Identification and Survival of Microbial Isolates Collected from the Viking Spacecraft Surfaces Subjected to Simulated Environmental Conditions of Mars and a Conceptualized Model for the Advancement of Connecting Stakeholders in Science, Technology, Engineering, and Mathematics (STEM) Education

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Authorization to Submit Dissertation

This dissertation of Alissa K. Korsak, submitted for the degree of Doctorate of Philosophy with a Major in Environmental Science and titled "Identification and Survival of Microbial Isolates Collected from the Viking Spacecraft Surfaces Subjected to Simulated Environmental Conditions of Mars and a Conceptualized Model for the Advancement of Connecting Stakeholders in Science, Technology, Engineering, and Mathematics (STEM) Education," has been reviewed in final form. Permission, as indicated by the signatures and dates below, is now granted to submit final copies to the College of Graduate Studies for approval.

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Abstract

Existing risk for potential contamination of celestial bodies via space exploration—in addition to the risk for returning potential extant life to Earth—prompted implementation of planetary protection policy for all outer space missions. Planetary protection helps to mitigate these risks and is comprised of an international treaty, international and national planetary protection policies, and numerous requirements that are implemented at the national level. Mars is of particular interest, since it may have regions that could potentially host microbial life. Contamination of Mars could alter its natural state and compromise any current or future life detection missions. Mars-bound spacecraft must comply with requirements pertaining to the microbial bioburden on their surfaces prior to launch. Consequently, the Viking spacecraft surfaces (NASA) were required to undergo a comprehensive microbial sampling prior to a terminal sterilization cycle and launch to enumerate the spacecraft bioburden. Approximately 1,300 microorganisms, constituting the Viking microbial archive, collected from the Viking surfaces in the late 1970s were tentatively identified, lyophilized, and stored for future studies.

This study was undertaken to begin to address whether microorganisms transported from Earth during space exploration pose a potential contamination risk to planetary protection efforts. The objectives of this study were (1) to identify the 1,300 isolates within the Viking microbial archive, (2) to determine which isolates might withstand simulated Martian surface conditions and if isolates could use energy sources potentially available on Mars or other celestial bodies, (3) to determine whether Viking isolates capable of tolerating 20% NaCl conditions can survive and grow in $MgSO_4$ at various temperatures and pH levels, and (4) to construct a conceptualized model related to partnerships within P-20

science, technology, engineering, and mathematics (STEM) education in informal and formal spaces. To promote engagement in STEM disciplines amongst students in formal and informal spaces, a conceptualized model is presented for consideration by practitioners and researchers. In the United States, student interest and achievement in STEM education is lagging behind other nations. Increasing interest and achievement in STEM disciplines is imperative for a strong future workforce in STEM fields including those pertaining to space exploration and planetary protection.

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Dedication

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List of Abbreviations

CME: coronal mass ejections

COSPAR: Committee on Space Research

DHMR: dry heat microbial reduction

GCR: galactic cosmic rays

MSL: Mars Science Laboratory

NASA: National Aeronautics and Space Administration

P-20: Pre-school through undergraduate education

PPP: planetary protection policy

SEP: solar energetic particles

STEM: science, technology, engineering, and mathematics

UV: ultraviolet

UV-C: ultraviolet C or shortwave UV

CHAPTER 1: INTRODUCTION

Contextual Background

The significance of science, technology, engineering, and math (STEM) is truly highlighted when these disciplines are discussed in respect to the future of an entire nation. STEM fields are key components of the United States' future not only because they generate innovative ideas and new businesses directly related to the growth and stability of the U.S. economy, but also help further understanding of ourselves, our planet, and the universe (President's Council of Advisors on Science and Technology, [PCAST] 2010). Space exploration is one of the many great achievements accomplished within the STEM fields and has generated a vast amount of knowledge pertaining to the universe. Preserving the natural state of planetary bodies is important for the sake of current and future life detection missions and other scientific investigations in extraterrestrial environments.

The Martian environment has proven to be an attractive place for scientists to search for extant life within the solar system. The surface of Mars hosts a number of harsh environmental conditions including extremely low water activity, very cold temperatures, high salt concentrations, and intense radiation due to a thin atmosphere (Horneck, 2008; Schulze-Makuch et al., 2008). While these conditions may seem unfavorable for most forms of life, microbial life has been found to be quite formidable and thrives in numerous extreme environments found on Earth. Consequentially, due to the risk of transporting terrestrial microorganisms to Mars or other target bodies via missions to space, all outbound spacecraft must adhere to stringent requirements pertaining to the microbial bioload on spacecraft prior to their launch (COSPAR, 2002; NASA, 2011). The United Nations Outer Space Treaty of 1967 introduced planetary protection policy (PPP) and provided a broad requirement

pertaining to the avoidance of harmful contamination of celestial bodies during all space exploration (United Nations, 1967). It is the responsibility of the interdisciplinary Committee on Space Research (COSPAR) to maintain an international PPP (Rummel & Billings, 2004). 'Forward contamination' is the process of transporting microbial life via spacecraft to celestial bodies, and 'backward contamination' is the act of bringing extant life back to Earth from spacecraft return missions (Conley & Rummel, 2010). COSPAR provides specific planetary protection requirements categorized by mission type and destination. These requirements include strict microbial reduction procedures and extensive sampling regimes of the spacecraft to ensure the number of microorganisms residing on spacecraft is below an allowable limit (COSPAR, 2002). NASA's (2011) planetary protection requirements are in compliance with the guidelines set forth by COSPAR.

The NASA Viking mission to Mars consisted of two spacecraft and the goals of the mission were, (1) to capture high-resolution images of the surface of Mars, (2) to study the characteristics of the surface and atmosphere, and (3) to investigate the potential for extant life (NASA, 2016). The Viking 1 spacecraft was launched on August 20, 1975 and Viking 2 was launched on September 9, 1975. In compliance with NASA's planetary protection requirements (NASA, 2011), over 1,300 microbial isolates were collected from the surfaces of the Viking 1 and 2 spacecraft during assembly and testing to enumerate the biological burden residing on the spacecraft prior to a terminal sterilization cycle and launch. These isolates were tentatively identified using classical bacteriological methods, lyophilized, and stored for future studies (Puleo et al., 1977). The microbiological archive collected by Puleo et al. (1977) was sent to the University of Idaho for further identification and characterization studies in collaboration with Jet Propulsion Laboratory. The research

presented in chapters three and four of this dissertation is a report on the findings from studies conducted on the microbial isolates collected from the Viking 1 & 2 spacecraft surfaces. The implementation and preservation of PPP is critical for mitigating the risk of contaminating Mars or other potentially habitable planets, and subsequently compromising any current or future life detection missions. Continued studies involving the analyses of spacecraft-associated microorganisms are needed to further the knowledge in this field and to maintain effective microbial reduction efforts in relation to planetary protection.

Recognizing the importance of bringing current and engaging scientific research content into the P-12 classroom as a way to strengthen the STEM workforce, the researcher in this study reviewed the literature surrounding partnerships and connectivity within P-20 education. As a graduate research assistant in the field of science, the author saw the need to promote STEM fields for the future of scientific endeavors and research and partnered with a faculty from the college of education and a secondary science teacher to co-create and implement a STEM-related curriculum unit in a local public school. A conceptualized model to increase connectivity between P-20 education in formal and informal spaces is presented in chapter five of this dissertation, and was realized through the author's own experiences and self-reflection as a graduate research assistant in a science discipline promoting STEM education in P-12 classrooms.

Statement of the Problem

The efforts carried out in relation to PPP help protect against forward and backward contamination. Contamination of solar system bodies could potentially hinder the ability to study celestial habitats in their natural state and may compromise any current or future life detection endeavors in addition to other scientific investigations within our solar system.

Despite the strict requirements for reducing microbial bioloads found on pre-launch spacecraft, microorganisms are still prevalent on spacecraft surfaces and in spacecraft-associated clean rooms (La Duc et al., 2007; La Duc et al., 2004; Vaishampayan et al., 2010; Moissl-Eichinger et al., 2012; Benardini et al., 2011; Ghosh et al., 2010).

Investigations into the physiological capabilities of spacecraft-associated microorganisms provide the necessary knowledge to effectively maintain regulations influencing planetary protection, since these organisms may end up on spacecraft surfaces and potentially inhabit Mars or other celestial bodies. Understanding the properties and contamination potential of these microorganisms is important for determining the risk they pose to current and future planetary protection efforts. Many studies have primarily focused on microorganisms isolated from spacecraft-associated clean rooms (Link et al., 2004; Newcombe et al., 2005; Nicholson et al., 2000; Setlow, 2006; Vaishampayan et al., 2012; Horneck et al., 2012; Stieglmeier et al., 2009; Probst et al., 2010) and relatively few studies exist that utilize microorganisms collected directly from Mars-bound spacecraft surfaces (Puleo et al., 1977; La Duc et al., 2003; Smith et al., unpublished). Insight into the diversity and capabilities of microorganisms collected directly from Mars-bound spacecraft surfaces can provide the information needed to help support the existing knowledge base related to planetary protection efforts for all current and future space missions and future manned missions to Mars or other celestial bodies.

The research presented in chapters three and four of this dissertation is related to planetary protection and is just one example of the many areas in which STEM education is critical for sustaining these scientific endeavors. Without increasing interest and achievement in P-20 STEM education, the assurance of a strong future workforce in STEM

fields including those related to NASA space exploration and planetary protection may be compromised. According to the PCAST report (2011), student interest and achievement in STEM fields in the United States is lacking. A sustainable program of STEM education is important not only in the United States, but also on a global level.

Purpose of the Study

The study was undertaken to begin to address the issue of whether microorganisms transported from Earth during space exploration missions pose a potential risk to planetary protection efforts. It is the first time molecular techniques have been utilized for the identification of the Viking microbial archive, and it is the first comprehensive study to address whether these microorganisms can tolerate simulated Martian conditions. The objectives of this study were (1) to identify over 1,300 microorganisms collected prior to a terminal sterilization and launch in the mid 1970s from the surfaces of the Viking landers and the Viking Precursor Lander and from the Viking 1 and 2 orbiters and shrouds, (2) to determine which isolates might withstand the harsh physical conditions on the Martian surface and if isolates could use energy sources potentially available on Mars as well as other celestial bodies, (3) to determine whether a subset of Viking isolates capable of tolerating 20% NaCl conditions can survive and grow in $MgSO_4$ at various temperatures and pH levels, and (4) to construct a conceptualized model related to partnerships within P-20 STEM education in informal and formal spaces.

Significance

The information generated from the scientific studies presented in chapters three and four of this dissertation provides a more thorough understanding of the diversity and physiological capabilities of the bacterial isolates collected from the surfaces of Mars-bound spacecraft.

The studies deliver additional insight needed for the protection of potentially habitable celestial environments from forward contamination. The generated results can aid in performing better evaluations of the various sterilization methods and techniques currently used in spacecraft associated settings and provide insight as to whether the bacteria collected from spacecraft surfaces may pose a risk to efforts in planetary protection. In a recent review of the MEPAG Report on Mars Special Regions (Committee to Review the MEPAG Report on Mars Special Regions et al., 2015), the authors issued a statement concerning the current bioburden assays. Their request was to have the bioburden assays altered to generate more information on non-spore-forming microorganisms that have the physiological potential of surviving conditions on Mars. Since non-spore-formers constitute a significant portion of the Viking microbial archive (Puleo et al., 1977), the experimental findings generated from this study will help fulfill the recently desired information on non-spore-forming microorganisms residing on spacecraft surfaces. On a broader scale, findings in this study may provide new insights for those who administer PPP. Current knowledge is necessary to help maintain related policy to preserve the natural state of celestial bodies, which will help to ensure the integrity of current and future scientific investigations.

Aside from the application to PPP and forward contamination, the generated information from the characterization studies of the microbial isolates collected from the Viking spacecraft surfaces can be applied to multiple fields of study. Since the microorganisms were collected from the surfaces of spacecraft residing in clean room facilities, the findings pertaining to the microbial isolates can be applied to other industries utilizing sterile conditions or clean room environments. Due to their harsh conditions for life, Venkateswaran et al. (2001) defined clean rooms as extreme environments. Such places

utilizing clean room facilities or similar sterile environments include operating rooms in hospitals and clean rooms in the pharmaceutical, food, or other technology industries. Departments and personnel focused on public health and related disinfection efforts may benefit from the findings presented in this dissertation as well. UV-C radiation is a widely used disinfection technique in a number of industries including wastewater treatment, and findings from a portion of the microorganisms subjected to UV-C radiation are presented in chapter three. Results pertaining to resistant *Staphylococcus* species are reported in chapters three and four and may be of significance when applied to healthcare settings and public health fields. The applicability of the information connected with the Viking microbial archive isolates is significant and may prove to be beneficial in a number of various fields not listed above.

The conceptualized model supported by the integrative literature review in chapter five can serve as a framework to help advance STEM education to further promote these fields and help build a strong STEM workforce for the nation's future. It is intended to increase engagement in STEM fields and provide access to STEM education opportunities by presenting an approach to create sustainable partnerships between individuals across various departments and institutions and from different disciplines.

Research Questions

Chapter 1: What does the literature surrounding PPP, potential for life on Mars, and environmental conditions on Mars today suggest about current and future PPP and implications for microbial life to survive on Mars?

Chapter 2: What is the diversity of bacterial organisms within the microbial archive collected from the Viking 1 & 2 surfaces and the Viking Precursor surfaces? What do the

results of the characterization studies suggest about the current and future efforts in planetary protection for Mars and other potentially habitable planets?

Chapter 3: Can the microorganisms within the Viking archive that are capable of tolerating 20% NaCl concentrations tolerate Mg salt concentrations? What are the effects of multiple stressors on these microorganisms?

Chapter 4: What is the current understanding of partnerships between P-12 education and post-secondary education to advance science, technology, engineering, and mathematics (STEM) education in informal and formal spaces?

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CHAPTER 2: A REVIEW OF CURRENT PLANETARY PROTECTION POLICY AND ENVIRONMENTAL CONDITIONS ON MARS

This dissertation involves an investigation of microbial isolates collected from surfaces of the Mars-bound Viking 1 and 2 spacecraft and its relation to the potential risk of interplanetary transfer of bacteria via spacecraft mission. This review of literature focuses on the existing policy, regulations, and requirements pertaining to planetary protection with an emphasis on missions to Mars and is presented for a more thorough understanding of the overall purpose of this research. To understand the contamination risk associated with bacteria residing on Mars-bound spacecraft surfaces, the currently known environmental conditions of Mars and the potential of microbial life for surviving these conditions is provided in this review. The scientific literature related to the potential for microbial life on Mars detailed in this chapter is categorized into the conditions tested in the characterization studies outlined in chapters three and four. This review of literature will provide a basis for the PPP and forward contamination risk associated with missions to Mars and will help to better understand the research presented in chapters three and four. The topics reviewed are: (1) current planetary protection policy, (2) currently known conditions of the Martian environment, (3) the potential for microbial life on Mars, and (4) literature pertaining to spacecraft-associated microorganisms.

Planetary Protection Policy

The Outer Space Treaty and COSPAR

Due to the risk of potential microbial contamination of Mars and other celestial bodies via space exploration, various requirements and protocols pertaining to PPP are implemented pre-launch to help mitigate the risk associated with all outbound spacecraft. A vast amount

of information exists on past and current PPP, therefore only the most pertinent and current requirements will be discussed. The Treaty on Principles Governing the Activities of States in the Exploration and Use of Outer Space, including the Moon and Other Celestial Bodies, more commonly known as the Outer Space Treaty, introduced planetary protection into international law. It was approved by the United Nations General Assembly in 1966 on the 19th of December and employed on October 10, 1967 (United Nations [UN], 1967). Planetary protection is discussed in Article IX of the Treaty, which states:

> States Parties to the Treaty shall pursue studies of outer space, including the Moon and other celestial bodies, and conduct exploration of them so as to avoid their harmful contamination and also adverse changes in the environment of the Earth resulting from the introduction of extraterrestrial matter and, where necessary, shall adopt appropriate measures for this purpose. (UN 1967)

The Treaty presented a general guideline to follow, making it necessary for other organizations and committees to provide an overall framework for more specific PPP. An interdisciplinary committee created in 1958 by the International Council of Scientific Unions (ICSU) called the Committee on Space Research (COSPAR) became the focal point for international activities regarding planetary protection. Both COSPAR and the International Astronautical Federation (IAF) confer about issues relating to planetary protection with the United Nations Committee on the Peaceful Uses of Outer Space (COPUOS). The responsibility of COSPAR is to maintain an international PPP (Rummel & Billings, 2004), and the Panel on Planetary Protection of COSPAR creates and issues recommendations to it as needed. In order to provide spacefaring nations an international reference for planetary protection and guidelines for maintaining compliance of international treaties and agreements, COSPAR administers and maintains PPP. COSPAR's policy states,

> The conduct of scientific investigations of possible extraterrestrial life forms, precursors, and remnants must not be jeopardized. In addition, the Earth must be protected from the potential hazard posed by extraterrestrial matter carried by a spacecraft returning from an interplanetary mission. Therefore, for certain space mission/target planet combinations, controls on contamination shall be imposed in accordance with issuances implementing this policy. (COSPAR, 2002, p. 1)

To support the policy, COSPAR developed five distinct categories that describe different target body/mission type combinations and specific requirements that are associated with each category. These requirements provide more of an in depth interpretation of the Outer Space Treaty guidelines on planetary protection. Below is a summarized version of Categories I-V of the COSPAR PPP (COSPAR, 2002):

Category I

Missions that fall under Category I include all of which are destined to a target body that is not of direct interest for the understanding of chemical evolution processes or the origin of life (COSPAR, 2002).

Category II

Category II missions are those going to target bodies that are of direct interest to understanding chemical evolution processes and the origin of life, but only have a small chance of contamination from spacecraft exploration compromising future investigations. Requirements for missions under this category entail simple documentation only (COSPAR, 2002).

Category III

The missions that fall into the third category are mainly flyby and orbiter missions destined for target bodies that are of relative interest to understanding processes of chemical evolution and the origin of life that have a significant chance of contamination, possibly

compromising scientific investigations in the future. Requirements for missions in this category include more documentation than Category II, trajectory biasing, clean rooms during assembly and testing, and possible bioburden reduction (COSPAR, 2002).

Category IV

Category IV missions are mainly lander and probe missions going to target bodies that are of interest for chemical evolution processes and the origin of life, which have a significant chance of contamination from exploration that could possibly compromise future scientific investigations. Various requirements for this category involve more detailed documentation than required for Category III missions, and include a bioassay for the enumeration of the bioburden, a contamination analysis probability, and an inventory of the bulk constituent organics. In addition, there is an increase in the number of implementation procedures from Category III, and may include trajectory biasing, clean rooms, bioburden reduction, and possibly a partial sterilization of the direct contact hardware and the hardware bioshield (COSPAR, 2002).

Category V

Missions designated under Category V include all Earth-return missions, and the primary concern with missions in this category is the protection of the terrestrial system, the Earth and the Moon. A subcategory labeled “unrestricted Earth return” is indicated for solar system bodies that are scientifically determined to have no indigenous lifeforms. For missions in this subcategory, planetary protection requirements are required on the outbound phase only and are specific to the category of that phase type. All other missions in Category V fall under the subcategory described as “restricted Earth return”. The primary concern is to avoid the destructive impact upon an Earth return mission making various containment

and sterilization procedures crucial for implementation of procedures. Throughout the return phase, any hardware that directly contacted the target body or any unsterilized material from the target body must be contained. In addition, all unsterilized samples collected from the target body returned to Earth must also be contained and analyses of the sample must be conducted using highly sensitive techniques. The return sample must either remain contained or be sterilized if there is any sign discovered suggesting the presence of an extraterrestrial replicating entity (COSPAR, 2002).

NASA's Planetary Protection Requirements

The planetary protection efforts carried out by the United States is embodied through policies administered by the National Aeronautics and Space Administration (NASA). The Associate Administrator for Space Science at NASA is responsible for the administration's planetary protection, while the Office of Space Science's Planetary Protection Officer manages the policy and administers the specific requirements pertaining to each mission. Maintaining compliance with NASA policy, the requirements for each mission are supported by the most up to date scientific knowledge available about the planetary target bodies corresponding to the specific mission. The US National Research Council's Space Studies Board (SSB) assists the Planetary Protection Officer by contributing recommendations on the requirements for a specific target body or bodies associated with a mission (Rummel & Billings, 2004).

NASA PPP is consistent with the previously described COSPAR policy (Conley & Rummel, 2008). Both policies are organized into five different categories, each pertaining to a distinct target body (destination) and mission type. Category IV covers the missions that are designed to make direct contact with Mars, and the constraints relating to biological

contamination are typically more severe. Since the conditions on Mars suggest the potential for the spread of terrestrial microorganisms on the planet, restrictions may include a complete decontamination and sterilization of the intended spacecraft (Rummel & Billings, 2004). The NASA Policy Directive 8020.12D, Planetary Protection Provisions for Robotic Extraterrestrial Missions, provides NASA's requirements for robotic planetary flight programs (NASA, 2011). The planetary protection requirements to be implemented for a specified space mission are categorized by target body and mission type with varying degrees of severity. Below is a summarized version of the planetary protection requirements outlined in the directive (NASA, 2011), which is organized into requirements for Mars, icy satellites, small solar system bodies, and Category V missions (Earth return missions).

Requirements for Mars

A mission destined for Mars would be categorized under Category III or IV because of the characteristics of the target body. For example, a mission that has no direct contact with the planet, such as an orbiter or flyby, would be placed into Category III. A mission that has direct contact with Mars, such as a lander or probe mission, would be defined under Category IV (Meltzer, 2011). Mission types in Category III consist of cruise stage, flyby, or orbiter, and requires those missions to meet either 1) an impact probability of nothing less than 0.99 for 20 years after launch and 0.95 for 20-50 years post launch, or 2) a required sterilization limit for its total surface, mated, and encapsulated bioburden level of 5×10^5 spores (NASA, 2011). The requirements for Category IV depend on the nature of the mission and are divided into three different subsections: IVa, IVb, and IVc. Lander missions not carrying life detection equipment are placed into Category IVa and the requirements state that spacecraft must meet a total surface biological burden level of 3×10^5 spores and

an average of 300 spores per square meter of exposed internal and external spacecraft surfaces. Category IVb defines lander missions that intend to investigate extant Martian life and must be in compliance with all the required planetary protection protocols for Category IVa in addition to one of the following: 1) a surface bioburden level of 30 spores on the entire landed system or a level of bioburden reduction and protection from recontamination that correlates with the sensitivity of the life-detection investigations, whichever are more stringent or 2) a life-detection subsystem instrument (those directly involved in the acquisition, analysis, and delivery of samples) sterilization cycle to these outlined levels. Any lander mission with or without life detection equipment that intends to investigate special regions must limit the entire landed system to a surface biological burden of 30 spores. The Earth return segment of a sample return from Mars is designated as "Restricted Earth return" and requires specific containment measures outlined in in Chapter 5 of the NASA Policy Directive 8020.12D (NASA, 2011).

Requirements for Icy Satellites

Chapter 5 of the NASA Policy Directive 8020.12D indicates Category II, III, and IV flyby, orbiter, and lander missions to icy satellites must implement planetary protection requirements including bioburden reduction to reduce the probability of contamination of an ocean or other liquid water body to less than 1×10^{-4} per mission. To meet the required probability calculation, planetary protection would likely involve bioburden reduction for Category III orbiters and Category IV landers with the use of clean room technology, cleaning of spacecraft associated parts prior to assembly, and monitoring of the spacecraft assembly facilities in order to gain an understanding of the existing microbial diversity. All

Category V Earth return missions for the icy satellites, Europa and Enceladus, are defined as "Restricted Earth return" and require specific containment measures (NASA, 2011).

Requirements for Small Solar System Bodies

The planetary protection requirements for Category I, II, III, or IV missions with an intended target of a small solar system body are on a case-by-case basis and are not required to have imposed forward contamination controls. These missions are typically defined as Category I or II mission types. Category V Earth return missions for small solar system bodies are determined as "Restricted Earth return" or "Unrestricted Earth return" utilizing a framework that is outlined in the 1998 report of the U.S. National Research Council's Space Studies Board. If the mission is categorized as "Restricted Earth return", specific containment measures are required (NASA, 2011).

Bioreduction

To ensure adherence to the bioburden restrictions mentioned above, various pre-launch procedures and sterilization techniques are required by NASA to achieve appropriate bioreduction levels. NASA utilizes Class 100,000 clean rooms, which act as an important defense for limiting the contamination of spacecraft. Class 100,000 clean room facilities control the particle ($\geq 0.5\mu m$) density at a level of 3,520,000 particles/m^3. To further reduce microbial contamination on surfaces and floors, cleaning by the use of various compatible organic solvents and detergents is implemented in clean rooms. Another precaution taken to protect against any microbial contamination during the assembly, testing, and launch procedures is the requirement of appropriate protective garments for individuals working in the clean rooms (NASA, 2016).

Dry heat microbial reduction (DHMR) for spacecraft sterilization has been used since the Viking Mars landers in the 1970s. The Viking landers were cleaned prior to launch in order to reduce their total biological burden to the required level and then packed into a totally enclosed bioshield. The landers were then heat sterilized for a total of 30 hours in an oven set at 111.7 degrees Celsius (NASA, 2016). This method, however, could damage the heat-sensitive equipment on present-day spacecraft. A new method, called vapor phase hydrogen peroxide (VHP), is certified by NASA for sterilization of exposed spacecraft surfaces (NASA, 2016).

Bioassay Techniques

To survey the microbial bioburden, extensive sampling during the assembly, testing, and launch operations of a spacecraft is conducted prior to launch. Similar to the sterilization method, the current standard assay method used by NASA stems from the Mars Viking spacecraft bioassay work done pre-launch in the 1970s (Puleo et al., 1977). The targets for the NASA Standard Assay are spore-forming organisms that survived heat shock, and they are obtained by the use of a cultivation-based technique (NASA, 2016). However, it must be noted, approximately half of the samples selected for the Viking microbial archive were not heat shocked during the NASA Standard Assay (Puleo et al., 1977). A single sterile cotton swab wetted with sterile water is used to collect samples off of a single area of no more than 25 cm^2. Once the sample has been collected, the swab is inserted into a test tube containing sterile water. The sample undergoes an extraction step by the use of vortex and sonication, and is then heat shocked at a temperature of 80°C. Next, the solution is then plated onto a nutrient agar and incubated at 32°C for 72 hours. The microbial colonies that grew on the

plate are counted, and contamination load levels are estimated from these numbers. The organisms collected off the spacecraft are typically preserved and stored for later studies.

Molecular-based techniques for bioburden assays include the Total Adenosine Triphosphate (ATP) assay and the Limulus Amebocyte Lysate (LAL) assay (NASA, 2016). The recent MEPAG Report on Mars Special Regions (Committee to Review the MEPAG Report on Mars Special Regions et al., 2015) requested that the current bioburden assays be altered in order to generate additional information on the non-spore-forming genera residing on spacecraft surfaces that may have the physiological potential of surviving on Mars. The authors suggested taking new molecular based techniques into consideration, as these techniques will recover a wider range of microorganisms than previous methods including non-spore-forming extremophiles (Committee to Review the MEPAG Report on Mars Special Regions et al., 2015).

Spacecraft-Associated Microorganisms

Bacteria Isolated from Spacecraft Clean Rooms

Even though the strategies discussed above, which include microbial barriers, sterilization processes, and rigorous cleaning regimes are employed in clean room facilities (Frick et al., 2014; NASA, 2016), microorganisms continue to be isolated from spacecraft clean rooms (La Duc et al., 2007; Moissl-Eichinger et al., 2012; Ghosh et al., 2010; Benardini et al., 2011; Vaishampayan et al., 2010). Venkateswaran (2001) indicated clean rooms are considered to be extreme environments due to factors such as controlled air circulation, desiccation, moderately high temperature, and low-nutrient conditions. Literature involving microorganisms isolated from spacecraft clean rooms directly relates to the studies presented in chapters three and four of this dissertation because these microorganisms may end up on

the surfaces of spacecraft during assembly and testing. Studies involving characterization of these hardy microbes provide insight into their biological capabilities and their potential risk to current and future planetary investigations.

Bacterial analyses of spacecraft-associated clean rooms indicate a wide range of microorganisms have been isolated from these environments including non-spore-forming and spore-forming organisms (K. Venkateswaran et al., 2001; La Duc et al., 2007; La Duc et al., 2009; Moissl et al., 2007; Schwendner et al., 2013; Stieglmeier et al., 2009). K. Venkateswaran et al. (2001) examined microbial populations within the Jet Propulsion Laboratory's Spacecraft Assembly Facility (JPL-SAF) by using witness plates made out of spacecraft materials, and exposed them for a time period of 7 to 9 months within the facility. The research group then collected culturable aerobic heterotrophs and heat-tolerant (80°C for 15 min) spore-forming bacteria. The results showed that mainly Gram-positive microbes and spore-forming *Bacillus* species reside in the JPL-SAF and most of the isolates were resistant to 80°C for 15 minutes. 16s rDNA showed the collected isolates belonged to the following clades: *Bacillus licheniformis, B. pumilus, B. cereus, B. circulans, Staphylococcus capitis, Planococcus sp. and Micrococcus lylae.* Studies such as these reveal the types of microorganisms found in clean room environments, thus, revealing which organisms are capable of surviving the harsh conditions found within them.

Due to their ability to survive in the extreme conditions, sporulating microorganisms are of primary interest to NASA, as they are the main targets of the NASA Standard Assay method (NASA, 2016). Previous studies have tested members of *Bacillus* genus isolated from spacecraft clean rooms to simulated extreme environments found on the surface of Mars (Link et al., 2004; Newcombe et al., 2005; Nicholson et al., 2000; and Vaishampayan

et al., 2012). Link et al. (2004) tested bacteria isolated from spacecraft clean room environments to one of the harshest environmental conditions on the surface of Mars, UV radiation. The researchers found spores of *B. pumilus* SAFR-032 isolated from the Spacecraft Assembly Facility at NASA's Jet Propulsion Laboratory (JPL-SAF) demonstrated significantly elevated UV resistance.

The study conducted by La Duc et al. (2007) involved testing both non-spore-forming and spore-forming microorganisms to analogous conditions of Mars. The researchers discovered some non-spore-forming bacteria were able to tolerate the environmental stresses tested in the study and they identified a diverse group of bacteria other than *Bacillus* species capable of surviving the harsh clean room environment. Stieglmeier et al. (2009) and Probst et al. (2010) have addressed metabolic capabilities of microbial communities isolated from spacecraft-associated clean rooms. In the study conducted by Stieglmeier et al. (2009), the researchers identified a wide range of facultatively anaerobic bacteria within European spacecraft-associated clean rooms and the Herschel Space Observatory. Strictly anaerobic bacteria, *Clostridium* and *Propionibacterium*, were isolated from spacecraft-associated clean room environments for the first time.

Bacteria Isolated from Spacecraft Surfaces

Studies involving microorganisms collected from the surfaces of spacecraft are directly related to the scientific studies presented in the next two chapters of this dissertation. Fewer studies exist that address the microbial isolates residing on spacecraft surfaces as compared to studies involving microorganisms isolated from spacecraft clean room facilities. Nonetheless, the information provided by these studies is valuable to efforts in planetary protection for current and future missions.

In the study conducted by La Duc et al. (2003), microorganisms isolated from the Mars Odyssey spacecraft were identified as members of the following genera: *Acinetobacter, Bacillus, Curtobacterium, Microbacteriumm Delftia, and Ralstonia.* A novel species, *Bacillus odyssey sp. nov,* was isolated from the Mars Odyssey spacecraft and was found to be resistant to desiccation, H_2O_2, and UV and gamma radiation (La Duc et al., 2004). Studies conducted by Newcombe et al. (2005) and Horneck et al. (2012) investigated the physiological capabilities of microbial isolates collected from spacecraft surfaces. Newcombe et al. (2005) subjected spores collected from spacecraft surfaces and various assembly facilities to UVA, UVA+B, and the full UV spectrum. The researchers reported that 19 isolates out of the 43 *Bacillus* spore lines tested by the researchers demonstrated UVC resistance at doses of 1,000 J m^{-2}. *B. pumilus* was found to be the most resistant of the *Bacillus* species tested.

Comprehensive studies conducted on the microbial archives collected directly from spacecraft surfaces are limited (Smith et al., unpublished; Puleo et al., 1977), but provide a more comprehensive view of the cultivable microorganisms residing on spacecraft surfaces. A study conducted by Puleo et al. (1977) in the 1970s involved the comprehensive sampling (6,683 samples total) of the microorganisms collected from the Viking 1 & 2 spacecraft (landers, orbiters, and shrouds) and Viking Precursor (lander and orbiter) surfaces and the identification of the resulting 1,294 microbial isolates by classic bacteriological methods used at that time. The 1,294 microbial isolates are the same microorganisms that constitute the Viking microbial archive, which are used in the studies presented in this dissertation. Puleo et al. (1977) found the majority (75%) of microorganisms collected from the surfaces of the Viking spacecraft were associated with the human body including genera from

Micrococcus, Staphylococcus, and *Corynebacterium-Brevibacterium.* This is likely due to the large number of people that are in and out of the clean room facility during the assembly and testing of a spacecraft. Technicians, engineers, and other personnel are continuously working in the clean room prior to launch and possibly shedding microorganisms from their skin that could end up on the surfaces of the spacecraft. A total of 343 isolates were identified as members of the *Bacillus* genus, and the study indicated a higher number of spores were found on the Viking Lander Capsules (landers) than found on the orbiters. Chapter three of this dissertation discusses the results in depth of both this current study conducted on the microorganisms in the Viking microbial archive and those of the past study by Puleo et al. (1977).

The Martian Environment

The potential for microorganisms that reside on the surfaces of outbound spacecraft being transported to space via space missions creates the existing risk for the possible contamination of Mars and other celestial bodies. To help mitigate any risk associated with space exploration, planetary protection requirements are implemented for all outbound missions. The requirements for Mars are based on the existing knowledge of the Martian environment. The most recent environmental conditions are taken into consideration along with the existing knowledge of the physiological abilities of terrestrial microorganisms to develop recommendations pertaining to planetary protection. Since the microorganisms utilized in the studies presented in this dissertation were collected from the Mars-bound Viking spacecraft and may pose a threat to planetary protection efforts for Mars missions, the literature regarding the environmental conditions on Mars will be reviewed in the following section.

General Environment

Mars hosts an extremely frigid environment, capable of maintaining below freezing temperatures with an average surface temperature of -65°C that can range anywhere from -10°C to -76°C (Horneck, 2008; Schofield, 1997). The major component of the Martian atmosphere is CO_2 at 95.3% with N_2 content coming in second at 2.7%. The O_2 levels are the lowest making up only 0.1% of the Martian atmosphere. On Earth, the atmosphere differs immensely from that of Mars, consisting of 0.03% CO_2, 78.1% N_2, and 20.9% O_2. Hassler et al. (2014) and Horneck (2008) indicated that since Mars has a thin atmosphere (less than 1% of Earth's atmosphere) in addition to lacking a global magnetic field, it does very little to protect against high-energy particles. The cosmic and particular radiation found on Mars is much higher than that found on Earth. The radiation comes from two different sources, galactic cosmic rays (GCRs) and solar energetic particles (SEPs). Due to their high energies, GCRs can penetrate as much as several meters into the regolith of Mars and therefore, are difficult to ward off. SEP producing events, such as flares, coronal mass ejections (CMEs), and the shocks associated with those, originate from the Sun and are often difficult to predict (Hassler et al., 2014). Because of the thin atmosphere, Mars hosts a wide spectral range of solar UV radiation that includes UV-C. Since the CO_2 on Mars is a primary absorber of short wavelength UV radiation, >200 nm wavelengths impact the Martian surface (Horneck, 2008).

Adding to the complexity of the high-energy radiation on Mars, secondary particles can form when GCRs and SEPs are energetic enough to interact with the Martian soil or regolith (Hassler et al., 2014). Horneck (2008) calculated the annual dose rate of radiation on Mars to be approximately 100 times higher than on the surface of Earth. Hassler et al.,

(2014) presented radiation dose measurements from galactic cosmic rays and solar energetic particles taken via the Radiation Assessment Detector (RAD) on the Mars Science Laboratory's (MSL) Curiosity rover. The researchers found an average total GCR dose rate of 0.210 ± 0.040 mGy/day at Gale crater on the Martian surface, and a dose rate of 0.48 ± 0.08 mGy/day measured during the cruise to Mars inside the spacecraft. The two dose rates differed in value because of several influences, but a reduction in the dose rate by a factor of ~2 was observed due to the planetary shielding of the lower hemisphere. The actual absorbed dose on Mars was 76 mGy/year at the surface.

In addition to the multiple forms of radiation present on the Mars, the Martian environment is also quite arid, which is another condition that is not conducive toward harboring life. The Viking 1 & 2 landers found water contents in the Martian regolith ranging from 1-3 wt% (Anderson & Tice, 1979), and just recently, Curiosity discovered a similar figure of 2.25 wt% water in the soil at the Gale Crater site (Meslin et al., 2013). While these figures expose the hydration levels of the soil on the Martian surface, there is still potential for additional sources of water below the surface. Many studies (Malin & Edgett, 2000; Malin & Edgett, 2003; McEwen et al., 2011; McEwen et al., 2014; Martínez & Renno, 2013) have provided evidence that liquid water had once existed or still exists on Mars today. It is unknown whether there may be subsurface sources of water, however there is much speculation suggesting the possibility for them. Martínez & Renno (2013) suggest the possibility of liquid water and liquid brines in the shallow subsurface of Mars since only a thin layer of soil is needed to create a barrier against sublimation, at least temporarily. Another possible location for liquid water on Mars is even deeper below the surface where there is the potential for low enough soil conductivity blanketing this area, in turn, causing

ice deposits to melt (Martínez & Renno, 2013). A recent study by Ojha et al. (2015) has produced the strongest evidence to date that liquid water exists on Mars today. By analyzing data collected via the Compact Reconnaissance Imaging Spectrometer for Mars Instrument located on the Mars Reconnaissance Orbiter, the researchers discovered evidence for the presence of hydrated salts at four different locations where recurring slope lineae are most extensive. The study strongly supports the notion of contemporary water activity on Mars as the explanation of the recurring slope lineae.

There is evidence the Martian environment contains high levels of salts (Sawyer, 2000; Treiman, 1999; Vaniman et al., 2004; Wang et. al. 2006), and any life form that could potentially exist on Mars must be able to tolerate the high salt conditions in order to survive and grow. The salts present in the Martian environment are in the forms of MgSO4, CaSO4, FeSO4, MgCl, NaCl and CaCl, however, the Mg, Na, or Ca chlorides exist at concentrations significantly lower than the various forms of sulfates (4:1 ratio) (Crisler et al., 2012). The potential for melting water ice on Mars exists, which may form saline flows within the permafrost (McEwen et al., 2011), so any potential life would be required to tolerate high salt concentrations (Crisler et al., 2012; Landis, 2001).

The results from the Wet Chemistry Laboratory on the Phoenix Mars Lander showed the Martian soil at the Vastitas Borealis site was moderately alkaline with a pH of 7.7 ± 0.5 (Hecht et al., 2009). However, from the recent Curiosity mission, preliminary data provides evidence of potential ancient acidic conditions on Mars (NASA, 2015). Although there is still much to learn about the various pH levels on Mars, bacteria capable of withstanding a wide range of pH would likely have a greater chance of survival in the Martian landscape. The regolith on Mars consists of olivine-rich basaltic rock as well as jarosite, a ferric sulfate

mineral, and gypsum, a calcium sulfate-containing mineral. Additionally, there is likely a sufficient amount of nitrogen and sulfur in the soils on Mars to potentially sustain microbial life (Crawford & Newcombe, 2008). The Wet Chemistry Laboratory on the Phoenix Mars Lander discovered 0.4 to 0.6% perchlorate (ClO_4) by mass in each sample from the Vastitas Borealis region (Hecht et al., 2009). The minerals and compounds described above have the possibility to be utilized as an electron acceptor to help to generate energy for various genera of bacteria. Although it may seem virtually impossible for any form of life to persist in some of the extreme environmental conditions described above, microbial life has shown to be surprisingly tough and capable of surviving extreme conditions on Earth.

Special Regions

COSPAR defines a Special Region as, "a region within which terrestrial organisms are likely to replicate" or "any region which is interpreted to have a high potential for the existence of extant martian life forms" (COSPAR, 2015, p. A-4). Special Regions are defined in relation to the knowledge surrounding terrestrial organisms, and include areas with sufficient water activity and adequately warm temperatures to allow Earth microbial life to replicate. COSPAR provides a lower limit of 0.5 and an upper limit of 1.0 for water activity, and a lower limit of -25°C for temperature. No upper limit for temperature is defined. Various features with a strong potential for liquid water to persist should be defined as special regions and include gullies, bright streaks associated with gullies, pasted-on terrains, and subsurface below 5 meters. Other possible sites may include dark streaks, geothermal sites, fresh craters with hydrothermal activity, modern outflow channels, or areas with recent seismic activity (COSPAR, 2002). These regions are the most likely places

within the Martian environment for life to exist, and special planetary protection requirements for current and future missions are critical.

Potential for Life on Mars

Microorganisms are found in nearly every type of environment on Earth, and the terrestrial life able to withstand extreme environmental conditions on Earth, serve as a valuable indicator for what type of life may potentially survive the harsh conditions of Mars. Scientific information surrounding the topics of terrestrial microorganisms capable of tolerating extreme environments and conditions on Earth will help to understand the contamination risk posed by the bacterial isolates residing in clean rooms and on spacecraft surfaces. Understanding the physiological capabilities of terrestrial extremophiles is crucial for maintaining appropriate planetary protection measures for space exploration. This review of literature is divided into sections pertaining to the simulated conditions of Mars tested in the studies presented in chapters three and four.

High Salt Tolerance

Halophilic microorganisms inhabit a wide range of terrestrial environments including those with low salt concentrations to some of the most hypersaline environments that are known to exist on Earth. Although there is no exact definition for the term 'halophilic' (Oren, 2008), there are specific categories that were defined by Kushner (1978) over thirty years ago that are still widely used to explain the relation between a microorganism and salt. Kushner's categories consist of extreme halophiles, which grow best in media with 2.5-5.2 M salt, borderline extreme halophiles, which grow best in media with 1.5-4.0 M salt, and moderate halophiles, which grow best in media with 0.5-2.5 M salt. Halotolerant microorganisms, as Kushner (1978) defines them, are those that don't demonstrate a need for salt in order to

grow, but can grow up to very high salt conditions (>2.5 M is considered extremely halotolerant) (Kushner, 1978). Halophilic and highly halotolerant microorganisms have been discovered in all three domains of life consisting of Archaea, Eucarya, and Bacteria (Oren, 2002).

Chaotropic salts are strong inhibitors of cellular systems due to their ability to destabilize biological macromolecules and activate a strong stress response (Hallsworth et al., 2003). Hallsworth et al. (2007) has shown $MgCl_2$ to be an exceptionally chaotropic solute, which makes an $MgCl_2$ rich environment seem nearly uninhabitable. However, a study conducted by van der Wielen et al. (2005) reported on the discovery of a metabolically active microbial community in deep hypersaline anoxic basins in the Mediterranean Sea that are nearly saturated with $MgCl_2$ (5M), making it one of the most extreme saline environments known on Earth. Aside from the microorganisms' remarkable ability to grow in such an extreme hypersaline environment, they were also part of sulfate reducing methane generating heterotrophic activity within the deep basins. The microbial community discovered consisted of a wide diversity of prokaryotes that also included a new and deeply branching order within the *Euryarchaeota* different from any communities within seawater. These results provide evidence for potential microbial life that exists in other environments with similar extremes to those found on Mars.

In order to tolerate various salt concentrations on Earth, microorganisms have developed specific mechanisms that help them adjust to the specific salt levels present in their surrounding environment. Oren (1999) explains how microorganisms thriving at high salt concentrations may be required to keep their cytoplasm at least isosmotic with their extracellular environment. This is because cells cannot maintain water activity of their

cytoplasm higher than their salty environment because biological membranes are permeable to water and would result in water loss out of the cell into the environment. The author goes on to describe two strategies microorganisms use to tolerate the osmotic stress associated with environments containing high salt concentrations. The first, called the "salt-in" strategy, is where the cell sustains a high intracellular salt concentration that is at least equivalent to the osmolality of the external concentrations, and therefore, must adjust its intracellular systems accordingly. The second strategy is called the "compatible-solute" strategy in which low salt concentrations are sustained within the cell and the osmotic potential is balanced with organic compatible solutes making there no need for any adaptation of intracellular systems (Oren, 1999). Whether a microorganism can thrive in high salt is determined by two main factors: "(i) the amount of energy generated during its dissimilatory metabolism [and] (ii) the mode of osmotic adaptation used" (Oren, 2011, p. 1908). When the "salt-in" strategy is compared to the "compatible-solute" strategy, it has a relatively low energetic cost (Oren, 2011).

Choi et al. (2014) investigated the characteristics of halotolerance in a strain of *Staphylococcus* isolated from fermented seafood containing a high concentration of NaCl via enzymatic, genomic and transcriptomic analyses. The strain, *Staphylococcus* sp. OJ82, grew at and retained β-galactosidase activity up to 25% NaCl concentrations in rich media and genes concerning cell membranes, transport, osmotic stress, ATP synthesis, and translation were highly expressed under high salt conditions. The data revealed the strain adapted to high salt conditions through the expression of core cellular pathways consisting of translation and ATP production; and defense genes, including membrane synthesis, compatible solute transports, and the ribulose monophosphate pathway. The data showed

Staphylococcus sp. OJ82 cells are protected from bacteriocin under high salt conditions (NaCl 11%), but were not protected at 1% NaCl concentrations (Choi et al., 2014). Studies pertaining to microorganisms thriving in extreme saline environments on Earth promote the notion that life may be able to exist elsewhere in the Universe, such as Mars, where similar high salt conditions are likely present.

Desiccation

For microorganisms to possibly pose a risk to efforts made in planetary protection, the ability to survive extreme desiccation is crucial. The microorganisms must survive in the moderately dry clean room environment on the surface of the spacecraft for days, months or even years before it is launched. With controlled air circulation, desiccation, moderately high temperature, and low-nutrient conditions, clean rooms are considered to be extreme environments (K. Venkateswaran et al, 2001) that are created by humans to control microbial contamination. Secondly, any organisms residing on the external surface of spacecraft that survived the clean room environment must also endure the 9 month long trip to Mars exposed to high radiations, intense space vacuum and below freezing temperatures, and, if able to survive the trip, they are faced with the challenge of the harsh desiccating environment that exists on the surface of Mars.

Dehydration can cause extensive damage to the cell components of a microorganism ultimately impairing their function, including alterations in the permeability of a cell's membrane, inhibited or altered enzyme or other protein activity and function, and changes in DNA genetic information (Horneck, 2010). Resistance of endospores to desiccation and space vacuum is largely due to factors including the cortex, spore coat layers, a dehydrated core encased by a thick envelope for protection, and DNA protection from small proteins

(Nicholson, 2000; Horneck, 2010; Checinska et al., 2015). Even though they are non-sporulating, due to the strength of their oxidative stress resistance mechanisms, *Deinococcus radiodurans* species are capable of surviving all reactive oxygen species (ROS), including desiccation and ionizing radiation (Slade & Radman, 2011). In a study by Dose et al., (1995), *D. radiodurans* species were tested under extreme open space conditions. The cells were subjected to the intense dehydrating conditions of open space for 7 months within the Exobiology and Radiation Assembly (ERA) onboard the European Retrievable Carrier (EURECA), which was designed to study effects of space on biological systems (Dose et al., 1995). Researchers concluded the fate of the *D. radiodurans* cells tested was a complete loss in viability due to strong DNA lesions. As part of another ERA experiment, Horneck et al., (1995) studied spores of *Bacillus subtilis* after they were dried in monolayers and exposed to a space vacuum environment for 10 days. The research team found that the wild-type strain of *B. subtilis* showed 70% survival after subjected to the previously mentioned environmental conditions of space.

Radiation

Studies (Mattimore & Batista, 1996; Musilova et al., 2015) have found a direct correlation between radiation and desiccation resistance in microorganisms. Musilova et al. (2015) discovered that a 5-day increase in microbial desiccation resistance coincides with a 1 kGy irradiation survival at room temperature. A. Venkatswaran et al. (2001) studied the survival of *Deinococcus radiodurans* under extreme continuous radiation conditions and found the bacterium was capable of surviving 6,000 rad/h (60 Gy/h) in rich medium. Abrevaya & Paulino-Lima (2011) tested *D. radiodurans* under vacuum UV irradiation conditions, and the survival of *D. radiodurans* was 1% after a vacuum UV radiation exposure of 1350 J/m^2.

As previously discussed, *D. radiodurans* species are capable of surviving such extremes such as desiccation and ionizing radiation due to their exceptional oxidative stress resistance mechanisms (Slade & Radman, 2011). Lockhart & DeVeaux (2013) found that the high radiation resistance seen in *D. radiodurans* and normal cellular metabolism is partially attributable to the expression of the gene *ssb.* The researchers found tolerances to both ultraviolet and ionizing radiation were both significantly impacted in *D. radiodurans* by reduced *ssb* expression.

Spore-forming bacteria are known for their resistance to a variety of environmental extremes (Nicholson et al., 2000). Wassmann et al. (2012) found that 100% of *Bacillus subtilis* MW01 spores were capable of surviving simulated Martian conditions if shielded against the solar irradiation. Interestingly, researchers have found there is an increased microbial radiation resistance at -79°C than when observed at higher temperatures (Musilova et al., 2015; Dartnell et al., 2010). Musilova et al. (2015) found a nine-fold increase in radiation resistance at -79°C. *D. radiodurans* only showed a slight decrease in viable population when exposed to radiation doses of 15,200 Gy, which was the maximum dose tested (Dartnell et al., 2010).

Cold Temperatures

Psychrophilic microorganisms, which include those referred to as obligate psychrophiles, are defined as having an optimal temperature for growth at approximately 15°C or lower, with maximal and minimal temperatures of 20°C and 0°C or lower, respectively. The term ‘psychrotroph’ is used for the microorganisms that do not adhere to the exact definition of ‘psychrophile’ (Morita, 1975). On Earth, approximately 85% of the biosphere is continuously exposed to temperatures below 5°C making it a seemingly impossible

landscape to thrive in. However, a wide range of microorganisms can be found living at extremely cold temperatures, including bacteria, archaea, yeasts, filamentous fungi and algae (Margesin & Miteva, 2011).

Yukimura et al. (2009) isolated and identified spore-forming moderately halophilic bacteria in the Arctic terrains of Greenland. Ten strains were isolated from a glacial moraine in Qaanaaq, Greenland, where temperatures generally stay below 0°C and can fall to -30°C. The strains were identified by 16S rRNA gene sequencing and of the ten strains, five were most closely related to the genus *Oceanobacillus*, two to the *Bacillus* genus, one to *Omithinibacillus,* one to *Gracilibacillus,* and one to *Virgibacillus.* Aside from supporting the salt tolerating (all multiplied in 12% NaCl medium) and cold-enduring nature of these microorganisms, the results also support the idea of potential long-range transportation, such as atmospheric transport, since two of the near full-length 16S rRNA gene sequences from the Arctic were a 100% match to strains found in desert sand in China, which were most closely related to *Bacillus lichenformis* and *Oceanobacillus picturae* (Yukimura et al., 2009). Isolated from a permafrost borehole in northeastern Siberia, six bacterial isolates were found to be capable of growth at 0°C, low pressure (7 mbar), and a CO_2-enriched anoxic atmosphere. Permafrost is considered a terrestrial analog to Mars since most of the Martian water is frozen within the regolith. The six strains were identified by 16S ribosomal DNA analysis and all were most closely related to the genus *Carnobacterium*, with five most closely related to *C. inhibens* and one to *C. viridans*. In addition to these six isolates, further growth assays showed all nine type species of *Carnobacterium* were capable of growth under cold, low-pressure, and anoxic conditions (Nicholson, 2013). A bacterium isolated from high Arctic permafrost, *Planococcus halocryophilus* strain Or1, was able to

grow and divide at -15°C (Mykytczuk et al., 2013). The researchers found that the bacterium's increased flexibility and stability of proteins and a unique cell enveloping membrane are responsible for its growth in subzero temperatures.

Microorganisms, such as the ones mentioned above, have made various adaptations and developed mechanisms to be able to survive in such cold environments. Studies performed by Mitsuya et al. (2013) show *Shewanella frigidimarina* K14-2, a psychrophile isolated from a fish spoilage test during low-temperature storage by Fujii (1988) and was active at near freezing temperatures, has evolved characteristics providing conformational flexibility of proteins enabling their catalytic activity in low temperatures (Mitsuya et al. 2013). Studies performed on psychrophilic microorganisms found on Earth can help to assess what types of life could potentially survive in the cold environments found Mars.

Heat

Each of the Viking landers was subjected to a terminal dry-heat sterilization prior to launch, and consisted of a cycle at 111.7 ± 1.7°C for 23 to 30 hours after the coldest point reached a temperature of 111.7°C (Puleo et al., 1977). Dry-heat microbial reduction is still considered the 'gold standard' for microbial reduction and it is the only sterilization method approved by NASA for reducing the encapsulated bioburden of spacecraft. A D-value corresponds to the measurement indicating the time needed for one log reduction of the total microbial population, and temperatures used range from 100-200°C (Frick et al., 2014). Although it seems extreme heat would be lethal for many types of microorganisms, as previously mentioned, bacterial spores are known for their impressive resistance to various environmental extremes (Nicholson et al., 2000). K. Venkateswaran et al. (2001) investigated the microbial diversity of the Jet Propulsion Laboratory Spacecraft Assembly

Facility (JPL-SAF), and the research team found that most of the microorganisms isolated were resistant to 80°C temperatures. The isolates represented 4 different genera and consisted of *Bacillus licheniformis, B. pumilus, B. cereus, B. circulans, Staphylococcus capitis, Planococcus sp.* and *Micrococcus lylae* (K. Venkateswaran et al., 2001). Schubert & Beaudet (2011) subjected spores of the *Bacillus* strain ATCC 29669, a heat resistant microorganism isolated from a spacecraft assembly area, to constant temperatures within the range of 125°C to 200°C. The researchers determined the D-values obtained for the *Bacillus* strain ATCC 29669 were 20 to 50 times longer than the values obtained for *B. atrophaeus* ATCC 9372, which is used as a standard indicator spore by NASA. Schubert & Beaudet (2011) concluded strain ATCC 29669 could serve as an adequate future model for heat-tolerant bacterial spores.

Alkaline pH

A range of bacterial strains are capable of tolerating alkaline pH levels indicating the potential for terrestrial microorganisms to tolerate the pH conditions found on Mars. Roadcap et al. (2006) found a variety of alkaliphilic β-Proteobacteria, *Bacillus,* and *Clostridium* species capable of growth in pH levels up to 13.2. *Bacillus firmus* OF4, a facultative alkaliphile, is capable of growing well within a range of pH 7.5 to 10.5 (Guffanti & Hicks, 1991).

Krulwich et al. (2011) explains how bacteria can overcome the challenges of varying levels of pH in their environment by maintaining a pH homeostasis. The first strategy the authors describe involves bacteria utilizing various active strategies for maintaining a cytoplasmic pH homeostasis that include use of transporter systems including proton-pumping respiratory chain complexes, proton-coupled ATPases, and cation-proton

antiporters. These systems perform active proton uptake or efflux depending upon the external proton concentrations present in a given environment. The second strategy involves the use of metabolic pathways to either consume or generate protons within the cytoplasm of the bacterium to support pH homeostasis. For example, the expression of specific cytoplasmic proton consuming enzymes including various hydrogenases and amino acid decarboxylases is increased when bacteria are subjected to acidic environmental conditions (Slonczewski et al., 2009; Krulwich et al., 2011; Maurer et al., 2005; Stancik et al., 2002). Bacteria have developed strategies to tolerate pH fluctuations in their environment and this capability would be required for microorganisms to survive the various pH levels encountered on Mars.

Alternative Energy Sources

The ability to utilize alternative energy sources is important for microorganisms to increase their chances of survival in environments with limited nutrients, such as those of various celestial bodies including Mars. As indicated by the literature surrounding this topic, a range of bacteria exists that are capable of growth by utilizing alternative sources for energy. As previously mentioned, 0.4 to 0.6% perchlorate (ClO4) by mass was discovered in samples taken from the Vastitas Borealis region (Hecht et al., 2009). Bacteria capable of reducing (per)chlorate isolated thus far represent a phylogenetically diverse group (Coates et al., 2004). Coates et al. (1999) determined that dissimilatory reduction of (per)chlorate was more diverse and expansive than what was believed about the process before the study. The researchers identified bacteria capable of reducing (per)chlorate from the genera *Pseudomonas* and *Azospirllum* as well as other organisms that represent new genera within the *Proteobacteria.* Crawford & Newcombe (2008) discussed the possibility of methanogens

on Mars, and they concluded the CO/H_2 combination required for the growth of methanogens is likely available within the Martian environment. A study conducted by Kral et al. (2003) reported on three different methanogen species that were capable of growth on simulated Martian soil with the addition of carbon dioxide, hydrogen, and water. The soil in the Martian environment also contains forms of iron (Crawford & Newcombe, 2008), and Lee (2013) indicates a wide range of dissimilatory iron-reducing bacteria (DIRB) exists. The reduction of Fe(III) within the genus *Shewanella* occurs as the result of various electron transport systems including direct electron transfer via an Mtr pathway, indirect electron transfer by the "electron shuttling mechanism", and direct electron transfer by the pili (Esther et al., 2014). Studies have shown bacteria have developed strategies to survive in environments with limited nutrients, and the possibility of bacteria utilizing various electron acceptors for energy in the Martian environment is plausible.

Planetary Protection Considerations

The existing risk for potential contamination of celestial bodies derives from the potential of microorganisms residing on spacecraft surfaces being transported to space via spacecraft missions. As previously mentioned, there is a wide range of resistant bacteria that inhabit spacecraft clean rooms and reside on spacecraft surfaces, and because of this, current and future PPP must be strictly adhered to and effectively maintained. The potential contamination of Mars or another celestial body poses a threat to the natural state of the planet and hinders any current or future life detection missions. Mars is especially of interest regarding the planetary protection precautions due to the various environmental conditions and specific regions that could potentially harbor microbial life. When considering future manned missions to Mars, it is important for policies and objectives related to robotic

missions delineated in the Outer Space Treaty to be followed with utmost concern because of the potential contamination risks that are involved with human exploration in space. However, it is also stated that space exploration with human astronauts makes some degree of forward contamination unavoidable (Conley & Rummel, 2008). New insight on planetary protection protocols and requirements will be increasingly important as the possibility of a manned mission to Mars approaches in the near future.

With the recent requests from the authors of the MEPAG Report on Mars Special Regions (Committee to Review the MEPAG Report on Mars Special Regions et al., 2015), implementation of the new molecular based techniques will allow for the study of a wide range of microorganisms including non-spore-forming extremophiles. Instead of only focusing on a small portion of the overall bioburden, it will allow for even more studies to be conducted on resistant, non-spore-forming microorganisms collected from spacecraft studies. This new approach within the niche of planetary protection will likely have a profound impact on the related research conducted from here on out. A significant portion of the Viking microbial archive, which was utilized in the studies presented in this dissertation, consists of non-spore-forming bacteria (Puleo et al., 1977). The information from the identification and characterization studies presented in chapters three and four will contribute to the recently requested knowledge pertaining to non-spore-formers residing on the surfaces of spacecraft. More studies, such as those presented in this dissertation, should be conducted to continue to learn about the microbial diversity and physiological capabilities of those microorganisms residing on spacecraft surfaces. In light of the ever-expanding knowledge of the Martian environment and the terrestrial microbial world, PPP will continue to be modified and maintained for ongoing and future missions.

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CHAPTER 3: IDENTIFACTION AND CHARACTERIZATION OF MICROBIAL ISOLATES COLLECTED PRE-LAUNCH FROM THE SURFACES OF SPACECRAFT DESTINED FOR MARS: VIKING 1 AND 2 LANDERS, ORBITERS, AND SHROUDS AND THE VIKING PRECURSOR LANDER AND ORBITER

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Abstract

The Martian environment is home to a wide range of extreme environmental conditions including frigid temperatures, desiccation, high salt concentrations, and intense radiation. However, Mars also harbors specific niches that could support microbial life. To help diminish the risk of terrestrial organisms being transported to Mars by spacecraft, Mars-bound landers, orbiters, and shrouds must meet strict pre-launch planetary protection requirements regarding the microbial bioload on spacecraft surfaces. In accordance with planetary protection regulations, the Viking 1 and 2 spacecraft (landers, orbiters, and shrouds) and Viking Precursor surfaces underwent extensive sampling to evaluate the microbial bioburden prior to a terminal sterilization cycle and launch. The goal of this study was to identify the microbial isolates collected from the surfaces of the Viking 1 & 2 spacecraft and Viking Precursor and determine survival after exposure to conditions similar to those on Mars. Another important aspect of this study was to generate microbial stocks to support ongoing studies and for the continued long-term preservation of these historical isolates.

[1]Manuscript in Preparation

Introduction

Over the past decades, the planet Mars has continued to gain attention as a desirable place to search for extraterrestrial life within our solar system. Although we are still learning about the Martian environment, we do know that it contains the specific requirements that can support microbial life (Horneck, 2008; McKay, 2004). Earlier studies (Malin & Edgett, 2000; Malin & Edgett, 2003; McEwen et al., 2011; McEwen et al., 2014; Martínez & Renno, 2013) suggested liquid water, a key requirement for life, had once existed on the planet and may still exist on present-day Mars. A recent publication by Ojha et al. (2015) provided the strongest evidence to date that liquid water exists on present-day Mars. However, the Martian surface is also subjected to a variety of harsh conditions including extremely low water activity, very cold temperatures, high salt concentrations, and intense radiation due to a thin atmosphere (Horneck, 2008; Schulze-Makuch et al., 2008). The average surface temperature of Mars is -65°C and can range anywhere from -10°C to -76°C (Horneck, 2008; Schofield et al., 1997). In addition, numerous data show the Martian surface contains elevated salt levels (Sawyer et al., 2000; Treiman, 1999; Vaniman et al., 2004; Wang et al., 2006) and could form flows of briny solutions due to melting water ice in Mars permafrost (McEwen et al., 2011). Any potential life on Mars must be able to grow in (or at least tolerate) the presence of high salts (Crisler et al., 2012; Landis, 2001) as well as the other environmental extremes known to impact the surface of Mars.

Even though the harsh environmental conditions on Mars make it seem nearly impossible for the hardiest of organisms to persist, life on Earth is common in extreme environments such as hydrothermal vent ecosystems and Arctic permafrost. Since terrestrial life has been discovered to be surprisingly robust, there is concern that microorganisms from

Earth residing on spacecraft surfaces traveling to Mars would survive in the Martian environment and confound ongoing and future investigations of extraterrestrial life. To maintain compliance with the United Nations Outer Space Treaty of 1967 (United Nations, 1967), spacecraft going to Mars must follow rigid guidelines established by the Committee on Space Research (COSPAR) prior to launch (Kminek & Rummel, 2015), which include rigorous microbial reduction procedures and extensive sampling of the spacecraft surfaces to ensure that the number of microorganisms transported to space is below the allowable limit. Consistent with COSPAR's guidelines, NASA's (2011) PPP limits a Mars lander without instruments for investigating potential extant life to a total biological burden level of 3×10^5 spores on its surfaces, and those with the intent to investigate extant Martian life must maintain a surface biological burden level of 30 spores on the entire spacecraft.

Even implementing rigorous microbial reduction regimes (Frick et al., 2014), microorganisms continue to be present in spacecraft-associated clean rooms and on spacecraft surfaces (La Duc et al., 2007; Moissl-Eichinger et al., 2012; Ghosh et al., 2010; La Duc et al., 2004; Benardini et al., 2011; Vaishampayan et al., 2010). Studies pertaining to bacterial analyses of clean room environments directly relate to this study because bacteria residing in spacecraft clean rooms may end up on spacecraft and have the potential travel to Mars. Since spore-forming microorganisms are known to endure harsh environments, a number of studies have been done on the survival of various spore-forming *Bacillus* species isolated from spacecraft associated clean rooms and spacecraft surfaces to conditions similar to those found on the surface of Mars (Link et al., 2004; Newcombe et al., 2005; Nicholson et al., 2000; Setlow, 2006; Vaishampayan et al., 2012; Horneck et al., 2012). In fact, many previous studies testing the survivability to Mars-like conditions (Horneck, 1993; Nicholson

et al., 2000; Riesenman & Nicholson, 2000; Schuerger et al., 2003; Tauscher et al., 2006; Zenoff et al., 2006; Fajardo-Cavazos et al., 2010) have focused on spore-forming microorganisms from the *Bacillus* genus. In comparison, limited studies exist using microorganisms directly isolated from the surface of a Mars-bound spacecraft (Puleo et al., 1977; La Duc et al., 2003; Smith et al., unpublished).

Studies have found that a wide range of organisms from genera other than *Bacillus* inhabits spacecraft-associated clean rooms and spacecraft surfaces (La Duc et al., 2007; La Duc et al., 2009; Berry et al., 2010; Moissl et al., 2007; Moissl-Eichinger et al., 2012, Schwendner et al., 2013; Stieglmeier et al., 2012; Vaishampayan et al., 2010). Puleo et al., (1977) conducted one of the first studies aimed at collecting the microorganisms present on the surfaces of pre-launch spacecraft. Puleo et al. identified non-spore-forming microorganisms within the microbial archives collected from the Mars-bound Viking spacecraft. Relatively few studies have attempted to determine the survival of non-spore forming microorganisms when exposed to harsh conditions similar to those found within the Martian environment (La Duc et al., 2007; Osman et al., 2008, Berry et al., 2010). Stieglmeier et al. (2009) and Probst et al. (2010) assessed the metabolic capabilities of spacecraft-associated clean room microbial communities. Investigations into the potential of spacecraft-associated bacteria to utilize various energy sources known to exist on Mars are lacking and should be conducted. More inclusive studies of all organisms able to survive extreme environmental conditions are warranted (Nicholson et al., 2013).

In the 1970s, Puleo et al. (1977) performed a comprehensive sampling regime of the surfaces of the Viking 1 and 2 spacecraft and Viking Precursor, a flight orbiter and lander that was used pre-launch to check various spacecraft test operations at the launch site.

Through this process, the microbial bioburden was determined prior to an extensive terminal sterilization cycle and launch. From the samples, 1,294 of the isolates were selected and later identified using classical bacteriology methods in use at the time. These isolates were then lyophilized and stored as a microbial archive for future studies.

The purpose of the current study was to further characterize the approximately 1,294 microbial isolates collected from the surfaces of the Viking 1 and 2 landers, orbiters, and shrouds archived by Puleo et al. (1977). This study reports the identification and characterization results of both 1) the isolates collected from the Viking 1 and 2 landers as well as 2) the isolates collected from the Viking 1 and 2 orbiters and shrouds. Since the landers have different planetary protection restrictions than the orbiters and shrouds, the results section is separated into two parts. Due to the volume of samples, microbial isolates were tentatively identified using comparative sequence analysis of small subunit rRNA genes. Isolates were also screened for growth in conditions similar to the surface of Mars, and although negative and inconclusive results were obtained, only positive results are presented in this article. Less than 1% of the results from the growth studies were found to be inconclusive. Results obtained from this study will help determine whether terrestrial bacteria residing on spacecraft surfaces could threaten any current and future planetary protection efforts implemented to prevent forward contamination of celestial bodies within our solar system.

Materials and Methods

Sample, Collection, and Growth

As described by Puleo et al. (1977), bioassay teams used swabs to collect samples from the surfaces of the Viking spacecraft. Approximately 1,300 colonies were picked from the

resulting culture plates, identified using classical determinative bacteriological methods (Bergey's Manual), lyophilized, and stored (Puleo et al., 1977). In 2011, the lyophilized culture samples were shipped to the University of Idaho and isolates were grown as described by Smith et al. (unpublished). Briefly, isolates were suspended in 1 mL of Tryptic Soy Broth (TSB) and incubated with shaking at 30°C for 24-48 hours. The culture was plated onto Tryptic Soy Agar (TSA) and incubated at 30°C for 24-48 hours. The isolates that grew were re-streaked three times for purity and two glycerol stocks were made for each. Any plates consisting of two or more different colony types were separated and all unique colonies were streaked onto separate plates labeled as a, b, c, etc. beside the original sample name. For the aerobic, anaerobic, and desiccation studies as well as the identification of the isolates, isolates in pure culture were inoculated into 96-well plates with TSB and incubated with shaking for 24-48 hours at 30°C. All of the characterization experiments were conducted separately from one another making the results distinct for each condition tested.

Identification

Identification of the isolates was accomplished by amplifying and sequencing the 16S rRNA gene. Two different DNA extraction methods were used. One method utilized a commercially available DNA extraction kit, Wizard SV Genomic DNA Purification System (Promega) and the other is described herein. Cells were collected by centrifugation at 4000 X g for 8 minutes and re-suspended in 200 µl of sterile dH_2O. Cell suspensions were subjected to three freeze/thaw cycles at temperatures of -80°C and 80°C for 15 minutes each. Once the freeze/thaw cycles were complete, silica beads were added to the cells and the suspension was rapidly mixed for approximately two minutes. Cell debris was pelleted by centrifugation at 4000 X g for two minutes and the resulting supernatant (lysate) was

transferred to a new tube. The bacterial universal primers 8F (Reysenbach et al., 1994) and 1525R (Suzuki et al., 1996) were used for amplifying the 16S rRNA gene using PCR. PCR was carried out as previously described by Smith et al. (2009) by mixing 25 µl of Dream Green Taq 2X Master Mix (Fermentas), 2.5 µl of 12.5 µmol primer 8F, 2.5 µl of 12.5 µmol primer 1525R and 10 µl of dH_2O (or 19 µl of dH_2O if the Wizard SV Genomic DNA Purification System was used) into each well or tube. Depending upon which extraction method was used, either 10 µl (lysate) or 1 µl (commercial kit) of chromosomal DNA was added to the 50 µl reaction. The PCR conditions included an initial denaturation step at 94 °C for 5 minutes followed by 32 cycles of 95 °C for 1 minutes, 51.4 °C for 1.5 minutes, and 72 °C for 1.5 minutes. After the completion of the 32 cycles, a final elongation step at 72 °C for 5 minutes was done. For purification of PCR amplified fragments, Exonuclease I (1 µl) and Antarctic Phosphatase (0.8 µl) per 10 µl were added to the amplified PCR product. The final reactions were heated for 15 minutes at 37˚C followed by heat-inactivation of the enzymes for 15 minutes at 80˚C.

The purified 16S rRNA PCR products were sequenced by a commercial facility using bacterial primer 27F (Lane, 1991). Gene sequences were aligned and trimmed to 450 bp and analyzed using the HiSTA analysis pipeline (available at http://www.ibest.uidaho.edu/tools) as described (DeGelder et al., 2005). BLAST (Altschul et al., 1997) was used to identify sequences to the closest relative amongst eubacterial type strains in the Ribosomal Database Project (RDP) (Cole et al., 2003). For each input sequence, the RDP sequence for the closest relative was distinguished and incorporated into the analyses that followed. Using ClustalW (Thompson et al., 1994), all of the input sequences and their closest relatives along with a selected outgroup sequence were aligned. The genetic distances were determined using the

Jukes and Cantor method (Jukes & Cantor, 1996).

Aerobic Growth Assays (pH, Temperature, NaCl)

To study the growth of isolates in alkaline pH conditions, aliquots (5 µl) of cells were inoculated into 96-well plates containing either TSB (1200 µl) at pH 7 or buffered media at pH 8, 9, 10, 11, and 12. Buffers used in the pH 8-10 media (100 mM final concentrations) were previously described by Nielsen *et al*., (2005), while the pH 11 buffer used was 100 mM Na_2HPO_4 and the pH 12 buffer used was 100 mM KCl. Each experiment was performed in duplicate. For growth at high salt concentrations, cells (5 µl) were inoculated into 96-well plates containing either TSB with 5, 10, or 20% (w/v) NaCl. Growth was measured by monitoring turbidity at 600 nm on days 0, 1, 2, 3, and 7. To investigate whether isolates were capable of growth at 4°C temperatures, cells (5 µl) were inoculated into 96-well assay plates containing TSB at neutral pH and incubated at 4°C. Turbidity measurements were taken at days 0, 1, 3, 7, 14, and 28 to determine growth.

Anaerobic Growth Assays

Anaerobic growth assays were performed in an anaerobic chamber (Coy Laboratory Products Inc., Grasslake, MI) with an atmosphere of 90:10 nitrogen to hydrogen. Cells were inoculated into 96-well plates containing ATCC #2106 medium that had been amended with a specific electron donor and acceptor pair (Table 3.1). Each experiment was performed in duplicate. Turbidity measurements were taken at intervals of 0, 7, 14, 21, and 28 days for cultures containing perchlorate, arsenate, and sulfate. For the selenate and selenite assays, growth was scored positive by the formation of a red precipitate in the well. For iron reduction, ferric citrate was used and reduction of Fe(III) to Fe(II) was visually determined by a color change in media from amber to a darker brownish black hue.

Table 3.1 List of the various electron acceptor and donor pairs used in anaerobic studies.

Media	Electron acceptor	Electron donor
1	Arsenate, $Na_3AsO_4^-$ (10 mM)	Acetate (10 mM)
2	Arsenate, $Na_3AsO_4^-$ (10 mM)	Lactate (20 mM)
3	Perchlorate, $NaClO_4^-$ (10 mM)	Acetate (20 mM)
4	Perchlorate, $NaClO_4^-$ (10 mM)	Lactate (20 mM)
5	Sulfate, Na_2SO_4 (50 mM)	Acetate (20 mM)
6	Sulfate, Na_2SO_4 (50 mM)	Lactate (20 mM)
7	Fe(III), Fe_2O_3 (80 mM)	Acetate (20 mM)
8	Fe(III), Fe_2O_3 (80 mM)	Lactate (20 mM)
9	Selenite, Na_2O_3Se (5 mM)	Acetate (20 mM)
10	Selenite, Na_2O_3Se (5 mM)	Lactate (20 mM)
11	Selenate, Na_2O_4Se (10 mM)	Acetate (10 mM)
12	Selenate, Na_2O_4Se (10 mM)	Lactate (20 mM)

Desiccation Studies

Cells grown up for 24-48 hours were collected by centrifugation at 4000 X g for 8 minutes and washed 2-3 times in 1200 µl of phosphate-buffered saline (PBS). Washed cells (30 µl) were added to empty assay plates and plates were covered with a breathable seal and left in the biological safety hood overnight so the PBS would evaporate. Experiments were performed in duplicate and the two assay plates were stacked inside a glass desiccation chamber containing dried silica desiccant for 14 days. Plastic partitions were used inside the chamber to separate the plates 1-2 inches apart from each other during the desiccation period. Inside the chamber, humidity levels remained below 5% relative humidity and the temperature was maintained close to average room temperature at approximately 23.5°C. After two weeks, cells were rehydrated with 200 µl of TSB and suspensions were mixed. Immediately following rehydration, turbidity was measured (day 0) at 600 nm and again on days 1, 2, 3, and 5 days.

UV-C and Heat Characterization

Isolates were selected to undergo heat and UV-C studies and were chosen based on their

tolerance to multiple extreme conditions during the other characterization studies previously described in this section. Five Viking lander isolates were selected to undergo heat characterization and eight were selected to study tolerance to UV-C irradiation.

For UV survival, stationary phase liquid cultures were serially diluted from 10^0 to 10^{-5}, spotted onto square grid plates containing TSA, and the spots were allowed to dry completely prior to UV-C exposure. A UVP multiple ray lamp (UVP, Upland, CA) with a UV-C bulb (Sankyo Denki G8T5) was utilized for all UV-C treatments, and all treatments were conducted entirely in a dark room in order to minimize the effects of light-dependent repair mechanisms potentially inherent in the strains. Prior to treatment, the lamp was turned on and allowed to stabilize for approximately twenty minutes. Lamp output was measured using a UVP MS-100 optical radiometer with a 254nm sensor attached (UVP, Upland, CA). Exposure time (in seconds) to achieve targeted fluence in J/m^2 was calculated by dividing target dose by the meter reading (mW/cm^2) multiplied by ten (T_s=target fluence/[meter*10]). Plates were uncovered and exposed to UV-C for appropriate times, then immediately wrapped with aluminum foil and placed in a 30°C incubator (Smith et al., unpublished).

For heat tolerance, isolated colonies of each strain were streaked onto TSA plates. Each plate was placed at a designated temperature (37°C, 42°C, 45°C, 47.5°C, 48.5°C, 49.5°C, 50.5°C, 51.5°C, 52.5°C, and 54°C) for 24 hours, and scored for presence or absence of growth.

Results Part I: Viking 1 & 2 Landers and Viking Precursor Lander

Identification

A total of 1,323 isolates were recovered from their lyophilized state from the Viking archive. Of the 1,323 total isolates revived from the archive, 586 of those were collected

from the Viking landers, and 563 of the 586 isolates were identified by 16S rRNA sequence analysis (Table 4). Spore-forming microorganisms constitute the majority (81%) of identified isolates, and included the genera *Paenibacillus* (4%), *Sporosarcina* (<1%), *Geobacillus* (<1%) and the most prevalent, *Bacillus* (95%). There were 26 different species represented within the *Bacillus* genus though 126 additional isolates were not identified to the species level. The species most commonly identified within the *Bacillus* genus was *B. pumilus* (22%) followed by *B. subtilis* (12%). Other species represented were *B. amyloliquefaciens* (6%), *B. megaterium* (6%), *B. cereus* (6%), *B. aerophilus* (3%), *B. lichenformis* (3%), and *B. mojavensis* (2%). Figure 3.1 portrays the most commonly represented genera identified within the microbial isolates collected from the Viking landers and Precursor lander.

The remaining isolates (19%) included non-spore-forming organisms and the majority was from the *Staphylococcus* genus (54%). Species represented in the *Staphylococcus* genus include *S. epidermis* (39%), *S. hominis* (9%), *S. capitis* (5%), *S. haemolyticus* (4%), and an additional 25 organisms that were not identified to the species level. Other genera identified within the non-spore-forming organisms included *Micrococcus* (28%), *Paracoccus* (2%), *Kocuria* (2%), *Rothia* (2%), *Enterobacter* (1%), *Streptococcus* (1%), and *Gordonia* (1%). A complete list of these identification results is represented in Table A3.

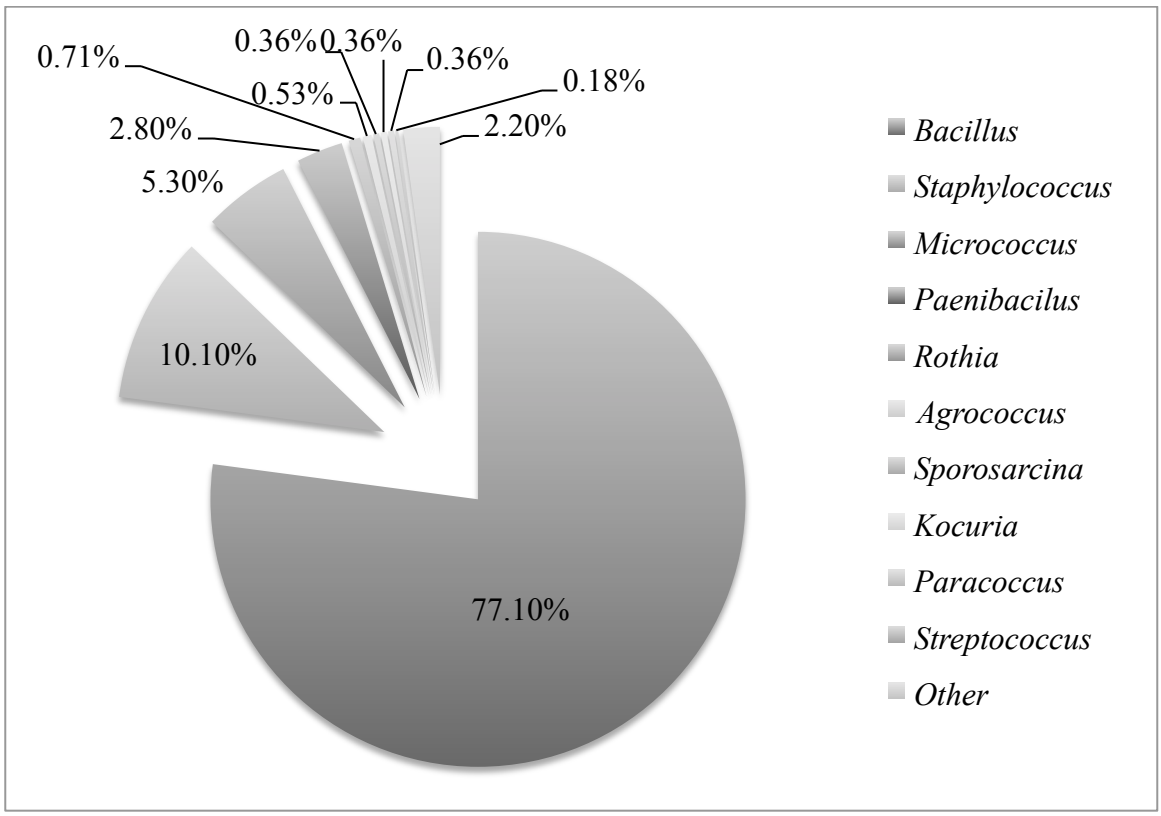

Figure 3.1 Most commonly represented genera of microbial isolates collected pre-launch from the surfaces of the Viking 1 & 2 landers and Viking Precursor lander.

Growth in Alkaline pH, High Salt, and 4°C

All 586 microbial isolates were tested for growth in alkaline pH, high salt, or at 4°C. Since media at neutral pH was used during the NASA Standard Assay, all 586 organisms were capable of growth at pH 7. Most (75%) of the isolates were able to grow at pH 8, and 58% grew at pH 9. There was a significant decline in the number of organisms capable of growth at pH 10 or above. Only 16% of the organisms were capable of growth at pH 10, while <3% grew at pH 11 or above.

The majority (81%) of isolates grew in media containing 5% NaCl and 383 (65%) of the isolates grew at 10% NaCl concentrations. Forty-five (8%) of the isolates grew when tested in media containing the highest salt concentration used, 20% NaCl. Of those 45

isolates, over two-thirds (69%) were identified as *Staphylococcus.* Only 12 isolates were identified as members of the spore-forming genus *Bacillus*, while just 1 isolate was identified as *Micrococcus* and another as *Streptococcus*. Out of the 586 isolates tested, just 29 (5%) grew at 4°C, but a wide range of genera was represented within this group. The majority of 29 isolates that grew at 4°C were identified as members of the *Bacillus* genus (19), followed by *Paenibacillus* (2), *Cellulomonas* (1), *Gordonia* (1), *Arthrobacter* (1), *Agrococcus* (1), *Staphylococcus* (1), *Sporosarcina* (1), *Rhodococcus* (1), and 1 isolate that has not yet been identified.

Anaerobic Assays

All 586 isolates were tested for the ability to utilize arsenate (As^{+5}), perchlorate (ClO_4^-), sulfate (SO_4^{-2}), iron (Fe^{+3}), selenite (Se^{+4}), and selenate (Se^{+6}) as terminal electron acceptors under anaerobic conditions. Of the isolates collected from the surfaces of the Viking landers, only 46 (8%) grew using the various electron acceptor and donor pairs tested, which are listed in Table 1. Thirty-five isolates grew with Se^{+4}, 16 using lactate, 5 using acetate, and 14 using either lactate or acetate as the electron donor. Fourteen isolates grew using Fe^{+3} as a terminal electron acceptor. Ten isolates grew with Fe^{+3} using either acetate or lactate as the electron donor, while 2 used only lactate and 2 used only acetate.

Only 4 isolates grew with perchlorate, and all 4 also used lactate as the electron acceptor. Two of the perchlorate reducers were most closely related to members of the *Micrococcus* genus, 1 to *Paenibacillus*, and 1 to *Bacillus*. One isolate, identified as a member of the *Micrococcus* genus, was capable of growth using arsenate as the terminal electron acceptor and only grew with lactate as the electron donor. None of the isolates were able to utilize perchlorate with acetate in place of lactate. None of the 586 isolates showed

detectable growth with selenate or sulfate as terminal electron acceptors.

Seven isolates were capable of using two or more electron acceptors tested (Table A1). Of these, 1 was a member of the *Bacillus* genus, 1 was a *Micrococcus*, and 5 were *Paenibacillus*. Only 1 isolate (LA 067b in Table 1) identified as *Paenibacillus lautus,* was capable of utilizing 3 of the 6 different electron acceptors tested.

Desiccation Studies

All of the 586 isolates collected from the Viking landers and Viking Precursor lander were tested for their resistance to desiccating conditions. Most (89%) of the isolates demonstrated growth after exposure to 2 weeks of desiccation and re-suspension. Spore-forming microorganisms were the majority (77%) of desiccation resistant isolates; 95% of those isolates were *Bacillus*. Other spore-forming organisms represented were identified as members of the genera *Paenibacillus* (4%), *Sporosarcina* (<1%) and *Geobacillus* (<1%). However, slightly under a quarter of the isolates (20%) that survived the desiccation period were identified as non-spore-forming organisms. The most abundant were members of *Staphylococcus* (55%) and *Micrococcus* (27%). Some of the other non-spore-forming genera included *Paracoccus* (2%), *Rothia* (2%), *Kocuria* (2%), *Methylobacterium* (<1%), *Enterobacter* (<1%), *Macrococcus* (<1%), *Gordonia* (<1%), *Arthrobacter* (<1%), and *Cellulomonas* (<1%). A total of 17 isolates have yet to be identified.

UV-C and Heat Characterization

The 5 isolates selected for heat tolerance studies were identified as spore-forming bacteria and represented *Bacillus* (2), *Paenibacillus* (2), and *Sporosarcina* (1). Two isolates, a *Sporosarcina* and a *Paenibacillus,* could tolerate temperatures up to 42°C. One isolate identified as *Paenibacillus lautus* (LA 067b) previously mentioned in the anaerobic results

section was capable of tolerating a slightly higher temperature of 45°C. Both of the *Bacillus* isolates were capable of growth at temperatures of 52.5°C. No isolate grew at the highest temperature tested, 54.5°C.

Members of *Bacillus* (3), *Staphylococcus* (3), *Micrococcus* (1), and *Cellulosimicrobium* (1) represented the eight isolates chosen for UV-C studies. All 8 isolates were able to withstand 100 J/m^2 of UV-C irradiation, but only 2 of these isolates, a *Bacillus* and a *Micrococcus*, survived when exposed to 500 J/m^2. None of the Viking landers isolates tolerated UV-C doses of 2000 J/m^2, the highest levels tested.

Multiple Tolerances

A small proportion (10%) of the isolates grew in more than one of the aerobic conditions tested (pH 9 or above, 20% NaCl, or 4°C). These results are summarized in Table A2. Interestingly, approximately 62% of those organisms were identified as non-spore-formers, while only 36% were identified as spore-forming organisms, with the remaining 2% of the organisms not yet identified.

Of the non-spore-forming organisms that showed multiple tolerances, the majority (86%) was identified as *Staphylococcus*. Other genera represented in this group were *Streptococcus, Cellulomonas, Gordonia, Arthrobacter,* and *Rhodococcus*. Within the spore-forming isolates demonstrating growth under more than one extreme condition, *Bacillus* made up the majority (90%) of isolates. Only two other genera were represented in this group, *Paenibacillus* and *Sporosarcina*, and one isolate not yet identified. All isolates grew in 2 out of 3 conditions tested (pH 9 or above, 20% NaCl, and 4°C). None of the isolates were able to grow in all 3 conditions tested.

Results Part II: Viking 1 & 2 Orbiters and Shrouds and Viking Precursor Orbiter Identification

A total of 737 isolates out of the 1,323 revived from the archive were collected from the Viking 1 & 2 orbiters and shrouds. From the 737 isolates collected from the Viking 1 & 2 orbiters and shrouds, a total of 706 isolates were successfully identified by 16S rRNA sequence analysis (Table 4).

Over half (56%) of the identified isolates were non-spore-forming microorganisms. The most commonly identified genus was *Staphylococcus,* constituting approximately 80% the non-spore-formers. Some of the species most commonly represented within the *Staphylococcus* genus included *S. epidermidis* (31%), *S. haemolyticus* (15%), *S. hominis* (11%), *S. saprophyticus* (3%), *S. pettenkoferi* (2%), and an additional 102 isolates that were not identified to the species level. There were an additional 18 non-spore-forming genera identified, with *Micrococcus* (8%) as the next most commonly identified genus, followed by others including *Acinetobacter* (3%), *Corynebacterium* (2%), *Brachybacterium* (1%), *Brevibacterium* (1%), *Agromyces* (1%), *Kocuria* (1%), and *Pseudomonas* (1%).

Spore-forming organisms constitute the remaining percentage (44%) of the identified isolates, spanning 5 different genera. The genera represented within the spore-forming organisms include *Paenibacillus* (5%), *Lysinibacillus* (2%), *Sporosarcina* (<1%), *Terribacillus* (3%), and the most commonly identified genus, *Bacillus* (90%). A total of 24 species were represented in the *Bacillus* genus with an additional 100 organisms not identified to the species level. Some of the more commonly represented species within the *Bacillus* genus included *B. pumilus* (17%), *B. subtilis* (13%), *B. megaterium* (7%), *B. cereus* (5%), and *B. lichenformis* (3%). Table A4 shows a complete list of genus and species results

broken down by spacecraft and Figure 3.2 depicts the most commonly represented genera identified within the microbial isolates collected from the Viking orbiters and shrouds and Viking Precursor orbiter.

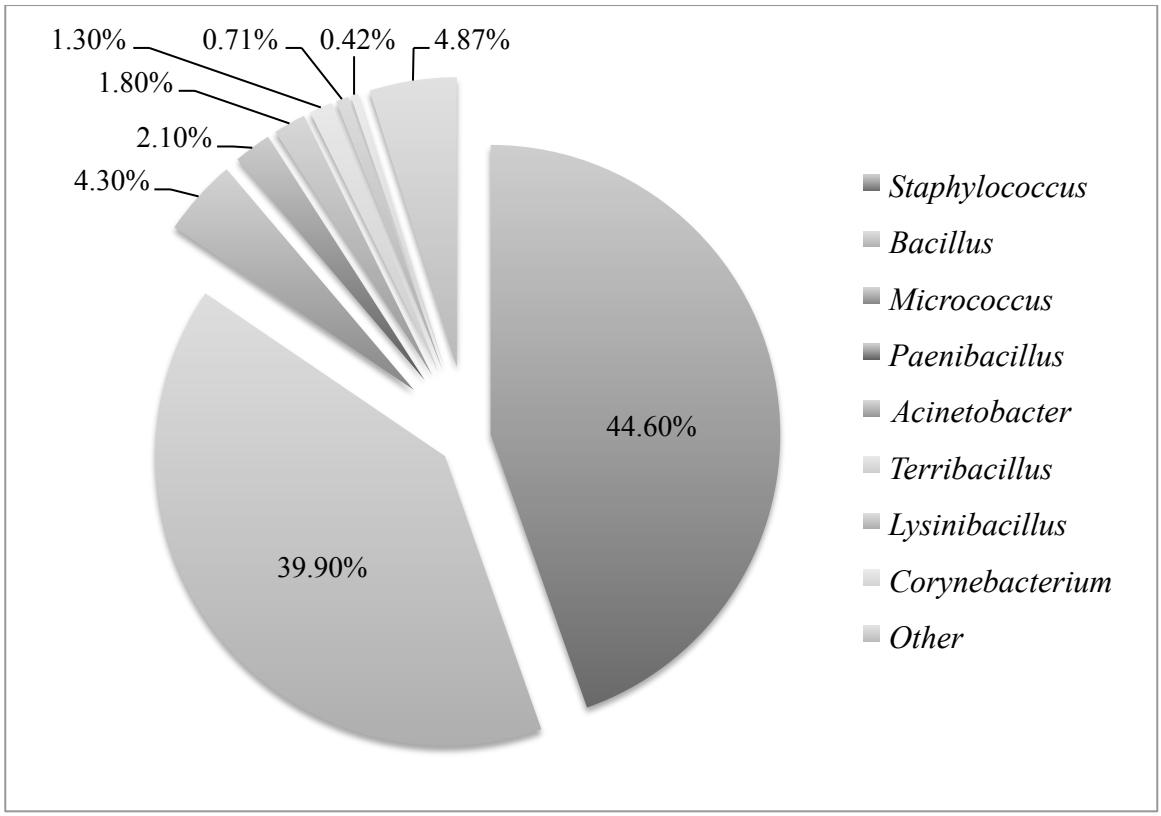

Figure 3.2 Most commonly represented genera of microbial isolates collected pre-launch from the surfaces of the Viking 1 & 2 orbiters and shrouds and Viking Precursor orbiter.

Growth in Alkaline pH, High Salt, and 4°C

All of the 737 isolates collected from the Viking 1 and 2 Orbiters and Shrouds were grown in alkaline pH levels, various NaCl conditions, or at low temperatures. All 737 isolates were able to grow at pH 7 since the media used during the NASA Standard Assay was at neutral pH. The majority (76%) of these isolates were capable of growth when tested at pH 8, and a

comparable number (73%) grew at pH 9. A significant decline in the number of isolates that grew at pH 10 and above was observed. Approximately 8% of the isolates grew at pH 10, and less than 1% grew at pH 11 or above.

All 737 isolates were tested under high salt conditions including 5%, 10% and 20% NaCl concentrations. Approximately 81% grew at 5% NaCl concentrations, and 69% grew at 10% NaCl concentrations. When tested at 20% NaCl concentrations, 27% of the isolates were capable of growth. Of those that grew at 20% NaCl concentrations, approximately 97% were identified as *Staphylococcus*, with the remaining isolates representing the *Bacillus* and *Micrococcus* genera as well as a fraction of unidentified isolates. Only 12 isolates were identified as members of the spore-forming genus *Bacillus*, while just 1 isolate was identified as *Micrococcus* and another as *Streptococcus*. Out of the 737 isolates tested at 4 degree Celsius temperatures, approximately 3% were capable of growth. A total of 6 different genera were represented within this group of isolates, and included *Staphylococcus, Acinetobacter, Bacillus, Micrococcus, Microbacterium,* and *Pseudomonas.*

Anaerobic Assays

The 737 microbial isolates were tested under anaerobic conditions for the ability to utilize perchlorate (ClO_4^-), arsenate (As^{+5}), iron (Fe^{+3}), sulfate (SO_4^{-2}), iron (Fe^{+3}), selenite (Se^{+4}), and selenate (Se^{+6}) as terminal electron acceptors with either lactate or acetate as electron donors. A total of 33 isolates out of the 737 collected from the surfaces of the Viking 1 & 2 orbiters and shrouds were capable of growth utilizing the different pairs of electron donors and acceptors shown in Table 1. A total of 31 isolates were capable of growth utilizing Se^{+4}, 14 using lactate as the electron donor, 6 using acetate as the electron donor, and 11 using either electron donor. One isolate was capable of growth utilizing Fe^{+3} as a terminal electron acceptor with either lactate or acetate as the electron donor. One isolate grew using As^{+5} and

acetate, and another grew using SO_4^{-2} and lactate.

None of the isolates tested showed detectable growth utilizing ClO_4^- or Se^{+6} as terminal electron acceptors. One isolate, a member of the *Paenibacillus* genus, was capable of utilizing two of the six electron acceptors tested. This *Paenibacillus* isolate was able to utilize either Se^{+4} or Fe^{+3} as the terminal electron acceptor with either lactate or acetate as the electron donor.

Desiccation Studies

All 737 isolates collected from the Viking orbiters and shrouds were subjected to desiccating conditions and approximately 86% of the isolates were capable of growth after the two-week period. Approximately 37% of the desiccation resistant microbial isolates collected from the orbiters and shrouds were identified as spore-forming organisms. Of the desiccation resistant spore-formers, approximately 92% were *Bacillus.* Other genera included *Paenibacillus* (5%), *Lysinibacillus* (2%), *Terribacillus* (<1%), and *Sporosarcina* (<1%). Non-spore-forming organisms constituted approximately 60% of the desiccation resistant microbial isolates and *Staphylococcus* was the most prevalent totaling 81% of the group. *Micrococcus* members were the next most prevalent genus, totaling 8% of the non-spore-formers. A total of 23 isolates have yet to be identified.

UV-C and Heat Characterization

A total of 7 isolates collected from the orbiters and shrouds were chosen for heat tolerance studies and represented 4 different genera including *Bacillus* (3), *Staphylococcus* (2), *Micrococcus* (1), and *Brachybacterium* (1). Four isolates, a *Micrococcus,* a *Staphylococcus,* and 2 *Bacillus,* were capable of tolerating temperatures up to 42°C. The isolate identified as *Brachybacterium* tolerated temperatures up to 37°C. The other *Staphylococcus* isolate was

capable of tolerating temperatures up to 45°C. The remaining *Bacillus* isolate tolerated temperatures up to 52.5°C, and no isolates were capable of tolerating the highest temperatures tested, 54°C.

Twelve isolates collected from the Viking 1 & 2 orbiters and shrouds were chosen for UV-C studies and represent five different genera. Those genera include *Bacillus* (2), *Staphylococcus* (4), *Microbacterium* (2), *Brevibacterium* (2), and *Micrococcus* (2). All twelve isolates were capable of withstanding 100 J/m^2 of UV-C irradiation. Four isolates, 2 *Microbacterium* and 2 *Micrococcus*, were able to withstand 500 J/m^2, and out of those isolates, the 2 *Microbacterium* were capable of surviving the highest level tested, 2000 J/m^2.

Multiple Tolerances

Over a quarter (26%) of the 737 isolates collected from the surfaces of the Viking 1 & 2 orbiters and shrouds were capable of growth in more than one of the aerobic conditions tested (pH 9 or above, 20% NaCl, or 4°C).

Of the 26% of microbial isolates that showed multiple tolerances, almost 93% were identified as *Staphylococcus,* with the remaining percentage belonging to *Bacillus, Acinetobacter, Micrococcus, Pseudomonas, Microbacterium,* and one isolate not yet identified. The majority of isolates grew in only 2 of the 3 conditions (pH 9 or above, 20% NaCl, and 4°C), but 4 isolates were able to grow under all 3 conditions. Three of the 4 isolates were *Staphylococcus* and the remaining isolate was identified as *Bacillus.*

Discussion: Viking 1 & 2 Landers, Orbiters, and Shrouds, Viking Precursor Lander and Orbiter

The study was undertaken to begin to address the issue of whether microorganisms transported from Earth during space exploration missions pose a potential risk to planetary

protection efforts. The overall goals of the study were (1) to identify over 1,300 microorganisms collected prior to a terminal sterilization and launch from the surfaces of the Viking landers launched in the mid 1970s and the Viking Precursor Lander and from the Viking 1 & 2 orbiters and shrouds and Viking Precursor Orbiter, (2) to determine which isolates might withstand the harsh physical conditions on the Mars surface, and (3) to determine if isolates could use energy sources potentially available on Mars as well as other celestial bodies. The study is unique because it is the first time molecular techniques have been used to identify the microorganisms collected pre-launch from the Viking 1 & 2 surfaces, and it is the only comprehensive analysis addressing whether these isolates can grow in extreme, Mars-like conditions. Only one other study (Puleo et al. 1977), conducted in the 1970s, involves the bacteria collected from the Viking spacecraft surfaces. Puleo et al. focused on the comprehensive sampling (6,683 samples total) of the Viking spacecraft, which included the surfaces of the Viking 1 and 2 landers, orbiters, and shrouds as well as the Viking Precursor orbiter and lander surfaces, and the identification of 1,294 microbial isolates constituting the Viking microbial archive using standard bacteriological methods. This study builds upon the findings of Puleo et al. (1977) by utilizing molecular tools to positively identify isolates collected from the Viking spacecraft and by determining the isolates that can grow in physical conditions analogous to those on Mars. We report on the identities of 563 of the 586 archived isolates that were collected from the surfaces of the Viking Precursor lander and the Viking 1 and 2 landers as well as the 706 of the 737 archived isolates collected from the surfaces of the Viking 1 & 2 orbiters and shrouds and compare our findings to those of Puleo et al. (1977) to better understand the microorganisms that may be leaving Earth on spacecraft.

The study by Puleo et al. (1977) showed there were low levels of microbial contamination on the Viking spacecraft despite rigorous cleaning protocols. The majority (75%) of the isolates found on the surfaces of the Viking 1 and 2 landers, orbiters, and shrouds were microorganisms associated with the human body including the *Micrococcus, Staphylococcus,* and *Corynebacterium-Brevibacterium* genera. Of the total number of isolates in the Viking archive (1,294), Puleo et al., (1977) reported that nearly three-quarters (951) of the isolates were vegetative microorganisms and only approximately a quarter (343) were members of the *Bacillus* genus though it was noted that a high number of spores were present on the Viking Lander Capsules (landers) relative to the orbiters. In comparison, our study found that the majority (81%) of the archived isolates collected from the Viking landers were spore-forming organisms and a smaller proportion (44%) of the isolates collected from the surfaces of the Viking orbiters and shrouds were spore-forming genera, similar to findings in the study by Puleo et al. Researchers in this study determined the remaining 19% of the isolates from the Viking 1 and 2 landers and the majority (56%) of those collected from the orbiters and shrouds were identified as non-spore-forming organisms with the two most prominent genera being *Staphylococcus* and *Micrococcus,* both of which were identified by Puleo et al. Of the archived microorganisms collected from the Viking 1 spacecraft surfaces, 55% identified by Puleo et al. were from either the *Staphylococcus* or *Micrococcus* genus, and the number of microorganisms associated with dust and soil (*Bacillus* and *Actinomycetes*) were relatively low when compared to the profiles of other automated spacecraft. Similar results were found when the organisms collected from the Viking 2 landers, orbiters, and shrouds were analyzed (Puleo et al., 1977).

It is recognized that salts are present in many areas on the Martian surface. Any life capable of surviving on Mars must be able to grow in or tolerate the presence of high salts (Crisler et al., 2012; Landis, 2001). On Mars, salts exist as $MgSO_4^-$, $CaSO_4$, $FeSO_4$, $MgCl_2$, NaCl and $CaCl_2$, however, higher concentrations of sulfate salts are found when compared to the various chloride forms by a 4:1 ratio (Crisler et al., 2012). Results from our study showed approximately 8% of the Viking landers isolates and 27% of Viking orbiters and shrouds isolates grew in media containing 20% NaCl, and of those, approximately 92% were members of the *Staphylococcus* genus. *Staphylococcus* species are known to tolerate a range of salt concentrations with some able to grow in media adjusted with concentrations as high as 19% NaCl (Vilhelmsson et al., 1997, Parfentjev & Catelli, 1964, Taponen et al., 2012, Tanasupawat et al., 1992). Recently, Choi et al. (2014) showed that *Staphylococcus* sp. OJ82 was able to grow at 25% NaCl in rich media. A study conducted by Kunin et al. (1991) found high levels of glycine betaine and K^+ in *Staphylococcus* species both in the presence and absence of osmotic stress, suggesting that these elevated concentrations may be responsible for their high salt tolerance and high turgor pressure. Though this study did not test for growth of Viking isolates in the presence of $MgSO_4$, the salt is likely to be predominant on Mars. Kilmer et al. (2014) report that *Staphylococcus* isolates from an epsomite lake grew in media supplemented with up to 2M $MgSO_4$.

Arguably one of the toughest conditions present on the surface of Mars is the intense radiation. Of the five Viking landers isolates and twelve Viking orbiters and shrouds isolates described in this study, two isolates, both identified as non-spore-forming *Microbacterium,* survived the highest UV-C doses tested, 2000 J/m^2. Typically, studies testing the UV resistance of microorganisms involve only members of the spore-forming *Bacillus* genus

(Link et al., 2004, Setlow et al., 2001, Setlow et al., 2006, Vaishampayan et al., 2010, Newcombe et al., 2005). A study by Osman et al. (2008) investigated the UV resistance of spacecraft-associated spores as well as four non-spore-forming microorganisms. Osman et al. found 2 non-spore-forming isolates, a *Microbacterium* and an *Arthrobacter*, survived 254-nm UV irradiation at doses comparable to those survived by *Bacillus pumilus* spores. As Osman et al. (2008) suggested, the desiccation tolerance of both of these non-spore-forming genera could be related to their ability to withstand UV radiation. Interestingly, *Microbacterium, Arthrobacter,* and *Micrococcus* are all actinomycetes, which have been found to have an increased desiccation resistance (Davet, 2004). As indicated by previous and current studies, further research involving the UV resistance of non-spore-forming microorganisms is warranted.

Additional findings in this study showed a portion of the isolates from the Viking landers, orbiters, and shrouds was found to grow in anaerobic conditions. Electron acceptors used included perchlorate and iron, compounds that are available in some form on Mars' surface. The isolates that grew in anaerobic conditions are not strict anaerobes, as all the Viking isolates were originally obtained after they grew on aerobic media (Puleo et al., 1977). Several studies have provided evidence of anaerobic bacteria populations residing in clean rooms and other spacecraft-associated surfaces. Stieglmeier et al. (2009) used a variety of anaerobic media formulations based on known Martian chemical conditions and isolated a broad range of facultative anaerobes, as well as strict anaerobes including *Clostridium* and *Propionibacterium,* from samples of spacecraft and surfaces in European clean rooms. Another study by Probst et al. (2010) found that highly diverse populations of anaerobes inhabiting clean rooms remain even after strict maintenance programs are implemented.

Spore-forming *Bacillus* species are commonly isolated from spacecraft-associated environments. Studies focused on testing the survival of *Bacillus* species isolated from spacecraft-associated surfaces (Horneck et al., 2012; Vaishampayan et al., 2012) found these isolates capable of surviving multiple simulated Martian conditions. *Bacillus* accounted for a little over a third (36%) of the Viking landers isolates that grew in multiple extreme conditions, though a range of microorganisms other than *Bacillus* were revealed that could tolerate more than one extreme condition, including *Staphylococcus*, *Streptococcus, Rhodococcus, Cellulomonas, Gordonia, Arthrobacter, Paenibacillus,* and *Sporosarcina*. Non-spore-forming *Staphylococcus* represented the majority (93%) of the Viking orbiters and landers isolates capable of growth in multiple extreme conditions. La Duc et al. (2007) reached a similar conclusion and found that genera other than *Bacillus* isolated from various clean room environments were capable of growth when subjected to extreme environmental conditions. La Duc et al. found that 32 isolates could grow in at least one of the extreme conditions tested (pH 10.6, anaerobic, 5% liquid H_2O_2, 65°C, or 1000 J m^{-2} UV-C doses) and isolates were representative of 14 different genera. Their study also concluded that only spore-forming microorganisms were capable of surviving the highest temperature tested, 65°C, similar to the most heat-tolerant isolates from the Viking landers identified in this study. In the study conducted by La Duc et al., further characterization identified 5 of the 32 isolates as the isolates that were capable of tolerating the broadest range of conditions (>65°C, 25% NaCl, 20% $MgCl_2$, pH 3, pH 9, and pH 10.6).

In summary, 586 isolates collected pre-launch from the Viking 1 and 2 landers and 737 collected from the Viking 1 and 2 orbiters and shrouds before a terminal sterilization in the mid-1970s were characterized for tolerance to Mars' surface conditions. A total of 563

Viking landers isolates and 706 Viking orbiters and shrouds isolates were successfully identified by comparative sequence analysis and identities of the isolates were remarkably similar to the identities of the same isolates determined by Puleo et al. (1977) using classic bacteriological methods. Many of the isolates grew in media adjusted for increased salinity, pH and temperature and several isolates used perchlorate, iron or selenite as the electron acceptor. In light of new knowledge about the existence of liquid water on Mars, additional studies are needed. Further investigations into other physiological capabilities of spacecraft-associated bacteria, namely non-spore-forming microorganisms, relevant to surviving on Mars will prove valuable for the implementation of appropriate planetary protection measures in future space missions. The knowledge generated from this study allows for a more comprehensive understanding of bacteria residing on spacecraft surfaces, which provides crucial insight for any current and future endeavors for the prevention of forward contamination of celestial habitats. Information from studies such as this one can be utilized in a way to better evaluate sterilization techniques used in spacecraft associated settings and whether any of these organisms could potentially pose a risk to planetary protection efforts.

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CHAPTER 4: A SUBSET OF HIGH SALT TOLERATING MICROBIAL ISOLATES COLLECTED FROM THE SURFACES OF MARS-BOUND VIKING SPACECRAFT TESTED UNDER $MGSO_4$ CONDITIONS

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Abstract

Characterization and identification studies have been done on approximately 1,300 microbial isolates collected pre-launch from the Mars-bound Viking spacecraft. The purpose of these studies was to screen for spacecraft-associated microorganisms that were able to survive extreme environmental conditions similar to those found on Mars (e.g. high salt conditions, low temperatures, extreme desiccation, high pH) and to determine if they have the ability to utilize potential terminal electron acceptors (e.g. As^{+5}, ClO_4^-, Fe^{+3}, SO_4^{-2}) that may be readily available on Mars, compounds which could serve as possible energy sources. Recent data indicates that if water is available on Mars, it is likely to be a briny solution with magnesium (Mg) ions being the most dominant. Therefore, tolerance to salt is a crucial requirement for the survival of microbes in the harsh salt environment found on Mars. The purpose of this study is to determine whether the Viking isolates that demonstrated the ability to survive and grow in 20% NaCl can grow in magnesium (Mg) containing salts at various temperatures and pH levels. Results from these multiple stress studies will help begin to determine if microorganisms residing on spacecraft surfaces pose a contamination risk to ongoing and future life detection missions.

*Manuscript in Progress

Introduction

Various extreme conditions exist on the surface of Mars, including alkaline pH, high salt concentrations, low temperatures, extreme desiccation, and radiation, which limit the likelihood of survival by organisms (Schulze-Makuch et al., 2008). There is still a risk of microorganisms residing on spacecraft surfaces being transported to outer space via space travel and possibly inhabiting another planets. Numerous policies, regulations, and requirements, which are collectively known as PPP, are carried out on both an international and national level to help address these concerns and mitigate any risks associated with outer space missions. The act of transporting microbial life via spacecraft to celestial bodies is called 'forward contamination', whereas bringing extant life back to Earth via spacecraft return missions is called 'backward contamination' (Conley & Rummel, 2010).

The planetary protection measures implemented for a specific space mission are categorized by target body destinations and mission types and consist of varying degrees of severity. Due to the characteristics of the target body, a mission to Mars would fall under Category III or IV. A mission with no direct contact, such as an orbiter or flyby, would fall under Category III, while a lander or probe mission with direct contact of Mars would be categorized under Category IV (Meltzer, 2011). A Category III cruise stage, flyby, or orbiter mission would need to meet either 1) a required impact probability of no less than 0.99 for 20 years after launch and a probability of no less than 0.95 for 20-50 years post launch, or 2) a sterilization requirement limiting its total surface, mated, and encapsulated bioburden level to 5×10^5 spores (NASA, 2011).

Category IV requirements are divided into three different subsections dependent upon the nature of the mission. Category IVa requirements are designated for lander

missions not carrying life detection equipment and must meet a total surface biological burden level of 3×10^5 spores and an average of 300 spores per square meter of exposed internal and external spacecraft surfaces. Lander missions intended to investigate extant Martian life fall under Category IVb and must be in accordance with all planetary protection requirements for Category IVa as well as one of the following requirements: 1) a surface biological burden level of 30 spores on the entire landed system or a level of biological burden reduction and protection from recontamination that correlates with the sensitivity of the life-detection investigations, whichever are more stringent or 2) the sterilization of the life-detection subsystem instruments directly involved in the acquisition, analysis, and delivery of samples to these outlined levels. Lander missions, even without life detection equipment, investigating special regions on Mars must limit the entire landed system to a surface biological burden of 30 spores (NASA, 2011). The definition of a "special region" according to Meltzer (2011) is, "a region within which terrestrial organisms are likely to propagate *or* a region that is interpreted to have a high potential for the existence of extant Martian life-forms" (p. 464). Contamination of Mars and other celestial bodies could possibly hinder the ability to study celestial habitats in their natural state and impede any current or future life detection endeavors.

For a microorganism to pose a risk to planetary protection efforts, it would have to survive the harsh conditions present on the surface of Mars. Many studies indicate Mars contains elevated levels of salts (Sawyer, 2000; Treiman, 1999; Vaniman et. al, 2004; Wang et. al. 2006), and the salts found on the planet contain a large amount of sulfur compounds as sulfates of Mg, Ca, and Fe (Clark & van Hart, 1981; Clark, 1993; Wanke et al., 2001; Clark et al., 2005). Also present in the Martian soils is chlorine in the form of Mg, Na, or Ca

chlorides or perchlorates, but at concentrations significantly lower than those of sulfate salts (Crisler et al., 2012). McEwen et al. (2011) reported melting ice in Mars permafrost could possibly form flows of briny solutions on Martian soil thus any potential life on Mars must be able to grow in (or at least tolerate) the presence of high salt concentration (Crisler et al., 2012; Landis, 2001). Chaotropic agents are strong inhibitors of cellular systems due to their ability to destabilize biological macromolecules and activate a strong stress response (Hallsworth et al., 2003). Hallsworth et al. (2007) has shown $MgCl_2$ is an exceptionally chaotropic agent, which makes an $MgCl_2$ rich environment nearly uninhabitable. Cold temperatures and varying pH ranges are additional environmental challenges that life would have to endure on the Martian surface. Results from the Phoenix Mars Lander indicated the soil on Mars at the Vastitas Borealis site was found to be slightly alkaline (Hecht, 2009), but recent preliminary data from the Curiosity mission suggests the possibility of ancient acidic conditions on Mars (NASA, 2015). Frigidly cold temperatures are present on Mars, with -65°C as the average surface temperature, ranging from -10°C to -76°C (Horneck, 2008; Schofield, 1997).

Previous studies have tested the growth of microorganisms under NaCl conditions (Vilhelmsson et al., 1997, Taponen et al., 2012, Tanasupawat et al., 1992, Lim et al., 2006; Carrasco et al., 2007; Amoozegar et al., 2009), but scientific studies testing microorganisms in $MgSO_4$ conditions are lacking (Crisler et al., 2012; Kilmer et al., 2014). More specifically, studies testing members of the *Staphylococcus* genus for growth in $MgSO_4$ are limited (Kilmer et al., 2014), making this study important for a more thorough understanding of the physiological characteristics of high salt tolerating *Staphylococcus spp.* residing on spacecraft surfaces. A study conducted by van der Wielen et al. (2005) reported

on the discovery of a metabolically active microbial community in a deep hypersaline anoxic basin that is nearly saturated with $MgCl_2$ (5M), indicating evidence for possible microbial life in extreme environments. Several studies have described moderately halophilic *Bacillus* species that can grow in 20% NaCl (Lim et al., 2006; Carrasco et al., 2007; Amoozegar et al., 2009), but only one study has reported growth of *Bacillus* in high Mg^{+2} media (Crisler et al., 2012). With evidence pointing to microorganisms surviving such extreme conditions on Earth, the notion that life may be able to exist elsewhere in the Universe, such as Mars, where similar high salt conditions are likely present is not unreasonable. Determining whether microorganisms collected directly from spacecraft surfaces can grow in high salt environments will provide insight as to whether microorganisms could possibly tolerate conditions found on Mars or other potentially habitable celestial bodies.

Previous studies discussed in chapter three investigated the ability of over 1,300 microorganisms, collected pre-launch from the surfaces of the Viking landers and the Viking Precursor and from the Viking 1 and 2 orbiters and shrouds, to survive extreme conditions similar to those found on Mars and other potentially habitable celestial bodies (UI-JPL Collaborators). The Viking Precursor consisted of a flight orbiter and lander that was used prior to launch to check various spacecraft test operations at the launch site (Puleo et al., 1977). The shroud, or in some cases termed bioshield, was an elliptical shaped contamination control barrier to protect against microorganisms during and after sterilization (Meltzer, 2011). The results generated from these studies determined if the microbes present on the surfaces of launched spacecraft present a risk to planetary protection and future life detection missions. Identification and characterization studies have been performed on these

isolates, and many microorganisms have been identified that exhibit growth under extreme environmental conditions. These microorganisms were able to survive and grow after exposure to various single extreme conditions including high salt concentrations, low temperatures, extreme desiccation, and high pH, and many can utilize potential electron acceptors, which may be readily available on Mars and other planets (e.g. As^{+5}, ClO_4^-, Fe^{+3}, SO_4^{-2}). A number of microbial isolates collected from pre-launch spacecraft were capable of growth in 20% NaCl. What is not known is if these halotolerant isolates can grow in the presence of Mg salts, an aspect of their physiology that is critical for a more in depth understanding of their potential to survive and grow in the high salt environment on Mars.

Determining whether isolates from the previous characterization study in chapter 3 that were able to grow in 20% NaCl concentrations can grow in media containing increasing amounts of magnesium salts would greatly contribute to the research in planetary protection. Since the Mars surface presents multiple extreme conditions for growth of any life form, studies testing growth in the presence of Mg salts will be performed at a range of pH (6-9) and at low temperatures (in contrast to the studies in chapter three in which isolates were exposed to only 1 condition at a time). There are currently >1,300 isolates within the Viking archive, however, the study outlined in this article focused solely on the 244 isolates that were capable of growth in 20% NaCl. The majority (92%) of the 244 microbial isolates used in this study are *Staphylococcus*. The goal of this study is to determine whether the subset of Viking isolates capable of tolerating 20% NaCl conditions can survive and grow in $MgSO_4$ at various temperatures and pH levels. These studies are vital to the NASA Planetary Science Office, which oversees matters involving planetary protection.

Materials and Methods

Sample Preparation

The selection of microorganisms for this study was based on whether isolates were capable of growth in media containing 20% NaCl in previous studies presented in chapter three. Those capable of growth at 4°C temperatures within the subset that can grow at 20% NaCl conditions were selected to undergo testing at low temperatures. Selected isolates were streaked onto Tryptic Soy Agar (TSA) plates from their respective glycerol freezer stocks and then inoculated into 1 mL Tryptic Soy Broth (TSB). Pure culture was inoculated into 96-well plates containing neutral TSB and incubated with shaking for 24-48 hours at 30°C. Each of the characterization experiments were conducted separately from one another making the results distinct for each condition tested.

Table 4.1 List of $MgSO_4$ containing media employed at various pHs and temperatures

Media	Salt/pH	Temperatures
1	1M $MgSO_4$ at pH 6	30°C
2	1M $MgSO_4$ at pH 7	4°C and 30°C
3	2M $MgSO_4$ at pH 7	4°C and 30°C
4	1M $MgSO_4$ at pH 8	4°C and 30°C
5	1M $MgSO_4$ at pH 9	4°C and 30°C

Aerobic Growth Assays

All growth studies were done under aerobic conditions. The selected isolates were grown in TSB media containing $MgSO_4$ concentrations at varying pH levels. Aliquots (5 µl) of cells were transferred into 96-well assay plates containing either TSB (1200 µl) at pH 7 or buffered media at pH 6, 7, 8, or 9 containing 1M or 2M $MgSO_4$. The buffer used in the 2M

$MgSO_4$ media adjusted to pH 7 and all media at pH 8 levels was a 10 mM HEPES solution. The pH 6 buffer used was 10 mM MES and the pH 9 buffer used was 10 mM glycine. Each experiment was performed in triplicate. Growth was measured by monitoring turbidity at 600 nm using a spectrophotometer on days 0, 1, 2, 3, and 7. Isolates selected for low temperature studies were also incubated at 4°C. Turbidity measurements were taken at 600 nm using a spectrophotometer days 0, 1, 3, 7, 14, and 28 to determine growth.

Results

1M $MgSO_4$ and 2M $MgSO_4$ Conditions at Various pH Levels

All 244 isolates were tested under 1M $MgSO_4$ concentrations at pH levels of 6, 7, 8, or 9. A total of 78% of the isolates grew when subjected to 1M $MgSO_4$ concentrations at neutral pH. Testing the microbial isolates to 1M $MgSO_4$ concentrations in a slightly acidic (pH 6) environment resulted in growth of 78% of the isolates. There was a significant decline in the percentage of isolates capable of growth when subjected to $MgSO_4$ concentrations in alkaline conditions. A total of 37% of the isolates tested were capable of growth in 1M $MgSO_4$ concentrations at pH 8, and 8% grew in 1M $MgSO_4$ concentrations at pH 9. A total of 244 isolates were tested under 2M $MgSO_4$ concentrations at neutral pH. Of the microbial isolates subjected to these conditions, 75% were capable of growth.

A total of 18 isolates were capable of growth of all five conditions discussed in this section (1M $MgSO_4$ at pH 6, 7, 8, or 9 and 2M $MgSO_4$ at pH 7; all tested at 30°C) and are listed in Table 4.2. Members of the *Staphylococcus* genus represented the majority of this group, accounting for 13 of the 18 isolates. *Bacillus* represented 3 of the isolates, and 1 isolate was identified as *Streptococcus.* Table 1 shows the various media and temperature combinations employed.

Table 4.2 Microbial isolates demonstrating growth under all conditions tested at 30°C

Isolate	Genus	1M $MgSO_4$ pH 6, 30°C	1M $MgSO_4$ pH 7, 30°C	1M $MgSO_4$ pH 8, 30°C	1M $MgSO_4$ pH 9, 30°C	2M $MgSO_4$ pH 7, 30°C
LA 085	*Streptococcus*	+	+	+	+	+
LA 096b	*Bacillus*	+	+	+	+	+
LA 211	*Staphylococcus*	+	+	+	+	+
LA 318	*Bacillus*	+	+	+	+	+
LA 342	*Bacillus*	+	+	+	+	+
LA 351	*Bacillus*	+	+	+	+	+
OA 056	*Staphylococcus*	+	+	+	+	+
OB 078	*Staphylococcus*	+	+	+	+	+
OB 203	*Staphylococcus*	+	+	+	+	+
OB 205	*Staphylococcus*	+	+	+	+	+
OB 207	*Staphylococcus*	+	+	+	+	+
OB 234	*Staphylococcus*	+	+	+	+	+
OB 238	*Staphylococcus*	+	+	+	+	+
OB 239	*Staphylococcus*	+	+	+	+	+
OB 240	*Staphylococcus*	+	+	+	+	+
OB 241	*Staphylococcus*	+	+	+	+	+
OB 242	*Staphylococcus*	+	+	+	+	+
OB 243	*Staphylococcus*	+	+	+	+	+

4°C Temperatures

Four microbial isolates were selected for the low temperature studies based on previous growth study data provided by UI-JPL collaborators. Isolates were subjected to 1M $MgSO_4$ concentrations at pH 7, 8, or 9 and 2M $MgSO_4$ concentrations at neutral pH. All experiments were performed at 4°C temperatures. None of the isolates were capable of growth under any of the multiple stress conditions described. Table 4.1 shows the various media and temperature combinations employed.

Discussion

The study was undertaken to begin to address the issue of whether microorganisms transported from Earth during space exploration missions pose a potential risk to planetary protection efforts. The objectives were to determine whether the Viking isolates capable of growth in media containing 20% NaCl during the previous study presented in chapter three

could grow in media containing $MgSO_4$ at various temperatures and pH levels. Results from these multiple stress studies are important for understanding whether these resistant organisms residing on spacecraft surfaces could possibly inhabit Mars or other potentially habitable celestial bodies and whether these organisms pose a contamination risk to current and future life detections missions. The microbial isolates used in this study were collected directly from the surfaces of the Viking 1 and 2 landers, orbiters, and shrouds and from the Viking Precursor lander and orbiter prior to a terminal sterilization and launch. Generated information will help support NASA mission goals for planetary exploration by providing additional information about these organisms that will assist in the optimization of strategies related to meeting rigid mission requirements pertaining to microbial reduction efforts.

The majority (92% and 93%, respectively) of the microorganisms used in this study were members of the *Staphylococcus* genus and were capable of growth at pH 9 in previous studies (chapter 4). Approximately 78% of these microorganisms grew at 1M MgSO4 concentrations at neutral pH and 75% grew at 2M MgSO4 concentrations at neutral pH. While there was a slight decline (3%) in the number of microbial isolates that were capable of growth at the higher concentration of $MgSO_4$, there was an initial assumption there would be a larger disparity between the two concentrations. Due to higher water activity, sulfate-dominated ecological salt systems present a more hospitable environment for life when compared to chloride-dominated salt systems at the same concentrations (Marion et al., 2003). Our previous sodium chloride findings suggested that since the subset of microorganisms used in this study were able to tolerate 20% (3.42M) NaCl concentrations, they would likely be able to grow in both concentrations (1M and 2M) of MgSO4 tested in this study. *Staphylococcus* species are known to be capable of tolerating NaCl

concentrations (Ventosa et al., 1998; Vilhelmsson et al., 1997; Parfentjev & Catelli, 1964; Taponen et al., 2012; Tanasupawat et al., 1992). Although few studies exist that are focused on determining magnesium tolerance of *Staphylococcus* species, Kilmer et al. (2014) reported the majority of approximately 100 microorganisms isolated from an epsomite lake that represented a diversity of genera including *Staphylococcus* were capable of growth in media supplemented with up to 2M $MgSO_4$. In the presence and absence of osmotic stress, Kunin et al. (1991) discovered elevated levels of glycine betaine and K^+ inside the cells of some *Staphylococcus spp*., indicating elevated concentrations of these compounds could potentially be responsible for the organism's high salt tolerance and high turgor pressure.

In addition to the high salt concentrations, any life on Mars would have to tolerate very cold temperatures, since -65°C is the average surface temperature, and can range from -10°C to -76°C (Horneck, 2008, Schofield et al., 1997). Microorganisms would also need to tolerate a range of pHs since slightly alkaline soil conditions have been discovered on Mars (Hecht et al., 2009), and new preliminary evidence suggests ancient acidic conditions may have also existed (NASA, 2015). Multiple stressors, specifically alkaline conditions or low temperatures, paired with elevated $MgSO_4$ concentrations significantly challenged growth of microorganisms in this study. A significant decline in the number of isolates capable of growth was observed when 1M $MgSO_4$ was tested at elevated pH levels. Approximately 78% of isolates grew at 1M $MgSO_4$ at neutral pH, while just 37% grew at 1M $MgSO_4$ at pH 8 and 8% at pH 9. None of the isolates were capable of growth when $MgSO_4$ concentrations at pH 6-9 were tested at 4°C. The findings from this current study correlate with those found by a study conducted by Crisler et al. (2012) that tested $MgSO_4$ and NaCl concentrations at various pH levels and temperatures. These researchers found evidence indicating, "a greater

number of simultaneous stressors reduces growth more than fewer stressors" (Crisler et al., 2012, p. 103). Further studies involving multiple stressors that help push the physiological boundaries of microorganisms are warranted, since they can better simulate the stress of a natural environment. The information from multiple stress studies on bacteria isolated directly from spacecraft surfaces will help advance clean room technology and methods involving microbial contamination prevention.

While the study by Crisler et al. (2012) investigated the growth of microorganisms in multiple environmental stressors, the researchers cited Shukla (2007), Mattimore & Battista (1996), Billi et al. (2000), and Battista et al. (2001) for finding evidence supporting different environmental stressors can cause similar damage to microorganisms, and their survival appears to depend on recombination repair and oxidative-stress resistance. *Deinococcus radiodurans* is known for its remarkable survival to all reactive oxygen species (ROS), including desiccation and ionizing radiation, due to its strong oxidative stress resistance mechanisms that shelter proteins from oxidative damage and DNA repair process (Slade & Radman, 2011).

The studies presented in this chapter are necessary to gain a better understanding of the salt tolerance of microorganisms collected directly from Mars-bound spacecraft landers, orbiters, and shrouds. This information is particularly important for the NASA Planetary Science Office, which oversees matters involving planetary protection, because the generated data will provide valuable information about highly salt tolerant microorganisms residing on spacecraft surfaces, which could potentially pose a risk to any future life detection missions. Insight into the physiological capabilities of microorganisms collected

directly from the surfaces of outbound spacecraft will help to provide a better understanding of their potential risk to current and future planetary protection efforts.

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CHAPTER 5: CONNECTING THE FUTURE: AN INTEGRATIVE LITERATURE REVIEW AND CONCEPTUALIZED MODEL FOR CO-CREATING STEM PARTNERSHIPS IN FORMAL AND INFORMAL SPACES

Abstract

The future well being of our nation and the economy is dependent, in part, on a strong workforce in the fields of science, technology, engineering, and math (STEM). Connecting P-12 education to postsecondary institutions in formal and informal spaces can provide the resources, knowledge, and skills necessary for advancing STEM education. The model presented in this article was conceptualized through reflexive engagement and is intended to increase overall engagement in STEM fields, benefitting students and ensuring a more skilled workforce in the fields of science, technology, engineering, and math. Provided in the text is an integrative literature review to help support the conceptualized model presented in this chapter. The insight delivered within this model may create opportunities for STEM access and promote sustainable partnerships needed for the future of STEM education in both formal and informal environments. The author is presenting this conceptualized model for further consideration so that individuals in science and education disciplines can unite in promoting STEM education opportunities for all learners.

Introduction

Workers in science, technology, engineering, and math (STEM) fields are essential to the future of our nation because they are continuously creating new ideas, companies, and industries, all of which play key roles in the growth and stability of the United States' economy (Langdon, McKittrick, Beede, Khan, & Doms, 2011; President's Council of Advisors on Science and Technology, [PCAST] 2010). The President's Council of Advisors on Science and Technology reported STEM education at the elementary and secondary levels in our nation is lagging behind other nations creating a lack of proficiency and interest in STEM-related fields among students. The United States consistently falls somewhere in the middle of the spectrum or lower when our students' performance in math and science are compared worldwide, and only approximately one third of bachelor degrees earned in the United States are in STEM fields (PCAST, 2010). Additionally, the United States continues to face a large gap in interest and achievement within some groups in STEM resulting in an underrepresentation of African Americans, Hispanics, Native Americans, and women in STEM fields. Rogers-Chapman (2014) indicates access to STEM-focused schools across the nation is limited and "exclusive schools are more likely to have fewer students from disadvantaged backgrounds" (p. 726). There is and will continue to be a relentless obligation to expand STEM initiatives throughout P-12 schools in the United States. President Obama's administration has called for an education reform through the Race to the Top program with the purpose of advancing STEM education in the United States (U.S. Department of Education, 2012). The model presented in this article supports the five priority STEM education investment areas of the Federal STEM Education 5-year Strategic Plan: (1) to improve P-12 STEM instruction; (2) to increase and sustain youth and public engagement in STEM; (3) to improve undergraduate STEM education; (4) to better serve

groups historically underrepresented in STEM fields; and (5) to design graduate education for today's STEM workforce (National Science and Technology Council, 2013, pp. 8-12).

STEM-related interests such as choosing career paths (Wang, 2013; Robnett & Leaper, 2013) and math and science achievement (Singh, Granville, & Dika, 2002) are directly influenced by student motivation. According to Singh et al. (2002), motivation is a variable considered to be responsive to change and suggests more positive experiences at school and improved instructional approaches. An increasing number of opinions surround the belief that students' overall lack of interest in science education stems from the methods educators are utilizing to teach science content (Havasy, 2001; Edwards, 2014; Krajcik, Czerniak, and Berger, 2003). The development of STEM curriculum focused on supporting meaning and relevance could affect school-related motivation and science attitude. Havasy (2001) urged science educators to help learners connect science to the real world. Administrators from six specialized STEM high schools indicated student research experiences, "allowed their students real-world opportunities and better prepared them for the real-world expectations of their future careers in STEM fields" (Tofel-Grehl & Callahan, 2014, p. 257). Knowledge in STEM disciplines is always on the forefront and current techniques and information should continuously be incorporated into P-20 curriculum to better prepare future generations to join the STEM workforce. Rull (2014) points out, "The deliberate and consistent addition of new scientific knowledge to enhance education might seem an obvious application of science, but it is often ignored" (p. 921). Cutting edge science, technology, engineering, and math information implemented into educational curriculum is more engaging and relevant, potentially helping to attract and retain more students in STEM fields.

Various approaches can also be taken to help reach specific groups of students. Ferrini-Mundy (2013) recognizes the need to promote ways in which to advance diversity and inclusion in the STEM workforce and suggests the creation of "widespread opportunity to engage in authentic, inspiring STEM learning inside and outside of school" (p. 278). Establishing partnerships and creating innovative and engaging STEM learning opportunities that attract a wide range of populations, including underrepresented populations such as students from rural, low-income families, and gender specific populations, could help to generate a diverse future workforce in STEM fields. Promoting female role models and employing successful outreach campaigns could be used for recruiting women into STEM fields (Milgram, 2011). A report by the National Resource Council recommended that universities promoting positive images of women in STEM fields would be a beneficial resource to P-12 schools. Guest lectures, visits, after-school programs, and summer camps involving female college students, faculty, and alumni in STEM fields can be a way to promote positive images of women in STEM fields to P-12 students (National Resource Council, NRC, 2006). In a recent publication by Young, Rudman, Buettner, and McLean (2013), researchers found "identifiable female role models in STEM fields can increase a woman's implicit identification with science, while simultaneously decreasing, and indeed inverting, implicit gendered stereotypes about science" (p. 290). By promoting the expansion of science learning within diverse populations, there is a greater potential for an increased diversity of perspectives within the nation's workforce, and therefore, innovative and advanced ideas in science and technology fields that will help sustain not only the future well-being of the United States, but the global society and economy as well (Langdon et al., 2011; PCAST, 2010). Policy helps to

transform research into practice, and a recent National Science Foundation (NSF) solicitation called NSF INCLUDES is working to increase diversity in STEM disciplines. The goal of the new solicitation is to significantly transform STEM over the next ten years to further expand inclusiveness in these technical disciplines. The solicitation calls for specific types of partnerships in order to meet the foundation's stated goal:

> Collaborative alliances, spanning education levels, public and private sectors, and including new partners, will need to be developed, expanded, organized and built by leveraging state-of-the-art knowledge on scaling and social innovations. (NSF, 2016, p. 2)

Connecting educators to communities in the real world can help to bring forth resources, knowledge, and skills needed to generate new teaching methods and engaging curriculum in STEM fields. Giving educators the knowledge and access to the resources to effectively implement these practices within their own classrooms is imperative, and partnerships between business leaders, educators, and administrators should be established to create these opportunities to help reform science teaching (Havasy, 2001).

Connections for Advancing STEM

Workplace isolation is all too common in P-12 schools (Snow-Gerono, 2005) and higher education (Hadar, 2010), and is not conducive to sharing ideas and knowledge or developing innovative teaching methods and curriculum. Teachers in the study by Snow-Gerono (2005) indicated collaboration was a key factor within a school learning community. This was mainly due to a shift away from the commonly seen isolation in schools to a more community-based environment, and in turn, an improved access to people. Professional development is an important factor for educational reform by allowing for continuous improvement of educators' knowledge and skills. Robinson et al. (2014) attributed teacher

professional development and a problem-based/inquiry curriculum as two important factors for students to successfully advance their science capabilities. When discussing effective professional development, Darling-Hammond and McLaughlin (1995) state, "it must be collaborative, involving a sharing of knowledge among educators and a focus on teachers' communities of practice rather than on individual teachers." (p. 598). Establishing a collaborative, community-based environment within a school can help to break away from the isolated workplace environments in education and promote sharing amongst educators to create innovative teaching methods and engaging curriculum. Professional development does not only have to occur inside the classroom or school building, but connections should exist outside these boundaries between schools and universities, in teacher-to-teacher and school-to-school networks, with neighborhood-based youth organizations, and between district, regional, or national activities (Darling-Hammond & McLaughlin, 1995).

Particularly, teachers who teach within the STEM areas must stay current since knowledge and skills in these disciplines is always changing. Current information in these fields should be implemented into P-12 curriculum so students can be more prepared to pursue a career in a STEM field. For example, establishing a connection between educators and university researchers—as in the model presented in this article—may provide teachers with current and engaging scientific content to implement into their P-12 classrooms and principals with effective STEM professional development. Making these connections and establishing networks of communities allows for many diverse backgrounds and expertise to come together and generate innovative ideas for educators. Tomanek (2005) defines a partnership as involving "two or more people, each with expertise or skills to contribute, working toward a common goal" (p. 28). The model described in this article allows all of the

involved participants to combine each of their individual expertise to generate an effective and meaningful STEM outreach for P-12 students. Sharing responsibility for teaching STEM content in informal or formal environments and supporting STEM career fields—with P-12 student learning at the center—is the underpinning of the model presented in this article.

Distributed Leadership

A way to promote shared responsibility is to adopt a leadership style that is shared or distributed. Distributed leadership has been shown to bolster academic capacity in schools over time as a way to improve student-learning outcomes (Heck & Hallinger, 2009). Literature points to a form of shared or distributed leadership as an effective type of leadership to support various educational partnerships (Bullough, 2008; Coleman, 2011; Thorton & Cherrington, 2014; Firestone, 2002). Spillane's (2001) perspective of distributive leadership is used as the foundation for the leadership style and management practice in the model presented in this integrative literature review. Spillane (2001) defined distributive leadership as "identification, acquisition, allocation, coordination, and use of the social, material, and cultural resources necessary to establish the conditions for the possibility of teaching and learning" (p. 24). In his original article, Spillane (2001) was mostly interested in developing a distributive practice for leadership that transformed teaching and learning. Spillane, Halverson, and Diamond (2004) expanded Spillane's original framework (2001) on distributed leadership to include four central themes: (1) leadership tasks and functions, (2) task-enactment, (3) social distribution of task-enactment, and (4) situational distribution of task-enactment. More recently, Spillane (2015) reiterated the importance of educational leaders to fulfill both leadership and management roles, stressing the need to "work in

tandem in day-to-day practice in organizations" (p. 280).

Spillane (2001) explained how distributed leadership involves multiple leaders and how leadership is "stretched over" principals, assistant principals, curriculum specialists, and teachers in order to carry out specific tasks to achieve various goals in a school system. An important role teacher leaders carry out is the "translation of the principles of school improvement into the practices of individual classrooms" (Harris, 2003, p. 316). Empowering teachers as leaders may help to ensure these important duties are implemented within P-12 classrooms. Distributed leadership can be important when attempting to lead a partnership where there is not a single designated leader for all parties involved (Firestone, 2002), such as one between postsecondary institutions and P-12 schools or an informal learning center. Results from a study conducted by Mebane and Galassi (2000) showed university and public school participants were more satisfied with their co-led groups and overall experience as compared to those in singly led groups. In their study, Hudson, English, Dawes, and Macri (2012) described how distributed and self-activated leadership were utilized in a university-school STEM partnership. Hudson et al. (2012) found,

> Importantly, continuous consultation processes were at the [center] of distributed and self-activated leadership practices, ensuring open and informative communication between all partners. Project partners were willing to share information and were flexible by undertaking self-activated leadership roles that lead more readily to successful outcomes. (p. 783)

A distributed form of leadership can help to increase participation and engagement, as well as make participants feel more comfortable and validated when sharing their expertise with others. Open communication is imperative toward leading a collaborative team consisting of many different areas of expertise. Hallinger (2003) described transformational leadership as

a type of shared or distributed leadership and warns the personal capabilities needed to carry out transformational leadership are difficult to learn through training. Harris (2003) indicates, "While distributed leadership does not equate with 'delegation', it also does not represent a form of leadership that is so diffuse that it loses its distinctive qualities" (p. 319). Firestone (2002) cautions how distributed leadership can end up becoming dispersed leadership when a 'shared understanding of the common good' is lacking. He warns that creating shared values for professional development between a university-school partnership can be challenging due to the many different perspectives and interests of those involved, but remains hopeful for smaller communities within larger partnerships.

Conceptualizing a Model for Improving Outreach

Hoyle & Kutka (2008) point out there is a limited number of models for learning communities that attempt to cross the borders established between lower and higher education. The main goal of this article is to present an integrative literature review that supports the conceptualized model for P-20 connections that is intended to help reform science, technology, engineering, and math (STEM) teaching methods and make content more relevant for students. The model can be employed by a number of people in a wide range of fields including P-20 educators, scientists, researchers, directors of informal learning spaces, policymakers, and students. This conceptualized model is based on the notion everyone involved is practicing some form of shared or distributed leadership (Spillane et al., 2001).

The conceptualization for the model presented in this article came about through the author's own experiences and self-reflection as a graduate research assistant in the field of science. Having been a part of smaller STEM educational outreach projects in the

community, the author partnered with a faculty from the college of education and a secondary science teacher to co-create and implement STEM-related curriculum in a local public school. This process of self-reflecting on one's own practical experiences, also known as reflexive engagement was defined by Ravitch & Riggan (2012) as "thinking iteratively about the connections between our own interests and values, what we are learning in the field and from our data, and what that tells us about the topic or phenomenon we are trying to understand" (p. 143). By employing the approach of an integrative literature review (Torraco, 2005), the conceptualized model presented in this chapter has a structured foundation of literature related to the respective topics. This model was conceptualized with the intent to increase overall engagement in STEM fields, benefitting students and ensuring a more skilled workforce in the fields of science, technology, engineering, and math. The opportunities described in this model may provide access and promote sustainability needed for future STEM generations. The author is presenting this conceptualized model for consideration so that others may join the efforts in advancing the efforts in promoting STEM education. The conceptualized model for consideration includes the following eight components: (1) Directors of Informal Learning Centers as Collaborators in STEM Partnerships, (2) Informal Learning Centers as Facilitators of STEM Learning, (3) University Faculty as Collaborators in STEM Partnerships, (4) STEM Postsecondary Students as Liaisons for Sharing Content Knowledge, (5) P-12 Teachers as Experts of Facilitating Student Learning, (6) P-12 Administrators Practicing Distributed Leadership to Advance STEM Education, (7) P-20 Administrative Officials Supporting STEM Partnerships, and (8) Supporting Agencies.

Informal Spaces

Informal science learning involves some element of science learning within a non-school environment such as a museum, science center, state park, various media outlets, etc (National Research Council [NRC], 2009). The NRC indicates science learning experiences in informal environments are described as "learner-motivated, guided by learner interests, voluntary, personal, ongoing, contextually relevant, collaborative, nonlinear, and open-ended" (p. 11). Various informal science-learning experiences are believed to help facilitate more questioning from students, overall enjoyment, and a personal connection, creating a much more fulfilling, relevant, and positive experience for the student. Informal education can take place in numerous settings, which the NRC separates into three main categories: everyday and family learning, after-school and adult programs, and designed environments. Examples of designed environments can include places such as aquariums, museums, science centers, or libraries in which learners are guided with signs or other means, yet still maintain free choice of which exhibit or feature they visit (NRC, 2009). By still maintaining the learner-motivated nature of informal science learning as previously described, designed informal environments could potentially serve as an effective alternative for engaging a diverse range of students in science related content than traditional science education.

The potential effectiveness of learning scientific education in designed environments, such as science centers, has been recognized for over forty years (Kimche 1978). Wellington (1990) describes how an interactive science center can help to develop interest and motivation for STEM learning, which are both crucial components to ultimately understanding science. The author describes how students who attend a science center display specific characteristics such as interest, enthusiasm, motivation, eagerness to learn,

awareness and general openness and alertness, which are often lacking in traditional science education (Wellington, 1990). That being said, a recent study conducted by Holmes (2011) discovered museum-based learning had only minimal effects on students' science motivation and achievement. Findings on factors other than interest and motivation to learn science have shown learner engagement in scientific reasoning processes in informal learning environments. Kisiel et al. (2012) discovered families who visited four live marine species touch-tank exhibits demonstrated engagement in making claims, challenging claims, and confirming claims surrounding the activities of the live exhibit. Other scientific reasoning related conversation was revealed during the study, which included such things as applying prior knowledge, making and testing predictions and hypotheses, and constructing arguments (Kisiel et al., 2012). Ramey-Gassert (1997) states, "Science centers are envisioned to entice learners to go beyond their present knowledge and to construct a newer, larger vista of scientific thinking" (Ramey-Gassert, 1997, p. 436). In an article by Jones (1997), the author supports the idea of informal science learning as an effective method for extending scientific content to normally underserved populations in science education, which could potentially be the key to achieving diversity amongst the nation's future STEM workers.

Connecting informal learning centers with postsecondary institutions may provide the necessary cutting edge STEM content for developing educational activities for P-12 students. Various discovery or science centers and museums have the staff and knowledge for building exciting hands-on activities to engage students in learning. This could be an effective way to help attract and retain students in STEM disciplines.

Postsecondary Institutions

Postsecondary Faculty as Collaborators in STEM Partnerships

All faculty members at postsecondary institutions have an obligation to seek opportunities to partner with local school districts or informal learning centers to promote STEM education. Possibilities for collaborative outreach within a community are endless, and faculty members at the postsecondary level have access to the unique resources and engaging STEM knowledge required to create rich and fulfilling experiences for P-12 students. Komoroske et al. (2015) suggests,

> By incorporating the key elements of adaptability, flexibility, and creativity into their K-12 educational collaborations, scientists can achieve effective and rewarding IBL science outreach while advancing their research and fulfilling their other commitments. (p. 320)

Participating in outreach initiatives is achievable if it is planned and coordinated in a strategic manner. University faculty members are often times inundated with research, teaching, university service, and other responsibilities, making it difficult to co-create partnerships with P-12 school districts, informal learning centers, or even other local colleges. If time constraints are limiting potential outreach efforts for faculty members, they can empower graduate and undergraduate students to take the lead on co-created STEM activities within their communities. University and college students can develop activities that engage P-12 students in STEM learning and exploration of careers in STEM fields. As the model presented in this article suggests, graduate or undergraduate students could act as liaisons between the postsecondary institution and P-12 schools or informal learning centers to fulfill the project implementation and outreach efforts.

Graduate and Undergraduate Students as Liaisons for Sharing Content Knowledge

Graduate and undergraduate students studying in STEM fields are often involved with research at their university and can be great sources of knowledge in their areas of study. A graduate student or undergraduate pursuing a STEM-related major acting as a liaison between postsecondary institutions and P-12 schools or informal learning centers has the potential to transmit an immense amount of current knowledge, skills, and techniques to P-12 education. When discussing a National Science Foundation-funded GK-12 project where graduate student fellows "serve as conduits between university and school cultures" (p. 29), Tomanek (2005) credits the graduate student fellows with enabling the partnership between the university and school. The author further revealed the collaboration and partnership would not have existed if the graduate student fellows were not part of the project.

Postsecondary students studying in STEM fields can also act as influential role models for P-12 students. As previously mentioned, female college students pursuing a STEM field are amongst the groups of females within higher education that can help promote positive images of women in STEM fields by serving as role models for P-12 students. Female students who interacted with a female role model within the scientific community were more inclined to pursue their science interests (NRC, 2006). The role of graduate and undergraduate students acting as liaisons between higher education and P-12 schools or informal learning centers not only directly benefits P-12 students, but also provides the participating college students with valuable professional skills. Illingworth and Roop (2015) observe,

> For those scientists who wish to pursue a career outside of academia at the end of their Ph.D. or postdoctoral positions, these outreach activities provide communication and other key transferable skills. The initiative shown in

> participating in these activities can also look encouraging to employers, and demonstrates that the candidate has experience of working outside of academia, and communicating with the real world. (p. 11)

Not only are they practicing skills in scientific or technical communication, but they are also learning valuable presentation skills and teaching techniques within their subject area. College students would also have the experience of learning how to form professional connections and gain valuable practice acting as representatives of an institution. Ideally, college students will apply the philosophy described in the model for co-creating partnerships in their future careers whether it is as a scientist, researcher, schoolteacher, school leader, or in industry. The model presented in this article may provide helpful insights for future generations to expand their collaborative capabilities needed to promote STEM fields.

Formal Environments

P-12 Administrators Practicing Distributed Leadership to Advance STEM Education

In an article describing a university-school professional learning community, Bullough (2008) explains "developing professional learning communities and supporting educational renewal across institutions require new forms of leadership" (p. 292). P-12 administrators should practice distributed leadership (Spillane, 2001) and be open to partnerships focused on advancing STEM education. A shift in the role of the principal is required in distributed leadership. Harris (2012) suggests the principal's role in distributed leadership must be redefined with the purpose "to orchestrate the talent and leadership capability of others to move the school forward" (p. 15). To gain access to real-world applications of STEM content, P-12 administrators should remain open to both internal and external partnerships with their school. Connecting schools with external entities such as a science center,

museum, or university can prove to be extremely rewarding for P-12 students. As previously mentioned, a partnership with an external institution such as a university can supply valuable resources including professional development for educators, peer mentors for students, and current STEM knowledge, skills and techniques to be implemented into curriculum.

For advancing the learning and success of all P-12 students, principals must advocate a shared expectation regarding student performance (Lunenburg, 2010). Harris & Jones (2011) define a professional learning community as "a group of connected and engaged professionals who are responsible for driving change and improvement within, between and across schools that will directly benefit learners" (p. 173). Principals, as instructional leaders, are responsible for providing professional development with a clearly articulated objective (Bambrick-Santoyo, 2012). Real-world objectives and current scientific research incorporated into STEM curriculum makes for a much more engaging experience for the students. Observing faculty and teachers engaged in improving student learning transcends to P-12 students becoming more engaged in the content in which they are learning. Principals should be open to connecting with postsecondary faculty for professional development and to develop partnerships for other opportunities.

P-12 Teachers as Experts of Facilitating Student Learning

Teachers are experts in curriculum at the grade level and subject(s) for which they teach. Methods for teaching and content are continuously being improved; so staying informed with the latest STEM teaching methods is an important duty all teachers must fulfill. This can be done through professional development in the form of workshops, curriculum development, seminars, and continuing education courses. Herbers et al., (2011) indicated,

> The CoP is a community of scholar-practitioners whose individual members, through interactions of participation and reification, engage in meaningful discussions to convert abstract formulations into practical actions resulting in a common goal. (p. 93)

This type of reflection could help to share and advance STEM teaching methods in classrooms, ultimately benefitting the student learners. As experts in delivering content knowledge, teachers can advance student learning by continuously searching for current and engaging content to implement into curriculum for their students. A strategy Penuel, Harris, & DeBarger (2015) suggested for effectively implementing the Next Generation Science Standards (NGSS) in P-12 classrooms is to ensure teachers and students have access to high-quality curriculum materials. Previously mentioned in this article and as the model presented in this article demonstrates, connections made with higher education institutions could provide P-12 teachers and students with current and engaging STEM content for curriculum. Teachers should work to co-construct partnerships with available entities in their community such as higher education institutions, museums, libraries, science centers, and even STEM related industries.

P-20 Administrative Officials Supporting STEM Partnerships

Outreach is still undervalued within academic institutions (Komoroske, Hameed, Szoboszlai, Newsom, & Williams 2015), causing a lack of internal support and professional recognition for outreach efforts from within the universities (Illingworth & Roop, 2015). Without some form of encouragement from within the universities, outreach will remain undervalued and unsupported amongst higher education institutions. Komoroske, Hameed, Szoboszlai, Newsom, and Williams indicated incentives and rewards would follow once the benefits of collaborative outreach become recognized amongst faculty members and institutions. In fact, all P-20 administrative leaders should be collaborating and supporting

these university-school partnerships (Goodlad, 1993; De Bevoise, 1986; Dyson, 1999). Sirotnik & Goodlad (1988) in Goodlad (1993) indicate, "University presidents and deans, school superintendents, executive directors—these leaders need to be visible and clearly supportive of the [school-university] partnership concept and effort" (p. 31). The authors also see the need for a shared type of leadership within a university-school partnership and do not want it to be viewed as contradictory to the importance of top-level leadership and related support for the partnership.

Cultivating collaborative environments requires the continued support of school leaders; both school and university administrators can provide leadership that will support teachers, faculty and students to make the necessary connections and obtain resources to allow these relationships to be sustainable. To achieve advances in STEM education, the transition from the commonplace practice of isolated teaching to a more collaborative, community-based teaching environment must be made. Collaboration within an institution requires support from different departments including the necessary resources and frameworks to sustain these partnerships (Dolan & Tanner, 2005; Magolda, 2001). However, according to Dolan & Tanner (2005), before these resource commitments and infrastructure will be established, educational partnerships must be viewed as an important method to generate internally valuable knowledge. Similarly, Kezar (2005) finds that benefits of collaboration helpful to the goals of the campus must be understood before further steps can be taken to develop a collaborative environment. Although the authors suggest tenured professors would be the best candidates to lead outreach efforts and promote resulting benefits, all faculty members have a responsibility to promote and participate in outreach and collaboration with P-12 schools in some way.

Conceptualized Model for Advancing STEM Education Opportunities in Formal and Informal Spaces Through P-20 Partnerships

The model shown in Figure 5.1 is presented for consideration with the intent to increase overall engagement in STEM fields in formal and informal environments, which will help to promote student learning and help to bolster the United States' future STEM workforce. With sustained support and collaboration from P-20 leaders at the top, other participants in the model including postsecondary faculty, postsecondary students, school principals, P-12 teachers, directors of informal learning centers, and informal learning center staff can build

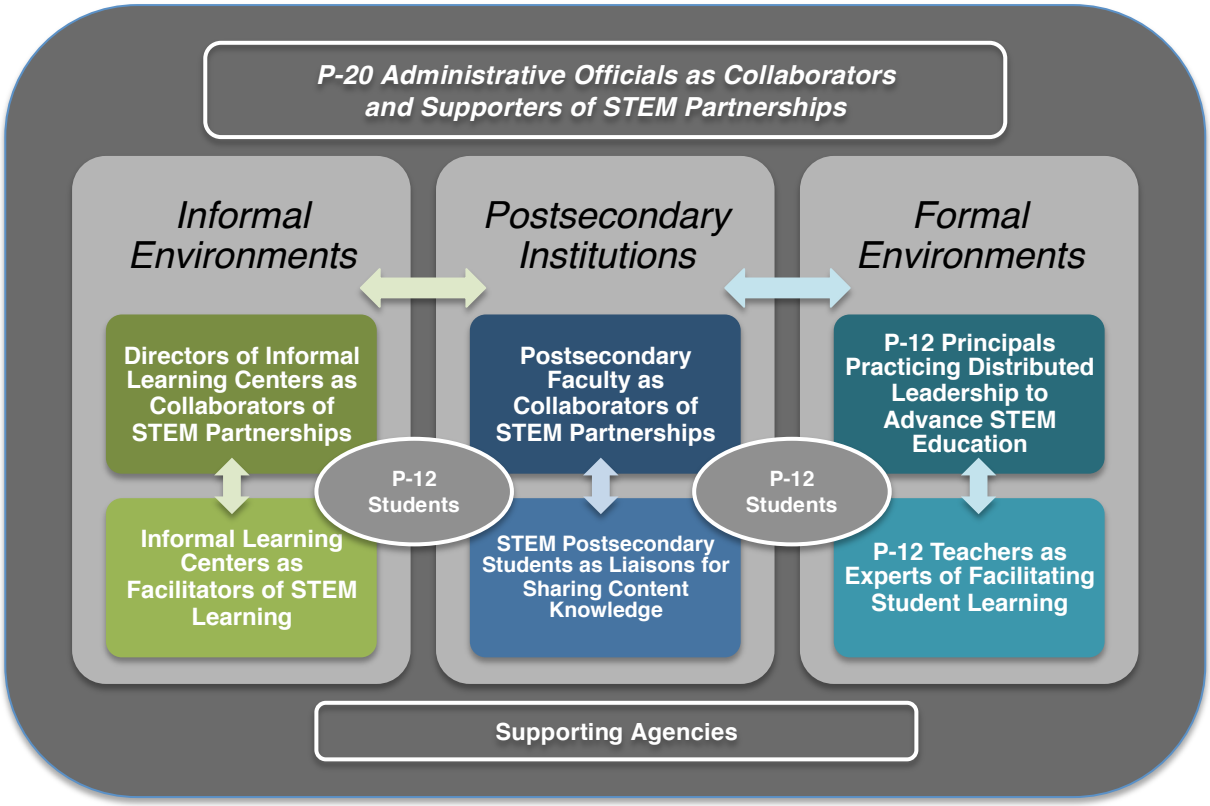

Figure 5.1 Conceptualized model for advancing STEM education opportunities in formal and informal spaces through P-20 partnerships

partnerships and employ his or her specialized expertise to advance STEM education for P-

12 student learners. Under the assumption all involved participants will be practicing distributive leadership, all groups will have the potential to take on a leadership role and assume responsibility for accomplishing tasks associated with the shared goal or vision of the project.

Discussion

Support and collaboration from university presidents and deans, school superintendents and executive board members is imperative for success of a university-school partnership. As previously described, distributive leadership has proven to be an effective form of leadership for educational partnerships and is utilized in the model shared in this article. Postsecondary faculty should act as collaborators and co-creators of STEM partnerships and empower undergraduate and graduate students to perform outreach activities within P-12 schools in the local community. Acting as liaisons between postsecondary institutions and P-12 schools, postsecondary students should share STEM content knowledge with P-12 teachers and students that they have learned from their respective degree coursework and/or research. As experts in content delivery and curriculum for their grade level, P-12 teachers should guide the development of engaging grade-specific STEM curriculum. P-12 principals should practice distributive leadership and focus on empowering leadership in others within the school and provide effective professional development for teachers to advance STEM education. Dolan & Tanner (2005) indicated sustaining partnerships between K-12 institutions and universities requires grant funding. All members participating in the partnership should share the responsibility of obtaining grant funding. Other support could stem from postsecondary institutions such as credit hours for graduate students.

A change in the perception of collaborative outreach within higher education must

occur before sustainable STEM partnerships can be established between P-12 schools and postsecondary institutions. Universities can offer an abundance of knowledge and resources to create rich experiences through collaborative outreach to help improve P-12 students' interest and achievement in STEM fields. Some of the resources universities can offer during STEM school partnerships include current STEM knowledge, scientific role models, laboratory resources, grant funding potential, industry connections, professional development, and peer role models such as undergraduates or graduate students. According to a recent article (Blank & Villarreal, 2015), growth of university-assisted community schools is on the rise. Recognizing the potential benefits of these partnerships, numerous university-school connections to promote STEM content and initiatives have been carried out since the turn of the century (Hudson et al., 2012; Hardre et al., 2013; Scott et al., 2011; Elgin, Flowers, & May, 2005). Markowitz (2004) reports on the impact of a university-developed high school summer outreach program on students' interest and perceived abilities in science. The study found students who participated in the outreach program reported a positive influence on their performance in the advanced science courses in which they are enrolled as well as a strong positive influence in their passion to pursue a science career. Weinstein, Whitesell, and Schwartz (2014), examined the impact of a long-term, collaborative partnership between the New York City Department of Education and eight informal science education institutions had on science education in New York City. The researchers found evidence supporting the program implemented improved performance in science. Collaboration between the U.S. Department of Agriculture, Texas A&M University, and regional P-12 schools generated effective and engaging educational STEM outreaches for P-12 students and provided internships for undergraduate students (Scott et al., 2011).

Partnerships are essential for providing students with real-life examples for learning STEM content, giving them an authentic and relevant environment for engaging in the curriculum.

Not only are university-school partnerships beneficial to the participating P-12 schools, but are also rewarding to the participating university (Walsh & Backe, 2013). P-12 schools provide school and classroom environments where university-enrolled student teachers and administrators gain practical knowledge in internships. When university representatives provide services co-created with local school districts, "the endeavor can become a 'win-win' for both partners, yielding advances in practice, research, and theory" (p. 596). Murray and Gurbisz (2012) address a partnership between scientific researchers, students, and educators. Just as the conceptualized model presented below in this article, the team chose scientists currently conducting scientific research to participate in the partnership. By doing this, it ensures current, ongoing scientific knowledge and techniques will be learned by the students and teachers involved. This study found that collaborative partnerships between these groups led to multiple positive outcomes for everyone involved, including strengthening research scientists' and graduate students' communication skills to convey their research to larger audiences, equipping educators with scientific research experience and knowledge to bring back into their classroom, and allowing undergraduates the opportunity to enhance their research and career backgrounds (Murray & Gurbisz, 2012). When a partnership is mutually beneficial for all involved parties, it can help aid in sustaining the collaboration (Duffield, Olson, & Kerzman 2013).

The conceptualized model is intended to promote engagement in STEM fields and help build a stronger workforce in the fields of science, technology, engineering, and mathematics for the future. This model will hopefully expand and create engaging

opportunities for STEM outreach and help promote sustainability within university-school partnerships. There is a need for further studies, including research involving the application of this model. Ideally, individuals from all disciplines will join in the related efforts for advancing STEM education and build upon the model presented for consideration within this review.

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CHAPTER 6: CONCLUSIONS

Conclusion

Nearly all of the microorganisms collected prior to a terminal sterilization and launch from the surfaces of the Viking landers, orbiters, and shrouds and from the Viking Precursor lander and orbiter were successfully identified. This was the first time molecular based tools were utilized to identify the microorganisms within the Viking microbial archive. A total of 563 of the 586 archived microbial isolates collected from the Viking landers and 706 of the 737 archived isolates collected from the Viking orbiters and shrouds were successfully identified by comparative sequence analysis. Results have determined over three-quarters of the Viking landers isolates were from spore-forming genera and slightly less than half of the Viking orbiters and shrouds isolates were spore-forming organisms. The remaining percentage of landers isolates and slightly over half of the orbiters and shrouds isolates were identified as non-spore-forming organisms with *Staphylococcus* and *Micrococcus* as the most represented genera. When compared to the identification results conducted by Puleo's research team in which classical bacteriological methods were utilized to identify these Viking microbial isolates, the identities of the isolates were remarkably similar.

All of the microbial isolates available within the Viking archive were subjected to simulated environmental conditions of the Martian surface and tested for their ability to utilize alternative energy sources potentially available on Mars. Many isolates were able to grow in media with adjusted salinity or pH, and some of the isolates were able to use perchlorate, iron, or selenite as a terminal electron acceptor. A small number of the selected microbial isolates that underwent additional testing were able to survive elevated temperatures and even high doses of UV-C radiation. A total of 244 isolates within the

Viking microbial archive that were capable of growth in 20% NaCl from the characterization studies in chapter three were tested in media containing $MgSO_4$ at various pH levels and temperatures. Over three-quarters of the isolates were capable of growth in media containing 1M and 2M $MgSO_4$ at neutral pH levels, but when the lowest level of $MgSO_4$ media tested was adjusted to pH 8 and pH 9, there was a significant decline in the number or organisms capable of growth. None of the isolates were capable of growth in any of the media tested at 4°C. It is apparent multiple stresses at a given time had a significant impact on the physiological capabilities of the microorganisms tested in this study. Further multiple stress studies of spacecraft associated microorganisms can help provide a more complete understanding of these microorganisms.

The study was undertaken to begin to address the issue of whether microorganisms transported from Earth during space exploration missions pose a potential risk to planetary protection efforts. Generated knowledge from all of the physiological studies conducted on the Viking microbial archive allows for a more thorough understanding of the bacteria residing on the surfaces of spacecraft. The information presented in this dissertation can help determine whether the sterilization methods used in planetary protection efforts are acceptable for current and future space exploration missions. The high percentage of non-spore-formers within the Viking microbial archive allows for insight into the resistance of non-spore-forming genera residing on spacecraft surfaces. This information helps to fulfill the request made in the recent review of the MEPAG Report on Mars Special Regions referring to further studies on non-spore-forming microorganisms collected from spacecraft surfaces. As the results from the physiological research presented in this dissertation suggest, many non-spore-forming organisms within the Viking microbial archive were

capable of surviving multiple extreme conditions. These findings support the conclusions drawn in the review of the MEPAG Report and warrant further, more in-depth studies on non-spore-forming microbes isolated from spacecraft surfaces. The suggested use of the molecular based assay techniques by the authors of the review of the MEPAG Report on Mars Special Regions will shed new light on a large portion of the bioburden residing on spacecraft surfaces, more specifically non-spore-forming organisms. This will most likely have a significant impact on any ongoing or future planetary protection research and bolster the existing body of research pertaining to planetary protection for future manned missions to Mars.

The literature pertaining to establishing and sustaining partnerships within P-20 education to advance STEM education in informal and formal spaces was reviewed and served to support the conceptualized model presented in chapter five. The intention of the conceptualized model is to promote and increase the overall engagement in STEM fields amongst students by improving partnerships within P-20 institutions to ensure a strong workforce in the fields of science, technology, engineering, and mathematics for the future of the United States. Ideally, the insight this model contributes will help promote the practice of sustainable P-20 partnerships in formal and informal spaces to create engaging opportunities in STEM education for a diversity of learners. Space exploration and the related planetary protection research would not be possible without a strong workforce in the fields of science, technology, engineering, and mathematics. Continuous efforts in the advancement of STEM education is imperative for a sustainable future of space exploration and to ensure the necessary planetary protection research is conducted to help preserve the natural state of celestial bodies for the sake of future scientific investigations.

APPENDIX A

Table A1 Organisms demonstrating the ability to utilize multiple electron donors and acceptors.

Isolate	**Genus**	**Acetate**	**Lactate**
LA 038	*Paenibacillus*	Fe^{+3} Se^{+4}	Fe^{+3} Se^{+4}
LA 040	*Bacillus*	Fe^{+3} Se^{+4}	Fe^{+3} Se^{+4}
LA 067b	*Paenibacillus*	Fe^{+3} Se^{+4}	ClO_4^- Fe^{+3} Se^{+4}
LA 288	*Paenibacillus*	Fe^{+3} Se^{+4}	Fe^{+3} Se^{+4}
LB 125	*Paenibacillus*	Fe^{+3} Se^{+4}	Fe^{+3} Se^{+4}
LB 152	*Micrococcus*	-	As^{+5} ClO_4^-
LB 177	*Paenibacillus*	Fe^{+3} Se^{+4}	Fe^{+3} Se^{+4}

Table A2 Organisms demonstrating aerobic growth under multiple extreme conditions.

Isolate	Genus	Highest pH	NaCl 20%	4°C
LA 002	*Bacillus*	9	-	+
LA 072	*Bacillus*	9	+	-
LA 085	*Streptococcus*	10	+	-
LA 091	*Staphylococcus*	9	+	-
LA 093b	*Bacillus*	9	-	+
LA 094	*Bacillus*	9	+	-
LA 102	*Staphylococcus*	9	+	-
LA 104	*Staphylococcus*	9	+	-
LA 110	*Staphylococcus*	9	+	-
LA 114	*Bacillus*	9	-	+
LA 116	*Bacillus*	9	+	-
LA 119	*Staphylococcus*	10	+	-
LA 120	*Staphylococcus*	9	+	-
LA 123	*Staphylococcus*	9	+	-
LA 126	*Bacillus*	9	-	+
LA 128	*Bacillus*	9	-	+
LA 138a	*Cellulomonas*	9	-	+
LA 138b	*Gordonia*	9	-	+
LA 143	*Staphylococcus*	9	+	-
LA 165b	*Bacillus*	9	-	+
LA 170	*Bacillus*	9	-	+
LA 178	*Arthrobacter*	9	-	+
LA 198	*Bacillus*	9	-	+
LA 208	*Bacillus*	9	+	-
LA 211	*Staphylococcus*	9	+	-
LA 247	*Bacillus*	9	-	+
LA 318	*Bacillus*	10	+	-
LA 342	*Bacillus*	9	+	-
LA 351	*Bacillus*	9	+	-
LA 352	*Rhodococcus*	10	-	+
LA 354	*Staphylococcus*	9	-	+
LA 368	*Staphylococcus*	9	+	-
LA 376	*Staphylococcus*	9	+	-

Table A2 (cont.) Organisms demonstrating aerobic growth under multiple extreme conditions.

Isolate	Genus	Highest pH	NaCl 20%	4°C
LA 415	*Bacillus*	9	+	-
LB 060	*Paenibacillus*	9	-	+
LB 095	*Staphylococcus*	9	+	-
LB 100	*Staphylococcus*	9	-	+
LB 102	*Staphylococcus*	9	+	-
LB 105	*Staphylococcus*	9	+	-
LB 112	*Staphylococcus*	9	+	-
LB 122	*Staphylococcus*	9	+	-
LB 124	*Staphylococcus*	9	+	-
LB 126	*Bacillus*	9	-	+
LB 151a	*Not Identified*	9	-	+
LB 151b	*Staphylococcus*	9	+	-
LB 202	*Sporosarcina*	9	-	+
LB 209	*Staphylococcus*	9	+	-
LB 219	*Staphylococcus*	9	+	-
LB 220	*Staphylococcus*	9	+	-
LB 238	*Staphylococcus*	9	+	-
LB 239	*Staphylococcus*	9	+	-
LB 244	*Staphylococcus*	9	+	-
LB 245	*Staphylococcus*	9	+	-
LB 246	*Staphylococcus*	9	+	-
LB 247	*Staphylococcus*	9	+	-
LB 253	*Staphylococcus*	9	+	-

- = Negative for growth

Table A3 Identification results from the total number successfully identified from the Viking landers isolates (563) broken down by spacecraft

16S Identification	Lander 1	Lander 2	Precursor Lander
Agrococcus			
A. citreus	0	0	1
A. jenensis	0	1	0
Arthrobacter citreus	0	0	1
Bacillus			
B. aerophilus	14	0	0
B. altitudinis	0	0	1
B. amyloliquefaciens	5	5	15
B. anthracis	0	0	1
B. aryabhattai	1	0	0
B. atrophaeus	1	0	0
B. cereus	13	8	4
B. circulans	2	1	1
B. clausii	1	1	0
B. flexus	1	2	1
B. gibsonii	0	0	1
B. infantis	1	0	0
B. koreensis	0	0	1
B. licheniformis	1	11	2
B. nealsonii	2	1	6
B. megaterium	4	4	17
B. methylotrophicus	1	0	2
B. mojavensis	1	0	6
B. mycoides	1	0	0
B. pumilus	26	32	39
B. safensis	2	6	2
B. selenatarsenatis	0	1	0
B. sp	30	44	52
B. subtilis	24	15	15
B. tequilensis	1	1	0
B. thuringiensis	1	1	0
B. vallismortis	0	1	1
Cellulomonas flavigena	0	0	1
Cellulosimicrobium sp.	0	0	1
Corynebacterium minutissimum	0	0	1
Enterobacter cloacae	0	0	1
Geobacillus stearothermophilus	0	1	0
Gordonia terrae	0	0	1
Kocuria sp.	0	1	1

Table A3 (cont.) Identification results from the total number successfully identified from the Viking landers isolates (563) broken down by spacecraft

16S Identification	Lander 1	Lander 2	Precursor Lander
Macrococcus sp.	1	0	0
Methylobacterium sp.	0	0	1
Micrococcus			
M. luteus	2	14	0
M. sp	3	7	0
M. yunnanensis	1	3	0
Paenibacillus			
P. xylanilyticus	0	2	0
P. favisporus	0	1	1
P. sp	4	2	2
P. lautus	1	2	1
P. campinasensis	0	0	1
P. taiwanensis	0	0	2
P. pabuli	0	0	1
Paracoccus yeei	0	2	0
Rhodococcus sp.	1	0	0
Rothia			
R. aeria	1	0	0
R. dentocariosa	1	0	0
Sporosarcina sp.	0	1	1
Staphylococcus			
S. capitis	0	1	2
S. epidermidis	0	18	4
S. haemolyticus	0	1	1
S. hominis	3	1	1
S. sp	7	11	7
Streptococcus sanguinis	0	0	1
Streptomyces sp.	0	0	1
TOTALS	158	203	202

Table A4 Identification results from the total number successfully identified from the Viking orbiters and shrouds isolates (706) broken down by spacecraft

16S Identification	Orbiter 1	Orbiter 2	Shroud 1	Shroud 2
Agromyces				
A. sp.	3	0	0	0
Acinetobacter				
A. schindleri	4	0	0	0
A. sp.	3	1	1	0
A. radioresistens	0	4	0	0
Arthrobacter				
A. sp	1	0	0	0
Bacillus				
B. amyloliquefaciens	6	1	0	1
B. anthracis	1	0	2	0
B. arsenicus	1	0	0	0
B. aryabhattai	1	2	0	3
B. barbaricus	1	0	0	0
B. cereus	7	2	4	0
B. circulans	0	0	0	1
B. clausii	1	0	0	0
B. firmus	0	0	0	1
B. flexus	0	0	1	0
B. idriensis	3	0	0	0
B. jeotgali	1	0	0	0
B. licheniformis	1	1	5	2
B. megaterium	6	8	3	3
B. methylotrophicus	0	1	1	0
B. mojavensis	3	0	0	0
B. nealsonii	2	0	2	2
B. okhensis	0	1	0	0
B. pumilus	25	10	9	5
B. safensis	6	1	0	1
B. sp.	45	32	10	13
B. subtilis	24	10	1	1
B. tequilensis	2	0	0	0
B. thioparans	0	1	0	0
B. thuringiensis	3	1	1	0
Brachybacterium				
B. sp.	1	0	2	0
Brevibacterium				
B. casei	1	0	0	0
B. sp	2	0	0	0

Table A4 (cont.) Identification results from the total number successfully identified from the Viking orbiters and shrouds isolates (706) broken down by spacecraft

16S Identification	Orbiter 1	Orbiter 2	Shroud 1	Shroud 2
Cellulomonas				
C. hominis	1	0	0	0
Chungangia				
C. sp.	0	0	0	1
Clostridium				
C. tertium	0	1	0	0
Corynebacterium				
C. aurimucosum	1	0	0	0
C. glaucum	0	0	1	0
C. imitans	0	1	0	0
C. singulare	1	0	0	0
C. sp.	0	0	3	0
Kocuria				
K. palustris	0	1	0	0
K. sp.	2	0	0	0
Lysinibacillus				
L. sphaericus	0	4	1	0
Methylobacterium				
M. sp.	1	0	0	0
Microbacterium				
M. sp.	0	2	0	0
Micrococcus				
M. luteus	14	5	0	0
M. sp.	7	2	1	0
M. yunnanensis	1	0	0	0
Moraxella				
M. osloensis	1	0	0	0
Nocardioides				
N. sp.	0	1	0	0
Paenibacillus				
P. lactis	0	0	0	1
P. pabuli	1	0	0	0
P. sp.	2	6	3	0
P. woosongensis	2	0	0	0
Paracoccus				
P. yeei	0	0	1	0
Pseudoclavibacter				
P. alba	2	0	0	0
Pseudomonas				
P. sp.	0	1	0	0
P. stutzeri	2	0	0	0

Table A4 (cont.) Identification results from the total number successfully identified from the Viking orbiters and shrouds isolates (706) broken down by spacecraft

16S Identification	Orbiter 1	Orbiter 2	Shroud 1	Shroud 2
Sporosarcina				
S. sp.	0	2	0	0
Staphylococcus				
S. capitis	2	1	2	0
S. epidermidis	48	36	6	9
S. saprophyticus	3	6	0	0
S. haemolyticus	10	35	2	1
S. sp.	42	35	18	7
S. hominis	8	9	16	2
S. pettenkoferi	2	4	0	0
S. kloosii	2	0	0	0
S. warneri	0	4	0	0
S. pasteuri	0	1	1	0
S. cohnii	0	2	0	0
S. xylosus	0	1	0	0
Terribacillus				
T. goriensis	0	1	0	0
T. saccharophilus	0	2	0	0
T. sp.	1	5	0	0
Zimmermannella				
Z. alba	0	1	0	0
TOTALS	310	245	97	54