Filamentation Is Associated with Reduced Pathogenicity of Multiple Non-albicans Candida Species

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ABSTRACT Candidiasis affects a wide variety of immunocompromised and medically compromised patients. Candida albicans, a major human fungal pathogen, accounts for about 50% of all cases, while the remainder are caused by the less pathogenic non-albicans Candida species (NACS). These species are believed to be less pathogenic, in part, because they do not filament as readily or robustly as C. albicans, although definitive evidence is lacking. To address this question, we used strains for two NACS, Candida tropicalis and Candida parapsilosis, which were genetically engineered to constitutively express the key transcriptional regulator UME6 and drive strong filamentation both in vitro and during infection in vivo. Unexpectedly, both strains showed a dramatic reduction in organ fungal burden in response to UME6 expression. Consistent with these findings, we observed that a C. tropicalis hyperfilamentous mutant was significantly reduced and a filamentation-defective mutant was slightly increased for organ fungal burden. Comprehensive immune profiling generally did not reveal any significant changes in the host response to UME6 expression in the NACS that could explain the increased clearance of infection. Interestingly, whole-genome transcriptional profiling indicated that while genes important for filamentation were induced by UME6 expression in C. tropicalis and C. parapsilosis, other genes involved in a variety of processes important for pathogenesis were strongly downregulated. These findings suggest that there are fundamental evolutionary differences in the relationship between morphology and pathogenicity among Candida species and that NACS do not necessarily possess the same virulence properties as C. albicans.

IMPORTANCE Many immunocompromised individuals, including HIV/AIDS and cancer patients, are susceptible to candidiasis. About half of all cases are caused by the major fungal pathogen Candida albicans, whereas the remainder are due to less pathogenic non-albicans Candida species (NACS). Generation of filamentous cells represents a major virulence property of C. albicans, and the NACS are believed to be less pathogenic, in part, because they do not filament as well as C. albicans does. To address this question, we determined the pathogenicity of two NACS strains that have been genetically engineered to promote filamentation during infection. Surprisingly, these strains showed a dramatic reduction in pathogenicity. The host immune response did not appear to be affected. However, unlike C. albicans, filamentation of the NACS was associated with downregulation of several genes important for pathogenicity processes. Our results suggest that there are fundamental evolutionary differences in the relationship between filamentation and pathogenesis in NACS compared to C. albicans.

KEYWORDS candidiasis, infectious disease, mycology, morphology, pathogenicity, Candida species, evolution, filamentation, gene expression

Candida species account for the large majority of human fungal infections. These species can cause both mucosal infections, such as oral and vaginal thrush, and more serious life-threatening systemic bloodstream infections (1–3). Individuals with a
compromised immune system, including cancer patients on chemotherapy, organ transplant recipients, and HIV/AIDS patients, are highly susceptible to infection (2, 3). Approximately 50% of all Candida bloodstream infections in the United States can be attributed to Candida albicans, while the remainder are due to a variety of inherently less pathogenic non-albicans Candida species (NACS) (4). Infections by NACS are on the rise, and several of these species show increased resistance to commonly used antifungal therapies (5).

C. albicans possesses a variety of virulence traits, including the ability to undergo a reversible morphological transition from single budding yeast cells to filaments, which are best described as elongated cells attached end to end (6). Multiple independent lines of evidence have suggested a strong association between the C. albicans yeast-filament transition and virulence (7–11), including our previous demonstration that constitutive high-level expression of the filament-specific transcriptional regulator UME6 promotes this morphological transition and enhances C. albicans virulence, as well as tissue invasion, in a mouse model of systemic candidiasis (12).

Considerably less research has focused on NACS. These species are thought to be less pathogenic than C. albicans for a variety of reasons, including a reduced ability to adhere to host cells, secrete degradative enzymes, and form biofilms (13, 14). They are also generally more sensitive to cell stresses encountered in the host environment and do not filament as readily or robustly as C. albicans (14). We have previously shown that certain C. albicans morphological regulatory functions are evolutionarily conserved in several NACS, including Candida tropicalis and Candida parapsilosis (15). As in C. albicans, orthologs of UME6 are transcriptionally induced in these species during filamentation, although at a reduced level and, in the case of C. parapsilosis, with delayed timing. In addition, as is the case for C. albicans, constitutive high-level expression of UME6 orthologs is sufficient to promote strong filamentation in C. tropicalis and C. parapsilosis; orthologs of several, but not all, C. albicans filament-specific genes were also induced in response to UME6 expression in these species (15).

In order to determine the specific effect of filamentation on the pathogenicity of C. parapsilosis and C. tropicalis during infection in vivo, we used our previously constructed tetO-CTUM6 and tetO-CpUME6 strains (15). In the absence of doxycycline (Dox), a repressor of the tetracycline operator (tetO), one allele of UME6 in these strains is expressed at constitutive high levels, generating a highly filamentous morphology, whereas in the presence of Dox this allele is shut off and cells grow as yeast. Both C. tropicalis and C. parapsilosis tetO-UME6 strains were used to inoculate female BALB/c mice by tail vein injection. Half the mice in each group were supplied with drinking water containing Dox. At day 16 postinfection, all mice were sacrificed and kidneys were harvested for fungal burden analysis. Unexpectedly, as shown in Fig. 1A, there was a significant reduction in fungal burden in the −Dox versus +Dox group for both species. Indeed, fungal burdens in the −Dox groups were below the limit of detection, suggesting that the infections had cleared. In a similar experiment, mice sacrificed at 24 h postinfection showed equivalent kidney (Fig. 1B) and brain (data not shown) fungal burdens in both +Dox and −Dox groups for each species. In order to determine the time course for organ clearance and whether clearance occurred in multiple organs, we next performed a timed-sacrifice experiment using both C. parapsilosis and C. tropicalis tetO-UME6 strains and examined fungal burdens in both kidneys and brains at different days postinfection (Fig. 1C). Interestingly, at the day 3 postinfection time point the +Dox and −Dox groups inoculated with the C. parapsilosis tetO-UME6 strain showed roughly equivalent kidney fungal burdens, although fungal burden was partly reduced in the brains of −Dox versus +Dox animals. At day 6 and day 10, there was a dramatic reduction in both kidney and brain fungal burden in the −Dox versus +Dox groups. As in the previous experiment, fungal burdens for the −Dox groups at these later time points were below the limit of detection. A similar trend in results, though less pronounced, was observed for the C. tropicalis tetO-UME6 strain (Fig. 1C). Interestingly, these results suggested that constitutive high-level UME6 expression is sufficient to significantly reduce both C. parapsilosis and C. tropicalis fungal burden, eventually
FIG 1 Filamentation is associated with reduced fungal burden of *C. tropicalis* and *C. parapsilosis* in a mouse model of systemic candidiasis. (A) *C. tropicalis* ($4 \times 10^4$ CFU) and *C. parapsilosis* ($4 \times 10^6$ CFU) tetO-UME6 strains were used to inoculate female BALB/c mice (6 to 8 weeks old) by tail vein injection. Half

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leading to clearance of the infection from multiple organs. Histological analysis of infected kidneys was performed to confirm that UME6 expression was able to promote filamentation during infection in vivo. As shown in Fig. 1D, both strains were observed to grow primarily in the yeast form in infected kidneys from the +Dox group. In contrast, in the absence of Dox the C. tropicalis tetO-UME6 strain was found as a mixture of yeast and filaments and the C. parapsilosis tetO-UME6 strain grew primarily as filaments (Fig. 1D). In order to determine whether organ clearance was more generally associated with morphology, mice were inoculated with either a hyperfilamentous C. tropicalis nrg1Δ/Δ mutant (15) or a filamentation-defective C. tropicalis hgc1Δ/Δ strain (16). Consistent with our previous results, the nrg1Δ/Δ mutant showed significantly reduced fungal burden compared to the wild-type (WT) strain in both the kidneys and brain (Fig. 1E). Also, in contrast to most filamentation-defective mutants of C. albicans, the C. tropicalis hgc1Δ/Δ mutant showed slightly increased organ fungal burdens compared to those of the WT control (Fig. 1F).

We next sought to determine whether organ clearance that is observed in response to UME6 expression in C. tropicalis and C. parapsilosis occurs as the result of an altered host response. Kidney homogenates were prepared from mice placed on drinking water in the presence or absence of Dox, inoculated with C. tropicalis and C. parapsilosis tetO-UME6 strains, and sacrificed at day 1 postinfection when fungal burdens are equivalent in +Dox and −Dox groups. These homogenates were then used to carry out comprehensive multianalyte profiling of over 50 different cytokines, chemokines, and other host markers of infection. In general, we did not detect significant differences in the levels of these analytes when comparing −Dox with +Dox groups, which could indicate an altered immune response (see Fig. S1 in the supplemental material). However, we did observe a significant reduction in both the C. tropicalis and C. parapsilosis −Dox versus +Dox ratio for myoglobin. Given that myoglobin levels are known to be correlated with tissue damage (17), these results are consistent with our previous findings and suggest that greater tissue damage occurs in response to C. tropicalis and C. parapsilosis yeast than to filamentous cells.

We were also interested in gaining a better understanding of the basis for organ clearance in response to UME6 expression in C. tropicalis and C. parapsilosis. Thus, we performed whole-genome transcriptional profiling experiments. Both tetO-CtUME6 and tetO-CpUME6 strains were grown in the presence and absence of Dox to generate yeast and filaments (Fig. S2), respectively, and cells were harvested for RNA preparation and transcriptome sequencing (RNA-seq) analysis. As indicated in Fig. 2A as well as Tables S1 and S2, we observed that large sets of genes showed significantly increased and

**FIG 1 Legend (Continued)**

the mice were placed on drinking water with 2 mg/ml Dox (n = 5 mice/group). All mice were sacrificed at 16 days postinfection, kidneys were harvested, and fungal burdens were determined. For both species, the reduction in fungal burden in the −Dox versus +Dox groups was statistically significant (*) as determined by a Mann-Whitney test. (B) The experiment in panel A was repeated using 2.1 × 10^6 CFU of C. tropicalis and 4.2 × 10^6 CFU of C. parapsilosis tetO-UME6 strains, and all mice were sacrificed at 24 h postinfection for kidney fungal burden determination. (C) The experiment in panel A was repeated for the C. parapsilosis C. tropicalis tetO-UME6 strains using inoculum sizes of 4.5 × 10^6 CFU and 3.0 × 10^6 CFU, respectively. Mice were sacrificed at the indicated postinfection time points, and fungal burdens were determined for the indicated organs (*, P < 0.05, using a Mann-Whitney test). Please note that the fungal burden value for kidneys infected with the C. tropicalis tetO-UME6 strain in the absence of Dox at day 6 represents the lower limit of detection. (D) Examples showing the effect of UME6 expression on C. tropicalis and C. parapsilosis morphology during infection in vivo. Kidneys from mice infected with tetO-UME6 strains from the indicated species were harvested, fixed, embedded in paraffin, stained with Grocott-Gomori methenamine silver (GMS), and visualized by light microscopy (fungal cells shown in black). Black arrows indicate yeast cells. (E) The C. tropicalis WT (3.7 × 10^4 CFU and nrg1Δ/Δ (3.5 × 10^5 CFU) strains were used to inoculate female BALB/c mice (6 to 8 weeks old) by tail vein injection (n = 5). All mice were sacrificed at 6 days postinfection, kidneys were harvested, and fungal burdens were determined. The reduction in fungal burdens in mice infected with nrg1Δ/Δ versus WT strains was statistically significant (*, P < 0.01) as determined by a Mann-Whitney test. (F) The experiment in panel E was repeated using 2.9 × 10^4 and 3.1 × 10^4 CFU of C. tropicalis WT and hgc1Δ/Δ strains, respectively. The increase in kidney fungal burden in mice infected with hgc1Δ/Δ versus WT strains was statistically significant (*, P < 0.05) as determined by a Mann-Whitney test.
Transcriptional profile of *C. tropicalis* and *C. parapsilosis* in response to UME6 expression. (A) Scatter plots showing gene expression changes for *C. tropicalis* and *C. parapsilosis* tetO-UME6 strains grown in the absence versus presence of doxycycline (Dox). Axes represent log2(averaged normalized read counts) for /H11001Dox and /H11002Dox. Transcripts that are differentially expressed ≥2-fold (dashed lines) are indicated in red (upregulated) and green (downregulated). Transcripts of interest are labeled in black. (B) GO slim mapper (www.candidagenome.org) analysis showing percent representation of process gene classes in the sets of genes induced ≥2-fold in response to UME6 expression in *C. tropicalis* and *C. parapsilosis*. Gene classes showing less than 4% representation are not shown. (C) Venn diagrams showing overlap of gene orthologs that are upregulated or downregulated in response to UME6 expression in *C. tropicalis, C. parapsilosis, and C. albicans*. *C. albicans* genes showing differential expression in response to UME6 induction have been described previously (18).
decreased expression in response to UME6 induction in both strains. In contrast, as expected, very few genes showed significant expression changes in the absence versus presence of Dox for both C. tropicalis and C. parapsilosis WT control strains. A gene ontology (GO) analysis identified several common gene classes that were induced in response to UME6 expression in both C. tropicalis and C. parapsilosis (Fig. 2B and Data Sets S1 and S3). In addition to filamentous growth, as expected, these gene classes included organelle organization, stress response, cell cycle, and transport. In C. parapsilosis, genes involved in amino acid metabolism were overrepresented compared to the genome as a whole, whereas genes associated with mitosis and the cell cycle were overrepresented in C. tropicalis. Among downregulated genes, those involved in carbohydrate metabolism were overrepresented in both C. tropicalis and C. parapsilosis, whereas genes associated with lipid catabolic processes were strongly overrepresented only in C. parapsilosis (Data Sets S2 and S4).

Orthologs of several genes known to be induced in response to C. albicans UME6 expression (18) were also induced in both C. tropicalis and C. parapsilosis. As an independent confirmation of our RNA-seq results, we have previously demonstrated by Northern analysis that a few of these genes (e.g., UME6, HGC1, and PHR1) are induced in both NACS (15). In addition to HGC1, which encodes a cyclin-like protein important for septin phosphorylation (19), a number of other genes that play direct roles in the filamentation process, including the CDC37 septin and SHE3 mRNA-binding protein, were induced in all three Candida species (Fig. 2A and Data Sets S1 and S3); the CDC11 septin, previously shown to be induced by UME6 in C. albicans (18), was also induced in C. tropicalis. Interestingly, however, orthologs of several C. albicans genes involved in processes important for pathogenesis were significantly downregulated in response to UME6 expression in both C. tropicalis and C. parapsilosis but not C. albicans (Fig. 2A and Data Sets S2 and S4) (18). These genes included SOD2, a superoxide dismutase important for combating oxidative stress, and YHB1, important for adapting to nitrosative stress, as well as a variety of genes important for iron, zinc, and copper transport/regulation (e.g., CTR1, ZRT1, ZRT2, and FRE9). Multiple members of the secreted aspartyl protease (SAP) gene family as well as the PLB3 phospholipase, which is likely important for host cell degradation, were also downregulated in response to UME6 expression in both C. tropicalis and C. parapsilosis. As in C. albicans (18), CNRG1, previously shown to function as a repressor of filamentation in C. tropicalis (15), was downregulated in response to CtUME6 expression, which is again consistent with the notion that mechanisms important for induction of filamentation by UME6 are conserved among Candida species. A three-way comparison of gene orthologs regulated by UME6 in C. tropicalis, C. parapsilosis, and C. albicans (Fig. 2C) revealed that orthologs of about one-third of C. albicans genes are upregulated in C. tropicalis and/or C. parapsilosis whereas orthologs of over one-half of C. albicans genes downregulated by UME6 were also downregulated in C. tropicalis and C. parapsilosis. In the future, it will be useful to determine whether similar gene expression patterns are observed in response to expression of other activators of filamentation in NACS.

In this study, we have used a variety of approaches to more specifically define the relationship between morphology and pathogenicity in two NACS, C. tropicalis and C. parapsilosis. Surprisingly, unlike the situation in C. albicans, we find that under our experimental conditions the transition from yeast cells to filaments is associated with reduced pathogenicity as well as reduced expression of certain genes involved in pathogenicity-related processes. At this point, however, it is unclear whether the clearance effect is due to filamentation per se or some other process associated with filamentation (e.g., lowered expression of pathogenicity genes). With respect to C. parapsilosis, our results are consistent with those of a previous study demonstrating that the ability of this species to invade the oral epithelium does not correlate with formation of pseudohyphae (20). Also in support of our findings, a different study has found that a hyperfilamentous C. parapsilosis mutant is significantly attenuated for pathogenicity in both a Galleria model and a mouse model of candidiasis (21). While previous reports have suggested a correlation between C. tropicalis filamentation ability
and the ability to invade/damage epithelial cells, it is important to note that these experiments were carried out using in vitro systems rather than animal models (22, 23). A previous study showing this correlation during infection used immunosuppressed animals and a significantly larger inoculum size (24). We have also observed that increasing inoculum size for the tetO-CtUME6 strain reduces the organ clearance effect (our unpublished results). An independent previous study has shown, by histological analysis, that C. tropicalis cells injected at smaller inoculum sizes equivalent to those used in our experiments typically grow in the yeast form, whereas greater inoculum sizes of C. tropicalis are correlated with an increased proportion of filamentous cells in kidneys (25). Interestingly, our results suggest that forcing filamentation of C. tropicalis at smaller inoculum sizes, at which cells would otherwise grow in the yeast form during infection in vivo, leads to organ clearance. Importantly, these findings suggest not only that filamentation can confer an evolutionary disadvantage for C. tropicalis and C. parapsilosis during infection but also that there are fundamental evolutionary differences in the relationship between morphology and pathogenicity among Candida species. More specifically, in NACS other processes that are independent of the morphological transition are likely to play a more prominent role in pathogenesis. On a broader level, our findings are significant because they suggest that not everything we learn about pathogenicity and virulence-related processes in C. albicans can be directly applied to NACS, but rather the virulence traits of these species need to be studied in their own right.

For a detailed description of experimental procedures used in this study, please see Text S1. All research using animals was approved by the Institutional Animal Care and Use Committee at The University of Texas at San Antonio.

Data availability. Raw RNA sequencing data for this study have been deposited at the NCBI Gene Expression Omnibus (GEO) database (accession number GSE134321).

SUPPLEMENTAL MATERIAL
Supplemental material for this article may be found at https://doi.org/10.1128/mSphere.00656-19.

TEXT S1, DOCX file, 0.04 MB.
FIG S1, PDF file, 0.01 MB.
FIG S2, PDF file, 0.6 MB.
TABLE S1, DOCX file, 0.02 MB.
TABLE S2, DOCX file, 0.02 MB.
TABLE S3, DOCX file, 0.01 MB.
DATA SET S1, XLSX file, 0.3 MB.
DATA SET S2, XLSX file, 0.2 MB.
DATA SET S3, XLSX file, 0.1 MB.
DATA SET S4, XLSX file, 0.2 MB.

ACKNOWLEDGMENTS
We thank Brian Wickes for fruitful discussions during the course of the experiments and for critical reading of the manuscript as well as Yidong Chen for bioinformatics assistance. We are also grateful to Guanghua Huang for providing C. tropicalis strains.

This work was supported by NIH grants R01AI127692, R21AI130668, R21AI12988, R21AI142560, and R21AI117299 to D.K. Additional support was provided by an Innovation Challenge Award from the San Antonio Life Sciences Institute (to D.K. and J.L.L.-R.) and by the Margaret Batts Tobin Foundation (to J.L.L.-R.). RNA-seq data were generated in the Genome Sequencing Facility, which is supported by UT Health San Antonio and NIH grants P30CA054174 and UL1TR002645 as well as CPRIT grant RP160732.

The content is solely the responsibility of the authors and does not necessarily reflect the official views of the National Institutes of Health, National Cancer Institute, or the National Institute of Allergy and Infectious Diseases.
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