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Methane Production on Mars-Relevant Clay Minerals and Simulant Regolith

Rebecca L. Mickol [1,2,*], W. Hunter Waddell [3], James Wray [3], Ryan Pohlkamp [3], Chandler Kern [3] and Timothy A. Kral [2,3,*]

[1] US Naval Research Laboratory, 4555 Overlook Ave SW, Washington, DC 20375
[2] Arkansas Center for Space and Planetary Science, University of Arkansas, 332 N. Arkansas Ave, Fayetteville, AR 72701
[3] Department of Biological Sciences, University of Arkansas, Science and Engineering Room 601, Fayetteville, AR 72701
* Correspondence: rebecca.l.mickol.civ@us.navy.mil (R.L.M.); tkral@uark.edu (T.A.K.)

Abstract

The presence of atmospheric carbon dioxide and potential subsurface molecular hydrogen (H_2), in addition to potential subsurface liquid water sources, suggest that the martian subsurface may currently be habitable, particularly to autotrophic chemosynthetic microorganisms. In addition, the widespread nature of clays and other minerals on Mars could provide sufficient nutrients to support microbial life. Here we tested four methanogenic species (*Methanosarcina barkeri*, *Methanobacterium formicicum*, *Methanothermobacter wolfeii*, and *Methanococcus maripaludis*) in the presence of illite, nontronite, and one martian regolith simulant, Mojave Mars Simulant (MMS), in their optimal growth medium. We aimed to determine whether the presence of certain clay minerals and regolith simulants inhibited, promoted, or had no effect on methane (CH_4) production by these microorganisms. We also tested the same methanogens in the presence of montmorillonite, H_2, sodium sulfide (Na_2S), and bicarbonate buffer to determine if this clay could support growth (as measured by CH_4 production). Results indicated that three of the four methanogens tested, *M. barkeri*, *M. formicicum*, and *M. wolfeii*, were capable of growth in the presence of both clay minerals and MMS, although most cultures demonstrated lower CH_4 production compared to growth in optimal media without clay minerals. Additionally, all three methanogens were capable of CH_4 production in cultures containing only 10% (w/v) montmorillonite, H_2, Na_2S, and bicarbonate buffer. Conversely, *M. maripaludis*, a halophile, showed the greatest sensitivity of the four methanogens tested, being unable to produce any CH_4 in cultures containing standard methanogenic growth medium and 2% (w/v) illite or in bicarbonate buffer containing H_2, Na_2S, and 10% (w/v) montmorillonite. However, in one experiment assessing the minimum medium requirements for this organism, the presence of 5% (w/v) montmorillonite enabled greater CH_4 production compared to cultures containing the organism's standard growth medium alone. Overall, these results suggest that the presence of clay minerals on Mars does not preclude the survivability and growth of methanogens in a potential subsurface habitat on the planet. In fact, these geological components may provide sufficient nutrients to support growth and survivability.

Keywords: Mars; methanogens; clay minerals; montmorillonite; nontronite; illite; habitability; astrobiology

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1. Introduction

Continued exploration of the martian surface, near-subsurface, and atmosphere have served to provide increasing amounts of evidence that Mars may once have been habitable, or may even still be habitable today, at least to microbial life. Reports of methane (CH_4) in the martian atmosphere [1–12] would seem to support this possibility. However, it should be noted that non-detections of CH_4

have also been reported [13–20]. Still, it is hard not to consider methanogenesis as a possible metabolism on early or extant Mars [21–37], considering carbon dioxide (CO_2) in the atmosphere, and likely, subsurface sources of molecular hydrogen (H_2) [24,25,36,38–44]. The further evidence of organic carbon [45–49] and the widespread nature of clays [50–52], such as nontronite [53–57] and montmorillonite [58–70], on the planet have bolstered the idea that Mars may once have hosted microbial life.

Nontronite and montmorillonite are iron (Fe)- and aluminum (Al)-containing, respectively, dioctahedral smectites, types of phyllosilicates, formed through interaction with liquid water [67,71]. On Earth, Al- and Fe-containing smectites are often created through the alteration of basalt in environments with high water/rock ratios and thus, are typically found in marine and lacustrine environments [67]. The presence of these clays on Mars is considered to have required a volume of water seven times larger than that currently held in the martian polar caps [72]. In addition, water adsorbed to these clays is considered to be an amount comparable to that of Earth, given Mars' smaller size [73]. The clays, and the water they may once have held or may still hold, is important to understanding the current and past habitability of Mars [67,74], considering that water is a necessity for all life on Earth as we know it [75–77].

On Earth, microorganisms and clay minerals are ubiquitous across the globe and interactions between the two affect both the biosphere and the geosphere. One particular aspect of clays that forms the basis of the experiments conducted here is the cycling of numerous elements, including Al, silicon (Si), magnesium (Mg), Fe, phosphorus (P), sulfur (S), carbon (C), and nitrogen (N), through interactions between clays and microorganisms [78,79]. With smectites, in particular, such as nontronite and montmorillonite, interlayers are occupied by cations, usually K^+, Na^+, Ca^{2+}, NH_4^+, or H_3O^+. The octahedral cation is typically Al^{3+}, Fe^{3+}, Mg^{2+}, or Fe^{2+}, but can also be less frequent elements such as lithium (Li), manganese (Mn), zinc (Zn), chromium (Cr), or titanium (Ti) [67]. In these clays, high Fe content can create unique redox conditions that microorganisms, including methanogens, can utilize as electron acceptors (*e.g.*, Fe^{3+}, see Fisk and Giovannoni [75], Zhang *et al.* [80], Zhang *et al.* [81]). In addition, the changing oxidation state of Fe in the soil can increase the bioavailability of other nutrients such as K^+, Ca^{2+}, Cu^{2+}, Zn^{2+}, and NH_4^+ [78]. The dissolution of smectites is typically controlled by bacterial metabolism under acidic conditions [78], which could be relevant to certain environments on Mars [67,82–86]. This dissolution releases Fe, Al, and Si, as well as Na^+ and Ca^{2+}, which can serve as nutrients and promote microbial absorption to the clay minerals themselves [78].

Illites have also been detected on Mars [63,87] and illitization (*i.e.*, the transition of smectite to illite) can be facilitated through microbial reduction of Fe^{3+} to Fe^{2+} [67,88], although this process is reversible (see Li *et al.* [78] for a comprehensive review of microbe-clay interactions). Illitization can also occur abiotically, typically through diagenesis (the conversion of sediment to sedimentary rock), which is dependent on both time and depth (*i.e.*, burial) [87,89]. On Mars, the dominance of basalt suggests that illite was formed either through diagenesis or hydrothermal activity; however, illite is less widespread or common than either montmorillonite or nontronite, and cannot be distinguished from muscovite, another potassium mica, through spectral data alone [63,87]. In contrast to smectites, illites have non-expandable basal spacing, a property which affects swelling capacity (for example, the amount of water in the interlayer), as well as molecule adsorption, including CH_4 [78,90]. The basal spacing of illites is ca. 10 Å and lower, which could correspond to martian data indicating similar basal spacing [88], although most interpretations believe basal spacings of 10 Å and lower to be due to collapsed smectites (due to dehydration) [54,91–93]. Ultimately, the widespread presence of these clays on Mars indicates significant aqueous alteration and serves to promote the habitability of clay-rich environments on the planet [50–52,56,57,59,60,62,63,65–69].

The multiple detections of CH_4 in the martian atmosphere from Earth-based telescopes [4,11,12], martian orbiters [5–9], and martian landers [1–3,10] all provide convincing evidence for extinct or extant methanogenesis on the planet. On Earth, over 80% of atmospheric CH_4 results from the biosphere [94], including CH_4 produced by the metabolism of microorganisms from the domain Archaea, known as methanogens. Certain methanogens are capable of using carbon dioxide (CO_2) as a carbon source and H_2 as an energy source to produce CH_4 [95]. Methanogens have been considered model microorganisms for past or present life on Mars for over 20 years [22,24,96–99]. While today there are many (potentially) biocidal or inhibitory factors that would affect the ability of life to survive and thrive on the surface of the planet, such as radiation [100,101] or the lack of liquid water [102], among others [103–105], the non-photosynthetic nature of methanogens enables them to potentially persist in a subsurface habitat [23–25,27,28,32,35,37,95,106–113]. Additionally, their ability to use inorganic carbon (*i.e.*, CO_2) for their metabolism removes the constraint that the presence of other forms of life is necessary for their survival.

Research over the past 22 years has focused on various environmental and/or physiological conditions that any extant methanogens on Mars may endure, such as desiccation [99], clays and martian regolith simulants [98,114–117], perchlorates [118–120], carbonates [121,122], radiation [123,124], low temperatures [96,125,126], low pressure [108,109,113], high pressure [110,111], or combinations of these conditions [97,127–133]. While many of these factors have been tested individually, overall results indicate that these factors may not prohibit the past or present existence of microbial life on Mars [134], and that particular niches with increased habitability, such as the subsurface [23–25,27,28,32,37,44,75,106,107,112,113,135–141], may provide sufficient conditions for microbial life to develop and perhaps, still exist, on the planet.

Here we tested the ability of four methanogenic species to grow in the presence of two common clays found on Mars, nontronite and illite, and the martian regolith simulant, Mojave Mars Simulant (MMS) [142], in their respective growth media. For simplicity, henceforth, the clays and MMS will be referred to as Mars simulants. We aimed to determine if any of these Mars simulants were inhibitory, had no effect, or were possibly stimulatory to growth, as measured by CH_4 production. Previous research has determined that these same methanogens are capable of growth in the presence of the martian regolith simulant, JSC Mars-1 [98,115,116], the igneous rock, basalt [117], and the clay, montmorillonite [115,116].

Another major goal of these experiments was to determine if these same methanogens could derive their nutrient requirements (other than water, CO_2, H_2, and Na_2S) from montmorillonite. Both Chastain and Kral [116] and Sinha and Kral [115] previously determined that montmorillonite could support the growth of *M. barkeri*, *M. formicicum*, and *M. wolfeii* when cells were washed to remove residual growth medium prior to inoculation. However, these experiments included only a single washing of cells, resulting in concern that carry-over of residual nutrients from the original growth media might have allowed for growth of the methanogens. Here we report on CH_4 production following two additional transfers in order to rule out this possibility. Lastly, an experiment was performed assessing the growth medium for the halophile, *M. maripaludis*, and whether montmorillonite could provide sufficient nutrients in place of certain medium components.

2. Materials and Methods

2.1. Cultures and Growth Media

Methanogens were initially obtained from the Oregon Collection of Methanogens (OCM), Portland State University, Oregon, USA or the American Type Culture Collection (ATCC). Each methanogen was grown in its own anaerobic medium and at its own growth temperature: *Methanosarcina barkeri* (OCM 38, ATCC 43569), 37 °C, MS medium (yeast extract, trypticase peptone, mercaptoethanesulfonic acid, potassium phosphate, ammonium chloride, magnesium chloride, calcium chloride, and additional trace minerals) [99,143]; *Methanobacterium formicicum* (OCM 55, ATCC 33274), 37 °C, MS medium supplemented with sodium formate (designated MSF medium) [143]; *Methanothermobacter wolfeii* (OCM 36, ATCC 43096), 55 °C, MM medium (a minimal medium containing the same components as MS medium except yeast extract, trypticase peptone, and mercaptoethanesulfonic acid) [99,144]; and *Methanococcus maripaludis* (OCM 151, ATCC 43000), 22 °C, MSH medium (MS medium containing additional sodium chloride, magnesium chloride, and potassium chloride) [145]. All media were created in bicarbonate buffer (4 g/L sodium hydroxide (NaOH) saturated with CO_2). These media and temperatures were used for growth of the organisms and are not meant to mimic available nutrients or environmental conditions on Mars. Growth was measured by increases in CH_4 production over time via gas chromatography (CP-4900, Varian Micro Gas Chromatograph). Typically, CH_4 concentrations of 0.1% or higher increasing with time [116] were considered to be an indication of growth.

2.2. Mars Simulants

Three clays were used in the experiments described here that are akin to those commonly identified on the surface of Mars. Nontronite, an Fe-smectite (NAu-1, Uley Mine, South Australia; Keeling *et al.* [146], Gates *et al.* [147]), and illite, an Al-phyllosilicate (IMt-1/2, Silver Hill, Montana, USA), were obtained from the Clay Minerals Society Source Clays Repository. Montmorillonite, an Al-smectite, was obtained from Ward's Natural Science (Clay Spur, Wyoming, USA). Mojave Mars Simulant (MMS) [142] is a basaltic martian simulant regolith selected for the similarity of its chemical and physical characteristics to those measured by various martian rovers. General chemical compositions for the Mars simulants are given in Table 1.

2.3. Experimental Procedures

2.3.1. Methane Production in the Presence of Illite, Nontronite, or Mojave Mars Simulant in Standard Growth Media

Growth experiments in the presence of illite were performed with the addition of either 1% (w/v) or 2% (w/v) illite clay to each methanogens' respective anaerobic growth medium (see *2.1 Cultures and growth media*). More specifically, growth media were prepared in flasks and transferred to a Coy anaerobic chamber (90:10 CO_2:H_2; Coy Laboratory Products Inc., Grass Lake Charter Township, MI) to deoxygenate for 24-36 hours. Either 0.1 g illite [1% (w/v)] or 0.2 g illite [2% (w/v)] were added to each of five Balch tubes, after which 10 mL growth medium were distributed to each tube for each organism (n = 3-5; Table 2). The tubes were capped with butyl rubber stoppers and aluminum crimps and autoclaved (121 °C, 15 psi, 30 min). Afterward, ca. 0.125 mL 2.5% Na_2S were added to each tube to remove residual oxygen. Each tube was inoculated with 0.5 mL of culture containing the respective methanogen, pressurized with 2 bar H_2 gas, and kept at the organisms' standard growth temperature. Tubes were monitored for CH_4 production (measured via gas chromatography) over 29-31 days. Positive control tubes containing solely 10 mL growth medium without illite (n = 3-5) were also monitored for CH_4 production over time (Table 2).

Experimental procedures for the nontronite and MMS experiments were identical to those for the illite experiments, except for the amount of nontronite or MMS and medium in each tube. For the nontronite experiments, three sets of tubes were inoculated: 0 g nontronite in 10 mL medium (n = 3; positive control tubes), 1 g nontronite in 9 mL medium (11.1% (w/v), n = 3) or 2.5 g nontronite in 7.5 mL medium (33.3% (w/v), n = 3). Methane production was monitored via gas chromatography over 35 days (Table 2). For the MMS experiments, tubes contained 10 g MMS and 10 mL medium (100% (w/v), n = 3). Positive control tubes contained solely 10 mL medium (no MMS, n = 2). Tubes were monitored for methane production for 140 days (Table 2).

Table 1. Mars simulant physical and chemical data.

Clay	Nontronite[1,2]	Illite[1,2]	Montmorillonite[1,2,3]	Mojave Mars Simulant[4]
Type	Iron smectite	Aluminum phyllosilicate	Aluminum smectite	Martian regolith simulant
Identifier	NAu-1	IMt-1/2	SWy-1/2/3	MMS
Origin	South Australia	Silver Hill, Montana, United States	Wyoming, United States	Mojave Desert, California, United States
SiO_2	53.33	49.3	62.9	49.4
Fe_2O_3	34.19	7.32	3.35	10.87
Al_2O_3	10.22	24.25	19.6	17.1

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CaO	3.47	0.43	1.68	10.45
MgO	0.27	2.56	3.05	6.08
Na_2O	0.08	0	1.53	3.28
K_2O	0.03	7.83	0.53	0.48
TiO_2		0.55	0.090	1.09
P_2O_5		0.08	0.049	0.17
MnO		0.03	0.006	0.17
FeO		0.55	0.32	
F			0.111	
S			0.05	
SO_3				0.10
Cr_2O_3				0.05

[1] Data from van Olphen and Fripiar [148] and available from clays.org/sourceclays_data/. [2] Data is unofficial and is meant to be used as a guideline and not as an analytical certification. [3] Chemical composition not available from Ward's Natural Science. Example chemical components from montmorillonite sourced globally given from [1] and [2]. [4] General chemical composition from Peters *et al.* [142].

Table 2. Experimental conditions and maximum methane concentrations produced by *Methanobacterium formicicum*, *Methanosarcina barkeri*, *Methanothermobacter wolfeii*, and *Methanococcus maripaludis* grown in the presence of Mars simulants in standard methanogenic growth media.

Experiment	Organism	Clay Concentration (% w/v)	Clay Concentration (g added / mL culture)	Number of Replicates	Total Length of Incubation (days)	Length of Incubation to Maximum Methane Concentration (days)	Maximum Methane Concentration (% headspace)
Illite	*M. formicicum*	0	0 / 10	4	31	31	7.4 ± 0.5
		1	0.1 / 10	4		16	24.6 ± 5.2
		2	0.2 / 10	3			26.7 ± 2.6
Illite	*M. barkeri*	0	0 / 10	5	31	31	1.2 ± 0.3
		1	0.1 / 10	4			1.1 ± 0.3
		2	0.2 / 10	5			1.4 ± 0.5
Illite	*M. wolfeii*	0	0 / 10	4	31	24	22.5 ± 1.0
		1	0.1 / 10	3	29	29	29.6 ± 2.8
		2	0.2 / 10	3	31	16	24.9 ± 0.6
Illite	*M. maripaludis*	0	0 / 10	4	31	31	12.7 ± 3.2
		1	0.1 / 10	5			13.6 ± 6.0
		2	0.2 / 10	4			0
Nontronite	*M. formicicum*	0	0 / 10	3	35	35	44.3 ± 3.4
		11.1	1 / 9				17.9 ± 15.3
		33.3	2.5 / 7.5				2.4 ± 1.9
Nontronite	*M. barkeri*	0	0 / 10	3	35	35	16.1 ± 3.9
		11.1	1 / 9				8.8 ± 7.4
		33.3	2.5 / 7.5				13.3 ± 14.7
Nontronite	*M. wolfeii*	0	0 / 10	3	35	35	41.0 ± 3.0
		11.1	1 / 9				23.0 ± 18.1
		33.3	2.5 / 7.5				12.1 ± 19.4
Nontronite	*M. maripaludis*	0	0 / 10	3	35	35	42.3 ± 6.6
		11.1	1 / 9				26.3 ± 14.8
		33.3	2.5 / 7.5				28.5 ± 7.2
Mojave Mars Simulant	*M. formicicum*	0	0 / 10	2	140	52	29.9 ± 0.7
		100	10 / 10	3			25.9 ± 1.5
Mojave Mars Simulant	*M. barkeri*	0	0 / 10	2	140	52	4.8 ± 1.6
		100	10 / 10	3			15.1 ± 1.0
Mojave Mars Simulant	*M. wolfeii*	0	0 / 10	2	140	9	26.8 ± 2.1
		100	10 / 10	3		52	20.9 ± 1.7
Mojave Mars Simulant	*M. maripaludis*	0	0 / 10	2	140	24	28.6 ± 0.2
		100	10 / 10	3			8.4 ± 10.4

2.3.2. Methane Production with Montmorillonite as the Sole Nutrient Source

Experimental procedures for the montmorillonite experiments were conducted to remove any residual growth media from the inoculated tubes, forcing the methanogens to obtain any necessary nutrients from the clay mineral itself. Cells grown in their respective anaerobic growth media were centrifuged at 5000 rpm for 15 minutes, the supernatant was discarded, and the pellet was re-suspended in sterile bicarbonate buffer. This wash procedure was repeated two more times to rid the cells of any nutrients contained in their original respective growth medium [149]. Next, 0.5 mL aliquots of washed cells were added to tubes containing sterile montmorillonite (1 g) and 10 mL bicarbonate buffer (10% (w/v), n = 3 for each organism), then pressurized with 2 bar H_2 gas. The tubes were monitored for CH_4 production over time for six weeks. In order to ensure that no residual nutrients remained in the experimental tubes following inoculation, two transfer sets were also created: at six week intervals, a new set of tubes containing 1 g montmorillonite and 10 mL bicarbonate buffer were inoculated with 0.5 mL culture (*i.e.*, cells in buffer with 1 g montmorillonite) from the corresponding tube in the previous set. This resulted in three transfers over 18 weeks. For clarity, Set A was inoculated with 0.5 mL washed cells and CH_4 was measured for six weeks. At six weeks, 0.5 mL culture from Tube 1 from Set A were used to inoculate Tube 1 from Set B. Both Sets A and B were monitored for CH_4 production for an additional six weeks. At six weeks from the time that Set B was inoculated, 0.5 mL culture from Tube 1 from Set B were used to inoculate Tube 1 from Set C and Sets A, B, and C were monitored for CH_4 production for an additional 16 weeks (Table 3).

Table 3. Experimental conditions and maximum methane concentrations produced by *Methanobacterium formicicum*, *Methanosarcina barkeri*, *Methanothermobacter wolfeii*, and *Methanococcus maripaludis* grown in bicarbonate buffer containing molecular hydrogen, sodium sulfide, and montmorillonite.

Experiment	Organism	Clay Concentration (% w/v)	Clay Concentration (g added / mL buffer)	Number of Replicates	Total Length of Incubation (days)	Length of Incubation to Maximum Methane Concentration (days)	Maximum Methane Concentration (% headspace)
Montmorillonite	*M. formicicum*	10 (Set A)	1 / 10	3	198	42	14.8 ± 4.0
		10 (Set B)			156	156	39.1 ± 10.1
		10 (Set C)			114	114	11.7 ± 11.7
Montmorillonite	*M. barkeri*	10 (Set A)	1 / 10	3	198	84	7.8 ± 1.0
		10 (Set B)			156	156	18.5 ± 8.6
		10 (Set C)			114	114	19.0 ± 6.0
Montmorillonite	*M. wolfeii*	10 (Set A)	1 / 10	3	198	84	23.5 ± 19.4
		10 (Set B)			156	156	49.3 ± 25.3
		10 (Set C)			114	114	49.3 ± 4.4
Montmorillonite	*M. maripaludis*	10 (Set A)	1 / 10	3	198	198	0
		10 (Set B)			156	156	0
		10 (Set C)			114	114	0

2.3.3. Minimal Medium Requirements for *Methanococcus maripaludis*

Following from the inability of *M. maripaludis* to grow in cultures containing montmorillonite as the sole nutrient source (Table 3), a separate experiment was conducted to determine the minimal medium requirements for this organism and whether montmorillonite could serve as a replacement for any necessary medium components. Thus, seven variations of MSH medium with montmorillonite (Table 4) were tested to determine which medium components are vital to the growth of *M. maripaludis* and whether montmorillonite could provide certain necessary nutrients. Experimental procedures were identical to those performed for the illite, nontronite, and MMS experiments, except that tubes contained 0.5 g montmorillonite and 10 mL of each medium variation (5% (w/v), n = 3, Table 4). Three tubes used as positive controls contained MSH medium and no montmorillonite. All tubes were incubated at room temperature (ca. 22 °C) for three weeks and periodically monitored for CH_4 production via gas chromatography.

Table 4. Components for eight variations of MSH medium with and without montmorillonite.

Per 100 mL	Control (MSH medium)	Mont^1 +Salt	MSH+Mont-SolnA	MSH+Mont-SolnB	MSH+Mont-SolnD	MSH+Mont-Salt	Mont+Buffer^2 Only	Mont+MSH
0.5 g montmorillonite (per 10 mL)	--	X	X	X	X	X	X	X
Solution A^3, 500 µL	X	--	--	X	X	X	--	X
Solution B^4, 100 µL	X	--	X	--	X	X	--	X
Solution C^5, 100 µL	X	--	X	X	X	X	--	X
Solution D^6, 50 µL	X	--	X	X	--	X	--	X
0.1 g yeast extract	X	--	X	X	X	X	--	X
0.1 g trypticase peptone	X	--	X	X	X	X	--	X
0.025 g mercaptoethane sulfonic acid	X	--	X	X	X	X	--	X
1.475 g NaCl	X	X	X	X	X	--	--	X
0.085 g $MgCl_2$	X	X	X	X	X	--	--	X
0.025 g KCl	X	X	X	X	X	--	--	X
Buffer^5	X	X	X	X	X	X	X	X

^{1}Mont = montmorillonite. ^{2}Bicarbonate buffer: 4 g/L NaOH saturated with CO_2. ^{3}Solution A, per L: 100 g NH_4Cl, 100 g $MgCl_2 \cdot 6H_2O$, 40 g $CaCl_2 \cdot 2H_2O$. ^{4}Solution B, per L: 200 g $K_2PO_4 \cdot 3H_2O$. ^{5}Solution C, per L: 0.5 g resazurin. ^{6}Solution D, per L: 500 mg Na_2-EDTA$\cdot 2H_2O$, 150 mg $CoCl_2 \cdot 6H_2O$, 100 mg $MnCl_2 \cdot 4H_2O$, 100 mg $FeSO_4 \cdot 7H_2O$, 100 mg $ZnCl_2$, 40 mg $AlCl_3 \cdot 6H_2O$, 30 mg $Na_2WO_4 \cdot 2H_2O$, 20 mg $CuCl_2 \cdot 2H_2O$, 20 mg $NiSO_4 \cdot 6H_2O$, 10 mg H_2SeO_3, 10 mg H_3BO_3, 10 mg $Na_2MoO_4 \cdot 2H_2O$.

3. Results

3.1. Methane Production in the Presence of Illite, Nontronite, and Mojave Mars Simulant

When grown in the presence of 1% (w/v) or 2% (w/v) illite, both *M. wolfeii* and *M. formicicum* showed enhanced growth (higher CH_4 production) in the presence of the clay mineral than in their standard anaerobic growth medium (Figure 1A,C, Table 2). Methane concentrations reached a maximum of 29.6 ± 2.8 % headspace after 29 days for *M. wolfeii* grown with 1% (w/v) illite and a maximum of 24.9 ± 0.6 % headspace after 16 days with 2% (w/v) illite. *M. wolfeii* cultures grown in their optimum growth medium (MM medium) without clay minerals reached a maximum CH_4 concentration of 22.5 ± 1.0 % headspace after 24 days' incubation. *M. formicicum* cultures reached a maximum CH_4 concentration of 24.6 ±5.2 % headspace after 16 days in the presence of 1% (w/v) illite and 26.7 ± 2.6 % headspace after 16 days in the presence of 2% (w/v) illite. In comparison, cultures of *M. formicicum* grown in MSF medium without clay minerals reached a CH_4 concentration of 7.4 ± 0.5 % headspace after 31 days' incubation. For *M. barkeri*, CH_4 production was not affected by the presence of illite with maximum CH_4 concentrations ca. 1 % headspace after 31 days' incubation amongst all conditions (Figure 1D, Table 2). When grown with 1% (w/v) illite, *M. maripaludis* showed CH_4 production similar to the control tubes (ca. 13 % headspace); however 2% (w/v) illite proved inhibitory as no CH_4 production occurred in these cultures (Figure 1B, Table 2).

In the nontronite experiments, all four methanogens were capable of CH_4 production in cultures containing up to 2.5 g nontronite [33.3% (w/v)], although CH_4 production was greatly delayed and reduced for *M. formicicum* at this concentration (Figure 2C). After 35 days, CH_4 concentrations reached 2.4 ± 2.9 % headspace for *M. formicicum* grown in the presence of 2.5 g nontronite whereas cultures grown in MSF medium without clay minerals reached a maximum concentration of 44.3 ± 3.4 % headspace CH_4 (Figure 2C, Table 2). Similar to *M. formicicum*, CH_4 production decreased for cultures of *M. wolfeii* with increasing nontronite concentration: CH_4 reached concentrations of 41.0 ± 3.0 % headspace for cultures without nontronite, 23.0 ± 18.1% headspace for cultures grown with 1 g nontronite, and 12.1 ± 19.4 % headspace for cultures grown with 2.5 g nontronite (Figure 2A, Table 2). For *M. maripaludis*, CH_4 production was similar between experimental tubes containing either 1 g or 2.5 g nontronite (ca. 26-28 % headspace), but was higher for cultures grown without clay minerals

(42.3 ± 6.6 % headspace; Figure 2B, Table 2). Methane production was also highest for *M. barkeri* in cultures without clay minerals (16.1 ± 3.9 % headspace), but average CH_4 concentration was higher in cultures grown with 2.5 g nontronite (13.3 ± 14.7 % headspace) than with 1 g nontronite (8.8 ± 7.4 % headspace; Figure 2D, Table 2). Notably, CH_4 production amongst replicates for the same organism and same conditions varied greatly, resulting in large error bars (Figure 2).

For *M. wolfeii* and *M. formicicum,* growth was similar in both the presence and absence of MMS, but with slightly higher CH_4 production in tubes without MMS (Figure 3A,C, Table 2). For *M. wolfeii*, CH_4 concentration peaked at 26.8 ± 2.1 % headspace after 9 days' incubation at 55 °C in cultures without MMS, while CH_4 concentration reached 20.9 ± 1.7 % headspace in cultures containing MMS after 52 days' incubation (Figure 3A, Table 2). Maximum CH_4 concentrations for *M. formicicum* were higher in cultures without MMS (reaching 29.9 ± 0.7 % headspace after 52 days' incubation at 37 °C), while cultures containing MMS reached 25.9 ± 1.5 % headspace after 52 days' incubation (Figure 3C, Table 2). *M. maripaludis* was the only organism where the presence of MMS significantly hindered CH_4 production by the organism (compared to growth in standard medium). Methane production peaked at 8.4 ± 10.4 % headspace after 24 days' incubation at 22 °C for *M. maripaludis* cultures containing MMS, but CH_4 concentrations between the three biological replicates were considerably different (20.3 % vs. 4.2 % vs. 0.8 % headspace). In cultures without MMS, *M. maripaludis* produced 28.6 ± 0.2 % headspace CH_4 after 24 days' incubation (Figure 3B, Table 2). In contrast to the other three organisms, *M. barkeri* was the only methanogen that produced greater amounts of CH_4 in the presence of MMS than in the absence of MMS. Methane production by *M. barkeri* peaked at 15.1 ± 1.0 % headspace in cultures containing MMS after 52 days' incubation, whereas cultures without MMS reached only 4.8 ± 1.6 % headspace CH_4 in the same amount of time (Figure 3D, Table 2).

3.2. Methane Production with Montmorillonite as the Sole Nutrient Source

In the montmorillonite experiments, each of the four methanogens was grown in bicarbonate buffer containing H_2, Na_2S, and 10% (w/v) montmorillonite (n = 3; Table 3). After six weeks, 0.5 mL culture was used to inoculate another tube containing 10% (w/v) montmorillonite in bicarbonate buffer. In addition, all tubes (from all sets) were pressurized with 2 bar H_2 at 0, 42, 84, and 144 days. This pressurization at 42 days likely results in the decrease in CH_4 as a percentage of the headspace (seen in Figure 4A–C). Data indicate that each of three methanogens (*M. wolfeii*, *M. formicicum*, and *M. barkeri*) were capable of CH_4 production in tubes containing solely bicarbonate buffer, montmorillonite, Na_2S, and H_2 gas (Figure 4, Table 3). In contrast, *M. maripaludis* was incapable of any CH_4 production (data not shown). For *M. wolfeii* and *M. barkeri*, cultures in Set A reached maximum CH_4 concentrations after 84 days' incubation (23.5 ± 19.4 % headspace and 7.8 ± 1.0 % headspace, respectively; Figure 4A,C, Table 3). *M. formicicum* cultures in Set A reached a maximum CH_4 concentration of 14.8 ± 4.0 % headspace after 42 days' incubation (Figure 4B, Table 3). For Set B cultures, CH_4 production reached higher concentrations than Set A cultures for each methanogen after 156 days' incubation: 49.3 ± 24.3 % headspace (*M. wolfeii*), 18.5 ± 8.6 % headspace (*M. barkeri*), and 39.1 ± 10.1 % headspace (*M. formicicum*; Figure 4, Table 3). Methane concentrations were only measured once after 114 days' incubation for Set C cultures. After 114 days' incubation, CH_4 concentrations for *M. wolfeii* and *M. barkeri* for Set C were similar to the concentrations reached for Set B (49.3 ± 4.4 % headspace and 19.0 ± 6.0 % headspace, respectively; Figure 4A,C, Table 3). Set C cultures for *M. formicicum* measured 11.7 ± 11.7 % headspace after 114 days' incubation (Figure 4B, Table 3).

3.3. Minimal Medium Requirements for Methanococcus maripaludis

The highest CH_4 concentration by *M. maripaludis* was achieved after 18 days' incubation at 22 °C (23.9 ± 14.8 % headspace) in cultures containing MSH medium with 0.5 g montmorillonite but without Solution B (200 g/L $K_2PO_4 \cdot 3H_2O$; Table 4, Figure 5). Cultures containing MSH medium and 0.5 g montmorillonite, but without Solution D (trace minerals; Table 4) achieved an average maximum CH_4 concentration of 19.5 ± 8.3 % headspace, also after 18 days' incubation (Figure 5). Methane production in MSH medium alone (control samples) also peaked at 18 days' incubation, reaching 9.4 ± 5.2 % headspace. Methane production in the cultures containing MSH with 0.5 g montmorillonite without Solution A or solely 0.5 g montmorillonite and the salt solution (per L, 1.475 g NaCl, 0.085 g $MgCl_2$, and 0.025 g KCl; Table 4) reached similar CH_4 concentrations near 3-4 % headspace after 18-22 days' incubation (Figure 5). Cultures grown in solutions consisting of MSH medium with 0.5 g montmorillonite but without the salt solution and solely bicarbonate buffer with 0.5 g montmorillonite failed to produce any CH_4 (data not shown).

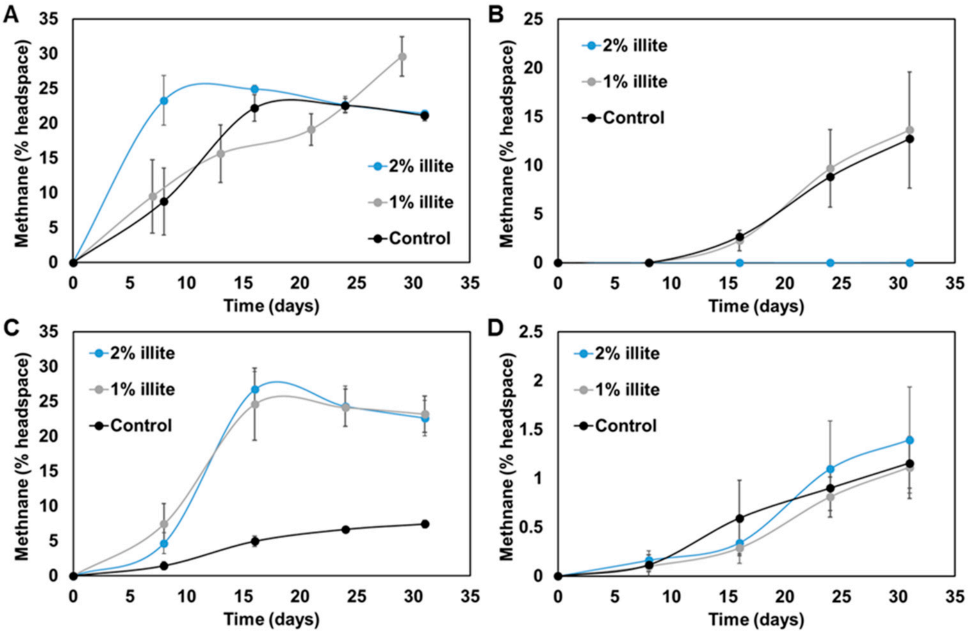

Figure 1. Methane production (% headspace) over time for four methanogens grown in their respective anaerobic growth medium with either 0% (control), 1% (w/v), or 2% (w/v) illite clay: **A)** *Methanothermobacter wolfeii* (55 °C, MM medium); **B)** *Methanococcus maripaludis* (22 °C, MSH medium); **C)** *Methanobacterium formicicum* (37°C, MSF medium); and **D)** *Methanosarcina barkeri* (37 °C, MS medium). Data are the average of 3-5 replicates (Table 2). Error bars represent ± one standard deviation.

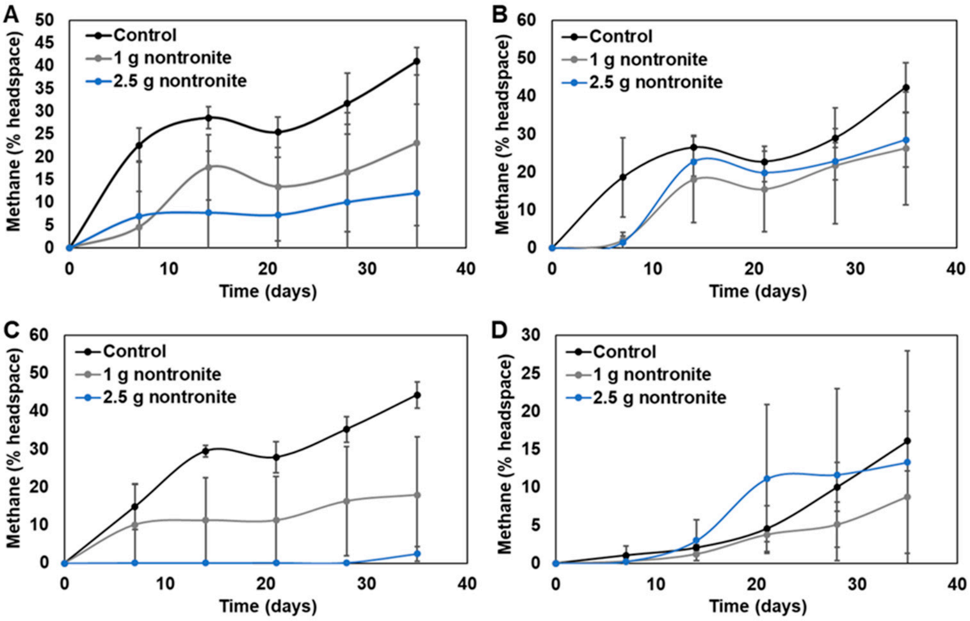

Figure 2. Methane production (% headspace) over time for four methanogens grown in their respective anaerobic growth medium with either 1 g (in 9 mL medium) or 2.5 g (in 7.5 mL medium) nontronite clay: **A)**

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Methanothermobacter wolfeii (55 °C, MM medium); **B**) *Methanococcus maripaludis* (22 °C, MSH medium); **C**) *Methanobacterium formicicum* (37°C, MSF medium); and **D**) *Methanosarcina barkeri* (37 °C, MS medium). Control tubes contain only 10 mL medium. Data are the average of three biological replicates (Table 2). Error bars represent ± one standard deviation.

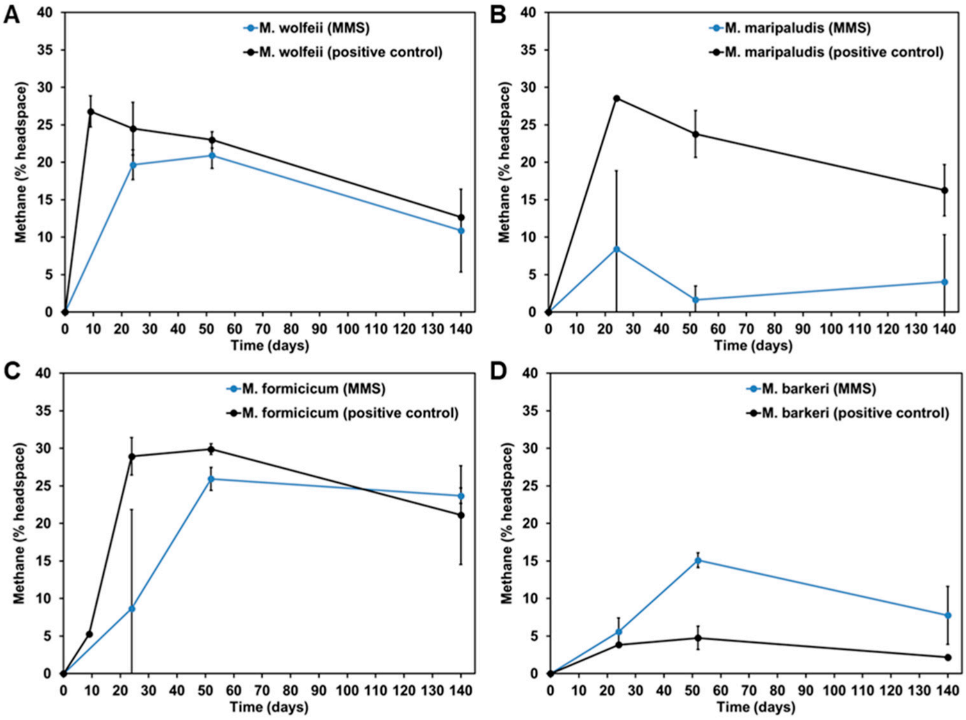

Figure 3. Methane production (% headspace) over time for four methanogens grown in their respective anaerobic growth medium with either 0% (positive control) or 100% (w/v) [10 g/10 mL] Mojave Mars Simulant (MMS) martian regolith simulant: **A**) *Methanothermobacter wolfeii* (55 °C, MM medium); **B**) *Methanococcus maripaludis* (22 °C, MSH medium); **C**) *Methanobacterium formicicum* (37°C, MSF medium); and **D**) *Methanosarcina barkeri* (37 °C, MS medium). Data are the average of either two (positive controls) or three (experimental tubes) biological replicates (Table 2). Error bars represent ± one standard deviation.

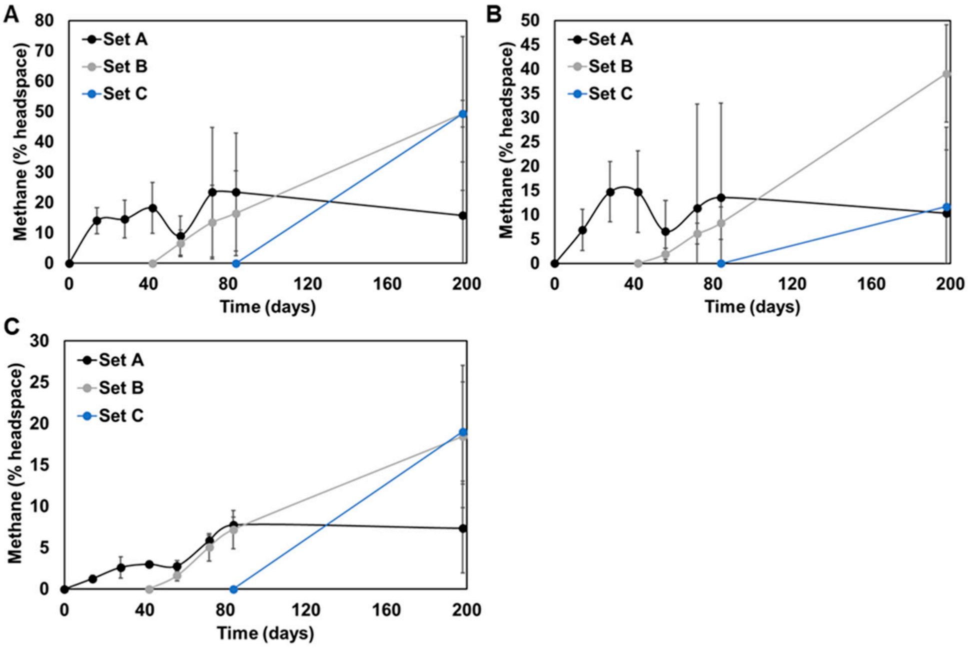

Figure 4. Methane production (% headspace) over time for three methanogens grown in 10 mL bicarbonate buffer with 1 g montmorillonite clay [10% (w/v)] and subject to two subsequent transfers to new tubes containing

1 g montmorillonite clay and 10 mL bicarbonate buffer: **A**) *Methanothermobacter wolfeii* (55 °C); **B**) *Methanobacterium formicicum* (37 °C); and **C**) *Methanosarcina barkeri* (37 °C). Initial inocula consisted of 0.5 mL washed cells (Set A). Each tube in the subsequent sets (Set B, Set C) was inoculated with 0.5 mL culture from the corresponding tube in the preceding set (*i.e.*, Tube B1 was inoculated with 0.5 mL culture from Tube A1). All tubes were pressurized with 2 bar H_2 on days 0, 42, 84, and 144. Data are the average of three biological replicates (Table 3). Error bars represent ± one standard deviation.

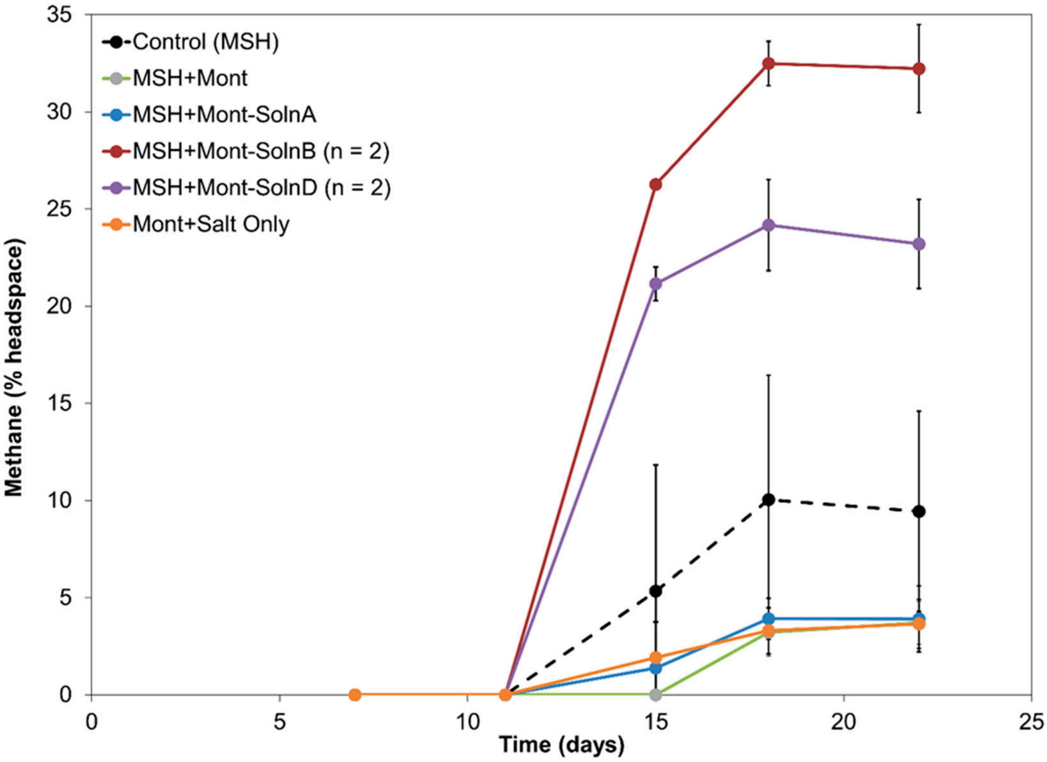

Figure 5. Methane production (% headspace) over time for *Methanococcus maripaludis* grown in eight variations of MSH medium at room temperature (ca. 22 °C). Conditions labeled "Mont" contain 0.5 g montmorillonite. Components of Solutions A, B, and D (SolnA, SolnB, SolnD, respectively) and the salt solution ("Salt") are given in Table 4. Data are not shown for conditions under which no methane was produced by any replicates (MSH+Mont-Salt [MSH medium containing 0.5 g montmorillonite and no salt solution] and Buffer+Mont [bicarbonate buffer containing 0.5 g montmorillonite only]). Data are the average of three biological replicates, unless otherwise indicated. Error bars represent ± one standard deviation.

4. Discussion

Here we tested the ability of four methanogens, often used to represent potential model organisms for life on Mars, to grow in the presence of two clay minerals (illite or nontronite) and one martian simulant regolith (MMS) in their standard growth media. The aim was to determine if the presence of the Mars simulant would inhibit, enhance, or have no effect on CH_4 production. Additionally, we tested the ability of these four methanogens to produce CH_4 using nutrients available solely within the clay mineral montmorillonite, without the addition of salts or trace minerals to the bicarbonate buffer. The goal of this experiment was to determine if a Mars simulant contains the necessary nutrients to support methanogen growth given the availability of H_2 and CO_2 as energy and carbon sources, respectively. Overall, the results shown here indicate that each of four methanogens was capable of growth in the presence of multiple Mars simulants, albeit in their standard methanogenic growth medium. When solely provided with H_2, CO_2 (in the form of bicarbonate buffer), Na_2S, and montmorillonite, only three of the four methanogens (*M. formicicum, M. barkeri, M. wolfeii*) were capable of growth. In all, aside from a few select cases (*i.e.*, *M. maripaludis* grown in the presence of 2% (w/v) illite or *M. maripaludis* grown in bicarbonate buffer containing 10%

(w/v) montmorillonite), none of the Mars simulants proved completely biocidal to the methanogens tested, with enhanced CH_4 production seen in certain instances. While CH_4 production varied amongst the four species and the Mars simulants tested, variation in CH_4 concentrations is not unusual, even amongst individual replicates [125,126]. The ability of these methanogens to metabolize in the presence of these Mars simulants adds further support to the possibility that methanogens may have existed or may still exist in a martian subsurface environment.

M. maripaludis was the methanogen that was most sensitive to the addition of Mars simulant to the growth medium or buffer, with all replicates failing to grow in cultures containing the organism's standard growth medium (MSH medium) with 2% (w/v) illite (Figure 1B) or in bicarbonate buffer containing 10% (w/v) montmorillonite (Table 3). Methane production by *M. maripaludis* was also more greatly affected by the presence of MMS than for either *M. wolfeii* or *M. formicicum*, which also saw decreases in CH_4 production in cultures containing MMS (Figure 3). The increased sensitivity of *M. maripaludis* to the presence of Mars simulants may be due to its halophilic nature. *M. maripaludis* was originally isolated from salt marsh sediment and found to require at least 5 mM Mg for growth [150]. Jones *et al.* [150] also discovered that neither sodium (Na) nor calcium (Ca) could substitute for this Mg requirement. Compared to the other methanogens tested here, the cell wall of *M. maripaludis* is a single, electron-dense, proteinaceous S-layer, lacking peptidoglycan molecules, which easily lyses [150,151] and likely contributes to the sensitivity seen here. In contrast, *M. barkeri* is considered to have a relatively thick and rigid cell wall [152], but also tends to form aggregates in culture, which may aid in survival [153–155]. Similarly, *M. formicicum* and *M. wolfeii* are described as having thick and rigid cell walls containing pseudomurein [156–158], also contributing to their robustness. Lastly, the genome for *M. wolfeii* encodes for a relatively large number of glycosyltransferases, which aid in the maintenance of cell integrity and stability, and are believed to contribute to the organism's ability to adapt to harsh environments [159].

Previous studies have also looked at the ability of methanogens to produce CH_4 in the presence of Mars simulants including nontronite, illite, montmorillonite, and MMS [80,81,115,116]. In experiments with *Methanosarcina mazei*, Zhang *et al.* [80] assessed both CH_4 production and Fe reduction in the presence of either nontronite or illite. The authors discovered that CH_4 production on these clays after 20 days' incubation at 37 °C was typically less than one-third the amount produced in cultures without clays (ca. 0.0015 mmol CH_4 vs. ca. 0.0055 mmol CH_4) and that CH_4 inhibition was directly correlated with the extent of bioreduction [80]. Importantly, however, and in contrast to the experiments conducted here, 0.5% (v/v) methanol was added to the cultures as a carbon source with the reduction of Fe^{3+} coupled to the oxidation of methanol [80]. Similarly, Zhang *et al.* [81] measured both CH_4 production and Fe reduction by *Methanothermobacter thermautotrophicus* in cultures containing either 5 g/L [0.5% (w/v)] montmorillonite or nontronite. With either clay, Zhang *et al.* [81] found CH_4 production by *M. thermautotrophicus* to be significant (ca. 0.12-0.16 mmol after 40 days' incubation at 65 °C). In comparison to the experiments conducted here, after 35 days' incubation in cultures containing 11.1% (w/v) nontronite, *M. barkeri* produced 8.8 ± 7.4 % headspace CH_4, *M. wolfeii* produced 23.0 ± 18.1 % headspace CH_4, and *M. formicicum* produced 17.9 ± 15.3 % headspace CH_4 (Figure 2, Table 2), equating to ca. 0.052 mmol, 0.13 mmol, and 0.11 mmol CH_4, respectively (see Appendix A for calculations). Methane production was similar for *M. wolfeii* and *M. formicicum*, compared to *M. thermautotrophicus*, despite differences in the final clay concentration in the medium and how the clay was provided to the methanogens.

All four methanogens proved capable of producing CH_4 in the presence of up to 33.3% (w/v) nontronite (Figure 2), which may not be surprising considering that nontronite is an iron (Fe) smectite, and Fe is critical for methanogenesis (as Fe/Fe or Ni/Fe clusters used by hydrogenases [95]). The lowest amount of CH_4 produced in the presence of 33.3% (w/v) nontronite in the experiments conducted here was by *M. formicicum* (Figure 2C) and averaged 2.4 ± 1.9 % headspace after 35 days' incubation at 37 °C. This is equivalent to 0.014 mmol CH_4 (see Appendix A for calculations), about ten times lower than the CH_4 produced by *M. thermautotrophicus* with 0.5% (w/v) nontronite [81]. However, the greatest amount of CH_4 produced in the presence of 33.3% (w/v) nontronite averaged

28.5 ± 7.2 % headspace, which is equivalent to ca. 0.18 mmol, and was produced by *M. maripaludis* after 35 days' incubation at 22 °C (Figure 2B). As mentioned above, the discrepancy between the amount of CH_4 produced between the two studies likely relates to the concentration of clays within the tubes [*i.e.*, 11-33% (w/v) vs. 0.5% (w/v)] and the mechanism of integration (*e.g.*, creating a slurry). Additionally, the lower CH_4 concentrations in cultures containing nontronite may be due to Fe reduction by the methanogen as suggested by Zhang *et al.* [81]. While direct comparison is difficult due to differences in experimental conditions (*i.e.*, the methanogens used, the optimal growth temperatures, specific medium components, how the clay minerals were added to the medium, etc.), both data provided here and data from Zhang *et al.* [81] indicate that certain methanogens are capable of growth (CH_4 production) in the presence of nontronite. Surprisingly, the greatest CH_4 concentrations in the presence of nontronite were produced by cultures of *M. maripaludis*, which showed the highest sensitivity (inhibition of CH_4 production) to the other Mars simulants tested.

Significant differences are seen in both CH_4 concentration and time to maximum CH_4 concentration amongst the three methanogens and three Mars simulants tested here and between the experiments conducted by Sinha and Kral [115], including amongst control tubes (Table S1). For control cultures, as well as samples containing montmorillonite, CH_4 concentrations were much higher and achieved much more quickly in experiments conducted by Sinha and Kral [115] (Table S1). However, variation amongst experimental procedures could explain the differences in observed CH_4 production. For example, Sinha and Kral [115] measured CH_4 production from serum bottles containing 3 g Mars simulant and 60 mL [5% (w/v)] bicarbonate buffer (leaving ca. 90 mL headspace). Here, experiments took place in Balch tubes (ca. 25 mL total volume) and contained variable amounts of Mars simulant and medium. Incubation temperatures and amount of H_2 provided (*i.e.*, 2 bar) were the same. However, one major factor that might be attributable to the differences in CH_4 production is that in neither study (Sinha and Kral [115] nor here) were inocula standardized to cell number. In Sinha and Kral [115], an unspecified volume of washed cells were resuspended in 15 mL bicarbonate buffer, and 1 mL of resuspended cells was used to inoculate each serum bottle. In contrast, the experiments conducted here typically used 0.5 mL culture as inocula (the montmorillonite experiment used 0.5 mL washed cells as the initial inoculum; Table S1). Further, for the nontronite and MMS experiments, the concentration of the Mars simulant were much higher in the experiments conducted here than in Sinha and Kral [115], which could affect CH_4 production; although, ultimately, this does not explain the differences in CH_4 concentration between the controls.

Chastain and Kral [116] looked at the ability of one methanogen, *M. wolfeii*, to utilize montmorillonite as a nutrient source without a reducing or buffering agent in the medium: the bicarbonate buffer was replaced with the liquid fraction of a 1% montmorillonite/deionized water suspension and no Na_2S was added to the medium. The authors found that, compared to buffered and reduced cultures, cultures containing solely H_2, CO_2, montmorillonite, and the liquid fraction of a montmorillonite-in-water suspension resulted in CH_4 production that was much slower and reached much lower concentrations (buffered/reduced: 26.2 ± 7.9% headspace CH_4 after 21 days' incubation vs. non-buffered/reduced: 10.2 ± 0.5% headspace CH_4 after 96 days' incubation) [116]. The authors attributed the lower CH_4 concentration to ‘simple metabolism’ (non-dividing, non-growing cells) compared to conventional microbial growth (*i.e.*, increasing cell number) [116]. Despite differences in CH_4 production amongst these various studies, which could be attributable to differences in cell number in the inocula as discussed above, CH_4 production by methanogens is possible using some Mars simulants, such as montmorillonite, as nutrient sources.

One factor not investigated in this study nor others mentioned here [80,81,115,116] is how the grain size, available pore space, or method of integration (of the clay mineral and the medium) affects CH_4 production or bioreduction. For example, Zhang *et al.* [81] first separated the 0.02-0.5 μm fraction from each clay, and then created individual slurries with final concentrations of 5 g/L [0.5% (w/v)]. These slurries were added to the methanogenic growth medium and supplemented with additional Al to promote illite formation [81]. In contrast, here, 1 g nontronite was added to tubes containing 9 mL medium, or 2.5 g nontronite was added to tubes containing 7.5 mL medium, resulting in much higher concentrations [11.1% and 33.3% (w/v)]; although the static incubation of these cultures and the limited dispersal mechanism (*i.e.*, no mixing of the clay and medium) may have reduced any negative impact that the presence of the clay may have had on the methanogens. More specifically, the slurry produced by Zhang *et al.* [81] likely resulted in more clay substrate being made available to the methanogenic population, which could either be beneficial in providing substate for metabolism (and/or bioreduction) and/or could have inhibited CH_4 production. While bioreduction was not measured here, results from Zhang *et al.* [80] and Zhang *et al.* [81] suggest that the intricacies of microbial metabolism (methanogenesis) and Fe reduction may serve to complicate the assessment of CH_4 production in the presence of these Mars simulants. The correlation between inhibition of CH_4 production and Fe reduction could also explain the inhibition of CH_4 production for certain methanogens as seen in many of the experiments conducted here; additional experiments would be necessary to confirm this, but could be an interesting avenue for future experiments.

It is important to note that Cervini-Silva *et al.* [160] conducted abiotic experiments in which they reacted nontronite with bicarbonate buffer and measured subsequent CH_4 production. The highest CH_4 concentration reached was 2040 ppbv (ca. 1.3 nmol CH_4; see Appendix A for calculations) after 60 days incubation at 25 °C. However, the authors also performed scanning electron microscopy (SEM) and noted the presence of hollow vesicles within the nontronite and attributed these to evidence of Fe^{2+}-oxidizing bacteria, as well as to evidence of gas trapping [160], which suggests that the experiments may not have been truly abiotic. The CH_4 concentration measured by Cervini-Silva *et al.* [160] (1.3 nmol CH_4) is well below that which we reliably consider to be the result of biotic methane production (*i.e.*, ca. 0.1 % headspace or ca. 0.61 µmol CH_4; see Appendix A, Chastain and Kral [116]). Thus, we can confidently attribute the CH_4 production shown in Figure 2 to methanogenesis and not to the reaction between nontronite and bicarbonate in the tubes.

Differences in experimental procedures limit the ability to directly compare the extent of the effects that these Mars simulants have on CH_4 production by methanogens. However, data provided here serve to confirm that while CH_4 production may be reduced in the presence of Mars simulants, these components are not completely inhibitory or biocidal for certain methanogens. Further, as evidenced by both the montmorillonite experiments and the ‘minimal medium requirements’ experiment conducted here, Mars simulants may be capable of providing certain nutrients to support or enhance microbial growth. For example, CH_4 production was higher for *M. barkeri* in standard methanogenic growth medium in the presence of MMS than without. In addition, CH_4 production was significantly higher for *M. maripaludis* cultures containing standard methanogenic growth medium without 200 g/L $K_2PO_4{\cdot}3H_2O$ (Solution B) or without trace minerals (Solution D), but with 0.5 g montmorillonite (Table 4, Figure 5). Future studies could further explore optimal and minimal medium requirements through additional experiments with and without various concentrations of salts, trace minerals, and other medium components. It would also serve future studies to include cell counts, of both inocula and samples taken during the length of the experiment, which could delineate between ‘simple metabolism’ and active growth. Additional data such as gene expression, as collected for *M. barkeri* by Harris *et al.* [119] and Harris and Schuerger [113], would also be extremely informative, but was outside the scope of the studies conducted here. Ultimately, the data shown here provide support for the possibility of past or present microbial life on Mars.

5. Conclusions

While these experiments did not aim to replicate current or past conditions on Mars, the ability of these four methanogens to grow in the presence of multiple Mars simulants suggests that martian regolith is not inherently biocidal. Further, the ability of *M. wolfeii*, *M. barkeri*, and *M. formicicum* to produce CH_4 in cultures containing solely bicarbonate buffer, H_2 gas, Na_2S, and montmorillonite, indicates that martian regolith, potentially, could have provided vital nutrients for past life or could currently support extant microbial metabolism(s) in the martian subsurface.

Overall, there were varied results among replicates and across species and Mars simulant; however, the actual CH_4 concentrations are less important than the fact that CH_4 was or was not produced. Future experiments including analyses such as cell counts may better enable more accurate comparisons against previous studies. While much more labor- and analysis-intensive, transcriptomics and/or proteomics approaches have been useful in more recent methanogen studies and could also help to elucidate metabolic effects of the Mars simulants. Additionally, the potential for bioreduction of Mars simulants, while also inhibiting CH_4 production, poses a unique opportunity to further explore the possibility of the existence of methanogenic Archaea on Mars.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Table S1: Maximum methane concentrations (% headspace) for *Methanothermobacter wolfeii*, *Methanosarcina barkeri*, and *Methanobacterium formicicum* grown on Mars simulants.

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Data Availability Statement: Data available upon request.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

Al	Aluminum
ATCC	American Type Culture Collection
C	Carbon
Ca	Calcium
CH_4	Methane
CO_2	Carbon dioxide
Cr	Chromium
Cu	Copper
Fe	Iron
H_2	Hydrogen
H_3O^+	Hydronium
K	Potassium
Li	Lithium
Mg	Magnesium
Mn	Manganese
MMS	Mojave Mars Simulant
N	Nitrogen
Na	Sodium
NaOH	Sodium hydroxide
Na_2S	Sodium sulfide
NH_4	Ammonium chloride
OCM	Oregon Collection of Methanogens
P	Phosphorus
S	Sulfur
SEM	Scanning electron microscopy
Si	Silicon
Ti	Titanium
Zn	Zinc

Appendix A

Calculations Converting Methane from % Headspace to Mol

For comparison to methane (CH_4) production measured in millimoles by Zhang *et al.* [80] and Zhang *et al.* [81], we have converted our headspace CH_4 data to moles (mol) using the ideal gas law (1) and the following conditions:

$$n\ (moles\ CH_4) = \frac{PV}{RT} \tag{1}$$

where P = pressure = 1 atm, V = the CH_4 percentage of the total internal headspace volume of the culture tube, T = temperature, and R is the ideal gas constant (0.0821 L-atm/mol-K). The total internal headspace volume of the culture tube is estimated to be 0.015 L given that the Balch tubes used have a total internal volume of 0.025 L and are filled with ca. 0.01 L medium. Additionally, although all CH_4 measurements were taken at room temperature (22 °C), the tubes were incubated at the organisms' respective ideal growth temperatures (22 °C, 37 °C, or 55 °C). These incubation temperatures were used to calculate moles CH_4 so as not to artificially inflate any concentrations. Thus, for moles CH_4 calculations for *M. maripaludis* and *M. wolfeii*, temperatures of 295 K (22 °C) and 328 K (55 °C), respectively, were used. For *M. barkeri* and *M. formicicum*, a temperature of 310 K (37 °C) was used. For example, for a % headspace concentration of 8.8 % CH_4 produced by *M. barkeri* at 37 °C, V = 0.088 * 0.015 L = 0.00132 L and T = 310 K, equating to 0.052 mmol CH_4. For comparison, using a temperature of 22 °C (295 K) equates to a CH_4 concentration of 0.055 mmol.

Chastain and Kral [116] measured 0.14 ± 0.05 % headspace CH_4 from *Methanothermobacter wolfeii* cultures containing carbon dioxide (CO_2), 1 g montmorillonite, and 6 mL buffer (derived from montmorillonite and deionized water), but without an energy source [*i.e.*, hydrogen (H_2)]. The authors thus considered headspace concentrations greater than 0.1% to be the result of methanogenesis. Using equation (1), this value corresponds to 0.61 μmol CH_4 in the experiments performed here, which we also consider to be the lower limit to attribute increases in CH_4 concentration to active methane production by methanogens.

Calculations Converting Methane from Ppbv to Mol

Cervini-Silva *et al.* [160] conducted abiotic experiments in which they reacted nontronite with bicarbonate buffer and measured subsequent CH_4 production. The highest CH_4 concentration reached was 2040 ppbv after 60 days incubation at 25 °C. These experiments were conducted in 25 mL bottles and contained 100 mg nontronite in 10 mL 1 mM sodium bicarbonate ($NaHCO_3$) with a nitrogen (N_2) headspace. Assuming 0.015 L total headspace within the bottles, the total amount of N_2 can be found using equation (1): with V = 0.015 L, P = 1 atm and T = 298 K, the total number of moles N_2 in the headspace of the bottles is 0.61 mmol. The concentration of CH_4 measured by Cervini-Silva *et al.* [160] (2040 ppbv CH_4) equates to a 2.04×10^{-6} mole fraction. This equates to a CH_4 concentration of 1.3 nmol, which is significantly lower than the value of 0.61 μmol CH_4 that we attribute to active methanogenesis.

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