Bacterial gene essentiality under modeled microgravity

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ABSTRACT

To support long-duration spaceflight, identifying genes that enable microbial survival and stability under space stressors is crucial, as these factors can affect both host health and in-space biomanufacturing efforts. Our objective was to determine what bacterial genes are required for growth in culture under modeled, or simulated, microgravity conditions compared to normal gravity controls. We focused on the marine bacterium *Vibrio fischeri*, which forms a monospecific symbiosis with the Hawaiian bobtail squid, *Euprymna scolopes*. The symbiosis has been studied during spaceflight and in ground-based modeled microgravity conditions. Using transposon insertion sequencing (INSeq), we identified dozens of genes that exhibited fitness defects under both conditions, yet we identified relatively few genes with differential effects under modeled microgravity or gravity specifically. We additionally compared RNA-seq and INSeq data and determined that expression under microgravity was not predictive of the essentiality of a given gene. In summary, empirical determination of conditional gene essentiality identifies few microgravity-specific genes for environmental growth of *V. fischeri*, suggesting that the condition of microgravity has a minimal impact on symbiont gene requirement during growth in media. These findings suggest that maintaining beneficial microbial communities during spaceflight, and enabling their use in biomanufacturing applications, may require minimal genetic adaptation, easing the challenges of supporting healthy symbiotic relationships on long-duration missions.

INTRODUCTION

Spaceflight alters microbial physiology and their interactions with eukaryotes, and as the number and duration of manned space missions expands beyond low Earth orbit, elucidating the underlying mechanisms of these changes is essential[1–10]. To address these issues, experiments are needed to examine bacterial growth, bacterial-host interactions, and host health under spaceflight-like conditions. Although previous studies using both natural and modeled microgravity conditions have resulted in a wide range of physiological and genetic responses, many initial studies primarily targeted pathogenic strains[11–13]. More recently, studies have begun to examine the effects of microgravity on beneficial microbes that associate with animals and how spaceflight impacts these interactions[4–6,10,14,15]. Given the importance of beneficial microbes to host health, we sought here to ask a fundamental question of what genes are required for bacterial growth in microgravity.

Due to the number of logistical constraints in conducting spaceflight experiments, several ground-based platforms exist to simulate the low-shear environment of microgravity[16]. Rotating cell culture systems with High Aspect-Ratio Vessels (HARVs) represent one such platform as they mimic the low-shear fluid conditions that occur in low Earth orbit and have been used for decades to model microgravity environments[17]. As cells grow in the HARVs, the hydrodynamic forces within each vessel offset the effects of gravity and the cells are essentially in “freefall” and maintained in a constant suspension (**Fig. 1**). Bacterial strains grown in these low-shear modeled microgravity (LSMMG) conditions can experience a wide range of different

physiological responses, including increases in growth rates, biofilm formation, secondary metabolite production, environmental stress responses, and antibiotic resistance[6,18–23].

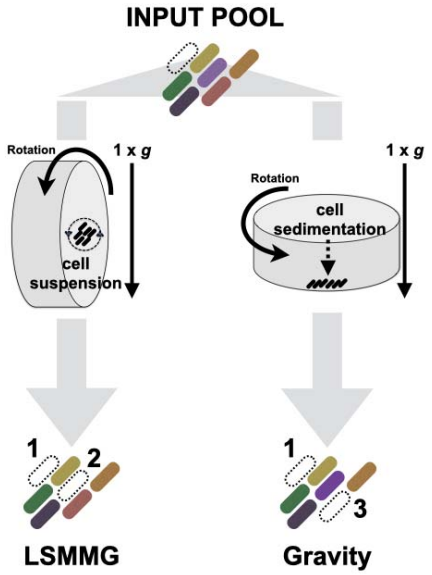

Fig. 1. Experimental setup for bacterial mutant enrichment in HARV vessels to model microgravity (LSMMG) and to have the control gravity condition. INSeq libraries were grown in HARV vessels that can simulate microgravity when placed perpendicular or gravity when horizontal. Mutants exhibited either no defects in either condition or exhibited (1) a growth defect in both conditions, (2) LSMMG defect, (3) or a gravity-specific defect.

The symbiosis of *Vibrio fischeri* colonization of the light organ of the Hawaiian bobtail squid, *Euprymna scolopes*, has emerged as a valuable model to study the effects of microgravity on host-microbe dynamics and function[1,10,24,25]. The squid hatches each generation without its bacterial partner and then proceeds to harvest *V. fischeri* from the seawater[26]. The bacteria colonize the dedicated symbiotic light organ of the host, where bioluminescence from the bacteria is projected downward and camouflages the host in the moonlight[27]. This system has

been especially valuable to understand the molecular basis by which animals acquire specific microbes from the environment[28]. The bacteria are genetically manipulatable, and the site of colonization can be imaged directly in live animals, enabling studies that have revealed much of the molecular dialogue between the two partners[29–32]. The specificity of the relationship, in which only certain strains of *V. fischeri* can colonize the squid host, provides a strong model system to study animal-microbiome formation, development, and evolution[26]. Furthermore, the small size of the host animal and simplicity of the symbiosis has contributed to the value of this system for studying host-microbe interactions during spaceflight[1,3,10,33].

In this study, we take a complementary approach to previous work on bacterial gene expression under simulated microgravity. Previous studies using the squid-vibrio system have focused extensively on the impact of simulated and actual spaceflight conditions on the host squid physiology in the presence and absence of *V. fischeri*[1,3,10,33,34]. Results have shown that under both simulated and actual spaceflight, the colonization of the host by *V. fischeri* results in the down-regulation of several host genes associated with oxidative and heat stress, suggesting that being in a symbiotic state is beneficial to the host during spaceflight. These studies do not, however, examine the impact of the space-like environment on the symbiont, specifically, what genes are required for survival under microgravity and modeled microgravity conditions. Other studies in the symbiont have applied a genome-scale genetic approach technology to globally identify *V. fischeri* genes that are essential, and conditionally essential, under specific environmental conditions and during growth in media under laboratory conditions[35]. Here, we

apply a similar global approach to determine what genes are required for bacterial growth under simulated microgravity conditions to ascertain the overall impact that microgravity-like conditions have on symbiont health and physiology.

RESULTS

Global analysis of bacterial genes required for growth in low-shear modeled microgravity. A number of studies have examined differential gene expression in beneficial microbes under microgravity or simulated microgravity conditions[36–39], including in the marine bacterium *V. fischeri*[39,40]. The current study asks a distinct, yet related, question of what genes are differentially required for growth under simulated microgravity conditions compared to a gravity control condition. To identify mutants with a competitive growth defect in simulated microgravity, we applied the transposon insertion sequencing (INSeq/Tn-seq) approach to *V. fischeri* strains cultivated under HARV conditions in both the low-shear modeled microgravity (LSMMG) and gravity positions (**Fig. 1**)[40,41]. We started with a characterized mutant library of over 40,000 transposon mutants in *V. fischeri* strain ES114[35]. The resulting “input” library was introduced into HARV vessels under either LSMMG or normal gravity conditions (1 x *g*). We simultaneously examined the mutant library grown in LSMMG and gravity control conditions after growth for approximately 15 generations, and each resulting “output” pool of mutants was frozen. A total of six LSMMG biological replicates and three gravity control replicates were compared to the input library. From each sample, DNA was isolated, from which INSeq libraries

were constructed[41]. The libraries were sequenced on an Illumina HiSeq 2000 instrument, and the location of each transposon insertion was mapped using the pyinseq Python package.

For each sample, we obtained between 6.65 - 8.18 x 10^6 Illumina reads (**Table S1**). We observed similar numbers and patterns of unique transposon hits across the samples tested, suggesting that the library did not undergo significant bottlenecks during the experiment (**Table S1**). Next, for each sample, we examined the normalized transposon insertion counts (CPM; counts-per-million Illumina reads) in each gene. We compared the similarity of these gene-level counts across the samples in the analysis using pairwise correlation analysis. All pairwise comparisons had a high level of similarity (Spearman $R^2 > 0.95$), and the HARV-grown samples were clearly distinguishable from the input libraries (**Fig. 2**). Examination of the heat map suggested little overall differentiation between the LSMMG and gravity samples that were otherwise grown similarly. We proceeded to compare individual genes that were depleted under LSMMG, gravity, or both conditions.

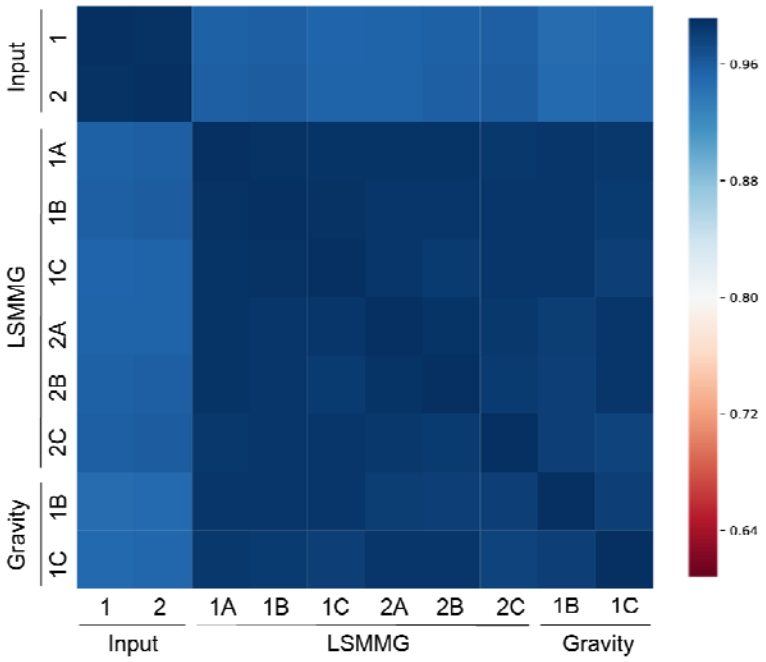

Fig. 2. Pairwise correlation between samples revealed a clear difference between input samples and output samples from the HARV conditions. Spearman correlations between samples, in which the normalized transposon insertion counts in each gene were compared. Input replicates were the original INSeq library grown for inoculation into the HARV, and low-shear modeled microgravity (LSMMG) and normal gravity replicates were outputs from the respective HARV-grown experiments.

To identify genes that had significant differential depletion under modeled microgravity we used DESeq2 to calculate the median representation of each mutant in the output LSMMG or gravity pools, and plotted those values compared to the input pool. We focused our analysis on genes that were depleted at least 2-fold in the different conditions. Genes that did not meet these criteria, genes that were poorly represented in the input pool, and genes for which a previous study suggested that mutants impaired bacterial growth were excluded from this analysis (**Fig. 3**; open black circles). A total of 109 genes exhibited depletion under both LSMMG and gravity conditions (**Fig. 3**; filled circles). Of these genes, most were similarly depleted under both conditions. However, there were genes in this group that had a ≥ 2-fold difference in one

condition (LSMMG or gravity) relative to the other, including 10 genes more depleted under gravity conditions and one gene (*rodA*) more depleted under LSMMG. Furthermore, there were two genes in the analysis that were significantly depleted ($p < 0.05$ from DESeq2 analysis) under gravity conditions and not under LSMMG conditions (*flgD, rfaD*). There were no genes that were only significantly depleted under LSMMG conditions.

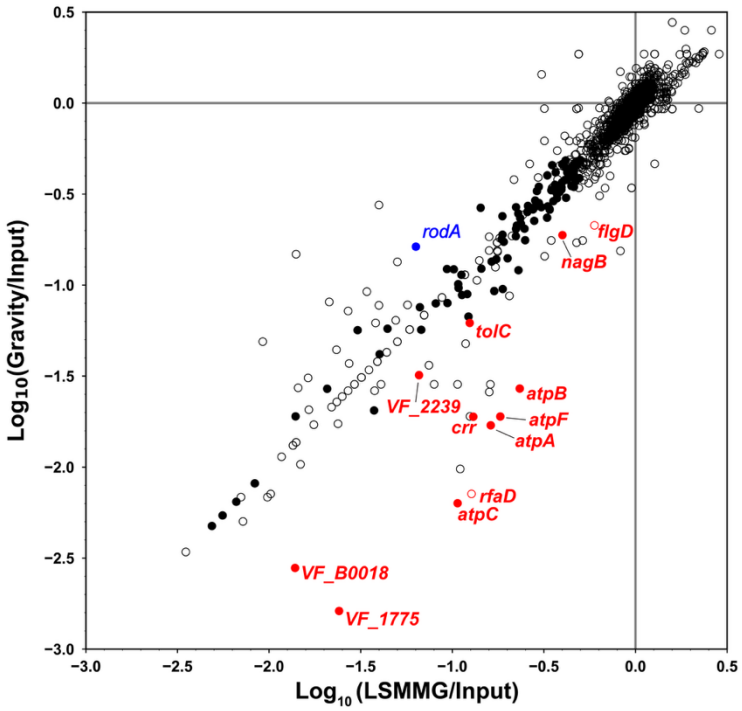

Fig. 3. Mutant behavior under LSMMG or gravity revealed fitness in each HARV condition, normalized to the input transposon sequencing library. A) Comparison scatter plot of microgravity (x-axis) and gravity (y-axis) Log(Fold-Change) values. Counts for each gene for each replicate were normalized and used to calculate Log_{10}(Fold-Change) values as described in Materials and Methods. Genes that were poorly represented in the input pool, did not exhibit significant depletion, or were previously determined to be growth deficient in LBS medium were designated as open black circles. Filled black circles are genes that were significantly (p-value < 0.05) depleted 2-fold change under both simulated microgravity and gravity conditions. Genes that are filled red were significant in both conditions but more depleted under gravity, whereas open red circles are exclusively significant in gravity. Filled blue genes are significant in both, but more depleted under simulated microgravity. Remaining genes are shown as open black circles.

Validation of INSeq results through defined mutant competitions. The above results were derived from an analysis of complex mutant pools with > 40,000 mutants. We therefore sought to determine whether we would observe the same behavior using one-versus-one competitions between defined mutant strains and the parental strain. For this analysis, we considered the entire data set (i.e., Table S3), and we proceeded to isolate mutant strains that had a depletion from the INSeq analysis, as well as mutants in two control genes that were not depleted under either condition (*brnQ* and *nhaR*). We examined mutants that had a range of fitness defects and that were represented in a mutant collection in the the laboratory. Each defined mutant strain was grown in culture, then competed in the HARVs against the parental strain that carries the LacZ-expressing plasmid pVSV103[42]. The input and output pools from each experiment were plated onto LBS-Xgal medium, and the ratios of blue:white colonies in the samples were calculated. A competitive index was calculated to determine the fitness of each mutant under each condition. The results of this analysis are plotted in **Fig. 4**. There was strong overall concordance between the INSeq and defined competition results. All the genes for which mutants were significantly depleted under INSeq were also substantially depleted in the defined competitions, and the control mutants exhibited no substantial depletion. Therefore, we conclude that the INSeq analysis can reliably predict conditional gene requirements in the simulated microgravity environment.

Examination of the relationship between bacterial gene requirement and gene induction in modeled microgravity. We recently examined bacterial genes for which mRNA levels are

induced in the modeled microgravity condition, compared to gravity controls[42]. We analyzed our current study of gene requirement (i.e., INSeq) with the previous dataset on gene expression

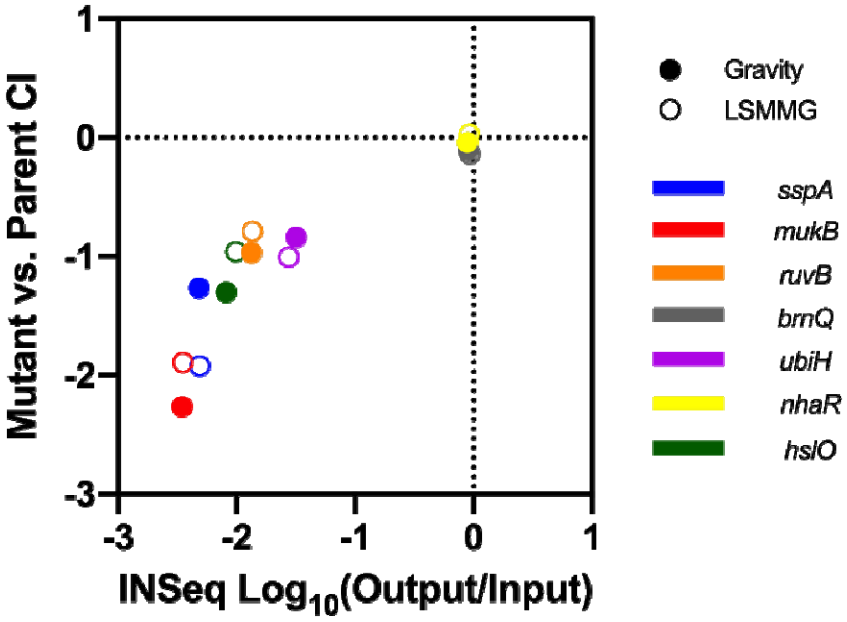

Fig. 4. Competition of defined mutants provided validation of mutant fitness from within the complex INSeq library. Mutants were competed against wild-type *V. fischeri* under simulated microgravity and gravity using the HARV vessels. Dots represent fold-change for 1:1 competition of mutant versus marked parental strain (MJM1575).

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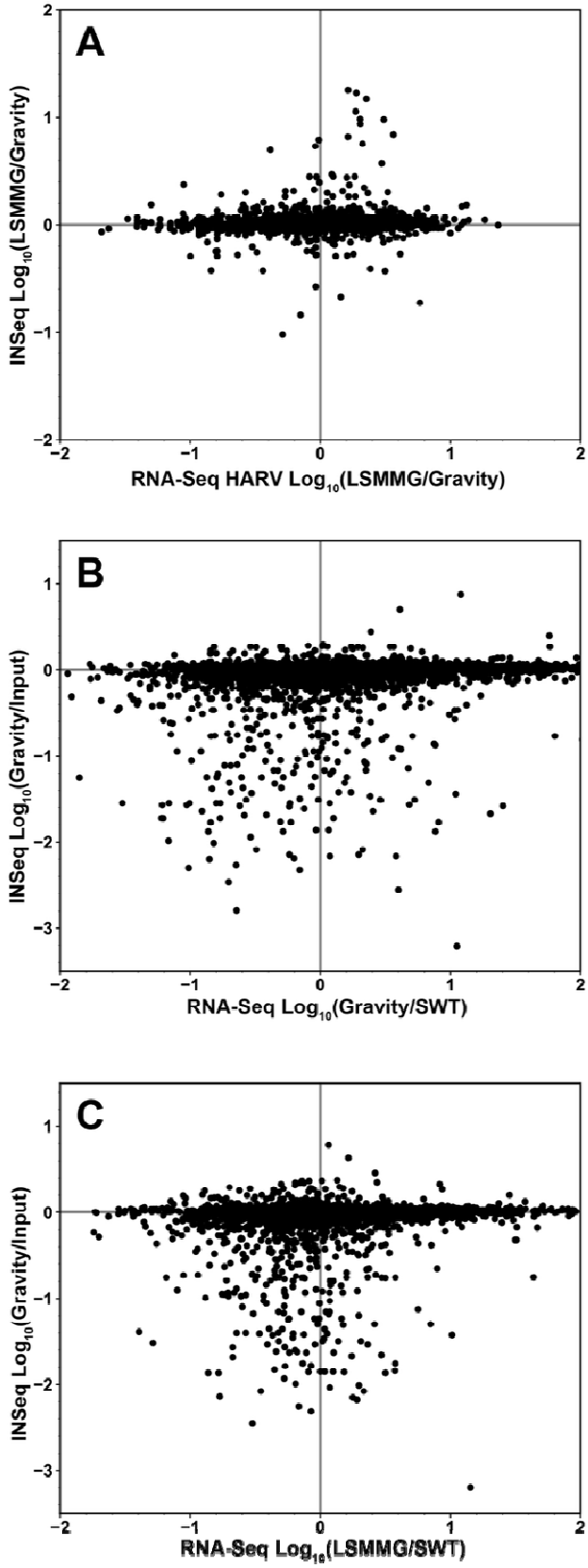
A
INSeq Log10(LSMMG/Gravity)
RNA-Seq HARV Log10(LSMMG/Gravity)
B
INSeq Log10(Gravity/Input)
RNA-Seq Log10(Gravity/SWT)
C
INSeq Log10(Gravity/Input)
RNA-Seq Log10(LSMMG/SWT)

Fig. 5. Gene expression (RNA-Seq) does not predict conditional gene requirement (INSeq) under LSMMG conditions. INSeq (y-axis) and RNA-Seq (x-axis) were compared by using log-normalized fold-change values for different experimental conditions. A) INSeq (LSMMG/Gravity) was compared to a previously published RNA-Seq (LSMMG/Gravity) under HARV conditions and had an R^2 of 0.08. We also calculated fold-changes for RNA-Seq HARV conditions using a dataset from SWT medium. B) INSeq (Gravity/Input) were compared to RNA-Seq (Gravity/SWT) and had an R^2 of 0.18. C) INSeq (LSMMG/Input) was compared to RNA-Seq (LSMMG/SWT) and had an R^2 0.11.(i.e., RNA-Seq) to ask whether there is a correlation between gene requirement and gene expression. As shown in **Fig. 5A**, there was no significant correlation between gene requirement and transcript induction (Pearson R^2 = 0.08). Our data above suggest that the HARV platform may play a more significant role in shaping mutant communities than the specific LSMMG or gravity conditions as has been determined for other bacterial strains[43]. Therefore, we explored whether genes required in the HARV are also induced in the HARV when normalized to non-HARV samples. For our INSeq data, we normalized to the input sample that did not experience the HARV condition, and for RNA-Seq, we normalized to published data on culture-grown *V. fischeri*[44]. As shown in **Fig. 5B-C**, there was a lack of overall correlation between the gene expression and gene requirement (Pearson R^2 = 0.18, 0.11 for **Fig. 5B, 5C**, respectively). Therefore, we conclude that gene requirement in the HARV cannot be predicted from transcriptome induction data, emphasizing the need for empirical determination of gene essentiality.

DISCUSSION

This study provides a global view of *V. fischeri* genes that are required for growth in conditions that simulate microgravity. Given the prominence of modeled microgravity platforms, such as HARVs, in examining the impact of spaceflight on animal-microbe interactions, a motivation for this work was to understand how the basic requirements for bacterial survival and growth are altered during growth in the microgravity analog platforms. A surprising finding was that there was little difference in gene requirement under these conditions, despite dramatic differences in gene expression. These findings, and their implications, are discussed in greater detail below.

The global data provided by INSeq, along with the comparison of the INSeq data with results from defined mutants, provided sensitive internal controls to examine growth in multiple HARV

samples. Overall, the data we obtained from HARV samples were highly consistent. As shown in the heat map in **Fig. 2**, the high correlation of INSeq replicates within a treatment (e.g., for LSMMG samples) suggests that there was no substantial bottleneck during inoculation or growth in the HARVs. Furthermore, a strong correlation between INSeq results and the results with defined mutants (**Fig. 4**) provides support that the global data are representative of gene-level data. In fact, the consistency we observed between vessel replicates was also observed between LSMMG and gravity samples in the HARVs. The heat map in **Fig. 2**, coupled with the tight correlation of the samples in **Fig. 3** (Pearson $R^2 = 0.96$), illustrates that there was little variation observed between the LSMMG and gravity conditions. A striking consistency between genes required for bacterial growth under gravity and those required for growth under modeled microgravity is the major finding from this study. As the *V. fischeri* system is used for more extensive research on microgravity and is emerging as a tool for space biomanufacturing[45], this result indicates there will not be a confounding effect of genes that are simply required for bacterial growth under microgravity. Put another way, in the future, if genes are identified that are required for colonization under microgravity, they are likely to be required specifically for interaction with the animal host, and not simply for growth under this altered gravity condition.

A common follow-up experiment for INSeq experiments is to validate the global data with individual gene mutants. We conducted such an experiment in **Fig. 4** and found that the results were consistent across over two orders of magnitude. Another common follow-up experiment is to complement back the wild-type allele for mutants under study to then demonstrate that the

wild-type phenotype is restored. Given the negative result that we observed—i.e., that mutants did not display different effects in simulated microgravity compared to the gravity control—there was not the impetus to conduct this experiment. However, as described above, we argue that this negative finding is an important one given the expectation of the experiment, and given the rigor with which we validated the results with the single-gene mutants in **Fig. 4**.

Despite the overall patterns of concordance between LSMMG and gravity, there were genes for which we observed differential effects between the two conditions, as plotted in **Fig. 3** and detailed in **Table S2**. For example, depletion in *rodA (mrdB)* under both LSMMG and gravity was observed, yet the gene was more significantly depleted under LSMMG. RodA is a SEDS-family peptidoglycan polymerase that has multiple effects on bacterial cell shape and division [46]. This result suggests that differences in bacterial morphology under the two different conditions may impact the genetic requirement for *rodA*, though we note that its absence does affect growth under both regimes.

We additionally identified several genes with mutants depleted under both conditions but that were more significantly depleted under gravity (**Fig. 3**, filled red dots). Notably, multiple genes for the F0F1-ATPase were depleted under both conditions, but more so under gravity. Interestingly, prior work in *Escherichia coli* demonstrated that a similar set of genes was essential for aerobic growth in minimal glycerol media, even though the metabolic model used predicted that they would not be required[47]. Together with our results, this suggests that this

subset of genes (*atpA, atpB, atpC, atpF*) may perform a function separate from their role in ATP synthesis. Finally, there were two genes for which mutants exhibited significant depletion under gravity but not LSMMG: *flgD* (encoding the hook capping protein FlgD) and *rfaD* (encoding the LPS biosynthesis enzyme ADP-L-glycero-D-mannoheptose 6-epimerase). Both genes affect the outer surface of the bacteria, and consistent with the *rodA* results above, support the idea that the bacterial envelope is most susceptible to differential effects of gravity.

It is also important to note that we observed dozens of genes for which mutants exhibited similar depletion under both LSMMG and gravity conditions (**Fig. 3**; black filled dots). We note that significant growth defects for mutants in these genes were not observed during 15 generations of growth in LBS medium under aerobic conditions[35]. Therefore, these genes are likely required for robust growth in the HARV, but not for growth in LSMMG versus gravity. Given the anaerobic environment of the HARV, it seems likely that many of these genes may be required for optimal growth under anaerobic conditions. This hypothesis is supported by the presence of four genes of the Na^+-translocating NADH:quinone oxidoreductase (*nqrA*, *nqrB*, *nqrD*, *nqrE*), which, in the related species *V. cholerae*, conducts 90% of the membrane NADH dehydrogenase activity under anaerobic conditions[48,49]. We also note the presence of some genes that are required for robust symbiosis in this category, including *degS*, *dnaJ*, and *ompU*[35,50]. Given that mutants in these genes exhibited growth defects in both LSMMG and gravity conditions in the HARVs, our results suggest that colonization in the HARV may proceed

differently than under standard laboratory conditions. Furthermore, we speculate that these genes may play a role in *V. fischeri* growth under anaerobic conditions.

A possible limitation of the current study is that the transposon library used was built on agar plates in a standard microbiology laboratory (i.e., under normal gravity conditions). However, were this a major limitation, then in **Fig. 3**, we would have observed many mutants with no defect in gravity but with a defect in LSMMG (e.g., in the top left of the figure). Not only did we not observe a substantial number of such genes, but they were outnumbered by the genes that fell in the bottom right. Therefore, we can conclude that the origination of the library under unit gravity conditions did not impair our ability to investigate this question.

We provide a comparison of our INSeq data with previously published RNA-Seq data. As shown in **Fig. 5**, there is little correlation between gene requirement (INSeq) and gene expression (RNA-Seq). It is important to consider both gene requirement and gene expression, as genes that are not induced in a condition may nonetheless be required for growth and/or survival. For example, a gene that is expressed at a constant level under two conditions may be required for survival in only one of those conditions. A broader analysis comparing gene requirement and expression has demonstrated that these categories are often unlinked[51]. Our results argue that genes that are induced in simulated microgravity are not preferentially required for bacterial growth under these conditions.

Overall, this work identifies genes required under exposure to LSMMG, unit gravity, or the general conditions within the analog HARV environment. Importantly, these results demonstrate that microgravity-induced transcripts do not predict which genes are conditionally essential for survival in this environment. Finally, we demonstrated that the HARV environment is amenable to high-throughput genetic experiments and was reproducible within treatments. By laying this groundwork, our findings enable deeper exploration of the genetic mechanisms underlying how microbes adapt and thrive during spaceflight. Such insights are crucial for maintaining healthy host-microbe associations and advancing space biomanufacturing, since enabling reliable biological production processes in reduced gravity is key to optimizing resource utilization and supporting long-term human missions beyond low Earth orbit.

MATERIALS AND METHODS

Media and growth conditions. *V. fischeri* strains (**Table 1**) were grown at 25°C in Luria-Bertani salt (LBS) medium (per liter, 10 g Bacto-tryptone, 5 g yeast extract and 20 g NaCl, 50 ml 1 M Tris buffer, pH 7.5, in distilled water). When appropriate, antibiotics or supplements were added to media at the following concentrations: erythromycin, 5 µg/ml; chloramphenicol, 5 µg/ml; X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), 80 µg/ml. Growth media were solidified with 1.5% agar as needed.

Table 1. *V. fischeri* strains used in this study.

Strain	Genotype	Reference/Source
MJM1100	Wild-type ES114	[52,53]
MJM1538	MJM1100/pLostfoX	[54]
MJM1575	MJM1100/pVSV103	[42,55]
MJM3801	MJM1100 *sspA*::Tn*erm* (transformed from VFS025D03)	This work
MJM3802	MJM1100 *mukB*::Tn*erm* (transformed from VFS001A10)	This work
MJM3804	MJM1100 *ruvB*::Tn*erm* (transformed from VFS020B09)	This work
MJM3805	MJM1100 *brnQ*::Tn*erm* (transformed from VFS022E03)	This work
MJM3807	MJM1100 *nhaR*::Tn*erm* (transformed from VFS022G01)	This work
MJM3808	MJM1100 *ubiH*::Tn*erm* (transformed from MJM1976)	This work
MJM3809	MJM1100 *hslO*::Tn*erm* (transformed from MJM1628)	This work

Preparation of input and output samples from HARV growth. The Lib04 library of pMarVF1 mariner transposon insertions in MJM1100 (ES114) was described previously[35]. Twenty-five microliters of the library (approximately 2 x 10^8 CFU) were inoculated into 5 ml LBS, and grown at 26 °C for approximately 5 h, to an OD_{600} of approximately 0.3. Aliquots (552 µl) of culture was

transferred to each of eight flasks containing 100 ml LBS (i.e., $2^{7.5}$-fold dilution) and mixed well; an additional 500 µl of input culture was frozen in 17% glycerol (v/v) as the input sample. From each flask, 50 ml was inoculated into each HARV: LSMMG vessels A, B, C, and D; and gravity vessels A, B, C, and D. Each vessel D was used to monitor growth rate. Samples were grown for approximately 5.25 h to an $OD_{600} \leq 0.3$; diluted another $2^{7.5}$-fold; grown to a final OD_{600} of approximately 0.3, then frozen as the 15-generation output samples. In some cases, HARV vessels leaked or had significantly reduced growth rate, and those samples were excluded from further analysis.

INSeq sample preparation. DNA from the input and output samples was prepared using the MoBio Biofilm Isolation Kit (Carlsbad, CA). Samples were prepared for INSeq analysis using the protocol of Goodman *et al.*[41], with double the BioSamA primer concentration. Samples were submitted to the Tufts University Core Facility (TUCF) for sequencing on the Illumina HiSeq 2500 (single-end 50 bp reads). The resulting reads were deposited at NCBI SRA under accession number SRR12394639.

Bioinformatic Analysis of INSeq data. Each sample was processed using the bioinformatic software pyinseq (https://github.com/mjmlab/pyinseq, v0.2.0) to quantify transposon insertions and then analyzed using Python visualization and statistical modules. Pyinseq starts by demultiplexing the raw reads using a barcode index and then maps them to the reference genome (ES114v2: CP000020.2, CP000021.2, CP000022.1) using the short-read aligner

software Bowtie[56] with parameters that allow a single base-pair mismatch. Reads with multiple alignments (e.g., to the 12 semi-redundant rRNA operons and in tRNA genes) were excluded. The output alignment files were used to quantify the frequency of reads at each transposon insertion site (TA dinucleotides, for the mariner transposon). Transposon-insertion sites were analyzed if they contain a minimum of three reads and have reads from both the left and right flanking sequence (with a maximum difference of 10-fold abundance for one side over the other). For each sample, a T50 value was calculated, which is defined as the minimum number of transposon insertion sites that account for 50% of the reads in that sample. Gene-level analysis consolidates the site-level data for insertions that fall in the 5'-most 90% of each gene (-d parameter of 0.9). The pyinseq summary gene table (**Table S3**) was further analyzed using pandas*,* a python module for manipulating large datasets [57,58]*.* In addition, pandas was used to calculate spearman correlation values of each pairwise sample comparison. The technical averages for each sample was further analyzed using DESeq2, an R package that normalizes the dataset and performs appropriate statistical tests on high-throughput count data that does not follow a normal distribution such as INSeq[59]. DESeq2 was used to estimate the library size, normalize sequencing depth of samples, and calculate variation of each gene for statistical testing.

Transformation of VFS into MJM1100 background. The VFS mutant collection was assembled by sequencing random pEVS170 transposon insertions into ES114[35,60]. Mutant alleles were moved into the MJM1100 background using transformation under *tfox* induction[61].

Selected VFS strains grown on LBS plates were verified with PCR amplification using locus-specific primers and transposon-anchored primers MJM-127 (pEVS170 transposon) or SamA (pMarVF1 transposon) (**Table 2**). Recipient strain MJM1538 (MJM1100 carrying *pLostfoX)* was grown overnight in 3 ml of LBS containing 2.5 µg/ml chloramphenicol and then subcultured 1:100 into 3 ml of Tris Minimal with *N*-acetylglucosamine (GlcNac) for overnight growth. The recipient was then subcultured 1:50 into fresh Tris-Minimal-GlcNac-Cam with aeration until the OD_{600} reached 0.2-0.3, when 500 µl of recipient culture was incubated with 2.4 µg of VFS donor DNA (prepared with the Qiagen Blood and Tissue kit, Gram-negative bacteria protocol), followed by a brief vortex, and then static incubation at room temperature for 30 min. One ml of LBS was added to culture, transferred to a glass culture tube, and incubated overnight with aeration. The culture was spun down (8000 x *g*, 1 min), 900 µl of supernatant was removed, and the pellet was resuspended in the remaining ~100 µl of LBS. Aliquots (50 µl) of each sample were plated on LBS-erythromycin (LBS-Erm; 5 µg/ml) and three candidates were selected. Colonies were restreaked on LBS-Erm plates and then patched on selective media to check for absence of *pLostfoX* (LBS-Cam^S) and the presence of the transformed DNA (LBS-Erm^R). Transformation was verified with PCR amplification using primers that target the transposon junction and the gene target (**Table 2**).

Table 2. Oligonucleotide primers

Name	Sequence (5`-3`)	Source
MJM-127	ACAAGCATAAAGCTTGCTCAATCAATCACC	[62]
SamA	AAGCAGAAGACGGCATACGAAGACC	[41]
EB01 (*sspA*)	AACGGAGCTAAGTAACAATCCACT	This work
EB02 (*mukB*)	CACCTAGTGTATCAGCAAGACCTT	This work
EB03 (*ruvB*)	TCACTCGCTTTCAATTAACTCAGC	This work
EB06 (*brnQ*)	CAGCAACTTTCGCAGCATCTAATA	This work
EB08 (*nhaR*)	TTGTAAGTCCATATAGACGCCCAC	This work
EB10 (*hslO*)	AACATGGTTGATGGAGGTAAAGTT	This work
EB11 (*ubiH*)	CAAATTCAATCTTAGCAAAGCTGTC	This work

Competitions of individual gene mutants with a marked parental strain in the HARVs.

The competitive fitness of seven transposon mutants (**Table 1**) was evaluated in one-on-one competition assays against the LacZ-expressing wild type, MJM1575, in the HARV vessels.

Overnight cultures of each strain were prepared in LBS with shaking at 25 °C and diluted 1:100 in fresh medium the following morning. After 2 h of growth at 25 °C with shaking, the mutant and wild-type subcultures were normalized by OD_{600} and combined at a 1:1 ratio in fresh LBS. The resultant starting culture was subsequently loaded into the HARV vessel. At this time, two samples of the mixed input culture were collected: the first was preserved in 33% glycerol (v/v) and stored at -80 °C for later analysis, whereas the second was serially diluted in PBS (pH 7.0) and plated on LBS-Xgal agar (2 mg/ml) to reveal the starting ratio of mutant:control, based on the proportion of white-to-blue colonies, as previously described [35]. In the HARVs, the mutant and marked wild type were grown in competition with each other under LSMMG or gravity conditions for approximately 10 generations at 25 °C and 13 rpm. After 10 doublings, which was determined by OD_{600}, samples of the output cultures were preserved in 33% glycerol (v/v) and stored at -80 °C. The input and output samples were plated on LBS-Xgal agar to calculate the competitive index for each sample. The competitive index is equal to the Log_{10} value of the mutant/wild type ratio after competition normalized to its measured ratio at the beginning of the competition.

DATA AVAILABILITY

Illumina data for the INSeq reads are available at NCBI SRA, Accession number SRR12394639.

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AUTHOR CONTRIBUTIONS

EB analyzed the data and wrote the paper. MMV conducted the HARV experiments. ER and MMB conducted the INSeq library preparation. MJM and JSF conceived of the study, obtained funding for the study, and wrote and edited the manuscript.

COMPETING INTERESTS

All authors declare no financial or non-financial competing interests.

SUPPLEMENTAL DATA

The file Supplemental_Tables.xlsx includes the following tables:

Table S1: INSeq sample details

Table S2: List of genes depleted as shown in Figure 3

Table S3: Summary gene table output from pyinseq

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