Growth of Cyanobacteria *Anabaena variabilis* on JSC-1A, a Martian Regolith Simulant

Abstract

Mars is a planet. Freezing temperatures, harsh solar, and a lack of water have left the surface of the planet sterile with no hope for even the hardiest of life forms to emerge. However, if Mars was warmer and had an atmosphere; could life emerge? In this project, we study how a martian regolith analog affects the growth of the cyanobacteria *Anabaena variabilis*. Our results indicate that in appropriate quantities, the martian material may aid growth by providing extra minerals in the growth media, however at higher concentrations these minerals become chemotoxic. We also test whether the martian regolith simulant aids growth by providing small particle ‘scaffolds’ to which the organism may adhere to form microbial communities. We demonstrate that in the presence of inert, fine glass beads, expression of genes related to biological processes of the cell are actually down regulated rather than kept at wild-type levels.

Introduction

Ever since man landed on the moon we have collectively wondered as a species how humankind could stretch beyond our current home and live elsewhere. When we do this, the first we turn our attention to is our nearby neighbor, Mars.

Currently, we can artificially sustain life for short periods of time on brief journeys into the cosmos. This includes launching dogs and monkeys into orbit, and sending humans as far as the moon and back. However, one of mankind’s lofty goals is to find a place that can permanently sustain life. If a planet can support even primitive life forms, it also will have the capacity to support more complex higher life forms. Therefore, in order to determine a planet’s ability to harbor even the most primitive life forms we need to ask the following questions: Was this planet able to support life in the past? Is it able to support life now? Could it support life in the future?

Requirements for life

Research suggests that there are four basic requirements a planet must have to first qualify as a life supporting planet; energy, carbon, liquid water, and a position in the habitable zone of its solar system. Using these metrics, we will examine the feasibility of life on Mars in the past, present, and future.

*Energy*

Life forms must harness and use energy in order to grow and replicate. On planets such as our own, the sun is the predominant provider of this energy, giving light to photosynthetic plants which in turn convert it into chemical energy. These plants in turn are consumed by other organisms which may also be used as energy for other living
things. Although the sun provides almost all of the energy for living things on earth, there are some exceptions. An example of one of these exceptions are deep-sea hydrothermal vents which provide energy in the form of heat and minerals. Some of earth's most ancient and primitive life forms still thrive near these vents, completely independent of the sun.

Mars nearly 1.5 times as far from the sun as earth does and therefore the surface receives about half the light that our planet does. Furthermore, frequent and sometimes planet-wide dust storms enshroud the planet reducing exposure. However, plants and algae on earth have been shown to survive with less than 5% of the sun’s light, meaning that although Mars receives reduced quantities of light from our sun, it would still be enough light satisfy photosynthetic activity.

There may also be alternate forms of energy beneath the surface of Mars’ crust. For many years, most researchers assumed that the core of Mars was solid and non-rotating due to the lack of any detectable magnetosphere. However, new research suggests that the core of Mars is completely molten, without any inner solid core. Heat currents provided by a molten core may provide alternative energy sources for budding organisms below the crust of the planet.

Carbon

Because of its unique chemistry, carbon serves as the backbone for all organic molecules. A common source of organic carbon is methane gas, which can be detected in the environment. On Mars, the Curiosity rover has not only detected varying concentrations of methane gas, but also long-chain organic molecules from solid soil and rock samples. Almost just as exciting as the discovery of organic molecules is the discovery of biologically accessible nitrogen in the soil. Although these finds do not confirm that life currently exists on the red planet, they could be remains of ancient life that once existed there. In summary, Mars possesses the carbon needed for organic molecules that are crucial to life forms.

Liquid water

Water serves as the solvent for all of the chemical reactions required for life. Liquid water’s properties, such as expansion upon freezing, low molecular weight, and abundance in the universe make it an ideal solvent for harboring the chemical reactions required for life.

For decades it was thought that liquid water could not exist on the surface of Mars. Images of the planet demonstrated that nearly all of Mars’ water is frozen underground or has evaporated away. One may think that this is a result of being colder and further from the sun but they would only be partially correct. The biggest reason almost all of the water on Mars is frozen and trapped underground is due to the fact that the planet lacks an atmosphere. Without a protective atmosphere, the planet is much colder than it otherwise could be and is also exposed to high levels of ionizing solar radiation. The lack
of an atmosphere on Mars caused most of the water to evaporate, and for the planet to become colder than it could be if it had an atmosphere. Despite the chilly climate, recent studies based on satellite imagery have proved that liquid water flows on Mars in small quantities and under the right conditions. This exciting new evidence satisfies another of the basic requirements for life.

**Habitable zone**

The most generic definition of the habitable zone of a solar system is the distance required from the sun where water can exist in liquid form. This means the planet is neither too hot nor too cold. How the habitable zone is defined can be somewhat ambiguous, although due to the evidence of liquid water on Mars, the red planet is currently placed near the outer edge of our solar systems habitable zone.

When examining the specifics of life on Mars we must also consider that despite the fact that Mars fulfills all of these requirements, it barely fulfills them. A big inhibitor to life on Mars is the fact that it possesses a very thin atmosphere and no magnetosphere. This leaves everything on the surface of the planet exposed to harsh UV and solar radiation. This radiation is so harsh that the entire surface of Mars is sterile, and not likely to change unless a significant atmosphere and magnetic field are generated.

**What organism would live on Mars?**

If life on Mars were to follow a similar evolutionary trajectory like Earth’s, it would most likely begin in an extreme environment below the surface of the planet. As mentioned previously, current conditions on Mars make life inhospitable on the cold sterile surface. However, underneath the crust may exist warmth and energy.

Many scientists speculate that the first living organisms on earth come from the domain *Archaea* and arose from deep-sea hydrothermal vents mentioned earlier. Diverse and abundant microbial communities have been discovered at depths of up to 500 meters on earth and more recently an in depth study demonstrated how a microbial environment can be created in deep rock layers under the Earths crust. Similar life-supporting conditions on Mars may have existed in the past or even in the present.

For our project, we decided to choose an organism that could live on the surface of the planet assuming it had an earth-like atmosphere. We selected the free-living cyanobacteria *Anabaena variabilis*. *Anabaena* is a blue-green cyanobacteria that grows in freshwater. Cyanobacteria are more primitive life forms than even other microbes such as *E. coli* and it is thought that cyanobacteria were the first organisms on earth that began to fill the atmosphere with oxygen. We chose *Anabaena* due to its ability to photosynthesize, making it independent of other food sources. As a cyanobacteria, we were also able to study how a more primitive life form interacted with martian regolith.
These factors made *Anabaena variabilis* a good candidate to study the effects of martian surface material on growth.

**Materials & Methods**

The strain of *Anabaena* used for this study is called PCC 7120. PCC 7120 is well studied and has been sequenced. Anabaena was grown in the growth media BG-11 and BG-11(0). The difference between these two media is simply the absence of biologically available nitrate in BG-11(0). For most experiments, including RT-PCR, *Anabaena* was grown in BG-11(0) to ensure it retained nitrogen fixing activity. Growth of *Anabaena* was performed at 30° and 32° C in a shaking incubator kept at 125 rpm. White light was provided at surface level of 30 µmol/m²/sec.

To study the effects of Martian soil on this organism we purchased a Martian regolith simulant from Orbitec known as JSC-1A. JSC-1A is a spectral and chemical analog to the regolith that exists on the planet currently. We found that at 30°C, including JSC-1A in the growth media from a starting culture led to a lack of growth of *Anabaena*. To overcome this, we grew *Anabaena* in liquid culture until cell density was sufficient then spiked the culture with 0.1g of sterile JSC-1A. Growth was then allowed un-interrupted for two days to ensure at least two separate cell divisions. Growth issues in the presence of JSC-1A seemed to be resolved when the temperature was increased from 30° to 32° C however we still preferred to spike the culture as any increase or decrease in growth could mostly be avoided and cell cultures could harvested at the same time. We avoided increasing temperatures above 32°C as it has been shown to impede growth of *Anabaena*.

To test the effect of small particulate matter in the growth media, we also measure *Anabaena* growth in the presence of 0.1 g of fine glass beads (ThermoFisher). These beads were added in the same fashion as JSC-1A as part of a 0.1g spike.

Targets for qPCR were determined by analyzing the entire genome of *Anabaena* using blast2go software. After blasting every gene in the genome of PCC 7120, we used gene ontology (GO) annotations to group each gene three different biological categories: cellular component, molecular function, and biological process. An explanation of these three categories can be found in Table 1 and a diagram specific to *Anabaena* is provided in supplemental figure 1. We chose to examine genes that were placed in the biological

<table>
<thead>
<tr>
<th>Gene Ontology domain</th>
<th>Definition</th>
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<tbody>
<tr>
<td>Cellular component</td>
<td>The parts of a cell or its extracellular environment</td>
</tr>
<tr>
<td>Molecular function</td>
<td>Elemental activities of a gene or its product. Examples include molecule binding of catalysis.</td>
</tr>
<tr>
<td>Biological process</td>
<td>Operations or sets of molecular events that have a defined beginning and ending. Examples include the Kreb cycle, or the oxidative chain in mitochondria.</td>
</tr>
</tbody>
</table>
process category. The reason for this is because the GO definition of biological process is an operation, or set of molecular events that has a defined start and finish. Because of this we felt RT-PCR results of genes in this category would provide a better idea of what was going on more generally rather than focusing on one gene. Where possible, targets unique only to their specific biological category were used. We also selected two genes to study changes in iron transportation. A summary of all targets used for RT-PCR can be seen in Table 2.

RNA was isolated following a hot-phenol protocol. We found that despite the large

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Gene Function</th>
<th>Biological Process</th>
</tr>
</thead>
<tbody>
<tr>
<td>all4191</td>
<td>α subunit of RNA polymerase</td>
<td>Reference for RT-PCR</td>
</tr>
<tr>
<td>alr1128</td>
<td>chromosome segregation protein</td>
<td>multicellular organismal process</td>
</tr>
<tr>
<td>all0910</td>
<td>ABC phosphate transport system permease protein</td>
<td>multi-organism process</td>
</tr>
<tr>
<td>all0284</td>
<td>WD-40 repeat protein</td>
<td>Signalling</td>
</tr>
<tr>
<td>all3600</td>
<td>two-component sensor histidine kinase</td>
<td>developmental process</td>
</tr>
<tr>
<td>alr0652</td>
<td>Mrp protein homolog</td>
<td>cell organization/biogenesis</td>
</tr>
<tr>
<td>alr0483</td>
<td>GTP binding protein</td>
<td>localization</td>
</tr>
<tr>
<td>alr4550</td>
<td>hypothetical protein*</td>
<td>response to stimulus</td>
</tr>
<tr>
<td>all2375</td>
<td>similar to bacterioferritin comigratory protein</td>
<td></td>
</tr>
<tr>
<td>alr4359</td>
<td>hypothetical protein*</td>
<td></td>
</tr>
<tr>
<td>alr0950</td>
<td>cytochrome c oxidase subunit II</td>
<td>single-organism process</td>
</tr>
<tr>
<td>alr2082</td>
<td>cobalamin biosynthetic protein CobD</td>
<td></td>
</tr>
<tr>
<td>all1642</td>
<td>hypothetical protein*</td>
<td>metabolic process</td>
</tr>
<tr>
<td>all2533</td>
<td>prolyl endopeptidase</td>
<td></td>
</tr>
<tr>
<td>all1272</td>
<td>probable glycogen phosphorylase</td>
<td></td>
</tr>
<tr>
<td>all0453</td>
<td>cobalamin biosynthesis precorrin-3 methylase</td>
<td></td>
</tr>
<tr>
<td>all1588</td>
<td>33kD chaperonin, heat shock protein HSP33</td>
<td>cellular process</td>
</tr>
<tr>
<td>alr3661</td>
<td>chaperonin GroES</td>
<td></td>
</tr>
<tr>
<td>alr8073</td>
<td>hypothetical protein*</td>
<td></td>
</tr>
<tr>
<td>all2162</td>
<td>methyl-accepting chemotaxis protein</td>
<td></td>
</tr>
</tbody>
</table>

**Iron transport**

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Gene Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>alr2118</td>
<td>iron(II) transporter</td>
</tr>
<tr>
<td>all0387</td>
<td>iron(III) ABC transporter, permease protein</td>
</tr>
</tbody>
</table>

*All hypothetical proteins were predicted to be involved in their corresponding biological process by GO annotations*
variety of commercial kits, hot-phenol isolation isolated higher quantities of RNA and at higher qualities. Following isolation, conversion of RNA into cDNA was performed using SuperScript IV reverse transcriptase enzyme (ThermoFisher).

RT-PCR was performed using a StepOne Plus real time instrument (ThermoFisher). PowerUp SYBR (ThermoFisher) was used to perform quantitation during real-time experiments. Data from RT-PCR experiments was analyzed using the ΔΔCt method. As a reference, we used the alpha subunit of RNA polymerase to make all of our measurements. All error measurements were made using 95% confidence intervals rather than the standard error of the mean.

**Results**

Upon analysis of the genome of PCC 7120, it is interesting to note that over half of the genes return no BLAST results. Without a more complete picture, there is still much to learn about the genes needed for life on Mars. Of the 2986 genes with blast hits, 2897 were able to be mapped to the genome and 2471 had GO annotations available.

Growth of *Anabaena* was inhibited in the presence of JSC-1A and at a temperature of 30°C (data not shown). Initially we thought this was due to chemotoxicity of JSC-1A towards the organism, however growth in the presence of JSC-1A seemed to be restored when the temperature was raised to 32°C. We conclude that most likely the inhibition of growth at lower temperatures was more likely due to less light exposure caused by JSC-1A in the liquid media. We also noticed accumulation of *Anabaena* on the edges of the flask and on fine layers of JSC-1A (Fig. 1).

Surprisingly, RT-PCR results indicate that nearly every gene or function tested displayed an increase in expression (Fig. 2). Due to these results we decided to test whether adding inert fine particles somehow enhanced growth. We tested this by repeating the experiment using fine glass beads in place of JSC-1A. Contrary to the results from the first experiment, expression of genes involved in each biological process was down-regulated, suggesting that JSC-1A enhances expression by providing extra mineral content to the growth media. However, if the concentration of JSC-1A is too high, the organism dies off. This suggests that in lower quantities JSC-1A provides extra nutrients but at higher concentrations these nutrients and minerals are toxic to the cells. Alternatively, JSC-1A may be light-starving *Anabaena*, leading to the lack of growth.
observed when higher concentrations of JSC-1A are used. This light-dependent hypothesis may explain why other studies have shown that life forms as complex as green plants are able to grow in JSC-1A without being killed.

**Discussion**

On a simple level, this study shows that microbial life on earth is not completely incompatible with martian regolith in low quantities, however future studies involving *Anabaena* would benefit greatly from a well-characterized and annotated genome.

Other studies have examined survival of microbial life and even plant life on martian regolith. One study demonstrated the ability of four different species of methanogens were able to survive in differing concentrations of JSC-1A.\(^{19}\) Unlike *Anabaena*, these methanogens do not photosynthesize and make their energy chemically. Several other studies have examined the properties of Martian and even lunar regoliths as a medium for plants to grow in.\(^{20,21}\) Unrelated studies have examined the viability of spores on
spacecraft surfaces on Mars. All of these studies indicate that the surface material of Mars is not inherently toxic to living organisms. Although the current conditions on Mars are inhospitable, this will most likely change in the future due to the following factors.

*Mars will heat up*

As our sun ages it will get hotter. The habitable zone will shift more in the direction of Mars, heating up both Earth and the red planet. Although this is bad news for Earth, it will make surface temperatures on Mars much more temperate instead of freezing cold.

*Mars could regain an atmosphere*

If the core of Mars is completely molten, its gradual solidification could restart the dynamo responsible for the electromagnetic currents needed to form the planet’s magnetosphere. This would shield the planet from harmful solar radiation that burned away the planets ancient atmosphere. With the protection of a new magnetosphere, the atmosphere will begin to thicken with gases that will in turn protect the planets surface from toxic levels of UV radiation.

*Liquid water could exist on Mars again*

Hotter temperatures and thicker atmospheres on Mars will allow trapped and frozen liquid water to make its way to the surface and flow freely again. Once this occurs all the necessary elements for a life supporting planet are in place.

In summary, life cannot exist on the surface of Mars presently. However, our study and others has demonstrated that the surface material of Mars is not toxic and that life can grow on it. Mars is just outside the edge of habitability. However, if we were to look into the future nearly a billion year, instead of seeing a red planet, we might see a green one.

**References**


Supplemental Figure 1. Biological processes specific to *Anabaena*. These nodes represent the second level of GO annotation provided by blast2go software. Each node is colored based on the number of sequences for each category.