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Authors

Fan, Shuru
Yue, Huizhen
Zheng, Qiushi
et al.

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Filamentous growth is a general feature of *Candida auris* clinical isolates

Shuru Fan[1], Huizhen Yue[2], Qiushi Zheng[1], Jian Bing[1], Sufei Tian[3], Jingjing Chen[3], Craig L. Ennis[4,5], Clarissa J. Nobile[5], Guanghua Huang[1] and Han Du[1,*]

[1]Department of Infectious Diseases, Huashan Hospital, State Key Laboratory of Genetic Engineering, School of Life Sciences, Institutes of Biomedical Sciences, Fudan University, Shanghai, China, [2]State Key Laboratory of Mycology, Institute of Microbiology, Chinese Academy of Sciences, Beijing, China, [3]Department of Laboratory Medicine, The First Affiliated Hospital of China Medical University, Shenyang, China, [4]Department of Molecular and Cell Biology, University of California, Merced, Merced, USA and [5]Quantitative and Systems Biology Graduate Program, University of California, Merced, Merced, USA

*To whom correspondence should be addressed. Han Du, PhD, State Key Laboratory of Genetic Engineering, School of Life Sciences, Fudan University, Shanghai 200438, China. Tel: +86-21-31246792; Fax: +86-21-31246792; E-mail: handu@fudan.edu.cn

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Abstract

A striking feature of pathogenic *Candida* species is morphological plasticity that facilitates environmental adaptation and host infection. *Candida auris* is an emerging multidrug-resistant fungal pathogen first described in Japan in 2009. In this study, we demonstrate that clinical isolates of *C. auris* have multiple colony and cellular morphologies including the yeast, filamentous, aggregated, and elongated forms. This phenotypic diversity has been observed in eight clinical isolates of *C. auris* representing four major genetic clades, suggesting that it could be a general characteristic. We further demonstrate that different cell types of *C. auris* exhibit distinct antifungal resistance and virulence properties in a *Galleria mellonella* infection model. Our findings imply that morphological diversity is an important biological feature of *C. auris* and could be a contributor to its emergence and rapid prevalence worldwide.

Lay Summary

Candida auris is an emerging multidrug-resistant fungal pathogen. Morphological analyses indicate that filamentation is a general feature of clinical isolates of *C. auris*. This ability is associated with antifungal resistance and virulence.

Key words: *Candida auris*, filamentation, morphological plasticity, virulence, antifungal resistance.

Introduction

Candida auris is an emerging multidrug-resistant threat worldwide.[1,2] This nosocomial fungal species was first described in 2009 in Japan from the ear discharge of a hospitalized woman.[3] To date, *C. auris* has been implicated in infections in at least 32 countries with predominance in hospital Intensive Care Units (ICUs). Recently, several hospital outbreaks with a range of *C. auris* infections including bloodstream infections, wound infections, genital and respiratory tract infections, and otitis media have been reported.[4–8] Due to its innate antifungal resistance, invasive *C. auris* infections are challenging to treat and are associated with high mortality rates. *C. auris* cells are also extremely difficult to remove from surfaces using standard disinfection methods, such as wipes containing quaternary ammonium compounds. The ability of this fungus to survive in the

environment facilitates its persistence and transmission in hospital settings. Recently, it was reported that an outbreak of *C. auris* in the Oxford University Hospitals was linked to the use of reusable skin surface axillary temperature probes.[6]

Morphological plasticity is a strategy used by fungi to adapt to different ecological niches and changing environments.[9] Cell types of a certain morphology often have advantages over other cell types in specific niches. For example, the major human fungal pathogens, *Candida albicans* and *Candida tropicalis*, undergo morphological transitions between a single-celled yeast form and a multicellular filamentous form in response to specific environmental cues,[9] and this plasticity is critical for virulence in the host.

C. auris is known to produce rudimentary pseudohyphae, cellular aggregates, and biofilms under specific environmental conditions.[10,11] Aggregative and nonaggregative *C. auris* cells differ in their virulence, antifungal susceptibilities, and abilities to form biofilms.[11–13] It had long been thought that *C. auris* was unable to undergo filamentation (to form elongated hyphal and pseudohyphal cells). One recent study, however, found that the *C. auris* strain BJCA001, isolated from China, was able to develop filaments after passage through the mouse.[14] Here, we demonstrate that several morphological phenotypes exist for *C. auris* and that all tested clinical isolates encompassing four major genetic clades are capable of developing long filaments under specific environmental conditions. This morphological diversity could, therefore, be a general feature of *C. auris* clinical isolates.

Methods

Strains and culture conditions

Candida auris strains were routinely grown in YPD medium (20 g/L peptone, 10 g/L yeast extract, 20 g/L glucose; for solid medium, 20 g/L agar was added). To facilitate our observation of different *C. auris* morphologies, the red dye phloxine B (5 μg/mL) was added to the solid medium. Colonies of different cell types could be differentially stained due to their distinct cell wall structures and permeabilities.

For the morphological assays, *C. auris* cells were initially grown on YPD medium at 25°C for 5 days. For the *in vitro* induction of morphological changes, fungal cells from several colonies were re-plated onto YPD medium containing phloxine B and cultured at 25°C for 7–10 days. To induce yeast-to-filament switching *in vivo*, *C. auris* cells were injected into mice via the tail vein and subsequently recovered from the mouse organs. Fungal cells were then plated onto YPD medium containing phloxine B and cultured at 25°C for 7–10 days. Determinations for the robustness of filamentation was based on the average length of the filamentous cells for each sample.

Microscopy assays

Cells grown on YPD medium were collected and used for morphological analyses as described previously.[14,15] Briefly, differential interference contrast (DIC) optics were used for standard cellular morphology assays. The 4′,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, Inc., Beijing) was used for nuclear staining, and Calcofluor white (Sigma-Aldrich, Inc., Beijing) was used for septa/chitin staining. Scanning electron microscopy (SEM) assays were performed as previously described.[14,15]

Animal experiments

All animal experiments were performed according to the guidelines approved by the Animal Care and Use Committee of the Institute of Microbiology, Chinese Academy of Sciences.

Before being used for morphological analyses, cells of *C. auris* strains BJCA001, CBS 12 373, and CBS 12 766 were passaged through the mouse and recovered from the liver or kidney as described previously.[14] *C. auris* cultures with different morphologies were first diluted to an OD_{600} of 1.0. Samples of equal volumes were spun down, resuspended in 1× PBS, and used for virulence assays. Cells of *C. auris* (~2×10^7 cells) were suspended in 250 μL 1× PBS and intravenouslyinjected into each mouse. Following 24 h of infection, mice were humanely euthanized. Fungal cells recovered from the kidney and liver samples of intravenously infected mice were used for *in vitro* morphological analyses.

Minimal inhibitory concentration assays

Minimal inhibitory concentrations(MICs) of different cell types were determined according to the CLSI guidelines document M27-A2 and a previous report.[16] Liquid RPMI-1640 medium (w/v, 1.04% RPMI-1640, 3.45% MOPs, NaOH used for pH adjustment to 7.0) containing different antifungals in a series of concentrations was used. *C. auris* cells with different morphologies were first diluted to an OD_{600} of 1.0. Samples of equal volumes were spun down, re-suspended in fresh medium, and used for MIC assays. *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22 019 strains served as controls.

Galleria mellonella infection model

Virulence assays were performed as previously described.[15] *Galleria mellonella* in the final instar larval stage were purchased from Tianjin Huiyu biological technology Co. LTD. (Tianjin, China). Larvae with a similar size (0.3–0.4 g) were used for infection assays. Different types of *C. auris* cells were cultured on YPD medium at 30°C for 2 days and then transferred to 25°C for 5 additional days of incubation. Cells (1×10^6 in 10 µL 1× PBS) were then collected, washed twice with 1× PBS, and injected into each larva. After injection, the larvae were placed in plastic culture dishes and incubated at 30°C and 37°C in the dark.

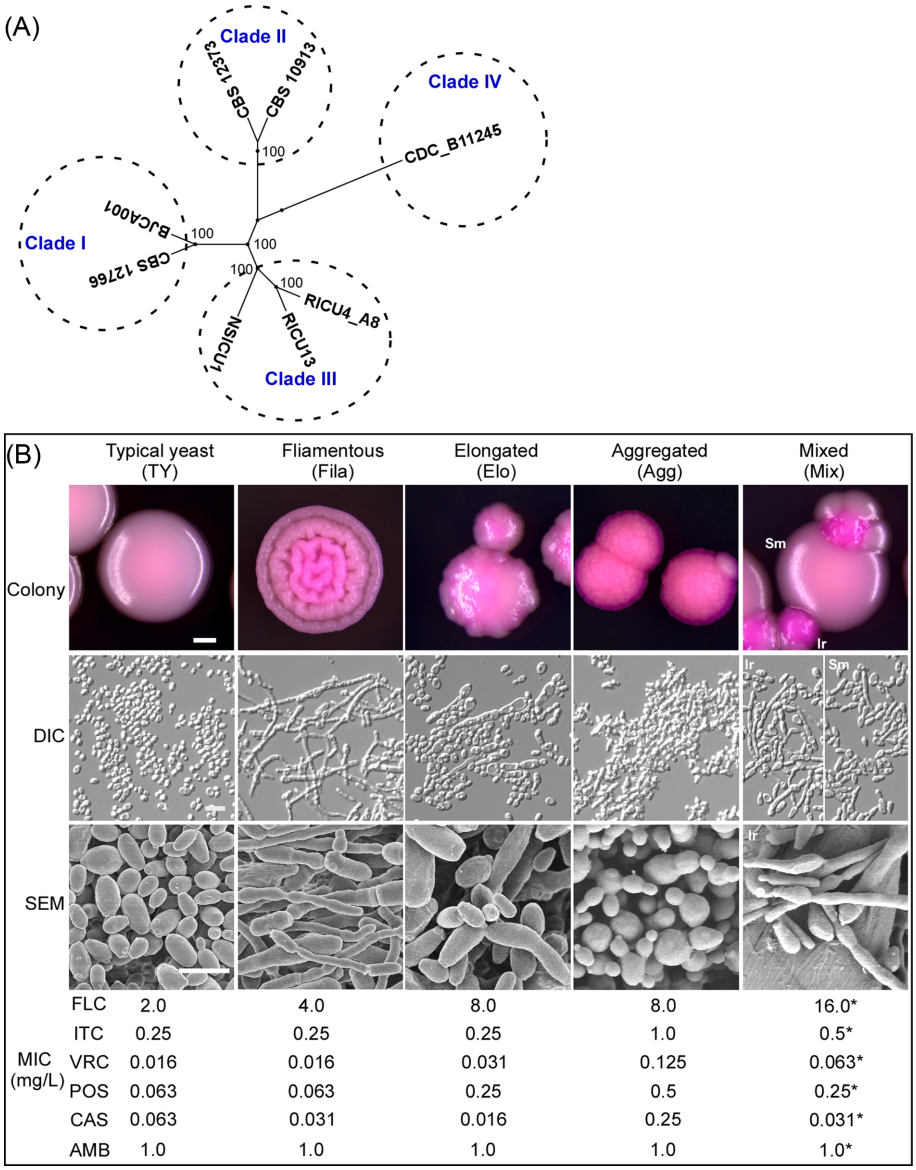

Figure 1. Morphological analysis of clinical isolates of *C. auris*. (A) Phylogenetic tree of representative isolates of four clades. The phylogenetic tree was generated by the Maximum-Likelihood (ML) method based on the internal transcribed spacer (ITS) sequences. (B) Colony and cellular morphologies of strain BJCA001. Fungal cells were grown at 25°C for 7 days. Colonies were imaged and fungal cells were subject to differential interference contrast (DIC) and scanning electron microscopy (SEM) imaging. MIC values of different cell types with different antifungals are indicated below the corresponding images. Ir, irregular phenotype; Sm, smooth. Scale bars: for colonies: 1 mm; for cells (DIC): 10 μm; for cells (SEM): 5 μm. FLC, fluconazole; ITC, itraconazole; VRC, voriconazole; POS, posaconazole; CAS, caspofungin; AMB, amphotericin B. MIC, minimal inhibitory concentration (μg/mL).

Results and discussion

Morphological phenotypes of clinical isolates

We used eight clinical isolates of *C. auris* representing four distinct genetic clades and two mating types (*MTL***a** and *MTLα*) and performed morphological analyses (Figures 1 and 2, and Table S1). Strains BJCA001 and CBS 12766 belong to clade I (South Asian); CBS 12373 and CBS 10913 belong to clade II (East Asian); RICU4, RICU13, NSICU1 belong to clade III (African), and B11245 belongs to clade IV (South American). The mating type of strains BJCA001, CBS 12766, and B11245 is *MTL***a**; the mating type of strains RICU4, RICU13, NSICU1, CBS 10913, and CBS 12373 is *MTLα*.

Before being used for morphological analyses, cells of *C. auris* isolates were passaged through the mouse and recovered from tissues as described previously.[14] Recovered cells were plated on YPD medium for seven days of growth at 30°C. Cells of single colonies with distinct appearances were then replated onto YPD medium containing 5 μg/mL phloxine B. After 7 days of incubation at 25°C, colony and cellular morphologies were examined (Figures 1 and 2). As shown in Figure 1B, five distinct types of colonies and three distinct cellular morphologies were

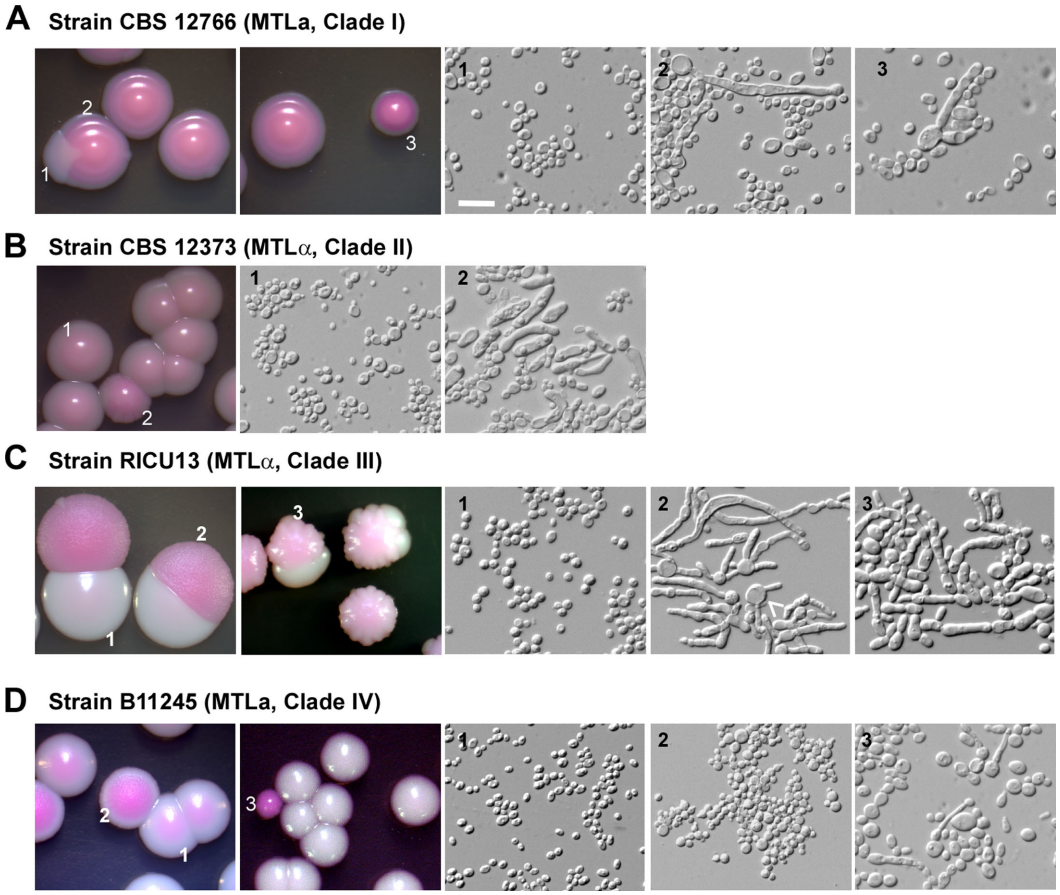

Figure 2. Colony and cellular morphologies of four clinical isolates of *C. auris*. Before being used for morphological analysis, fungal cells were grown on regular laboratory medium. Cells were incubated on YPD medium containing 5 μg/mL red dye phloxine B at 25°C for 7 days. (A) Strain CBS 12766. (B) Strain CBS 12766. (C) Strain RICU13. (D) Strain B11245. Numbers indicate distinct colony phenotypes and corresponding cellular morphologies. Scale bar, 10 μm.

observed in strain BJCA001 by light microscopy and scanning electron microscopy (SEM). Cells of the typical yeast (TY) form were round and morphologically similar to yeast-form cells of *C. albicans* and formed smooth colonies; filamentous (Fila) cells were long and resembled true hyphae of *C. albicans* and formed highly wrinkled colonies; elongated (Elo) cells were larger and longer than TY cells. Interestingly, the *C. auris* colonies of different morphotypes often contained a homogeneous cell population. Although it could switch to a different phenotype at a very low frequency under *in vitro* culture conditions, *C. auris* often maintained the original phenotype for many generations. This observation is consistent with our previous report on the yeast-filament switch in *C. auris*.[14] It remains to be investigated whether the switch among different morphotypes is genetically or epigenetically regulated.

Calcofluor White staining assays indicated that there are chitin rings in filamentous and elongated cells of *C. auris*. DAPI staining assays indicated that filamentous cells often contained two or more nuclei (Figure 3), suggesting that *C. auris* is capable of developing multicellular forms.

Morphological plasticity is independent of host induction

To investigate whether the process of passaging through the animal body can induce morphological changes in *C. auris* clinical isolates, we performed *in vitro* induction assays with the eight *C. auris* clinical isolates. Yeast-form cells were first grown on YPD medium at 25°C for 10 days. Cells of single colonies with distinct morphologies were replated on YPD medium (Figure 2 and Table S1). Two or more cellular morphologies were observed in different clinical isolates after seven days of growth at 25°C. Morphologies of one representative strain for each genetic clade are shown in Figure 2. Detailed results for all isolates are summarized in Table S1. Strain BJCA001 formed aggregated (Agg) and morphologically irregular colonies. Cells of the aggregated phenotype were tightly linked and formed rough and red colonies (in the presence of phloxine B), whereas colonies with the irregular phenotype contained both yeast and elongated cells (mixed type, Mix). All strains underwent filamentation at 25°C either after being passaged through the mouse or

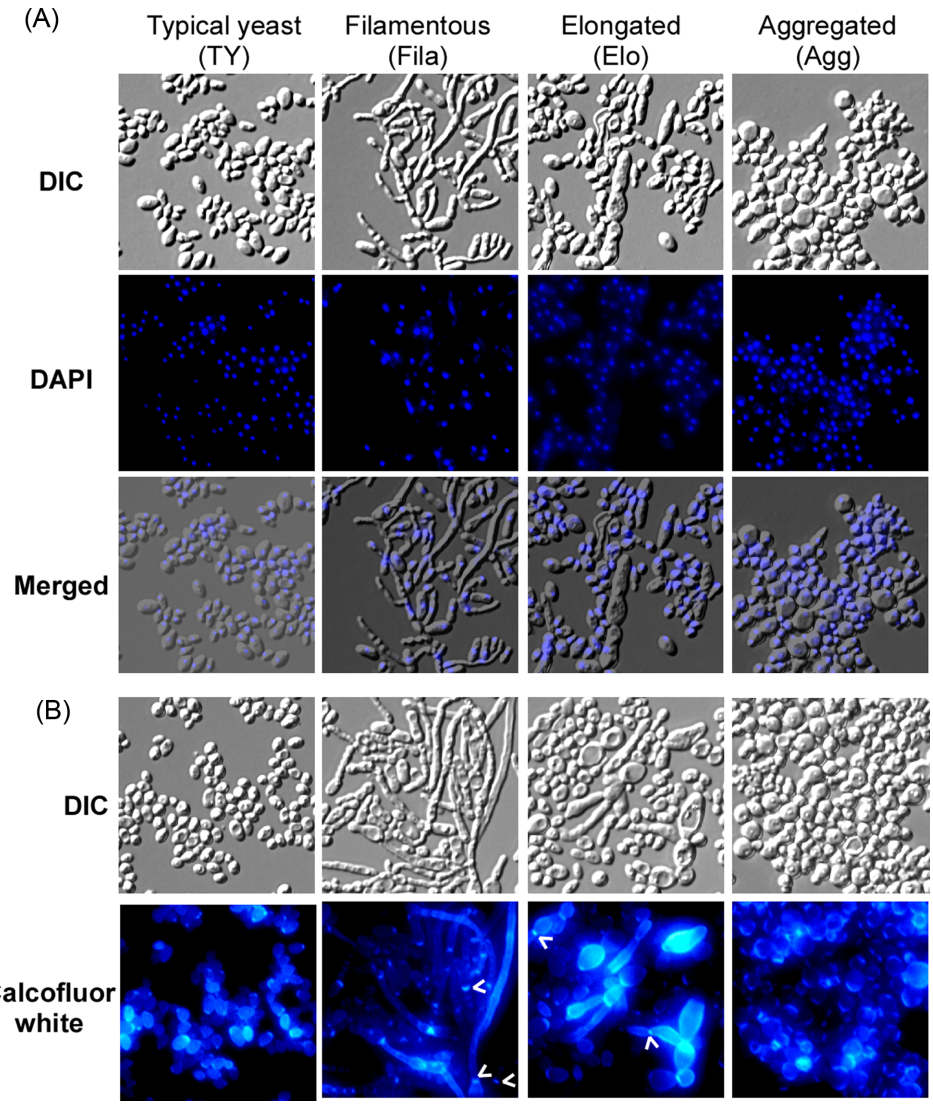

Figure 3. Calcofluor White and DAPI staining of *C. auris* cells.Cells of different morphological types were grown on YPD medium at 25°C for seven days and stained with DAPI and Calcofluor White. White arrows indicate septin rings. TY, typical yeast; Fila, filamentous; Elo, elongated form; Agg, aggregated form. DIC, Differential interference contrast.

induced by the *in vitro* medium (Figure 2 and Table S1), suggesting that filamentous growth in *C. auris* could be independent of host induction.[14] Interestingly, a small portion of cells of RICU13 exhibited a unique morphology, where two or multiple hyphae were often observed to bud from a single round yeast cell (Figure 2C).

Morphological diversity is associated with antifungal resistance and virulence

We previously demonstrated that cells of the TY form of strain BJCA001 were sensitive to all antifungal drugs tested.[15] Antifungal susceptibility tests showed that different cell types of BJCA001 exhibited a variation of MICs of six different antifungals (Figure 1B). For example, the MICs for fluconazole varied from 2.0 to 16 mg/L, whereas those of posaconazole were from 0.063 to 0.5 mg/L. These results suggest that morphological diversity is associated with antifungal resistance.

We next performed virulence assays with different cell types of strains BJCA001 and RICU13 using a *Galleria mellonella* infection model at 30°C and 37°C.[15] As shown in Figure 4, filamentous or elongated cells of both strains exhibited a higher killing effect on the *G. mellonella* larvae at both temperatures. However, compared to the other two cell types, both the aggregated (Agg) and the yeast (TY) forms of BJCA001 showed a decreased level of virulence. Consistently, for strain RICU13, cells of the yeast form also exhibited a lower level of virulence than that of the two observed filamentous cell types. These results are consistent with those of a previous study demonstrating that aggregate-forming isolates of *C. auris* were less virulent than nonaggregating isolates in a *G. mellonella* infection

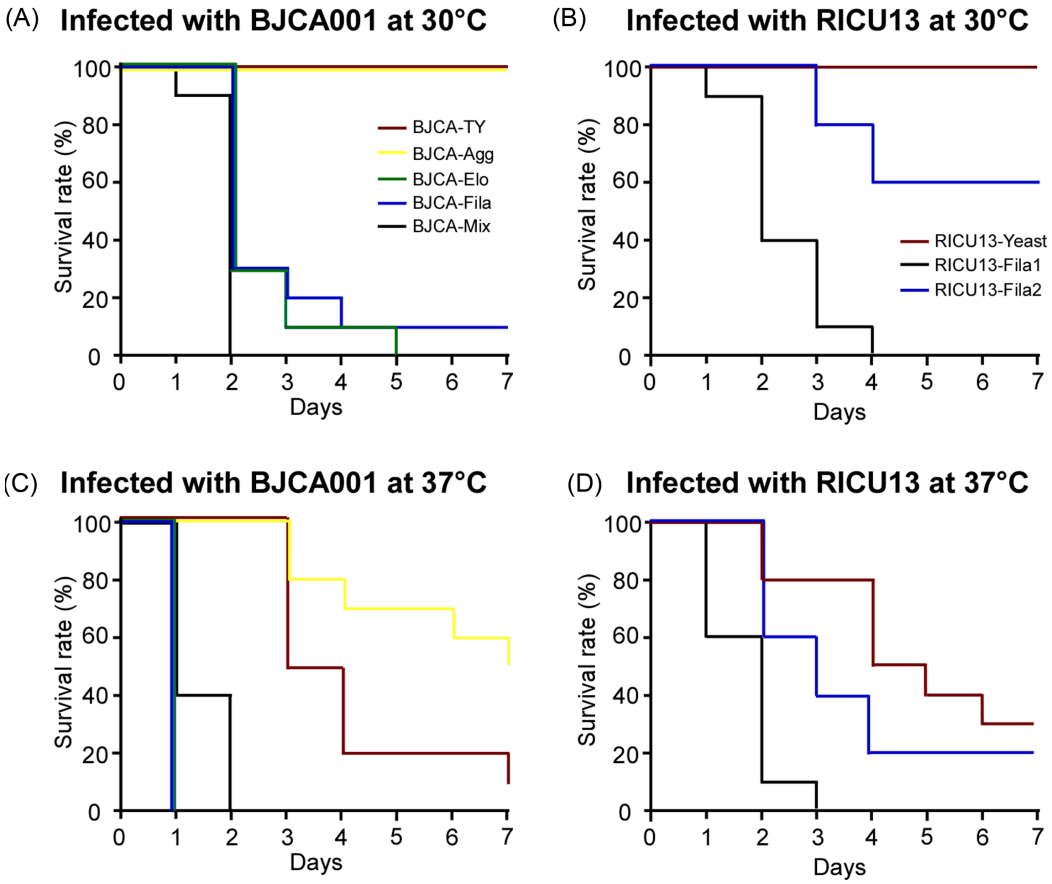

Figure 4. Virulence of different cell types of *C. auris* in a *G. mellonella* infection model. (A and C) Survival curves of *G. mellonella* infected strain BJCA001 (*MTL***a**) at 30°C and 37°C. TY, typical yeast; Agg, aggregated form; Elo, elongated form; Fila, filamentous; Mix, yeast-filament mixed. (B and D) Survival curves of *G. mellonella* infected strain RICU13 (*MTL*α) at 30°C and 37°C. Yeast, yeast cells; Fila1, filamentous form 1; Fila2, filamentous form 2. Fila1 and Fila2 represent two distinct filamentous cell types. 1×10^6 fungal cells of each cell type were injected into each larva of *G. mellonella*. A filament may represent multiple cells. The injected amounts of fungal cells were normalized to that of the yeast form based on cell concentrations (determined by OD600). Ten larvae were injected for each strain.

model.[15] Interestingly, all morphological phenotypes exhibited a higher virulence at 37°C than at 30°C. This increased virulence for *C. auris* could be due to an increase in the overall growth rate or to the secretion of virulence factors that is enhanced at 37°C.[14]

C. auris belongs to the *Metschnikowiaceae* family of the CTG clade. It is emerging as a significant threat worldwide due to its recalcitrance to disinfectants and its multidrug-resistant properties. The biological features contributing to its prevalence and rapid emergence remain largely unknown.

Morphological plasticity plays a critical role in adaptation to environmental changes and virulence in pathogenic fungi. In this study, we investigated the morphological plasticity, specifically filamentous growth, of *C. auris* and found that it has multiple cellular morphologies. The filamentous phenotype we observed for *C. auris* appeared phenotypically similar to the filaments of *C. albicans*. Combined with its antifungal resistance properties, the ability of *C. auris* to undergo morphological transitions could be a contributing factor in its emergence and rapid prevalence worldwide.

Supplementary material

Supplementary data are available at *MMYCOL* online.

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Declaration of interest

Clarissa J. Nobile is a cofounder of BioSynesis, Inc., a company developing inhibitors and diagnostics of biofilm formation.

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