmorillonite absorb water within their structural layers and have been used as a nutrient source for microorganisms. (Craig et al., 2017). Basalt is a volcanic igneous rock that is crystallized from magma formation and dominates Mars's soil and meteorite composition (Mcsween et al., 2003).

Methanogens

Methanogens are possible life forms in Mars's subsurface (Kral et al., 2011). If methanogens are present on Mars, they could not be on the surface due to the radiation as explained above (Kral et al., 2011). Methanogens are chemoautotrophs that consume molecular hydrogen and carbon dioxide and emit methane as a waste product during metabolism (e.g. Kral et al., 1998; Kral et al., 2004). Methane has been detected in the atmosphere of Mars which suggests that methanogens may exist in the subsurface (Formisano et al., 2004). Hydrogen could be obtained through geothermal sources such as volcanic and hydrothermal activity or subsurface liquid water (Kral et al., 2004). Many methanogens on Earth are known to be adaptive to extreme environmental conditions and occupy most of Earth's anaerobic habitats such as deep-sea hydrothermal vents and subsurface hot springs (Kral et al., 2004). These anaerobic Earth communities are comparable to the Mars subsurface which sustains seasonal liquid or freezing water (e.g. Kral et al., 2004; Boston et al., 1992). Since the Mars environment will undergo freeze/thaw cycles, the microorganisms would have to adapt to limited water access (e.g. Kendrick & Kral, 2006; Mickol et al., 2018). This survival withstanding limited water can be replicated in the laboratory by determining how long the microorganisms can survive while desiccating on Mars soil simulants (Kendrick & Kral, 2006).

Purpose

In this experiment, the hypothesis focuses on methane production that increases with time from any of the methanogens following desiccation on soil simulants. Given the uncertainty of life on Mars, any observed growth from these methanogens serves as an indication of potential life forms in the subsurface of Mars (Kral et al., 1998). The desiccation process and anaerobic

conditions were suitable for replicating the Martian environment (Kendrick & Kral, 2006). The two different time variables were useful when understanding how long these methanogens can undergo harsh environmental conditions. Based on the Mars simulant soil the methanogens survive on, this could also indicate a geographical marker of where the methanogen could be found on Mars.

Materials and Methods

Media preparation

In this experiment, four different methanogens were used and grown in their respective media: MM for *Methanothermobacter wolfeii*, MS for *Methanosarcina barkeri*, MSF for *Methanobacterium formicicum* and MSH for *Methanococcus maripaludi*. Please refer to the Appendix for the contents of the media and the incubation temperatures. Five Mars simulant soils, substitutes for Martian soil, were used: JSC Mars-1 (JSC.), montmorillonite (Mont.), basalt (Bas.), jarosite (Jar.), and nontronite (Non.). In addition to these 5 simulant soils, a control containing no soil was included. This control was used to determine the efficacy of the desiccation process with or without the presence of simulant soil.

With the use of an electronic balance, medium components for 200 mL of media (specific to all four mediums) were poured into four different 250 mL flasks. The flasks of media were transferred to the Coy anaerobic chamber and inside, 200 mL bicarbonate buffer were added. The Coy anaerobic chamber consists of approximately 90% carbon dioxide and 10% hydrogen. Sterile bicarbonate buffer was prepared by dissolving 4g of NaOH in 1L of deionized water. This solution was saturated with carbon dioxide gas using a gassing manifold. The media were left for 24 hours to deoxygenate. Following deoxygenation, media were poured into anaerobic culture

bottles (100 mL per bottle). Each bottle was sealed with a rubber stopper and then removed from the chamber. After removing the media, the bottles were crimped and sterilized with an autoclave for 30 minutes at 121 ℃ and 15 psi.

Before inoculation, 1.5 mL of 2.5% sterile sodium sulfide solution were injected into each bottle to remove any residual oxygen. Methanogen stock cultures were transferred into the fresh media (1.0 mL per bottle). After inoculation, the tubes were pressurized with 200 kpa of H_2 gas. Molecular hydrogen serves as the energy source for the methanogens while the carbon dioxide in the buffer serves as carbon source (Kral et al., 2016) After inoculation and pressurization, the cultures were incubated at their optimal growth temperatures (see Appendix).

Desiccation

One gram of each soil simulant was added to a 1.5 mL plastic microcentrifuge tube. In total, there were 96 tubes: 24 tubes of each methanogen (not including the 4 tubes that were created as a control to confirm the media would support growth). There were two tubes for each organism, soil simulant and time period. Figure 1 illustrates the experimental design. The microcentrifuge tubes were numbered, closed, and then sterilized in the autoclave (30 minutes at 121 ℃ and 15 psi).

After letting the organisms in the bottles grow for a week, culture samples were removed and placed into sterile centrifuge tubes. The tubes were centrifuged at 5000 rpm for 20 minutes followed by pouring off the supernatant. Sterile buffer was added to each pellet that remained after centrifugation, the pellet was suspended in the buffer, and then the suspensions were centrifuged again. The supernatants were poured off again. The remaining cell pellets were suspended in sterile buffer again. This procedure washed the cells of residual nutrients and

concentrated the cells. The washed cells and microcentrifuge tubes containing the sterile soil simulants were placed into the anaerobic chamber. A couple of drops of each organism were placed into their designated microcentrifuge tube with a sterile syringe. Each inoculated microcentrifuge tube was placed into one of two desiccators in the anaerobic chamber. One desiccator was for the 8-month experiment, the other for the 15-month experiment. The microcentrifuge tubes remained open in the desiccators to ensure complete desiccation for the duration of the experiments.

Transferring Desiccated Cultures

The 8-month and 15-month tubes had the same preparation for transferring and inoculating to new media. Media were made in the same process mentioned above. After the combined organisms and soil simulants had their allocated time in the desiccators, the freshly made media were dispersed into 52 tubes for each time-period experiment (including four controls used for media growth confirmation, one for each organism), crimped, and autoclaved. Once inside the Coy anaerobic chamber, 1 ml of sterile buffer was injected into the plastic tubes containing the desiccated organism/soil simulant mixture by syringe. The soil and buffer were stirred until mixed with a syringe needle and then 0.5 ml of that combination was taken and transferred to the corresponding anaerobic tube of medium. Once the transfer was complete, the tubes were pressurized with H_2 as described previously, and then incubated at the appropriate temperatures. This procedure was performed following 8 months of desiccation, and then again after 15 months.

Methane Measurements

To determine if the organisms had survived the desiccation on soil simulants, 1 mL headspace gas samples was removed from each tube after three weeks of incubation and then injected into a gas chromatograph (GC; Varian Micro-GC, model CP-4900) to analyze for methane. This was repeated every week thereafter up until the 9-week time. Increases in methane concentration with time indicated that the organism had survived the procedure.

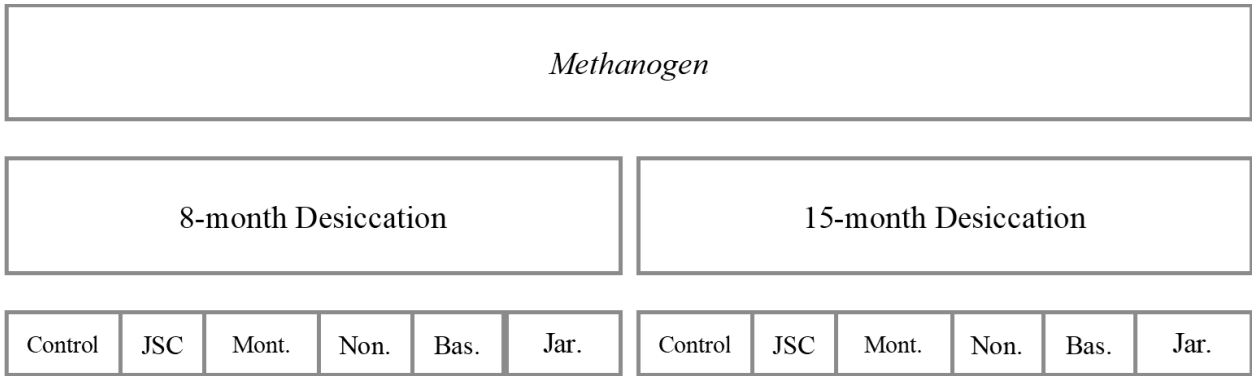

Figure 1. This flow chart illustrates the experimental design for this study. With each methanogen, there are two time periods of desiccation: 8 months and 15 months. Each time period has five soil simulant groups and a control. There were two organisms for each variable.

Results

Table 1 includes the highest methane production percentage for all four methanogens that were desiccated on the five different Martian soil simulants for 8 months. The week of the highest methane peak is shown. Data collection started after the organisms grew for three weeks and readings were taken up to nine weeks. If oxygen contaminated a tube turning it pink, that tube was eliminated from further measurements. Figures 2-10 show data from the 8 months of desiccation and the relationship between the methanogens and the soil simulants. Figures 2-4 focus on the individual methanogen's growth depending on the Martian soil simulant. Figures 5-10 show a selected soil simulant and the methane production from all the methanogens.

8-month Time Period

The 8-month time group showed sufficient evidence of growth based on methane gas in the gas chromatograph readings. Increases in methane concentration are a proxy for growth in methanogen studies. The control had growth with all four methanogens which shows the desiccation procedure was successful and lacked major oxygen infiltration (Figure 5).

M. wolfeii had the most prolific growth with each soil simulant. *M. wolfeii* grew abundantly on montmorillonite and basalt with the amount of methane exceeding 16% (Table 1 and Figure 2). *M. wolfeii* grew less efficiently on JSC- Mars, nontronite, or jarosite but still achieved more than 1% methane concentration. *M. wolfeii* showed desiccation tolerance to the 8-month desiccation regardless of the soil simulant.

M. barkeri exhibited growth when on soil simulants JSC- Mars and montmorillonite, but had zero methane production when grown on nontronite, basalt, or jarosite. *M. barkeri* in the control produced up to 5.20% methane.

M. formicicum had significant growth with JSC Mars-1, montmorillonite, and jarosite (Figures 6,7,10). *M. formicicium* had methane concentrations of 25.35% on JSC- Mars, 17.53% on jarosite, and 7.76% on montmorillonite (Table 1 and Figure 3). However, methane production from *M. formicicum* was reduced when desiccated on nontronite or basalt to 0% (Table 1 and Figure 4). *M. maripaludis* had no significant growth regardless of the soil.

Cells desiccated on nontronite had the least growth compared to the other soil simulants (Figure 8). The highest methane was taken from *M. wolfeii*, measured at 1.42%. *M. wolfeii* desiccated on basalt soil simulant had a peak of 24.98% methane (Figure 9).

15-month Time Period

It appears that the methanogens did not survive the 15-month desiccation exposure time. No methane was detected in any of the experimental tubes after weeks of GC analyses.

Species and Soil Simulant	Top Methane %	Week #
M. wolfeii, control	26.38%	4
M. wolfeii, JSC-mars	3.48%	4
M. wolfeii, montmorillonite	16.97%	4
M. wolfeii, nontronite	1.42%	8
M. wolfeii, basalt	24.98%	4
M. wolfeii, jarosite	4.49%	4
M. barkeri, control	5.20%	3
M. barkeri, JSC- Mars	0.06%	8
M. barkeri, montmorillonite	0.0828	3
M. barkeri, nontronite	0%	
M. barkeri, basalt	0%	
M. barkeri, Jarosite	0%	
M. formicicum, control	22.80%	8
M. formicicum, JSC- Mars	25.35%	8
M. formicicum, montmorillonite	7.76%	9
M. formicicum, nontronite	0.00%	
M. formicicum, basalt	0%	
M. formicicum, jarosite	17.53%	8
M. maripaludis, control	0.11%	8
M. maripaludis, JSC- Mars	0.00%	
M. maripaludis, montmorillonite	0.00%	
M. maripaludis, nontronite	0.00%	
M. maripaludis, basalt	0.00%	
M. maripaludis, jarosite	0.00%	

Table 1. Top methane production from each methanogen and the corresponding soil simulant after the 8-month desiccation period. If the methanogen grew, the week number of the peak growth % is also shown.

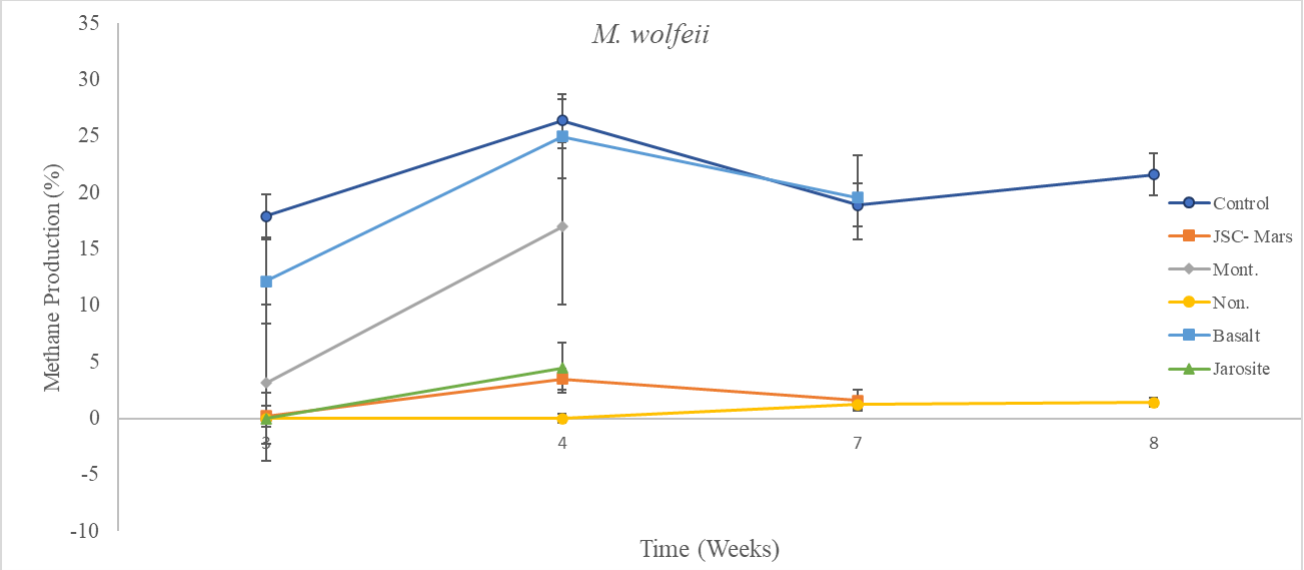

Figure 2. Methane production by *Methanothermobacter wolfeii* after 8 months of desiccation on the control (no soil simulant), JSC Mars-1, montmorillonite, nontronite, basalt, and jarosite.

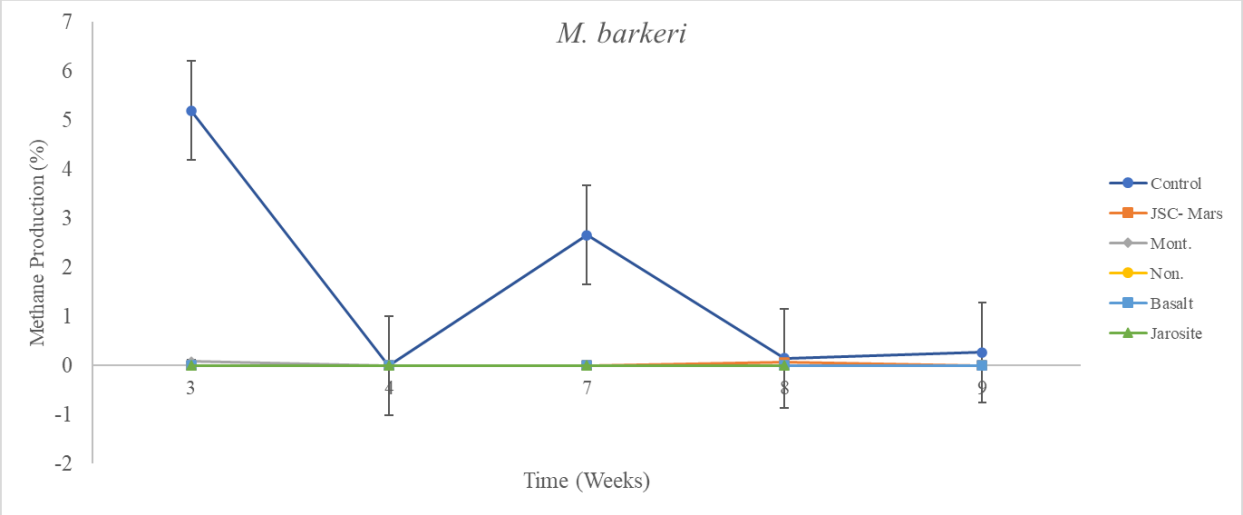

Figure 3. Methane production by *Methanosarcina barkeri* after 8 months of desiccation on the control (no soil simulant), JSC Mars-1, montmorillonite, nontronite, basalt, and jarosite.

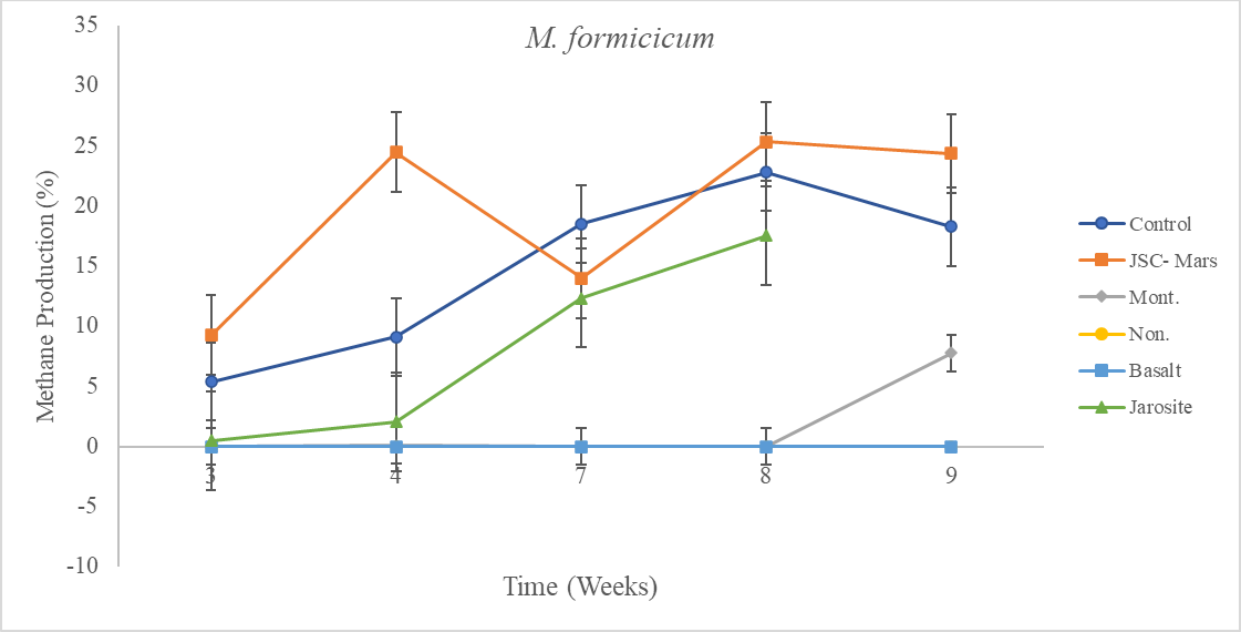

Figure 4. Methane production by *Methanobacterium formicicum* after 8 months of desiccation on the control (no soil simulant), JSC Mars-1, montmorillonite, nontronite, basalt, and jarosite.

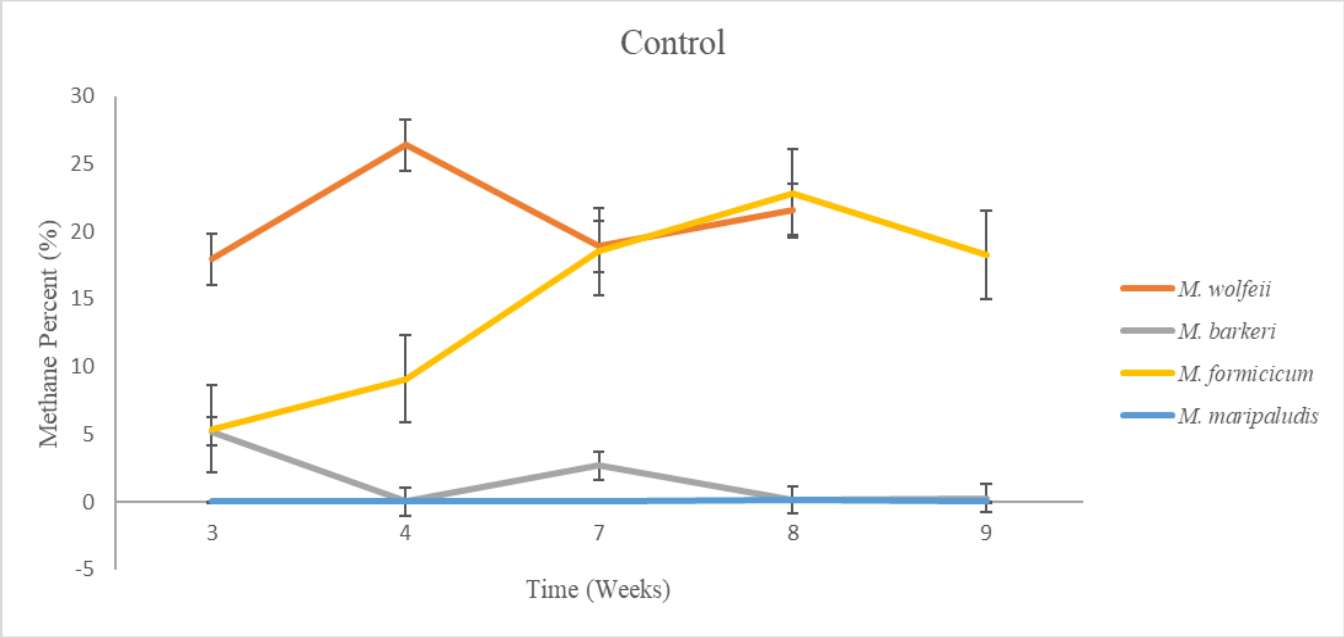

Figure 5. Methane production by *Methanothermobacter wolfeii, Methanosarcina barkeri, Methanobacterium formicicum, and Methanococcus maripaludi* after 8 months of desiccation on no soil simulant (control).

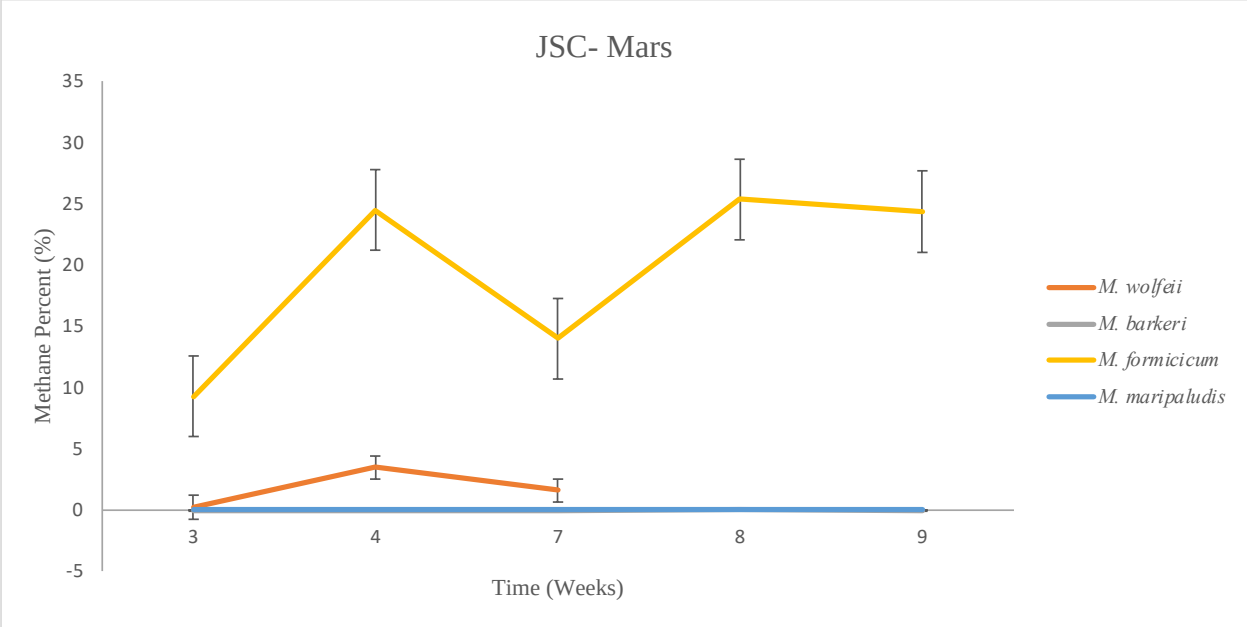

Figure 6. Methane production by *Methanothermobacter wolfeii, Methanosarcina barkeri, Methanobacterium formicicum, and Methanococcus maripaludi* after 8 months of desiccation on the JSC- Mars.

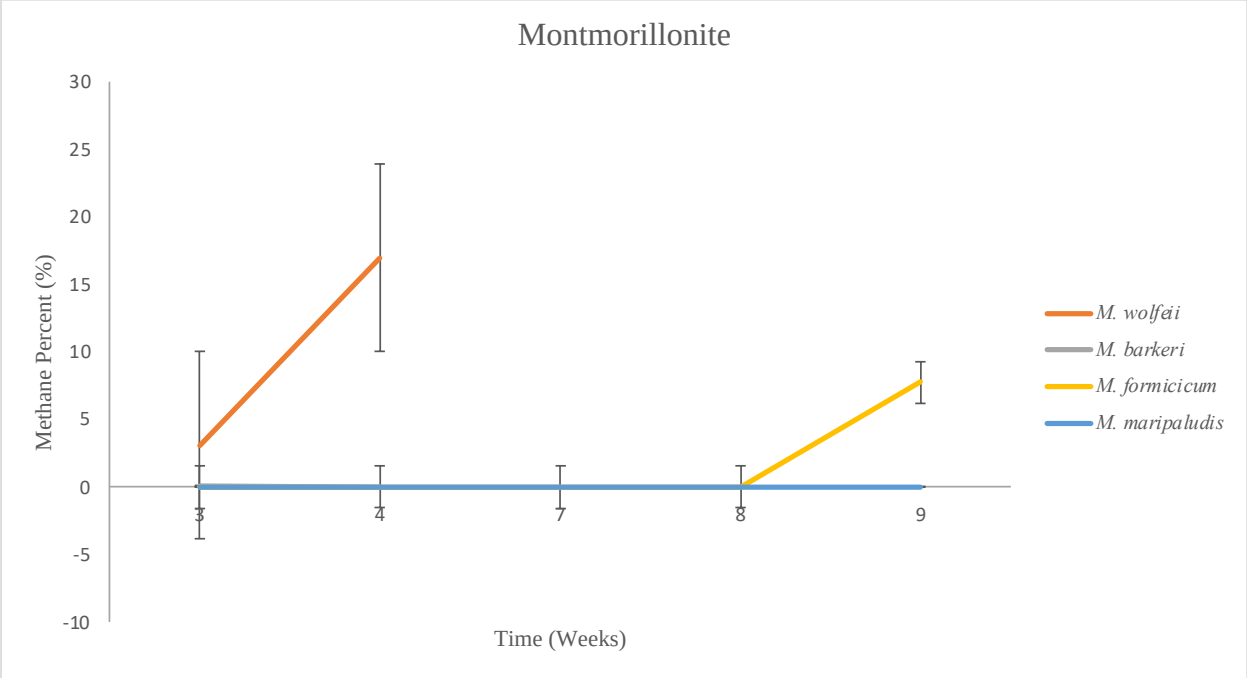

Figure 7. Methane production by *Methanothermobacter wolfeii, Methanosarcina barkeri, Methanobacterium formicicum, and Methanococcus maripaludi* after 8 months of desiccation on montmorillonite.

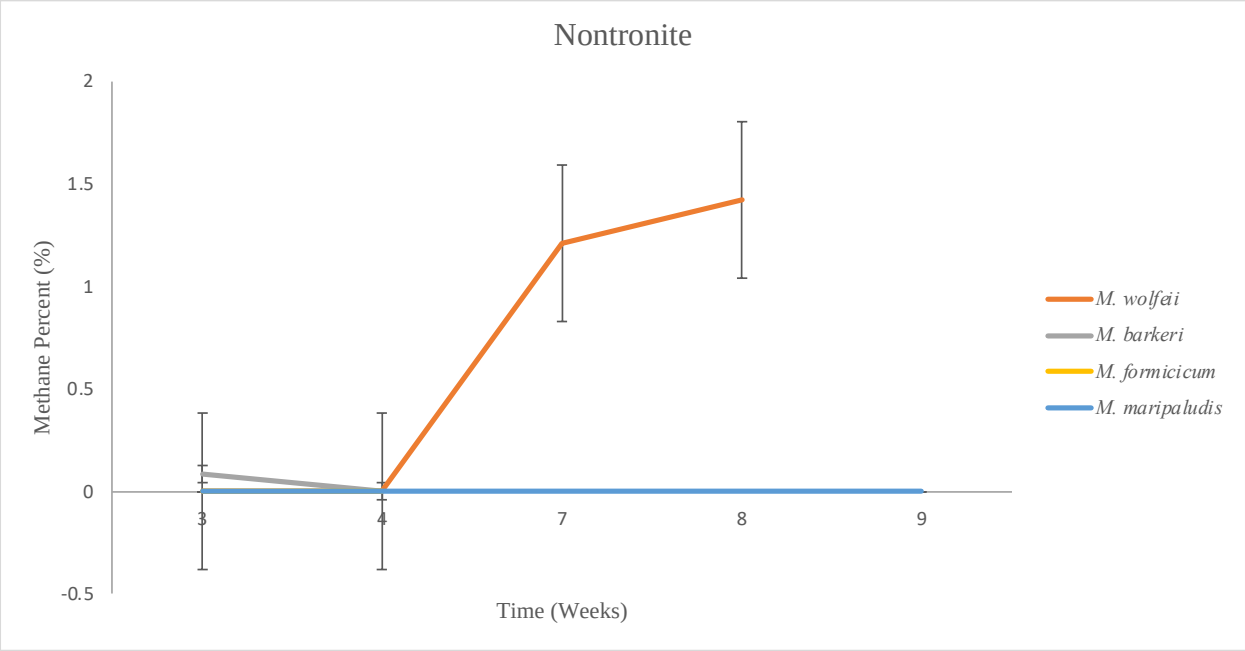

Figure 8. Methane production by *Methanothermobacter wolfeii*, *Methanosarcina barkeri*, *Methanobacterium formicicum, and Methanococcus maripaludi* after 8 months of desiccation on nontronite.

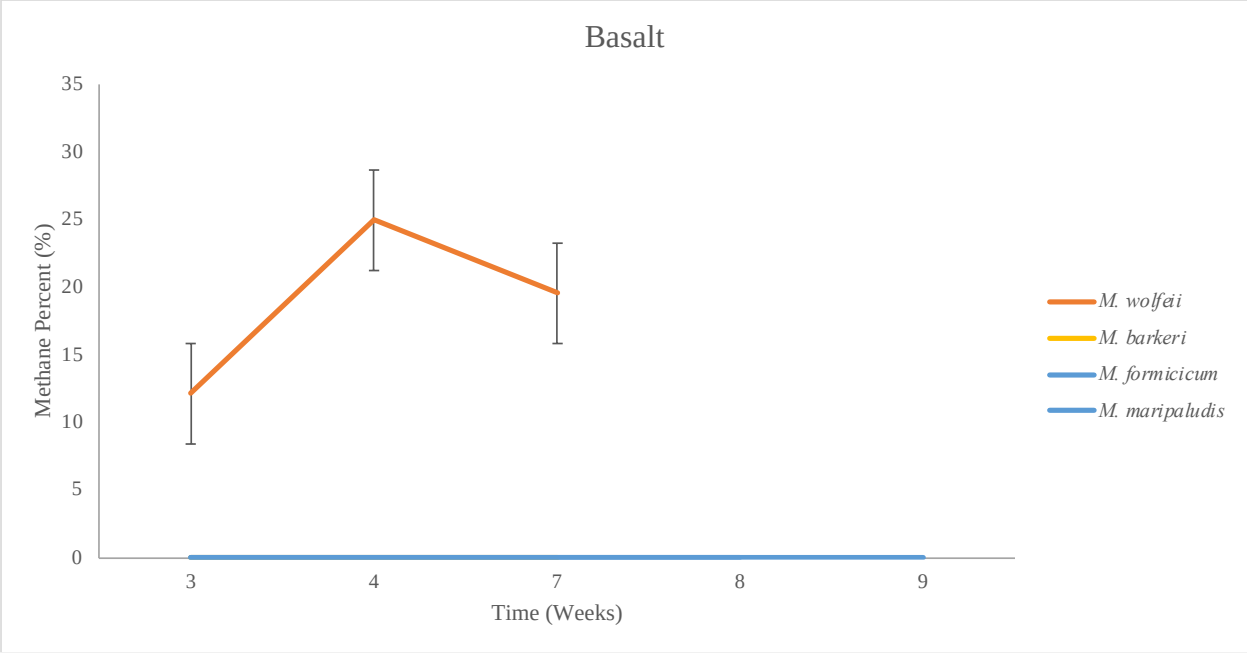

Figure 9. Methane production by *Methanothermobacter wolfeii*, *Methanosarcina barkeri*, *Methanobacterium formicicum, and Methanococcus maripaludi* after 8 months of desiccation on basalt.

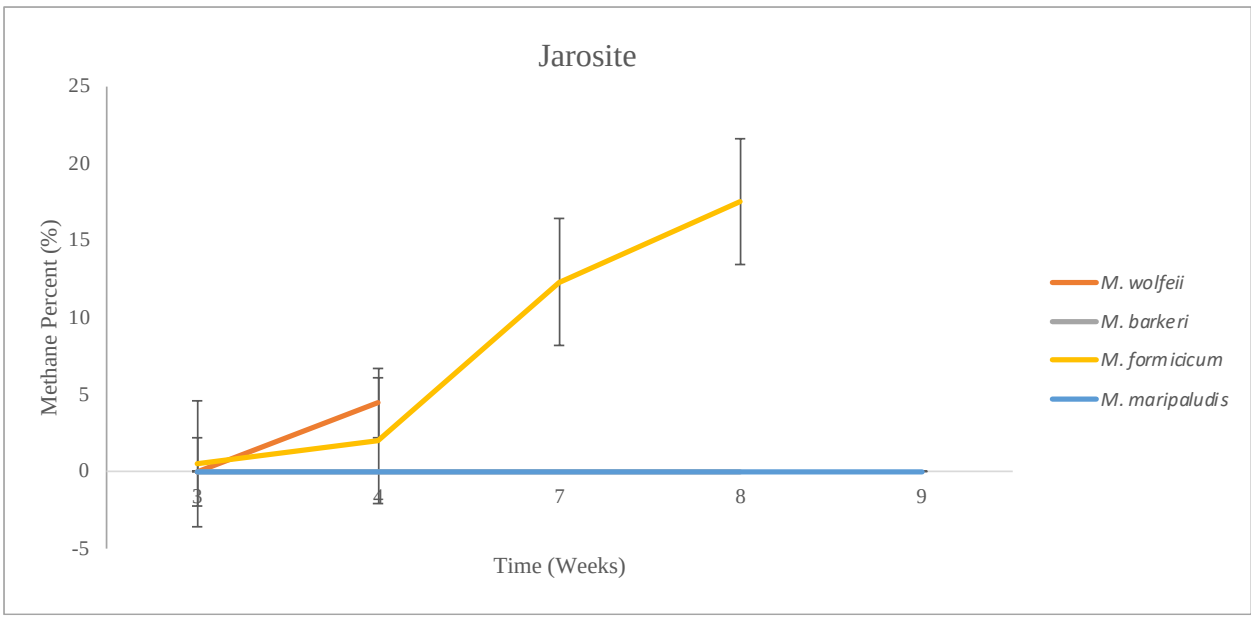

Figure 10. Methane production by *Methanothermobacter wolfeii, Methanosarcina barkeri, Methanobacterium formicicum, and Methanococcus maripaludi.*after 8 months of desiccation on jarosite.

Discussion

When desiccated for 8 months, all four methanogens experienced methane production, some following desiccation on soil simulants. This project used five different Martian soil simulants. Each methanogen exhibited distinct methane production depending on the soil simulant used, except for nontronite, which demonstrated minimal to no growth. The relationship between the methanogens and their respective soil simulants is a representation of the probable habitats for these organisms on Mars. Why there is a difference in responses to the various soil simulants is unknown. In previous research, it has been demonstrated that these methanogens do not grow well in the presence of nontronite, and they did not survive well in its presence here.

To withstand Mars's harsh dry environment, organisms would have to be able to survive long periods without any water. After desiccating for 8 months, *M. wolfeii* and *M. formicicium* showed more resistance to the extreme environmental factors compared to *M. barkeri* and *M. maripaludi. M. maripaludi* did not produce any methane when grown on any of the soil simulants. This may be related to its halophilic nature. It requires salt to grow, yet there was salt present in its growth medium following desiccation.

It should be noted that methane measurement at weeks 5 and 6 have been eliminated from this study. Many of the measurements showed no methane following a weekly reading where methane was present. This was most likely due to problems with the calibration of the gas chromatograph.

None of the methanogens survived after desiccating for 15 months. The media that the desiccated cells were transferred into supported the growth of control cells, so it was not a problem with faulty media. One explanation for the lack of growth from the 15-month desiccated methanogens was the prolonged dry period. Since the methanogens endured the 8-month long

desiccation process, but did not survive the 15 months, the organisms most likely reached a survival threshold sometime after the initial 8 months.

Prior to running the experiments reported here, an entire set of identical experiments were performed except for one factor. In the earlier experiments the desiccated cells were stored in incubators with the microcentrifuge tubes closed, but in the presence of atmospheric oxygen. Apparently, the oxygen was able to infiltrate the tubes and kill the desiccated cells, and no methane was produced by any of the experimental cultures.

In regard to determining where these methanogens could specifically survive on Mars, the research reported here suggests that some methanogens may be able to survive for a limited time while desiccated on a number of components found in the Martian environment. Future experiments with other Mars soil simulants and time periods longer than 8 months should be conducted to contribute to a more complete story.

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Appendix

Medium	Methanogen	Incubation Temperature (°C)
MM	*Methanothermobacter wolfeii*	55
MS	*Methanosarcina barkeri*	37
MSH	*Methanobacterium formicicum*	37
MSF	*Methanococcus maripaludi*	23

Components for Standard 100 mL Media:

Ingredient	MM Medium	MS Medium	MSF Medium	MSH Medium
1000 uL Solution A	*x*	x	x	x
200 uL Solution B	*x*	x	x	x
200 uL Solution C	x	x	x	x
100 uL Solution D	x	x	x	x
0.2 g Trypticase Peptone		x	x	x
0.2 g Yeast Extract		x	x	x
0.05 g Mercaptoethane Sulfonic Acid		x	x	x
1000 uL Sodium Formate (2.5%)			x	x
2.95 g NaCl				x
0.17 g $MgCl_2$				x
0.050 g KCl				x

Solution Composition:

Solution A:

100 g l^{-1} NH_4Cl

100 g l^{-1} $MgCl_2 \cdot 6H_2O$

40 g l^{-1} $CaCl_2 \cdot 2H_2O$

Solution B:

200 g l^{-1} $K_2HPO_4 \cdot 3H_2O$

Solution C:

0.5 g l^{-1} Resazurin

Solution D:

500 mg l^{-1} Na_2-EDTA·$2H_2O$

150 mg l^{-1} $CoCl_2·6H_2O$

100 mg l^{-1} $MnCl_2·4H_2O$

100 mg l^{-1} $FeSO_4·7H_2O$

100 mg l^{-1} $ZnCl_2$

40 mg l^{-1} $AlCl_3·6H_2O$

30 mg l^{-1} $Na_2WO_4·2H_2O$

20 mg l^{-1} $CuCl_2·2H_2O$

20 mg l^{-1} $NiSO_4·6H_2O$

10 mg l^{-1} H_2SeO_3

10 mg l^{-1} H_3BO_3

10 mg l^{-1} $Na_2MoO_4·2H_2O$