

Increased Filamentous Growth of *Candida albicans* in Simulated Microgravity

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Knowledge of simulated microgravity (SMG)-induced changes in the pathogenicity of microorganisms is important for success of long-term spaceflight. In a previous study using the high aspect ratio vessel bioreactor, we showed that the yeast species *Saccharomyces cerevisiae* underwent a significant phenotypic response when grown in modeled microgravity, which was reflected in the analysis of gene expression profiles. In this study, we establish that *Candida albicans* responds to SMG in a similar fashion, demonstrating that there is a conserved response among yeast to this environmental stress. We also report that the growth of *C. albicans* in SMG results in a morphogenic switch that is consistent with enhanced pathogenicity. Specifically, we observed an increase in filamentous forms of the organism and accompanying changes in the expression of two genes associated with the yeast-hyphal transition. The morphological response may have significant implications for astronauts' safety, as the fungal pathogen may become more virulent during spaceflight.

Key words: yeast, *Candida albicans*, SMG, HARV, HWP1, YWP1

Introduction

Microgravity presents a unique environmental stress on prokaryotic and eukaryotic organisms that have evolved under normal gravity on the Earth. Determining the effects of this stress on cellular function during spaceflight is important to better understand the physiologic responses of humans and microorganisms that are potential human pathogens. Microgravity has been shown to alter the phenotype of several microorganisms. For example, the pathogenicity of *Salmonella* increases in microgravity, and *Escherichia coli* demonstrates an increased resistance to hyperosmosis, low pH, and antibiotics, as well as increased formation of biofilms (1–3).

Ground-based methods have been developed to mimic the microgravity conditions that exist in space. One such method simulates microgravity by rotating bioreactors around a plane vertical to the normal gravitational vector. This system does not remove the force of gravity, but creates a state of “functional weightlessness” by randomizing the gravitational effect and minimizing turbulence (shear) over the surface of the cell. The gravitational vector pre-

sent in the bioreactor is averaged over time to near zero (2–5). The high aspect ratio vessel (HARV) bioreactor in this simulated microgravity (SMG) system enables sufficient movement of the cells to allow for continuous exchange of dissolved gases through a permeable membrane and the exchange of nutrients and waste in the medium within the vessel (2, 4, 5).

In previous studies from our laboratory, we examined the effects of SMG at both the phenotypic and genotypic levels in *Saccharomyces cerevisiae* using the HARV bioreactor (6). *S. cerevisiae* cells grown under the influence of SMG displayed a random budding phenotype compared with the typical bipolar budding pattern of cells grown under normal conditions. In addition, we observed an increase in the number of cells found in clumps or aggregates. Consistent with these phenotypic alterations, there were significant changes (≥ 1.5 fold) in the expression of genes associated with bipolar budding and cell separation. In a separate but related study, genome-wide microarray analysis revealed significant microgravity-induced changes in the expression of genes that have a role in environmental stress responses, as well as in genes that may represent novel responses to microgravity (7).

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Based on the results obtained on *S. cerevisiae*, we questioned whether other yeast species undergo similar changes when exposed to SMG. *Candida albicans* is an opportunistic human pathogen responsible for a wide array of diseases, such as oral thrush, vaginitis, biofilm-associated infections, and disseminated candidiasis. In an immunocompromised host, the normally commensal fungus can spread rapidly, causing serious infections of the blood stream and internal organs. The mode(s) of infection employed by *C. albicans* has been widely studied in an effort to develop effective anti-fungal therapies (8). The morphogenic state of *C. albicans* can transit from a budding yeast form to a filamentous form (germ tubes, pseudohyphae, or hyphae). This morphogenic change is responsive to environmental stressors and contributes to the organism's virulence as an opportunistic pathogen (9, 10). It is hypothesized that the filamentous morphology is advantageous and/or required for adhesion to host tissues.

The objective of the current study was to determine the effects of SMG on *C. albicans* and discover whether the response is comparable to that observed in *S. cerevisiae*. Specifically, we examined whether a subset of genes conserved between *S. cerevisiae* and *C. albicans* respond in a similar fashion when grown under SMG conditions. In addition, we examined whether the phenotypic response to SMG observed in *S. cerevisiae* was mimicked by a morphogenic response in *C. albicans*, particularly to the response that might induce a pathogenic response. The results from this study provide evidence that there is a conserved response to SMG between the two dissimilar yeast species and the changes that occur in *C. albicans* may predict an increased pathogenicity. A

spaceflight-associated increase in the virulence of opportunistic human pathogens combined with an observed decrease in the human immune response is a significant concern that will require further analysis (2).

Results

Effects of SMG on cell budding

One of the first visual indications that yeast cells may respond to growth in the SMG environment came from the aberrant budding and cell clumping of *S. cerevisiae* (6). In order to find whether *C. albicans* has a comparable response, cells were cultured in HARVs under experimental (grown in HARVs on a vertical plane) or control conditions (grown in HARVs on a horizontal plane or in a conventional shaker) and evaluated microscopically. Cultures were maintained at 30°C in rich media for continuous log growth. In control culture conditions, *C. albicans* budded in axial or bipolar patterns (Figure 1, panels 1–4). In contrast, cells subjected to SMG underwent normal (Figure 1, panels 5 and 6) or significant abnormal budding (Figure 1, panels 7 and 8). Budding was considered random or abnormal when bud scars or daughter cells were observed at medial sites on the mother cell. Microscopic analyses also revealed clumps of morphogenically diverse cells in cultures following exposure to SMG (data not shown). The yeast cells that were associated with the clumps often displayed a random budding phenotype. The clumping trend was not observed in the shaker control samples, although the HARV control samples with filamentation displayed similar but less frequent clumping tendencies.

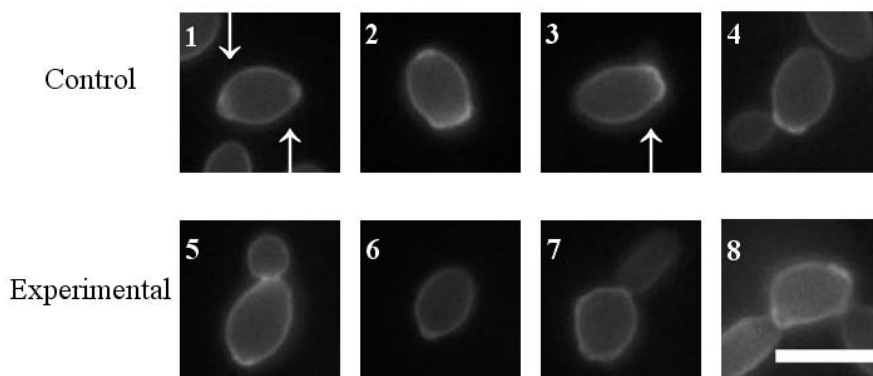


Fig. 1 *C. albicans* budding patterns under normal and SMG conditions. Panels 1–4: Cells cultured in shaker (Control) exhibit either bipolar (1 and 2) or axial (3 and 4) budding; Panels 5–8: Cells cultured in HARVs under SMG conditions (Experimental) exhibit bipolar (5), axial (6), or random budding (7 and 8). The arrows in panels 1 and 3 point to bipolar and axial budding sites, respectively. Scale bar = 2 μm (1000 \times magnification).

Effects of SMG on gene expression

In our previous study, the random budding and cell clumping responses to SMG observed in *S. cerevisiae* were accompanied by differential gene expression in pathways consistent with the phenotypes observed (6). Notably, the genomic response analyzed by functional gene categories such as MIPS (Munich Information Center for Protein Sequences) indicated a significant effect of genes involved in cell polarity and budding. We reasoned that if the *C. albicans* response was comparable to that observed in *S. cerevisiae*, then genes conserved between the two organisms might respond in an analogous fashion. Table 1 lists the *S. cerevisiae* genes identified by microarray analysis with significant SMG-induced changes in expression (≥ 1.5 fold) and whether or not there are *C. albicans* homologs. The *S. cerevisiae* genes listed in Table 1 are included in the MIPS functional category of budding and cell polarity. Table 2 illustrates the *S. cerevisiae* genes involved in cell budding and separation targeted in the SMG study (6) and their *C. albicans* homologs. In order to find whether a comparable SMG-induced genetic response exists between the two yeast systems, we evaluated the expression of two homologous genes, *Dse1* and *Rax2*, using real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) (Table 3). RNA was prepared from cells in log and stationary phases under SMG conditions. The results between the two organisms for both genes are strikingly similar. *S. cerevisiae* and *C. albicans* both display a down-regulation of *Dse1* in late log phase (*S. cerevisiae* 1.50 ± 0.30 ; *C. albicans* 1.49 ± 0.45) and late stationary phase (*S. cerevisiae* 2.50 ± 0.50 ; *C. albicans* 2.56 ± 0.82). *Rax2* expression in *C. albicans* was up-regulated (1.54 ± 0.23) in late log phase but no change was reported in *S. cerevisiae*. However, *Rax2* was down-regulated in both organ-

isms at late stationary phase (*S. cerevisiae* 2.00 ± 0.40 ; *C. albicans* 2.21 ± 0.50). This pattern of gene expression establishes a conserved response between the two yeast systems.

Table 1 *S. cerevisiae* genes with significant SMG-induced changes and their *C. albicans* homologs*

<i>S. cerevisiae</i> gene	<i>C. albicans</i> homolog
BUD4	+
BUD5	+
BUD9	-
BUD22	-
BUD23	-
ERV15	-
HKR1	-
RAX1	+
RAX2	+
RSR1	+
TOS2	-
TWF1	+

*“+” indicates there are *C. albicans* homologs and “-” indicates no *C. albicans* homologs.

Table 2 *S. cerevisiae* genes involved in cell budding and separation and their *C. albicans* homologs

<i>S. cerevisiae</i> gene	<i>C. albicans</i> homolog
BUD5	+
BUD25	-
EGT2	-
DSE1	+
DSE2	-
RAX1	+
RAX2	+

Table 3 Relative differences in expression of conserved candidate genes sensitive to SMG in *S. cerevisiae* and *C. albicans*

Gene	Species	Change in expression (n-fold)*	
		Late log phase	Late stationary phase
<i>Dse1</i>	<i>S. cerevisiae</i>	1.50 ± 0.30 (↓)	2.50 ± 0.50 (↓)
<i>Dse1</i>	<i>C. albicans</i>	1.49 ± 0.45 (↓)	2.56 ± 0.82 (↓)
<i>Rax2</i>	<i>S. cerevisiae</i>	No change	2.00 ± 0.40 (↓)
<i>Rax2</i>	<i>C. albicans</i>	1.54 ± 0.23 (↑)	2.21 ± 0.50 (↓)

*The data are the mean \pm standard error for control cells versus cells grown in SMG. “↑” indicates up-regulation and “↓” indicates down-regulation.

Effects of SMG on morphology

In addition to budding abnormalities, we observed an increased frequency of filamentous forms in *C. albicans* under SMG conditions relative to controls. Figure 2 illustrates the nearly uniform presence of yeast forms in the shaker cultured inoculum for each

subsequent condition (A1–3) and the following 25 generations of growth in the shaker (B1–3). Photomicrographs of the HARV control (Figure 2, C1–3) and HARV experimental samples (Figure 2, D1–3) revealed an increased prevalence of filamentous forms in SMG.

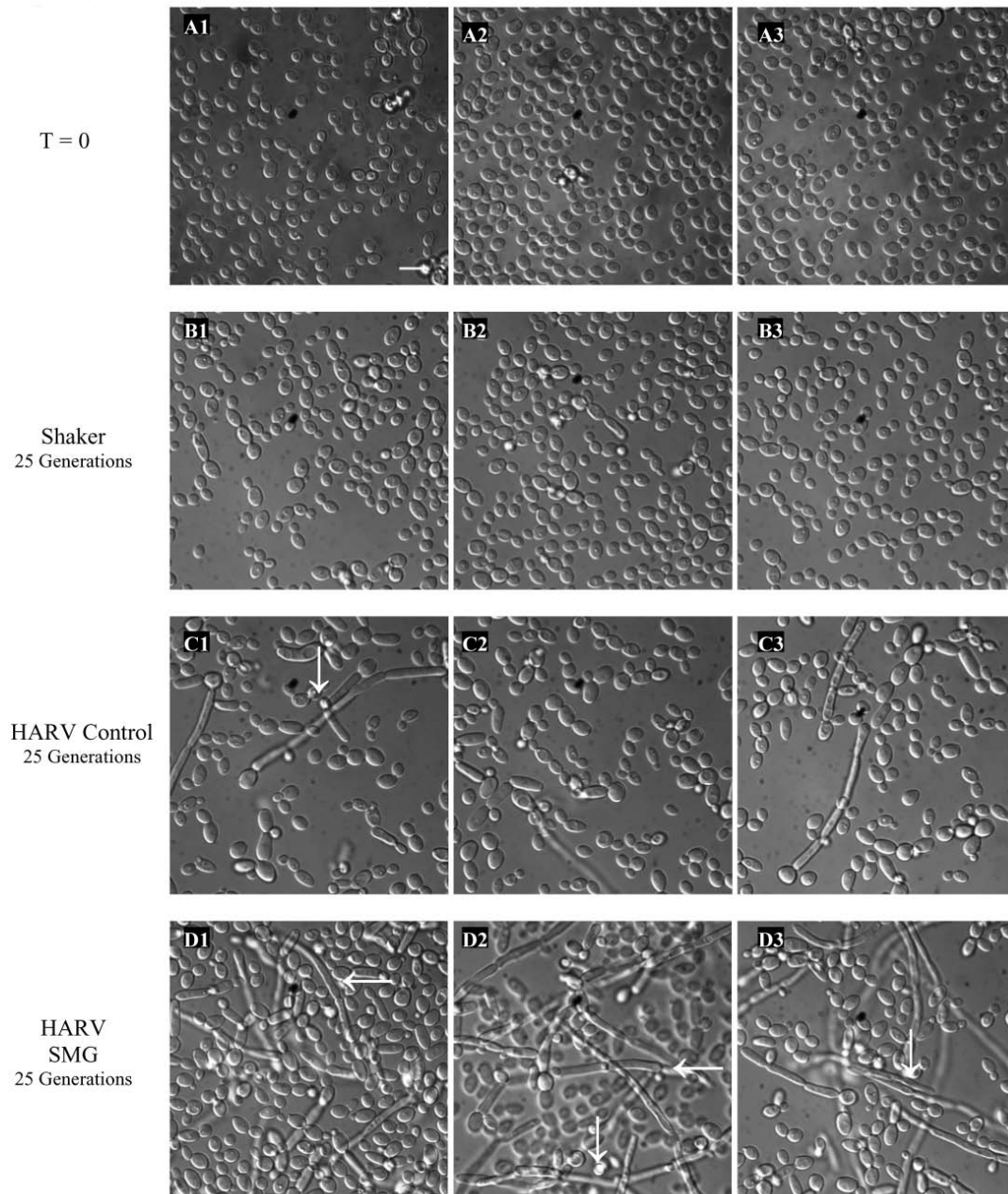


Fig. 2 Cell morphology of *C. albicans* under normal and SMG conditions. All samples were grown at 30°C in YPD media for continuous log growth. A1–3: Cell morphology at the beginning of growth (T=0) under normal gravity. B1–3: Cell morphology after 25 generations of growth in shaker under normal gravity. C1–3: Cell morphology after 25 generations of growth in HARV under normal gravity. D1–3: Cell morphology after 25 generations of growth in HARV under SMG conditions. Examples of filamentous morphology are noted with white arrows. Scale bar = 6 μm (630 \times magnification).

To obtain filamentous control samples as a comparison for the HARV SMG filamentation, the yeast-hyphal transition was induced at 37°C for 6 h. Figure 3 shows a comparison between the filamentous control (A1–3) and SMG (B1–5) samples after Calcofluor staining of chitin to identify bud scars. Both types of samples display a variety of morphologic forms in clusters, with random budding and filamentous cells. The similarity between the filamentous control and SMG samples indicates that the morphologic change observed under SMG conditions is comparable to the induced transition seen under normal gravity conditions.

Gene expression changes in *C. albicans* are consistent with morphology

To further establish a filamentous transition of *C. albicans* upon exposure to SMG, expression of genes associated with yeast and hyphal forms was assessed. RNA was prepared from cells grown under SMG and normal conditions. The expressions of a hyphae-specific gene, hyphal wall protein 1 (HWP1), and a yeast-specific gene, yeast wall protein 1 (YWP1),

were evaluated by qRT-PCR (11, 12). Figure 4 demonstrates a 2-fold increase in HWP1 expression and a 25-fold decrease in YWP1 expression following exposure to SMG for 40 generations of log growth relative to the expression in shaker controls. The gene expression data are consistent with the observed morphogenic changes presented in Figures 2 and 3. Cumulatively, these data support a hypothesis that the environmental stress of modeled microgravity induces conserved responses in yeast species, and in the case of *C. albicans* may lead to morphogenic alterations consistent with increased pathogenicity.

Discussion

Spaceflight presents a novel condition for organisms that have evolved under the normal gravity on the earth. It is clear, through ground- and flight-based experiments, that eukaryotic and prokaryotic organisms undergo physiological changes affecting a variety of mechanisms when exposed to microgravity conditions (1, 2, 13–19). Some of the affected physiologic systems in humans include reduced immune function, decreased bone and muscle mass, and cardiovascular

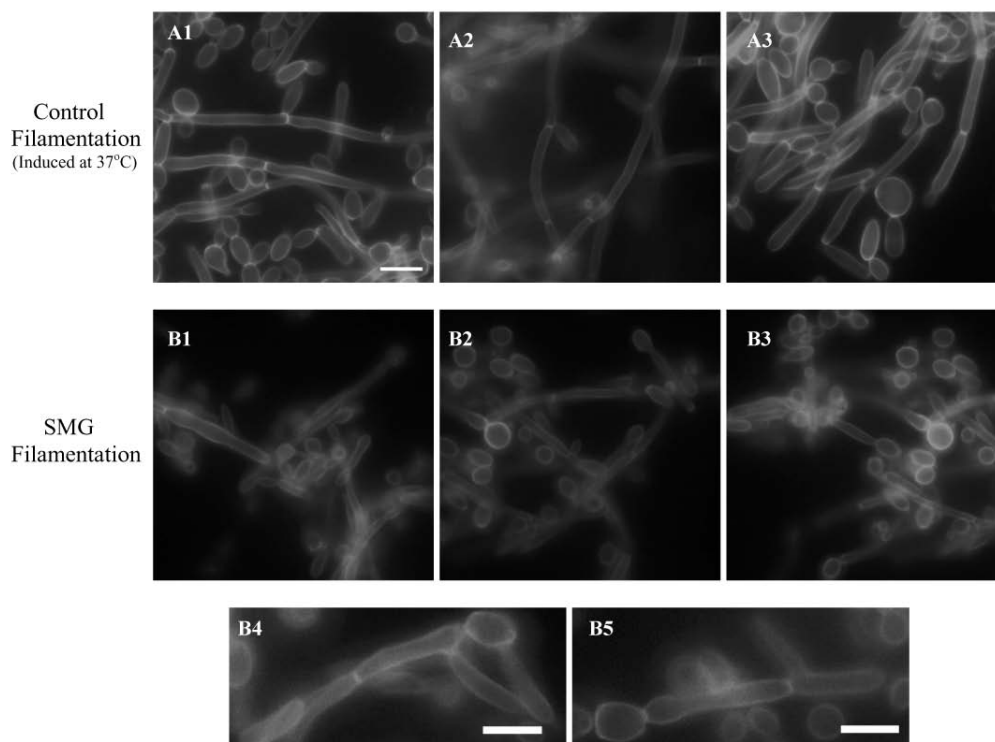


Fig. 3 Comparison of hyphal forms induced under normal and SMG conditions. A1–3: Epifluorescence visualization of Calcofluor white staining of control (temperature-induced) filamentation. B1–5: Epifluorescence visualization of Calcofluor white staining of SMG filamentation. Scale bar = 4 μm (1000× magnification).

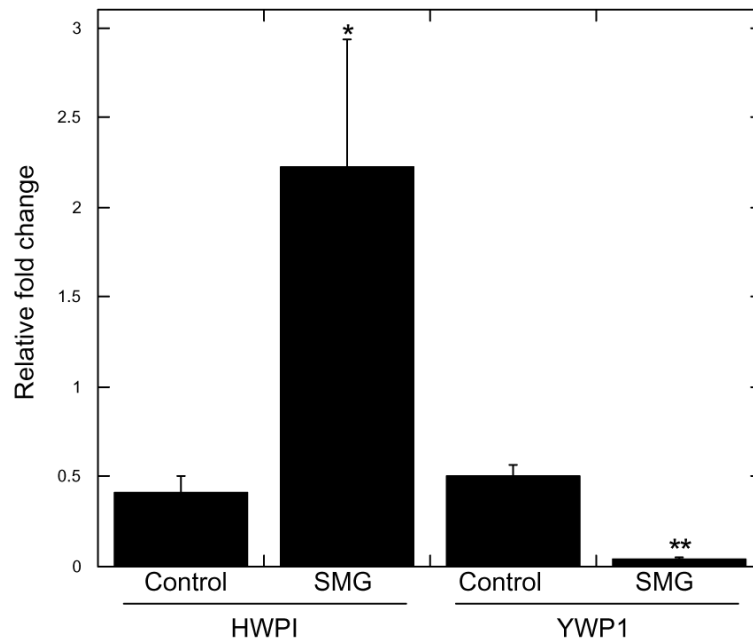


Fig. 4 Gene expression changes in *C. albicans* correlated with morphology. The relative expression of HWP1 and YWP1 under normal gravity (Control) and SMG conditions after 40 generations is shown as determined by qRT-PCR analysis ($n = 4$, $*p = 0.04$, $**p = 0.0002$).

irregularities (15, 20). For prokaryotes, the affected systems include increased pathogenicity and virulence as well as increased resistance to antimicrobial agents (1, 13). Notably, Lynch *et al* reported an increase in resistance to penicillin G and chloramphenicol in *E. coli* biofilms that were cultured under SMG conditions (21). The physiological changes that occur in the human immune system in combination with the changes in human pathogens are of particular concern for long-term exposure to microgravity. Remarkably, astronauts have experienced a variety of in-flight bacterial and viral infections (14, 15, 20; http://exploration.nasa.gov/articles/immune.12-2002_lite.html). Exchange of microflora between astronauts on board the shuttle has been reported, which serves as an indication that pathogenic outbreaks on the spacecraft are a viable concern (20). As an opportunistic pathogen, *C. albicans* remains a significant health risk.

Pathogenicity and virulence of *C. albicans* have been associated with the ability to alter morphology from a round, budding yeast to elongated, filamentous forms (germ tubes, pseudohyphae, and hyphae). Studies indicate that transition to filamentous forms contributes to virulence. For example, mutants defective in hyphal growth are less effective in causing disease (10, 22, 23). The exact mechanism of the morphologic switch is unknown, but it is thought that

filamentous forms may be better suited to adhere, penetrate, and colonize host tissues and organs, while the yeast form plays a major role in dissemination through the blood stream (10, 23, 24). Importantly, all morphologic forms play a role in the formation of *C. albicans* biofilms, which are clinically relevant fungal communities causing infections that are difficult to treat pharmacologically (25, 26). There is documentation of bacterial biofilms in space, specifically on the Mir space station, which experienced corrosion and blockage of the water purification system (21). The presence of bacterial biofilms in space suggests that fungal biofilms, specifically of *C. albicans*, may present a medical concern during long-term spaceflight.

Although several studies involving the microbial response to SMG have been reported, it is not clear whether the response is conserved, even among closely related species. For example, a recent study comparing *E. coli* (MG1655) with *Salmonella enterica* serovar Typhimurium showed that there were no similarities in the genes that were significantly up- or down-regulated, despite culturing in nearly identical conditions (27). In contrast, our data suggest that the phenotypic and genotypic responses in *C. albicans* and *S. cerevisiae* are very similar. *S. cerevisiae* displayed a random budding and cell clumping phenotype when cultured under SMG conditions

(6), and the current study presents evidence that a similar budding phenotype occurs in *C. albicans*. Interestingly, cells exhibiting random budding were often found in clusters composed of a variety of morphologic forms, including filamentous forms. In particular, two conserved genes, *Rax2* and *Dse1*, were also similarly regulated, thus indicating conservation of the genetic response between the two distantly related yeast species.

The phenotypic and genotypic responses observed under SMG conditions suggest that *C. albicans* may have a distinct advantage as a pathogen during long-term spaceflight when there is also reduced immune response in astronauts. Our future studies will explore the extent of the genomic response and how that response correlates to pathogenicity in humans, specifically in invasive biofilm infection and antibiotic resistance over long-term exposure to microgravity.

Materials and Methods

Strains and growth conditions

C. albicans strain SC5314 was used throughout the study. Overnight liquid cultures with optical density (OD) 600 nm in readings of 2.0–2.6, propagated from a single colony and incubated at 30°C in yeast peptone dextrose (YPD) on a rollerdrum, were inoculated (1:30) into fresh YPD liquid media and aseptically loaded into sterile HARVs and flasks. HARVs were attached to the Synthecon rotary units (Synthecon, Houston, TX, USA) and incubated at 30°C throughout the duration of the study. Experimental (SMG) and control (HARV normal gravity) vessels were rotated either vertically (with the vessel parallel to the gravitational vector) or horizontally (with the vessel perpendicular to the gravitational vector). Shaker cultures were maintained in a floor shaker (New Brunswick Scientific, Edison, NJ, USA) at 30°C as a baseline control for both experimental and control HARV samples. Cultures were maintained in log growth (confirmed by OD 600 nm in readings of 1.5–2.0) with sample collection points at approximately 0, 25, and 40 generations. Aliquots from each vessel and flask were removed for sample collection. Each aliquot was mounted on glass coverslips and examined microscopically on a Zeiss Axiovert microscope (Carl Zeiss, Göttingen, Germany) to ensure culture purity and to observe morphological changes. Samples were collected at 0, 25, and 40 generations as indicated and centrifuged at 5,000 rpm for 5 min at ambient

temperature, respectively. In an effort to reduce any potential effects of centrifugation on gene expression, samples were spun for 5 min to obtain a solid pellet. The cell pellets were then stored immediately at –80°C for further analyses. Positive controls for hyphal induction were obtained in YPD cultures grown under normal gravity conditions at 37°C for 6 h.

Calcofluor staining

Cell pellets were resuspended in phosphate buffered saline (PBS) and aliquoted into 100 μ L samples. Each sample was fixed with 4% paraformaldehyde at 30°C for 30 min. Cells were washed twice and resuspended in PBS. Calcofluor white (Molecular Probes, Carlsbad, CA, USA) was added to a final concentration of 25 μ M and incubated at 30°C for 30 min. The samples were visualized by epifluorescence microscopy.

RNA isolation and qRT-PCR

Total RNA was extracted using the Qiagen RNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA) according to manufacturer's instructions, with the following mechanical disruption protocol. Cells were disrupted using the Savant Fast Prep bead beater (Savant Instruments Inc., Farmingdale, NY, USA) for six cycles of 20 s, each at 4,500 rpm with cooling on ice after every other cycle. RNA quality and quantity were determined by spectrophotometry and gel electrophoresis. qRT-PCR analysis was performed using the SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit (Invitrogen, Carlsbad, CA, USA). Twenty-five microliter reaction volumes were used with 0.2 mM of each dNTP, 0.5 μ M sense and antisense primer, 0.5 μ L RT-Taq mixture, and 0.4 μ g RNA. All primers (20 bp) were designed by primer3 software (<http://frodo.wi.mit.edu/cgi-bin/primer3/primer3>) with amplicon sizes ranging from 78–183 bp. Fragments were amplified by incubation at 50°C for 30 min (cDNA synthesis); 94°C for 2 min; 40 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 90 s; followed by the final extension at 72°C for 5 min. Replicate PCR analyses were performed on two independent experiments containing internal biological replicates. Fold changes were calculated by normalizing expression levels to Pda1 as an internal standard also used in the *S. cerevisiae* studies (6, 7), and were quantified by the $2^{-\Delta\Delta CT}$ method (28).

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Authors' contributions

SDA collected microscopy data and wrote the manuscript. SMNP collected PCR data and co-wrote the manuscript. LEH supervised the project and co-wrote the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors have declared that no competing interests exist.

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