Bacterial gene essentiality under modeled microgravity

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Short title: *Vibrio fischeri* modeled microgravity mutant analysis

Keywords: modeled microgravity, essential genes, symbiosis, *Vibrio fischeri*, *Aliivibrio fischeri*, *Euprymna scolopes*, INSeq, Tn-seq

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ABSTRACT

The health of eukaryotic hosts is tightly connected to relationships with symbiotic microorganisms, yet how these relationships develop and evolve during long-duration spaceflight is not well understood. In this study, we asked what bacterial genes are required for growth under modeled, or simulated, microgravity conditions compared to normal gravity controls. To conduct this study, 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. We employed a library of over 40,000 *V. fischeri* transposon mutants and compared the fitness of mutants in modeled microgravity compared to the gravity controls using transposon insertion sequencing (INSeq). We identified dozens of genes that exhibited fitness defects under both conditions, likely due to the controlled anaerobic environment, yet we identified relatively few genes with differential effects under modeled microgravity or gravity specifically: only mutants in *rodA* were more depleted under modeled microgravity, and mutants in 12 genes exhibited greater depletion under gravity conditions. 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.

65 **IMPORTANCE**

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67 There is substantial evidence that both the host immune system and microbial physiology are
68 altered during space travel. It is difficult to discern the molecular mechanisms of these
69 processes in a complex microbial consortium and during the short durations of experiments in
70 space. By using a model organism that is amenable to high-throughput genetic approaches, we
71 have determined that *V. fischeri* does not require a separate genetic repertoire for media growth
72 in modeled microgravity versus gravity conditions. Our results argue that future studies on how
73 this organism forms a specific and stable association with its animal host will not be confounded
74 by growth effects in the environment. The identification of similar genetic requirements under
75 modeled microgravity and gravity suggest that fitness pressures on microbiome growth in space
76 may be similar to those on Earth and may not negatively impact their animal hosts during long-
77 duration spaceflight.

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INTRODUCTION

During spaceflight, microbes--and microbe-host relationships--are altered, and understanding the mechanisms of these effects are critical as people, plants, and animals spend more time in space (1–7). To address this issue, experiments are needed to examine bacterial growth, bacterial-host interactions, and host health. Although previous studies using both natural and modeled microgravity conditions have resulted in a wide range of physiological and genetic responses, most have primarily targeted pathogenic strains (8, 9). While fewer studies have examined the effects of microgravity on beneficial microbes, there are recent studies that have begun to examine this issue (4–6). 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 mimic the low-shear environment of microgravity (10). Rotating High Aspect-Ratio Vessels (HARVs) represent one such platform; they mimic the low-shear fluid conditions that occur at low Earth orbit, and have been used for decades to simulate microgravity environments (11). 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, 12–16).

The model 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 microbiome assembly and function (1). The squid hatches each generation without its bacterial partner, and then proceeds to harvest *V. fischeri* from the seawater (17). 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 (18). This system has been especially valuable to understand the molecular basis by which animals acquire specific microbes from the environment. 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 (19–22). The specific relationship--only *V. fischeri*, and only specific strains of *V. fischeri*, can colonize the squid host--provides a strong model system to study animal-microbiome formation, development, and evolution (17). Furthermore, the small size of the host animal and simplicity of the symbiosis has contributed to the value of this system for studying microbiome formation during spaceflight (1, 23).

In this study, we take a complementary approach to previous work on bacterial gene expression under simulated microgravity to ask what genes are required for survival under this condition.

Our lab has previously published a study to globally identify *V. fischeri* genes that are essential, and that are conditionally essential under specific environmental conditions (24). 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

A number of studies have examined differential-gene expression in beneficial microbes under microgravity or simulated microgravity conditions, including in the marine bacterium *V. fischeri (6)*. 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 LSMMG and gravity positions (Fig. 1) (25, 26). We started with a characterized mutant library of over 40,000 transposon mutants in *V. fischeri* strain ES114 (24). 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

144 sample, DNA was isolated, from which INSeq libraries were constructed (26). The libraries were
145 sequenced on an Illumina HiSeq 2000 instrument, and the location of each transposon insertion
146 was mapped using the pyinseq Python package.
147
148 For each sample, we obtained between 6.65 - 8.18 x 10^6 Illumina reads (Table S1). We
149 observed similar numbers and patterns of unique transposon hits across the samples tested,
150 suggesting that the library did not undergo significant bottlenecks during the experiment (Table
151 S1). Next, for each sample, we examined the normalized transposon insertion counts (CPM;
152 counts-per-million Illumina reads) in each gene. We compared the similarity of these gene-level
153 counts across the samples in the analysis using pairwise correlation analysis. All pairwise
154 comparisons had a high level of similarity (Spearman $R^2 > 0.95$), and the HARV-grown samples
155 were clearly distinguishable from the input libraries (Fig. 2). Examination of the heat map
156 suggested little overall differentiation between the LSMMG and gravity samples that were
157 otherwise grown similarly. We proceeded to compare individual genes that were depleted under
158 LSMMG, gravity, or both conditions. To identify genes that had significant differential depletion
159 under modeled microgravity we used DESeq2 to calculate the median representation of each
160 mutant in the output LSMMG or gravity pools, and plotted those values compared to the input
161 pool. We focused our analysis on genes that were depleted at least 2-fold in the different
162 conditions. Genes that did not meet these criteria, genes that were poorly represented in the
163 input pool, and genes for which a previous study suggested that mutants impaired bacterial
164 growth were excluded from further analyses (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: this includes 10 genes more depleted under gravity 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.

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. 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*). Each defined mutant strain was grown in culture, then competed in the HARVs against the parental strain that carries the LacZ-expressing plasmid pVSV103 (27). The input and output pools from each experiment were plated onto LBS-Xgal medium, and the ratio of blue:white colonies in the samples were calculated. A competitive index was calculated to determine the fitness of each mutant under each condition. Plotted in Figure 4 are the results of this analysis. Although some differences were observed between the massive INSeq competition and the defined one-vs-one competitions, there was strong concordance between the INSeq and defined competition results. All of the genes for which mutants were significantly depleted under

186 INSeq were also substantially depleted in the defined competitions, and the control mutants
187 exhibited no substantial depletion. Therefore, we conclude that the INSeq analysis can reliably
188 predict conditional gene requirements in the HARV environment.
189
190 We recently examined bacterial genes for which mRNA levels are induced in the modeled
191 microgravity condition, compared to gravity controls (6). We analyzed our current study of gene
192 requirement (i.e., INSeq) with the previous dataset on gene expression (i.e., RNA-Seq) to ask
193 whether there is a correlation between gene requirement and gene expression. As shown in
194 Figure 5A, there was no significant correlation between gene requirement and transcript
195 induction (Pearson $R^2 = 0.08$). Our data above suggest that the HARV platform may play a
196 more significant role in shaping mutant communities than the specific LSMMG or gravity
197 conditions. Therefore, we asked whether genes required in the HARV are also induced in the
198 HARV, when normalized to non-HARV samples. For our INSeq data, we normalized to the input
199 sample that did not experience the HARV condition, and for RNA-Seq we normalized to
200 published data on culture-grown *V. fischeri* (28). As shown in Figure 5B-C, there is similarly a
201 lack of overall correlation between the gene expression and gene requirement (Pearson $R^2 =$
202 0.18, 0.11 for Fig. 5B, 5C, respectively). Therefore, we conclude that gene requirement in the
203 HARV cannot be predicted from transcriptome induction data, emphasizing the need for
204 empirical determination of gene essentiality.
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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 HARV platforms. A surprising finding was that there is 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 was highly consistent. As shown in the heat map in Figure 2, the high correlation of INSeq replicates within a treatment (e.g., for LSMMG samples) argues that there was no substantial bottleneck during inoculation or growth in the HARVs. Furthermore, 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 HARV LSMMG and gravity samples. The heat map in Fig. 2 coupled with the tight correlation of the samples in Figure 3 (Pearson $R^2 = 0.96$) illustrate that there was little variation observed between the LSMMG and gravity conditions. A striking consistency between genes

228 required for bacterial growth under gravity and those required for growth under modeled
229 microgravity is the major finding from this study. As the *V. fischeri* system is used for more
230 extensive research on microgravity, this result indicates there will not be a confounding effect of
231 genes that are simply required for bacterial growth under microgravity. Put another way, in the
232 future, if genes are identified that are required for colonization under microgravity, they are likely
233 to be required specifically for interaction with the animal host, and not simply for growth under
234 this altered gravity condition. Our findings, in concert with previous studies showing limited
235 changes in gene expression during spaceflight, suggest that spaceflight missions will have
236 minimal negative consequences on the microbiome.
237
238 Despite the overall patterns of concordance between LSMMG and gravity, there were genes for
239 which we observed differential effects between the two conditions, as plotted in Figure 3 and
240 detailed in Table S3. For example, depletion in *rodA (mrdB)* under both LSMMG and gravity
241 was observed, yet the gene was more significantly depleted under LSMMG. RodA is a SEDS-
242 family peptidoglycan polymerase that has multiple effects on bacterial cell shape and division
243 (29). This result suggests that differences in bacterial shape under the two different conditions
244 may impact the genetic requirement for *rodA*, though we note that its absence does affect
245 growth under both regimes. We additionally identified a number of genes with mutants depleted
246 under both conditions but that were more significantly depleted under gravity (Figure 3, filled red
247 dots). Notably, multiple genes for the F0F1-ATPase were depleted under both conditions, but
248 more so under gravity. Interestingly, prior work in *Escherichia coli* demonstrated that a similar

249 set of genes were essential for aerobic growth in minimal glycerol media, even though the
250 metabolic model used predicted that they would not be required (30). Together with our results,
251 this suggests that this subset of genes (*atpA, atpB, atpC, atpF*) may perform a function separate
252 from the role in ATP synthesis. Finally, there were two genes for which mutants exhibited
253 significant depletion under gravity but not LSMMG: *flgD* (encoding the hook capping protein
254 FlgD) and *rfaD* (encoding the LPS biosynthesis enzyme ADP-L-glycero-D-mannoheptose 6-
255 epimerase). Both genes affect the outer surface of the bacteria, and consistent with the *rodA*
256 results above, support the idea that the bacterial envelope is most susceptible to differential
257 effects of gravity.
258
259 It is also important to note that we observed dozens of genes for which mutants exhibited similar
260 depletion under both LSMMG and gravity conditions (Fig. 3; black filled dots). We note that
261 significant growth defects for mutants in these genes was not observed during 15 generations of
262 growth in LBS medium under aerobic conditions (24). Therefore, these genes are likely required
263 for robust growth in the HARV, but not for growth in LSMMG versus gravity. Given the
264 anaerobic environment of the HARV, it seems likely that many of these genes may be required
265 for optimal growth under anaerobic conditions. This hypothesis is supported by the presence of
266 four genes of the Na^+-translocating NADH:quinone oxidoreductase (*nqrA*, *nqrB*, *nqrD*, *nqrE*),
267 which, in the related species *V. cholerae*, conducts 90% of the membrane NADH
268 dehydrogenase activity under anaerobic conditions (31, 32). We also note the presence of some
269 genes that are required for robust symbiosis in this category, including *degS*, *dnaJ*, and *ompU*

(24, 33). 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 Figure 3 we would have observed a large number of 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 in the gravity condition 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 Figure 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. Broader analysis comparing gene requirement and expression have demonstrated that these categories are often unlinked (34). Our results argue

290 that genes that are induced in simulated microgravity are not preferentially required for bacterial
291 growth under these conditions.
292
293 Overall, this work establishes which genes were impacted by growth in LSMMG, gravity, or the
294 HARV environment generally. We additionally determined that microgravity-induced transcripts
295 do not predict which genes are conditionally essential under this condition. Finally, we
296 demonstrated that the HARV environment is amenable to high-throughput genetic experiments
297 and was reproducible within treatments. This study, therefore, provides a solid foundation for
298 future studies that seek to identify the genetic basis by which bacteria and animals form specific,
299 robust interactions under conditions of reduced gravity.
300
301 **MATERIALS AND METHODS**
302
303 **Media and growth conditions.** *V. fischeri* strains were grown at 25°C in Luria-Bertani salt
304 (LBS) medium (per liter, 10 g Bacto-tryptone, 5 g yeast extract and 20 g NaCl, 50 ml 1 M Tris
305 buffer, pH 7.5, in distilled water). When appropriate, antibiotics or supplements were added to
306 media at the following concentrations: erythromycin, 5 μg/ml; chloramphenicol, 5 μg/ml; X-gal
307 (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), 80 μg/ml. Growth media were solidified
308 with 1.5% agar as needed.
309

Preparation of input and output samples from HARV growth. The Lib04 library of pMarVF1 mariner transposon insertions in MJM1100 (ES114) was described previously (24). 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. (26), 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 (35) 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 S2) was further analyzed using pandas, a python module for manipulating large datasets (36, 37). 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 (38). 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 (24, 39). Mutant alleles were moved into the MJM1100 background using transformation under *tfox* induction (40). 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

371 (LBS-Erm^R). Transformation was verified with PCR amplification using primers that target the
372 transposon junction and the gene target (Table 2).
373
374 **Competitions of individual gene mutants with a marked parental strain in the HARVs**.
375 The competitive fitness of seven transposon mutants (Table 1) was evaluated in one-
376 on-one competition assays against the LacZ-expressing wild type, MJM1575, in the
377 HARV vessels. Overnight cultures of each strain were prepared in LBS with shaking at
378 25 °C and diluted 1:100 in fresh medium the following morning. After 2 h of growth at 25
379 °C with shaking, the mutant and wild-type subcultures were normalized by OD_{600} and
380 combined at a 1:1 ratio in fresh LBS. The resultant starting culture was subsequently
381 loaded into the HARV vessel. At this time, two samples of the mixed input culture were
382 collected: the first was preserved in 33% glycerol (v/v) and stored at -80 °C for later
383 analysis, whereas the second was serially diluted in PBS (pH 7.0) and plated on LBS-
384 Xgal agar (2 mg/ml) to reveal the starting ratio of mutant:control, based on the
385 proportion of white-to-blue colonies, as previously described (24). In the HARVs, the
386 mutant and marked wild type were grown in competition with each other under LSMMG
387 or gravity conditions for approximately 10 generations at 25 °C and 13 rpm. After 10
388 doublings, which was determined by OD_{600}, samples of the output cultures were
389 preserved in 33% glycerol (v/v) and stored at -80 °C. The input and output samples
390 were plated on LBS-Xgal agar to calculate the competitive index for each sample. The

391 competitive index is equal to the Log_{10} value of the mutant/wild type ratio after

392 competition normalized to its measured ratio at the beginning of the competition.

393

394 **DATA AVAILABILITY**

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

396 SRR12394639.

397

TABLES

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

Strain	Genotype	Reference/Source
MJM1100	Wild-type ES114	(41, 42)
MJM1538	MJM1100/pLostfoX	(43)
MJM1575	MJM1100/pVSV103	(27, 44)
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

404 **Table 2. Oligonucleotide primers**

Name	Sequence (5`-3`)	Source
MJM-127	ACAAGCATAAAGCTTGCTCAATCAATCACC	(45)
SamA	AAGCAGAAGACGGCATACGAAGACC	(26)
EB01 (*sspA*)	AACGGAGCTAAGTAACAATCCACT	This work
EB02 (*mukB*)	CACCTAGTGTATCAGCAAGACCTT	This work
EB03 (*ruvB*)	TCACTCGCTTTCAATTAACTCAGC	This work
EB06 (*bmQ*)	CAGCAACTTTCGCAGCATCTAATA	This work
EB08 (*nhaR*)	TTGTAAGTCCATATAGACGCCCAC	This work
EB10 (*hslO*)	AACATGGTTGATGGAGGTAAAGTT	This work
EB11 (*ubiH*)	CAAATTCAATCTTAGCAAAGCTGTC	This work

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410

411 **FIGURES**

412

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

418

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

425

426 **Figure 3. Mutant behavior under LSMMG or gravity revealed fitness in each HARV**
427 **condition, normalized to the input transposon sequencing library.** A) Comparison scatter
428 plot of microgravity (x-axis) and gravity (y-axis) Log(Fold-Change) values. Counts for each gene
429 for each replicate were normalized and used to calculate Log10(Fold-Change) values as
430 described in Materials and Methods. Genes that were poorly represented in the input pool did
431 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.

Figure 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 vs. marked parental strain (MJM1575).

Figure 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.

SUPPLEMENTAL DATA

The file Supplemental_Tables.xlsx includes the following tables:

Table S1: INSeq sample details

Table S2: Summary gene table output from pyinseq

Table S3: List of genes depleted as shown in Figure 3

ACKNOWLEDGMENTS

We acknowledge Cheryl Whistler and Randi Foxall for VFS Library mutants and A. Murat Eren and his laboratory for technical advice and mentorship. Funding was provided by NASA's Illinois Space Grant Consortium Research Seed Grant (M.J.M.), NIGMS R35 GM119627 (M.J.M.), an American Society for Microbiology Undergraduate Research Fellowship (E.B.), and the McNair Scholars Program (E.B.). The project was also supported in part by a NASA Space Biology award 80NSSC18K1465 (J.S.F.).

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