DETERMINING THE SENSITIVITY OF PCR TO DETECT METHANOGENS IN VARYING SOIL TYPES.

Travis Altheide[1], Jennifer Lewter[2], Timothy Kral[3], and Allen Szalanski[2], University of Arkansas, [1]Arkansas-Oklahoma Center for Space and Planetary Sciences, [2]Department of Entomology, [3]Department of Biological Sciences, Fayetteville, AR.

Introduction: Methanogens are a group of anaerobic micro-organisms that produce methane from hydrogen gas and carbon dioxide as a result of cellular metabolism [3]. They will usually not tolerate even brief exposure to oxygen. These organisms live in a range of anaerobic environments, such as marine and fresh-water sediments, intestinal tracts of animals, ocean vents, and hot springs. Methanogens belong to the Archaea domain, one of the three domains life, so they should not be confused with bacteria. There ability to inhabit extreme environments is of great interest to astrobiologists who search for the possibility of life on other planets. Mars has become the main area of focus for the search of life in space due to its unique environment which closely resembles many of Earth's extreme habitats. Recently, a trio of research teams independently probing the martian atmosphere for signs of methane have confirmed the presence of the gas. The source of the methane can either be from volcanism or, what many scientists hope, from subterranean microbes.

PCR, as a molecular diagnostics test, has proven to be an effective protocol for detecting methanogens present in a martian soil stimulant [2]. The ability to detect specific methanogens in a soil sample by using PCR is a great advantage for researchers, because it can work on extracted DNA from living or dead orgamisms. This of course is of real value to scientists, who in the future, will be analyzing actual soil samples returned from Mars.

Objectives: I would like to accomplish two things with this research: To determine the sensitivity of the PCR technique by quantifying the number of microbes in a soil sample necessary for detection, and to test this technique on other soil types, such as sand and potting soil, to determine its versatility and to establish a range of varying simulants that support methanogen growth.

Methods and Materials: Four strains of methanogens were obtained from stock cultures. These four strains are *Methanobacterium formicicum, Methanogenium frigidum, Methanococcus maripaludis,* and *Methanothermobacter wolfeii*. Microscopic views of four of the five methanogens used in the experiment are shown below Figure 2. The organisms were grown in specific media. For *M. wolfeii*, "MM" and "MS" media were used. MS media requires 0.4 grams of yeast extract, 0.2 grams of Trypticase peptones, and 0.2 grams of Mercaptoethanesulfonic acid in addition to the ingredients needed to make MM media. Seven tubes of varying concentrations were accomplished by the serial dilution method. This gave a range of dilution from undilute to 10-6. This method was used only for *M. wolfeii* in the martian soil stimulant.

All four strains were tested in a medium of sand and potting soil. Here, the dilution method was not used, but instead the organisms were placed directly onto the soil samples and were allowed to incubate for five days. This particular procedure was only tested twice. For the other three strains of methanogens, "MH" and "MSH" media were used . MH media is the same as MS media but with 87.75 grams of sodium chloride, five additional grams of $MgCl_2 \cdot 6H_20$ and 1.5 grams of KCl added per liter. MSH media is a mixture of 2 parts MS medium with 1 part MH media.

DNA extraction and PCR. The Puregene protocol for DNA extraction was used in this experiment. Figure 1 shows all the primers that were used of PCR. The ME 1 and ME 2 primers are the universal methanogen primers used to distinguish them from all other organisms. Mfrig, Mwolf and Mform are the reverse diagnostic primers specific for each strain. The actual PCR program goes through 40 complete cycles, with each cycle doubling the amount of DNA synthesized in the previous cycle. The first three cycles of the process are depicted in Figure 2.

Primer	**Sequence**
ME1	5'GCMATGCARATHGGWATGTC
ME2	5'TCATKGCRTAGTTDGGRTAGT
Diagnostic primer	**Sequence**
Mfrig-R	5'GCTGCGGGGTCCGGGTT
Mwolf-R	5'TGTTCAAGTCCGTAGAAC
Mform-R	5'TGTTGCCATACATACACCA

Figure 1: The five primers used to amplify specific base pairs of the MCR α-subunit gene of all strains except *M. barkeri*.

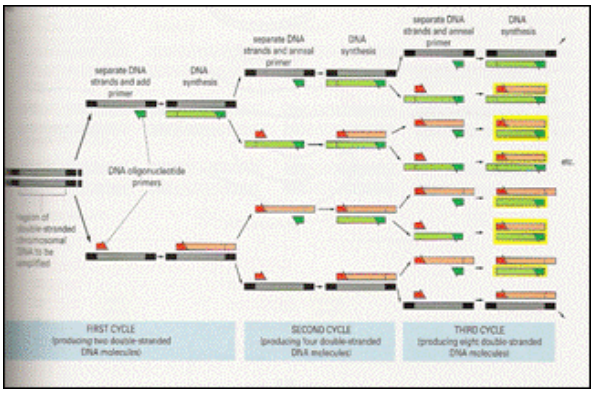
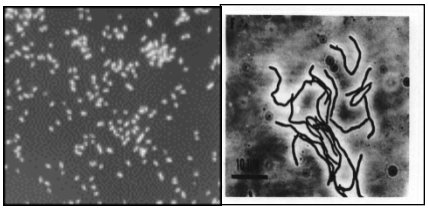
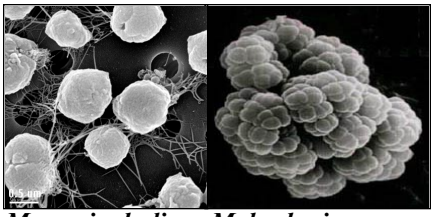


Figure 2: This demonstrates three cycles of the PCR procedure. Note how each cycle doubles the amount of DNA from the previous one. By the time 40 cycles are complete, an amplification factor of around one billion can be obtained [1].



M. formicicum ***M. wolfeii***



M. maripaludis ***M. barkeri***

Results: The tests conducted concerning *M. wolfeii* in the martian soil stimulant, using the dilution method, yielded positive results with only the undilute samples. Upon electrophoresis, the diluted samples produced no bands at the appropriate base pair marking. Figure 3 shows the results of an agarose gel which was negative for the presence of *M. wolfeii* from the martian soil simulant.

The results from the second experiment, using sand and potting soil, were more observerable. Both *M. frigidum* and *M. wolfeii* were genetically identified in the sand samples using PCR. On the other hand, they were not found in the potting soil samples. *M. maripaludis* and *M. formicicum* were not identified in any of the sand and potting soil samples. Figure 4 displays the results from this test.

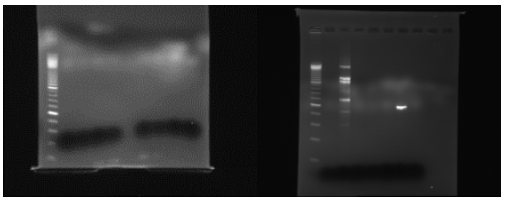


Figure 3 and Figure 4: Methanogen diagnostic gel

Discussion: There are a few reasons why the diluted samples producted no positive results. First and foremost, the samples could very well have contained too low of a concentration for the PCR to detect. If this is the case, then the sensitivity of PCR concerning the diluted samples has been roughly estimated. Second, the method for which the samples were mixed with the soil simulant could have removed enough of the organisms to produce a negative test. Third, the fact that *M. wolfeii*, in the dilution tests, was not grown in the soil samples, but instead was mixed with the soil after the organisms were killed, could also have had a negative effect on the numbers of cells present to give a positive result. A more plausible reason explaning these results is a combination of all three factors listed above. For future work using the dilution method, obtaining an optical density reading and/or an approximate cell count for each dilution

Because only two trials were carried out with the sand and potting soil, I should refrain from making any concrete conclusions, though, the fact that PCR was able to identify two of the organisms in a sand environment, clearly adds to the versatility of this technique. I am comfident that if more trials were carried out, all of the tested organisms will be identified using PCR. Concerning the potting soil samples, it is interesting to note how the organics of this soil type could have interfered with either the growth of the methanogen or with the DNA extraction and PCR. Testing the role of organic material in soil as a possible growth regulatory in methanogens should be further explored.

Acknowledgements: I would like to thank Carrie Owens for all her help with the equipment in the Entomology Department and with questions in general that I had during the course of the summer. I would also like to acknowledge Kathy for her help in Dr. Kral's laboratory, specificly, making media, washing the materials I needed and answering my many questions.

References: [1] B. Alberts. (2004) *Essential Cell Biology.*, 349. [2] J. Lewter. (2003) *Molecular Diagnostics of Methanogens in a Mars Soil Simulant* [3] K. Talaro and A. Talaro. (1999) *Foundations in Microbiology.*, 197.