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Low Pressure Tolerance by Methanogens in an Aqueous Environment: Implications for Subsurface Life on Mars

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Abstract The low pressure at the surface of Mars (average: 6 mbar) is one potentially biocidal factor that any extant life on the planet would need to endure. Near subsurface life, while shielded from ultraviolet radiation, would also be exposed to this low pressure environment, as the atmospheric gas-phase pressure increases very gradually with depth. Few studies have focused on low pressure as inhibitory to the growth or survival of organisms. However, recent work has uncovered a potential constraint to bacterial growth below 25 mbar. The study reported here tested the survivability of four methanogen species (*Methanothermobacter wolfeii*, *Methanosarcina barkeri*, *Methanobacterium formicicum*, *Methanococcus maripaludis*) under low pressure conditions approaching average martian surface pressure (6 mbar – 143 mbar) in an aqueous environment. Each of the four species survived exposure of varying length (3 days – 21 days) at pressures down to 6 mbar. This research is an important stepping-stone to determining if methanogens can actively metabolize/grow under these low pressures. Additionally, the recently discovered recurring slope lineae suggest that liquid water columns may connect the surface to deeper levels in the subsurface. If that is the case, any organism being transported in the water column would encounter the changing pressures during the transport.

Keywords Methanogens · Mars · Methane · Low pressure · Survival

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Introduction

The potential discovery of methane in the martian atmosphere by both space-based missions (Fonti and Marzo 2010; Formisano et al. 2004; Geminale et al. 2008; Geminale et al. 2011; Maguire 1977) and ground-based telescopes (Krasnopolsky et al. 1997; Krasnopolsky et al. 2004; Mumma et al. 2009) has fueled the study of methanogens as ideal organisms for life on Mars. While there are possible abiotic sources for the methane on Mars (Chassefière and Leblanc 2011; Chastain and Chevrier 2007; Lyons et al. 2005; Maguire 1977; Onstott et al. 2006; Oze and Sharma 2005), a biological source cannot be ruled out. Although Curiosity initially failed to detect methane in the martian atmosphere (Webster et al. 2013), previous reports note very localized sources of methane on the planet (Fonti and Marzo 2010; Mumma et al. 2009). However, more recent results released by the Mars Science Laboratory team have illustrated an increase in methane abundance over time (Webster et al. 2015).

Methanogens are microorganisms within the domain Archaea that produce methane. Some methanogens are chemoautotrophic, producing methane through the metabolism of hydrogen (H_2) as an energy source and carbon dioxide (CO_2) as a carbon source. Methanogens can be considered ideal organisms for life on Mars because they are anaerobic, do not require organic nutrients, and are non-photosynthetic, indicating they could exist in a subsurface environment. Methanogens have previously been shown to metabolize or survive under various martian conditions, including metabolism at low pressure [50 mbar (Kral et al. 2011)], metabolism on JSC Mars-1, a martian soil simulant (Kral et al. 2011; Kral et al. 2004), and survival following desiccation at Earth and Mars surface pressures (Kral and Altheide 2013; Kral et al. 2011). A distinction between growth, metabolism and survival should be noted. Growth is typically thought of as an increase in size or numbers in the case of microorganisms (Tortora et al. 2015). Growth typically accompanies metabolism, and in the research reported here, prior to and following exposure to low pressure, they are occurring concomitantly. Survival would indicate that the organism has remained viable (capable of metabolism/growth when more favorable conditions are restored) during challenging conditions, but may not have demonstrated any measureable metabolism/growth during those challenging conditions. The experiments conducted in the research reported here were testing for survival only under low-pressure conditions.

The surface pressure of Mars is approximately 1/100th the surface pressure of Earth, averaging between one and ten millibar over one martian year over the martian surface, based on differences in topography and the exchange of CO_2 between the atmosphere and the polar caps (Hess et al. 1979; Hess et al. 1980; Spiga et al. 2007). There are no locations on Earth's surface that reach such low levels (the pressure at the top of Mount Everest is 330 mbar; Fajardo-Cavazos et al. 2012), thus there are no surface environments on Earth within which organisms could adapt to low pressure. It is possible, however, that low-pressure atmospheric environments exist that house microorganisms. At sufficiently high altitudes (~20 km), the atmospheric pressure is low enough to be Mars-like (~5 mbar). Studies by Griffin (2004, 2008) and Smith et al. (2010) have collected air samples at these heights which contain microorganisms including bacteria, fungi and viruses capable of growth, isolation and identification under Earth-normal lab conditions between 22 and 30 °C. Various mechanisms can transport bacteria from Earth's surface through the highest reaches of the atmosphere, but general atmospheric retention time (3–10 days) and cold temperatures (−75 °C) suggest that these altitudes do not comprise permanent ecosystems (Smith et al. 2010). Additionally, the studies by Griffin (2004, 2008) and Smith et al. (2010) did not include archaeal identification. In a recent review, Gandolfi et al. (2013) note that of eight studies that did include archaeal sequencing, only one

sequence (*Euryarchaeota*) was retrieved. Thus, in terms of atmospheric biology and low pressure environments, more data including archaeal species is needed.

Schuerger et al. (2013) cited 17 biocidal/inhibitory factors that any extant life on Mars would need to endure in order to remain viable. Although the synergistic effects of these biocidal factors are not explored within the experiments conducted here, certain assumptions can be made to increase the validity of these studies: 1. The organisms are protected from UV radiation. 2. There is H_2 gas available for metabolism. 3. There is sufficient liquid water for active metabolism. These three assumptions are not improbable when a subsurface environment is considered. In regard to UV radiation, Schuerger et al. (2012) note that a one-millimeter thick layer of crushed basalt (analog martian regolith) provides sufficient attenuation of UV radiation allowing for the survival of *Bacillus subtilis* HA101 endospores and *Enterococcus faecalis* ATCC 29212 cells. Although H_2 has only been detected in the upper atmosphere (Krasnopolsky and Feldman 2001) and not definitively identified at the surface, it is believed to exist on Mars and is incorporated into a number of atmospheric models (Atreya and Gu 1994; Krasnopolsky 1993; Nair et al. 1994). Possible sources of H_2 on Mars include downward diffusion from the upper atmosphere (Weiss et al. 2000), volcanic and hydrothermal activity (Boston et al. 1992; Wray and Ehlmann 2011), radiolysis of subsurface ice and water (Onstott et al. 2006), and water-rock interactions, specifically, serpentinization (Atreya et al. 2007; McCollom and Bach 2009; Oze and Sharma 2005). Significant H_2 can be produced through serpentinization, but reaction rates are severely limited at low temperatures (< ~200 °C). However, at low temperatures conducive to life (< 130 °C), a steady source of H_2 may result from the decomposition of Fe-rich brucite, as H_2 is lost from the system. In this scenario, the total amount of H_2 produced could eventually equal the amount produced at high temperature, although the production would be very gradual over time (McCollom and Bach 2009).

It is important to note that the absence of detection of H_2 does not necessarily rule out its existence in the martian atmosphere or within the subsurface. Kral et al. (1998) have shown that *Methanobacterium formicicum* is capable of H_2 uptake at levels down to 15 ppm. The low concentration of H_2 on Mars may not be detectable when the entire atmosphere is taken into account. Thus, the absence of H_2 in the martian atmosphere may be more consistent with the presence of methanogens than with their absence. However, in the case that H_2 is not available on Mars, carbon monoxide has also been reported in the martian atmosphere (Barth et al. 1969; Clancy et al. 1983; Krasnopolsky 2007; Lellouch et al. 1991), which certain methanogens can use in place of H_2 as an energy source (Daniels et al. 1977; O'Brien et al. 1984). Recently, King (2015) has demonstrated the ability of two microorganisms to oxidize carbon monoxide at concentrations much lower than that contained in the martian atmosphere, under conditions of high salt and low water activity. One of these organisms, *Halorubrum* str. BV1, is a member of the Euryarchaeota, a phylum to which methanogens also belong (King 2015). Lastly, there is evidence that there is water, albeit frozen, on Mars in the near subsurface (Boynton et al. 2002; Feldman et al. 2002; Haberle et al. 2001; Malin and Edgett 2000; Mitrofanov et al. 2002; Rennó et al. 2009; Smith et al. 2009). The presence of recurring slope lineae (RSLs) has reignited the idea that there is liquid water, in the form of brines, available on or near the planet surface (Grimm et al. 2014; McEwen et al. 2014; McEwen et al. 2011; Ojha et al. 2015; Stillman et al. 2014). More recently, thermodynamic modeling, in conjunction with temperature and humidity measurements at the martian surface, suggest that nighttime transient brines may form in the very near subsurface (< 5 cm). However, the nighttime temperatures and the water activity of the brines are likely much too low to support life as we know it (Martín-Torres et al. 2015). Additionally, meteoritic evidence suggests the presence of a subsurface water reservoir either in the form of a hydrated crust or embedded ground ice (Usui et al. 2015).

Of the various conditions on Mars that contribute to its seeming inhospitality, low pressure is typically included in Mars simulation experiments, but the effect of low pressure itself is often overlooked when compared to more lethal effects, such as UV radiation and desiccation. However, the low pressure environment cannot be ignored, as the atmospheric gas-phase pressure increases only very gradually with depth and there appears to be a "25 mbar limit" below which many bacteria fail to grow (Schuerger et al. 2013).

This research encompasses seven experiments testing the survival of four species of methanogens (*Methanothermobacter wolfeii*, *Methanosarcina barkeri*, *M. formicicum*, *Methanococcus maripaludis*) at low pressures approaching 6 mbar, the average surface pressure on Mars, in liquid media.

Methods

Cultures and Growth Media

Methanogen cultures were originally obtained from the Oregon Collection of Methanogens, Portland State University, Oregon. Each methanogen was grown in its own anaerobic medium for optimum growth: *Methanosarcina barkeri* [OCM 38], MS medium [yeast extract, trypticase peptone, mercaptoethanesulfonic acid, potassium phosphate, ammonium chloride, magnesium chloride, calcium chloride, and additional trace minerals (Boone et al. 1989; Kendrick and Kral 2006)]; *Methanobacterium formicicum* [OCM 55], MS medium supplemented with sodium formate [designated MSF medium; (Boone et al. 1989)]; *Methanothermobacter wolfeii* [OCM 36], MM medium [a minimal medium containing the same components as MS medium except yeast extract, trypticase peptone and mercaptoethanesulfonic acid (Kendrick and Kral 2006; Xun et al. 1988)]; and *Methanococcus maripaludis* [OCM 151], MSH medium [MS medium containing additional sodium chloride, magnesium chloride and potassium chloride (Ni and Boone 1991)]. These media provide the nutrients and minerals necessary for growth, and are not intended to represent the available concentration of nutrients on Mars.

Microbial procedures for each of the seven experiments were as follows: Growth media were prepared under anaerobic conditions in a 90:10 CO_2:H_2 gas Coy Anaerobic Chamber (Coy Laboratory Products Inc., Grass Lake Charter Township, MI) following the procedure of Kendrick and Kral (2006). Ten milliliters of each of the four media were added to each of five anaerobic culture tubes, for a total of twenty tubes (see Table 1). This provided five replicates for each of the four methanogen species for each of the seven experiments. The tubes were fitted with rubber stoppers and aluminum crimps (Boone et al. 1989), sealing the tubes under anaerobic conditions and eliminating exposure to the ambient atmosphere. A sterile solution of ~125 μL of 2.5 % sodium sulfide was added to the media following sterilization via autoclave (Boone et al. 1989). Each culture tube was inoculated with 0.5 mL of the corresponding methanogen. The anaerobic nature and slow doubling time of methanogens makes them difficult to grow on agar or to provide accurate cell counts without the use of expensive and/or involved techniques. Common methods used to determine methanogen growth are optical density measurements and methane measurements using gas chromatography (Sowers and Schreier 1995). In all experiments explained here, 0.5 mL of culture was used as a standard inoculum. The tubes were pressurized with 2 bar H_2 gas and placed at their respective incubation temperatures (24 °C for *M. maripaludis,* 37 °C for *M. barkeri* and *M. formicicum*,

Table 1 Experimental conditions for each of seven experiments, including pressures, time punctured and time exposed to low pressure. Each experiment consisted of five replicates for each of four methanogen species in 10 mL of their respective anaerobic growth medium. Incubations both pre- and post-exposure were conducted at the methanogens' respective growth temperatures (*M. barkeri*, 37 °C, MS medium; *M. formicicum*, 37 °C, MSF medium; *M. wolfeii*, 55 °C, MM medium; *M. maripaludis*, 24 °C, MSH medium)

Expt.	Amount of regolith analog per tube	Time for equilibration of chamber[a] (days)	Pressure range during exposure[b] (mbar)	Time exposed to low pressure (days)	Time between filling chamber with CO_2 and removing tubes[c] (days)	Average temperature inside Pegasus Chamber during exposure (°C)	Overall time punctured (days)	Incubation times following exposure (days)
1	NA[d]	1	138 ± 5	8	NA	28	8	96
2	NA	2	69 ± 3	11	4	30	12	111
3	NA	2	69 ± 3	11	5	30	14	37
4	NA	2	35 ± 3	6	3	31	7	44
5	NA	1	8 ± 2	3	1	30	4	56
6	5 g JSC Mars-1	1	14 ± 7	5	3	27	7	40
7	NA	1 h	49.8 ± 0.1	21	1 h	28	1 h	157

[a] The time for equilibration corresponds to the length of time between when the chamber was set at the desired pressure and the tubes were punctured

[b] A pressure range is given based on the capabilities of our chamber

[c] The chamber was filled with CO_2 before removal of the needles from the tubes to ensure that the tubes were not under negative pressure when removed from the chamber

[d] *NA* Not Applicable

55 °C for *M. wolfeii*). This incubation period allowed for the initial growth of the organisms for use in each experiment, which was verified via methane detection by gas chromatography.

Pegasus Planetary Simulation Chamber and Experimental Procedures

Seven low pressure experiments were conducted in the Pegasus Planetary Simulation Chamber, previously described (Kral et al. 2011). In Experiments 1 through 5 and Experiment 7, anaerobic tubes contained only cultures in liquid media, as prepared above (Section Cultures and Growth Media). The major variable was pressure.

In Experiment 6, cultures consisted of liquid media, as prepared above (Section Cultures and Growth Media), with an additional five grams of JSC Mars-1 regolith simulant situated atop a sterile cotton plug (Fig. 1a). This served to keep the liquid cultures separated from the regolith, in order to eliminate the possibility of soil-water interactions or clumping of cells adhering to soil particles.

For the preparation of the cultures for Experiment 6, five grams of JSC Mars-1 simulant regolith were placed into each of twenty empty, anaerobic culture tubes and sterilized via autoclave. Previously prepared growth media containing cultures of each methanogen species (10 mL of liquid media) and the tubes containing the sterilized regolith simulant were placed into a Coy Anaerobic Chamber. Within the chamber, the aluminum crimps and rubber stoppers were removed from each of the twenty tubes containing the methanogens. A sterile cotton ball was placed into each of these tubes above the liquid medium. Five grams of sterile regolith simulant were transferred to each tube and allowed to sit on top of the cotton. The tubes were re-stoppered with their original stoppers and re-crimped with new crimps (Fig. 1a).

For Experiments 1 through 6, before being placed in the Pegasus Planetary Simulation Chamber, cultures were tested for methane production (Varian Micro-GC, model CP-4900, Palo Alto, CA) and optical density (600 nm; Spectronic 20D+, Spectronic Instruments, USA; Experiments 5, 6 only) to confirm active metabolism and growth. Methane production and optical density are typically used as a proxy for methanogen growth when both are seen to increase over time (Sowers and Schreier 1995). The tubes were placed into the chamber with a palladium

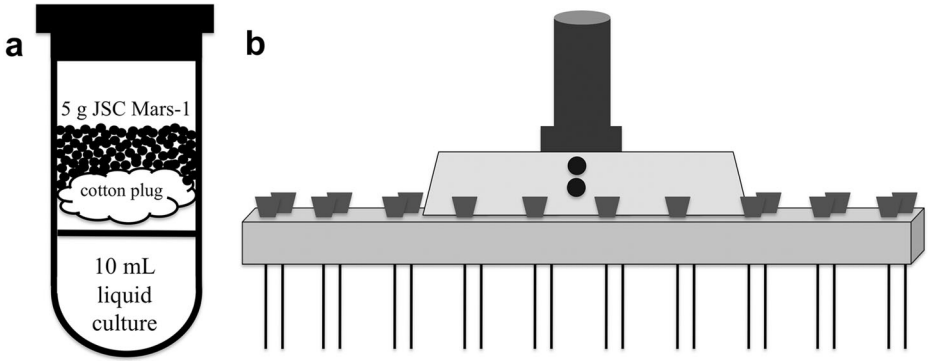

Fig. 1 **a** Diagram illustrating anaerobic tube contents for Experiment 6 only. Tubes were sealed with a rubber stopper and crimp, and contained 10 mL liquid culture, a cotton plug situated just above the liquid, and five grams JSC Mars-1 atop the cotton **b** Diagram of the specialized puncture device. Twenty holes were cut into a piece of Plexiglas within which one-inch 22-gauge syringe needles were inserted and removed for each experiment. The device was connected to a cylindrical manipulator via two screws. The cylindrical manipulator was fitted through one of the top ports of the Pegasus Planetary Simulation Chamber, and allowed for manual operation of the device (puncture of tubes, removal of needles) during experiments

catalyst box to remove residual oxygen. Within the chamber, the twenty tubes for each experiment were situated inside a test tube rack, sorted randomly, but grouped by species (five replicates for each of the four species). A second test tube rack was placed over the top of the tubes to secure their position for use with a specialized puncture device (Fig. 1b). The chamber door was closed and duct seal putty (Rainbow Technology, Pelham, AL) was applied around the seal as a further safeguard against oxygen contamination. The chamber was evacuated, filled with 80:20 H_2:CO_2 gas and evacuated again. This cycle was repeated three times to ensure removal of the ambient atmosphere. On the third cycle, H_2/CO_2 gas was added to the chamber in a continuous flow while under vacuum for a total of three minutes to ensure removal of the atmosphere. The chamber was then set at the desired pressure for the duration of the experiment. Pressure setpoints (Table 1) were maintained using a DU 200 capacitive sensor and Center One controller (Oerlikon Leybold Vacuum, Export, PA). Following a prescribed time for each experiment, the tubes were punctured with a specialized device containing one-inch, 22-gauge syringe needles (Fig. 1b) to allow equilibration between the chamber pressure and the pressure inside the tubes. The seal between the puncture device and the chamber was also covered with duct seal putty to minimize oxygen contamination. All experiments were conducted at room temperature. Pressures and exposure times are seen in Table 1.

Following the prescribed exposure times (limited by evaporation of the liquid media), the needles were removed from the tubes using the same device. The puncturing of the tubes and removal of the needles before and after exposure to low pressure were performed in order to limit oxygen exposure to the methanogens, which are strict anaerobes. This also limited exposure to the ambient atmosphere, keeping the methanogens in contact with solely H_2/CO_2 gas. After another set of designated times (Table 1), the chamber was filled to atmospheric pressure with CO_2. Following removal from the chamber, additional 2.5 % sodium sulfide solution (~125 μL) was added to each test tube to remove residual oxygen. A second set of sterile methanogen growth media was prepared as above (five test tubes for each of the four types of media). Each of these twenty tubes was inoculated with 0.5 mL of methanogen media from one of the original tubes (e.g., 0.5 mL from original tube #1 was used to inoculate transfer tube #1). Both the original and transfer sets were pressurized with 2 bar H_2 gas and kept at the organisms' respective incubation temperatures (Table 1). For each experiment, growth was monitored by methane production (gas chromatography) and optical density (Experiments 5, 6 only).

For Experiments 1 through 6, following designated post-exposure incubation periods (Table 1), electrical conductivity, salinity and total dissolved solids (TDS) were measured for both the original and transfer sets for each experiment (Tables 2, 3 and 4), where applicable, using an EcoSense EC300 Conductivity/Temperature probe (YSI Inc., Yellow Springs, OH). In order to have sufficient liquid for measurements, tubes within each set, for each experiment, were combined.

In Experiment 7, in order to limit evaporation and extend the length of the experiment, a different procedure was used. Media in anaerobic test tubes were prepared as above (Section Cultures and Growth Media). As with Experiments 1 through 6, the test tubes were measured for methane production via gas chromatography (Shimadzu Scientific Instruments Inc., model GC-2014, Columbia, MD), as well as optical density (Expts. 5 through 7 only) before the start of the experiment. The tubes were placed into the Pegasus Planetary Simulation Chamber with a palladium catalyst box to remove residual oxygen. Within the chamber, the twenty tubes were situated inside a test tube rack, sorted randomly (five replicates for each of the four species). A second test tube rack was placed over the top of the tubes to secure their position for use with a specialized puncture device (Fig. 1b). The chamber door was closed,

Table 2 Electrical conductivity measurements (milliSiemens/cm; mS/cm) for four methanogens for Experiments 1 through 6, including uninoculated media. Measurements are from the combined media from each culture tube for each set, (original and transfer) and were taken following both exposure to low pressure (original cultures) and inoculation of methanogens from original to transfer cultures

Expt.	*M. barkeri*		*M. formicicum*		*M. wolfeii*		*M. maripaludis*	
	O[a]	T[b]	O	T	O	T	O	T
1	10.6	10.66	11.5	11.11	9.65	9.52	47.76	49.95
2	10.45	9.41	10.68	11.2	10.32	9.97	47.42	52.46
3	10.12	10.39	10.42	12.15	9.66	9.83	50.52	48.7
4	10.78	10.74	12.68	11.89	11.05	10.23	53.6	50.64
5	ND[c]	9.02	ND	ND	ND	10.99	ND	58.4
6	ND	11.8	ND	13.33	ND	ND	ND	57.6
Media	9.6		10.18		9.68		44.9	

[a] *O* original cultures

[b] *T* transfer cultures

[c] *ND* No Data

and the chamber was evacuated to 50 mbar while 80:20 H_2:CO_2 gas was bled into the chamber. Pressure setpoints (Table 1) were maintained using a MKS Type 651C pressure controller and MKS Type 253B throttling valve (MKS Instruments Inc., Andover, MA). After 30 min, the test tubes were punctured with the specialized device mentioned above (Fig. 1b) to allow equilibration between the chamber pressure and the pressure inside the tubes. This equilibration period lasted for 1 h, after which the needles were removed from the tubes, effectively creating "micro-environments" within each culture tube. The 80:20 H_2:CO_2 gas source continued to bleed into the chamber during the 1 h period of equilibration. The tubes remained sealed within the chamber for the duration of the experiment, which dictates that the

Table 3 Salinity measurements (parts per thousand; ppt) for four methanogens for Experiments 1 through 6, including uninoculated media. Measurements are from the combined media from each culture tube for each set (original and transfer), and were taken following both exposure to low pressure (original cultures) and inoculation of methanogens from original to transfer cultures

Expt.	*M. barkeri*		*M. formicicum*		*M. wolfeii*		*M. maripaludis*	
	O[a]	T[b]	O	T	O	T	O	T
1	6.1	6.0	6.5	6.3	5.3	5.2	31.6	33.2
2	5.9	5.2	6.0	6.3	5.7	5.5	31.3	35.0
3	5.6	6.0	6.0	7.1	5.3	5.4	33.6	32.3
4	6.1	6.1	7.3	6.8	6.3	5.7	36.1	ND
5	ND[c]	5.2	ND	ND	ND	6.2	ND	39.6
6	ND	6.7	ND	7.6	ND	ND	ND	39.1
Media	5.5		5.9		5.5		29.3	

[a] *O* original cultures

[b] *T* transfer cultures

[c] *ND* No data

Table 4 Total dissolved solids (TDS) measurements (g/L) for four methanogens for Experiments 1 through 6, including uninoculated media. Measurements are from the combined media from each culture tube for each set (original and transfer), and were taken following both exposure to low pressure (original cultures) and inoculation of methanogens from original to transfer cultures

Expt.	*M. barkeri*		*M. formicicum*		*M. wolfeii*		*M. maripaludis*	
	O[a]	T[b]	O	T	O	T	O	T
1	7.16	6.94	7.46	7.18	6.38	6.28	31.09	32.55
2	6.80	6.93	6.91	7.25	6.77	6.45	30.90	34.17
3	6.58	6.79	6.80	7.92	6.25	6.36	32.95	31.82
4	6.92	6.94	8.25	7.73	7.22	6.64	35.2	ND
5	ND[c]	6.00	ND	ND	ND	7.01	ND	38.2
6	ND	7.73	ND	8.7	ND	ND	ND	37.7
Media	6.24		6.67		5.5		28.96	

[a] *O* original cultures

[b] *T* transfer cultures

[c] *ND* No data

actual pressure within the tubes was dependent upon the temperature of the tubes within the chamber. However, the resulting vapor pressure based on the temperature within the chamber (27–30 °C) was calculated to be between 36 and 42 mbar, lower than the experimental pressure (50 mbar) initially sealed in the tubes. Therefore, the chamber temperature would not have increased the pressure in the tubes. There is a somewhat foolproof aspect of this procedure. If there is a leak in the septum following the removal of the needle, the pressure will remain in equilibrium with the low pressure in the chamber. If the leak is large enough to allow any measureable evaporation, it will be visually obvious.

After 21 days (Table 1), the chamber was filled to atmospheric pressure with CO_2 gas and the test tubes were re-punctured to equilibrate them with the chamber environment. After 20 min, the needles were removed from the test tubes and the tubes were removed from the chamber. Following removal, additional 2.5 % sodium sulfide solution (~125 μL) was added to each test tube to remove residual oxygen. A second set of sterile methanogen growth media was prepared as above (five test tubes for each of the four types of media). Each of these twenty tubes was inoculated with 0.5 mL of methanogen media from one of the original tubes (e.g., 0.5 mL from original tube #1 was used to inoculate transfer tube #1). Both the original and transfer sets were pressurized with 2 bar H_2 gas and kept at the organisms' respective incubation temperatures (Table 1). Growth was monitored over 157 days by methane production (gas chromatography) and optical density.

Results

For each of the seven experiments, viable cells of each of the four methanogen species (*M. barkeri*, *M. formicicum*, *M. maripaludis*, *M. wolfeii*) were successfully transferred to new media (transfer cultures) following exposure to low pressure. Methane production was generally similar between original and transfer cultures in Experiments 1–4 and 7, although *M. formicicum* and *M. maripaludis* experienced slightly higher methane production within

transfer tubes than in original tubes for all seven experiments (Figs. 2-4). *M. formicicum* produced the highest and most consistent amounts of methane across all seven experiments (40–60 % headspace; Figs. 2-4).

Experiment 1: 133–143 mbar

For each species, at least one original culture continued to show an increase in methane production during the post-exposure incubation period (Fig. 2). However, at least one original culture for each species also failed to produce any significant methane following exposure to low pressure (> 1 %).

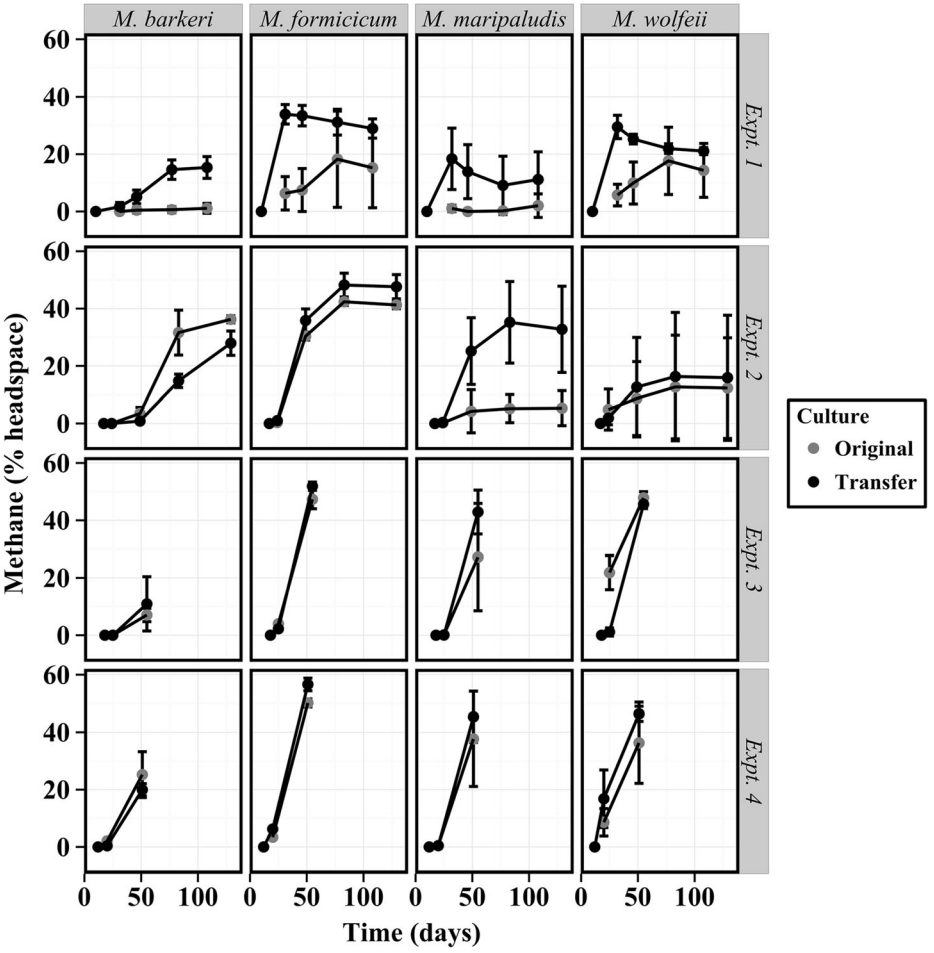

Fig. 2 Average methane (% headspace) produced for four methanogen species (*M. barkeri*, *M. formicicum*, *M. maripaludis* and *M. wolfeii*) after exposure to low pressure for four separate experiments (Experiment 1: 133–143 mbar, Experiments 2, 3: 67–72 mbar, Experiment 4: 33–38 mbar). Original tubes (gray circles) contained active cultures producing methane before being placed into the Pegasus Planetary Simulation Chamber (Day 0). Transfer cultures (black circles) were inoculated on the day the original tubes were removed from the chamber. Prior to and following the low-pressure exposure period, cultures were kept at the organisms' growth temperatures (24 °C for *M. maripaludis,* 37 °C for *M. barkeri* and *M. formicicum*, and 55 °C for *M. wolfeii*). Error bars indicate +/− one standard deviation

Transfer cultures for each species produced greater amounts of methane than the original cultures during the post-exposure incubation period (Fig. 2), with methane being produced in all five transfer culture replicates. Methane produced in transfer cultures of *M. wolfeii* and *M. formicicum* was initially high (29.5 ± 4.1 % and 33.9 ± 3.4 %, respectively). Electrical conductivities, salinities and total dissolved solids were relatively similar between original and transfer cultures for *M. barkeri*, *M. formicicum* and *M. wolfeii* (Tables 2, 3 and 4).

Experiment 2: 67–72 mbar

All five original culture replicates for *M. barkeri*, *M. formicicum* and *M. maripaludis* continued to produce methane following exposure to low pressure (Fig. 2). Only two original cultures for *M. wolfeii* produced significant methane (>1 %) during the post-exposure incubation period. For *M. maripaludis*, one original culture continued to produce methane but to a much lesser extent than the other four cultures (~10 % vs. ~40 %).

Only two transfer cultures for *M. wolfeii* produced significant methane during the post-exposure incubation period, which are sub-cultures from the two original cultures mentioned above.

Electrical conductivities, salinities and TDS differed between original and transfer cultures for each methanogen, although there was not significant evaporation within the original cultures during this experiment. All three measurements were slightly higher in transfer cultures than in original cultures for *M. formicicum*, whereas measurements were higher in original cultures than transfer cultures for *M. wolfeii*. Electrical conductivity and salinity were higher in original cultures than transfer cultures for *M. barkeri* (Tables 2, 3 and 4).

Experiment 3: 67–72 mbar

All five original cultures and all five transfer cultures for all four species produced methane after the start of the experiment.

Electrical conductivity, salinity and TDS were greater in transfer cultures than original cultures for both *M. formicicum* and *M. maripaludis*, despite insignificant evaporation during the experiment. Measurements were relatively similar between original and transfer cultures for *M. barkeri* and *M. wolfeii*, with slightly higher values in transfer cultures (Tables 2, 3 and 4).

Experiment 4: 33–37 mbar

All five replicates for both original and transfer cultures for all four species produced methane after the original cultures were removed from the chamber and the transfer cultures were inoculated (Fig. 2).

Varying rates of evaporation occurred within original cultures throughout the experiment (0.5–5.6 mL decrease in volume of liquid media over six days). Evaporation was three times greater in the front row of cultures tubes than in the back row (decrease in volume of liquid media of 3.83 ± 1.33 mL vs. 1.15 ± 0.53 mL).

Electrical conductivity, salinity and TDS were greater in original cultures than transfer cultures for *M. formicicum* and *M. wolfeii*. Measurements were essentially identical between original and transfer cultures for *M. barkeri*. Electrical conductivity was higher in original cultures than transfer cultures for *M. maripaludis* (Tables 2, 3 and 4; salinity and TDS were not measured in transfer cultures).

Experiment 5: 6–10 mbar

All four methanogen species survived three days' exposure to 8 mbar, close to the average martian surface pressure (Fig. 3), despite heavy evaporation (~10 mL in 3 days). Sufficient liquid remained to perform transfers from at least two original cultures to fresh media for each of the four species. However, original tubes were depleted of liquid media after transfer, measured 0 % methane after 16 days, and were discarded.

Electrical conductivity, salinity and TDS were only measured in transfer cultures for *M. barkeri*, *M. wolfeii* and *M. maripaludis* (measurements were not taken in transfer cultures for *M. formicicum* due to an insufficient amount of liquid following evaporation in original

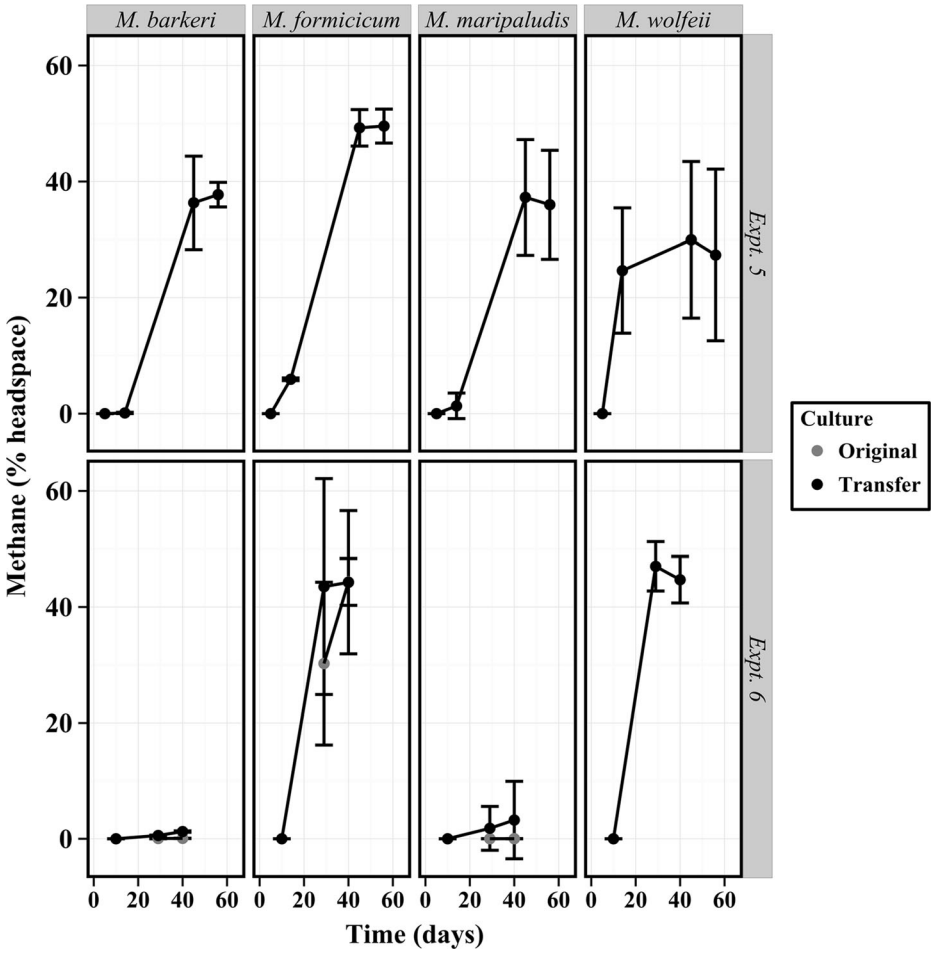

Fig. 3 Average methane (% headspace) produced for four methanogen species (*M. barkeri*, *M. formicicum*, *M. maripaludis* and *M. wolfeii*) after exposure to low pressure for two separate experiments (Experiment 5: 6–10 mbar, Experiment 6: 7–20 mbar). Original tubes (gray circles) contained active cultures producing methane before being placed into the Pegasus Planetary Simulation Chamber (Day 0). Transfer cultures (black circles) were inoculated on the day the original tubes were removed from the chamber. Prior to and following the low-pressure exposure period, cultures were kept at the organisms' growth temperatures (24 °C for *M. maripaludis,* 37 °C for *M. barkeri* and *M. formicicum*, and 55 °C for *M. wolfeii*). Error bars indicate +/− one standard deviation

cultures). Measurements for *M. barkeri* cultures were comparable to measurements in uninoculated MS medium. Electrical conductivity, salinity and TDS were all higher in transfer cultures for *M. wolfeii* and *M. maripaludis* than for uninoculated MM (*M. wolfeii*) and MSH (*M. maripaludis*) media (Tables 2, 3 and 4).

Transfer cultures for each of the four species all produced methane after inoculation. Optical density values within transfer cultures increased for all four methanogens after inoculation (data not shown). Measurements were comparable to pre-exposure values for *M. barkeri*, *M. maripaludis* and *M. formicicum* (~0.07, ~0.1, and ~0.2 respectively). *M. wolfeii* transfer cultures displayed greater optical density values, as compared to pre-exposure numbers (0.12 ± 0.02 vs. 0.05 ± 0.01).

Experiment 6: 7–20 mbar

All four methanogen species survived five days' exposure to pressures approaching average martian surface pressures (Fig. 3). JSC Mars-1 was utilized as a diffusion barrier, which prolonged the experiment by two days (compared to Experiment 5). Although each culture experienced heavy evaporation (~10 mL in 5 days), at least three replicates for each species retained a sufficient amount of culture to perform transfers to fresh media following exposure to low pressure.

Due to a lack of media as a result of evaporation in the original cultures, electrical conductivity, salinity and TDS measurements were only taken in transfer cultures of *M. maripaludis*, *M. barkeri* and *M. formicicum* (measurements were not possible in original cultures for all four methanogens, nor in transfer cultures for *M. wolfeii*). Values for electrical conductivity, salinity and TDS were all higher in transfer cultures than for uninoculated media (Tables 2, 3 and 4).

Original cultures of *M. maripaludis* ($n = 4$), *M. barkeri* ($n = 3$) and *M. formicicum* ($n = 2$) retained enough liquid culture following transfer to continue being monitored for methane production during the post-exposure incubation period. The original cultures of *M. maripaludis* and *M. barkeri* produced methane to a much lesser extent than cultures in previous experiments, but contained methane amounts similar to transfer cultures of the same experiment (Fig. 3). Original cultures of *M. formicicum* initially produced high amounts of methane after being removed from the chamber (~30–45 %). *M. formicicum* and *M. wolfeii* transfer cultures initially produced high amounts of methane (~45 %) after inoculation (Fig. 3).

Optical density values within transfer cultures for *M. barkeri*, *M. formicicum*, and *M. wolfeii* were initially higher than the values measured in the original cultures before the start of the experiment (data not shown). All three transfer cultures for *M. wolfeii* increased in optical density after inoculation, whereas only two of five transfer cultures for *M. maripaludis* increased in optical density during the post-exposure incubation period.

Experiment 7: 49–50 mbar

All five original culture replicates for *M. barkeri*, *M. formicicum* and *M. wolfeii* survived 21 days at 50 mbar and produced methane following exposure to low pressure (Fig. 4). Only one original culture of *M. maripaludis* produced significant methane (~8 %) at the beginning of the post-exposure incubation period, and continued to increase in methane abundance over this period. One original culture of *M. maripaludis* did not begin to produce significant methane (~1 %) until the second measurement taken on Day 64 (Fig. 4). The other three original cultures of *M. maripaludis* failed to produce any methane during the post-exposure incubation period.

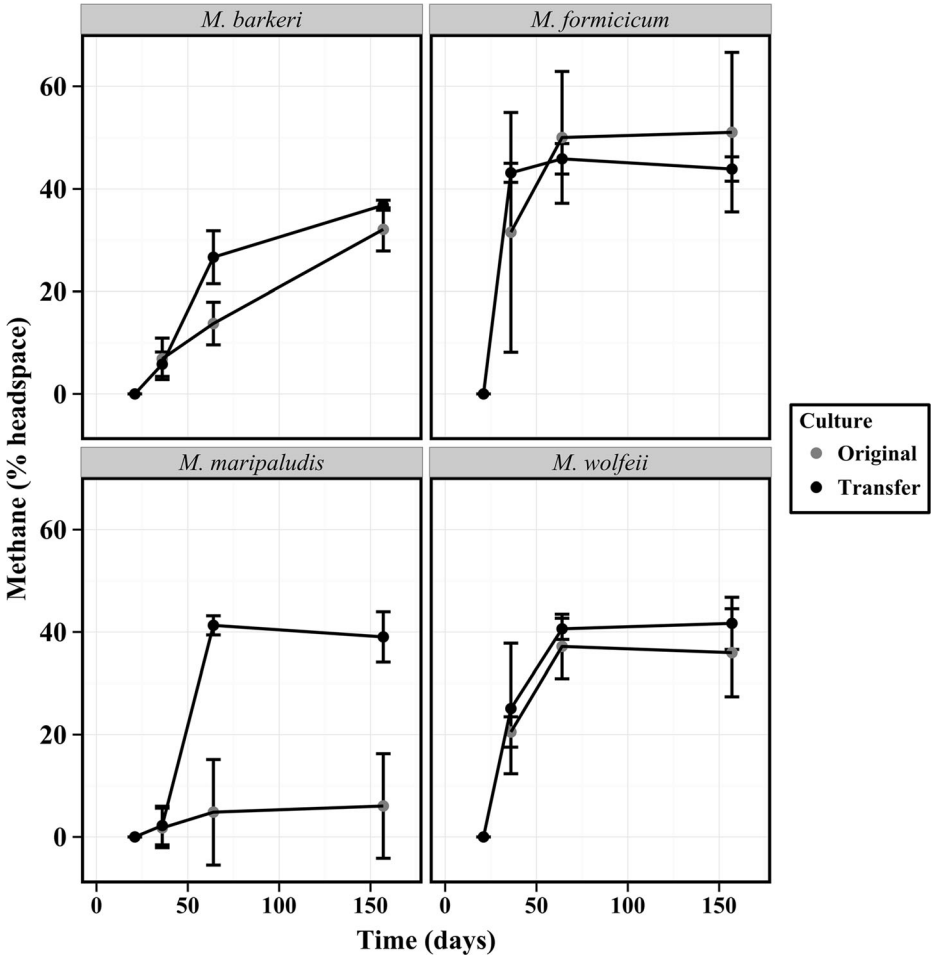

Fig. 4 Average methane (% headspace) produced for four methanogen species (*M. barkeri*, *M. formicicum*, *M. maripaludis* and *M. wolfeii*) after exposure to low pressure (Experiment 7: 21 days, 49–50 mbar). Original tubes (gray circles) contained active cultures producing methane before being placed into the Pegasus Planetary Simulation Chamber (Day 0). Transfer cultures (black circles) were inoculated on the day the original tubes were removed from the chamber. Prior to and following the low-pressure exposure period, cultures were kept at the organisms' growth temperatures (24 °C for *M. maripaludis,* 37 °C for *M. barkeri* and *M. formicicum*, and 55 °C for *M. wolfeii*). Error bars indicate +/− one standard deviation

All five transfer cultures for all four species produced methane after inoculation from original cultures following exposure to low pressure. Transfer cultures containing *M. formicicum* initially produced high amounts of methane (~43 %, Fig. 4). After 157 days of post-exposure incubation, transfer cultures of all four species produced ~40 % methane. Original cultures of *M. barkeri*, *M. formicicum* and *M. wolfeii* produced methane amounts similar to transfer cultures. Transfer cultures of *M. maripaludis* produced significantly more methane than original cultures (~40 % vs. ~5 %, Fig. 4).

Optical density values (data not shown) for both original and transfer cultures verify the growth demonstrated via methane production above.

Discussion

The results shown here indicate that methanogen cells within aqueous media can remain viable after exposure to a low pressure environment, as well as the consequent evaporation of the liquid media, for the time periods tested. While survival during relatively long-term desiccation at low pressures has already been shown (Kral and Altheide 2013; Kral et al. 2011), the effect of low pressure on cells in aqueous media represents novel research and an important stepping stone toward observing active growth of methanogens at low pressure. The results reported here are also important from the standpoint of methanogens possibly inhabiting a liquid water column below the surface of Mars. The recently discovered recurring slope lineae (Grimm et al. 2014; McEwen et al. 2014; McEwen et al. 2011; Stillman et al. 2014) suggest that liquid water is moving from the subsurface to the surface and, very likely, vice versa. That being the case, those methanogens might be encountering substantially different pressures, and survival at those varying pressures would be paramount for their continued existence.

The assumptions made in these experiments (availability of water, protection from UV radiation, availability of H_2) are not unreasonable with respect to Mars when considering a subsurface environment. Similar to Earth, it is possible that Mars contains deep subsurface habitats conducive to life. These habitats may contain H_2 and warmer temperatures due to geothermal or volcanic activity (Boston et al. 1992). As such, these experiments provide possible insight into the survival of methanogens under subsurface martian conditions.

Few studies have characterized the effects of low pressure on the growth and survivability of microorganisms (Fajardo-Cavazos et al. 2012; Nicholson et al. 2013; Schuerger et al. 2013), whereas the only studies investigating the effects of martian conditions on methanogens at Mars surface pressures have used desiccated cells (Johnson et al. 2011; Kral and Altheide 2013; Kral et al. 2011) or pelleted cells (Morozova et al. 2007). A recent study by Schirmack et al. (2014) incorporated in situ measurements of methane production by methanogens under Mars-like conditions, but the pressure used was 500 mbar, which the authors cite as an achievable pressure in the near subsurface (< 20 m), based on modeling by Jones et al. (2011). However, Schuerger et al. (2013) described two separate pressure models for the martian subsurface using either the lithographic pressure (of the overlying rock) or the gas-phase pressure (due to void spaces within the regolith). The gas-phase model predicts that pressure within the martian subsurface increases only slightly with depth, reaching 25 mbar at 13.9 km (Schuerger et al. 2013). In contrast, the lithographic pressure requires complete seclusion from the atmosphere, such as within rock and ice grains, and reaches 25 mbar at only 19.5 cm below the surface. The model by Jones et al. (2011) agrees with the lithographic pressure model described by (Schuerger et al. 2013), indicating that any microorganisms at this pressure would need to be completely shielded from the martian atmosphere.

Morozova et al. (2007) have previously demonstrated the effects of a simulated Mars environment on pelleted cells of three methanogen strains isolated from Siberian permafrost (*Methanosarcina* spec. SMA-21, *Methanosarcina* spec. SMA-16, *Methanosarcina* spec. SMA-23) compared to three non-permafrost reference methanogens (*Methanobacterium* spec. MC-20, *Methanosarcina barkeri* [DSM 8687], and *Methanogenium frigidum* [DSM 16458]). Morozova et al. (2007) discovered that the methanogens isolated from permafrost habitats showed increased survival under martian conditions when compared to the reference organisms (60–90 % vs. 0.3–5.8 %). Interestingly, whereas *M. barkeri* exhibited the least resistance to martian conditions (0.3 % survival) during the Morozova et al. (2007) experiments, during our low pressure experiment at 8 mbar (Experiment 5), *M. barkeri* produced similar amounts of methane post-exposure as

compared to pre-exposure values (Fig. 3). In addition, for Experiments 5, 6 and 7, the optical density values for *M. barkeri* post-exposure were equal to or greater than the values pre-exposure (data not shown). Although the survival of *M. barkeri* cannot be directly compared between the Morozova et al. (2007) experiment and this paper due to differences in methods and experimental setup, these conflicting results illustrate the need for further study of microorganisms under martian conditions, taking into account martian environmental factors (temperature, pressure, etc.), as well as cell state (desiccated, pellet, active, etc.).

Considering that hydrated cells, as opposed to desiccated cells, were exposed to the low pressure environment, it is not extreme to suggest that methanogens *might* be able to metabolize under these conditions, given the availability of liquid water. However, it is more likely, given the relatively slow metabolism of methanogens and thus, long generation times, that the cells simply entered an inactive state for the short duration of the experiments. Additionally, it could be considered a large assumption to expect that liquid water is consistently available in the martian subsurface, either in terms of prolonged availability or suitable salt concentration.

These experiments were conducted in anaerobic culture tubes, within which the liquid media initially formed a 10 mL water column. The hydrostatic pressure of the water column could have increased the pressure at the bottom of the liquid environment. Eq. 1 gives the formula for the pressure at a given depth in a static liquid, where P_{atm} is the pressure of the atmosphere acting on the liquid, ρ is the density of the liquid, g is gravity (9.8 m/s^2) and h is the height of the liquid:

$$P = P_{atm} + \rho g h \tag{1}$$

The height of the 10 mL water column within the tubes was about 6 cm. Using the density of water and substituting the appropriate values into Eq. 1, the hydrostatic pressure of the liquid medium, before any evaporation, is 5.9 mbar. As such, should the methanogen cells have collected at the bottom of the test tube during each experiment (the tubes were kept upright during the course of the experiments), the minimum pressures for each experiments are, in reality, ~6 mbar greater then the pressures quoted in each experiment. Thus, the minimum pressure for Experiments 5 and 6, which aimed to incorporate the average martian surface pressure of 6 mbar, was initially slightly higher (~12–13 mbar). However, as the liquid continued to evaporate, the decrease in the height of the water column would also decrease the hydrostatic pressure. Although these pressures are slightly higher than desired, they are still comparable to pressures at the martian surface, specifically in the Hellas basin region (Spiga et al. 2007).

The evaporation of the liquid media [~2 mm/h at 7 mbar and 20 °C (Sears and Moore 2005)] constitutes the limiting factor to experiment length for experiments below 37 mbar (Expts. 4, 5, 6). This necessitates either the replenishment of liquid water throughout the duration of the experiment or the use of diffusion barriers to slow the rate of evaporation (as in Experiment 6). The use of five grams JSC Mars-1 in Experiment 6 did prolong the experiment by two days (compared to Experiment 5), though the pressure was slightly higher in Experiment 6 (7–20 mbar vs. 6–10 mbar). One option to reduce the rate of evaporation is to reduce the temperature of the chamber to 0 °C. Another option would be to use brines, such as magnesium sulfate ($MgSO_4$) or calcium chloride ($CaCl_2$), which can significantly reduce the rate of evaporation (Altheide et al. 2009; Sears and Chittenden 2005), although these would both introduce additional stressors to the methanogens.

In order to assess the effect of evaporation, the electrical conductivity, salinity and TDS of the remaining liquid media within the original cultures, as well as the transfer cultures, were

measured. Significant evaporation occurred in Experiments 4, 5, and 6, as expected, but sufficient liquid remained in Experiment 4 to measure both the original and transfer cultures. The twenty culture tubes in Experiment 4 experienced variable rates of evaporation, with greater evaporation occurring in the front row of test tubes. This discrepancy in evaporation may be due to the airflow through the chamber as a result of the fan within the palladium catalyst apparatus. The original cultures in Experiments 5 and 6 retained sufficient liquid media to perform transfers to new media, although electrical conductivity, salinity and TDS measurements were not possible in these tubes (Tables 2, 3 and 4). For Experiments 1–4, there was no clear trend or increase in electrical conductivity, salinity or TDS values between the original and transfer cultures, or between experiments. The electrical conductivity, salinity and TDS of uninoculated media were also measured for comparison. Values for original and transfer cultures within each experiment were typically greater than uninoculated media. In general, differences in values between original and transfer cultures within each experiment did not vary greatly (Expts. 1–4; Tables 2-4). The greatest differences occurred in cultures of *M. maripaludis*, which is expected due to the higher salt content of this medium.

The most significant evaporation occurred in Experiments 5 and 6 and insufficient liquid remained to analyze the resulting salinity of the media within the original cultures, although it is certain the salinities would increase. Electrical conductivity, salinity and TDS values for transfer cultures of Experiments 5 and 6 (where applicable) were typically higher than values for original and transfer cultures of Experiments 1–4, as well as values for uninoculated media. A better assessment of methanogen tolerance to brines, however, requires a much more in-depth assessment of electrical conductivity, salinity, and TDS values over time, in conjunction with methane production.

The creation of low-pressure "micro-environments" in Experiment 7 eliminated the risk of evaporation and allowed for a much longer exposure period (21 days), compared to the other six experiments (Table 1). Future experiments attempting to demonstrate survival at pressures lower than 50 mbar would require adequate temperature control to also maintain the vapor pressure of the liquid media.

The four methanogens tested in these experiments were chosen as the type strains of their species while also representing three (*Methanobacteriales*, *Methanococcales*, and *Methanosarcinales)* of the seven methanogenic orders. The aim was to address the possible stress responses to low pressure from a variety of methanogenic Archaea. However, methane production was relatively similar for both pre-exposure and post-exposure cultures for each of the four species. Previous studies in this lab have demonstrated the hardiness of *M. barkeri*, *M. formicicum*, and *M. wolfeii* when exposed to relatively harsh conditions, whereas *M. maripaludis* often displays lower tolerability (Kendrick and Kral 2006; Kral and Altheide 2013; Kral et al. 2011; Kral et al. 2004). Previously, Kral et al. (2011) investigated the effect of low pressure on active and desiccated cells. *M. barkeri*, *M. wolfeii*, and *M. formicicum* all produced methane at both 400 mbar and 50 mbar on JSC Mars-1, although methane production was reduced at 50 mbar compared to 400 mbar. In terms of desiccation at 1 bar, *M. barkeri* survived 330 days, *M. wolfeii* survived 180 days, *M. formicicum* survived 120 days and *M. maripaludis* did not survive at all. At 6 mbar, desiccated cells of *M. barkeri*, *M. wolfeii*, and *M. formicicum* survived 120 days desiccation, while *M. maripaludis* only survived for 60 days (Kral et al. 2011). The differences in survivability may be attributable to the differences in cell wall composition and morphology of the cells. *Methanosarcina* species are known for their large genomes with many redundant coding sequences (Anderson et al. 2012). These redundancies are believed to be responsible for the organisms' abilities to endure

a broader range of environments through both the ability to use multiple substrates for metabolism (H_2/CO_2, carbon monoxide, methanol, methyl compounds, acetate), as well as the formation of complex structures that could aid in protection. For example, *Methanosarcina* are the only methanogens that typically form multicellular aggregates embedded in an extracellular polysaccharide, which aids in protection against desiccation and oxygen exposure (Anderson et al. 2012). Additionally, *Methanosarcina* have thick (~0.18 μm) and rigid cell walls (Kandler and Hippe 1977). In contrast, the cell wall of *M. maripaludis* consists of a single electron dense layer (~10 nm) and the cell envelope is relatively fragile (Jones et al. 1983). The apparent higher sensitivity of *M. maripaludis* to low pressure as seen here (see Figs. 3, 4; Experiments 6, 7), and desiccation, as in previous studies (Kral and Altheide 2013; Kral et al. 2011), may be attributable to the relatively weaker cell wall, whereas *Methanosarcina* and *Methanobacterium* both contain thick, rigid cell walls composed of specific polymers (Kandler and König 1978). Overall, however, archaeal lipid membranes typically have higher rigidity and stability, as well as lower permeability to protons and higher salt tolerance, as compared to those of bacteria and eukarya, which promotes tolerance of harsher environments (van de Vossenberg et al. 1998).

Methanogens also contain a number of unique mechanisms for dealing with osmoadaptation and osmoregulation. Even in low-salt conditions, Archaea typically contain high concentrations of intracellular potassium ions (K^+). As such, many Archaea have evolved salt-tolerant enzymes that consist of mainly acidic amino acids, which gives an overall negative charge to the protein and prevents folding unless K^+ is available (Martin et al. 1999). Aside from the typical response of osmosis in order to counterbalance salt concentration, many methanogens also incorporate compatible solutes, or osmolytes, as a long-term adaptation technique. However, due to the normally high concentration of K^+ ions in the cell, potassium is an inadequate compatible solute and other solutes are typically used (Martin et al. 1999). There are two main ways that compatible solutes stabilize proteins. First, osmolytes tend to destabilize the unfolded protein compared to the folded structure, which keeps the protein intact. Also, osmolytes utilize differences in physical properties, such as the density of water, to maintain equilibrium at interface regions (Roberts 2004).

The lack of experiments studying the effects of low pressure on growth, metabolism and survival of organisms suggests that low pressure has not necessarily been deemed an important biocidal factor when considering life on other planets, specifically Mars. Few studies have very recently begun to address the issue of low pressure with the conclusions noting that pressure may have more of an affect on growth than has previously been believed (Fajardo-Cavazos et al. 2012; Nicholson et al. 2013; Schuerger et al. 2013). Schuerger et al. (2013) tested the ability of 26 strains of 22 bacterial species to grow under low pressure (7 mbar), low temperature (0 °C), and a CO_2-dominated anoxic atmosphere. Of these 26 strains, only *Serratia liquefaciens* ATCC 27592 exhibited obvious growth under these conditions. Although the synergistic effects of pressure, temperature, and anoxia may have contributed to the death of many of these species, Schuerger et al. (2013) also discovered that when looking at pressure separately, most species were inhibited at 25 mbar. In addition, of the six bacterial strains that grew at 25 mbar, all of them exhibited smaller colonies compared to those grown at 1013 mbar or 100 mbar (Schuerger et al. 2013). The inability for a number of strains to grow at low pressure, along with changes in colony morphology, signify the importance of studying the effects of low pressure as an inhibitor of growth and survival.

The recent and ongoing studies of low pressure focus on various bacterial strains commonly found on spacecraft or in clean rooms, in terms of planetary protection. However, the

synergistic effects of multiple potentially biocidal factors (low pressure, low temperature, CO_2 atmosphere) can overwhelm the organism, resulting in death (Schuerger et al. 2013). Methanogens are ideal candidates for life on Mars because they are anaerobic, non-photosynthetic, and do not require organic nutrients. These factors alone warrant further investigation into the survivability and growth of methanogens under martian conditions. In the work of Schuerger et al. (2013), 12 of the 26 bacterial strains tested were unable to grow in a CO_2 atmosphere at any pressure. The anaerobic nature of methanogens removes the CO_2 atmosphere of Mars as a potential biocidal factor considering the fact that many methanogens require CO_2 as a carbon source. Although this characteristic also makes methanogens unlikely to persist on spacecraft within clean rooms, if cells did remain on spacecraft prior to launch, survival following long-term desiccation of these microorganisms has already been shown (Kral and Altheide 2013; Kral et al. 2011). The ability of methanogens to remain viable following desiccation and actively grow under CO_2 atmospheres warrants further investigation in terms of planetary protection, mainly forward contamination, alone.

As stated previously (Kral and Altheide 2013; Kral et al. 2014; Kral et al. 2016), these experiments were not intended to mimic actual martian conditions, but rather, in this case, to study survival of methanogens exposed to pressures approaching those at the martian surface.

Future work will attempt in situ methane measurements within the Pegasus Planetary Simulation Chamber to determine if the methanogens are actively metabolizing under low pressure conditions. These experiments will make use of one or more of the options above (low temperature, regolith as diffusion barrier, brines, "micro-environments") in order to slow the evaporation rate of the liquid media and prolong the experiment.

Conclusions

Four species of methanogen (*M. barkeri*, *M. formicicum*, *M. wolfeii*, *M. maripaludis*) were tested for their ability to survive pressures approaching average martian surface pressures. Hydrated cells from all four methanogen species survived varying lengths of exposure (3 days – 21 days) to pressures between 6 mbar and 143 mbar. The limiting factor in most of the experiments was the evaporation of the liquid media. Future work will attempt to prolong experiment length (by decreasing the rate of evaporation) through the use of brines and analog regolith as diffusion barriers, and/or creating "micro-environments" as described.

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