# Space flight alters bacterial gene expression and virulence and reveals a role for global regulator Hfq

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A comprehensive analysis of both the molecular genetic and phenotypic responses of any organism to the space flight environment has never been accomplished because of significant technological and logistical hurdles. Moreover, the effects of space flight on microbial pathogenicity and associated infectious disease risks have not been studied. The bacterial pathogen Salmonella typhimurium was grown aboard Space Shuttle mission STS-115 and compared with identical ground control cultures. Global microarray and proteomic analyses revealed that 167 transcripts and 73 proteins changed expression with the conserved RNA-binding protein Hfq identified as a likely global regulator involved in the response to this environment. Hfq involvement was confirmed with a ground-based microgravity culture model. Space flight samples exhibited enhanced virulence in a murine infection model and extracellular matrix accumulation consistent with a biofilm. Strategies to target Hfq and related regulators could potentially decrease infectious disease risks during space flight missions and provide novel therapeutic options on Earth.

microgravity | Space Shuttle | low shear modeled microgravity | rotating wall vessel | Salmonella

**E** nvironmental conditions and crew member immune dysfunction associated with space flight may increase the risk of infectious disease during a long-duration mission (1-4). However, our knowledge of microbial changes in response to space flight conditions and the corresponding changes to infectious disease risk is limited and unclear. Elucidation of such risks and the mechanisms behind any space flight-induced changes to microbial pathogens holds the potential to decrease risk for human exploration of space and provide insight into how pathogens cause infections in Earth-based environments. Numerous logistical and technological hurdles exist when performing biological space flight experimentation, and an extremely limited number of opportunities to perform such research are available. Accordingly, comprehensive analysis of cells, including pathogenic microbes, at the molecular and phenotypic level during space flight offers a rare opportunity to examine their behavior and response in this environment.

Previous studies using the enteric bacterial pathogen *Salmo-nella enterica* serovar Typhimurium showed that growth in a ground-based space flight analog bioreactor, termed the rotating wall vessel (RWV), induced global genotypic and phenotypic changes in this organism (5–7). Specifically, *S. typhimurium* grown in this space flight analog culture environment, described

as low-shear modeled microgravity (LSMMG), exhibited increased virulence, increased resistance to environmental stresses (acid, osmotic, and thermal), increased survival in macrophages, and global changes in gene expression at the transcriptional and translational levels (5–7). Collectively, these results suggested the potential that the true space flight environment could globally alter bacterial genotypic and phenotypic responses. Thus, we designed an experimental approach to test our hypothesis, specifically to culture *S. typhimurium* during space flight and evaluate changes in microbial gene expression and virulence in response to this environment.

Our experiments were flown on Space Shuttle Atlantis Mission STS-115 (September 2006). In this experiment, cultures of *S. typhimurium* were activated to grow in space for a specific time period and then either fixed in an RNA/protein fixative or supplemented with additional growth media after this time period [supporting information (SI) Fig. 3]. At 2.5 h after landing at Kennedy Space Center, the culture samples were recovered and subsequently used for whole-genome transcriptional microarray and proteomic analysis (fixed samples) or for infections in a murine model of salmonellosis (media-supplemented samples). In each case, the flight culture samples were compared with culture samples grown under identical conditions on the ground at Kennedy Space Center using coordinated activation

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Abbreviations: LSMMG, low-shear modeled microgravity; RWV, rotating wall vessel; FPA, fluid processing apparatus; Km, konamycin.

Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE8573).

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**Fig. 1.** Data from STS-115 *S. typhimurium* experiments. (*A*) Map of the 4.8-Mb circular *S. typhimurium* genome with the locations of the genes belonging to the space flight transcriptional stimulon indicated as black hash marks. (*B*) Decreased time to death in mice infected with flight *S. typhimurium* as compared with identical ground controls. Female BALB/c mice per-orally infected with  $10^7$  bacteria from either space flight or ground cultures were monitored every 6-12 h over a 30-day period, and the percent survival of the mice in each group is graphed versus the number of days. (*C*) Increased percent mortality of mice infected with space flight cultures across a range of infection dosages. Groups of mice were infected with increasing dosages of bacteria from space flight and ground cultures and monitored for survival over 30 days. The percent mortality (calculated as in ref. 23) of each dosage group is graphed versus the dosage amount. (*D*) Decreased LD<sub>50</sub> value (calculated as in ref. 23) for space flight bacteria in a murine infection model. (*F*) SEM of space flight and ground *S. typhimurium* bacteria showing the formation of an extracellular matrix and associated cellular aggregation of space flight cells. (Magnification: ×3,500.)

and termination times (by means of real-time communications with the Shuttle crew) in an insulated room that maintained temperature and humidity identical to those on the Shuttle (Orbital Environment Simulator). The culture experiments were loaded into specially designed hardware [termed fluid processing apparatus (FPA)] to facilitate controlled activation and fixation of the cultures while maintaining suitable culture containment requirements (SI Fig. 4).

To our knowledge, our results are the first documentation of changes in bacterial gene expression and microbial virulence in response to culture during space flight. Specifically, these findings demonstrate that the space flight environment imparts a signal that can induce molecular changes in bacterial cells. Furthermore, these results also provide direct evidence that this signal can alter the virulence of a microbial pathogen. Our collective data indicate that the conserved RNA-binding protein Hfq plays a central regulatory role in the microbial response to space flight conditions. Evaluation of microbial changes in response to this unique environment has the potential to provide heretofore unavailable insight into microbial response mechanisms to Earth-based environments, including those encountered by pathogens during the natural course of infection.

# Results

Whole-Genome Transcriptional and Proteomic Analysis of Space Flight and Ground Cultures. To determine which genes changed expression in response to space flight, total bacterial RNA was isolated from the fixed flight and ground samples, qualitatively analyzed to ensure lack of degradation by means of denaturing gel electrophoresis, quantitated, and then reverse-transcribed into labeled, single-stranded cDNA. The labeled cDNA was cohybridized with differentially labeled *S. typhimurium* genomic DNA to whole-genome *S. typhimurium* microarray slides. The cDNA signal hybridizing to each gene spot was quantitated, and the normalized, background-subtracted data were analyzed for statistically significant 2-fold or greater differences in gene expression between the flight and ground samples. We found 167 genes differentially expressed in flight as compared with ground controls from a variety of functional categories (69 up-regulated and 98 down-regulated) (SI Table 2). The proteomes of fixed cultures were also obtained by means of multidimensional protein identification analysis. We identified 251 proteins expressed in the flight and ground cultures, with 73 being present at different levels in these samples (SI Table 3). Several of the genes encoding these proteins were also identified by means of microarray analysis. Collectively, these gene expression changes form the first documented bacterial space flight stimulon indicating that bacteria respond to this environment with widespread alterations of expression of genes distributed globally throughout the chromosome (Fig. 1A).

Involvement of Hfq in Space Flight and LSMMG Responses. Identification of one or more regulators of the space flight stimulon represents an important step in understanding the nature of this unique environmental signal. Our data indicated that a pathway involving the conserved RNA-binding regulatory protein Hfq played a role in this response (Table 1). Hfq is an RNA chaperone that binds to small regulatory RNA and mRNA molecules to facilitate mRNA translational regulation in response to envelope stress (in conjunction with the specialized  $\sigma$ factor RpoE), environmental stress (by means of alteration of RpoS expression), and changes in metabolite concentrations, such as iron levels (via the Fur pathway) (8–12). Hfq is also involved in promoting the virulence of several pathogens including *S. typhimurium* (13), and Hfq homologues are highly con-

Table 1. Space flight stimulon genes belonging to Hfq regulo	'n
or involved with iron utilization or biofilm formation	

# Table 1. (continued)

Gene     change     Function       Hft regulon genes (up-regulated)     opp     0.421     Similar to CQA, not colsinduced miph     0.435     Upper protein upp       Outer membrane porin ompA     2.45     Outer membrane porin ompA     ygaC     0.457     Putative cytopiamic protein ygaC       Plasmid transfer apparatus     3.41     Outer membrane porin ompA     ygaC     0.453     Putative cytopiamic protein ygaC       Plasmid transfer apparatus     3.42     Conjugative transfer transfer     ppiB     0.428     Membrane-bound ATP synthase transfer       Trab     4.24     Conjugative transfer trab     0.428     Conjugative transfer     traf     0.482     Membrane-bound ATP synthase trats       Trab     2.23     Conjugative transfer     traf     0.656     Ribosome-associated factor       Trat     2.32     Conjugative transfer     mpA     0.554     DAta-infrator       Trat     2.32     Conjugative transfer     mpA     0.554     DAta-infrator       Trat     2.32     Conjugative transfer     mpA     0.554     DAta-infrator       Trat     2.33     Conjug		Fold			Fold	
Hig regulon genes (up-regulated)     cpD     0.411     Gmata     CpD     CpD     0.411     Gmata     CpD	Gene	change	Function	Gene	change	Function
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Outer membrane porin     ygaC     0.451     Putative incer membrane porin       ompC     2.44     Outer membrane porin     ygaM     0.453     Putative incer membrane porin       pmpD     0.447     AE2 (putamate/aspartate)     transportate       PURP     Conjugative transfer     ppB     0.452     Pethod (Putamate/aspartate)       PURP     Conjugative transfer     apE     0.452     Putative incer membrane porin       trans     3.14     Conjugative transfer     apE     0.452     Membrane bound ATP synthase       trans     3.14     Conjugative transfer     nFM     0.453     Nitroredoni 1, redox factor       track     2.47     Conjugative transfer     nFB     0.514     Anti-se factor       track     2.33     Conjugative transfer     mFB     0.554     NNase P, protein in morphoni Regulation and transfer in mark in	Outer membrane proteins	J		daln	0.435	Lipoprotein-34
0.11,2     2.24     Outer membrane porten     ygaM     0.453     Putzetie inner membrane porten       0.11,2     Outer membrane porten     gH     0.479     AEC glutamettex inner membrane porten       11,2     Conjugative transfer     apE     0.428     Peptid/ipro/ipro/ip isomerse B       11,3     A24     Conjugative transfer     apE     0.482     Peptid/ipro/ipro/ip isomerse B       11,4     A24     Conjugative transfer     trA     0.482     Ribosome binding factor       11,4     Conjugative transfer     trA     0.483     Ribosome binding factor       11,4     Conjugative transfer     trA     0.483     Ribosome binding factor       11,4     Conjugative transfer     trA     0.538     Ritzen protein       11,4     Conjugative transfer     trA     0.554     NAx-binding protein       11,6     Conjugative transfer     trA     0.554     NAx-binding protein       11,6     Conjugative transfer     trans     0.554     NAx-binding protein       11,6     Conjugative transfer     trans     0.554     NAx-binding protein		2.05	Quter membrane perin	vgaC	0.451	Putative cytoplasmic protein
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Tram     2.39     Conjugative transfer     yiaG     0.522     Protein the transcriptional regulator       traT     2.34     Conjugative transfer     ompX     0.514     Protein component       traG     2.21     Conjugative transfer     Inn     0.554     PNA-binding protein       traF     2.11     Conjugative transfer     Inn     0.554     PNA-binding protein       traF     1.71     Conjugative transfer     Inn     0.556     Ribosome modulation factor       various cellular functions     opigentive transfer     Inn     0.566     Ribosome modulation factor       sipC     6.27     Cell invasion protein     Transcriptional replication protein     Inn       sipC     6.27     Cell invasion protein     Inn     Inn     Inn       sipC     6.27     Cell invasion protein     Inn     Inn     Inn       sipC     6.27     Cell invasion protein     Inn     Inn     Inn       sipC     2.36     Giverophosphodisetrase     adhE     2.74     Z-4     Inn       sipC     6.26	trbC	2.68	Conjugative transfer	rseB	0 514	Anti- <i>a</i> E factor
traX     2.3     Conjugative transfer     Jusc     Teal (a) (b) (b) (c) (c) (c) (c) (c) (c) (c) (c) (c) (c	traH	2.59	Conjugative transfer	viaG	0.578	Putative transcriptional regulator
traft     2.34     Conjugative transfer     Only is provided in the provide in opponent of the provide in the provide in the provide in the provide in opponent of the provide in theprovide in the provide in theprovide in the provide in theprovi	traX	2.37	Conjugative transfer	omnX	0.520	Outer membrane protein
trag     2.32     Conjugative transfer     Inp.     0.254     DNA: binding protein       trag     2.21     Conjugative transfer     IamB     0.566     Phage A receptor protein       traf     2.11     Conjugative transfer     IamB     0.566     Phage A receptor protein       various cellular functions     rmf     0.566     Thiol peroxidase     Transport       gapA     7.67     Glyceraldehyde dehydrogenase     prill     0.571     Primosomal replication protein N       adhE     4.75     Fe-dependent dehydrogenase     adhE     4.76     Fe-dependent dehydrogenase       filC     2.11     Flagellin, structural protein     mid     0.767     Mol Score ductase       smA     1.67     ABC superfamily transporter     Iligase     1ligase     1ligase       Hfq regulon genes (down-regulated)     dmsC     0.499     Transcriptional regulator, frac     0.494       rmaseP     0.305     Small RNA     regulatory     Fe-binding     Fe-binding       cr8     0.318     Small RNA     romg NA     0.474     Contremerbine Norage	traT	2.34	Conjugative transfer	rnnA	0.547	RNase B. protein component
trag     2.21     Conjugative transfer     Ins.     0.24     Divention in protein       trag     1.79     Conjugative transfer     Ims     0.566     Ribosome modulation factor       yarious cellular functions     conjugative transfer     rmf     0.566     Ribosome modulation factor       gapA     7.67     Glyceraldehyde dehydrogenase     pril8     0.571     Primosomal replication protein       sipC     6.27     Fe-dependent dehydrogenase     pril8     0.571     Primosomal replication protein       gipQ     2.58     Glycerophosphodiesterase     adhE     4.76     Fe-dependent dehydrogenase       ginQ     2.58     Glycerophosphodiesterase     adhE     4.76     Fe-dependent dehydrogenase       smal     1.67     ABC superfamily transporter     ligase     1/34     Frait center)       MdR     0.305     Small RNA     fnr     0.497     Anaerobic DMSO reductase       smal RNA     0.305     Small RNA regulatory     fdrH     0.495     Fe-5 formation protein       rpsS     0.305     Small RNA     Small RNA     gmat <td< td=""><td>trbB</td><td>2.32</td><td>Conjugative transfer</td><td>hns</td><td>0.554</td><td>DNA-binding protein</td></td<>	trbB	2.32	Conjugative transfer	hns	0.554	DNA-binding protein
traf 2.11 Conjugative transfer mf 0.566 Ribesome modulation factor traR 1.79 Conjugative transfer mf 0.566 Ribesome modulation factor trax 0.566 Thiol peroxidase gapA 7.67 Glyceraldehyde dehydrogenase sjpC 6.27 Cell invasion protein adhE 4.75 Fe-dependent dehydrogenase glpQ 2.58 Glycerophosphodiserses adhE 4.75 Fe-dependent dehydrogenase fliC 2.11 Flagellin, structural protein sbmA 1.67 ABC superfamily transporter Hfg regulon genes (down-regulated) Hfg regulon genes (down-regulated) Hfg regulon genes (down-regulated) MmSC 0.497 Anaerobic DMSO reductase nflU 0.495 Fe-S (duster formation protein) maseP 0.306 Small RNA for dustory crasP 0.306 Small RNA regulatory fraC 0.411 Fumarate reductase, anaerobic oxyS 0.422 Small RNA regulatory res 0.499 Small RNA Ribosomal protein str2 rpsS 0.229 Jos ribosomal subunit protein str2 rpsS 0.229 Jos ribosomal subunit protein str2 rpsS 0.229 Jos ribosomal subunit protein str2 various cellular functions rpsF 0.401 Jos ribosomal subunit protein trp 4. 0.422 Sor formal subunit protein str2 various cellular functions rpsF 0.401 Jos ribosomal subunit protein trp 4. 0.423 Strase response protein and ferritin rpsL 0.251 Jos ribosomal subunit protein str2 various cellular functions rpsF 0.401 Jos ribosomal subunit protein trp 4. 0.423 Strase response protein and ferritin rpsL 0.251 Jos ribosomal subunit protein str2 various cellular functions rpsF 0.401 Jos ribosomal subunit protein trp 4. 0.423 Strase response protein and frift varia 0.207 Outer membrane protein varia 0.207 Putative glycosyl transferase rp 4. 0.423 Strase response protein finH 1.64 Putative cyclosyl transferase rp 4. 0.423 Strase response protein manC 1.71 Mannose guanylythransferase rp 4. 0.203 Fibosomal subunit protein finH 1.64 Putative cyclositasis finH 1.64 Putati	traG	2.21	Conjugative transfer	lamB	0.554	Phage ) recentor protein
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Various cellular functions     UpX     0.360     Thild pertoxidase       gapA     7.67     Glyceraidehyde dehydrogenase     priB     0.710     Prinsoomal replication protein N       adhE     4.75     Fe-dependent dehydrogenase     Iron utilization/storage genes     Iron utilization/storage genes       fliC     2.11     Flagellin, structural protein     entE     2.23 dihydroxybenzoate-AMP       sbmA     1.67     ABC superfamily transporter     hydN     2.03     Electron transport (FeS center)       small RNAs     0.305     Small RNA regulatory     frd     0.495     Fe-5 cluster formation protein       rmaseP     0.305     Small RNA regulatory     frd     0.444     Transcriptional regulator,       csB     0.318     Small RNA regulatory     frd     0.448     Fe-5 formate dehydrogenase-N       tke1     0.427     Small RNA     mpW     0.276     Outer membrane protein N       rmsse     0.305     Small RNA regulatory     frdC     0.411     Fumarsereducase, anaerobic       cyS     0.432     Small RNA regulatory     frdC     0.411     Fer-shormate dehyd	traR	1.79	Conjugative transfer	1111	0.500	This is a service as
gapA     7.67     Glyceraldehydrogenase     prb     0.371     Primosonian replication forderin in       sipC     6.27     Cell invasion protein     Iron utilization/Storage genes       glpQ     2.58     Giycerophosphodiesterase     adhE     4.76     Fe-dependent dehydrogenase       glpQ     2.58     Giycerophosphodiesterase     adhE     4.76     Fe-dependent dehydrogenase       shmA     1.67     ABC superfamily transporter     entE     2.24     2.3-dihydrosybenzoate-AMP       shmA     1.67     ABC superfamily transporter     hydN     2.03     Electron transport (FeS center)       maseP     0.305     Small RNA     fnr     0.497     Anaerobic DMSO reductase       rnaseP     0.305     Small RNA regulatory     fdnH     0.438     Fe-S formate dehydrogenase-N       rgpL     0.432     Small RNA     gpla     0.77     Outer membrane protein W       rgpS     0.432     Small RNA     gpla     0.77     Outer membrane protein W       rgpS     0.439     Sor ibosomal subunit protein L3     mar     2.30     Polyascharide expor protein<	Various cellular functions			tpx	0.566	Inioi peroxidase
sipC     6.27     Cell invasion protein adhE     Iron utilization/storage genes       glpQ     2.58     Glycerophosphodiesterase flC     adhE     4.76     Fe-dependent dehydrogenase adhE     2.24     2.3-dihydroxybenzoate-AMP ligase       flC     2.11     Flagellin, structural protein sbmA     1.67     ABC superfamily transporter     Iligase       flC     2.11     Flagellin, structural protein sbmA     1.67     ABC superfamily transporter     Iligase       flG     0.315     Small RNA structural protein     msC     0.497     Anaerobic DMSO reductase inf/U     0.497     Anaerobic DMSO reductase       rmaseP     0.306     Small RNA regulatory     fnr     0.497     Transcriptional regulator, forld     Terescriptional regulator, forld     Terescription rule, since regulatore, forld <td>gapA</td> <td>7.67</td> <td>Glyceraldehyde dehydrogenase</td> <td>рпв</td> <td>0.571</td> <td>Primosomal replication protein N</td>	gapA	7.67	Glyceraldehyde dehydrogenase	рпв	0.571	Primosomal replication protein N
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glpQ     2.88     Glycerophosphodiesterase     ahE     4.76     Fe-dependent dehydrogenase       filC     2.11     Flagelin, structural protein     entE     2.24     2,3 dihydroxybenzoate-AMP       iigase	adhE	4.75	Fe-dependent dehydrogenase		Iron utilization/	storage genes
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served across species of prokaryotes and eukaryotes (14). Our data strongly support a role for Hfq in the response to space flight: (i) The expression of hfq was decreased in flight, and this finding matched previous results in which S. typhimurium hfq gene expression was decreased in a ground-based model of microgravity (7). (ii) Expression of 64 genes in the Hfq regulon was altered in flight (32% of the total genes identified), and the directions of differential changes of major classes of these genes matched predictions associated with decreased hfq expression (see subsequent examples). (iii) Several small regulatory RNAs that interact with Hfq were differentially regulated in flight as would be predicted if small RNA/Hfq pathways are involved in a space flight response. (iv) The levels of OmpA, OmpC, and OmpD mRNA and protein are classic indicators of the RpoEmediated periplasmic stress response, which involves Hfq (15). Transcripts encoding OmpA, OmpC, and OmpD (and OmpC protein level) were up-regulated in flight, correlating with hfq down-regulation. (v) Hfq promotes expression of a large class of ribosomal structural protein genes (12), and we found that many such genes exhibited decreased expression in flight. (vi) Hfq is a negative regulator of the large tra operon encoding the F plasmid transfer apparatus (16), and several tra genes from related operons on two plasmids present in S. typhimurium  $\chi$ 3339 were up-regulated in flight. (vii) Hfq is intimately involved in a periplasmic stress signaling pathway that depends on the activity levels of three key proteins, RpoE, DksA, and RseB; differential expression of these genes was observed in flight (8, 12). (viii) Hfq regulates the expression of the Fur protein and other genes involved in the iron response pathway, and we observed several iron utilization/storage genes with altered expression in flight (9, 11). This finding also matched previous results in which iron pathway genes in S. typhimurium changed expression in a ground-based model of microgravity, and the Fur protein was shown to play a role in stress resistance alterations induced in the same model (7).

Given these findings, we designed experiments to verify a role for Hfq in the space flight response using a cellular growth apparatus that serves as a ground-based model of microgravity conditions termed the RWV bioreactor (SI Fig. 5). Designed by the National Aeronautics and Space Administration, the RWV has been extensively used in this capacity to study the effects of a biomedically relevant low-fluid-shear growth environment (which closely models the liquid growth environment encountered by cells in the microgravity environment of space flight as well as by pathogens during infection of the host) on various types of cells (6, 17–19). Studies with the RWV involve using two separate apparatuses: one is operated in the low shear modeled microgravity position (LSMMG), and one is operated as a control in a position (termed  $1 \times g$ ) where sedimentation due to gravity is not offset by the rotating action of the vessel.

LSMMG-induced alterations in acid stress resistance and macrophage survival of S. typhimurium have previously been shown to be associated with global changes in gene expression and virulence (5, 7). We grew WT and isogenic hfq mutant strains of S. typhi*murium* in the RWV in the LSMMG and  $1 \times g$  positions and assayed the acid stress response and macrophage survival of these cultures. Whereas the WT strain displayed a significant difference in acid resistance between the LSMMG and  $1 \times g$  cultures, this response was not observed in the hfq mutant, which contains a deletion of the hfq gene and replacement with a Cm-r cassette (Fig. 2A). Two control strains, hfq 3'Cm (containing an insertion of the Cm-r cassette just downstream of the WT hfq gene) and invA kanamycin (Km) (containing a Km-r insertion in a gene unrelated to stress resistance), gave the same result as the WT strain. We also observed increased intracellular replication of the LSMMG-grown WT (hfq 3'Cm) strain in infected J774 macrophages as compared with the  $1 \times g$  control, and this phenotype was not observed in the hfq mutant strain (Fig. 2B). Collectively, these data indicate that



**Fig. 2.** Hfq is required for *S. typhimurium* LSMMG-induced phenotypes in RWV culture. (*A*) The survival ratio of WT and isogenic *hfq*, *hfq* 3'Cm, and *invA* mutant strains in acid stress after RWV culture in the LSMMG and  $1 \times g$  positions is plotted (P < 0.05, ANOVA). (*B*) Fold intracellular replication of *S. typhimurium* strains *hfq* 3'Cm and  $\Delta hfq$  in J774 macrophages after RWV culture as above. Intracellular bacteria were quantitated at 2 h and 24 h after infection, and the fold increase in bacterial numbers between those two time periods was calculated (P < 0.05, ANOVA).

Hfq is involved in the bacterial space flight response as confirmed in a ground-based model of microgravity conditions. In addition, the intracellular replication phenotype inside macrophages correlates with the finding that space flight and LSMMG cultures exhibit increased virulence in mice (see next paragraph and ref. 5).

Increased Virulence of S. typhimurium Grown in Space Flight as Compared with Ground Controls. Because growth during space flight and potential global reprogramming of gene expression in response to this environment could alter the virulence of a pathogen, we compared the virulence of S. typhimurium space flight samples to identical ground controls as a second major part of our study. Bacteria from flight and ground cultures were harvested and immediately used to inoculate female BALB/c mice via a per-oral route of infection on the same day as the Shuttle landing. Two sets of mice were infected at increasing dosages of either flight or ground cultures, and the health of the mice was monitored every 6-12 h for 30 days. Mice infected with bacteria from the flight cultures displayed a decreased time to death (at the 10<sup>7</sup> dosage), increased percent mortality at each infection dosage, and a decreased LD<sub>50</sub> value compared with those infected with ground controls (Fig. 1 B-D). These data indicate increased virulence for space flight S. typhimurium samples and are consistent with previous studies in which the same strain of S. typhimurium grown in the RWV under

LSMMG conditions displayed enhanced virulence in a murine model as compared with  $1 \times g$  controls (5).

SEM of Space Flight and Ground Cultures. To determine any morphological differences between flight and ground cultures, SEM analysis of bacteria from these samples was performed. Although no difference in the size and shape of individual cells in both cultures was apparent, the flight samples demonstrated clear differences in cellular aggregation and clumping that was associated with the formation of an extracellular matrix (Fig. 1E). Consistent with this finding, several genes associated with surface alterations related to biofilm formation changed expression in flight (up-regulation of wca/wza colonic acid synthesis operon, *ompA*, *fimH*; down-regulation of motility genes) (Table 1). SEM images of other bacterial biofilms show a similar matrix accumulation (20, 21). Because extracellular matrix/biofilm formation can help to increase survival of bacteria under various conditions, this phenotype indicates a change in bacterial community potentially related to the increased virulence of the flight bacteria in the murine model.

## Discussion

To our knowledge, these results represent the first documented gene expression changes that occur in bacterial cells (and any microbial pathogen) during space flight and accordingly demonstrate that a microgravity growth condition provides an environmental signal that can induce molecular changes in bacterial cells. To our knowledge, these results also provide the first direct evidence that growth during space flight can alter the virulence of a pathogen; in this study, S. typhimurium grown in space flight displayed increased virulence in a murine infection model compared with identical ground controls. Importantly, these results correlate with previous findings in which the same strain of S. typhimurium displayed increased virulence in the murine model after growth in the low-shear microgravity-like conditions of the RWV bioreactor. In agreement with the increased virulence observed in the space flight samples, bacteria cultured in flight exhibited cellular aggregation and extracellular matrix formation consistent with biofilm production. Moreover, several Salmonella genes associated with biofilm formation changed expression in flight. In addition, the space flight analogue culture environment of the RWV was used to verify a mechanistic role for Hfq as a global regulator of microbial responses during growth in low-shear microgravitylike growth conditions similar to those found in space flight liquid culture.

Strategies designed to counteract the virulence-enhancing effects of space flight in microbes provide important potential benefits to crew health and open insight into novel antimicrobial strategies on Earth. Accordingly, the identification of global regulators, such as Hfq, that coordinate microbial responses to these biomedically relevant environments provides targets at which these strategies can be directed. Hfq is an RNA-binding global regulatory protein that is conserved in a wide range of organisms, both prokaryotes and eukaryotes, and primarily acts as a chaperone to stabilize interactions between small RNA and mRNA molecules (14). Further study is needed to determine whether changes in Hfq regulon expression under space flight and LSMMG space flight analogue conditions alter critical RNA interactions that control virulence and other microbial responses. Because low fluid shear is encountered by pathogens in the host, these responses may be important for bacterial reprogramming during transitions between environments of different physiological fluid shear levels (such as from the flow of a lumen to the protected environment between brush border microvilli), which results in enhanced survival and infection.

### **Materials and Methods**

Strains, Media, and Chemical Reagents. The virulent, mousepassaged S. typhimurium derivative of SL1344 termed  $\chi$ 3339 was used as the WT strain in all flight- and ground-based experiments (5). Isogenic derivatives of SL1344 with mutations  $\Delta hfq$ , hfq 3'Cm, and invA Km were used in ground-based experiments (13, 22). The  $\Delta hfq$  strain contains a deletion of the hfq ORF and replacement with a chloramphenicol resistance cassette, and the hfq 3'Cm strain contains an insertion of the same cassette immediately downstream of the WT hfq ORF. The invA Km strain contains a Km resistance cassette inserted in the invA ORF. Lennox broth was used as the growth medium in all experiments (5), and PBS (Invitrogen, Carlsbad, CA) was used to resuspend bacteria for use as inoculum in the FPAs. The RNA fixative RNA Later II (Ambion, Austin, TX), glutaraldehyde (16%; Sigma, St. Louis, MO), and formaldehyde (2%; Ted Pella, Redding, CA) were used as fixatives in flight experiments.

Loading of FPA. An FPA consists of a glass barrel that can be divided into compartments (by means of the insertion of rubber stoppers) and a lexan sheath into which the glass barrel is inserted (see SI Fig. 4). Each compartment in the glass barrel was filled with a solution in an order such that the solutions would be mixed at specific time points in flight via two actions: (i) downward plunging action on the rubber stoppers and (ii) passage of the fluid in a given compartment through a bevel on the side of the glass barrel such that it was released into the compartment below. Glass barrels and rubber stoppers were coated with a silicone lubricant (Sigmacote; Sigma) and autoclaved separately before assembly. A stopper with a gasexchange membrane was inserted just below the bevel in the glass barrel before autoclaving. FPA assembly was performed aseptically in a laminar flow hood in the following order: 2.0 ml of Lennox broth medium on top of the gas-exchange stopper, one rubber stopper, 0.5 ml of PBS containing bacterial inoculum ( $\approx 6.7 \times 10^6$  bacteria), another rubber stopper, 2.5 ml of either RNA fixative or Lennox broth medium, and a final rubber stopper. Syringe needles (gauge 25 5/8) were inserted into rubber stoppers during this process to release air pressure and facilitate assembly. To facilitate group activation of FPAs during flight and to ensure proper containment levels, sets of eight FPAs were loaded into larger containers termed group activation packs.

**Murine Infection Assay.** Six- to 8-week-old female BALB/c mice (housed in the Animal Facility at the Space Life Sciences Laboratory at Kennedy Space Center) were fasted for  $\approx 6$  h and then per-orally infected with increasing dosages of *S. typhimurium* harvested from flight and ground FPA cultures and resuspended in buffered saline gelatin (5). Ten mice per infectious dosage were used, and food and water were returned to the animals within 30 min after infection. The infected mice were monitored every 6–12 h for 30 days. The LD<sub>50</sub> value was calculated by using the formula of Reed and Muench (23).

**SEM.** A portion of cells from the viable, media-supplemented cultures from flight and ground FPAs was fixed for SEM analysis by using 8% glutaraldehyde and 1% formaldehyde and was processed for SEM as described previously (24).

**Microarray Analysis.** Total cellular RNA purification, preparation of fluorescently labeled, single-stranded cDNA probes, probe hybridization to whole-genome *S. typhimurium* microarrays, and image acquisition were performed as previously described (7) using three biological and three technical replicates for each culture condition. Flow cytometric analysis revealed that cell numbers in flight and ground biological replicate cultures were not statistically different (using SYTO-BC dye per the manu-

facturer's recommendations; Invitrogen). Data from stored array images were obtained with QuantArray software (Packard Bioscience, Billerica, MA) and statistically analyzed for significant gene expression differences by using the Webarray suite as described previously (25). GeneSpring software was also used to validate the genes identified with the Webarray suite. To obtain the genes comprising the space flight stimulon as listed in SI Table 2, the following parameters were used in Webarray: a fold increase or decrease in expression of 2-fold or greater, a spot quality (A value) of >9.5, and P value of <0.05. For some genes listed in Table 1, the following parameters were used: a fold increase or decrease in expression of value >1.6 or <0.6, respectively, an A value of 8.5 or greater, and P value of <0.1. The vast majority of genes listed in Table 1 had an A value of >9.0 (with most being >9.5) and a P value of 0.05 or less. The exceptions were as follows: sbmA (P = 0.06), oxyS (A = 8.81), rplY(A = 8.95), cspD(A = 8.90), yfiA(P = 0.08), ompX(P = 0.08))(0.09), hns (P = 0.08), rmf (A = 8.82), wead (P = 0.09), and fliE (A = 8.98). To identify space flight stimulon genes also contained in the Hfq regulon, proteins or genes found to be regulated by Hfq or RNAs found to be bound by Hfq as reported in the indicated references were scanned against the space flight microarray data for expression changes within the parameters above (8, 12, 13, 16, 26).

Multidimensional Protein Identification Analysis via Tandem MS Coupled to Dual Nano-Liquid Chromatography. Acetone-protein precipitates from whole-cell lysates obtained from flight and ground cultures (representing three biological replicates) were subjected to multidimensional protein identification analysis using the tandem MS-dual nano-liquid chromatography technique as described previously (27, 28). Tandem MS spectra of peptides were analyzed with TurboSEQUEST version 3.1 and XTandem software, and the data were further analyzed and organized by using the Scaffold program (29, 30). Please see SI Table 3 for the

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specific parameters used in Scaffold to identify the proteins in this study.

Ground-Based RWV Cultures and Acid Stress and Macrophage Survival Assays. S. typhimurium cultures were grown in the RWV in the LSMMG and  $1 \times g$  orientations and assayed for resistance to pH 3.5 and survival inside J774 macrophages as described previously (5), except that the RWV cultures were grown for 24 h at 37°C. For acid stress assays, the percentage of surviving bacteria present after 45-60 min of acid stress (compared with the original number of bacteria before addition of the stress) was calculated. A ratio of the percent survival values for the LSMMG and  $1 \times g$  cultures was obtained (indicating the fold difference in survival between these cultures) and is presented as the acid survival ratio in Fig. 2A. The mean and standard deviation from three independent experimental trials are presented. For macrophage survival assays, the number of bacteria present inside J774 macrophages at 2 h and 24 h after infection was determined, and the fold difference between these two numbers was calculated. The mean and standard deviation of values from three independent experimental trials (each done in triplicate tissue culture wells) are presented. The statistical differences observed in the graphs in Fig. 2 were calculated at P < 0.05.

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