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 researchers have extensively studied phage-host interactions 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 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 experiencing microgravity. This altered environment could change their microbiome composition and, consequently, their health. Secondly, spacecraft 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.

Due to physical and physiological factors, microgravity poses a challenge for phage predation and bacterial growth. From a physical perspective, gravity directly impacts the movement of phages and their ability to interact with their bacterial hosts. 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 movement of particles (both

phages and nutrients) in different directions, increasing fluid mixing. In microgravity, materials of different relative densities will evenly disperse, affecting nutrient diffusion 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 significant implications for the behavior of microbial communities in space environments.

In this study, we investigated the impact of microgravity on the interactions between T7 bacteriophage and non-motile *E. coli* BL21 aboard the International Space Station (ISS). Our evaluation of short-term (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 facilitate interaction with bacterial host receptors or otherwise improve phage infectivity, while bacteria acquired mutations in genes that may enhance 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 significant 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

We created two identical sets of 32 packaged, sealed tubes containing experimental samples. We designated one set for incubation in microgravity and one control set for incubation under terrestrial conditions in gravity (Figure 1). We divided 32 tubes into four prepackaged sets of eight for incubation at 37°C in each condition and incubated three sets at short-term time points of one, two, and four hours and one set for a long-term time point of 23 days. Each short-term prepackaged group contained three replicates of T7 and *E. coli* mixed at multiplicities of infection (MOIs) of 10^{-6} and 10^{-4} and two control sample controls with T7 phage only or *E. coli* only. 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 all samples containing bacteria. We chose the initial bacterial titer and phage MOI to allow for production of detectable changes in phage titer during incubation in microgravity after accounting for cell and phage viability loss during the experiment. The 25-day prepackaged group

contained three replicates of T7 phage and *E. coli* mixed at an MOI of 10^{-4}, three replicates of a DMS library of the T7 variants with *E. coli* at an MOI of 10^{-2}, and two control samples of T7 only and *E. coli* only. 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 that has been tested previously under terrestrial conditions [12].

The cryovial containers successfully passed biocompatibility and leakiness testing and 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 bacteria in Rhodium cryovials and immediately freezing samples at -80°C. The frozen samples were shipped to the National Aeronautics and Space Administration (NASA) Wallops Flight Facility in Virginia 24 days before the launch. 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, transported back to Earth, and shipped to our laboratory. We thawed the samples, immediately measured the phage and bacterial titers, sequenced the phage and bacterial genomes, and scored the DMS library (Figure 1). We recorded the duration of freezing and incubation onboard the ISS and then tested the second set of samples as terrestrial controls using identical incubation and freezing times.

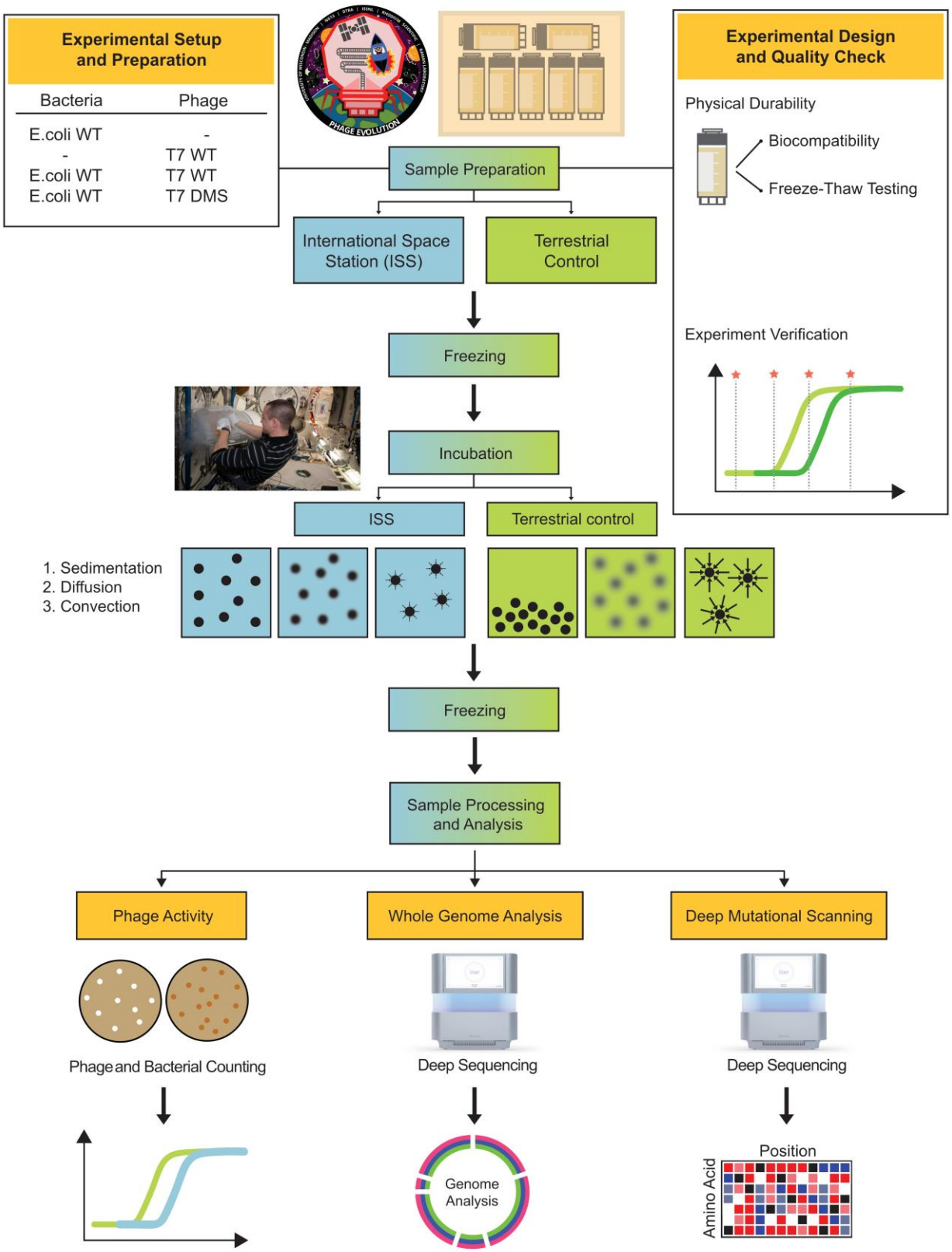

Experimental Setup and Preparation
Bacteria
Phage
E.coli WT
-
-
T7 WT
E.coli WT
T7 WT
E.coli WT
T7 DMS
PHAGE EVOLUTION
Experimental Design and Quality Check
Physical Durability
Biocompatibility
Freeze-Thaw Testing
Experiment Verification
Sample Preparation
International Space Station (ISS)
Terrestrial Control
Freezing
Incubation
ISS
Terrestrial control
1. Sedimentation
2. Diffusion
3. Convection
Freezing
Sample Processing and Analysis
Phage Activity
Whole Genome Analysis
Deep Mutational Scanning
Phage and Bacterial Counting
Deep Sequencing
Deep Sequencing
Genome Analysis
Position
Amino Acid

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

An illustration of the experimental process to prepare and test samples under terrestrial conditions and in microgravity onboard the International Space Station (ISS). We prepared samples on Earth and performed quality checks to ensure samples could not leak and cryovial integrity was 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 with sample mixing 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 in microgravity would be considerably slower 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 after one, two, four hours, and 23 days of incubation. 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.

Phage and bacteria were co-cultured at MOIs of 10^{-6} and 10^{-4} for short-term incubation time points (one-, two- and four-hours) and 10^{-4} for the long-term incubation (23-day) timepoint. An increase in phage titer and an initial decrease in bacterial host titer indicated successful phage activity. After 4 hours under terrestrial conditions, phage titers saw a significant 5-7 log fold increase, while bacterial titers saw a significant 4-5 log fold decrease, regardless of the MOI. These results show successful phage infection occurred between two and four hours for the terrestrial samples. The fact that we observed phage activity only at the four-hour timepoint in the terrestrial sample indicates that the experimental conditions delayed the infection cycle by approximately two hours but still allowed for successful phage replication.

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 significant 4-log increase in phage titer at the 23-day time point. This result indicates microgravity significantly delayed, but did not prevent, productive infection and lysis. The 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) also suggests that a phage-resistant bacterial population emerged in both conditions.

In samples with bacteria only, titer remained consistent at early time points under terrestrial incubation but saw 6-7 log-fold depletion at the 1- and 2-hour time points in microgravity. This result suggests the shock experienced by the bacteria from microgravity-induced stress negatively affected their ability to survive the following sample freeze in contrast to terrestrial incubation. Phage saw an overall decrease in viable titer in early time points in microgravity compared to terrestrial incubation but otherwise were more stable than bacteria, except for the 23-day timepoint, where we observed a seven- and four-log fold decrease in phage titers in microgravity and gravity respectively. 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.

In summary, these experiments demonstrate that microgravity presents a challenge to phage and bacterial viability. Microgravity delays phage infectivity compared to terrestrial conditions, but phages eventually overcome this disadvantage to productively infect their hosts. Future experiments focusing on intermediate time points will be essential in determining the precise latent period for the phage in such conditions.

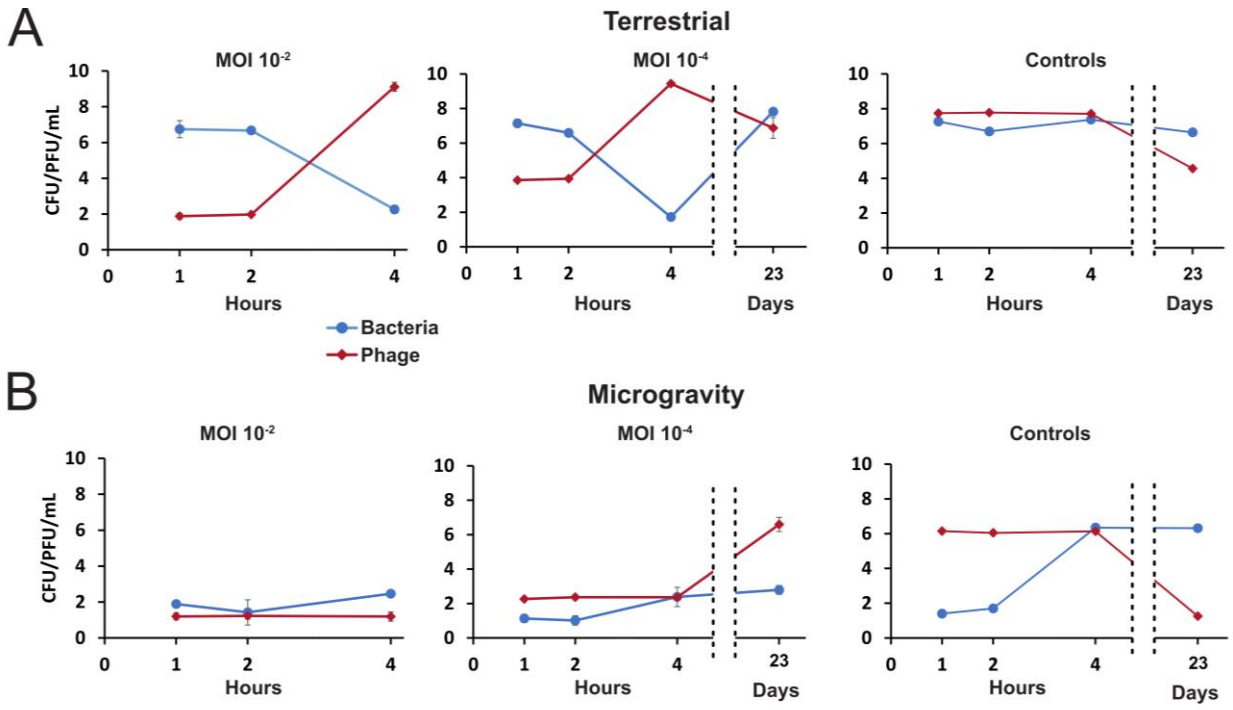


Figure 2. Bacteriophage T7 growth is inhibited by microgravity

The titer of bacteria (blue) and phage (red) samples ((log$_{10}$ PFU or CFU/mL) after **(A)** terrestrial incubation or **(B)** incubation in microgravity mixed at an MOI of 10^{-2} (left), 10^{-4} (middle) and for controls mixed separately (right). Triplicate samples are represented as mean +/- SD.

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

Next, we sought to identify the mutations that influenced phage-host relationships under microgravity by sequencing the genomes of bacteria and phage from the 23-day microgravity samples and terrestrial controls. Sequencing revealed several potential mutational hotspots on the T7 genome with 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 significantly 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 substantial 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 [15]. Though not fully characterized, structural gene *gp7.3* is likely injected into the cell upon adsorption. This gene may be a scaffolding protein or participate in adsorption to the host [16]. Although *gp7.3* is considered essential for the 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 well distributed throughout the protein (Supplementary File 2). These results suggest that single amino acid mutations arising in microgravity may either assist in the adsorption of the phage to the cell surface or promote scaffolding in this environment. At the same time, deletions under terrestrial selections may point advantages for shortened versions of this protein in prolonged terrestrial interactions.

We found mutations in only one class II gene, *gp4.*7, at substantially higher abundance under terrestrial conditions than in microgravity. Substitution T115A in *gp4.7* dominated the mutation pool with 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 [15].

Many mutations also accumulated in structural proteins, particularly in *gp11, gp12,* and *gp17*, forming the tail and tail fiber [17,18]. Mutations in these genes were more prevalent under terrestrial conditions than under microgravity. Mutations in *gp17* primarily occurred in the tip domain. Substitution D540G in a proximal β-strand was notably 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.

We saw mutations accumulating in *E. coli* BL21 samples mixed with phages at a much higher frequency than in controls without phages in both terrestrial and microgravity samples, indicating phages induce strong selective pressure in both conditions (Figure 3E-G, see Supplementary File 3). We found abundant deletions in *trxA* in both conditions (terrestrial: 44.4%, K53_V56d deletion, microgravity: 100%, I76 frameshift). *trxA* is a

processivity factor for T7 DNA polymerase and a known essential gene for phage activity [19,20]. The inactivation of this gene suggests that this gene remains essential to the phage in microgravity.

Bacterial genes highly enriched in microgravity were frequently associated with the outer membrane and related to cell stress management. Mutations that were only enriched in microgravity include *hldE* (56.5%, R232C) associated with catalyzing pathways for building the inner core of LPS [21], *mrcB* (51.6%, Q817 frameshift), related to cell wall synthesis and permeability [22,23], *yejM* (18.5%, L330 frameshift), a protein associated with outer membrane permeability [24], and *bipA* (96.5%, H16 frameshift) closely linked to LPS biosynthesis and temperature sensitivity. Researchers have previously associated this gene with truncated LPS [35-37]. The extremely high abundance of these mutations when bacteria are co-cultured with phages suggests that *bipA* might play a role in phage sensitivity in microgravity.

Genes mutated in microgravity more directly associated with cell stress response included *topA* (96.1%, V73G), a DNA topoisomerase associated with stress response [25,26], and *entS* (5.1%, A182 deletion), associated with enterobactin export and cell stress management [27,28]. Other mutants seen only in microgravity included *gltA* (85%, I138 frameshift), a citrate synthase [29], *mnmE* (10.3%, E280 frameshift), a GTPase [30], and *rpsF* (27.7%, V18 frameshift), a 30S ribosomal protein [31].

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 O-polysaccharide in other strains [32,33], *lptA* (100%, Q43H), responsible for LPS assembly [34], *hns* (66%, I70_L75 IS1 insertion), a global DNA-

binding protein responsible for regulating metabolism and nutrient acquisition [35], and *tufB* (18.9%, I120V; 13.9%, G123A), an elongation factor [36].

We identified several genes that 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 that triggers increasingly small, slow-growing persistor cell states, which could benefit bacteria during prolonged incubation [37]. Finally, intriguing indels arose in *mlaA* under 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. Samples without phages did not accrue either mutation.

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]. This gene has not yet been associated with changes in phage activity. Researchers previously characterized a mutant that results in the same F44_N45 deletion 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. Since 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 [40], but this mutation appears to be associated with increased survivability in this experiment. This difference may be due to the selection pressure imposed by the phage, 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.

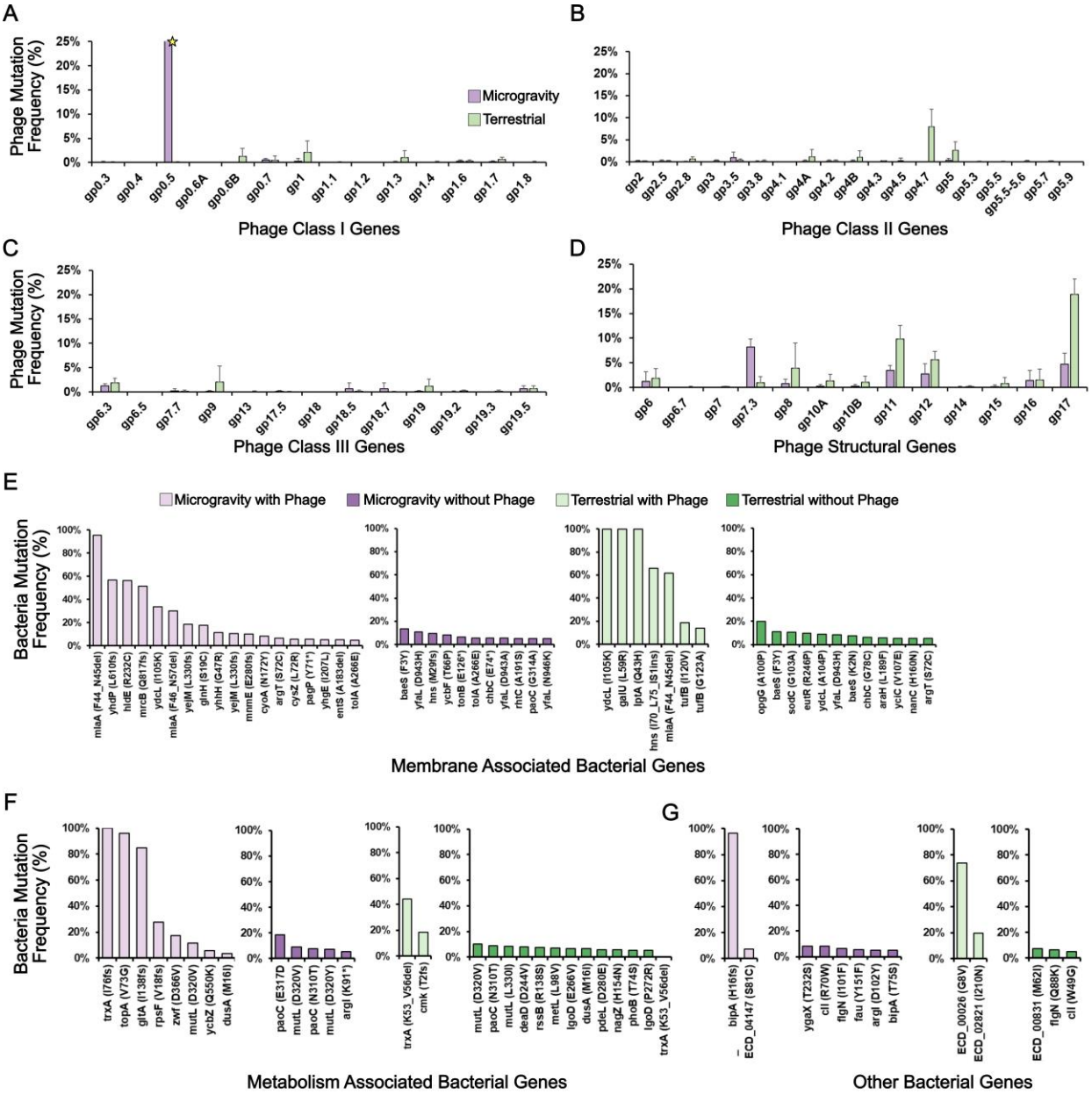

A
Phage Mutation Frequency (%)
Microgravity
Terrestrial
gp0.3 gp0.4 gp0.5 gp0.6A gp0.6B gp0.7 gp1 gp1.1 gp1.2 gp1.3 gp1.4 gp1.6 gp1.7 gp1.8
Phage Class I Genes
B
gp2 gp2.5 gp2.8 gp3 gp3.5 gp3.8 gp4.1 gp4A gp4.2 gp4B gp4.3 gp4.5 gp4.7 gp5 gp5.3 gp5.5 gp5.5-5.6 gp5.7 gp5.9
Phage Class II Genes
C
gp6.3 gp6.5 gp7.7 gp9 gp13 gp17.5 gp18 gp18.5 gp18.7 gp19 gp19.2 gp19.3 gp19.5
Phage Class III Genes
D
gp6 gp6.7 gp7 gp7.3 gp8 gp10A gp10B gp11 gp12 gp14 gp15 gp16 gp17
Phage Structural Genes
E
Microgravity with Phage
Microgravity without Phage
Terrestrial with Phage
Terrestrial without Phage
Bacteria Mutation Frequency (%)
mlaA (F44_N45del) yhdP (L610fs) hldE (R232C) mrcB (Q817fs) ydcL (I105K) mlaA (F46_N57del) yejM (L330fs) glnH (S19C) ynhH (G47R) yejM (L330fs) mmmE (E280fs) cyoA (N172Y) argT (S72C) cysZ (L72R) pagP (Y71*) yhgE (I207L) entS (A183del) tolA (A266E)
baeS (F3Y) yfaL (D943H) hns (M29fs) ycbF (T66P) tonB (E120*) tolA (A266E) chbC (E74*) yfaL (D943A) rhtC (A191S) paoC (G314A) yfaL (N946K)
ydcL (I105K) galU (L59R) lptA (Q43H) hns (I70_L75_IS1ins) mlaA (F44_N45del) tufB (I120V) tufB (G123A)
opgG (A100P) baeS (F3Y) sodC (G103A) eutR (R246P) ydcL (A104P) yfaL (D943H) baeS (K2N) chbC (G78C) araH (L189F) yciC (V107E) nanC (H160N) argT (S72C)
Membrane Associated Bacterial Genes
F
Bacteria Mutation Frequency (%)
trxA (I76fs) topA (V73G) gltA (I138fs) rpsF (V18fs) zwf (D366V) mutL (D320V) ycbZ (Q550K) dusA (M16I)
paoC (E317D) mutL (D320V) paoC (N310T) mutL (D320Y) argI (K91*)
trxA (K53_V56del) cmk (T2fs)
mutL (D320V) paoC (N310T) mutL (L330I) deaD (D244V) rssB (R138S) metL (L98V) lgoD (E266V) dusA (M16I) pdeL (D280E) nagZ (H154N) phoB (T74S) lgoD (P272R) trxA (K53_V56del)
Metabolism Associated Bacterial Genes
G
bipA (H16fs) ECD_04147 (S81C)
ygaX (T232S) cll (R70W) flgN (I101F) fau (Y151F) argI (D102Y) bipA (T75S)
ECD_00026 (G8V) ECD_02821 (I210N)
ECD_00831 (M62I) flgN (Q88K) cll (W49G)
Other Bacterial Genes
H
Pos30
Pos43
WT mlaA DPLEGFNRTMYNFNFN- - VLDPY
MlaA (F44_N45del) DPLEGFNRTMYNFN- - - - VLDPY
MlaA (F46_N47ins) DPLEGFNRTMYNFNFNFNVLDPY

Figure 3. Incubation in microgravity reveals novel mutations

(A-D) Total average percentage mutation frequency for bacteriophage T7 genes transcribed **(A)** early (class I), **(B)** middle (class II), **(C)** late in infection (class III), or **(D)** as structural genes after incubation in microgravity (purple) and under terrestrial conditions (green). Data is shown as the averaged sum of all mutation frequencies for each gene across the three replicates +/- SD. The star indicates *gp0.5*, which reached 100% frequency in two replicates. **(E-G)** Maximum abundance of mutations in the *E. coli* BL21 genome with >5% abundance after incubation in microgravity with phage (light purple), in microgravity without phage (dark purple), after terrestrial incubation with phage (light green), or after terrestrial incubation without phage (dark green) grouped by **(E)** membrane-associated genes, **(F)** metabolism-associated genes, or **(G)** other genes. **(H)** An 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. Bacterial response may be further exacerbated by stress caused by microgravity, leading to significant alterations in their proteome, including changes to the phage receptors. The change in the bacteria's surface receptor profile will result in mutational adaptations in the phage receptor binding protein (RBP). Microgravity provides a unique condition to explore phage RBP adaptations that may reveal novel interactions otherwise hidden under terrestrial conditions. To explore these novel interactions, we examined how individual substitutions of the tip domain of the RBP of T7 impacted phage viability. 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 significant determinant of phage activity and interacts with host receptor LPS to position the phage for successful, irreversible binding with the host [18,20,42–45]. 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 wildtype 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 S1) 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 in long-term infectivity on a 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.

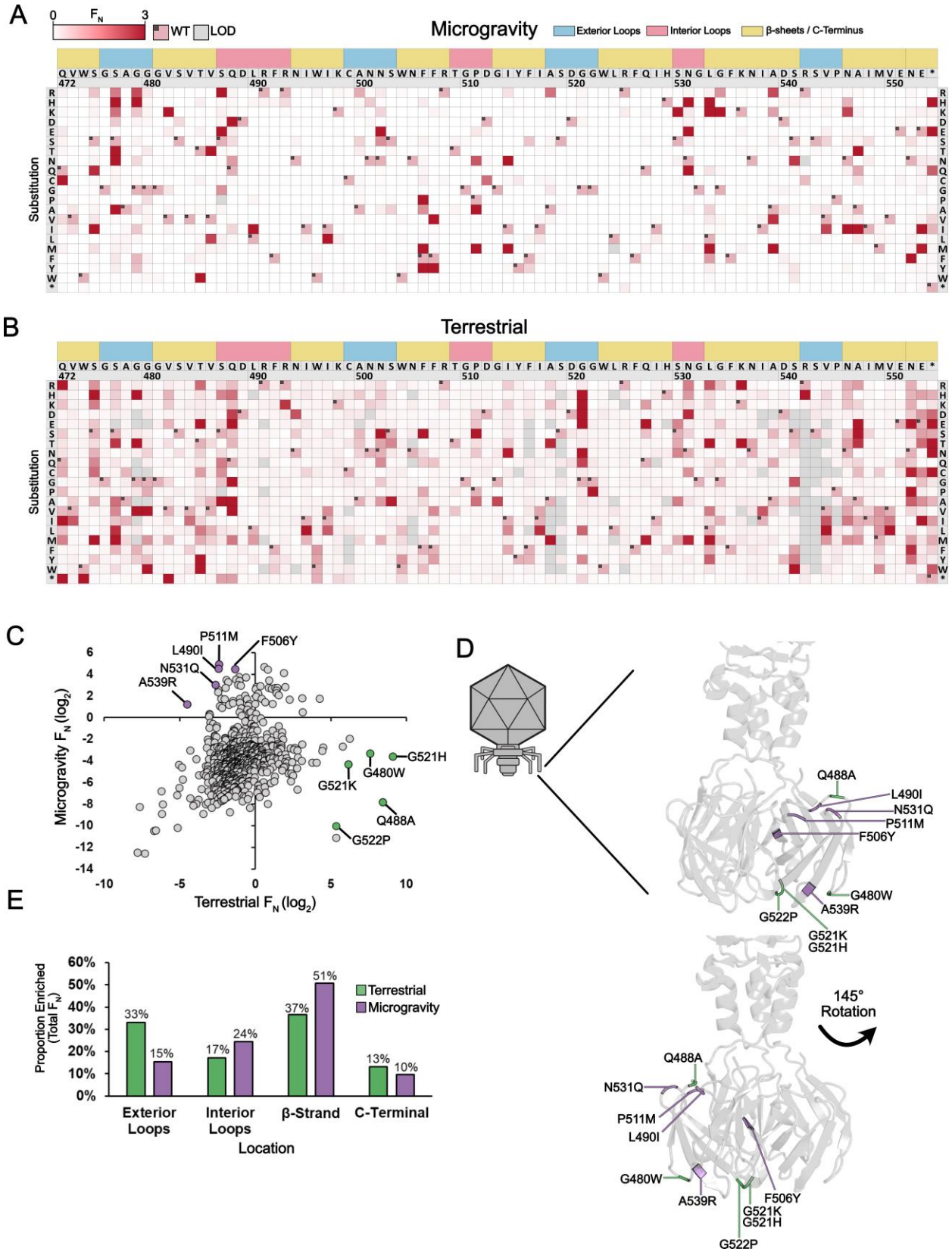

A
0
F_N
3
WT
LOD
Microgravity
Exterior Loops
Interior Loops
β-sheets / C-Terminus
Substitution
B
Terrestrial
C
P511M
F506Y
L490I
N531Q
A539R
G521H
G480W
G521K
Q488A
G522P
Microgravity F_N ($\log_2$)
Terrestrial F_N ($\log_2$)
D
Q488A
L490I
N531Q
P511M
F506Y
G480W
A539R
G522P
G521K
G521H
145°
Rotation
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. We chose thirteen substitutions 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 navigate sequencing space efficiently to find complex combinations that improved phage activity.

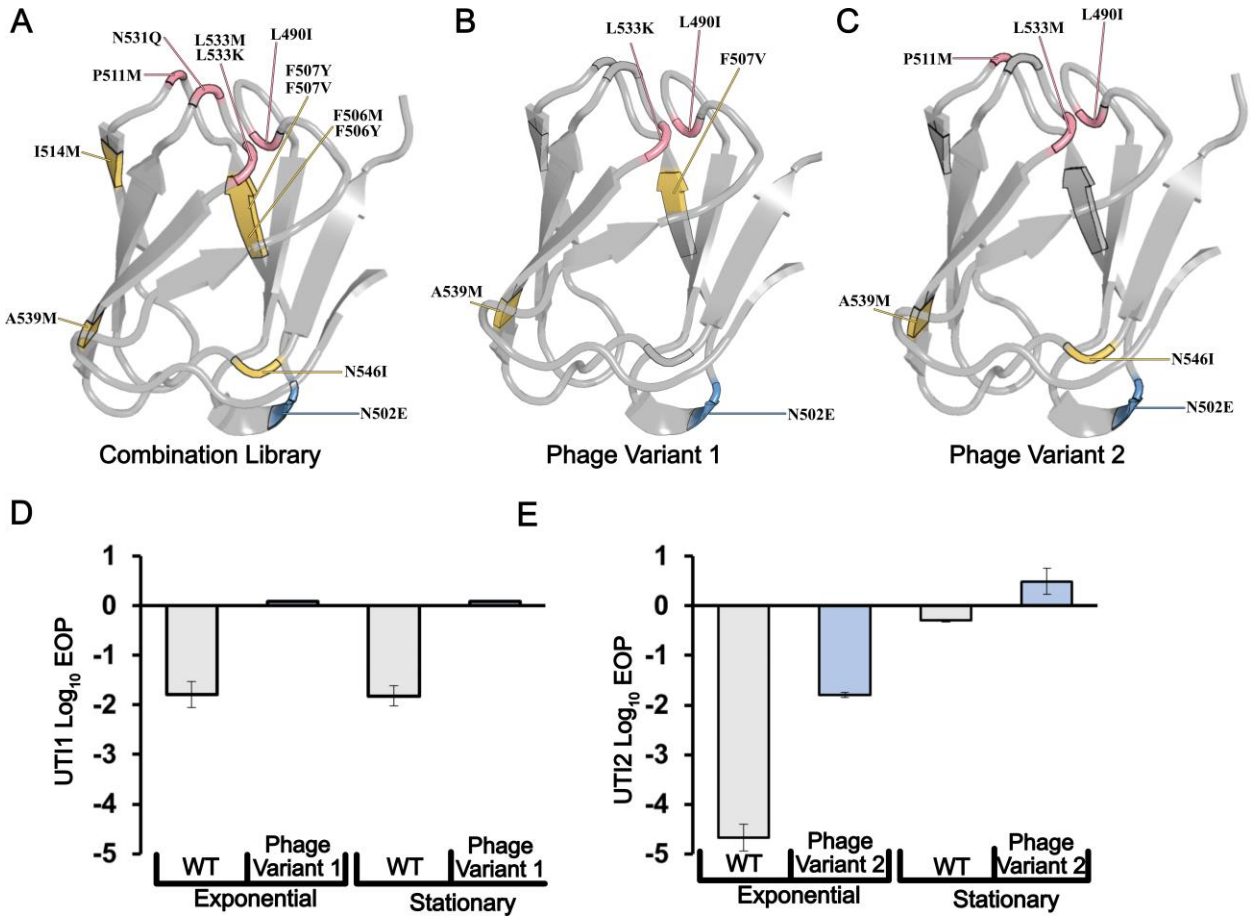


Figure 5. Microgravity-enriched variants enable activity on terrestrial pathogens

(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 bacterial physiology that can further alter this dynamic. Characterizing these interactions is essential for understanding how microbial communities adapt to microgravity and 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 successful replication cycle.

We identified many novel *de novo* mutations in phage and bacterial genes at high abundance, indicating a 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 important to fitness than previously understood [15]. Missense mutations and indels in *gp7.3* also highlight this scaffolding or adsorption-related protein as a potential key player [16]. 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 respond to stress, manage nutrient availability, and facilitate trans-membrane transport in this unique environment [11,46–49]. The high abundance of mutations in these genes suggests these mutations 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 navigate sequence space efficiently 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 of phage-host interactions and reveal substitutions and regions that can be leveraged to improve phage activity 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.

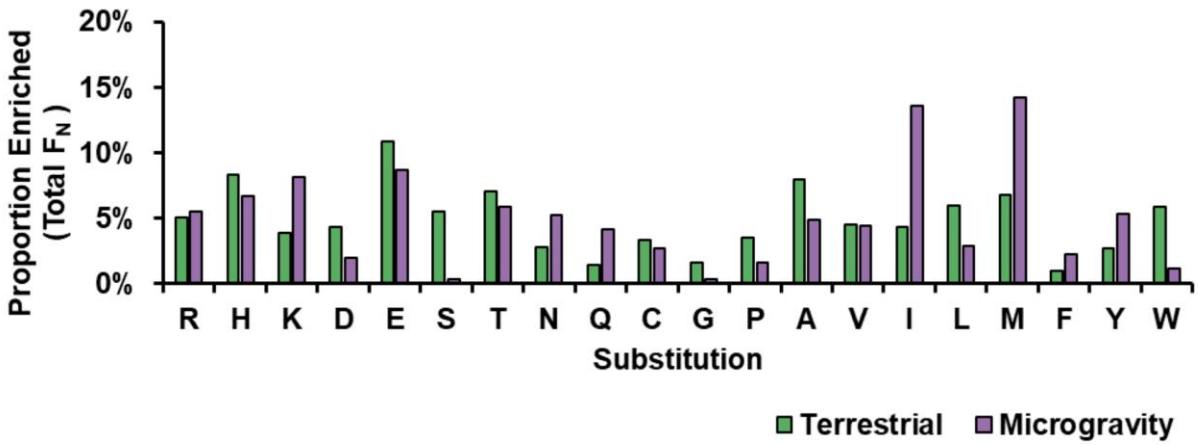
Supplementary Files

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. 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 4 mL of ~$1x10^8$ CFU/mL 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.

The initial time points for the experiment were 1, 2, and 3 hours and 25 days, with flexibility allowed to accommodate astronaut scheduling. Final time points were 1, 2, and 4 hours and 23 days. The duration of incubation for the experiments conducted in the ISS was recorded and terrestrial control experiments were carried out at that actual recorded, not the proposed, timepoints.

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 or whole plate assay averaging two replicates. 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]. Sequencing depth averaged ~49,000-fold per bp across the phage genome, allowing identification of lowly abundant mutations. Sequencing depth for the bacterial samples mixed with phage averaged ~250 reads per bp across the genome, while depth for bacterial samples without phage averaged ~1300 reads per bp across the genome, restricting analysis to more highly abundant mutations. Breseq was used to identify mutations for WGS [51]. For Figure 3 genes are grouped based on GO classifications [52]. Genes grouped as membrane-associated included genes with GO classifications for Membrane (GO:0016020), LPS Biosynthesis, (GO:0009103), Outer Membrane Bound Periplasmic Space (GO:0030288), and Periplasmic Space (GO:0042597). Genes grouped as metabolism-associated included genes with GO classifications for Metabolic Process (GO:0008152) and Regulation of metabolic process (GO:0019222).

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