National Aeronautics and Space Administration

A Researcher's Guide to:

INTERNATIONAL SPACE STATION

Microbial Research

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Cover and back cover:

a. Astronaut Dr. Peggy Whitson evaluates microbial growth on a surface sample kit (SSK) contact slide within the microgravity science glovebox (MSG) as part of the Genes in Space-3 investigation. The SSK contact slides are used as part of the Crew Health Care System's routine microbial monitoring of the ISS. In a spaceflight first, Dr. Whitson collected cells from the colonies and placed them into miniPCR for DNA extraction and gene amplification. Following this, she used the MinION (not shown) to sequence the DNA amplified from these organisms. The identifications obtained onboard the ISS matched those determined on the ground following nominal processing of the returned SSK slide. This marked the first time unknown organisms were collected, cultured, and identified off Earth.

b. Back Cover 1: Computer-generated bacteria image.

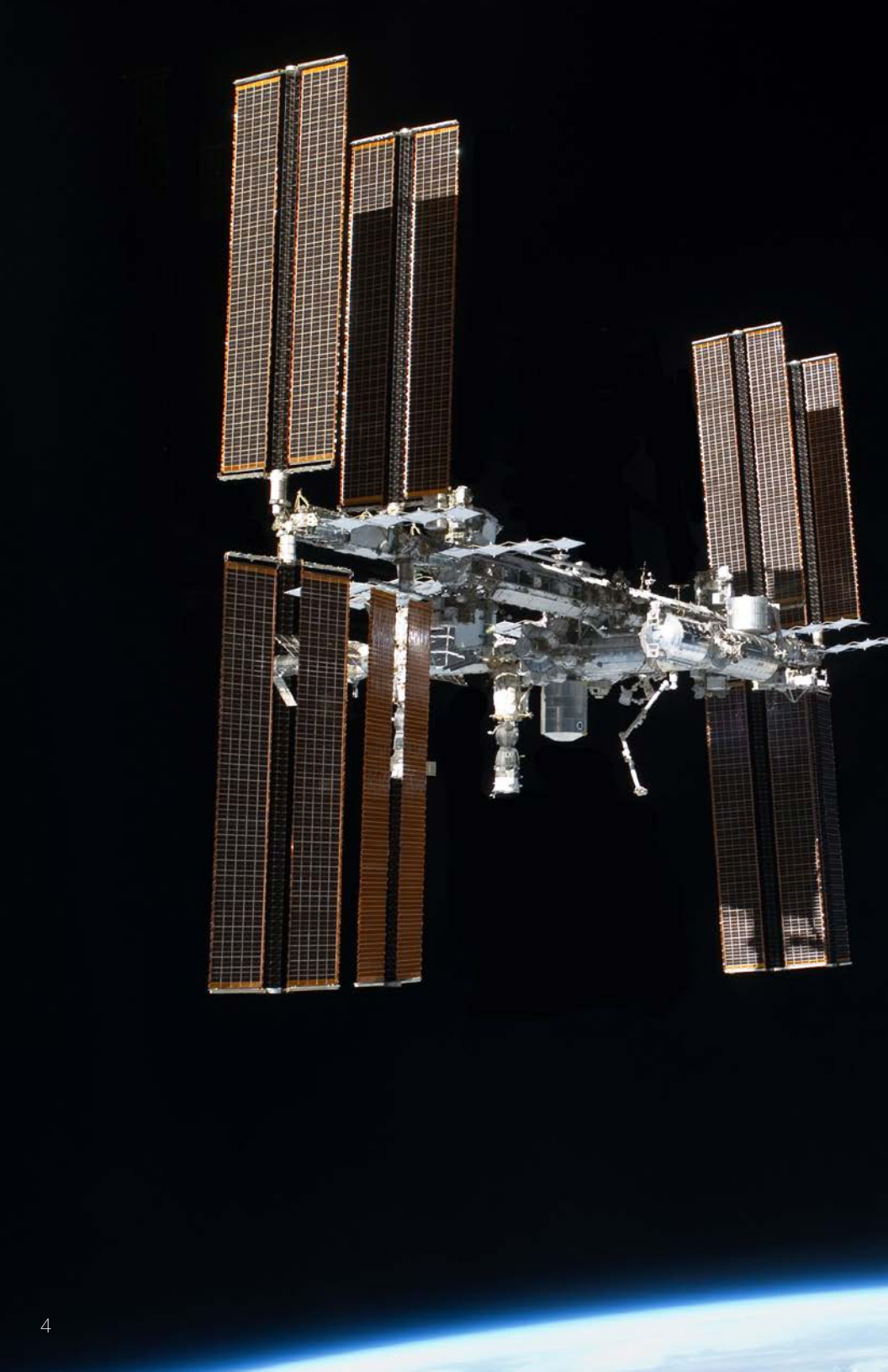
c. Back Cover 2: Computer-generated virus image.

The Lab is Open

Orbiting the Earth at almost 5 miles per second, a structure exists that is nearly the size of a football field and weighs almost a million pounds. The International Space Station (ISS) is a testament to international cooperation and significant achievements in engineering that has supported the advancement of scientific knowledge and technology development for over 19 years. The ISS is a truly unique research platform providing ground-breaking research opportunities for commercial, government and academic users. The possibilities of what can be discovered by conducting research on the ISS are endless and have the potential to contribute to the greater good of life on Earth and future long-duration deep space exploration missions.

As we increase utilization of ISS as a National Laboratory, now is the time for investigators to propose new research and to make discoveries unveiling novel responses that could not be defined using traditional approaches on Earth.

An astronaut holding a Microbial Air Sampler (MAS) Petri Dish on the ISS. The MAS is used for the collection of cabin air atmosphere for evaluation of the microbial load.



Unique Features of the ISS Research Environment

1. **Microgravity**, or weightlessness, alters many observable phenomena within the physical and life sciences. Systems and processes affected by microgravity include surface wetting and interfacial tension, multiphase flow and heat transfer, multiphase system dynamics, solidification, and fire phenomena and combustion. Microgravity induces a vast array of changes in organisms ranging from bacteria to humans, including global alterations in gene expression and 3-D aggregation of cells into tissue-like architecture.

2. **Extreme conditions** in the ISS environment include exposure to extreme heat and cold cycling, ultra-vacuum, atomic oxygen, and high energy radiation. Testing and qualification of materials exposed to these extreme conditions have provided data to enable the manufacturing of long-life reliable components used on Earth as well as in the world's most sophisticated satellite and spacecraft components.

3. **Low Earth orbit** affords ISS a unique vantage point with an altitude of approximately 240 miles (400 kilometers) and an orbital path over 90 percent of the Earth's population. This can provide improved spatial resolution and variable lighting conditions compared to the sun-synchronous orbits of typical Earth remote-sensing satellites.

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Microbiology Research Priorities on the ISS

We are on the cusp of the next giant leap in space exploration and related scientific research. The private sector has reinvigorated the space race, and several countries have affirmed their intentions of developing a robust human spaceflight program. The United States has targeted full utilization of the International Space Station (ISS) and set its exploration sights beyond low-Earth orbit. As we determine our destinations for the next generation of spaceflight, several questions remain unanswered as to the effects of the spaceflight environment on human physiology and the microorganisms that will inevitably accompany them. Previous spaceflight studies have demonstrated that microgravity can enable better understanding of fundamental biology and accelerate advancements in healthcare and medical technologies. These benefits are critical, not only for human deep-space exploration, but also for improving quality of life on Earth.

A human is both an individual organism and an entire ecosystem, including microorganisms in, on, and around the body, in which the microbial cells are roughly equal in number to the human cells. For the most part, these microorganisms are beneficial to their human host or are otherwise innocuous.

Given the right set of conditions, many otherwise benign microorganisms can become pathogenic. Therefore, potential pathogens have been present on all NASA missions (Rogers 1986, Castro 2004). Protective measures such as stringent microbial monitoring, prudent vehicle design, and preflight crew quarantine are used to decrease the risk of infectious disease during missions (Johnston 1969, Rogers 1986).

Over the past 50 years, a combination of operational experience, spaceflight and ground-based research have provided tremendous insight into infectious disease risk as well as necessary preventative measures (Johnston 1969, Taylor 1972, Taylor 1976, Facius 1978, Fang 1997, Nickerson 2004, Ott 2004). Significant strides to define and mitigate the source of microbial contamination aboard spacecraft and to document the responses of numerous microorganisms to the spaceflight environment have allowed the identification of critical gaps in our understanding of how this environment impacts microbial ecology, the microbial genotypic and phenotypic characteristics, and their interactions with plant and animal hosts.

As we look toward human interplanetary exploration, the importance of this knowledge is imperative. With the increases in both the occupancy and duration of humans aboard the ISS, these knowledge gaps are becoming better defined.

There is much to gain by employing the microgravity environment of spaceflight as a basic research platform. Life on Earth evolved in the presence of gravity. Therefore, performing research in the reduced gravity of spaceflight holds the potential to determine how this physical force shaped terrestrial life. Previous spaceflight and ground-based spaceflight analog research has established that even microorganisms, the smallest Earth-based life forms, are intrinsically able to respond to changes in this force (Dickson 1991, Mishra 1992, Nickerson 2000, Nickerson 2004).

While over 50 years of microbial research has been performed in spaceflight, a thorough understanding of microbial responses to spaceflight culture and how the spaceflight environment stimulates these responses is only beginning to be understood. Microgravity as a research tool, coupled with current molecular technology, provides researchers the opportunity to establish how variations in this physical force affect microbial life at the cellular, molecular and evolutionary levels. This potential is not surprising since innovative answers to complicated medical, environmental, and agricultural questions have arisen from assessing the properties of microorganisms in many extreme environments on Earth (Nickerson 2004).

Similarly, the study of microbes in the spaceflight environment holds considerable potential for future basic research and industrial applications. Investigations into microbial ecology, genotypic and phenotypic properties, and the infectious disease-causing potential of microorganisms in the spaceflight environment may unveil novel mechanisms that can not be elucidated using traditional approaches on Earth, where gravity may be restricting our discovery of unique cellular responses.

The ISS as a Microbial Research Platform

The ISS provides a unique research platform that enhances our knowledge of the effects of gravity on microorganisms. This unique environment allows a better understanding of microbial mechanisms and interdependencies that would normally be masked by gravity, enabling insight into fields including microbial physiology, microbial interactions, molecular microbiology and microbial ecology. The ISS is also an ideal setting to study microorganisms in a complex contained, isolated, “island-like” ecosystem. Many scientific studies have focused on either complex ecosystems that are not well controlled in a classical experimental sense or very simple ecosystems that are well controlled but severely limited in dimension and/or diversity. To date, complex controlled ecosystems have not persisted for

long periods, so studying microbial dynamics within them has been necessarily a short-term endeavor. Since its initial launch in the commencement of construction in orbit, ISS has been a relatively closed system with microbial exchange occurring during a small number of cargo resupply missions and crew changes. Factors influencing microbial growth and response are well monitored and recorded, including environmental conditions such as temperature and humidity as well as crew diet and activity. New ISS modules and transported cargo are also evaluated for microbial diversity and concentration.

Benefits of an ISS Microbial Research Platform

The ISS research platform provides an opportunity to broaden our understanding of the unique microbial responses of microorganisms cultured during spaceflight. This aspect of the ISS research platform distinguishes it from every other available facility since no other platform can provide this microgravity environment. As the microorganisms are adapting their responses to this novel environment, information can be gathered that provides unique insight into microbial regulation and function that cannot be discerned using traditional methods on Earth.

The use of the ISS as a microbial research platform drives experiments that could decrease infectious disease risk during the human exploration of space, advance the application of beneficial purposes for microorganisms (e.g., waste remediation, probiotics), and provide unique insight into basic microbial functions and interactions that could be translated to studies for scientists and commercial entities on Earth. Translation of spaceflight findings has already begun to take place as scientists and corporations investigate the use of ISS microbial findings to better understand virulence profiles, antibiotic and disinfectant resistance, biofilm formation, and biodegradation properties.

As NASA travels beyond low-Earth orbit to planets such as Mars, insight from ISS microbial research will influence our approach to exploration. NASA proposes to support advanced research focused on discovering and characterizing fundamental mechanisms used by microorganisms and microbial communities to adapt to the diverse challenges of the spaceflight environment highlighted in Table 1. Understanding how spaceflight and gravity alter microbial responses, their exchange of genetic material, and their expected concentrations and distribution is vital in the search for extraterrestrial while also protecting other planets from microbial transfer.

Table 1. Opportunities for Microbial Research on the ISS

Subject Area	Investigations of spaceflight environment alterations
Microbial Physiology	Microbial growth profiles Response to stressors Motility Microbial metabolism
Microbial Ecology	Relative roles of microbial ecology and evolution The human and plant microbiome as a subset of the ISS microbiome Microbial interactions with the environment over time Microbial population dynamics and dynamics of succession Stabilities of closed-model communities (not ambient) Mechanisms of community change/biogeography Selection pressure and generational aspects within selected microbes versus communities Microbial populations occurring naturally in the environment (air, surfaces, water) Spread of identified strains as a result of the spaceflight environment
Molecular Microbiology	Microbial genomic diversity and evolution Microbial sensing Microbial transcriptome, proteome, or metabolome
Microbial Interactions	Microbe-microbe interactions Host-microbe interactions Plant-microbe interactions Biofilm formation or function (single species and mixed populations)

The ISS also offers an unprecedented opportunity to advance indoor microbial ecology research. It provides an experimental platform for controlling two of the largest contributors to indoor microbial diversity: ventilation source and occupancy load. Research has shown that ventilation source significantly impacts microbial diversity indoors, with mechanically ventilated rooms harboring more potential airborne pathogens than naturally ventilated rooms (Kembel 2012). It has also been demonstrated that human occupancy load impacts the abundance and diversity of airborne microbes (Qian 2012).

Together, these findings suggest that tremendous knowledge would be gained by conducting experiments in a highly controlled environment like the ISS where the ventilation source and occupancy load can be systematically analyzed. By sampling the built environment microbiome and human microbiome in the ISS jointly, it would be possible to tease apart how microbes are exchanged among humans, indoor air, and indoor surfaces.

These findings are important for future spaceflight missions, as well as terrestrial homes and offices. Indoor microbial communities might be intimately connected to human health (Burge 1995, Mitchel 2007, Srikanth 2008), including the spread of acute respiratory disease (Cohen 2000, Smith 2000, WHO 2007, Glassroth 2008) and the increase in the occurrence of asthma symptoms (Ross 2000, Eggleston 2009, Schwartz 2009).

Another unique benefit of the ISS as an experimental, complex, closed ecosystem is that over time the microbial communities present on the space station are likely to become increasingly dominated by human-associated microbes. Within the confines of the ISS, the environmental control and life support system maintains a homeostatic environment suitable for sustaining the human crew. This environment also acts to sustain and select a human-associated microbiome that persists on the ISS across all missions.

As has been observed in other environmentally controlled and human-engineered constructs such as office buildings and airplanes, the microbiome will change over time in diversity (i.e., number of different types or species of microorganisms present) and structure (i.e., the relative composition of different types or species). The relative abundance of human-associated bacteria, including those that could potentially cause disease, is higher indoors than outdoors. Since the ISS is relatively closed, the microbial diversity is stable throughout the interior of the station such that the dispersion of new microorganisms can be tracked and the impact of their addition to the station microbial community can be evaluated. This premise may also make possible investigations into changes in the astronaut microbiome.

Unexpected Microbial Responses to Spaceflight Culture

The short generation time of microorganisms makes them uniquely suited for studies assessing responses to altered environmental conditions. Microbial cells were among the first Earth-based life forms to be sent into the microgravity environment of space. These early investigations established that bacteria and fungi remained viable and capable of reproducing while also setting a precedent for conducting research in the spaceflight microgravity environment. Although more than 100 spaceflight experiments involving microorganisms have been conducted over the past 50 years, significant gaps remain in our knowledge about how this environment impacts microbial ecology, microbial genotypic and phenotypic characteristics, and host-microbe interactions.

In 1960, prior to the flight of Yuri Gagarin, scientists from the Union of Soviet Socialist Republics (USSR) launched *E. coli*, *Aerobacter aerogenes*, and *Staphylococcus* species into orbit aboard an unmanned satellite (Zhukov-Verezhnikov 1962, Zhukov- Verezhnikov 1963). It was this experiment that lead to the conclusion that the microgravity environment of space did not affect the viability of the microorganisms (Zhukov-Verezhnikov 1962, Zhukov-Verezhnikov 1963). In an important subsequent experiment, the USSR launched *E. coli* aboard Vostok 2 in 1961, which resulted in the identification of a variant colony type that was reported to be a result of spaceflight factors (Klemparskaya 1964).

In 1967, NASA launched the unmanned Biosatellite 2, which exposed various biological specimens, including *E. coli* and *Salmonella enterica* serovar Typhimurium, to the microgravity environment of space for 45 hours (Mattoni 1968, Mattoni 1971). For both microorganisms, an increase in population density was noted for the in-flight samples (Mattoni 1968, Mattoni 1971). *Bacillus subtilis* was cultured aboard Apollo 16 and 17 and resulted in the finding that microgravity did not affect the developmental process of spore formation (Bucker 1975). However, when assessed after culture aboard the Apollo-Soyuz Test Project, the colony forming ability of *B. subtilis* spores was found to be reduced among spaceflight samples (Facius 1978). With evidence mounting that bacteria were able to sense and respond to the microgravity environment of spaceflight, the concern of both the U.S. and USSR space programs shifted to how these variations could impact crew health.

Over the course of numerous spaceflights, researchers from various countries have analyzed changes in antibiotic resistance in *E. coli* and *Staphylococcus aureus* (Tixador 1983, Tixador 1985, Tixador 1985, Lapchine 1987). The minimal inhibitory concentration (MIC) of oxacillin, chloramphenicol, and erythromycin for *S. aureus* and colistin and kanamycin for *E. coli* were evaluated among in-flight cultures as compared to controls (Tixador 1985, Tixador 1985). These investigations documented increased bacterial resistance to all antibiotics tested for both *S. aureus* and *E. coli*. The researchers observed a thickening of the cell wall that accompanied the increase in resistance of *S. aureus* once returned from flight (Tixador 1985, Tixador 1985). Various other microbial properties were recorded during this time, including increased conjugation in *E. coli* (Ciferi 1988) and increased growth kinetics in *B. subtilis* (Mennigmann and Lange 1986) in response to microgravity.

The space shuttle era brought an enhanced capability to perform biological research within the microgravity environment of space and to delve further into the implications for human health. In 2006, taking advantage of this opportunity, investigators launched several microorganisms including *S*. Typhimurium aboard Space Shuttle Atlantis (STS-115) in an attempt to define the impact of spaceflight culture on the disease-causing potential of the microorganisms (Wilson 2007, Crabbé 2011). The results of the study reported that mice infected with bacteria grown in flight displayed a significantly decreased time to death, increased percent mortality, and decrease in the lethal dose (Figure 1 D) (Wilson 2007). Analysis of the fixed returned samples revealed differential expression of a large number of genes and identified a regulatory protein that was mechanistically associated with the spaceflight response of the organism (Wilson 2007). This was the first report elucidating both the molecular response connected with a regulatory mechanism and alterations in bacterial virulence because of growth in the spaceflight microgravity environment.

To confirm these findings and further our understanding of factors influencing spaceflight culture-mediated changes in virulence, a follow-up investigation was performed on Space Shuttle Endeavor (STS-123) to again assess the response of *S*. Typhimurium to the spaceflight environment. This set of experiments included culturing *S*. Typhimurium in various different types of growth media in the spaceflight environment (Figure 1 E). The findings from this spaceflight experiment confirmed the previous reports of increased virulence of the bacteria

Figure 1. Astronauts A) Heidi Stefanyshyn-Piper activates S. Typhimurium to grow in flight during the MICROBE experiment on STS-115 and B) Don Gories activates S. Typhimurium to grow in-flight as part of the MDRV experiment on STS-123. After growth in the spaceflight environment, the bacterial samples were returned to Earth and immediately used in a mouse model of salmonellosis. The results from both experiments revealed that S. Typhimurium becomes more virulent as a result of growing in the microgravity conditions of space. The experiments also described the composition of the growth medium as an important factor controlling the change in virulence (Wilson 2007, Wilson 2008).

(Wilson 2008). Furthermore, the data from this assessment revealed that media ion concentration dramatically influences the spaceflight-related virulence response of *S*. Typhimurium (Wilson 2008).

In addition to microgravity, the spaceflight environment has a unique radiation background. Several spaceflight experiments have investigated the impact of this radiation on microbial organisms (de Serres 1969, Berry and Volz 1979, Bouloc and D'Ari 1991, Horneck 2010); however, topics such as alterations in mutational rates and how these mutations could alter the phenotype of the organisms is generally understudied.

Microbiology of the Built Environment

While the ISS is generally closed to external influences, microorganisms are continuously introduced on the ISS, providing a novel platform for the investigation of human and environmental microbiomes. Since the ISS is not a completely closed system, the low frequency of exchange of people and materials with the outside and the potential for characterization of the microbiology of materials brought to station makes control of microbial inputs to this unique system more achievable. The ISS offers opportunities to study the dynamics of microbial populations and communities in the absence of uncontrolled introduction of microorganisms from unknown sources. While other ecosystems, such as homes or submarines, have some of the characteristics of ISS, none can match this platform's unique isolation from contaminating contacts. This isolation provides the opportunity to gain insight into the interactions between humans and environmental organisms and changes in microbial communities through mutation or genetic exchange with minimal external interference.

Microbial diversity, concentration, and antibiotic resistance

Most of our understanding of the microbial diversity aboard spacecraft has relied on culturing microorganisms using a relatively few types of growth media. Generally, the environmental data indicate that the potable water, air, and surfaces to which the crew is exposed are free of obligate pathogens; however, opportunistic pathogens such as *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, and *S. aureus* are not uncommon (Pierson 1996, Castro 2004, Pierson 2012).

Spaceflight food is another potential source of microorganisms aboard spacecraft. While the incidence of contamination is low, preflight analyses of food samples have indicated the presence of organisms such as Salmonella enterica serovar Typhimurium (*S.* Typhimurium), *S. aureus*, *Enterobacter cloacae* and *Cronobacter sakazakii* (unpublished data). Figure 2 details the relative abundance of bacterial and fungal strains isolated from the air and surfaces of the ISS before and during flight. Since these findings are based on cultured organisms, only a part of the picture of the microbial diversity of spacecraft has been captured. By coupling current molecular methods with the ISS platform, a higher resolution of this picture has the potential to be viewed.

While multiple preflight measures are in place to limit microbial contamination aboard spacecraft, the limitations of these conventional monitoring methods were demonstrated by a comprehensive media-based and microscopic analysis of microorganisms isolated from free-floating water collected behind panels aboard

the Mir Space Station (Ott 2004). Several medically significant organisms that were not commonly isolated during standard operational monitoring were identified, including *Legionella* species, *Serratia marcescens*, and *E. coli*.

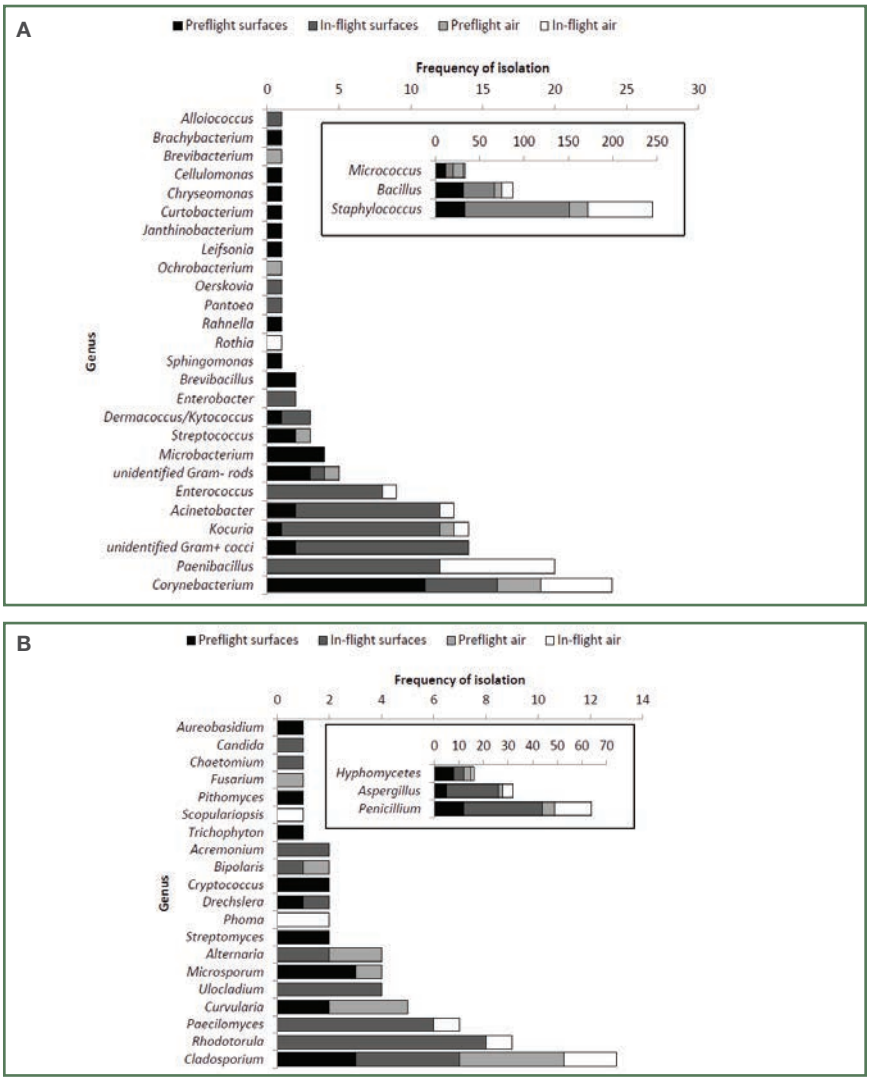

Figure 2. The abundance of A) bacterial and B) fungal strains isolated from air and surfaces from the International Space Station environment before and during flight. Isolates are categorized by genera and relative abundance (Pierson 2012).

Further microscopic examination of these samples revealed the presence of amoeba resembling *Acanthamoeba* or *Hartmanella* species and ciliated protozoa resembling *Stylonychia* species (Ott 2004). This finding reinforced the need for a more thorough investigation of the microbial diversity of spaceflight habitats, especially at time points later in their service life.

Recent studies have taken modern molecular approaches to characterize the microbial ecology of the built environment aboard the ISS. The ISS environment is predominantly composed of human-associated microorganisms (Mora 2019, Blaustein 2019). Findings also concluded that the microbial community composition on the ISS is not much different than terrestrial homes and persists across spaceflight expeditions even with the change of crew and payloads (Lang 2017, Singh 2018, Blaustein 2019).

Modern molecular approaches have also been used onboard the ISS with the goal of near real-time microbial profiling of the environment. The emergence of portable molecular biology tools such as miniPCR bio's miniPCR™ and Oxford Nanopore Technologies' MinION™ has made *in situ* analysis a reality with both systems being tested onboard the ISS in 2016 (Boguraev 2017, Castro-Wallace 2017).

With the validation of the first off-Earth polymerase chain reaction (PCR) and DNA sequencing, methods were developed to apply this technology to the identification of microbes onboard the ISS. In 2017, an astronaut collected cells from culture slides used for routine microbial monitoring, extracted and amplified DNA, generated sequencing libraries, and sequenced the resulting DNA with the MinION (Burton 2020). From the three colonies selected by the crewmember, two identifications were obtained. Upon return of the culture slide to the ground and nominal processing, the same identities were confirmed, as two of the colonies were the same organism (Burton 2020). This outcome resulted in the first collection, culture, and identification of unknown microorganisms off Earth.

To enable a more rapid assessment of the environment and to extend understanding beyond the limitations of traditional culture processes, a direct swab-to-sequencer, culture-independent method was developed and tested onboard the ISS beginning in 2018 (Stahl-Rommel 2021). As with previous investigations where samples were returned to Earth for molecular analysis, the data displayed a high similarity to that of the human microbiome. While unsurprisingly higher in diversity and difficult to culture organisms, the data also paralleled that of historic culture-based sampling (Figure 3.) (Stahl-Rommel 2021).

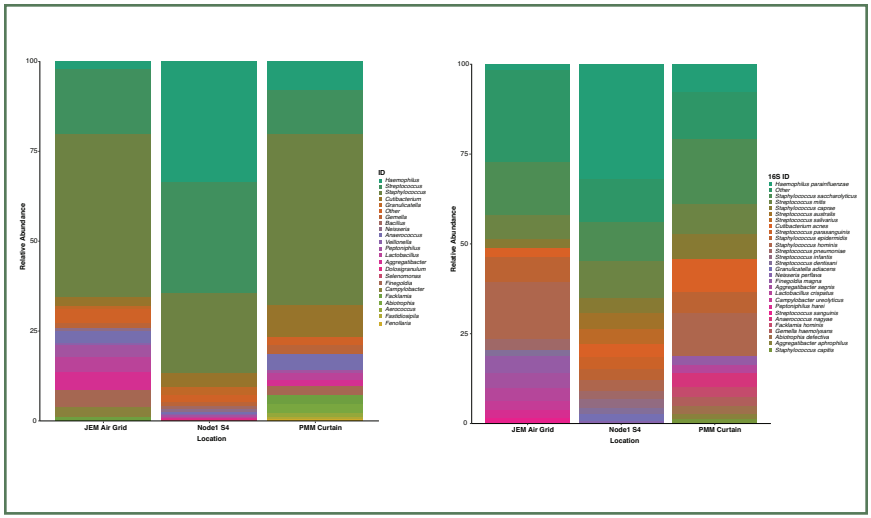

Figure 3. Microbial profiles generated from three locations onboard the ISS through use of the culture-independent, swab-to-sequencer method of the top 15 A) genus- and B) suggested species-level identifications. Data were generated onboard following sample collection, processing, and sequencing. Raw sequence data were downlinked to Earth for analysis. (Stahl-Rommel 2021)

Preventive measures during spaceflight missions continue to provide protection to the crew. Implementation of a preflight quarantine period, which began during the Apollo program, has significantly limited the number of obligate pathogens that are carried into the spacecraft by the crew. Other efforts to reduce the infectious disease risk to the crew include but are not limited to: routine screening of spaceflight food for the presence of harmful microorganisms, HEPA filters installed on the ISS, and treatment of water system with biocides. However, the risk of obligate and opportunistic pathogens on spacecraft has not been eliminated.

Biofilms and biofouling in water processing systems

The advancement of the current spacecraft water disinfection system is a major concern to take into consideration for future spaceflight missions. Biofilm formation aboard the ISS was first documented on STS-95 (Mclean 2001). Bacteria that form biofilms can often withstand environmental stressors such as disinfectants and antibiotics. The protective niche biofilms offer bacteria can also harbor infectious organisms that can potentially threaten crew health (Donlan 2002).

Microorganisms play a pivotal role in the functioning of key spacecraft systems such as the ISS water system (Pierson 2012). Microbial contamination due to biofilms within the water system could be catastrophic since the system has multiples uses such as providing potable drinking water, irrigating plants grown for consumption, and aiding in crew hygiene. Elevated bacterial concentrations have been detected in both the Russian and U.S. potable water systems (Bruce 2005, Pierson 2012). There are also reports of biofilm growth and associated biofouling of filters, membranes, and the stainless steel water line associated with spacecraft water systems (Carter 2017, Diaz 2019). These events further support the necessity to prevent or control microbial growth, inhibit or prevent biofilm formation, and prevent microbially-induced biofouling in design of future spacecraft systems.

Astronaut Microbiomes

With the exception of sending microorganisms into orbit for research purposes, significant care is taken to reduce the levels of microbes in the spaceflight environment. Stringent preflight microbiological monitoring and remediation of NASA spacecraft has been performed throughout the human spaceflight program (Johnston 1969, Rogers 1986, Castro 2004). However, the combination of the crew members' microbiota and the inability to ensure complete sterility of the craft and cargo results in the coexistence of humans and microorganisms in the spaceflight environment.

In-flight data acquired during Apollo, Skylab, and the Mir Space Station missions increased our knowledge of the impact of spacecraft habitation on the crew and vehicle microbiota. Findings from these early spaceflight programs were critical to the design of later spacecraft and in establishing microbiological acceptability limits for the in-flight environment. While this information and the insight gained from the space shuttle and ISS programs has proven critical in our approach to mitigating microbial risk to crewmembers and their vehicle, several questions still remain.

Microbiological evaluations of the crew members were in place since for the first manned Apollo flight with the goal of characterizing the microbial load of astronauts preparing for lunar surface exploration (Taylor 1972). During early Apollo missions, a thorough microbial baseline was established for each astronaut to facilitate the identification of any possible terrestrial contaminants in returned lunar samples. Studies conducted during the later Apollo missions were designed to identify and prepare for possible microbial-associated issues arising as a result of the lengthier Skylab program (Taylor 1972). The findings of these early investigations included identifying trends such as increases in the number of sites on a crew member's body that organisms were isolated from, the quantity of those organisms, and increased levels of microbes in the environment (Johnston 1969, Taylor 1972). These early studies also documented the incidence of microbial transfer between crew members and the spacecraft environment (Taylor 1972). The knowledge gained resulted in operational and engineering activities to control the environment of the crew concerning crew contacts (quarantine), food, water, and air.

More recent studies have collected data over longer time periods and sampling more body sites with the implementation of longer duration spaceflight missions. One study analyzed 16S and metagenomics sequencing to characterize astronauts' gut, skin, nasal and oral microbiomes over 6+ months before, during and after flight

(Voorhies 2017). Results from inflight samples revealed that the overall composition of bacterial communities from the gut, skin, and nose significantly change in space. Moreover, for some crew members, intestinal microbial diversity increased under microgravity conditions, most likely promoted by the new spaceflight diet and/or the crowded living conditions of the ISS that might favor a more fluid interchange of microbial flora among crew members (Voorhies 2017). The NASA Twins study also found more changes in microbial community composition and function were found during spaceflight (Garrett-Bakelman 2019). These findings warrant further investigation into longer duration spaceflight impacts on crew health.

Initiating Ground-Based Research – Spaceflight Analogs

Microgravity cannot be created on Earth; however, aspects of the microgravity environment can be mimicked by use of ground-based simulators. Numerous ground-based methods of simulating the microgravity environment of spaceflight have been developed and implemented to overcome the constraints that accompany biological gravitational research. Ground-based simulators have proven indispensable as tools for preparing spaceflight experiments and have generated independent investigations. For example, parabolic flights and drop towers are means of providing "free fall" for a limited amount of time.

To enable analysis of microbial response to aspects of the microgravity environment for greater amounts of time, other analogs have been developed using a variety of technology such as clinostats, rotating-wall vessels, random positioning machines, and magnetic levitation (Klaus 2001, Nickerson 2004, Herranz 2013). While these simulators do not eliminate the force of gravity, they reproduce many characteristics of the environment produced in true microgravity.

An Example of a Spaceflight Analog – The Rotating Wall-Vessel Bioreactor

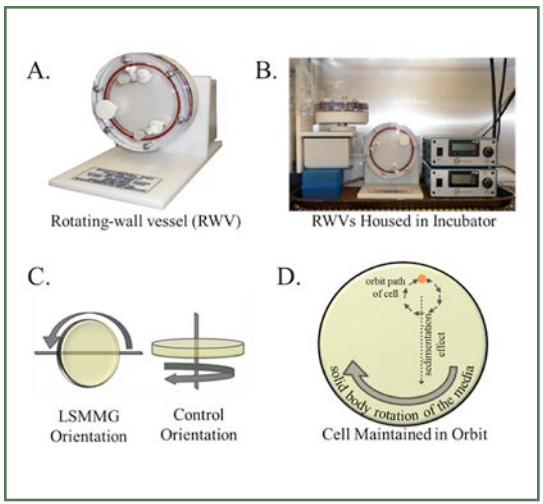

Figure 4. The Rotating-Wall Vessel Bioreactor (Synthecon, Houston, Texas). (A) Image of the NASA-designed RWV apparatus. (B) RWV culture system in the incubator with the respective base units and power supply systems. (C) The altered positioning of the RWV that results in the two culture orientations, depicting the axis of rotation. The LSMMG environment is achieved by rotation of the RWV on an axis parallel to the ground, whereas the axis of rotation in the control orientation is perpendicular to the ground. (D) Depiction of the orbital path of a cell when cultured in the LSMMG orientation. The combination of the clock-wise solid body rotation of the media and the sedimentation effect, whereby gravity and lack of motility causes a cell to settle to the bottom of the vessel, results in the continuous suspension of the cell in an orbit.

Each spaceflight analog system has unique advantages and disadvantages (Klaus 2001, Buels 2009, Dijkstra 2011). Of the simpler systems, the rotating-wall vessel

(RWV) bioreactor has been increasingly used to enhance our understanding of microbial responses that may be occurring during spaceflight (Fang 1997, Nickerson 2000, Lynch 2004, Nauman 2007, Crabbé 2008, Castro 2011). The RWV (Figure 4 A) is an optimized form of suspension culture in which cells are grown in physiologically relevant, low-fluid-shear conditions. These low-shear effects of the fluid on the cells has resulted in the adoption of the term "Low-Shear Modeled Microgravity" (LSMMG) for use in accurately describing the environment produced by the RWV bioreactor (Wilson 2002). A cell in liquid media in microgravity experiences two unique aspects important in modeling this environment: 1) remaining in a constant state of suspension and 2) experiencing a quiescent surrounding, devoid of shearing, turbulent forces (Klaus 1997). The RWV bioreactor effectively models these aspects of the microgravity culture environment.

Insights Gained from Microbial Culture Within the Rotating Wall-Vessel Bioreactor

The RWV bioreactors were initially intended as a spaceflight analog for eukaryotic cells (Wolf 1991, Hammond 2001) but have since been used to examine bacteria (Fang 1997, Nickerson 2000, Crabbé 2008, Castro 2011), fungi (Johanson 2002), and archaea (Dornmayr- Pfaffenhuemer 2011) in response to this environment. In the mid-1990s, Fang and colleagues were the first to put a bacterium inside the RWV. The team was primarily focused on the effects of LSMMG on secondary metabolite production (Fang 1997, Fang 1997). Over the course of their studies, they noted that the modeled microgravity environment of the RWV did not alter gramicidin production from *Bacillus brevis* (Fang 1997), decreased beta-lactam production by *Streptomyces clavuligerus* (Fang 1997), inhibited *Streptomyces hygroscopicus*' production of rapamycin (Fang 2000), and prevented microcin B17 production from *E. coli* (Fang 2000). A summary of certain bacterial, fungal, and archaeal responses to the simulated microgravity conditions within the RWV bioreactors since the work of Fang and colleagues can be found in Table 2.

Pioneering work by Nickerson and colleagues expanded this area of research by connecting the LSMMG response of an enteric pathogen, *S*. Typhimurium, to a human host and the spaceflight environment (Nickerson 2000, Wilson 2002, Wilson 2002). The conditions within the RWV were found to have profound effects on the behavior of *S*. Typhimurium, including an increase in its virulence potential (Nickerson 2000). Mice challenged with LSMMG-cultured *S*. Typhimurium suffered an increased percent mortality, increased time to death, and required a lower LD50 as compared to control cultures (Nickerson 2000). The

Table 2. Examples of Microbial Responses to Modeled Microgravity

Microorganism	Response to Modeled Microgravity within the RWV Bioreactor	Reference
S. Typhimurium 3339	- Increased: virulence in a mouse model; resistance to acid, thermal, and osmotic stress; macrophage survival - Decreased: LPS production; resistance to oxidative stress; Hfq expression - Differential gene expression	Nickerson, 2000 Wilson, 2002 Wilson, 2002b Wilson, 2007
S. Typhimurium 14028	- Increased: virulence in a mouse model and cellular invasion - Differential gene expression	Chopra, 2006 Pacello, 2012
S. Typhimurium D23580	- Altered virulence profile and pathogenesis-related stress responses	Yang, 2016
E. coli AMS6	- Increased biofilm formation and resistance to osmotic, ethanol and antibiotic stress	Lynch, 2006
E. coli E2348/69	- Increased intimin production	Carvalho, 2005
E. coli MG1655	- Decreased growth - Differential gene expression	Tucker, 2007
E. coli K12	- Differential gene expression	Vukanti, 2008 Tirumalai, 2017
E. coli 083:H1	- Increased resistance to thermal and oxidative stress and adhesion to epithelial cells	Allen, 2008
E. coli O157:H7	- Increased cell size; membrane fluidity - Decreased resistance to thermal stress	Kim, 2014 Kim, 2016
E. coli ATCC25922	- Increased expression of efflux pump genes; decreased antibiotic susceptibility	Xu, 2015
Klebsiella pneumoniae	- Thicker biofilm formation; increased productin of cellulose - Differential gene expression	Wang, 2016
Mycobacterium marinum	- Differential gene expression; Role for SigH identified - Increased sensitivity to oxidative stress	Abshire, 2016
P. aeruginosa PA01	- Increased: biofilm formation; elastase production, and rhamnolipid production; alginate production; resistance to oxidative and thermal stress; Hfq expression - Differential gene expression	Crabbé, 2008 Crabbé, 2010
Streptococcus pneumoniae TIGR4	- Differential gene expression	Allen, 2006 Allen, 2007
Streptococcus mutans	- Differential gene expression - Increased sensitivity to oxidative stress	Orsini, 2017

Microorganism	Response to Modeled Microgravity within the RWV Bioreactor	Reference
S. aureus N315	- Increased: biofilm formation; susceptibility to whole blood - Decreased: growth; carotenoid production; resistance to oxidative stress; Hfq expression	Castro, 2011
S. aureus RF1, RF6, RF11	- Decreased: carotenoid production; hemolytic activity - Differential gene expression	Rosado, 2010
S. aureus 25923	- Increased: growth and membrane integrity	Vukanti, 2012
Yersina pestis KIMD27	- Decreased: Hela cell rounding	Lawal, 2010
Haloferax mediterranei	- Increased antibiotic resistance - Differential pigment production and protein expression	Dornmayr-Pfaffenhuemer, 2011
Halococcus dombrowskii	- Decreased cell aggraegation - Differential pigment production and protein expression	Dornmayr-Pfaffenhuemer, 2011
Vibrio fischeri	- Differential gene expression - Altered host-symbiote relationships	Foster 2013 Casaburi 2017
Saccharomyces cerevisiae	- Increased aberrant budding - Differential gene expression	Johanson, 2002 Purevdorj-Gage, 2006 Sheehan, 2007
Candida albicans	- Increased: filamentous growth; biofilm formation; antimicrobial resistance; aggregation; random budding - Differential gene expression	Altenburg, 2008 Searles, 2011 Crabbé, 2013

success of the flight analog studies using the RWV resulted in the aforementioned two spaceflight experiments involving *S*. Typhimurium. One outcome of these investigations was the documented increased virulence of the bacterium in response to spaceflight, paralleling the bacterium's response to LSMMG as produced by the RWV (Nickerson 2000, Wilson 2007) and validating its use as a spaceflight analog.

The RWV bioreactor technology has also been used to develop 3-D intestinal cell culture models as predictive human surrogates to study host-enteric pathogen interactions to unveil novel infectious disease mechanisms and in vivo-like outcomes not observed using traditional cell cultures (Nickerson 2001, Barrila 2010, Drummond 2016, Barrila 2017). The RWV provides physiologically relevant low fluid shear conditions that enable 3-D architecture critical to recapitulate key aspects of the differentiated form and function of parental tissues in vivo (Barrila 2010). The first reported use of RWV-derived 3-D cell culture models of human intestinal epithelium was to study the early stages of *S*. Typhimurium-induced enteric salmonellosis (Nickerson 2001). These 3-D models were further advanced by incorporation of phagocytic macrophages, a critical immune cell in the

Salmonella infection process, to better reproduce the multicellular complexity of the parental tissue encountered during infection (Barrila 2017).

In addition to the similarities between *S*. Typhimurium cultured in-flight and within the RWV bioreactor, other commonalities have been demonstrated. For example, scanning electron microscopy images revealed an unidentified extracellular matrix around *S*. Typhimurium cells following spaceflight culture (Wilson 2007) in response to the modeled microgravity conditions within the RWV bioreactor (Figure 5 A). *P. aeruginosa*, *S. aureus*, *E. coli*, and *C. albicans* have all demonstrated increased biofilm formation (Lynch 2006, Crabbé 2008, Castro 2011, Searles 2011).

With multiple reports of changes in phenotype following exposure to both true microgravity and simulated microgravity, differences in gene expression in response to culture in these environments is not unexpected. The most surprising find was the identification of the involvement of Hfq, an RNA chaperone protein that exerts post-transcriptional regulation by binding messenger RNA with small non- coding RNA (Valentin-Hansen 2004), with the mechanism governing the spaceflight response of S. Typhimurium (Wilson 2007). The role for Hfq was validated with the RWV bioreactor and has since been shown to be involved in the modeled microgravity response of both *P. aeruginosa* and *S. aureus* (Crabbé 2010, Castro 2011). While first identified in spaceflight, the use of the RWV bioreactor on Earth produced evidence that suggests that the ability to sense and respond to mechanical stimuli such as microgravity and simulated microgravity may be evolutionarily conserved among structurally diverse prokaryotes.

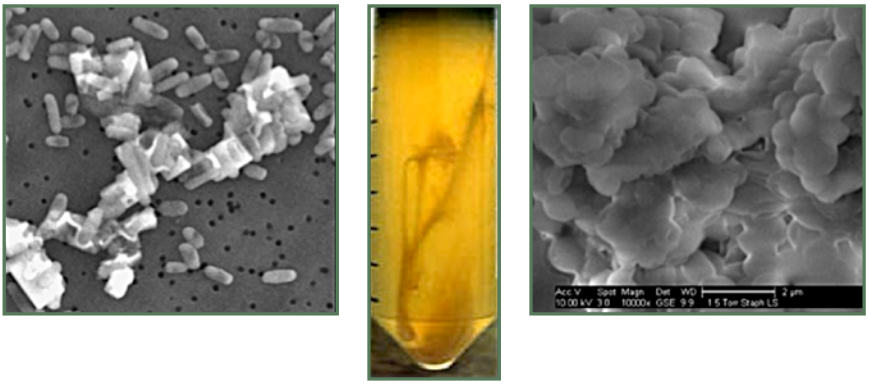

Figure 4. Increased extracellular matrix production as a result of A) spaceflight culture in S. *Typhimurium and modeled microgravity in B)* P. aeruginosa *and in C)* S. aureus *(Wilson et al. 2007, Crabbe et al. 2008, Castro et al. 2011).*

What Principal Investigators Should Know About Conducting Research on the ISS

Supporting research in science and technology is an important part of NASA's overall mission. NASA solicits research through the release of NASA Research Announcements (NRA), which cover a wide range of scientific disciplines. All NRA solicitations are facilitated through the web-based NASA Solicitation and Proposal Integrated Review and Evaluation System (NSPIRES) http://nspires.nasaprs.com/external/. Registering with NSPIRES allows investigators to stay informed of newly released NRAs and enables submission of proposals. NSPIRES supports the entire lifecycle of NASA research solicitations and awards, from the release of new research calls through the peer review and selection process.

In planning the scope of their proposal, investigators should be aware of available resources and the general direction guiding NASA research selection. NASA places high priority on recommendations from the latest National Research Council's NRC Decadal Survey. In addition, principal investigators (PIs) should be aware that spaceflight experiments may be limited by a combination of power, crew time, or volume constraints. Launch and/or landing scrubs, causing delays or rescheduling, are not uncommon, so alternative implementation scenarios should be considered in order to reduce any risk of pre-launch payload impacts or post-landing samples vulnerability from these scrubs. Preliminary investigations using ground-based simulators may be necessary to optimize procedures before spaceflight. In addition, many experiments require unique hardware to meet the needs of the spaceflight experiment.

To understand previous spaceflight studies, prospective PIs should familiarize themselves with the Space Station Research Explorer database (https://www.nasa.gov/stationexperiments). This database describes research conducted on the ISS, including that of International Partners [Canadian Space Agency (CSA), ESA (European Space Agency), Japan Aerospace Exploration Agency (JAXA), Roscosmos and others]. A detailed catalog of previous, current, and proposed experiments, facilities, and results, including investigator information, research summaries, operations, hardware information, and related publications is available.

Additionally, details pertaining to research supported by the Space Biology and Physical Sciences research areas within the Biological & Physical Sciences (BPS) Division of NASA's Science Mission Directorate, and the Human Research Program in NASA's Human Exploration and Operations (HEO) Mission Directorate can be located in the Task Book: Biological & Physical Sciences Division and Human Research Program in a searchable online database format at: https://taskbook.nasaprs.com/Publication/welcome.cfm.

When planning microbiology experiments bound for the ISS, it is important that PIs understand the exposure risks to the crew members and implement the required levels of containment. Only microorganisms with a biosafety level of 1 or 2 are allowed to be flown to the ISS. Biosafety level 1 organisms usually require only one level of containment. Biosafety level 2 organisms are divided into two categories: those that are moderate risk agents associated with human diseases and those for which primary exposure routes include percutaneous exposure, ingestion, and mucous membrane exposure. Microorganisms that meet this description generally require two levels of containment. Biosafety level 2 organisms associated with a higher risk of human diseases in which a lower infectious dose, the likelihood of aerosolization, and/or larger amounts of agent are present may require three levels of containment.

In order to fly any biological sample, an investigator must submit a biohazardous materials form through the NASA Biosafety Review Board (BRB). Information on flying biohazardous samples, including the form, can be found at: https://www.nasa.gov/feature/hazardous-material-summary-tables-hmsts.

Funding, Developing, and Launching Research to ISS

Several sources of funding are available to scientists to be used for research, payload development, payload processing at NASA facilities, on-orbit operation, and more. Once a payload has been selected for development, engineering and operations, personnel in the ISS Program Office are available to work with payload teams through the design, test, certification, build, and launch phases prior to beginning mission operations on ISS. More detailed information on this process is available at the ISS Research & Technology Opportunities web page at https://www.nasa.gov/stationopportunities.

In general, NASA funding for space station use is obtained through NASA Research Announcements (NRAs). Funding for other government agencies, private, non-profit, and educational use of the space station is obtained through research opportunities released by ISS U.S. National Laboratory. Space Station International Partner funding can be obtained through their respective processes.

Potential proposers to any NASA program announcement should contact the relevant Program Scientist to discuss the appropriateness of their research concept to the specific solicitation and for contacts within the ISS Program Office to discuss expected development costs for their proposal budgets.

For an overview of the types of considerations to make when planning and implementing a new payload, see The Quick Start Guide to Payload Design, found at https://www.nasa.gov/mission_pages/station/research/quick-start-guide.

ISS U.S. National Laboratory

In 2011, NASA finalized a cooperative agreement with the Center for the Advancement of Science in Space to manage the International Space Station U.S. National Laboratory (ISS National Lab). The independent, nonprofit research management organization ensures the station's unique capabilities are available to the broadest possible cross section of U.S. scientific, technological and industrial communities.

The ISS National Lab develops and manages a varied research and development portfolio based on U.S. national needs for basic and applied research. It establishes a marketplace to facilitate matching research pathways with qualified funding sources and stimulates interest in using the national lab for research and technology demonstrations and as a platform for science, technology, engineering and mathematics education. The goal is to support, promote and accelerate innovations and new discoveries in science, engineering and technology that will improve life on Earth.

More information on ISS National Lab, including proposal announcements, is available at www.issnationallab.org.

Other Government Agencies

Potential funding for research on the ISS is also available via governmental partnerships with ISS U.S. National Laboratory and includes (but is not limited to) such government agencies as:

- Defense Agency Research Projects Agency (DARPA)
- Department of Energy (DOE)
- Department of Defense (DOD)
- National Science Foundation (NSF)
- National Institutes of Health (NIH)
- U.S. Department of Agriculture (USDA)

International Funding Sources

Unique and integral to the ISS are the partnerships established between the United States, Russia, Japan, Canada and Europe. All partners share in the greatest international project of all time, providing various research and experiment opportunities for all. These organizations – Russian space agency Roscosmos, Japan Aerospace Exploration Agency (JAXA), Canadian Space Agency (CSA), and ESA (European Space Agency), which includes Italy's Agencia Spatiale Italiano (ASI), France's Centre National d'Etudes Spatiales (CNES), and Germany's Deutsches Zentrum für Luft- und Raumfahrt (DLR) or German Aerospace Center – provide potential funding opportunities for international scientists from many diverse disciplines.

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Acronyms

BRB	Biosafety Review Board
BRIC	Biological Research in Canisters
CASIS	Center for the Advancement of Science in Space
CSA	Canadian Space Agency
DNA	Deoxyribonucleic Acid
ESA	European Space Agency
EXPRESS	EXpedite the PRocessing of Experiments for Space Station
ISS	International Space Station
JAXA	Japan Aerospace Exploration Agency
LSMMG	Low-Shear Modeled Microgravity
MAS	Microbial Air Sampler
MIC	Minimal Inhibitory Concentration
NRA	NASA Research Announcements
NRC	National Research Council
NSF	National Science Foundation
NSPIRES	NASA Solicitation and Proposal Integrated Review and Evaluation System
PCR	polymerase chain reaction
PI	Principal Investigators
RWV	Rotating-Wall Vessel
USSR	Union of Soviet Socialist Republics

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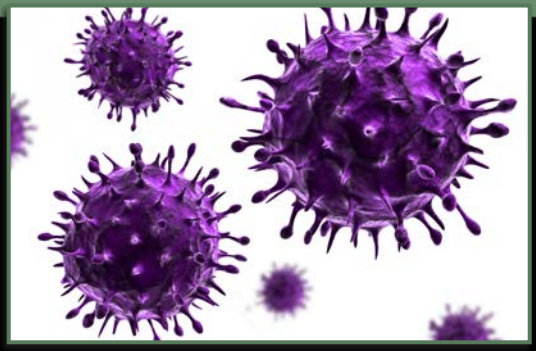

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