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Role of Hfq in an animal–microbe symbiosis under simulated microgravity conditions

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Abstract: Microgravity has a profound impact on the physiology of pathogenic microbes; however, its effects on mutualistic microbes are relatively unknown. To examine the effects of microgravity on those beneficial microbes that associate with animal tissues, we used the symbiosis between the bobtail squid *Euprymna scolopes* and a motile, luminescent bacterium, *Vibrio fischeri* as a model system. Specifically, we examined the role of Hfq, an RNA-binding protein known to be an important global regulator under space flight conditions, in the squid–vibrio symbiosis under simulated microgravity. To mimic a reduced gravity environment, the symbiotic partners were co-incubated in high-aspect-ratio rotating wall vessel bioreactors and examined at various stages of development. Results indicated that under simulated microgravity, *hfq* expression was down-regulated in *V. fischeri*. A mutant strain defective in *hfq* showed no colonization phenotype, indicating that Hfq was not required to initiate the symbiosis with the host squid. However, the *hfq* mutant showed attenuated levels of apoptotic cell death, a key symbiosis phenotype, within the host light organ suggesting that Hfq does contribute to normal light organ morphogenesis. Results also indicated that simulated microgravity conditions accelerated the onset of cell death in wild-type cells but not in the *hfq* mutant strains. These data suggest that Hfq plays an important role in the mutualism between *V. fischeri* and its animal host and that its expression can be negatively impacted by simulated microgravity conditions.

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Introduction

Space flight, in particular microgravity, imposes a wide range of physiological stresses on animals and their associated microbes. The adverse effects of microgravity have been well documented in animals and include bone loss, muscle atrophy and the dysregulation of the immune system (Sonnenfeld *et al*. 1998; Lynch & Matin 2005; Crucian *et al*. 2008). In addition, there have been several studies regarding the impact of microgravity on microbial physiology (for review, see Horneck *et al*. 2010), such as increases in growth rates in liquid media (Taylor 1974; Ciferri *et al*. 1986; Klaus *et al*. 1997), higher resistance to antibiotics (Tixador *et al*. 1985), as well as elevated rates of conjugation, genetic transfer (Ciferri *et al*. 1986) and phage induction (Mattoni 1968).

However, far less is known about the impact of the space environment on those microbes that comprise the healthy microbiome of animal hosts (McFall-Ngai *et al*. 2010). In humans, the microbiome is comprised more than 1000 different microbial species that interact with host cells and are essential for normal host physiology, such as maintenance of various metabolic activities and the regulation of the host immune system (Rakoff-Nahoum *et al*. 2004; Mazmanian *et al*. 2005; Dethlefsen *et al*. 2007; Human Microbiome Consortium 2012). Although most interactions between hosts and microbes do not result in disease (Dethlefsen *et al*. 2007), much of what has been learned about host–microbe interactions in microgravity is derived from the study of pathogenic microbes (e.g. Nickerson *et al*. 2000; Chopra *et al*. 2006; Wilson *et al*. 2007; Rosenzweig & Chopra 2012; Vukanti *et al*. 2012; Kim *et al*. 2013).

Several important studies have shown that microgravity can significantly impact the physiology of pathogenic microbes resulting in increases in virulence, resistance to environmental stress and increased survival in host macrophages (Nickerson *et al*. 2000, 2004; Wilson *et al*. 2002, 2007; Chopra *et al*. 2006; Crabbé *et al*. 2011). Previous studies have shown that these changes in virulence are environment-dependent and, in some cases, can be attenuated through media supplementation. For example, in *Salmonella typhimurium*, the concentration of phosphate ion in the surrounding media was correlated to the pathogenesis-related acid stress response (Wilson *et al*. 2008) suggesting that it is possible to mitigate potential harmful effects of pathogens in space flight (Sarker *et al*. 2010). These same studies have also determined that there are extensive changes in microbial gene expression both at the transcriptional and translational levels. One key finding is that microgravity alters the expression of the global regulator Hfq, an RNA-binding protein that binds to small regulatory RNA and message RNA (mRNA) molecules. These molecules foster the regulation of mRNA translation in response to environmental stress (e.g. acidic, osmotic, thermal), changes in metabolic concentrations (e.g. iron levels; Guisbert *et al*. 2007), as well as promote the virulence of several pathogens such as

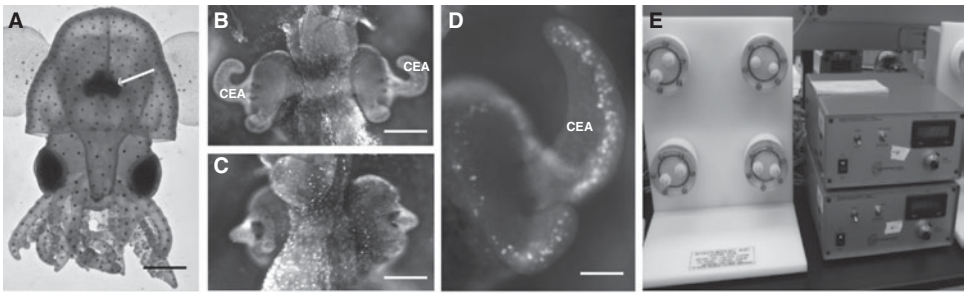

Fig. 1. Overview of the symbiosis between the host squid *Euprymna scolopes* and luminescent bacterium *Vibrio fischeri*. (A) Hatchling squid with pronounced light organ in the centre of mantle cavity (arrow; bar = 400 µm). (B) Micrograph of light organ in the absence of bacteria (i.e. aposymbiotic), depicting the pronounced ciliated epithelial appendages (CEA) extending from either side of the light organ (bar = 200 µm). (C) Light organ exposed to symbiosis-competent *V. fischeri* for 96 h showing the complete loss of the CEA (bar = 200 µm). (D) One half of the light organ stained with acridine orange depicting the pronounced pattern of dying pycnotic nuclei. The cell death event is triggered by exposure to *V. fischeri* and peaks 14 h after colonization under normal gravity conditions (bar = 75 µm). (E) High-aspect-ratio vessel bioreactors that can simulate microgravity conditions.

S. typhimurium (Wilson *et al.* 2007) and *Pseudomonas aeruginosa* (Crabbé *et al.* 2011). Approximately half of all eubacteria have a homologue to Hfq (Valentin-Hansen *et al.* 2004), including several animal and plant symbionts (Ruby *et al.* 2005; Torres-Quesada *et al.* 2010). In microgravity there is a down-regulation of *hfq* gene expression, which is thought to be involved in regulating virulence; however, the precise mechanisms are not known (Wilson *et al.* 2007). The *hfq* gene product has numerous regulatory functions that can differ significantly between species (for review Chao & Vogel 2010); however, the role of Hfq in beneficial animal–microbe interactions and the impact of space flight conditions on *hfq* expression in mutualistic bacteria remain unexplored.

To address the role of Hfq in beneficial host–microbe interactions and to assess the impact of microgravity on this important regulator, we used the well-studied symbiosis between the bobtail squid *Euprymna scolopes* and the luminescent bacterium *Vibrio fischeri* as a model (for review Stabb & Visick 2013). *V. fischeri* colonizes a specialized organ in the centre of the host mantle cavity called the light organ (Fig. 1(A)). Once infected the bacteria trigger a series of developmental events that result in the morphogenesis of the host light organ over the first 4 days of colonization (McFall-Ngai & Ruby 1991). Initially, in the juvenile squid there are fields of epithelial cells on either side of the light organ that form two appendage-like structures (Fig. 1(B)). These epithelial cells are covered in cilia that regularly beat to facilitate symbiosis-competent bacteria to aggregate, associate with the ciliated cells, and eventually enter pores found on the surface of the light organ (Montgomery & McFall-Ngai 1994; Nyholm *et al.* 2000; Altura *et al.* 2013). Persistent colonization of the light organ with *V. fischeri* for more than 12 h results in the onset of bacterial luminescence via quorum sensing (Lupp & Ruby 2005), as well as an irreversible loss of the ciliated epithelial appendage (CEA) structures over a 96-h period (Fig. 1(C); Doino & McFall-Ngai 1995). An early event triggered by the bacteria is the onset of apoptotic cell death throughout the CEA (Fig. 1(D); Montgomery & McFall-Ngai 1994; Foster & McFall-Ngai 1998), which is triggered by the synergistic effects of two *V. fischeri* microbe-associated molecular pattern (MAMP) molecules lipopolysaccharide (LPS) and a peptidoglycan derivative known as tracheal cytotoxin (Foster *et al.* 2000; Koropatnick *et al.* 2004). Typically, the cells undergoing cell death exhibit pycnotic (i.e., condensed) nuclei approximately 6 h after initial bacterial exposure. The number of pycnotic nuclei peaks 16 h after exposure to *V. fischeri* and slowly tapers off as the CEA structures begin to regress (Montgomery & McFall-Ngai 1994; Foster & McFall-Ngai 1998). The precise mechanism by which the CEA cells die and undergo regression is not completely understood; however, the regression occurs gradually over a 96-h period and is visible within 24 h after inoculation (Doino & McFall-Ngai 1995).

The monospecificity of the *Euprymna–Vibrio* symbiosis in conjunction with the rapid developmental time line of bacteria-triggered events has been shown to be an effective model to examine the effects of microgravity on host–microbe interactions (Foster *et al.* 2011, 2013). To simulate the microgravity environment we used high-aspect-ratio rotating wall vessel bioreactors (HARVs; Fig. 1(E)), which have been used for more than 20 years to mimic the low-shear environment of space flight (Wolf & Schwarz 1991; Schwarz *et al.* 1992; Nickerson *et al.* 2004; Horneck *et al.* 2010). The HARV chambers simulate the microgravity environment by maintaining the fluids in constant suspension. These hydrodynamic forces within the HARV offset the effects of gravity; thus the cell culture, or squid, remains at constant terminal velocity. Previous studies have shown that the low-shear environment generated in the HARV chambers is strongly correlated to the experimental results obtained in actual space flight (Nickerson *et al.* 2004; Wilson *et al.* 2007; Crabbé *et al.* 2011) and has been used to assess the effects of the space environment on a wide range of physiological and genetic responses in both microbes (e.g. Nickerson *et al.* 2000; Wilson *et al.* 2008; Mauclaire & Egli 2010; Pacello *et al.* 2012; Rosenzweig & Chopra 2012; Vukanti *et al.* 2012; Arunasri *et al.* 2013) and three-dimensional tissue cultures (e.g. Nickerson *et al.* 2003, 2004, 2007).

In this study, we assessed the impact of simulated microgravity on the expression of *hfq* in *V. fischeri* cultures. We also examined whether *V. fischeri* mutants defective in *hfq* were effective in colonizing and inducing normal light organ morphogenesis in the host squid. Both the juvenile squid and *V. fischeri* strains were co-incubated in the HARV chambers and the onset of apoptotic cell death and CEA regression, two of the most pronounced bacteria-induced phenotypes in light organ development, were assessed in the presence and absence of simulated microgravity. The resulting data provide evidence that the global regulating gene *hfq* is not required for host colonization but is required for normal light organ development. This study further confirms that mutualistic and pathogenic microbes often use similar mechanisms to interact with and colonize their animal hosts and that these symbiotic associations are susceptible to the effects of microgravity. By understanding the interactions of animals with their symbiotic microbiota in the space environment, we may be able to provide new insight into the mechanisms that maintain the balance between health and disease in animals.

Materials and methods

Host squid propagation

Specimen of adult *E. scolopes* were collected from O'ahu, Hawai'i and shipped to the Space Life Science Laboratory, Merritt Island, FL. The adult squid were maintained separately in 5-gallon aquaria at 23 °C on a 12 h light/dark cycle where they were mated bimonthly. The resulting egg clutches ranged in size from 150 to 300 eggs and were incubated in separate aquaria throughout their developmental period (~21 days). Immediately after hatching, juvenile squid (2–3 mm in size) were transported to individual vials containing 5 ml of filtered sterilized seawater (FSW).

Bacterial strains, media and host colonization

The motile, symbiosis-competent strain *V. fischeri* ES114, which was originally isolated from an adult squid light organ (Boettcher & Ruby 1990), was used for all experiments and is referred to as wild-type. *V. fischeri* ES114 also served as the parent strain for the *hfq* mutant construction. The *V. fischeri hfq* mutant was kindly donated by K. Visick, Loyola University Chicago. The insertional *hfq* mutant was confirmed using standard PCR regularly throughout the study.

Prior to exposure to the host animals, both the wild-type and *hfq* mutant were grown in seawater tryptone broth (SWT) containing 0.5% tryptone, 0.3% yeast extract and 0.3% glycerol in 70% seawater (Boettcher & Ruby 1990) at 23 °C. Juvenile squid were then inoculated with *V. fischeri* strains at concentrations of 1×10^5 cells ml^{-1} of FSW, replicating the approximate concentration of *V. fischeri* in the sand of the host squid habitat. The bacterial colonization of the host squid was monitored by measuring *V. fischeri* light production using a luminometer (GloMax 20/20, Promega Corp., Madison, WI). In all experiments, a subset of the juvenile squid was maintained without symbiosis-competent strains of *V. fischeri*, and these negative controls are referred as aposymbiotic animals.

Bacterial growth curves under simulated microgravity conditions

All *V. fischeri* cultures were grown in two rotary culture systems that contained eight 10 ml high-aspect-ratio vessels (HARVs, Synthecon, Houston, TX). The reactor vessels were positioned such that the axis of rotation was perpendicular to the direction of gravity, which simulated the low-shear microgravity conditions. For control purposes, HARVs were also positioned to replicate normal gravity conditions in which the axis of rotation was parallel to the gravity vector. The HARVs were inoculated with 1×10^6 cells/ml^{-1} of SWT, incubated at 23 °C with a rotation rate of 20 rpm. The cellular density of the cultures were monitored spectrophotometrically (DU800, Beckman Coulter, Fullerton, CA) in triplicate every 2 h at a wavelength of 600 nm. Three technical replicates were completed for each growth curve.

RNA isolation and quantitative real-time PCR

To monitor the effects of microgravity on the expression of *hfq* in *V. fischeri*, cells were cultivated for 12, 24, 48, 72 and 96 h in the rotating HARV chambers in the microgravity and gravity positions. There were eight replicate cultures for each time point grown under both microgravity and gravity conditions. At each time point, the cultures were immediately placed in RNAlater to arrest cellular activity and stabilize the RNA. The RNAlater–cell mixtures were then centrifuged at 3000 ***g*** for 10 min to pellet the cells. Total RNA was isolated from each replicate culture using a modified RNAzolRT protocol (MRC, Cincinnati, OH). Briefly, each pellet was resuspended in 1 ml of RNAzolRT reagent and transferred to a PowerBiofilm bead tube (MoBio, Carlsbad, CA) and vortexed for 5 min. The samples were centrifuged at 12 000 ***g*** for 5 min at 23 °C and the resulting supernatants were transferred to a new tube where the RNA was precipitated with 75% (w/v) ethanol. The precipitated RNA was rinsed twice in 75% (w/v) ethanol to remove any additional RNAzolRT reagent. The RNA was then treated with Turbo DNA-free DNase (Ambion, Austin, TX) to remove residual DNA and eluted with an RNeasy MinElute Cleanup Kit (Qiagen, Valencia, CA). RNA concentrations were determined spectrophotometrically (Qubit 2.0 Fluorometer, Invitrogen, Carlsbad, CA) and normalized to 10 ng/μl^{-1} of RNAse-free water.

The relative copy number of *hfq* genes in *V. fischeri* cells cultivated under both gravity and simulated microgravity conditions were assessed with quantitative real-time PCR (qPCR). The expressed *hfq* genes were amplified and quantified using the Superscript III Platinum SYBR Green One-Step RT-qPCR kit (Invitrogen, Carlsbad, CA) with a Roche, Light Cycler 480-platform (Roche, Indianapolis, IN). PCR reactions were run in triplicate in a total volume of 25 µl per reaction. Each reaction contained a final concentration of 1 × Superscript reaction master mix, 0.4 µgml^{-1} bovine serum albumin, 0.05 µM forward and reverse *hfq* primers and 50 ng RNA template. The primer set targeted the *hfq* gene found in

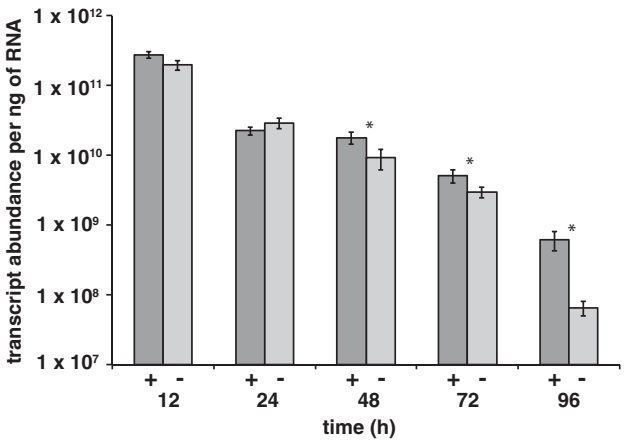

Fig. 2. Expression of gene *hfq* in *V. fischeri* cultures under gravity (+) and microgravity (−) conditions over time. Asterisk indicates significant difference between gravity and microgravity treatments with $p < 0.05$.

the *V. fischeri* genome (Ruby *et al.* 2005) and included the forward (5′-CGTCGTGAAAGGATTCCAGT) and reverse (5′-ACGAGCCGGAACAACAGTAG) primers. The reaction conditions included initial 3 min of incubation at 42 °C for reverse transcription and then 5 min of incubation at 95 °C for polymerase activation. The running protocol was followed by 40 cycles of 95 °C for 15 s, 58 °C for 30 s and a single elongation step at 40 °C for 1 min. To assess the disassociation characteristics of the PCR products and to ensure that there were no primer dimers, a melting curve analysis was performed at 95 °C for 30 s and then at 65 °C for 30 s with continuous measurements at 95 °C. The results were compared to a calibration curve that was prepared from RNA extracted from 24 h *V. fischeri* cultures. RNA was serially diluted to 0.001, 0.01, 0.1, 1 and 10 ng/μl^{-1} generating a five-point standard curve for comparison. The copy number of the *hfq* genes was calculated, based on the calibration curve (i.e. it was normalized to ng of RNA) and average molecular mass of double-stranded DNA, as previously described (Fierer *et al.* 2005).

Cell death determination and cellular regression

To visualize those cells of the CEAs undergoing apoptotic cell death, the juvenile squid were stained with the fluorescent marker acridine orange, which intercalates into the pycnotic (i.e. condensed) chromatin of the dying cells. Host squid were incubated in an equal mixture of 0.37 M $MgCl_2$ and FSW to anesthetize the squid before dissection. The squid were then stained with acridine orange at a concentration of 5 ng/ml^{-1} of FSW for 5 min. The mantle and funnel of the juvenile were removed and the exposed light organ was then visualized with epifluorescence microscopy (Zeiss Axioplan Microscopy, Zeiss, Jena, Germany). Pycnotic nuclei throughout superficial ciliated epithelium were quantified and statistically compared using a parametric Student's *t*-test, as previously described (Foster *et al.* 2000, 2013). A minimum of eight animals were used for each treatment at each time point.

To measure the extent of cellular regression of the ciliated epithelium on the surface of the light organ, host squid were anesthetized, as previously described, and examined with microscopy. The lengths of the anterior CEAs were measured using the Zeiss Micro Imaging software (Carl Zeiss, Jena, Germany) and statistically compared between treatments using pairwise comparisons. The lengths were then normalized to the anterior appendage of hatchling squid observed at the start of each experiment ($t = 0$ h). Results are reported as a per cent regression compared to the juvenile squid at $t = 0$ h.

Results

Expression of hfq under simulated microgravity conditions

To examine the impact of simulated microgravity on *hfq* gene expression in the symbiont *V. fischeri,* quantitative real-time PCR (qPCR) was used. Cultures of *V. fischeri* were grown within the HARV bioreactors under simulated microgravity and gravity control conditions for 12, 24, 48, 72 and 96 h and RNA concentrations from each treatment were normalized (i.e. 50 ng per reaction). There was an overall decrease in *hfq* transcript abundance in both simulated microgravity and gravity treatments detected throughout the 96 h observation period (Fig. 2). However, significant differences ($p < 0.05$) were observed between the *V. fischeri* parent and *hfq* mutant strain after 48 h incubation in simulated microgravity. The levels of *hfq* gene expression in cultures exposed to simulated microgravity continued to be significantly lower than normal gravity controls ($p < 0.05$) throughout the 96 h experiment (Fig. 2).

Growth and luminescence of V. fischeri *wild-type and* hfq *mutants under simulated microgravity conditions*

To address whether simulated microgravity impacts the growth of *V. fischeri*, motile cell cultures were incubated in HARV chambers and examined every 2 h spectrophotometrically. In addition to the wild-type strain of *V. fischeri,* an insertional mutant defective in *hfq* was also examined under identical growth conditions. There were few differences between wild-type and *hfq* mutant strains throughout the growth curves indicating that the lack of a functioning *hfq* gene did not impede normal growth (Fig. 3). However, differences were detected between simulated microgravity- and gravity-treated cells. Both strains had an altered growth rate under simulated microgravity conditions, beginning 10–14 h after initial inoculation. Although it was unclear whether the cultures were growing faster or experienced a shortened lag phase, the cultures reached cell densities that were higher than gravity-treated cells (Fig. 3).

In addition to the growth rate, the ability of the strains to colonize the host squid light organ and luminesce in simulated microgravity was also examined. Once symbiosis-competent strains of *V. fischeri* colonize the light organ and reach sufficient cell densities, the cells begin to luminesce through quorum sensing approximately 12 h after initial colonization (Boettcher *et al.* 1996; Lupp & Ruby 2005). Results indicated that there was no delay in the onset of luminescence under

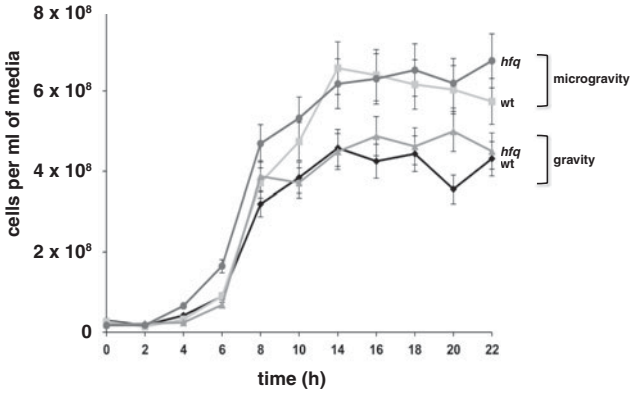

Fig. 3. Growth curve of wild-type *V. fischeri* (wt) and mutant strain defective in the gene *hfq* cultivated in HARV bioreactors under simulated microgravity and gravity conditions.

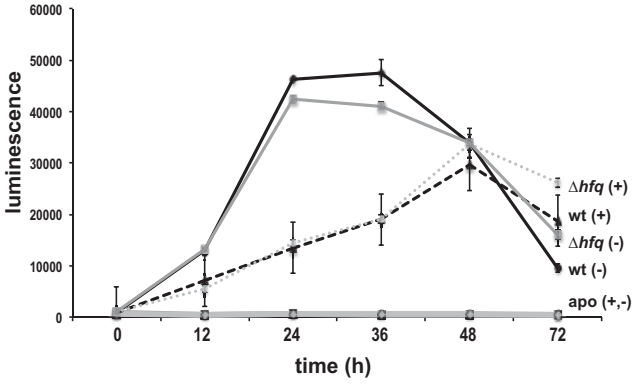

Fig. 4. Luminescence of host squid under microgravity (−) and gravity (+) conditions.
Squid colonized with *V. fischeri* wild-type (wt; black diamond) or mutant defective in *hfq* (grey square) under microgravity conditions were compared with those squid colonized with wild-type (black triangle) and hfq (grey circle) grown under 1 × g gravity. Control animals maintained aposymbiotically (apo) showed no luminescence under microgravity (grey diamond) nor gravity (black square) controls.

simulated microgravity conditions; additionally, there was a pronounced increase in light production in symbiotic squid exposed to simulated microgravity (Fig. 4). Between 24 and 36 h there was an increase in the light levels of squid infected with the wild-type strain; however by 48 h, all the host animals showed comparable levels of luminescence, regardless of strain or environmental treatment. In aposymbiotic animals, no light production was observed under either simulated microgravity or gravity treatments throughout the 72-h observation period (Fig. 4).

Bacteria-induced phenotypes in host light organ under simulated microgravity conditions

Although *hfq* mutants appear to colonize and initiate the symbiosis normally, there were differences observed in the onset of two key developmental phenotypes. Previous studies have shown that under simulated microgravity, the normal bacteria-induced apoptosis event that occurs along the surface epithelium of the light organ is accelerated and peaks several hours earlier than normal gravity conditions (Foster *et al.* 2013; Fig. 5). In mutants defective in *hfq*, however, this simulated microgravity-induced phenotype was lost and the apoptosis event peaked at the same time point as wild-type (i.e. 24 h) under the normal gravity conditions. In addition, the *hfq* mutants also induced lower levels of apoptotic cell death under both simulated microgravity and gravity conditions compared with wild-type, although pycnotic nuclei counts were generally higher under simulated microgravity (Fig. 5).

In addition to changes in the cell death event, there were also differences observed in the rates of cellular regression of the surface epithelium under simulated microgravity (Fig. 6). Previous results indicated that under simulated microgravity, the cells comprising the CEA structures on the surface of the light organ regress at a faster rate (Foster *et al.* 2013; Fig. 6). Mutants defective in *hfq* showed accelerated cellular regression of the ciliated fields, which was comparable with wild-type cells under simulated gravity conditions (Fig. 6). This trend continued throughout the 48-h observation period.

Discussion

This study examined the role of the global regulator Hfq in the mutualistic association between the bobtail squid *E. scolopes* and its luminescent symbiont *V. fischeri* under normal gravity and simulated microgravity conditions. The results of this study indicate that: (1) *hfq* gene expression is down-regulated in symbiosis-competent *V. fischeri* cultures under simulated microgravity conditions; (2) the *hfq* gene in *V. fischeri* is not required for normal colonization of the host squid light organ; (3) loss of Hfq reduces the microgravity-induced phenotype of early-onset apoptosis in the CEAs; and (4) the synergistic effects of simulated microgravity and the loss of Hfq in *V. fischeri* accelerates normal regression of the CEA within the first 24 h.

The RNA-binding protein Hfq is an important global post-transcriptional regulator that is present in approximately half of all bacteria, including the symbiont *V. fischeri* (Ruby *et al.* 2005; Chao & Vogel 2010). Hfq is essential for pathogenesis in several microbes such as, but not limited to, *Brucella abortus* (Robertson & Roop 1999); entero-toxigenic *Escherichia coli* (Shakhnovich *et al.* 2009); *P. aeruginosa* (Sonnleitner *et al.* 2003); *S. typhimurium* (Sittka *et al.* 2007); *Vibrio cholerae* (Ding *et al.* 2004); and *Yersinia pestis* (Geng *et al.* 2009). However, not all pathogens require Hfq and several strains show a wide range of virulence phenotypes in the absence of Hfq (for review, see Chao & Vogel 2010), with some strains such as the Gram-positive *Listeria monocytogens* (Christiansen *et al.* 2004) and *Staphylococcus aureus* (Bohn *et al.* 2007) having no Hfq phenotype.

Of the many microbes shown to possess an *Hfq* homologue, only two have been previously examined under a low-shear microgravity environment. In pathogenic strains of *S. typhimurium* and *P. aeruginosa*, there was a down-regulation of *hfq* expression under both actual and simulated microgravity

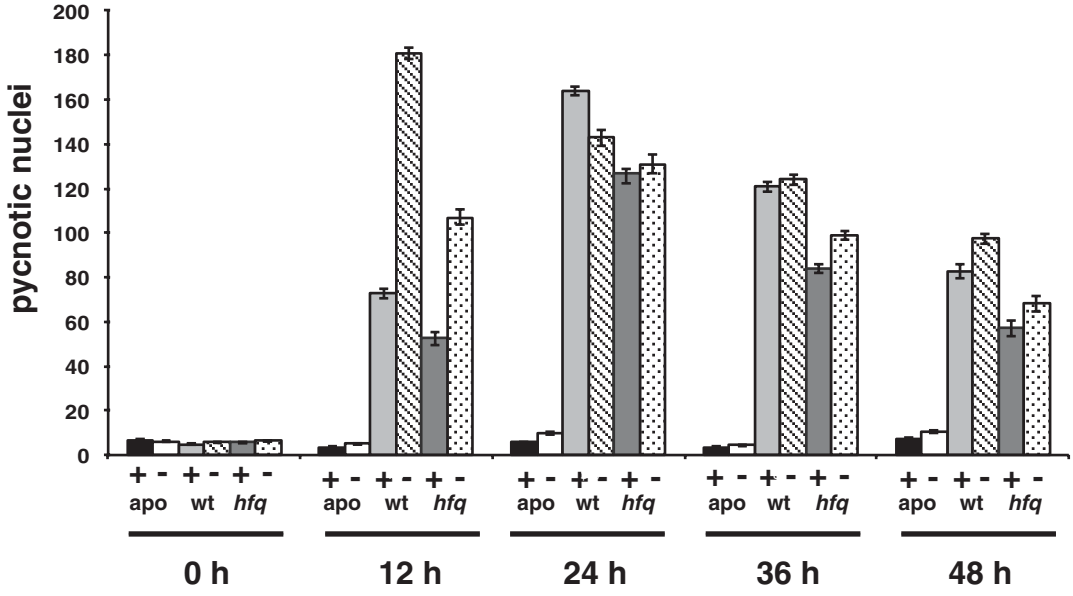

Fig. 5. Number of apoptotic pycnotic nuclei in host light organ under gravity (+) and microgravity (−) conditions over time. Host animals were either maintained without bacteria (apo) or inoculated with *V. fischeri* wild-type (wt) or mutants defective in the *hfq* gene (*hfq*).

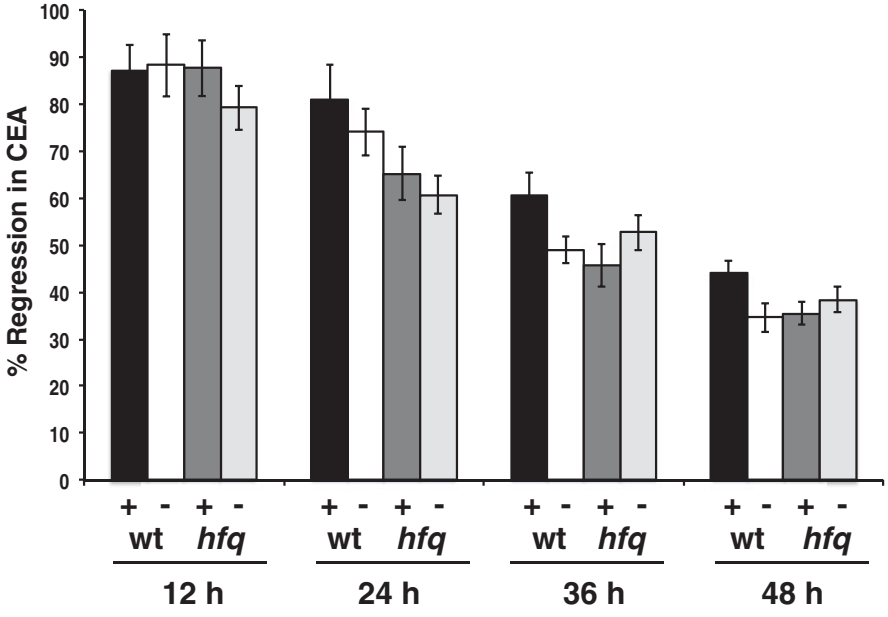

Fig. 6. Regression of the ciliated epithelial appendages (CEA) in the presence of *V. fischeri* wild-type (wt) and mutants defective in *hfq* (*hfq*) under 1 × gravity (+) and microgravity conditions (−) over time.

conditions (Wilson *et al.* 2002, 2007; Crabbé *et al.* 2011). Previous studies have shown that as much as 20% of the *S. typhimurium* genome is directly or indirectly regulated by Hfq (Sittka *et al.* 2008) and that 32% of this Hfq regulon is differentially regulated during space flight (Wilson *et al.* 2007). Similar results are seen in *P. aeruginosa* with approximately 15% of all genes disrupted in *hfq* mutants (Sonnleitner *et al.* 2006) and 23% of genes differentially regulated in space (i.e., space flight stimulon) can be attributed to the Hfq regulon (Crabbé *et al.* 2011). Although comparison of the two data sets showed a decrease in *hfq* expression in the two pathogens, only 16% of the down-regulated genes were shared between the two bacteria with no overlap in up-regulated genes, suggesting that the two pathogens have distinctive microgravity-induced stimulons (Crabbé *et al.* 2011). These results may reflect the dynamic and differing roles that Hfq plays in various host-associated microbes. The down-regulation of *hfq* expression in the symbiotic *V. fischeri* in simulated microgravity conditions further confirms the role of Hfq as an important microgravity-induced regulator of microbial gene expression and that it is

not limited to pathogens. However, future experiments that examine changes in the transcriptome of wild-type and *hfq* mutants will be required to fully assess the full role Hfq plays in the microgravity transcriptomic response of symbiotic *V. fischeri*, as well assess whether there is a core microgravity-induced stimulon response shared between mutualistic and pathogenic species.

Although Hfq appears to play an important role in the infection and virulence of a wide range of pathogens, the loss of Hfq function in *V. fischeri* did not impede normal colonization of the host light organ. The growth rates of *V. fischeri* with and without a functional *hfq* gene were comparable, suggesting that Hfq is not essential for *V. fischeri* cell growth. In addition, the onset of luminescence, a key indicator of light organ colonization, showed no statistical differences between the wild-type and *hfq* mutant, indicating that the loss of Hfq causes no initiation phenotype in the squid–vibrio symbiosis. A similar response has been seen in the nitrogen-fixing endo-symbiont *Sinorhizobium meliloti*, which form a functional symbiosis with leguminous alfalfa (Torres-Quesada *et al.* 2010). Mutant *S. meliloti* strains defective in Hfq are able to nodulate alfalfa roots at comparable levels to those found in the parent strain (Torres-Quesada *et al.* 2010); however, numerous downstream defects were observed, such as the inability of the nodules to undergo normal levels of nitrogen fixation and long-term survival within acidic membrane compartments of the host (Barra-Bily *et al.* 2010; Gao *et al.* 2010; Torres-Quesada *et al.* 2010). Together, these results suggest that Hfq may not be critical for the initiation of beneficial host–microbe associations, but rather that Hfq may be important in the maturation and development of the symbioses.

Growth between the parent and the mutant strains were similar; however, there were differences observed under simulated microgravity and normal gravity treatments. Both *V. fischeri* strains grew to higher cell densities under simulated low-shear microgravity conditions mirroring what is found in other modelled and actual microgravity studies (Mattoni 1968; Ciferri *et al.* 1986; Klaus *et al.* 1997; Foster *et al.* 2013). The modest increase in cell densities of both *V. fischeri* wild-type and *hfq* mutant compared to other bacterial species may reflect the motile nature of *V. fischeri* in culture. *V. fischeri* is motile in its free-living state; however, once it colonizes the light organ it loses its flagella and becomes non-motile (Ruby & Asato 1993). Growth changes of bacterial cultures under natural and modelled microgravity conditions have been correlated to motility, as motile bacteria can often disrupt the quiescent fluid and nutrient environment of the culture thus changing the physiology and growth rates of the microbes (Benoit & Klaus 2007).

The increase in luminescence observed in the host squid may also reflect the increased growth rate of *V. fischeri* strains under simulated microgravity conditions. Once the light organ has been colonized and *V. fischeri* cells reach a critical cell density ($\sim$ 12 h), the cells utilize two quorum-sensing systems (e.g. *ain* and *lux*) to regulate luminescence (Lupp & Ruby 2005). The *V. fischeri* cells continue to grow on nutrients provided by the host animal and reach maximum cell density approximately 24 h after initial inoculation (Graf & Ruby 1998). It is possible that under simulated microgravity conditions, the *V. fischeri* cells reached a higher cell density earlier than in the gravity controls and light production was subsequently elevated. By 48 h, the luminescence levels of both simulated microgravity and gravity controls were comparable. This decrease in light production during the second day of colonization may reflect the natural diel rhythm of the symbiosis (Boettcher *et al.* 1996). At dawn, the host squid vents 95% of the bacterial populations and recultivates the bacterial population from the remaining 5% (Lee & Ruby 1994). It is possible that during the regrowth of the bacterial population within the light organ the effects of modelled microgravity were no longer present. Future work that examines the expression of the *lux* operon under normal and modelled microgravity throughout the diel cycle is required.

Although the loss of Hfq in *V. fischeri* had no impact on the initiation of the symbiosis under normal or reduced gravity conditions, Hfq did appear to be required for the full onset of apoptotic cell death in the host light organ CEAs. Levels of pycnotic nuclei were significantly attenuated in *V. fischeri hfq* mutants under both normal and simulated microgravity conditions. Previous studies have shown that simulated microgravity conditions accelerate the onset of *V. fischeri* cell death in the host light organ with a significant increase in the numbers of pycnotic nuclei (Foster *et al.* 2013). The attenuated cell death response in *hfq* mutants under both gravity and simulated microgravity conditions may reflect an altered production of the microbe-associated molecular pattern (MAMP) molecule lipopolysaccharide (LPS). For example, examination of the transcriptome of several pathogens, such as *P. aeruginosa,* has shown that in *hfq* mutants there is a down-regulation of genes associated with cell wall, LPS and capsule production (Sonnleitner *et al.* 2006). Additionally, in *S. typhimurium* cultures exposed to simulated microgravity, there is a down-regulation of LPS O-antigen side chain synthesis and overall lower LPS production in microgravity (Wilson *et al.* 2002; Rosenzweig & Chopra *et al.* 2012). Previous studies have also shown that microgravity can increase the sensitivity of the CEA cells to LPS-induced apoptosis (Foster *et al.* 2013). Therefore, if levels of LPS are lower in *V. fischeri hfq* mutants, the increased sensitivity of CEA cells to LPS under microgravity conditions may play a factor in the attenuated cell death response seen in the host light organs. Further work is required to measure the levels of LPS in the symbiotic *V. fischeri* strains under normal and reduced gravity conditions.

Another important bacteria-induced development event that was altered under simulated microgravity conditions was the increased rate of regression of the CEA structures early on in light organ morphogenesis. Previous studies have shown that approximately 24 h post-inoculation of symbiotic *V. fischeri*, there is an irreversible loss of the CEA basement membrane, which provides structural support for the single layer of epithelial cells that comprise the CEA (Foster & McFall-Ngai 1998). It is thought that the loss of this structural membrane

hastens the regression of the cells that comprise the CEA (Foster & McFall-Ngai 1998). The results of this study suggest that *V. fischeri hfq* mutants underwent a faster rate of regression during the first 24 h of the symbiosis compared with the wild-type strains. This regression occurred faster under simulated microgravity conditions; however, by 36 h, the level of CEA regression in both the *V. fischeri* wild-type and *hfq* mutant were not statistically different in simulated microgravity. These results may suggest that *Hfq* plays a role in regulating the bacterial signals that trigger the collapse of the basement membrane and the subsequent regression of the CEA structures. *Hfq* has been identified in regulating the expression of several quorum-sensing pathways (Hammer & Bassler 2007) as well as several type III secretion systems that deliver effector molecules to the host cells (Sittka *et al*. 2007, 2008). The loss of *Hfq* in *V. fischeri* may disrupt the normal signal cascade required in the bacteria-induced light organ morphogenesis, thus accelerating the regression event. In addition, microgravity has also been shown to down-regulate the expression of genes associated with the extracellular matrix molecules, which are known to comprise the basement membrane structures (Zayzafoon *et al*. 2004, 2005). Furthermore, microgravity can alter and induce remodelling in the cytoskeleton in eukaryotic cells (Infanger *et al*. 2006). Together, the lack of Hfq and the effects of simulated microgravity may work synergistically to accelerate the regression of the CEA structures in symbiotic light organs.

In summary, the results of this study indicate that Hfq, an important post-transcriptional regulator of virulence in pathogenic microbes, is also an important molecule in symbiotic microbes that form associations with animal tissues. Although not essential for initiation of the symbiosis, Hfq does appear to play a critical role in two key developmental processes that restructure the host light organ tissues. The effects of simulated microgravity also appear to influence the expression of *hfq* in the symbiotic *V. fischeri*, suggesting that *hfq* may be an important component of the space flight stimulon in all host-associated microbes, not just pathogens. Further research is required to examine the influence of microgravity on the transcriptome of both the host squid and its symbiont in the presence and absence of Hfq. However, these results do indicate that the squid–vibrio symbiosis can serve as an important model to examine those molecular mechanisms required by both pathogens and mutualistic bacteria and whether the stress of the space flight environment can alter the regulation of these pathways, thus tipping the balance between health and disease in animal hosts.

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