Bacteriophage-host interactions in microgravity onboard the International Space Station

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Abstract

Bacteriophage-host interactions play a fundamental role in shaping microbial ecosystems. Although phage-host interactions have been extensively studied in terrestrial ecosystems, the impact of microgravity on phage-host interactions is yet to be investigated. Here, we report the dynamics of interactions between T7 bacteriophage and *E. coli* in microgravity onboard the International Space Station (ISS). We found phage activity was delayed but ultimately successful in microgravity. We identified several *de novo* mutations in phage and bacteria that improved fitness in microgravity. Deep mutational scanning of the receptor binding domain revealed substantial differences in the number, position, and mutational preferences between gravity and microgravity, reflecting underlying differences in the bacterial adaptations. Combinatorial libraries informed by microgravity selections gave T7 mutants with 100-10,000-fold higher activity on uropathogenic *E. coli* under terrestrial conditions than wildtype T7. Our findings lay the foundation for future research on the impact of microgravity on phage-host interactions and microbial communities.

Introduction

The interaction between bacteriophages (or ‘phages’) and their bacterial hosts plays a fundamental role in shaping microbial ecosystems both in humans and the environment. These interactions are influenced by the physical forces of fluid mixing and the underlying physiology of the bacterial host and the phage. Although phage-host interactions have been extensively studied in terrestrial ecosystems, the impact of microgravity on phage-host interactions is yet to be investigated. Understanding the role of microgravity on microbial behavior has gained significant importance in the context of space exploration. Firstly, long-term space missions, such as those planned for Mars, will require astronauts to live in a closed environment for extended periods of time experiencing microgravity. This altered environment could change their microbiome composition and, consequently, their health. Secondly, spacecraft themselves are complex ecosystems susceptible to colonization by microbes which may influence astronaut health. Finally, delving into the interplay between phages and hosts in microgravity might unveil novel mechanisms underlying these interactions, offering valuable knowledge applicable to Earth.

Microgravity might pose a challenge for both phage predation and bacterial growth due to physical and physiological factors. From a physical perspective, the movement of phages and their ability to interact with their bacterial host is directly impacted by gravity. Phages do not actively seek out their bacterial hosts. Instead, phages move randomly in fluid until they encounter a bacterial cell, after which local molecular forces such as van der Waals and electrostatics drive adsorption to the bacterial host [1,2]. Gravity induces fluid motion through natural convection, which improves fluid mixing. Additionally,

buoyancy and sedimentation caused by gravity result in the motion of particles (both phages and nutrients) in different directions, increasing fluid mixing. In microgravity, materials of different relative densities will evenly disperse, which affects the diffusion of nutrients to bacterial cells and cell motility [3–6]. Convection is contingent on temperature changes that cause changes in density and is therefore reliant on the presence of gravity [5,7]. From a physiological perspective, microgravity could itself be a stressor for bacteria. The absence of gravity can profoundly impact bacterial metabolism [7–9]. Biofilm formation and metabolic rates are both increased in microgravity [10,11]. Reduced mixing limits the movement of waste products away from the cell and reduces nutrient availability, resulting in overexpression of starvation-associated genes and increased membrane flux. Bacteria may adapt to the stress by changing their proteome, which may include changing host factors (e.g.: receptors) required by the phage to complete its infection cycle. In summary, microgravity is a unique environmental niche that could dramatically impact phage-host dynamics with major implications for the behavior of microbial communities in space environments.

In this study, we investigated the impact of microgravity on the dynamics of interactions between T7 bacteriophage and non-motile *E. coli* BL21 aboard the International Space Station (ISS). Our evaluation of short- (hours) and long-term (23 days) incubation of phage and host in microgravity showed significant differences in phage and bacterial viabilities, and phage infectivity compared to terrestrial controls. Mutations that have accumulated in phage over time in microgravity indicate genetic adaptations that may either facilitate interaction with bacterial host receptors or otherwise improve phage infectivity, while bacteria acquired mutations in genes that may improve fitness in

microgravity and counter phage predation. Deep mutational scanning (DMS) of the receptor binding protein (RBP) variants of the T7 phage on *E. coli* in microgravity revealed a fitness landscape very different from our terrestrial experiments, suggesting major differences in the host receptor profile and selection pressure under microgravity. Notably, a combinatorial library of RBP variants enriched in microgravity was able to improve activity on terrestrial *E. coli* responsible for urinary tract infections, hinting at possible similarities in the receptor profiles under both conditions, and potential application relevance of our space study to antibiotic-resistance on Earth. Overall, our findings lay a foundation for future research into the impact of phage-host interactions on microbial communities in microgravity and in the context of space exploration.

Results

Design of experiments for the International Space Station

The design of our experiments was guided by several considerations of what is and is not feasible in the ISS. First, since the space and time available to astronauts are extremely limited, the experimental workflow had to be kept very simple. Second, since open containers of any kind pose a risk to astronauts, all samples and their containers had to be kept sealed and required rigorous testing to ensure astronaut safety. As a result, conventional experiments to evaluate phage activity, such as plaque assays or growth curves, could not realistically be performed onboard the ISS without overwhelming cost. Third, the payload size and composition were fixed at 4 sealed bags of 8 sample tubes per bag, which limited the number of conditions that could be tested with replicates. Finally, samples had to be prepared on Earth and immediately frozen to facilitate travel to and

from the ISS, meaning samples had to tolerate two freeze-thaw cycles. Our experimental workflow was designed to accommodate these restrictions.

We created two identical sets of 32 packaged, sealed tubes containing experimental samples. One set was designated for incubation in microgravity and one control set for incubation under terrestrial conditions in gravity (Figure 1A). The 32 tubes were divided into four prepackaged sets of eight for incubation at 37°C – three sets for short-term timepoints of one, two and three-hours, and one set for a long-term timepoint of 25-days. The actual duration of incubation for the experiments conducted in the ISS was recorded and differed slightly from the proposed timepoints due to astronaut schedules, altering the 3-hour incubation time to 4 hours and the 25-day incubation to 23 days. Terrestrial control experiments were carried out at the actual recorded, not the proposed, timepoints.

For each timepoint, the samples were further divided into the following conditions. Each short-term prepackaged group contained one sample control with T7 phage only, one sample control with *E. coli* only, and three replicates of T7 and *E. coli* mixed at multiplicities of infection (MOIs) of 10^{-6} and 10^{-4}. We used 4 mL of log-phase (~OD_{600} 0.4) *E. coli* cells estimated at a titer of ~1-2x10^{8} CFU/mL in the experiment. The initial bacterial titer and phage MOI was chosen to allow for anticipated loss of cell and phage viability after multiple freeze-thaws while still producing detectable changes in phage titer during incubation in microgravity. Lower MOIs allowed for a wide range of detectable activity over several replication cycles. The 25-day prepackaged group contained one control sample of T7 only, one control sample of *E. coli* only, three replicates of T7 phage and *E. coli* mixed at an MOI of 10^{-4}, and three replicates of a DMS library of the T7 variants with

E. coli at an MOI of 10^{-2}. We chose a higher MOI for the DMS library to account for the lower abundance of each individual variant. The DMS library is a 1,660-member library consisting of phage variants with single-amino acid substitutions in the tip domain of the RBP. This library has been tested previously under terrestrial conditions [12].

The cryovial containers successfully passed the biocompatibility and leakiness testing, and samples passed experimental verification (see Supplementary File 1) to ensure sample integrity and to meet NASA standards for astronaut safety. Sample containers were validated by evaluating container structural integrity and the change in sample weight after a period of freezing and incubation. All samples were prepared on Earth by adding phage and/or bacteria in cryovials and immediately freezing samples at -80°C. The frozen samples were shipped to National Aeronautics and Space Administration (NASA) at the Wallops Flight Facility in Virginia, 24 days before the launch date. The samples were transported to the ISS by the Northrup Grumman NG-13 Cygnus rocket launched from Wallops Flight Facility in Virginia. After incubation in microgravity, the samples were frozen and transported back to Earth and shipped to our laboratory. We thawed the samples and immediately measured the phage and bacterial titers, sequenced the phage and bacterial genomes, and scored the DMS library (Figure 1B). The duration of freezing and incubation was recorded, and the second set of samples was tested as terrestrial controls using the same incubation and freezing times.

A

Sample Composition	1 Hour (1:12 Final)	2 Hours (2:11 Final)	3 Hours (4:17 Final)	25 Days (23:01:30 Final)
Phage / Bacteria – MOI 10^{-6}	3x	3x	3x	
Phage / Bacteria – MOI 10^{-4}	3x	3x	3x	3x
Bacteria Control (No Phage)	1x	1x	1x	1x
Phage Control (No Bacteria)	1x	1x	1x	1x
T7 tail fiber library (tip domain DMS, MOI 10^{-2})				3x

B

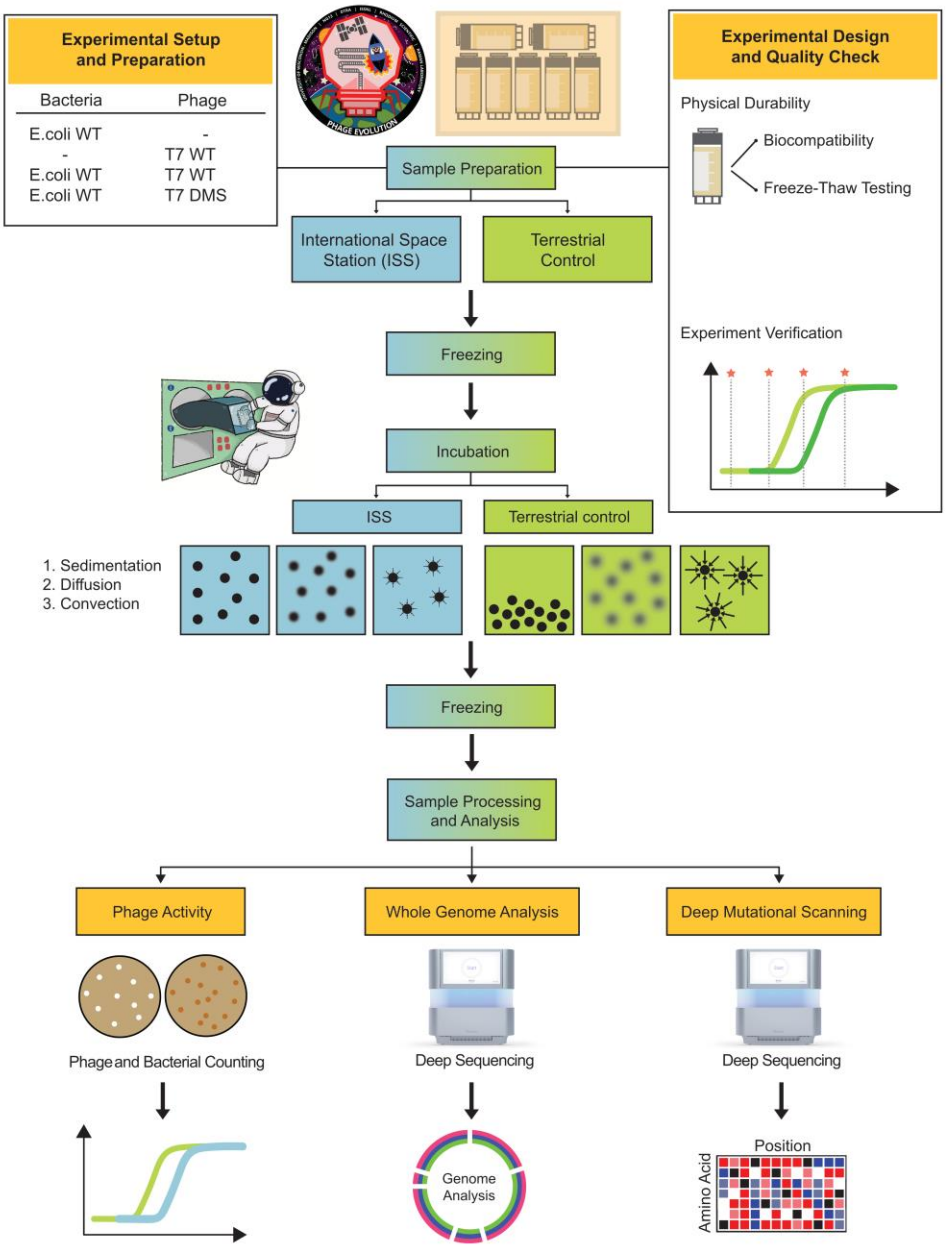


Figure 1. Experimental design to evaluate microgravity interactions on the ISS

(A) Summary of samples and timepoints used in experiments. Final time in parenthesis indicates actual incubation time on the ISS, which was subsequently used for terrestrial controls. **(B)** Illustration of experimental process to prepare and test samples under terrestrial conditions and in microgravity onboard the International Space Station (ISS). Samples were prepared on Earth, and quality checked to ensure samples cannot leak and cryovial integrity is unaffected by the freeze-thaw process. Two sets of samples were tested, one in microgravity in the ISS (left) and one on Earth (right). Samples were frozen, then thawed and incubated in either condition for specified intervals. Samples were then re-frozen and evaluated on Earth for phage and bacterial counts, for whole genome sequencing, and for deep mutational scanning of the T7 receptor binding protein tip domain. Image courtesy of NASA/Rhodium Scientific.

Bacteriophage T7 activity is reduced in microgravity

Under normal terrestrial conditions, the T7 phage life cycle and subsequent lysis of the *E. coli* BL21 host occur within 20 to 30 minutes, producing 100 to 200 progeny phages [26]. We hypothesized that the infection cycle would be considerably slower in microgravity due to poorer fluid mixing, leading to fewer productive collisions between phages and bacteria. Additionally, microgravity-induced stress could cause homeostatic disruption of the host, altering its receptor profile or intracellular processes making it difficult for the phage to complete its infection cycle. To test this hypothesis, we measured the titer of bacteria and phage in each sample at each time point of one, two, four hours, and 23 days. Since we had no initial indications regarding the extent of the delay in the phage replication cycle, if any, this approach allowed us to capture a wide range of potential delays in host lysis in microgravity.

In the bacteria-only samples, bacterial titer was reduced by approximately 6-7 logs in microgravity compared to 1-2 logs in terrestrial controls at the one- and two-hour time points, starting from an initial titer of 10^8 CFU/mL (Figure 2A). However, bacterial titers recovered in the four-hour and 23-day timepoints under microgravity with a 1-2 log loss relative to terrestrial controls. We hypothesize that this observed disparity can be attributed to the two freeze-thaw cycles. The 6-7 log loss in bacterial titer under microgravity suggested that the initial shock experienced by the bacteria from microgravity-induced stress prevented the bacteria from recovering effectively from the first freeze-thaw cycle, negatively affecting their ability to survive the second freeze-thaw cycle. However, after four hours of incubation, bacteria have sufficiently recovered from the initial shock of microgravity to better survive the second freeze-thaw cycle, resulting

in a 1-2 log reduction in titer. In general, the phages appeared to be less stable in microgravity than when incubated under terrestrial conditions but were otherwise less affected by the freeze-thaw cycles compared to the bacteria. In the phage-only samples, starting from a titer of approximately 10^8 PFU/mL, we observed a two-log loss in phage titer in microgravity at the one-, two- and four-hour timepoints compared to no appreciable loss in titer after terrestrial incubation. At the 23-day timepoint, we observed a seven- and four-log decrease in phage titers in microgravity and gravity respectively, a loss much greater than the 1-2 log loss we observed for bacterial titers. Phages are known to lose stability over time without a propagating host [13,14], and this effect appeared larger in microgravity compared to terrestrial incubation.

We had sent phage-bacteria co-cultures at two MOIs – 10^{-6} and 10^{-4}. Due to space constraints, we only had a long-term incubation (23-day) timepoint for the 10^{-4} MOI, but short-term incubation time points (one-, two- and four-hours) for both MOIs. The terrestrial samples had high bacterial titers at the one- and two-hour time points followed by a 4-5-log reduction at the four-hour timepoint, regardless of the MOI. Conversely, the phage titers were low under terrestrial conditions at the one- and two-hour time points, followed by a 6-7-log increase at the four-hour timepoint, regardless of the MOI. Given the loss of host and increase in phage titer, these results show that the phage infection occurred between two and four hours for the terrestrial samples. Under laboratory conditions without the freeze-thaw cycles, T7 phage mixed with *E. coli* at both MOIs would fully lyse the host within two hours. The fact that we observed phage activity only at the four-hour timepoint in the terrestrial sample suggests that the freeze-thaw cycles impeded the infection cycle by approximately two hours.

Under microgravity conditions, we observed no gain in phage titer at the one-, two- and four-hour time points at both MOIs. However, we observed a 4-log increase in phage titer at the 23-day time point. This indicates productive infection and lysis was delayed in microgravity but eventually occurred during the 23-day incubation. The loss of bacterial titer at the four-hour microgravity time point suggests phage replication and host lysis had begun but may not have been detectable due to the loss of titer from the freeze thaw cycle. Persistence of bacteria at the 23-day time points (10^7 CFU/mL for the terrestrial samples and 10^2 CFU/mL for microgravity samples), suggests that a phage-resistant bacterial population may have emerged.

In summary, these experiments demonstrate an initial delay in phage infectivity in microgravity compared to terrestrial conditions. However, over time, this disadvantage is overcome, leading to productive phage infection. These findings strongly suggest that phage predation can indeed take place in microgravity environments. Future experiments focusing on intermediate time points will be essential in determining the precise latent period for the phage in such conditions.

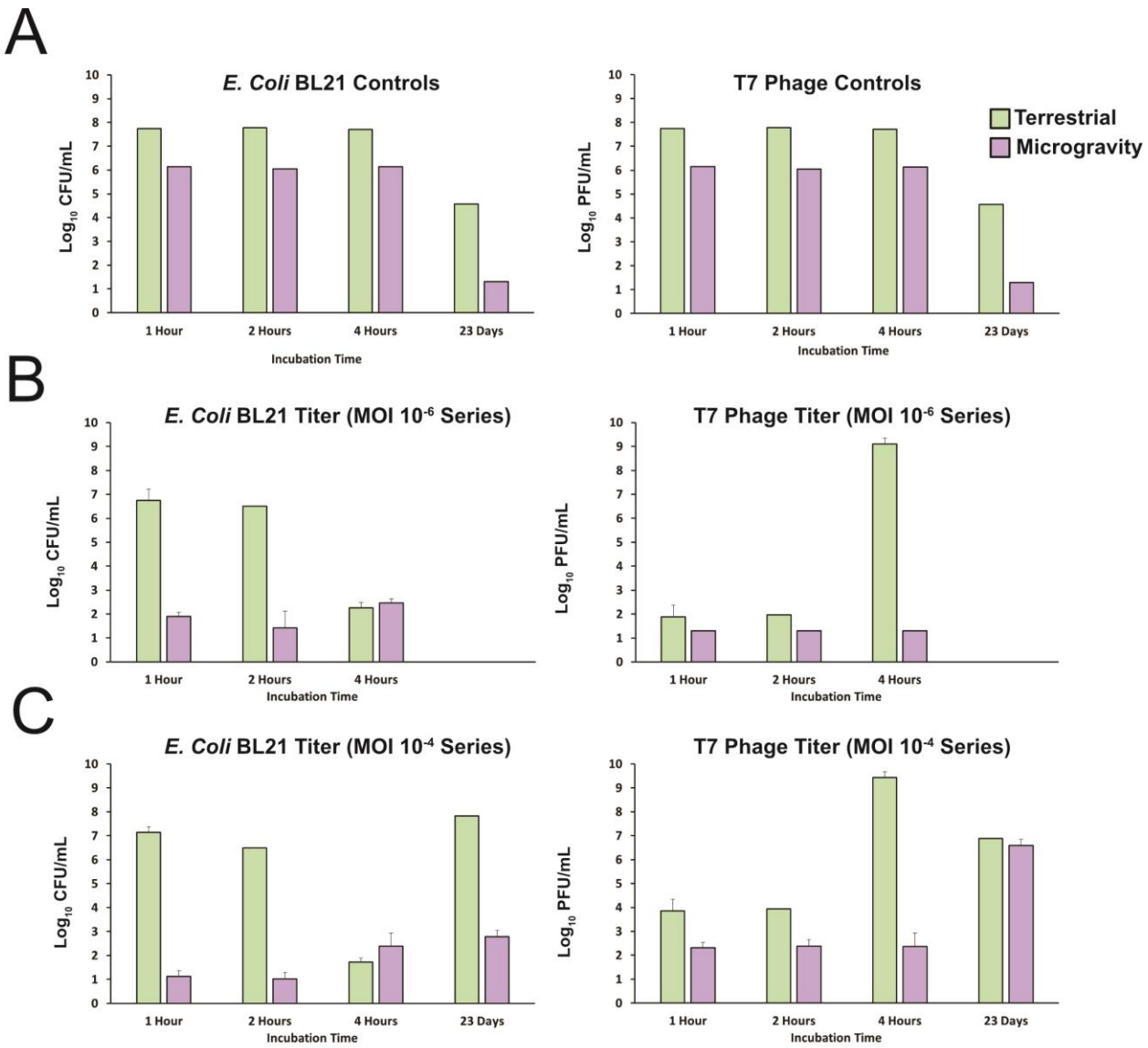


Figure 2. Bacteriophage T7 growth is inhibited by microgravity

(A-C) The titer of bacterial and phage samples at different incubation times ($\log_{10}$ PFU or CFU/mL) for terrestrial (green) and microgravity (purple) conditions for (A) control samples, (B) MOI^{-6} sample series, and (C) MOI^{-4} sample series. Triplicate samples are represented as mean +/- SD.

Microgravity Associated Mutations in T7 Bacteriophage and *E. coli* BL21

We next sought to identify the mutations that could influence phage-host relationships under microgravity. We sequenced the genomes of bacteria and phage from the phage-bacterial co-culture of the 23-day sample from microgravity and from terrestrial controls. With an average sequencing depth of >40,000-fold per bp across the entire phage genome, we could reliably identify low frequency mutations in the un-passaged controls and *de novo* mutations under selection (Figure S1A, Supplementary File 2).

Sequencing revealed several potential mutational hotspots on the T7 genome. The genes were grouped as transcribed early (class I), middle (class II), late in infection (class III), or as structural genes [15] (Figure 3A-D). We observed distinct mutations in the phage in both terrestrial and microgravity conditions and common mutations accumulating under both conditions. T7 genes *gp0.5* and *gp7.3*, were mutated substantially more in microgravity than under terrestrial conditions. V26I in *gp0.5* was the only mutation to sweep the entire phage population in two out of the three replicates, indicating a strong fitness benefit under microgravity. *Gp0.5* is an uncharacterized class I gene that may be associated with the host membrane based on the presence of a putative transmembrane helix [16]. Though not fully characterized, structural gene *gp7.3* is likely injected into the cell upon adsorption and is thought to play a role either as a scaffolding protein or to participate in adsorption to the host [17]. Although *gp7.3* is considered essential for activity of T7 on *E. coli* BL21 under terrestrial conditions, terrestrial samples contained 3- and 6-amino acid in-frame deletions between positions 39 and 47 in the protein sequence. In contrast, under microgravity conditions, single amino acid substitutions (missense mutations) were relatively abundant and were well distributed throughout the protein (see

Supplementary File 2). Notably, the deletions observed under terrestrial conditions were not seen in microgravity. These results suggest that single amino acid mutations arising in microgravity may either assist in adsorption of the phage to the cell surface or promote scaffolding in this environment, while deletions under terrestrial selections may point to interesting interactions yet to be characterized.

We found mutations in only one class II gene, *gp4.*7, at substantially higher abundance under terrestrial conditions than microgravity samples. These mutations in *gp4.7* were dominated by substitution T115A at 6%, 5% and 12% abundance in the three samples, respectively. No mutations were detected in this gene in any microgravity samples, suggesting the selection pressure may be specific to terrestrial conditions. The function of *gp4.7* remains unassigned, although BlastP matches protein homologs with approximately 40% homology to putative HNH endonucleases in *Klebsiella* and *Pectobacterium* phages [16].

Many mutations also accumulated in structural proteins, particularly in *gp11, gp12* and *gp17* which form parts of the tail and tail fiber [18,19]. Mutations in these genes were well distributed with a higher incidence under terrestrial conditions than under microgravity. Mutations in *gp17* primarily occurred in the tip domain. Notably, substitution D540G in a proximal β-strand was highly enriched across both conditions (terrestrial maximum abundance: 13.3%; microgravity maximum abundance: 5.3%). This region is known to be a lynchpin that can alter host range and efficacy terrestrially on other *E. coli hosts* [12], and these results indicate the region remains important in both conditions in prolonged incubation. These genes are promising targets for future mutational profiling to investigate changes in phage activity in either condition.

Characterizing mutations in the *E. coli* BL21 genome was challenging due to the loss of bacterial viability caused by phage predation in mixed samples. The bacterial population in mixed samples was greatly reduced, which impacted sequencing depth to less than 250 reads per bp on average across the genome. As a result, our analysis was limited to only to highly abundant mutations (Figure S1B). Despite these limitations, we identified several notable host mutations in samples mixed with phages indicating a strong selective pressure on the host (Figure 3E-F, see Supplementary File 3).

Bacterial genes with highly enriched mutations seen only in microgravity include *topA* (96.1%, V73G), a DNA topoisomerase [20], *hldE* (56.5%, R232C) associated with catalyzing pathways for building the inner core of LPS [21] and *entS* (5.1%, A182 deletion), associated with enterobactin export and cell stress management [22,23].

Other deletion mutants inducing a frameshift or disrupting bacterial genes only in microgravity included *gltA* (85%, I138 frameshift), a citrate synthase [24], *mnmE* (10.3%, E280 frameshift), a GTPase [25], *rpsF* (27.7%, V18 frameshift), a 30S ribosomal protein [26], *mrcB* (51.6%, Q817 frameshift), related to cell wall synthesis and permeability [27,28], and *yejM* (18.5%, L330 frameshift), a protein associated with outer membrane permeability [29]. In microgravity conditions, a high abundance of *typA* mutants was observed in bacteria when co-cultured with phages, accounting for 96.5% of one population (H16 frameshift). In samples containing bacteria only, a substitution in this gene was found but at a lower abundance of 5.1% (T75S). The *typA* gene is closely linked to several pathways in *E. coli*, such as temperature sensitivity, motility, and LPS biosynthesis. Mutations in this gene have been previously associated with truncated LPS

[35-37]. The high abundance of these mutations when bacteria are co-cultured with phages suggests that *typA* might play a role in phage sensitivity in microgravity.

Bacterial mutations that arose only in the presence of phage under terrestrial conditions included *galU* (100%, L59R), involved in UDP glucose metabolism and associated with and O-polysaccharide in other strains [30,31], *lptA* (100%, Q43H), involved in LPS assembly [32], *hns* (66%, I70_L75 IS1 insertion), a global DNA-binding protein responsible for regulating metabolism and nutrient acquisition [33], and *tufB* (18.9%, I120V; 13.9%, G123A), an elongation factor [34]. Several genes were mutated in both conditions. A mutation in *ydcL* occurred in one terrestrial and one microgravity sample (I105K, terrestrial 100%, microgravity 33.9%). *ydcL* is a transcriptional regulator associated with triggering increasing small, slow growing persistors which could benefit bacteria over prolonged incubation periods like this experiment regardless of gravity [35].

Under microgravity and terrestrial conditions, in one out of three samples, we found abundant deletions in *trxA* (terrestrial: 44.4%, K53_V56d deletion, microgravity: 100%, I76 frameshift), a processivity factor for T7 DNA polymerase and a known essential gene required for phage activity under terrestrial conditions [36,37]. These samples had higher titers of bacteria, indicating poor phage replication and suggesting that this gene remains essential to the phage in microgravity conditions.

Intriguing indels arose in *mlaA* in bacteria mixed with phages under both terrestrial and microgravity conditions (Figure 3F). Two microgravity samples and two terrestrial samples had six-bp deletions that resulted in the loss of two amino acids, equating to F44 and N45, (abundance in microgravity: 95.5% and 16.5%, terrestrial: 61.5% and 24.9%). In contrast, the third microgravity sample saw a 6-bp insertion resulting in two new amino

acids, Asp and Phe, after N45 (F46_N47ins), effectively inserting and repeating the same two amino acids deleted in the other samples. Neither control sample saw mutations arise in this gene. *mlaA* is an outer member lipoprotein thought to remove phospholipids that have mis-localized to the outer membrane and move them back to the inner membrane [38]. Mutations in this gene have not yet been associated with changes in phage activity. A mutant that results in the same F44_N45 deletion has been previously characterized in *E. coli* MC4100, an MG1655 derivative [39,40]. This deletion increased outer membrane permeability, the quantity of outer membrane phospholipids and outer membrane vesiculation. Given that lipopolysaccharide is the receptor for T7 phage, these alterations could impact the ability of the phage to adsorb to the host and provide these mutants with a competitive advantage. A prior study noted that the F44_N45 deletion mutation eventually caused bacterial cell death, which contradicts our results. This difference may be due to variations in strain background or other uncharacterized suppressor mutations providing survival benefits. We also found a high abundance frameshift-inducing deletion in *yhdP* (56.9%, L610 frameshift) in one microgravity sample associated with the most abundant *mlaA* deletion. This gene is related to phospholipid transport to the outer membrane and loss of this gene has been shown to slow transport and cell death in F44_N45 deletion mutants [39], indicating it may act in a similar fashion to improve survivability in microgravity. These bacterial mutations that arose in microgravity could be interesting avenues of future study to elucidate phage-host interactions further and understand how bacteria adapt to microgravity conditions.

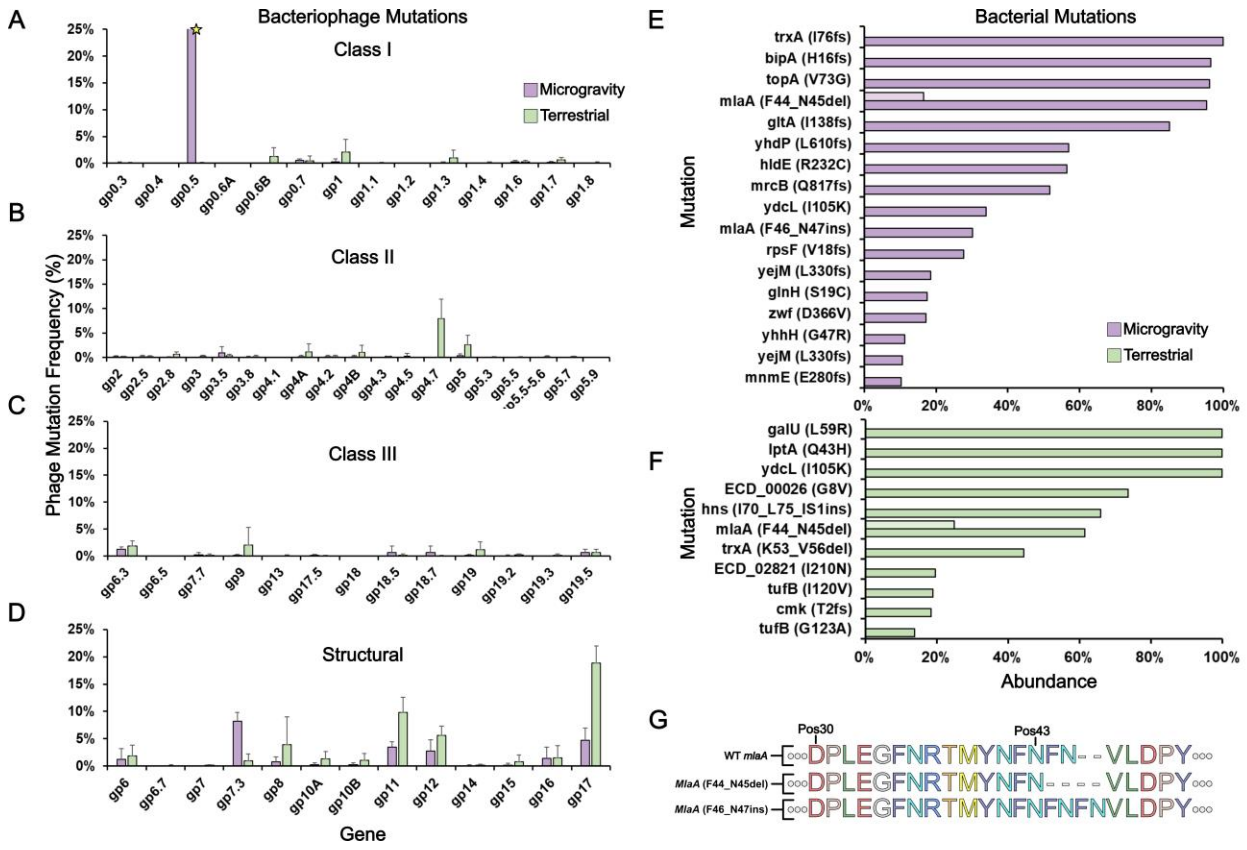


Figure 3. Incubation in microgravity reveals novel mutations

(A-D) Total average percentage mutation frequency for bacteriophage T7 in **(A)** class I, **(B)** class II, **(C)** class III and **(D)** structural genes after incubation in microgravity (purple) and under terrestrial conditions (green). Shown as the averaged sum of all mutation frequency for each gene across the three replicates +/- SD. Star indicates *gp0.5*, which reached 100% frequency in two replicates. **(E-F)** Abundance of all specific mutations in the *E. coli* BL21 genome with >10% abundance in any replicate after incubation in **(E)** microgravity (purple) and **(F)** under terrestrial conditions (green). Shown as abundance with multiple instances of specific mutations across replicates shown in lighter shading. **(F)** Illustration of the effect of noted *mlaA* deletions and insertion mutations on the protein.

Deep Mutational Scanning profiles beneficial substitutions in the RBP tip domain

Bacteria frequently respond to phage infection by either mutating or reducing the expression of the surface receptors that the phages rely on for infection. This response may be further exacerbated when bacteria experience stress caused by microgravity, leading to significant alterations in their proteome, including changes to the phage receptors. As bacteria's surface receptor profile changes, these alterations will be reflected by mutational adaptations in the phage RBP. Microgravity therefore provides a unique condition to explore phage RBP adaptations that may reveal novel interactions otherwise hidden under terrestrial conditions. We examined how individual substitutions of the tip domain of the RBP of T7 impacted phage viability to explore these novel interactions. The RBP of T7 phage consists of six short non-contractile tails that form a homotrimer composed of a rigid shaft ending with a β-sandwich tip domain [41]. This domain is a major determinant of phage activity and interacts with host receptor LPS to position the phage for successful, irreversible binding with the host [19,37,42–45]. We carried out comprehensive single-site saturation mutagenesis (1,660 variants) of the RBP tip domain spanning residue positions 472-554 in the RBP (residue numbering based on PDB 4A0T) and compared the differences in mutational profiles after selection under terrestrial conditions versus in microgravity at the 23-day time point. One sample for each condition had sufficient phage titer and sequencing quality to score the libraries post-selection. We have previously used this same library to functionally characterize the tip domain in terrestrial short passage experiments [12]. We scored each variant based on its relative abundance before and after selection normalized to wild type activity to

determine a normalized function score (F_N). Deep sequencing in each condition revealed selection, consistent with multiple rounds of replication over the extended 23 days of incubation. (Figure 4A-B, Supplementary File 4).

We observed substantial differences in the number, position and mutational preferences between gravity and microgravity, likely reflecting underlying differences in the bacterial adaptations to stress under both conditions. The tip domain can be broadly separated into exterior loops, facing the bacterial surface, β-sheets that form a sandwich structure, and interior loops facing the phage. Highly enriched variants under microgravity were distributed throughout the tip domain and were distinct from those variants that performed well during terrestrial incubation (Figure 4C-D). Fewer high scoring variants were present in exterior loops in microgravity compared to terrestrial incubation, with more successful substitutions in interior loops and β-sheets (Figure 4E), indicating these regions may be of increased importance for fitness in microgravity. Variants that performed well in microgravity frequently contained methionine and isoleucine substitutions (Figure S2) in the interior loops and β-sheets (P511M, F_N = 30.6; L490I, F_N = 23.1; N546I, F_N = 22.1; A539M F_N = 11.3; F506M F_N = 11.7), suggesting a preference for hydrophobic amino acids in these regions. Substitutions in these areas could influence the tip domain structure to facilitate adsorption or interaction with the host receptor in microgravity. Surprisingly, few of the highly enriched variants in microgravity were enriched on any *E. coli* strains previously tested under terrestrial conditions [12], indicating that the selection pressure experienced by phages in microgravity could be very different from terrestrial conditions and highlighting the adaptability of the receptor binding protein to different environments.

Under terrestrial conditions, top scoring variants included positively charged substitutions in the exterior loops that were consistent with our previous results on *E. coli* BL21 and 10G (G521H, F_N = 548.3; G521K, F_N = 71.6). Similarly, positively charged substitutions performed well in microgravity at one exterior loop but with much less enrichment (S477H F_N = 4.2, G479H F_N = 3.4). Negatively charged substitutions also performed well throughout the tip domain after terrestrial incubation (Q488E, F_N = 76.0; G521D, F_N = 21.4), as did substitutions to glycine that could result in structural changes to the tip domain (G480W F_N = 192.9; G522P F_N = 41.0). Enrichment of these variants was only seen with prolonged incubation with *E. coli* BL21, indicating these substitutions may play a functional role for long term infectivity in stationary-phase host that is not seen in shorter, nutrient rich incubations.

Finally, many mutations enriched in both conditions were present at C-terminus of the protein (Terrestrial N552E, F_N = 13.5; Microgravity E553I, F_N = 17.4) including substitutions that replaced the stop codon (*554E, Terrestrial F_N = 18.9, Microgravity F_N = 3.4), which results in a three amino acid (-DAR) extension to the protein. These results indicate this region can play a significant role in host recognition, and substitutions or extensions to this region could be one avenue for increasing phage activity in different environments.

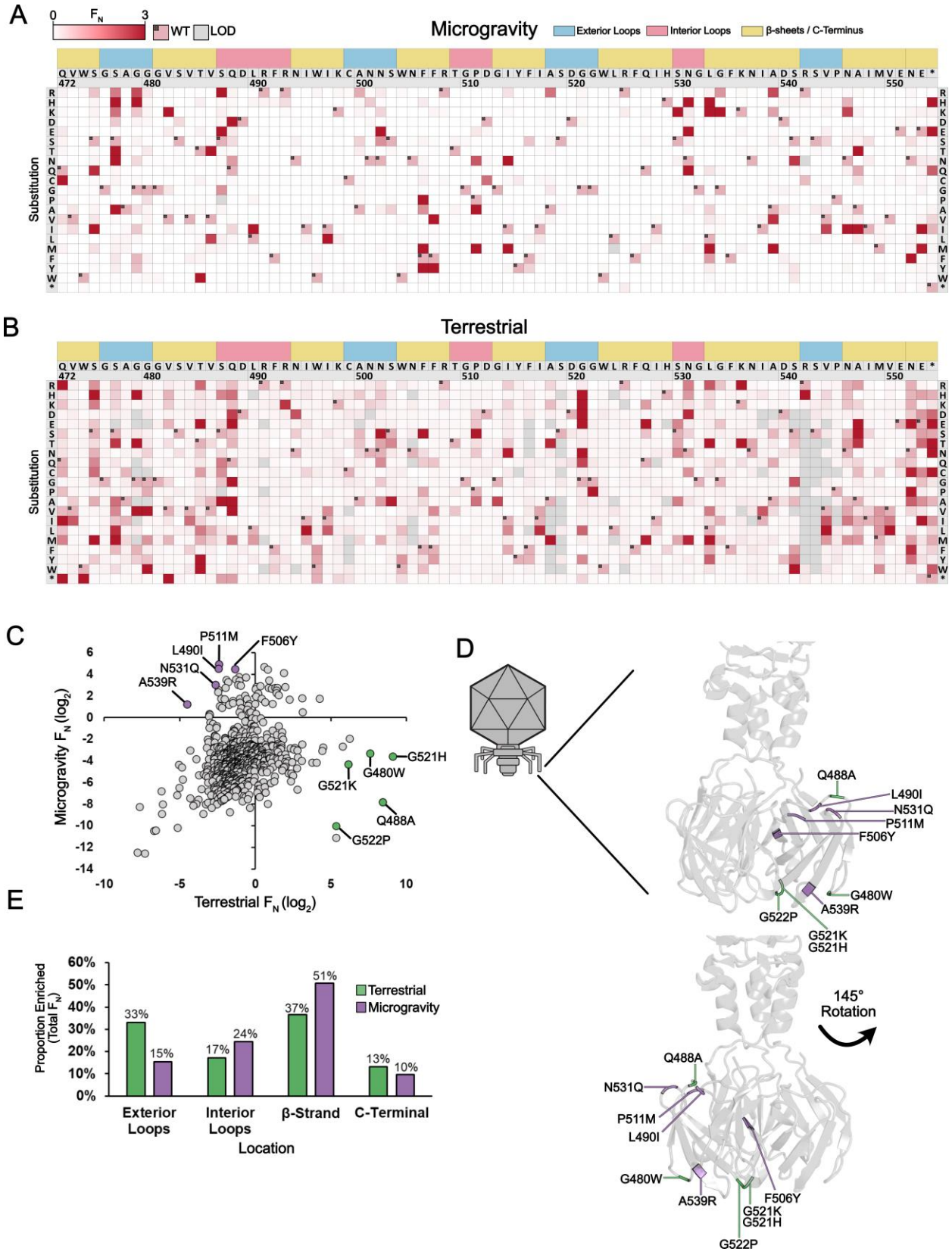

A
0
F_N
3
WT
LOD
Microgravity
Exterior Loops
Interior Loops
β-sheets / C-Terminus
Substitution
B
Terrestrial
C
P511M
F506Y
L490I
N531Q
A539R
G521H
G480W
G521K
Q488A
G522P
Microgravity F_N ($\log_2$)
Terrestrial F_N ($\log_2$)
D
Q488A
L490I
N531Q
P511M
F506Y
G480W
A539R
G522P
G521K
G521H
145°
Rotation
E
Proportion Enriched (Total F_N)
Terrestrial
Microgravity
33%
15%
17%
24%
37%
51%
13%
10%
Exterior Loops
Interior Loops
β-Strand
C-Terminal
Location

Figure 4. Deep Mutational Scanning profiles beneficial substitutions in the RBP tip domain

(A-B) Heatmaps showing normalized functional scores (F_N) of all substitutions (red gradient) and wildtype amino acids (F_N=1 and black dot upper left) at every position after incubation with *E. coli* BL21 in **(A)** microgravity and **(B)** after terrestrial incubation. Residue numbering (based on PDB 4A0T), wildtype amino acid and secondary structure topology (exterior loops in blue, interior loops in red, beta sheets and C-terminus in yellow) are shown above left to right, substitutions listed top to bottom. Substitutions below limit of detection (LOD) where a score cannot be called are shown in yellow. **(C)** Correlation plot of $\log_2$ transformed F_N after microgravity (purple) and terrestrial (green) incubation with example divergent substitutions colored accordingly. **(D)** Tip domain crystal structure (PDB: 4A0T) and secondary structure topology shown color coded with noted substitutions enriched in microgravity (purple) or terrestrial (green) incubation conditions. **(E)** Proportion of enriched substitutions based on total F_N by topological location throughout the tip domain in microgravity (purple) or terrestrial (green) incubation conditions.

Microgravity-enriched variants enhance activity on terrestrial pathogens

Screening unbiased phage variant libraries in microgravity revealed regions and substitution patterns distinct from those observed under terrestrial conditions. Identifying these functionally significant positions and substitutions across different environments enables a targeted approach for augmenting phage activity without the need to explore the entire combinatorial space of the gene. We hypothesized that phage mutants enriched in microgravity could hold the key to enhancing phage activity against human pathogens in terrestrial conditions. To evaluate this hypothesis, we combined high-performing mutants in microgravity to assemble a library of multi-mutant variants for screening against clinical isolates of uropathogenic *E. coli*.

We designed a combinatorial library consisting of every combination of thirteen unique substitutions that had the highest activity after incubation in microgravity. Thirteen substitutions were chosen to keep the library size relatively small at 3,638 variants. These substitutions (L490I, N502E, F506M, F506Y, F507V, F507Y, P511M, I514M, N531Q, L533K, L533M, A539M, and N546I) were spread throughout the tip domain (Figure 5A) with F_N scores ranging from approximately 7 to 30 (See Supplemental File 4). Variants were synthesized in an oligo pool and inserted into an unbiased phage library using ORACLE, then passaged terrestrially on two clinically isolated *E. coli* strains (designated UTI1 and UTI2) responsible for causing urinary tract infections.

We isolated several plaques from this experiment to confirm the improvement in activity on these hosts. On UTI1 we isolated a variant with the five substitutions L490I, N502E, F507V, L533K, and A539M (Figure 5B, phage variant 1). On UTI2 we isolated a variant with six substitutions, L490I, N502E, P511M, L533M, A539M, and N546I (Figure

5C, phage variant 2). These high-performing combinatorial variants showed approximately 100-fold or 10,000-fold higher efficiency of plating on each UTI strain compared to wildtype T7 (Figure 5D-E). The substitutions in these variants are distributed throughout the tip domain, including inside the interior loops and β-sheets, contrasting substitutions in the exterior loops that are typically enriched among high-activity variants under terrestrial selections [12]. These results confirmed our hypothesis that microgravity-enriched variants had revealed novel substitutions in the tip domain that can dramatically improve activity on terrestrial hosts. Incubation in microgravity identified hotspots that allowed us to efficiently navigate sequencing space to find complex combinations that were able to improve phage activity.

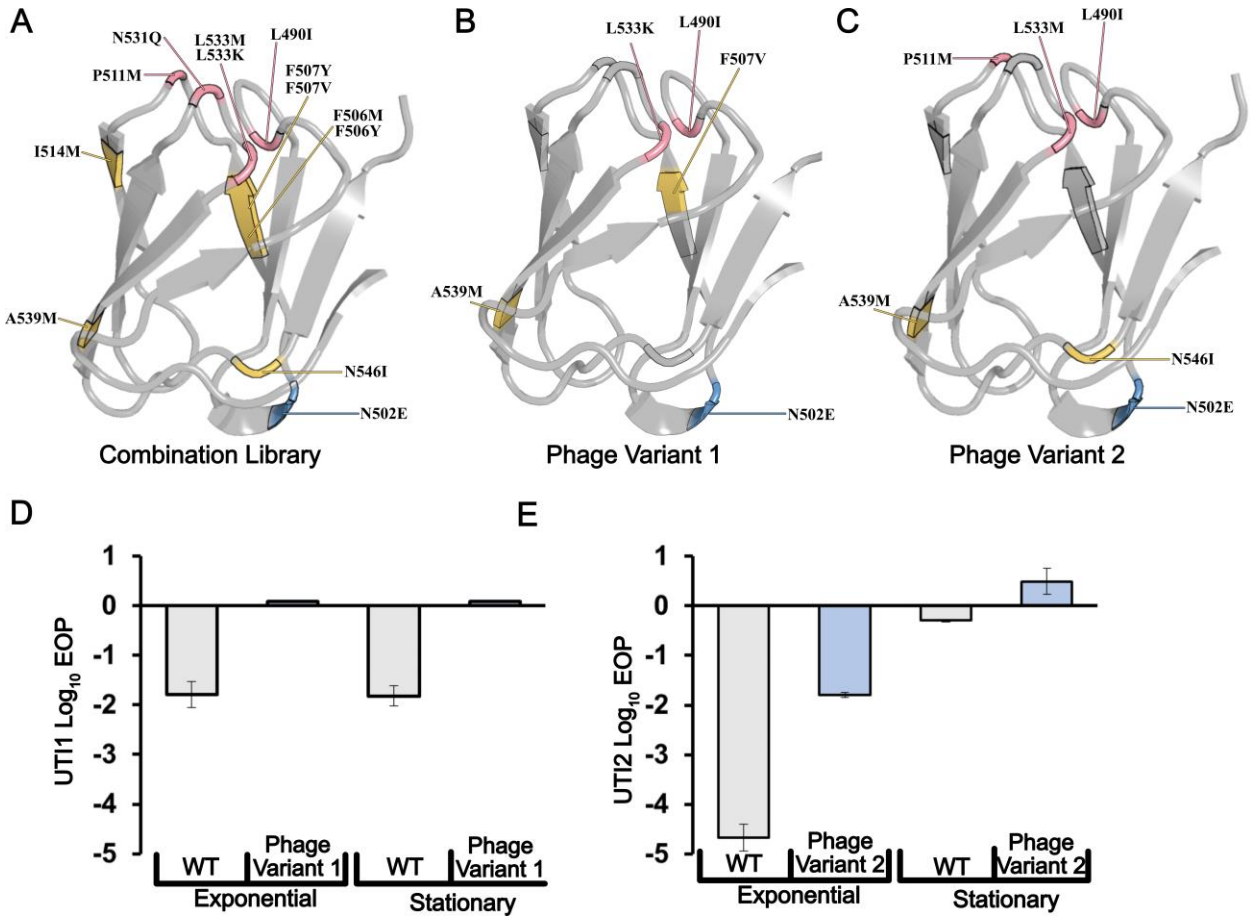


Figure 5. Microgravity-enriched variants enable activity on terrestrial pathogens

(A-C) Image of tip domain structure with substitutions noted for **(A)** the entire combinatorial library, **(B)** phage variant 1 isolated after passage on the *E. coli* UTI1 and **(C)** phage variant 2 isolated after passage on *E. coli* UTI2 . Structure is colored based on position at exterior loops (blue), beta sheets (yellow), or interior loops (red). **(D-E)** Efficiency of Plating (EOP) results for **(D)** *E. coli* UTI1 and **(E)** *E. coli* UTI2 comparing activity of each phage variant to wild type (WT) phage activity while the host is in exponential or stationary growth phase. Data shown as the average of three biological replicates +/- SD, normalized to BL21 in exponential growth phase.

Discussion

Interactions between phages and bacteria play a defining role in shaping microbial ecosystems, but this interplay has remained largely uncharacterized in microgravity. Microgravity dramatically alters how phages collide and interact with their bacterial hosts while also inducing changes in the bacterial physiological state that can further alter this dynamic. Characterizing these interactions is essential for understanding how microbial communities adapt to microgravity and for identifying unknown genes and novel mechanisms of phage-host interactions, which may have potential applications on Earth.

In this study, we measured bacterial and phage titers after incubation in microgravity to determine how phage infectivity is impacted by microgravity. We found that phage replication in microgravity was considerably delayed in microgravity past the 4-hour timepoint but ultimately successful at 23 days, indicating a markedly slower, but ultimately successful replication cycle.

We identified many novel *de novo* mutations in phage and bacterial genes at high abundance indicating strong selective advantage. Among phage genes, the preponderance of V26I in *gp0.5* suggests that the putative association of this protein with the host membrane may be more essential to fitness than previously understood [16]. Missense mutations and indels in *gp7.3,* also highlight this scaffolding or adsorption-related protein as a potential key player [17]. The distribution of mutations in structural proteins *gp11, gp12* and *gp17* point to regions that influence host range in different conditions that warrant further exploration by comprehensive mutational scanning.

Mutations seen in the bacterial host were predominantly in proteins associated with building or maintaining the outer membrane (such as *hldE, typA* and *mlaA*), outer membrane or cell wall permeability (such as *entS*, *mrcB, yejM)* or stress or nutrient

management (such as *entS, gltA, typA* and *ydcL*). These findings align with prior research, demonstrating the upregulation of comparable gene classes in closely related strains of *E. coli* under microgravity conditions. These genes play a role in responding to stress, managing nutrient availability, and facilitating trans-membrane transport in this unique environment [11,46–49]. Mutations in these genes may adversely impact phage infectivity, providing an additional selective advantage.

Results of the T7 receptor binding protein tip domain DMS library revealed novel regions and patterns of substitution that influence phage activity that were not visible in terrestrial selections [12]. Trends identified in microgravity selections allowed us to efficiently navigate sequence space to create complex, multiple mutation variants that gave ~100-fold to ~10,000-fold higher activity on uropathogenic *E. coli* under terrestrial conditions. Overall, these results demonstrate the unique potential for microgravity to improve our understanding phage-host interactions and reveal substitutions and regions that can be leveraged to improve phage activity which are otherwise hidden in terrestrial experiments.

Overall, our study provides a preliminary examination of the influence of microgravity on phage-host interactions. Investigating phages in new environments sheds light on different genes that can influence phage activity and provides avenues for engineering phages terrestrially in future experiments. The success of this approach lays the groundwork for future phage studies in microgravity aboard the ISS.

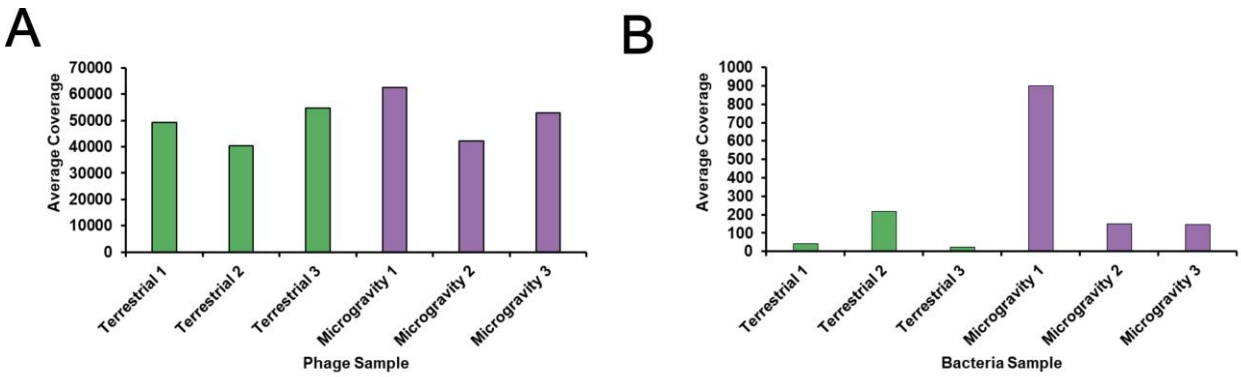
Supplementary Files

Supplementary File 1 – Summary of Cryotube Biocompatibility Testing, Cryotube Freeze-Thaw Testing and Experiment Verification Testing

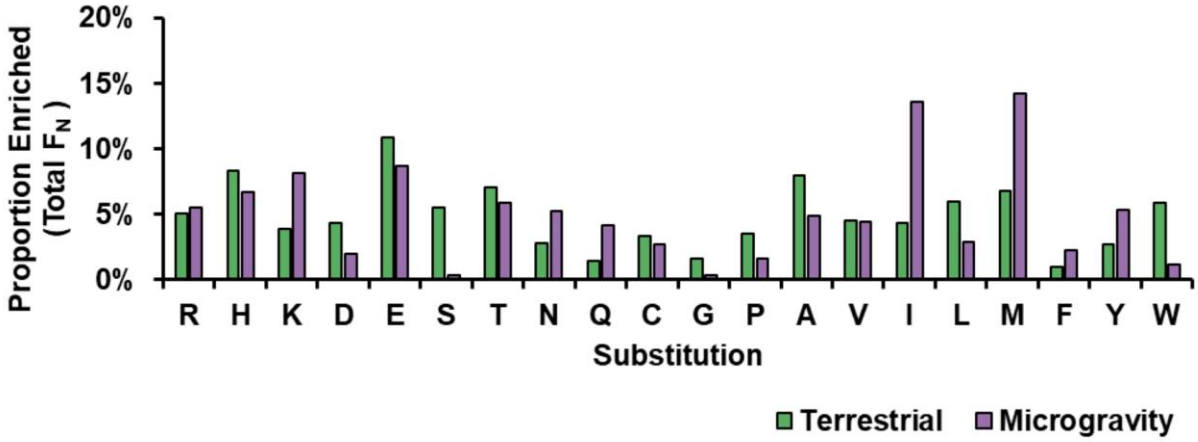
Supplementary File 2 – Summary of *de novo* Bacteriophage Mutations

Supplementary File 3 – Summary of *de novo* Bacterial Mutations

Supplementary File 4 – Deep Mutational Scanning F_N Scores



Supplementary Figure 1. Average read depth during whole genome sequencing for (A) phage and (B) bacteria.



Supplementary Figure 2. Proportion of enriched substitutions in the deep mutational scanning library based on total F_N in microgravity (purple) or in terrestrial (green) incubation conditions.

Contributions:

P.H.: Conceptualization, Data curation, Software, Formal analysis, Investigation, Visualization, Methodology, Writing - original draft, Writing - review and editing

C.C.: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing - original draft, Writing - review and editing

A.M.: Software

K.N.: Software

H.M., R.O., O.H.: Resources, Project administration, Methodology, Writing - review and editing

S.R.: Conceptualization, Resources, Supervision, Funding acquisition, Project administration, Writing - review and editing

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Methods

Microbes and Culture Conditions

T7 bacteriophage was obtained from ATCC (ATCC® BAA-1025-B2). The T7 DMS library is the same library stock as created previously [12]. T7 acceptor phages used for ORACLE for the combinatorial library were created previously [12]. *Escherichia coli* BL21 is a lab stock, UTI1 and UTI2 were obtained from Rod Welch (University of Wisconsin, Madison) and originate from a UTI collection [50].

All bacterial hosts are grown in and plated on Lb media (1% Tryptone, 0.5% Yeast Extract, 1% NaCl in dH2O, plates additionally contain 1.5% agar, while top agar contains only 0.5% agar) and Lb media was used for all experimentation and was used to recover hosts. All incubations of bacterial cultures were performed at 37°C without shaking in the relevant environment, besides the combinatorial library tested on UTI strains, which was incubated at 37°C shaking at 250 rpm. T7 bacteriophage was propagated using *E. coli* BL21 after initial receipt from ATCC and then as described on various hosts in methods. All phage experiments were performing using Lb and culture conditions as described for bacterial hosts. Phages were stored in Lb at 4°C. For freezing microbes were stored at -80°C in 100% Lb media.

General Cloning

PCR was performed using KAPA HiFi (Roche KK2101) for all experiments. The combinatorial library was prepared using the ORACLE method as described previously [12]. All cloning was performed according to manufacturer documentation. For WGS,

phage genomes were extracted using a Norgen Biotek Phage DNA Isolation Kit (Cat. 46800), bacterial genomes were extracted using a Norgen Biotek Bacterial Genomic DNA Isolation kit (Cat. 17900). Extracted genomes were prepared for WGS using an Illumina DNA Prep kit (Cat. 20060060) and sequenced on an Illumina Nextseq 1000. PCR reactions for the DMS and combinatorial library use 1 µl of undiluted phage stock as template directly with an extended 5-minute 95°C denaturation step. For analysis of the plaques on the UTI host, a small sample was picked off the plaque and used as template. Detailed protocols for cloning are available on request.

Sample Handling, Preparation, and Analysis

To establish titer, phage samples were serially diluted (1:10 or 1:100 dilutions made to 1 mL in 1.5 microcentrifuge tubes) in Lb to a 10^{-8} dilution for preliminary titering by spot assay. Spot assays were performed by mixing 250 µl of relevant bacterial host in stationary phase with 3.5 mL of 0.5% top agar, briefly vortexing, then plating on Lb plates warmed to 37°C. After plates solidified (typically ~5 minutes), 1.5 µl of each dilution of phage sample was spotted in series on the plate. Plates were incubated and checked after overnight incubation (~20-30 hours) to establish an estimate titer, after which titer was confirmed by whole plate plaque assay. To perform the whole plate EOP assay, 400 µl of bacterial host in exponential phase was mixed with between 5 to 50 µl of phages from a relevant dilution targeting 50 plaque forming units (PFUs) after overnight incubation. The phage and host mixture was briefly vortexed, briefly centrifuged, then added to 3.5 mL of 0.5% top agar, which was again briefly vortexed and immediately plated on Lb plates warmed to 37°C. After plates solidified (typically ~5 minutes), plates

were inverted and incubated overnight. PFUs were counted after overnight incubation (~20-30 hours) and the total overnight PFU count used to establish titer of the phage sample.

Bacterial concentrations were determined by serial dilution of bacterial culture (1:10 or 1:100 dilutions made to 1 mL in 1.5 microcentrifuge tubes in Lb) and subsequent plating and bead spreading of 100 µl of a countable dilution (targeting 50 colony forming units) on Lb plates. Plates were incubated overnight and counted the next morning. Three dilution series were performed for the *E. coli* BL21 host to establish concentration at different OD_{600} values to accurately target concentration for mixing with phage for sample preparation.

To prepare samples, phage and bacterial stocks were titered and samples were mixed by adding ~$1x10^8$ CFU of exponential phase *E. coli* BL21 with the noted amount of T7 phages in Rhodium Cryotubes and then immediately freezing at -80C. Phage and bacterial titer was confirmed after mixing samples. Samples were then shipped and incubated as described.

After incubation, samples were thawed at 37C and immediately split for phage and bacterial genomic extraction and phage and bacterial titering. Bacteria was titered by immediate serial dilution and subsequent plating and bead spreading of 100 µl on Lb plates. Plates were incubated overnight and counted the next morning. Phages were isolated by immediately spinning 1 mL of the sample at 16g for 1 minute and then filtering through a 0.22 uM filter and then counted by spot assay. MOI was calculated by dividing phage titer by bacterial concentration. MOI for the T7 DMS library was estimated using a helper plasmid as previously described [12].

Efficiency of Plating (EOP) was determined using *E. coli* BL21 as a reference host. EOP values were generated by taking the phage titer on the test host divided by the phage titer on the reference host, and this value was subsequently $\log_{10}$ transformed. Values are reported as mean ± SD. We used deep sequencing to evaluate phage populations as previously described [12]. Breseq was used to identify mutations for WGS [51].

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