

ORIGINAL ARTICLE

Phenotypic characterization of *Aspergillus niger* and *Candida albicans* grown under simulated microgravity using a three-dimensional clinostat

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ABSTRACT

The living and working environments of spacecraft become progressively contaminated by a number of microorganisms. A large number of microorganisms, including pathogenic microorganisms, some of which are fungi, have been found in the cabins of space stations. However, it is not known how the characteristics of microorganisms change in the space environment. To predict how a microgravity environment might affect fungi, and thus how their characteristics could change on board spacecraft, strains of the pathogenic fungi *Aspergillus niger* and *Candida albicans* were subjected to on-ground tests in a simulated microgravity environment produced by a three-dimensional (3D) clinostat. These fungi were incubated and cultured in a 3D clinostat in a simulated microgravity environment. No positive or negative differences in morphology, asexual reproductive capability, or susceptibility to antifungal agents were observed in cultures grown under simulated microgravity compared to those grown in normal earth gravity (1G). These results strongly suggest that a microgravity environment, such as that on board spacecraft, allows growth of potentially pathogenic fungi that can contaminate the living environment for astronauts in spacecraft in the same way as they contaminate residential areas on earth. They also suggest that these organisms pose a similar risk of opportunistic infections or allergies in astronauts as they do in people with compromised immunity on the ground and that treatment of fungal infections in space could be the same as on earth.

Key words fungi, microgravity, space environment, three-dimensional clinostat.

Humans living in space are exposed to space radiation and a microgravity environment and must live in small confined spaces for long periods. The pressurized living environment on board spacecraft is controlled to make it comfortable for astronauts, but the resultant environment is also ideal for microorganisms. Accordingly, a number of microorganisms progressively contaminate the living and working environments of spacecraft from the begin-

ning of construction (1). Researchers have found large numbers of microorganisms in the cabins of space stations, such as the Russian Mir Space Station and the ISS, some of which are pathogenic microorganisms, including opportunistic fungi (1–5). Because they are in a limited and confined space, astronauts and on board equipment would be continuously exposed to high concentrations of fungal spores. Microorganisms in the living space could

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List of Abbreviations: 3D, three-dimensional; 5-FC, flucytosine; AMPH-B, amphotericin B; *A. niger*, *Aspergillus niger*; *C. albicans*, *Candida albicans*; CFU, colony forming unit; CPFG, caspofungin; *D. discoideum*, *Dictyostelium discoideum*; FE-SEM, field emission-scanning electron microscopy; FLCZ, fluconazole; G, gravity; ISS, International Space Station; ITCZ, itraconazole; ITS, internal transcribed spacer; MIC, minimum inhibitory concentration; PDA, potato dextrose agar; RPMI, Roswell Park Memorial Institute; YPD, yeast extract-peptone-dextrose.

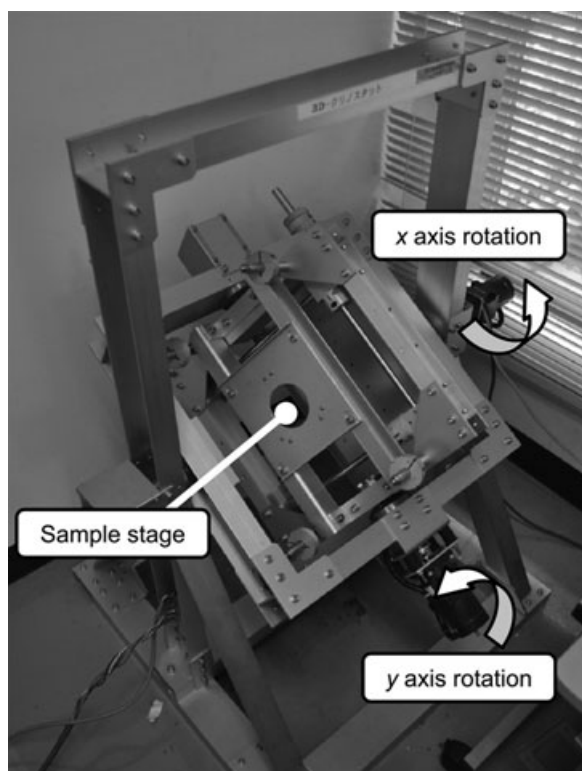


Fig. 1. 3D clinostat weightless microgravity simulator. This apparatus has two motors and rotates about the x and y axes independently. Samples are fixed on the center of the stage.

cause opportunistic infections and allergies in astronauts whose immunity has been compromised by prolonged stress, such as that due to space radiation, microgravity, or other factors associated with the space environment (6–8).

Environmental monitoring data have shown that the wide variety of microorganisms isolated from spacecraft are largely of human origin (1–5). Doctors and researchers are fully aware of the risk that fungal infection that could cause serious life-threatening disease in long-term space expeditions. Fungal flora isolated from samples collected from the Russian Mir Space Station consisted mainly of six strains. Morphological and molecular biological techniques based on 18S and ITS1 rDNA sequences resulted in identification of these isolates as *Penicillium chrysogenum*, *Aspergillus versicolor*, and *Penicillium* sp. (3). However, no information is available regarding how or whether the space environment affects and changes the characteristics of fungi. To evaluate the risks of fungi causing infections or allergies on board spacecraft, it is necessary to understand and predict how a microgravity environment could affect their growth and change their characteristics.

The 3D clinostat, an apparatus that nullifies the effect of gravity on the ground, is used in the field of space bi-

ology (Fig. 1) (9). A variety of substitution studies have utilized clinostats to conduct ground-based research on the effects of microgravity on biological events (10–20), and some organisms have been demonstrated to have the same characteristics under 3D clinostat rotation as they do in the microgravity environment. For example, cucumber seeds germinated under microgravity conditions during spaceflight produced some seedlings with two symmetrical protuberances, termed pegs, on each side of their transition zones (21). This phenomenon was expected because a prior ground-based experiment using a 3D clinostat had previously produced similar results (20). These findings indicated that ground-based experiments in 3D clinostats can provide very useful and reliable preliminary data that are helpful in predicting changes that will occur in terrestrial organisms in the space environment. In fungi, researchers have observed morphological and phenotypic changes in fruiting body formation in the basidiomycete *Pleurotus ostreatus* when it is cultured in the microgravity provided by 3D clinostat rotation (10). In the present study, which we designed to predict how fungi might change in the space environment, we assessed the effects of microgravity on fungi in a simulated microgravity environment in a 3D-clinostat, focusing on the major filamentous fungus *A. niger* and commensal yeast *C. albicans*, both of which may cause infection or act as allergens in the living environment (6–8).

MATERIALS AND METHODS

Strains

A. niger TIMM2911 and *C. albicans* TIMM1768, maintained at the Institute of Medical Mycology, Teikyo University, were used.

Evaluation of conidia formation and reproductive capability

Conidia of *A. niger* TIMM2911 were harvested and 1×10^6 cells spread on PDA (Difco, Franklin Lakes, NJ, USA) plates and cultured in a 3D clinostat at 27°C for 72 hr. Some plates were cultured simultaneously in ground gravity as controls. After cultivation, conidia were harvested using saline containing 0.05% Tween 80 and counted with a hemocytometer. Next, 1×10^2 harvested conidia cells were tested for their reproductive capability on PDA plates. *C. albicans* TIMM1768 was precultured in YPD liquid medium (2% bacto-tryptone, 1% bacto-yeast extract 2% glucose; Difco) and inoculated onto YPD agar plates. Single colonies were selected and 1×10^2 yeast cells from them inoculated onto YPD agar plates. These plates were incubated in the 3D clinostat or in ground gravity at

35°C for 24 hr. All colonies thus developed on the plates were counted. Cultivation using the 3D clinostat was performed as follows: agar plates were placed at the center of the 3D clinostat sample stage, which was then randomly and asymmetrically rotated in both *x* and *y* axial directions. The 1G control plates were firmly fixed and statically cultured in ground gravity.

Antifungal susceptibility testing

Susceptibility to antifungal agents was assayed in accordance with the standard protocol for Etest strips (BioMérieux, Durham, NC, USA) with slight modifications (22). Single colonies of *C. albicans* TIMM1768 were selected and each yeast suspension diluted and adjusted to MacFarland 0.5. Aliquots of 100 μ L of each solution were inoculated onto RPMI1640 with MOPS (3-(N-morpholino)propanesulfonic acid) agar plates containing AMPH-B, CPFG, 5-FC, FLCZ, and ITCZ. To determine the on-scale MIC of these antifungal agents, Etest strips were placed on the plates and the plates incubated at 35°C for 24 hr under 3D clinostat rotation or on the ground (1G). The results were assessed using Student's *t*-test.

RESULTS

Conidia formation and reproductive capabilities of *Aspergillus niger* under simulated microgravity

Conidia formation and reproductive capabilities of the filamentous fungus *A. niger*, which were expected to be affected by the space microgravity environment, were assessed by cultivating them in a simulated microgravity environment produced by the 3D clinostat. Conidia harvested from the culture were counted and tested for their reproductive capability to assessment their viability. As shown in Table 1, the number of conidia harvested from *A. niger* cultures incubated in the 3D clinostat was the same as that from cultures incubated in 1G on the ground. 1×10^2 conidia from each culture were spread on PDA plates and the numbers of colonies that developed were counted. There was no significant difference between them.

Stereoscopic and electron microscopic observation

Figures 2, 3 are stereoscopic microscope photographs of *A. niger* cultured in the 3D clinostat (Fig. 2) and in 1G (Fig. 3). After cultivation in the 3D clinostat, the aerial mycelia and conidiophores of *A. niger* grew and extended in a radial manner with an orderly arrangement similar to those that were cultured at 1G. Figures 4, 5 are FE-SEM photographs of each culture. Microscopic observations of

Table 1. Conidia formation and reproductive capability of *Aspergillus niger*

	Conidia formation ability (per plate)	Reproductive capability (per 1×10^2 cells)
3D clinostat culture group	$4.2 \times 10^7 \pm 3.8 \times 10^6$	89 ± 21
1G culture group	$4.3 \times 10^7 \pm 7.5 \times 10^6$	90 ± 16
<i>P</i> value	0.80	0.82

Conidia were harvested after incubation at 27°C for 72 hr and counted with a hemocytometer. Reproductive capability of 1×10^2 cells on PDA medium at 27°C was assessed after 72 hr. Data are presented as the mean $\pm$ SD.

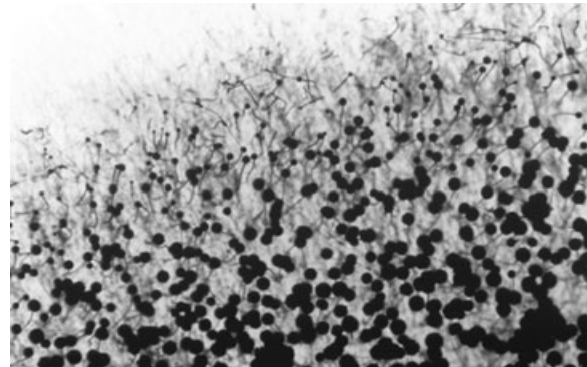


Fig. 2. Stereoscopic microscopic photograph of *A. niger* cultured in the 3D clinostat (100 $\times$ magnification).

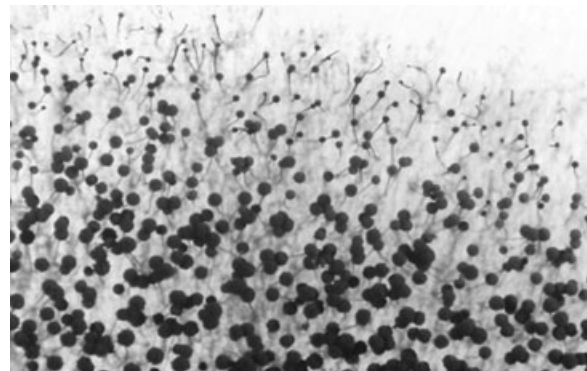


Fig. 3. Stereoscopic microscopic photograph of *A. niger* cultured on the ground (100 $\times$ magnification).

A. niger cultures grown in simulated microgravity and in 1G revealed no significant differences.

Reproductive capability of *Candida albicans* under simulated microgravity

Candida albicans TIMM1768 is an excellent experimental fungal strain for our experiments. It is a virulent strain

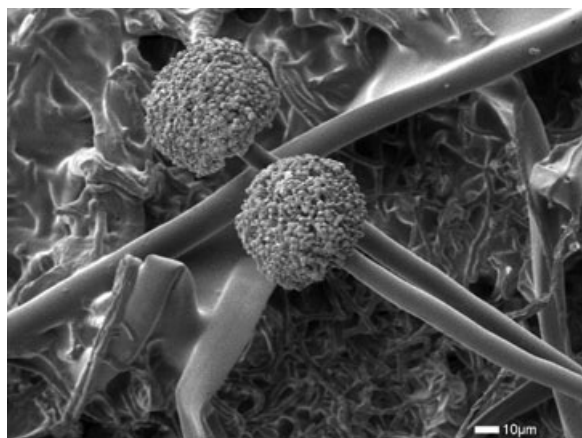


Fig. 4. FE-SEM photograph of *A. niger* cultured in the 3D clinostat. The white bar indicates 10 μm .

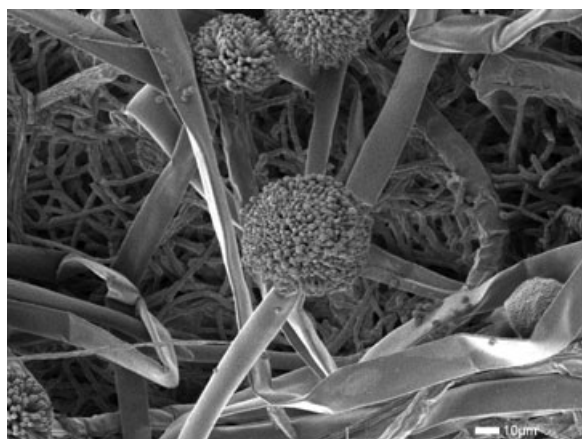


Fig. 5. FE-SEM photograph of *A. niger* cultured on the ground. The white bar indicates 10 μm .

that was isolated from a patient with deep-seated candidiasis and it possesses biological characteristics that are similar to those of standard strains of *C. albicans*. The reproductive capability of *C. albicans* was assessed using the 3D clinostat. Aliquots of 1×10^2 cells of TIMM1768 were spread on YPD plates and the colonies that developed after incubation were counted. As shown in Table 2, the number of colonies incubated in the 3D clinostat was the same as that incubated in 1G.

Antifungal susceptibility assessment for *Candida albicans* under simulated microgravity

To assess changes in antifungal susceptibility of *C. albicans*, the TIMM1768 strain was examined using Etest strips on agar plates containing AMPH-B, CPFG, 5-FC, FLCZ, and ITCZ. Macroscopic observations indicated that all these

agents were equally effective against *C. albicans* cultures incubated in the 3D clinostat and in 1G (data not shown). The MIC values of each agent for TIMM1768 are shown in Table 2. There were no significant differences between the cultures grown in simulated microgravity and those in 1G.

DISCUSSION

All terrestrial organisms are adapted to the 1G environment, and are subjected to very high levels of stress by other amounts of gravity, particularly the microgravity that is present on board spacecraft. Astronauts who have experienced microgravity have reported the stresses associated with weightlessness, and some on-board experiments performed on higher organisms have indicated that they experience stress from a microgravity environment and undergo morphological and/or phenotypic changes when incubated, cultured, or bred under such conditions (21, 23, 24). On the other hand, gene expression and enzyme activity of superoxide dismutase, catalase, and ascorbate peroxidase are reportedly not significantly different between barley grown on the ISS and that grown on the ground (25). Thus, stresses associated with the space environment do not necessarily cause significant changes in organisms at the molecular level.

In the present study, the filamentous fungus *A. niger* showed normal conidia formation and reproduction when cultured in a simulated microgravity environment, and both stereoscopic microscopy and FE-SEM revealed no significant differences between *A. niger* grown under in simulated microgravity and in 1G. Among the filamentous fungi, fruiting bodies of the basidiomycete *Pleurotus ostreatus* showed marked morphological and phenotypic changes in fruiting body formation when the mushroom bed was fixed and the mushrooms bred in a simulated microgravity environment in a 3D clinostat (10). On the other hand, no significant effect was found on fruiting body formation of the cellular slime mold *D. discoideum* in a microgravity environment in spacecraft (26). Possibly, the conidia of filamentous fungi and fruiting bodies of *D. discoideum* are too small and light to undergo morphological changes due to a reduction in gravity. These observations suggest that, like the cellular slime mold *D. discoideum*, *A. niger* would not undergo any changes in morphological characteristics in a microgravity environment. Previously, we performed morphological analyses of six strains of space fungi from samples collected and isolated from the Russian Mir Space Station, and found no significant morphological differences between these and ground-based controls (3). These observations strongly support our predictions that the same would apply for *A. niger*.

Table 2. Reproductive capability and antifungal susceptibility of *Candida albicans*

	Reproductive capability (per 1×10^2 cells)	Minimum inhibitory concentration ($\mu\text{g/mL}$)				
		AMPH-B	CPFG	5-FC	FLCZ	ITCZ
3D clinostat culture group	91 $\pm$ 27	0.102 $\pm$ 0.026	0.091 $\pm$ 0.024	0.099 $\pm$ 0.027	0.265 $\pm$ 0.327	0.068 $\pm$ 0.029
1G culture group	90 $\pm$ 26	0.109 $\pm$ 0.042	0.098 $\pm$ 0.038	0.099 $\pm$ 0.027	0.319 $\pm$ 0.289	0.085 $\pm$ 0.053
P value	0.89	0.55	0.47	0.99	0.58	0.23

Reproductive capability of 1×10^2 cells on YPD medium at 35°C was assessed after 24 hr.

Antifungal susceptibility testing was performed using Etest strips. Data are presented as the mean $\pm$ SD.

Growth ability and antifungal susceptibility of a highly virulent *C. albicans* strain were not changed by the high simulated microgravity stress generated by 3D clinostat rotation. These results strongly suggest that the microgravity environment on board spacecraft would allow the growth of potentially pathogenic fungi, which could contaminate the living environment and cause opportunistic infections or allergies on board as occur in residential areas on the earth. However, astronauts on board spacecraft who become infected with *C. albicans* could be treated with the antifungal agents assessed in this study.

It is not well known how the characteristics of microorganisms change in the space environment. With regard to bacteria, alterations in gene expression reportedly occurred in the pathogen *Salmonella typhimurium* grown on board the Space Shuttle; space flight samples exhibited enhanced virulence in a murine infection model and extracellular matrix accumulation consistent with biofilm formation *in vitro* (24). The present study shows that *A. niger* and *C. albicans* acquire neither increased nor reduced growth capability or altered antifungal agent susceptibility when cultured with 3D clinostat rotation. Although the simulated microgravity conditions produced by a 3D clinostat are not identical to the microgravity conditions on board spacecraft, the severely limited flight resources and necessity for safety in spacecraft make it difficult to perform space experiments using potentially pathogenic microorganisms. Therefore, it is necessary to perform various ground experiments under simulated space conditions produced by a 3D clinostat to predict how terrestrial organisms will change in the space environment. This study indicates the importance of ground experiments for predicting the problems that could be caused by microorganisms on board spacecraft, and to protect astronauts and spacecraft from severe infections or contamination by microorganisms.

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DISCLOSURE

The authors have no financial support or relationships that may result in conflict of interest with any company whose product figures prominently in the submitted manuscript or with any company making a competing product.

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