Comparison of Isolates Collected from Mars Bound Pre-Launch Spacecraft: Survival of Microorganisms Under Extreme Conditions

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Abstract

Planetary protection is governed by the Outer Space Treaty and includes the practice of protecting planetary bodies from contamination by Earth life. Mars is considered a likely place to look for extraterrestrial life, given its proximity to Earth, the presence of carbon and other essential elements, and the presence of water in some form. Although studies are constantly expanding our knowledge about life in extreme environments, it is still unclear whether organisms from Earth, traveling on Mars-bound spacecraft, can survive and grow in a Martian atmosphere where there is intense radiation, high oxidation potential and extreme desiccation. Knowing if microorganisms survive in conditions simulating those on the Mars surface is paramount to addressing the issue of whether microorganisms from Earth, traveling on spacecraft, could potentially pose a risk to future challenging planetary protection missions (i.e. a life detection mission).

Microbial isolates were collected from the Viking, Curiosity, Spirit, and Opportunity spacecraft prior to launch and tested under several extreme growth conditions. Conditions tested include high NaCl and pH, low temperatures, and anaerobic growth using various electron acceptors and carbon sources. Additionally, organisms were tested for their ability to persist after exposure to UVC radiation, desiccation, and 5% H_2O_2. It was determined that the majority of isolates belong to the *Bacillus* genus, although many of the organisms identified belong to non-spore forming genera such as *Moraxella, Staphylococcus,* and *Pseudomonas*. Most of isolates can grow in media containing 10% NaCl and in highly basic media. The majority of isolates were able to withstand desiccation for 2 weeks and many were resistant to UVC radiation. Interestingly, many isolates identified as belonging to the same genus and species showed different levels of survival under the same conditions.

The data being generated from these kinds of studies provides much needed basic information regarding the types and survivability of microorganism potentially entering space as a consequence of space exploration missions. The knowledge gained from these studies will provide a better understanding of the actual risk of forward contamination, and may lead to improved spacecraft manufacturing and sterilization methods for future missions.

Introduction

Mars is a likely candidate in the search for extraterrestrial life, both past and present, since it contains the basic requirements for life such as carbon, potential energy sources, and water in some form. Conditions on Mars are thought to be analogous to those of early Earth thus studies on the ability of organisms to survive extreme conditions such as those found on Mars is one approach to investigating the potential for life beyond Earth, and to understand how life may have evolved on Earth.

Planetary protection of Mars is governed by NASA and international policy, since microorganisms transported on the surface of spacecraft to Mars could hinder the search for past or present life on Mars. This policy restricts the spacecraft's exposed surface areas, mated surface areas, and total encapsulated volume to a bioburden level of less than or equal to 5 X 10^5 spores (NASA NPR 8020.12D). During the build up of a spacecraft, such as MSL, a microbial sampling campaign is undertaken to assess this bioburden throughout the mission build up and testing phases. The organisms that are enumerated from these campaigns are typically isolated and persevered for future study. There have been extensive studies to collect and identify the microbes that are associated with spacecraft surfaces such as that of the cleanroom facilities or ground support equipment (LaDuc et. al) however more studies looking at the microbes directly associated with the spacecraft are needed. It has been found that many of the microbes originating from cleanrooms and support equipment are resistant to extreme conditions similar to those found on Mars, but studies characterizing the ability of these organisms to utilize energy sources found on Mars are lacking. Understanding the biochemical potential and environmental tolerances of spacecraft-associated organisms would directly provide insight into whether these organisms could survive under Mars-like conditions thereby enhancing the scientific knowledge base for predictive risk assessments for future missions.

The goals of our project are to identify organisms isolated from the surfaces of the pre-launch spacecraft and investigate the potential of these organisms to withstand extreme conditions and utilize energy sources similar to those found on Mars. The information collected from this study should improve the knowledge base for predictive risk assessments for the survival of organisms to Mars and provide information as to whether organisms residing on pre-launch spacecraft are likely to survive Mars-like conditions.

Materials and Methods

Sample Collection and Identification

Samples were collected and processed by JPL's Planetary Protection Implementation Team. Samples for the NSA analysis were collected using cotton swabs (6" cotton tip applicators, 806-WC, Puritan Medical, Guilford, ME) and polyester wipes (9" ×9" ITW Alpha polyester wipes, Texwipe TX3211, Kernersville, NC), as detailed in the NASA technical handbook for the microbial examination of spacecraft hardware (NASA 2010). Swabs were then placed into water and sonicated to liberate microorganisms. Samples were heat shocked at 80°C for 15 minutes then plated onto Tryptic Soy Agar (TSA) and incubated for 3 days at 32°C. Colonies were isolated and stored. Isolates were taken from stocks, streaked onto TSA and incubated for 24-48 hours at 30°C. Chromosomal DNA was extracted and PCR was performed on the 16S rRNA gene using the universal primers 8F and 1525R. For phylogenetic analysis, 16S rRNA gene sequences were analyzed using the rRNA analysis pipeline (www.ibest.uidaho.edu/tools).

Anaerobic Growth Assays

Cells were inoculated into modified ATCC #2106 medium in an anaerobic chamber (Table 1). Growth was determined on perchlorate and arsenate cultures by performing spectrophotometer readings at 600 nm at various times over 28 days. Growth of cultures on selenite and selenate were observed visually by the formation of a red precipitate (Se°). Growth of cultures on Fe(III) were observed for visually for the reduction of Fe(III) to Fe(II) which results in a color change of the Fe from brown to black.

Aerobic Growth Studies (Temperature , pH, and NaCl)

To study growth of isolates in medium containing NaCl, aliquots of ells were inoculated into TSB without NaCl or containing 5, 10 or 20% NaCl. To determine growth of isolates at alkaline pH, cells were inoculated into buffered TSB media at pH 7-12. Cells were grown at 30°C and OD_{600} readings were taken at intervals over 28 days. To determine growth of isolates at low temperatures washed cells were inoculated into TSB and incubated at 4°C and OD_{600} readings were taken at intervals over 28 days.

Desiccation Studies

Cultures (30 µl) were added to 96 well assay plates, covered with a breathable film and left in the biosafety cabinet overnight to allow evaporation of the medium to occur. The plates were then placed into a desiccation chamber containing silica gel desiccant and left to dry for 14 days at <5% Rh. At the end of 14 days plates were removed and cells were rehydrated with 200 µl of TSB. Initial OD_{600} readings were taken immediately after rehydration and again at 24, 48, 72 and 96 hours to determine growth.

UVC Radiation Studies

Single colonies were inoculated into TSB and grown to a density of 10^8-10^9 CFU/mL. Strains were then serially diluted out to 10^{-5} and 10µL of each dilution from 10^{-5} to 10^0 were spotted onto square grid plates containing TSA. A UVP multiple ray lamp (UVP, Upland, CA) was utilized for all UV-C treatments. Output was measured using a UVP MS-100 optical radiometer with a 254nm sensor attached . Plates were then exposed uncovered for appropriate times, immediately wrapped with aluminum foil and incubated at 30°C in the dark for 24 hours.

Peroxide Tolerance Assays

Cells were grown overnight in 1 ml of TSB. Cells (93.3 µl) were transferred into 839.7 µl of PBS. H_2O_2 was added to cell suspensions (final conc = 5%) and incubated at room temperature with gentle mixing for 1 hr. Following peroxide exposure, a 100 µl aliquot of the sample was removed and 900 µl of bovine catalase (100 µg/ml) was added to the sample. After incubation for 1 hour, treated cells (10 µl) were inoculated into 240 µl of TSB and OD600 readings were taken immediately and at 24 and 48 hours. Catalase activity was confirmed by placing cells on microscope slides, adding 3% and 5 % H_2O_2 and observing for the formation of O_2.

Table 1: Anaerobic media used

Media	Electron Acceptor	Carbon Source
1	Perchlorate (10 mM)	Acetate (20 mM)
2	Perchlorate (10 mM)	Lactate (20 mM)
3	Arsenate (10 mM)	Acetate (10mM)
4	Arsenate (10 mM)	Lactate (20 mM)
5	Selenite (5 mM)	Acetate (20 mM)
6	Selenite (5 mM)	Lactate (20 mM)
7	Selenate (10 mM)	Acetate (10mM)
8	Selenate (10 mM)	Lactate (20 mM)
9	Sulfate (50 mM)	Acetate (20 mM)
10	Sulfate (50 mM)	Formate (20 mM)
11	Fe (III) (80 mM)	Acetate (20 mM)
12	Fe (III) (80 mM)	Lactate (20 mM)

Figure 2: Viking Lander . Photo Credit: NASA/JPL-Caltech/University of

Figure 3: Mars Exploration Rovers Spirit and Opportunity. Photo Credit: NASA

Figure 4: Mars Science Laboratory (Curiosity). Photo Credit: NASA/JPL-Caltech

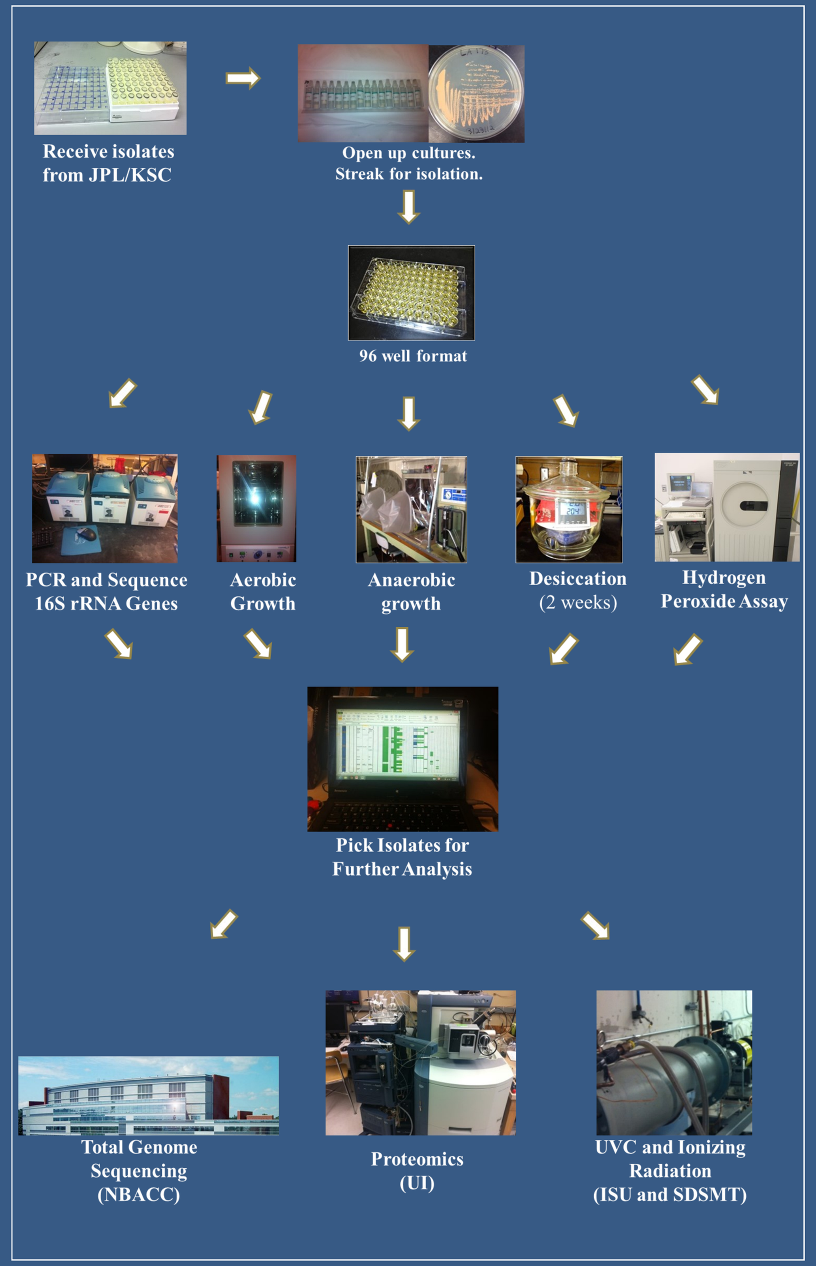

Figure 1: Process Flow

Results

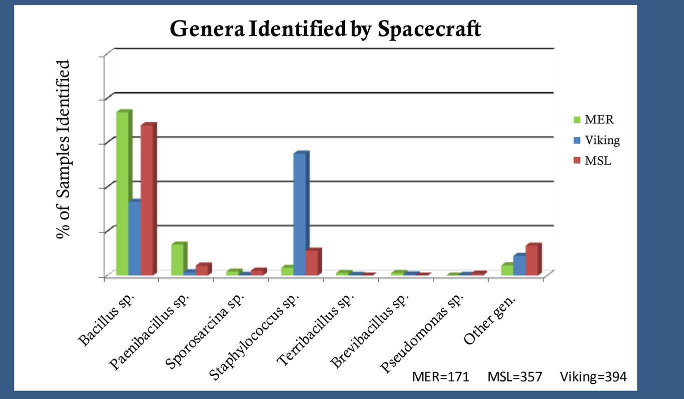

Figure 5: Genera identified on each spacecraft.

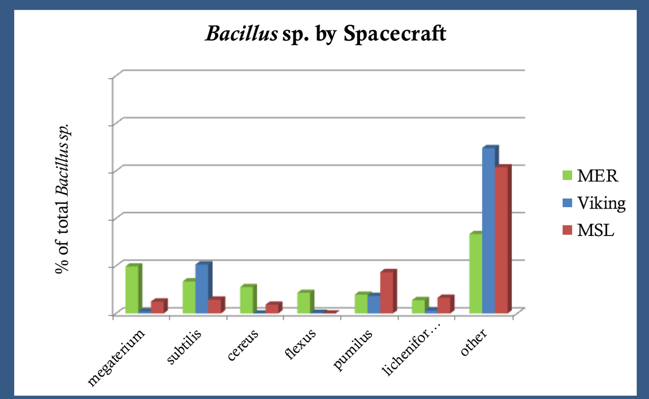

Figure 6: *Bacillus sp.* identified on each spacecraft.

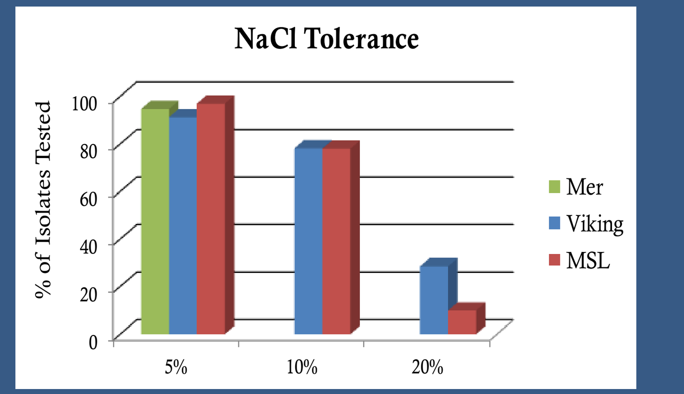

Figure 7: NaCl tolerance of isolates by spacecraft.

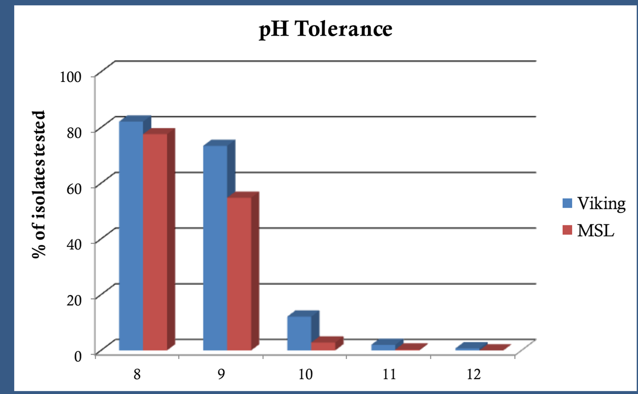

Figure 8: Percentage of isolates by spacecraft growing

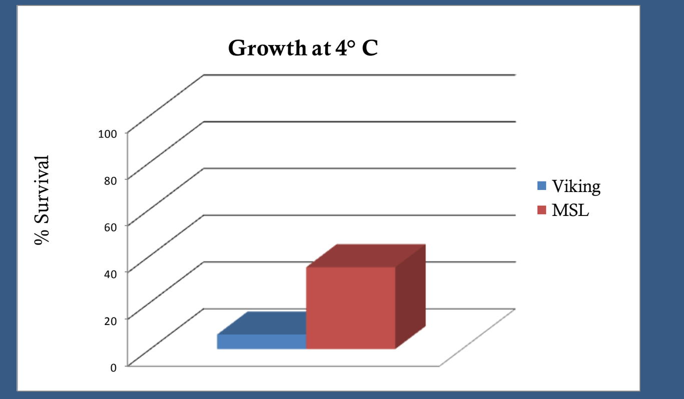

Figure 11: Percentage of organisms showing growth at 4° C.

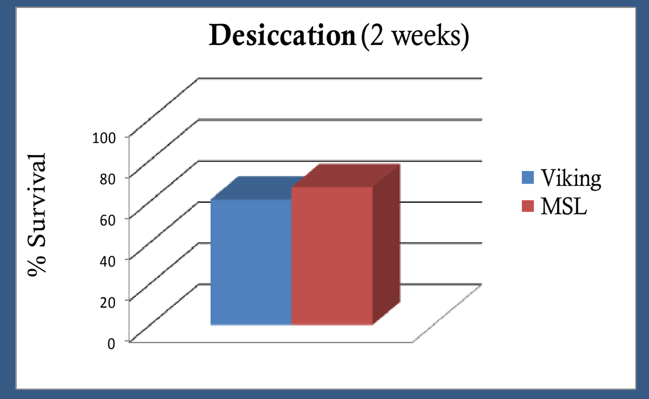

Figure 10: Percentage of organisms able to survive desiccation for 2 weeks.

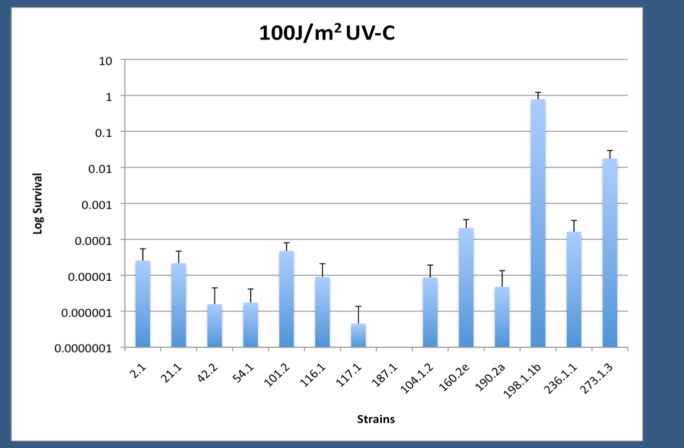

Figure 12: Survival of selected MSL isolates after exposure to 100J/m^2 UVC.

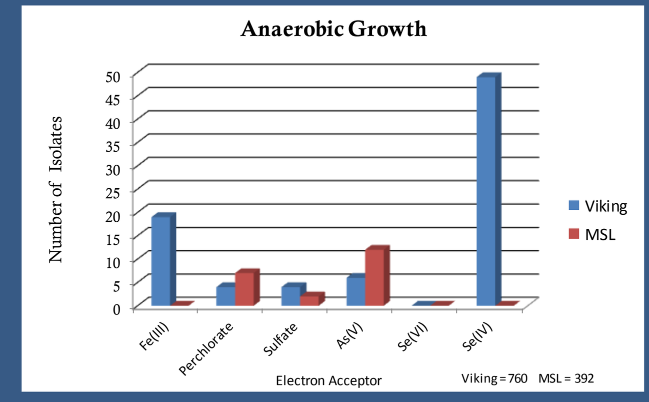

Figure 9: Number of organisms able to utilize potential electron donors available on Mars.

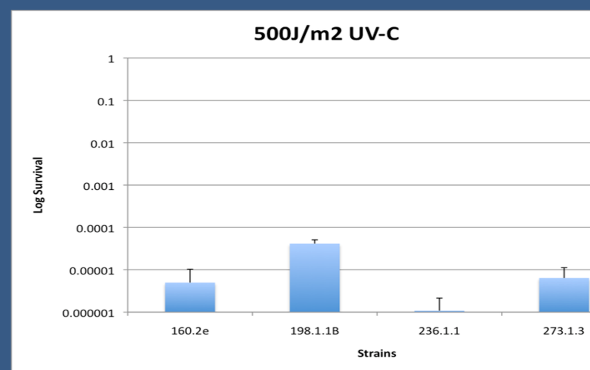

Figure 13: Survival of selected MSL isolates after exposure to 500J/m^2 UVC.

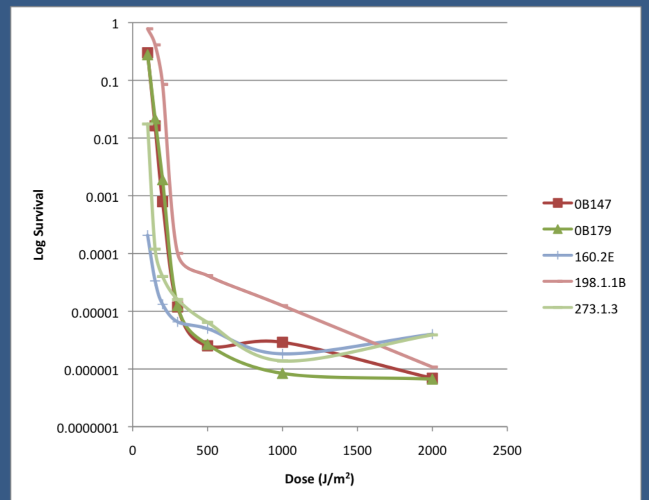

Figure 14: Survival of selected Viking and MSL isolates after exposure to 100-2000 J/m^2 UVC.

Table 2: Isolates of interest that have survived multiple extreme conditions

Isolate	Tentative ID	4° C	NaCl	pH	Desiccation	UVC (J/m^2)	5% H_2O_2	Anaerobic Growth
2.1	*Bacillus subtilis* (100%)	-	10	8	-	100	+	perchlorate
68.1	*Bacillus safensis* (99%)	+	20	9	+	NT	+	-
137.1	*Bacillus aerius* (96%)	+	20	9	+	NT	+	-
160.2E	*Bacillus amyloliquefaciens* (99%)	+	20	9	-	2000	-	-
164.1.2B	*Staphylococcus pasteuri* (99%)	+	20	9	+	NT	+	-
195.1A	*Paenibacillus lautus* (99%)	+	20	10	+	NT	+	-
198.1.1B	*Moraxella osloensis* (99%)	-	5	7	+	2000	-	-
236.1.1	*Bacillus pumilus* (100%)	-	20	10	-	300	+	sulfate
273.1.3	*Bacillus megaterium* (100%)	-	10	10	-	500	+	perchlorate
279.1.2B	*Gracilibacillus dipsosauri* (100%)	+	10	9	+	NT	-	perchlorate

Conclusions

The purpose of this project was to screen the isolates collected from pre-launch spacecraft to determine their ability to withstand extreme environmental conditions and utilize substrates for growth that are found on Mars and other celestial bodies. Although the majority of isolates are spore formers, many of these isolates (up to 25%) were found to belong to non-spore forming genera such as *Staphylococcus, Streptococcus, Moraxella, Leclercia* and *Pseudomonas* to name a few. Many of these isolates have shown ability to grow on substrates and are tolerant to simulated environmental conditions to those observed on Mars, other planetary bodies or icy moons. Of the isolates tested to-date, the anaerobic growth studies have shown that several isolates are able to grow using the limited carbon source and electron acceptor pairs employed. To further elucidate the isolates potential survivability on Mars, follow-up studies will be conducted with these isolates to verify that reduction of various compounds is directly linked to microbial growth. A majority of these isolates were able to grow in elevated salt concentrations with ~75% of the isolates able to grow in medium supplemented with 10% NaCl. Over half of the isolates can grow at pH 9, and an average of 20% of isolates show growth at low temperature (4°C).

Although the growth on Mars-simulated conditions (e.g. anaerobic substrates, salt, temperature and pH) provide a means to determine the potential ability to proliferate on Mars, the tolerance to harsh environmental stresses are equally important for the organism's survival, both in transit to Mars and on Mars. Over 60% of the organisms are able to survive desiccation for a period of 2 weeks. We wish to perform further studies to determine the maximum length of time each of these organisms can survive desiccation, since an organism traveling on spacecraft to Mars, would have to endure a 9 month trip without water before there is any potential for it to grow on Mars. Up to 20% of the MSL isolates survived treatment with 5% hydrogen peroxide (data not shown) indicating that many of these isolates would survive bioburden reduction methods which utilize hydrogen peroxide. Additionally, it has been reported that the surface ice of Europa contains as much as 0.13% hydrogen peroxide which is generated from radiolysis of ice (Johnson 2003). Thus organisms traveling to and surviving on distant planetary bodies would need the means to protect themselves from strong oxidants such as hydrogen peroxide. The ability of organisms to survive highly oxidizing conditions and UVC radiation make them more likely to survive on a spacecraft destined for Mars or other planetary bodies. In general, most of the previous studies have focused on the resistance of spore-forming isolates however, our studies show that there are several non-spore forming isolates which are able to survive multiple extreme environmental conditions. We wish to study these organisms further in hope that we can identify the resistance mechanisms utilized by these isolates that allows them to survive these extreme conditions.

These results provide an enhancement over the current knowledge base of microorganisms present and associated with spacecraft surfaces. This study also provides further detailed information regarding the physiological traits of those microorganisms and their ability to survive extreme environmental conditions analogous to those on Mars and other planetary bodies such as Europa. We expect that remaining studies will further identify organisms which exhibit unusually high resistance to stresses specific to the extreme outer planetary environment (e.g., ionizing radiation). Additional studies will also provide us with genomic and proteomic information that is invaluable for studies exploring the molecular mechanisms that could account for the stress resistances observed in our isolates. We anticipate that a basic overview will emerge thus highlighting some of the biochemical pathways and/or proteins that are recognized as important for the survival of microorganisms in harsh environments. It is also possible that we may gain insight regarding novel resistance mechanisms yet to be described. Despite undergoing bioburden reduction treatments, these isolates continue to persist on spacecraft and in clean room facilities. Currently, it is not known how the microbes adjust to such bioburden reduction technologies, and how the human-controlled environment may influence the overall evolution of the microbial population within this environment. The information collected from these studies will allow us to assess the current cleaning procedures of spacecraft components and will provide information on the ability of these isolates to withstand extreme conditions similar to those found during space travel and on Mars .

Selected References

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Acknowledgements

We would like to thank Celeste Brown at the University of Idaho for bioinformatics support. We wish to thank the non-authored MSL implementation team members, F. Morales, B,. Koukol. , M. La Duc and G. Kazarians, for the collection of these isolates, and J. Spry for his for his encouragement and management support for the work conducted at JPL . We extend our gratitude towards P. Grunther, and K. Buxbaum for their mentorship, programmatic oversight, and monetary assistance. This project was funded by NASA EPSCoR Grant # NNX11AQ30A, Idaho Space Grant Consortium (Research Initiation Grant & Fellowships), Amgen Scholars Program, National Center for Research Resources #5P20RR016448-10 and the National Institute of General Medical Sciences #8P20 GM103397-10.

For additional information on this project please visit posters 1050, 1187, 2030, and 1627.