# DISCOVERY OF SPACEFLIGHT-RELATED VIRULENCE MECHANISMS IN *SALMONELLA* AND OTHER MICROBIAL PATHOGENS: NOVEL APPROACHES TO COMMERCIAL VACCINE DEVELOPMENT

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## ABSTRACT

Understanding infectious disease risks during spaceflight is critical to provide safe passage for human space exploration and holds potential for innovations in infectious disease control for the general public. The key to this research is the novel way that cells adapt and respond to spaceflight, as they exhibit important biological characteristics that are directly relevant to human health and disease including changes in immune function, cellular stress responses, and infectious disease potential that are not observed using traditional experimental approaches. We discovered that spaceflight uniquely alters the virulence and gene expression of the bacterial pathogen *Salmonella typhimurium*, and that the conserved, small regulatory RNA-binding protein, Hfq, plays a central role in regulating the *Salmonella* spaceflight response. We have subsequently shown that spaceflight culture also alters the Hfq regulon in other bacterial pathogens. As Hfq regulation is often associated with ionic salt concentrations, we discovered that altering the concentration of certain ionic salts, like phosphates, in the growth media prevents the increased disease causing potential of *Salmonella* during spaceflight. Collectively, our results suggest that RNA binding regulatory proteins and their small RNA binding counterparts may be key to a conserved, common cellular spaceflight response mechanism in bacterial cells that can be manipulated by environmental salt/ion levels. The implications of our findings would affect NASA's approach to infectious disease risk assessment, development of biological processing systems for exploration, and other mission-related functions. Knowledge gained from this work will broaden our knowledge of microbial cells for both spaceflight and Earth based applications and holds translational potential for the development of vaccines and therapeutics for the general public.

Spaceflight offers a unique platform to study the effects of the microgravity environment on cell and tissue function, explore fundamental questions about human health, and advance our understanding of cellular and molecular processes in both normal and diseased cells. For example, spaceflight research into infectious disease holds tremendous potential to benefit both the crew and the general public on Earth. From a spaceflight perspective, human presence in space, whether permanent or temporary, is accompanied by the presence of microbes. However, the extent of microbial changes in response to spaceflight conditions and the corresponding changes to infectious disease risk is unclear. Previous studies have indicated that spaceflight weakens the immune system in humans and animals (Gueguinou et al, 2009). In addition, preflight and in-flight monitoring of the International Space Station (ISS) and other spacecraft indicates the presence of opportunistic pathogens and the potential for obligate pathogens. Altered antibiotic resistance of microorganisms in flight has also been shown (Klaus et al, 2006). As astronauts and cosmonauts live for longer periods in closed environments, especially those using recycled water and air, there is an increased risk to crewmembers of infectious disease events occurring in-flight. Therefore, understanding how the space environment affects microorganisms and their disease potential is critically important for spaceflight missions and requires further study.

From an Earth-based perspective, the eradication or control of many microbial diseases has dramatically improved public health and longevity, however, infectious diseases are still a leading cause of human death and illness worldwide. Infectious disease causes 35 percent of deaths globally, and is the world's biggest killer of children and young adults. Within the United States, infectious disease has a tremendous social, economic, and security impact. Total costs for infectious disease in the U.S. exceed $120 billion annually due to direct medical and lost productivity costs. Perhaps the greatest application from spaceflight life sciences research will not apply directly to spaceflight, but rather to improving the quality of life on Earth – for example, through the development of novel strategies to combat infection and disease. Internationally, we face many challenges to our health by microbial threats. Antibiotic resistant strains are on the rise, regional diseases are expanding to new locations, the threat of bioterrorism looms, and a multitude of infectious diseases have inadequate or no treatments. New diagnostic, treatment and prevention paradigms are thus urgently needed. The knowledge gained from spaceflight research can advance and accelerate therapeutic and vaccine development and implementation of new strategies for translation of this research into health benefits for the developing world. The costs of therapeutics and vaccine development can be prohibitively high. Bringing a new drug to market can cost in excess of one billion dollars and take over a decade before it reaches the patient. If the knowledge gained from spaceflight studies into the mechanisms of infection and disease provides even an incremental decrease in these costs and timelines (which studies strongly suggest is the case), then this research is of tremendous importance.

It should be noted, however, that the current goal of spaceflight research is not to produce vaccines in flight.

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Food and Drug Administration (FDA) approval for new drugs and therapeutics takes years and production cost can exceed one billion dollars on the ground. The additional costs and logistics that would be incurred for a similar effort in flight would be prohibitive from both a cost and schedule standpoint. Instead, the immediate goal is to identify target mechanisms in space and then investigate these mechanisms on Earth in terms of their potential to serve as novel drug/vaccine/therapeutic candidates to effectively protect against infection and disease. By understanding more fully how microbial pathogens respond and adapt to novel environmental stimuli (in this case, microgravity), we can develop new methods to treat and prevent the spread of infectious agents. The approach of utilizing extreme environments to provide new mechanistic insight into how biological systems adapt and respond is not new. Indeed, many breakthroughs in life sciences research have come from studying living systems in unique and extreme environments. It is from studying the response of biological systems under these environments that we have not only gained new fundamental insight into how they function and adapt to extreme conditions, but have also translated these findings into beneficial biotechnology and biomedical advances to improve our quality of life. Spaceflight is simply the next logical progression and extreme environment to study in this regard. Still, it is important to remember that while identification of novel vaccine targets holds tremendous potential to provide the next ground breaking advances in public health, the practical application of therapeutics derived from such discoveries will take several years for approval and translation to the clinical setting.

To investigate the effects of the microgravity environment of spaceflight on microbial pathogenesis, we chose the model enteric bacterial pathogen, *Salmonella typhimurium*, for both our spaceflight analogue and spaceflight studies, as it is among the best characterized pathogens and poses a risk to both the crew during flight and for the general public on Earth. *S. typhimurium* is one of the most readily and fully understood pathogens and belongs to a large group of bacteria whose natural habitat is the intestinal tract of humans and animals. This group includes most of the bacteria that cause intestinal and diarrheal disease, considered to be one of the greatest health problems globally. Indeed, *S. typhimurium* infection is one of the most common food-borne infections worldwide. In the United States, an estimated 2 million cases occur, resulting in 168,000 visits to physicians, 15,000 hospitalizations and 580 deaths annually. *S. typhimurium* accounts for approximately 30% of deaths caused by food-borne infections in the United States, and is even more detrimental in the developing world. The total cost associated with *S. typhimurium* infections in the US is estimated at three billion dollars annually.

Since spaceflight experiments are a rare opportunity, our initial studies into the effect of spaceflight on microbial virulence and gene expression began by using ground-based spaceflight analogue culture systems (Nickerson et al, 2000; Nickerson et al, 2004), which systematically led to our flight experiments (Wilson et al, 2007; Wilson, 2008) (**Figure 1**). These early experimental efforts concentrated on the use of a unique bioreactor, called the Rotating Wall Vessel (RWV), designed at the NASA Johnson Space Center in Houston as a ground-based spaceflight analogue. The RWV bioreactor allows for the culture of cells (microbial or mammalian) in the laboratory under conditions that mimic several aspects of the spaceflight environment, and can be used to induce many of the biological changes that occur to cells and organisms during spaceflight. Our initial ground-based studies showed that culture of *S. typhimurium* in this flight analogue environment globally alters the gene expression, stress resistance and virulence of this organism (Nickerson et al, 2000; Wilson et al, 2002a; Wilson et al, 2002b). In addition, by using mathematical modeling (Nauman et al, 2007), we found that culture of *S. typhimurium* in both spaceflight analogue conditions as well as true spaceflight, produces an environment that is relevant to conditions encountered by the pathogen during infection in the human host. Overall, the data from our RWV experiments led to a better understanding of the potential alterations in microbial virulence and gene expression in spaceflight and the relevance of our findings for the development of new strategies to combat infectious disease on Earth.

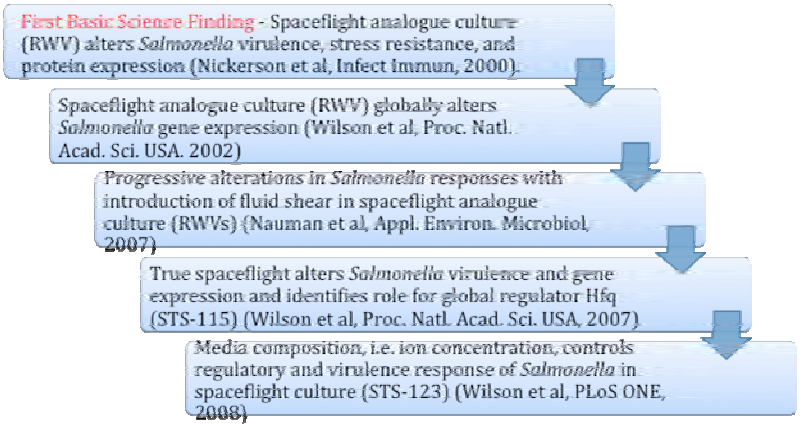


***Figure 1.*** *Discovery timeline:* S. typhimurium *investigations in space and spaceflight analogues*

The first investigation of alterations in microbial virulence in response to spaceflight was operationally designated MICROBE, and was flown aboard STS-115 in September, 2006. The purpose of the MICROBE experiment was to examine the global effects of spaceflight on microbial gene expression and virulence attributes. This experiment conducted by our lab was the first study to examine the effect of spaceflight on the virulence of a pathogen, and the first to obtain the entire gene expression response of a bacterium to spaceflight. In this experiment, we performed 1) whole genome microarray-mediated and proteomic gene expression profiling, and 2) virulence profiling of the microbial pathogens S. *typhimurium*, *Pseudomonas aeruginosa*, and *Candida albicans* in response to spaceflight as compared to identical synchronous ground controls. The model microorganisms were selected as they have been isolated from pre-flight or in-flight monitoring, represent different degrees of pathogenic behavior, are well characterized, and have sequenced genomes with available microarrays. In addition, the extensive studies of *S. typhimurium* by our lab using the RWV demonstrated important changes in the genotypic, phenotypic, and virulence characteristics of this pathogen resulting from exposure to a flight-like environment (i.e. modeled microgravity). Published results from MICROBE showed that *S. typhimurium* grown in rich LB media in spaceflight displayed increased virulence, biofilm-like formation, and global alterations in gene expression, as compared to synchronous ground controls (Wilson et al, 2007). A central role was identified for the conserved, small regulatory RNA-binding protein Hfq in regulating key aspects of the *S. typhimurium* spaceflight microgravity response. In addition, many genes encoding ion response pathways showed altered expression during spaceflight, reinforcing our earlier results observed in the spaceflight-analogue RWV bioreactor culture of *S. typhimurium* (Wilson et al, 2007).

In 2008, we were fortunate to have the opportunity to fly a second spaceflight experiment on STS-123, which was in combination with several other researchers' experiments investigating microbial responses to spaceflight. This experiment, operationally designated as MDRV (Microbial Drug Resistance and Virulence), allowed us to test our hypothesis that media ion concentrations could be manipulated to prevent/turn off the enhanced *S. typhimurium* virulence imparted during spaceflight. In addition, the MDRV experiment also provided us with the opportunity to independently validate our virulence and gene expression results obtained from MICROBE on an independent Shuttle mission. Consistent with our hypothesis, we found that spaceflight-induced increases in *S. typhimurium* virulence are regulated by media ion composition, and that phosphate ion is sufficient to alter related pathogenesis responses in a spaceflight analogue model (Wilson et al., 2008). Specifically, the increased *S. typhimurium* virulence observed with cultures grown in spaceflight in rich LB medium was not exhibited with cultures grown in M9 minimal medium. In the latter regard, we had previously shown that unlike *S. typhimurium* cultured in rich LB media during spaceflight, culture of this same organism in M9 minimal media in spaceflight did not result in an increase in virulence (Wilson et al., 2002). To determine the effect of inorganic ion concentration on the virulence of *S. typhimurium*, we also supplemented LB medium with inorganic ions to the same levels as those found in M9 medium and tested the effect on virulence during spaceflight. We found that this ion supplementation was sufficient to prevent the enhanced *S. typhimurium* virulence imparted by spaceflight culture (Wilson et al., 2008). Subsequent testing in ground-based RWV culture indicted that the altered acid tolerance exhibited by *S. typhimurium* during culture in LB alone was prevented with the addition of inorganic phosphate. These results demonstrated a direct correlation between phosphate ion concentration and the phenotypic pathogenesis-related acid stress response of *S. typhimurium* to the environment of spaceflight analogue culture. Moreover, a comparison of data from the spaceflight cultures to identical synchronous ground control cultures showed that spaceflight uniquely exacerbates *S. typhimurium* virulence effect in an ion-dependent fashion (Wilson et al., 2008). These findings confirmed that virulence in *S. typhimurium* can be influenced by the environmental stimulus of spaceflight, and that the response to this stimulus can be manipulated to improve astronaut health measures and exploited to better understand microbial pathogenesis and holds promise for the development of innovative therapeutics and vaccines for the general public on Earth.

Finally, the MICROBE and MDRV experiments indicated that despite profoundly different *Salmonella* virulence responses to spaceflight culture induced by the two media (LB and M9), we found several common genes and gene families were altered in expression in both media during spaceflight culture. As with spaceflight growth in rich LB media, *S. typhimurium* grown in minimal M9 media during flight displayed differential expression of many genes, including those associated with either the regulation of, or regulation by, the Hfq protein and small regulatory RNAs (Wilson et al., 2008). Moreover, in collaboration with our colleagues, we have also shown that both spaceflight and spaceflight analogue culture alters the Hfq regulon of other bacterial pathogens, including *Pseudomonas aeruginosa* (Crabbé et al, 2009; Crabbé et al, Manuscript submitted). Identification of these genes, whose expression is commonly regulated by the low fluid shear environment of spaceflight, provides key targets whose expression can be manipulated to control microbial responses, including potential use for development of vaccines and therapeutics.

Our current research efforts are focused on constructing a detailed "molecular roadmap" from this data to mechanistically explain how the observed microbial gene expression changes in-flight correlate with phenotypic responses, including changes in virulence. The results of these studies will provide important new insight for microbial risk assessment for both long and short duration spaceflight missions. In addition, our recent Shuttle spaceflight experiment on STS-131 in March 2010, operationally designated as STL-IMMUNE, characterized

the host-pathogen interaction when *both* the host and pathogen were *simultaneously* exposed to the microgravity environment of spaceflight. This was the first experiment to profile the infection process in human cells during spaceflight. We are currently analyzing the data from this study, including cellular responses of human intestinal and lung cells before and after infection with *S. typhimurium* or *Staphylococcus aureus*, respectively, during spaceflight, and are characterizing changes in immune function and cellular stress responses, cellular differentiation as well as targeted gene expression profiling.

Moreover, our research continues to expand toward translationally advancing the findings from our recent spaceflight experiments into novel clinical applications to combat infectious disease. Using the targets that we have found, we are collaborating with Dr. Roy Curtiss III, a pioneer and world leader in vaccine development, to develop innovative and effective vaccines and therapeutics to protect against infectious disease using the spaceflight discovery platform. These and other efforts hold potential for development of novel strategies to mitigate health risks to the crew during spaceflight and for translation to the clinical bedside to advance the health and quality of life for the general public.

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