EXTREMOPHILES FOR ECOPOIESIS: DESIRABLE TRAITS FOR AND SURVIVABILITY OF PIONEER MARTIAN ORGANISMS

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ABSTRACT

Humanity is on the verge of having the capability of constructively directing environmental changes on a planetary scale. Within the foreseeable future, we will have the technology to modify Mars' environment, and make it a habitable planet. However, we do not have enough information to determine the course of such an event. To our knowledge, no known terrestrial organism has the capability of living on Mars' surface under present conditions. However, with some modification, Mars' environment could be brought into the survival and growth range of currently known microorganisms. Using the SHOT Ecopoesis Testbed, we performed survival/growth experiments to determine the suitability of potential pioneering life forms for Mars. Included among the potential pioneers were five genera of cyanobacteria (Anabaena, Chroococcidiopsis, Plectonema, Synechococcus and Synechocystis), three partially-characterized Atacama Desert heterotrophic eubacterial strains, and several desert varnish isolates. Microorganisms were exposed to a present-day mix of martian atmospheric gases, but at a pressure of 100 mbar (10 times Mars' current atmospheric pressure). Cultures were inoculated into samples of JSC Mars-1 soil stimulant and exposed to full-spectrum simulated martian sunlight. Day/night temperature cycled from 26°C to -80°C and back. Preliminary results indicate that both autotrophic and heterotrophic bacteria can survive in the simulated engineered martian environment.

INTRODUCTION

In relation to terrestrial organisms, Mars’ environment is harsher than any on Earth. The combination of low temperature, low atmospheric pressure, low moisture, high atmospheric CO₂ fraction and high UV flux is not experienced by any known Earth organism. However, Earth organisms may be found in environments that include at least one of the martian environmental factors (although Mars is still harsher in most respects). The dry valleys of Antarctica provide a low temperature, low moisture, high UV environment. Chile’s Atacama Desert is arguably the driest place on Earth. Some hot springs and volcanic vents produce anoxic environments with very high CO₂ levels. Alpine and stratospheric environments possess low pressure, low temperature and high UV.

Each of these terrestrial environments harbors some form of life. Organisms living there have adapted to the extreme conditions, and those adaptations are genetically encoded. In theory, genes encoding favorable characteristics from one organism could be transferred to another organism, which already possesses some other characteristics for extreme environment survival. With enough genetic alterations, we could conceivably "build" a microorganism—a "marsbug”—that could survive, reproduce and grow in a somewhat modified martian environment.

Ten years ago, Hiscox and Thomas (Hiscox and Thomas, 1995) detailed some of the characteristics that an ideal marsbug might possess. Here, we elaborate upon and update these characteristics, and summarize some initial experiments in which organisms with some of these characteristics were tested under approximated martian conditions.

DESIRABLE TRAITS FOR PIONEER MICROORGANISMS

For the purposes of this paper, we assume that initial, non-biological, modification of Mars’ environment would occur before pioneer microorganisms are introduced. At minimum, Mars would have an atmospheric pressure of at least 25 mbar, significant periods of above-freezing temperatures, and significant bodies of liquid water (Thomas, 1995). At 25 mbar, water boils at 9°C, providing a narrow temperature window at which water remains liquid. Also, recent studies suggest that Earth bacteria do not grow at pressures below 25 mbar (Schuenger et al., 2006a; Schuenger and Nicholson, 2005). Even with initial modification, the martian environment would be at least as harsh as the least hospitable environments on Earth. Accordingly, pioneer microorganisms would require many, if not all, of the following characteristics (summarized in Table 1).

Autotrophy. Mars has no known reserves of organic materials. Pioneer organisms would need to make their
own biomolecules from inorganic constituents via photosynthesis or chemosynthesis. Photosynthesis would replace the CO$_2$ in Mars' atmosphere with O$_2$—a key process in planetary engineering (Graham, 2004; McKay, 1998; McKay et al., 1991; Thomas, 1995). Iron and manganese-oxidizing chemolithotrophs produce dark-colored byproducts (Dorn and Oberlander, 1981), which decrease the albedo of rock surfaces, and would aid in the heating of Mars (Boston et al., 2004).

**Antioxidants.** Oxygenic photosynthesis produces a variety of reactive oxygen species (ROS) as normal byproducts of electron transfer. In addition, Mars’ surface is currently a highly oxidizing environment, presumably due to UV-produced ROS (Hunten, 1979; Plumb et al., 1989; Yen et al., 2000). Currently, we do not know whether initial engineering efforts would change the oxidizing nature of Mars' surface. However, other environmental stresses, such as chilling, increase the amount of ROS produced by photosynthesis (Clare et al., 1984; Hodgson and Raison, 1991a; Hodgson and Raison, 1991b; Thomas et al., 1999; Wise, 1995; Wise and Naylor, 1987). Pioneer microorganisms would require robust antioxidant systems in order to detoxify both internally- and externally-generated ROS.

**Psychrophily.** Even under engineered conditions, Mars will be cold. Truly psychrophilic organisms have optimum growth temperatures below 20°C (Atlas, 1997). The ability to grow at low temperature requires an entire suite of enzymes that operate well at low temperature as well as highly unsaturated membrane lipids (Chattopadhyay and Jagannadham, 2001; Gerday et al., 2003; Golden and Yoon, 1998; Hodgson and Raison, 1991a; Hodgson and Raison, 1991b; Kraegeloh and Kunte, 2002; Northup et al., 2003; Thomas et al., 1999; Wise, 1995). Psychrophily will be cold. Truly psychrophilic organisms have optimum growth temperatures below 20°C (Atlas, 1997). The ability to grow at low temperature requires an entire suite of enzymes that operate well at low temperature as well as highly unsaturated membrane lipids (Chattopadhyay and Jagannadham, 2001; Gerday et al., 2003; Golden and Yoon, 1998; Hodgson and Raison, 1991a; Hodgson and Raison, 1991b; Kraegeloh and Kunte, 2002; Northup et al., 2003; Thomas et al., 1999; Wise, 1995).

**CO$_2$ tolerance.** Mars' atmosphere currently contains 95% CO$_2$, albeit at low pressure. We expect initial engineering
efforts to increase the total atmospheric pressure primarily through the release of additional CO$_2$ from the polar caps and from possible carbonate rocks, which would further increase the proportion of CO$_2$. At high CO$_2$ levels, decreased pH becomes a factor as well. When CO$_2$ dissolves in water, it forms carbonic acid with a pH of 5.5 - 6.0 at 1000 mbar. Fortunately, several cyanobacteria and at least one alga survive and grow in >20% CO$_2$ at 1000 mbar, and many microorganisms grow within the pH range of 5 - 6 (Negoro et al., 1991; Seckbach et al., 1970; Seckbach and Libby, 1970; Thomas et al., 2005).

**Hypoxia tolerance.** Mars' atmosphere contains very little O$_2$. Pioneer microorganisms will have to grow during extended periods of hypoxia or anoxia. Even though photosynthesis produces O$_2$, plants require at least 50 mbar pO$_2$ for proper development (due in part to the large proportion of non-photosynthetic tissues) (Alpi and Beavers, 1983; Atwell et al., 1982; Barclay and Crawford, 1982; Jackson and Drew, 1984). Most chemoheterotrophs also require O$_2$ in order to oxidize inorganic molecules for energy production (Atlas, 1997), thus limiting their initial growth on Mars. Fortunately, most of the CO$_2$-tolerant phototrophic microbes, cited previously, also grow anaerobically.

**Carbonate dissolution.** As mentioned previously, carbonates are a potential source of atmospheric CO$_2$. Many cyanobacteria and algae cause damage to limestone-based structures due to carbonate dissolution—the conversion of carbonate ions into CO$_2$ (Crispim et al., 2003). If Mars has extensive carbonate deposits, such organisms could speed the process of thickening Mars' CO$_2$ atmosphere while also providing oxygen. The Antarctic cyanobacterium, *Matteia*, has been specifically suggested for this purpose (Friedmann et al., 1993).

**Denitrification.** Although Mars' atmosphere could be thickened by the addition of CO$_2$, photosynthesis will remove the CO$_2$ and replace it with O$_2$. An atmosphere of pure O$_2$ would result in catastrophic combustion. On Earth, the atmosphere is buffered with N$_2$, which is largely nonreactive. If Mars possesses significant nitrate deposits, these can be converted to N$_2$ via the process of denitrification (Thomas, 1995). Denitrifying bacteria use nitrate in place of O$_2$ for respiration, and thus are usually hypoxia tolerant as well. *Pseudomonas aeruginosa*, a heterotrophic facultative anaerobe, grows well and denitrifies under conditions similar to those expected during ecopoiesis (Hart et al., 2000). Additionally, some chemoheterotrophs utilize nitrate in place of oxygen (Oremland et al., 2002), thus avoiding the problem of hypoxia and chemosynthesis mentioned previously.

**Nitrogen fixation.** Later in the process of planetary engineering, biologically-usable nitrogen will need to be recycled from the atmosphere. Nitrogen fixation (Mancinelli, 1996)—the opposite of denitrification—would be detrimental during the initial stages, but would be required later in order to stabilize biological communities (Thomas, 1995). *Azotobacter vinelandii* and *Azomonas agilis* have the capability of fixing nitrogen at pN$_2$ as low as 5 mbar (Klingler et al., 1989). Many genera of cyanobacteria also fix nitrogen (Böhme, 1998; Fay, 1983; Friedmann and Kibler, 1980; Golden and Yoon, 1998; Mitsui and Cao, 1988; Mulholland and Capone, 2000; Olson et al., 1998; Potts et al., 1983; Schneegurt et al., 2000; Zehr et al., 2001).

**Osmotic tolerance.** The initial bodies of water that form on Mars probably will contain significant amounts of dissolved salts; they may also be transient. Pioneer microorganisms will need to tolerate osmotic stress, and survive periods of desiccation. A large number of Gram-positive bacteria form endospores that allow long periods of dormancy under adverse conditions. Many non-spor-forming bacteria also survive periods of desiccation. The period between dormancy and the resumption of metabolism should be as short as possible for pioneering marsbugs so that they can take full advantage of periods of favorable conditions. Some cyanobacteria undergo seasonal desiccation and quickly become active during wet periods (de Winder et al., 1989; Hershovitz et al., 1991a; Hershovitz et al., 1991b; Scherer et al., 1984).

**Ultraviolet and ionizing radiation resistance.** Mars has very little free O$_2$ and only a very thin ozone layer that changes with season and latitude (Barth et al., 1974; Lefèvre et al., 2004), which would offer little or no protection to potential marsbugs. Even though Mars receives about half as much total solar radiation of Earth, the amount of UV at the surface is much higher. Sun-exposed surfaces on Mars receive sterilizing doses of UV radiation. However, presumed planetary engineering processes would release additional CO$_2$ into the atmosphere, and reduce the UV flux at the surface. For example, Mars currently receives approximately 3.5 Watts m$^{-2}$ UVC at the equator during the Vernal Equinox, but with an atmosphere of 500 mbar CO$_2$, the UVC flux drops to 0.9 Watts m$^{-2}$ (Cockell et al., 2000). Also, Mars doesn't internally generate a magnetic field like Earth's, and possesses only patchy remnant crustal magnetic fields (Acuna et al., 1999; Acuna et al., 1998; Connerney et al., 2004; Connerney et al., 2001). Although some localized remnant magnetic fields are up to 30 times stronger than those of Earth (Connerney et al., 2001), the lack of a global magnetic field allows the surface of Mars to receives more cosmic radiation than Earth – 20-30 centiSieverts/year (Cucinotta et al., 2001). A marsbug would need mechanisms to resist radiation damage, and repair any damage that occurs. Endolithic cyanobacteria, which have been suggested as models for martian microorganisms (Friedmann and Ocampo-Friedman, 1994; Friedmann and Ocampo-Friedmann, 1984; Thomas and Schimel, 1991), protect themselves from UV by their habitats (porous rocks) and by the production of photoprotective pigments (Villar et al., 2005). The bacterium, *Deinococcus radiodurans*, survives very high doses of ionizing radiation due to its efficient DNA repair mechanisms (Battista et al., 1999; Bauche and Laval, 1999; Levin-Zaidman et al., 2003; Venkateswaran et al., 2000). Desiccation resistant strains of the
cyanobacterium *Chroococcidiopsis* also exhibit resistance to ionizing radiation, presumably due to efficient DNA repair as well (Billi et al., 2000). Protective pigments and repair enzymes potentially could be genetically added to other microorganisms, increasing their abilities to live in the martian environment.

**Hypobaric tolerance.** Even after initial engineering efforts, the atmospheric pressure of Mars would be far lower than that of Earth. While many bacteria can survive the desiccation that may occur at low pressure, a marsbug would have to actively metabolize and grow. As mentioned previously, bacteria may have a 25 mbar lower limit for active growth (Schuerger et al., 2006a; Schuerger and Nicholson, 2005). Bacteria have been isolated from the upper atmosphere; however, their metabolic state remains controversial (Imshenetsky et al., 1977; Wainwright et al., 2003; Wainwright et al., 2004). They may be actively growing without ever "touching ground," or they may be dormant while being transported through the air. If these bacteria do indeed remain active while in the upper atmosphere, they may have physiological characteristics that could be useful on Mars.

"Switchable" genes. While the introduction of new genes into organisms is relatively easy, their regulation is more problematic. Many of the characteristics described here are metabolic opposites (e.g., respiration and photosynthesis, denitrification and nitrogen fixation). The ability to control pioneer microorganisms over large areas with the application of a very dilute controlling agent would be highly desirable. Large numbers of genes are switched on and off in organisms that have multiple energetic pathways (Garlick et al., 1977; Guay and Silver, 1975; Sorokin et al., 2000). "Switchable" genes are also desirable in genetically engineered agricultural crops to make them resistant to environmental stresses (Sanhar and Danyluk, 1998); research in this area may also benefit planetary engineering.

A relatively small number of experiments have examined the effects of parts of Mars' environment on potential pioneer organisms, including UV radiation (Cockell et al., 2005; Hansen et al., 2005; Nicholson and Schuerger, 2005; Schuerger et al., 2003), high CO$_2$ (Hart et al., 2000; Kanervo et al., 2005; Thomas et al., 2005), low pressure and hypoxia (Boston, 1981; Kanervo et al., 2005; Paul et al., 2004). However, these experiments only integrated 2-5 of the variable conditions that would occur during ecopoiesis on Mars. To address this problem, SHOT, Inc. built a simulator capable of specifically replicating most of the environmental parameters (with the notable exceptions of gravity and cosmic radiation) on Mars during all phases of planetary engineering (Thomas et al., in review).

**ECOPOESIS SIMULATIONS**

The SHOT Martian Environment Simulator has been operational since May 2005. As of October 2005, six experiments have been performed with durations of 24 hours to six weeks (Thomas et al., accepted for publication). The initial experiments were primarily to determine logistical and analytical needs for longer-duration research, but we also determined short-term survival and growth characteristics for a variety of heterotrophic and autotrophic bacteria.

A 24-hour day was used in all experiments. Illumination was provided by a xenon arc lamp (Sylvania 69263-0 Short Arc Lamp, XBO, 1000 W/HS OFR) fitted with a solar filter that provided a close approximation of solar radiation. Photosynthetically active radiation at sample level ranged from 15 μmol photons m$^{-2}$ s$^{-1}$ in the shaded region to 1000 μmol photons m$^{-2}$ s$^{-1}$ in direct light. Total ultraviolet radiation (250-400 nm) was 1.7 μmol photons m$^{-2}$ s$^{-1}$ in the shaded region, and 50 μmol photons m$^{-2}$ s$^{-1}$ in direct light. The temperature regime cycled from a low of -80°C to a high of 26°C, diurnally. This approximated the lower latitudes of Mars during the vernal equinox (Carr, 1996), but probably is more extreme than what would occur after the initial stages of planetary engineering. As of October 2005, all of the experiments were performed at an atmospheric pressure of 100 mbar—10 times Mars' current highest pressure, but only 10% of Earth's atmospheric pressure. For experiments of 14 days or less, we used simulated atmosphere of 95% CO$_2$, 2.7% N$_2$, 1.6% Ar and 0.13% O$_2$ (Owen et al., 1977). For longer-duration experiments, we used an atmosphere of 100% CO$_2$. In most of the experiments, water was added (1 mL per day or less) in order to maintain atmospheric water saturation.

Cyanobacteria stock cultures were grown in liquid BG-11 medium, and diluted to $A_{720} = 0.25$ with fresh BG-11. Heterotrophic bacteria stock cultures were grown in trypticase soy broth (TSB), and diluted to $A_{720} = 0.25$ with fresh TSB. Several sample configurations were tested, including open trays, multi-well tissue culture plates and arrays of individual sample containers. Individual liquid cultures or mixtures of cultures were added to sterile JSC Mars-1 simulant (5-15 g, depending on the container) to the point of saturation. Desert varnish and cave microorganisms were grown on BG-11 agar. Agar cultures were then macerated and mixed with JSC Mars-1. While samples were in the simulator, parallel control samples were kept at 4°C in darkness. In cases where samples could not be analyzed at the SHOT facility, the samples were placed on ice and transported by car of by overnight courier.

Samples were analyzed for esterase activity via an assay of fluorescein diacetate (FDA) hydrolysis (Adam and Duncan, 2001; Schnürer and Rosswall, 1982) at the beginning and end of each experiment. The FDA hydrolysis assay indicates microbial metabolism across a wide variety of taxa, and correlates well with assays of respiration. Subsamples of 0.5 - 1.0 g were taken from each sample before and after each experiment and transferred into 15 mL centrifuge tubes. 5 mL of 60 mM K$_2$PO$_4$ buffer (pH 7.6) was added to each tube and briskly shaken for 10-20 seconds. Ten μL FDA in ethanol (5 mg
(mL) was added to each tube, and then all tubes were incubated for 3-5 hours at 25°C on a rocker table. Following incubation, the samples were extracted by adding 5 mL 2:1 chloroform:methanol. The samples were centrifuged for 10 minutes at 1000 x g, and the supernatant was measured spectrophotometrically at 490 nm.

Chlorophyll a extractions were also used to determine the relative abundance of photosynthetic organisms (Bowles et al., 1985; Myers et al., 1980). Subsamples of 0.5 - 1.0 g were taken from each sample before and after each experiment and transferred into 15 mL centrifuge tubes. 5 mL of 80% ethanol was added to each tube. Tubes were placed in a -20°C freezer overnight, and then were centrifuged for 10 minutes at 1000 x g. The supernatant was measured spectrophotometrically at 664 nm.

For the first two experiments (24 hours and 14 days), plate counts (Atlas, 1997) and trypan blue live-dead stains (Sigma-Aldrich Co., 2005) were also used to determine survival. However, the tests were very time-consuming with ambiguous results, and were discontinued in favor of the FDA and chlorophyll assays.

The full results of the initial experiments are reported elsewhere (Thomas et al., accepted for publication). Here, we summarize the most relevant results.

In experiments of seven days or longer duration, a "water cycle" became evident within the test chamber as water tended to condense at the ends as the chamber cooled at "night," and the samples at the ends of the chamber tended to be moister than samples in the middle. While this was disconcerting at first, it probably approximates the conditions on a planet-wide scale—some areas of Mars will be wetter than others, and life will become distributed according to moisture levels.

Live-dead microscopic assays and plate counts showed survival of all of the organisms tested in the overnight and 14-day trials. However, because of the small sizes of the cells tested, the live-dead assays were prone to large errors. Non-cellular material was sometimes counted as cells, and some cells were incorrectly counted as debris. Plate counts of cyanobacteria took 1-2 weeks in order to grow countable colonies. In addition, Plecnonema and Anabaena, being filamentous, gave underestimated results from plate counts. Further use of these assays was discontinued.

![Figure 1](image-url)  
**Figure 1. FDA hydrolysis by eubacteria and cyanobacteria after a 14-day ecopoesis trial.** Hydrolysis rates are per gram of total soil simulant. Samples were placed in two 24-well tissue culture plates—one in direct light, the other in shadow. "Atacama," "Rock Garden," and "Yungay" refer to partially-characterized, heterotrophic Atacama Desert isolates. "Atacama 2002" is a strain of Klebsiella oxytoca; "Rock Garden-2" is a strain of Bacillus licheniformis; and "Yungay-2" is another strain of Bacillus. The other "Rock Garden" strains appear to be species of Staphylococcus (but not S. aureus). All heterotrophic bacteria were exposed in JSC Mars-1 simulant amended with trypticase soy broth. The cyanobacteria (five organisms on the right side of the graph) were grown in JSC Mars-1 simulant amended with BG-11 medium. Each bar represents a single sample.
The heterotrophic bacteria, *Bacillus* and *Klebsiella*, grew very well during short-duration experiments in which they were supplied organic nutrients (Figure 1). These results indicate the hardiness of both sporogenic (*Bacillus*) and non-sporogenic (*Klebsiella*) bacteria. These organisms were discontinued in later experiments since organic nutrients are not expected to be available during the early ecopoiesis of Mars. However, since both genera are commonly associated with humans, these results have relevance for planetary protection issues associated with both robotic and human exploration of Mars. In our experiments, heterotrophic bacteria survived at least 14 days in the simulator. Although *Bacillus* spores are quickly inactivated in the presence of Mars levels of UV (Newcombe et al., 2005; Schuerger et al., 2003; Schuerger et al., 2006b), spores beneath the regolith would be protected. Similar experiments to ours (without UV) have shown even longer survival times of *Bacillus* under present day martian conditions (Nicholson and Schuerger, 2005). While these results are encouraging from a planetary engineering perspective, the presence of terrestrial "hitch-hikers" in the martian regolith could interfere with astrobiological research on Mars.

Several genera of cyanobacteria have been tested with a wide range of survival (Figures 1 and 3). Cyanobacterial survival in the simulator was somewhat correlated with CO₂ tolerance (Thomas et al., 2005), but desiccation resistance also had a role. *Anabaena, Plectonema* and *Chroococcidiopsis* appear to be very good candidates for further study. One strain of *Chroococcidiopsis* in particular (CCMEE 029), has been shown previously to have higher UV resistance than *Bacillus* (Cockell et al., 2005). UV resistance is especially important for cyanobacteria on Mars since they need exposure to light for photosynthesis, but that same exposure causes damage from UV radiation. A fine covering of regolith may provide UV shielding while still allow enough photosynthetically active radiation to reach the cells. Further research in this area will prove beneficial both for ecopoiesis research and in understanding niches for possible extant life on Mars.

Of the desert varnish and cave bacteria (Figure 2), *Pedomicrobium* had very high esterase activity after five weeks of exposure. Even though all of these strains have long generation times, *Pedomicrobium* may also be a good candidate for further study. However, at this time, most of these strains have only been partially characterized and more information is needed about their metabolism and environmental limits.

**Figure 2.** FDA hydrolysis by desert varnish and cave bacteria after a 5-week ecopoiesis trial. Pure cultures from agar plates were mixed with BG-11 amended JSC Mars-1 simulant and placed in individual polypropylene containers in direct light. Strain 1 has been identified as *Pedomicrobium manganicum* isolated from desert varnish. The other strains are partially-characterized isolates from cave and desert varnish environments. Each bar represents the mean of three samples; error bars = s.d.
CONCLUSIONS

With the increasing availability of martian environment simulators, the science of planetary engineering is moving from the theoretical to the experimental realm. Although planetary-scale ecopoesis is still in the distant future, population and community level experiments are possible and are currently in progress. In addition to providing insight about possible future life on Mars, these experiments also tell us about the survivability of Earth organisms in the present martian environment.

As additional simulators become available, voluntary standardization of environmental parameters will be desirable. We envision a consortium that would set guidelines for planetary simulators so that experiments undertaken with different simulators would still be directly comparable. Efforts to form such a consortium are currently underway.

Ecopoesis also provides an exciting opportunity to engage students in science. While many students have heard of terraforming through Star Trek and other science fiction stories, they usually do not realize that serious science underlies the fiction. Ecopoesis can be used as a focal point for discussions of geology, environmental science, microbiology, ecology and other disciplines to show the interdisciplinary nature of planetary science—whether that planet is Earth, Mars or one that has not been discovered yet.

ACKNOWLEDGEMENTS

This research was funded under subcontract agreement 07605-003-026 with NASA's Institute for Advanced Concepts (NIAC). Additional support was provided by the Arkansas Space Grant Consortium. KAC's participation was supported in part by the Arkansas Supporting Teachers Research Involvement for Vital Education (STRIVE) Program.

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