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Role of filamentation in *Galleria mellonella* killing by *Candida albicans*

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Abstract

Candida albicans is an important cause of morbidity in hospitalized and immunosuppressed patients. Virulence factors of *C. albicans* include: filamentation, proteinases, adherence proteins and biofilm formation. The objective of this work was to use *Galleria mellonella* as a model to study the roles of *C. albicans* filamentation in virulence. We focused our study to five genes *BCR1*, *FLO8*, *KEM1*, *SUV3* and *TEC1* that have been shown to play a role in filamentation. Filaments are necessary for biofilm formation and evading interaction with macrophages in mammalian infections. Among the five mutant strain tested, we found that only the *flo8/flo8* mutant strain did not form filaments within *G. mellonella*. This strain also exhibited reduced virulence in the larvae. Another strain that exhibited reduced pathogenicity in the *G. mellonella* model was *tec1/tec1* but by contrast, the *tec1/tec1* strain retained the ability to form filaments. Overexpression of *TEC1* in the *flo8/flo8* mutant restored filamentation but did not restore virulence in the larvae as well as in a mouse model of *C. albicans* infection. The filamentation phenotype did not affect the ability of hemocytes, the immune cells of *G. mellonella*, to associate with the various mutant strains of *C. albicans*. The capacities of the *tec1/tec1* mutant and the *flo8/flo8* *TDH3-TEC1* strains to form filaments with impaired virulence suggest that filamentation alone is not sufficient to kill *G. mellonella* and suggest other virulence factors may be associated with genes that regulate filamentation.

Keywords

Candida albicans; *Galleria mellonella*; Filamentation; Hyphae

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1. Introduction

Candida albicans is the fourth most common cause of bloodstream infections in patients. *Candida* infections carry a mortality rate that can reach over 35% [1]. Infections can be provoked by the presence of medical implants that provide a niche for the development of a biofilm which is resistant to antifungal agents [1,2]. The development of a biofilm relies on filamentation whereby hyphae form and extend from a basal layer of cells followed by the production of an extracellular matrix. Several virulence traits are associated with *C. albicans* hyphal-development and biofilm development, including adherence proteins, proteinases, phospholipases, and filamentation [3,4].

A study by Brennan et al., (2002) demonstrated that genes important for the transition from yeast to hyphal forms could be tested for their role in virulence using a *Galleria mellonella* (Lepidoptera: the greater wax moth) infection system [5]. *C. albicans* causes a lethal infection in *G. mellonella* [6] and elicits an immune response [7,8] that is useful in evaluating differences in pathogenicity between *Candida* species [6]. We continued the pursuit of this model as a means of studying the role of filamentation in virulence.

Our study focused on a collection of genes previously shown to be necessary for filamentation during biofilm formation. These genes included *BCR1*, *FLO8*, *KEM1*, *SUV3* and *TEC1* [9-11]. Examination of the *kem1/kem1* mutant revealed it was capable of forming pseudohyphae rather than hyphae in an *in vitro* study by Richard and colleagues [10] and the abnormal filaments integrate into a biofilm [10]. The *suv3/suv3* mutant also exhibited hyphal-defects in the same study for which hyphae formation was blocked and produced only a thin layer of cells and occasionally pseudohyphae [10]. The transcription factor mutant strains *tec1/tec1* and *bcr1/bcr1* which were identified for their roles in filamentation as it pertains to biofilm development in a targeted screen by Nobile and Mitchell [9,10]. The *tec1/tec1* mutant was defective in hyphal-development, which resulted in a thin biofilm *in vitro* [9]. The *bcr1/bcr1* mutant produced hyphae, but these hyphae lacked the ability to adhere, which led to the formation of filaments that could not support biofilm development *in vitro* [9]. Flo8 was characterized in a separate study by Cao et al. and was described as being involved in hyphal-specific gene expression [11]. The *flo8/flo8* mutant was unable to form hyphae *in vitro* in hyphal-induction growth conditions [11]. Hence our virulence assay included mutant strains that represent a spectrum of filamentation development defects.

In the *G. mellonella* infection model, *bcr1/bcr1* and *suv3/suv3* strains were virulent and able to form filaments associated with *G. mellonella* internal structures. In contrast, the *flo8/flo8* mutant strain, which did not produce filaments within *G. mellonella*, exhibited reduced virulence. The mutant strains *tec1/tec1* and *kem1/kem1* exhibited reduced pathogenicity but unlike the *flo8/flo8* mutant the *tec1/tec1* and *kem1/kem1* strains retained the ability to form filaments within *G. mellonella*. To determine if these mutations affect the interactions between *C. albicans* and immune system of *G. mellonella*, we tested the association between hemocytes and the mutant strains of *C. albicans* *in vitro*. We found that all mutant strains included in our study associated with hemocytes in a similar capacity. Thus defects in *C. albicans* filamentation do not alter the capacity for hemocytes recognition of the pathogen and therefore, aspects of filamentation that lead to reduced pathogenicity in the *G. mellonella* model are associated with fungal cell virulence.

2. Materials and Methods

2.1. Strains and media

All fungal strains used in this work are listed in Table 1. *C. albicans* strains were grown in YPD (1% yeast extract, 2% peptone and 2% dextrose) at 30°C.

2.2. *C. albicans* strains construction

To construct BF131, a genomic fusion was created with the *TDH3* promoter to the *TEC1* coding region. For construction of the *flo8/flo8 TDH3-TEC1* strain, CA2 (previously reported as strain CCF3 *ura3::1 imm434/ura3::1 imm434 flo8::hisG/flo8::hisG-URA3-hisG*) [11] was transformed, with a PCR product generated from template *NAT1-TDH3* promoter plasmid pCJN542 [12] and primers EIMy 257 (5'-TCAGGTAGAAATTTACAAAGGCGACAAAAACCAAGAAGCAGAATAAGTTAAATGAAGTAAAGAAAAAATAAAGAGAGAGTAAAAAATGCACCATCAAGCTTGCCTCGTCCCC-3') and EIMy 258 (5'-TACCAATTGCAACATCAACAATTAATGGAAGCTTCTTGCCTTTCTTTTGTCCA TCGGCATTTCTCACGGGAGTAGCACTAGGAGTAGCTTGCGACATCATATTTGAATTC AATTGTGATG-3'). These primers amplified the *Ashbya gossypii* *TEF1* promoter, the *C. albicans* *NAT1* ORF, the *A. gossypii* *TEF1* terminator and the *C. albicans* *TDH3* promoter with 100 bp of hanging homology to 500 bp upstream into the promoter of *TEC1* and 100 bp of hanging homology from the start codon of the *TEC1* ORF. The generated PCR product provides the necessary sequence for homologous recombination of the entire cassette directly upstream of the *TEC1* locus so that *TEC1* was overexpressed with the *TDH3* promoter instead of its endogenous promoter. The cassette was transformed into CA2 using a standard *C. albicans* transformation protocol described previously [13]. Transformants were selected on YPD+clonNAT (1% yeast extract, 2% peptone, 2% dextrose and 400 µg/ml nourseothricin [NAT] [Werner Bioagents]). Integration of the construct was verified by PCR using primers EIMy 299 (5'-GGGAATTAATGGGCAATAGAAAGTG-3') annealing to the promoter and EIMy 216 (5'-GCAGTATCATCCAAAGTAGTA-3') annealing to a sequence within the *NAT1* gene.

2.3. *G. mellonella* survival assay

Inocula were prepared by growing *C. albicans* strains at 30°C overnight with agitation. Cells were collected with centrifugation and washed twice with PBS. Yeast cells were counted using a hemocytometer. *G. mellonella* larvae (Vanderhorst Wholesale) at their final instar stage were inoculated with 10⁶ cfu of *C. albicans* suspended in phosphate buffered saline (PBS). Each infection group contained 16 randomly chosen larvae of the appropriate weight (330 ± 25 mg). The inoculum was injected in a 10 µl volume directly to the last left pro-leg using a Hamilton syringe [14]. Larvae were incubated at 37°C and the number of dead larvae was scored daily. Killing curves were plotted and statistical analysis was performed by the Kaplan-Meier method using STATA 6 statistical software (Stata). Killing curves were performed in duplicate and a representative graphs were reported.

2.4. Fungal cell staining with Periodic Acid Schiff (PAS)

Larvae were infected as described above. Two larvae per infection group were cut open to release the internal solid structures into a collection tube. The structures were formalin fixed and embedded in paraffin. Sections were cut and then stained with PAS. The stained tissue was observed with microscopy at 60× magnification using an Olympus microscope.

2.5. Labeling of *C. albicans* with Fluorescein-5-iodothiocyante (FITC)

Overnight cultures of *C. albicans* grown at 30°C with agitation were collected with centrifugation and washed twice with PBS. *C. albicans* were counted with a hemocytometer and 10⁶ cells in PBS were incubated with 0.1 mg/ml FITC (Invitrogen, Molecular Probes) by adding 10 µl of 10 mg/ml FITC in DMSO to 990 µl of PBS. Cells were incubated for 30 min in the dark at room temperature. Cells were washed three times with PBS containing 1.5% fetal bovine serum (FBS).

2.6. Isolation of *G. mellonella* hemocytes

Using a procedure modified from Bergin and colleagues (2005) [15], healthy final instar larvae were bled by incision at the base of the right last true leg. Hemolymph was collected in tubes containing cold, sterile insect physiologic saline (IPS) (150 mM sodium chloride; 5 mM potassium chloride; 100 mM tris hydrochloride, pH 6.9 with 10 mM EDTA and 30 mM sodium citrate). The suspension was centrifuged at $700 \times g$ for five min at 4°C and washed twice with cold IPS then suspended in cold PBS containing 100 mg/ml calcium chloride, 100 mg/ml magnesium chloride and 5 mM glucose and then counted with a hemocytometer and used immediately.

2.7. Opsonization of fungal cells

Hemolymph was collected from larvae into cold IPS as described above. Cells were pelleted with centrifugation at $700 \times g$ for five min at 4°C. The supernatant was passed through a 0.45 µm filter and retained as cell-free hemolymph. FITC labeled *C. albicans* were then suspended in 10% cell-free hemolymph in IPS for 60 min at 37°C in the dark with agitation for opsonization.

2.8. Visualization of *C. albicans* co-incubated with hemocytes

Hemocytes were permitted to adhere to glass chamber slides by suspending 5×10^4 cells in 500 µl of Grace's media. After the hemocytes incubated for two h at 37°C on the glass chamber slides, the media was aspirated. Adherent hemocytes received 400 µl of Grace's media to the glass chamber slide. To the hemocytes, 100 µl of opsonized, FITC labeled *C. albicans* in 10% hemolymph diluted in IPS were added for an MOI of 2:1 (fungal cells: hemocytes) and incubated at 37°C for an additional two h. The chamber slides were then washed twice with PBS containing 100 mg/ml calcium chloride, 100 mg/ml magnesium chloride and 5 mM glucose and fixed overnight with 4% paraformaldehyde. The slides were viewed with a confocal laser microscope (TCS; NT Leica).

2.9. FACS

Hemocytes were isolated as described above suspended in PBS with 100 mg/ml calcium chloride, 100 mg/ml magnesium chloride and 5mM glucose; incubated with FITC labeled, opsonized *C. albicans* cells at a MOI of 2:1 (fungal cells: hemocytes) for 30 min at 37°C in the dark and then submitted to FACS. FACS analysis was performed with a Becton Dickinson FACSCalibur flow cytometer.

2.10. Mouse model of candidiasis

Mice were infected with *C. albicans* according to an established protocol [16]. Strains CAN14, *flo8/flo8* or *flo8/flo8 TDH3-TEC1* were used to infect mice. Cultures were grown overnight then washed twice with PBS. CD-1, six week old female mice were infected with 1.5×10^6 cfus suspended in PBS via a tail vein injection in a 100 µl volume. Twelve mice were inoculated per strain tested. Mice were observed daily. The murine protocol was approved by Massachusetts General Hospital, Subcommittee on Research Animal Care.

3. Results

3.1. Infection with hyphal-defective mutants

Our goal for this study was to investigate whether *G. mellonella* is a useful model to study the role of *C. albicans* filamentation in virulence. We focused our study to five genes that play various roles in filamentation. Four of the genes included in our study, *KEM1*, *SUV3*, *FLO8* and *TEC1* are required for hyphal morphology. The final gene included in our study, *BCR1*, is not required for hyphal morphology but does play a role in filament-associated adherence

during biofilm development. All of the genes included in this study play varying roles in filamentation in the context of biofilm development. We determined if these genes were also required for virulence by observing the survival of *G. mellonella* infected with *C. albicans* mutant strains and compared survival rates to infections by the appropriate reference strains or wild-type CAN14. The *bcr1/bcr1* and *tec1/tec1* strains were each derived from the prototrophic reference strain DAY185 and therefore were compared to DAY185 to determine changes to pathogenicity (Table 1). Strains *kem1/kem1* and *suv3/suv3* were constructed from the auxotrophic reference strain DAY286 and were thus compared to DAY286 to determine changes in pathogenicity caused by *KEM1* or *SUV3* deletions (Table 1). *G. mellonella* infected with the *flo8/flo8* strain was compared to the survival of *G. mellonella* infected with the wild-type strain CAN14.

By observing *G. mellonella* survival after *C. albicans* infection, we found that *TEC1* was associated with pathogenicity whereby the *tec1/tec1* mutant exhibited a significant reduction in virulence compared to the reference strain DAY185 ($P<0.01$) (Fig. 1A). *G. mellonella* infected with the *tec1/tec1* strain reached 50% mortality by 168 h post inoculation. Infection of *G. mellonella* with a complemented strain carrying an ectopic copy of *TEC1* (*tec1/tec1* + p*TEC1*) restored virulence to levels equivalent to the DAY185 reference strain with 50% mortality reached by 24 h (Fig. 1A). The *tec1/tec1* mutant strain remained less virulent when compared to the complement strain *tec1/tec1* + p*TEC1* ($P<0.01$). At 216 h post infection, when the experiment was concluded, 6 out of 16 *G. mellonella* in the *tec1/tec1* infected group (37.5%) remained alive.

The *kem1/kem1* strain demonstrated reduced pathogenicity when compared to the reference strain DAY286 ($P<0.0001$). The *kem1/kem1* infected *G. mellonella* exhibited 50% mortality by 72 h after infection (Fig. 1B).

FLO8 also contributes to pathogenicity. The *flo8/flo8* mutant exhibited a slower rate of killing *G. mellonella* compared to CAN14 ($P<0.001$) (Fig. 1C). To explore the roles of *TEC1* and *FLO8* in virulence in our model, we attempted to determine if they control a similar set of genes as transcription factors by overexpression of *TEC1* in the *flo8/flo8* mutant by generating the strain *flo8/flo8 TDH3-TEC1*. Expression of *TEC1* restored filamentation ability (see below) but did not restore virulence to the level of a wild type infection to the *flo8/flo8* strain in the *G. mellonella* model (Fig. 1C). The *flo8/flo8 TDH3-TEC1* strain was slightly more virulent than the *flo8/flo8* strain ($P>0.05$), but was still less virulent than CAN14 ($P=0.0001$). We confirmed that *flo8/flo8 TDH3-TEC1* did not restore virulence using a mouse model of candidiasis. All twelve *flo8/flo8 TDH3-TEC1* inoculated mice remained alive at 62 days post inoculation ($P<0.01$) compared to CAN14, which exhibited 100% mortality by day 9 post infection (Supplemental Fig. 1).

The deletion of either *SUV3* or *BCR1*, do not reduce *C. albicans* virulence in the *G. mellonella* model. The rate of killing by *suv3/suv3* and *bcr1/bcr1* was not significantly different from the reference strains DAY286 and DAY185 respectively. Strains *suv3/suv3* and *bcr1/bcr1* each reached 50% mortality by 24 h post infection (Fig. 1A and B).

3.2. Filamentation

We endeavored to determine if the less virulent mutant strains filament in *G. mellonella*. We observed that virulent strains CAN14 and DAY185 produced a mixture of yeast cells and filaments within *G. mellonella* (Fig. 2). Observations of *G. mellonella* tissue collected from larvae infected with *tec1/tec1*, *kem1/kem1*, *suv3/suv3* or *bcr1/bcr1* revealed that each of these strains was capable of forming filaments within *G. mellonella*. We observed a mixture of both yeast and filamenting cells within the tissue (Fig. 2).

By contrast, *flo8/flo8* did not form filaments within the *G. mellonella* body (Fig. 2). Thus, although all the genes examined play some role in hyphal-development in the context of biofilm formation only *FLO8* is necessary for filament formation in *G. mellonella* tissue.

The filamentation phenotype of the *flo8/flo8* mutant was recovered by overexpressing *TEC1* in the *flo8/flo8* mutant background, where we observed filamentation within *G. mellonella* (Fig. 2). We next endeavored to determine if *TEC1* overexpression could restore the ability of *flo8/flo8* to form a biofilm using silicone pads as a platform for *in vitro* biofilm growth according to a previously described method [10,17]. However, *TEC1* overexpression did not restore the biofilm phenotype (data not shown).

We extended our examination to *in vitro* conditions. To determine if our *C. albicans* strains of interest interact with hemocytes, we first determined if *C. albicans* were able to filament under the *in vitro* test conditions. FITC labeled *C. albicans* suspended in 10% hemolymph were added to glass slides containing adherent hemocytes for two h. The wild-type strain was indeed able to filament. We found that many of the strains included in our study were able to filament *in vitro* with a few notable exceptions. The *flo8/flo8*, *flo8/flo8 TDH3-TEC1* and *suv3/suv3* strains did not form hyphae (Fig. 3). The *flo8/flo8* strains appeared to remain in a yeast-like form as it did within the *G. mellonella* tissue. Unlike the *in vivo* conditions in which overexpression of *TEC1* recovered the filament phenotype of the *flo8/flo8* mutant, overexpression of the *TEC1* gene in the *flo8/flo8* mutant in the *in vitro* conditions did not restore the filamenting phenotype.

Since there were differences in filamentation between the various mutant strains we tested if the filamentation phenotype would affect the association between *C. albicans* strains and *G. mellonella* hemocytes, which could potentially be a factor in the reduced virulence of the *tec1/tec1* and *flo8/flo8* strains. Our observations for associations between *C. albicans* and hemocytes did not distinguishing between phagocytosis and adherence; both types of interaction were considered an association. FITC labeled *C. albicans* were co-incubated with hemocytes *in vitro* and observed with microscopy. All of the tested *C. albicans* strains were capable of associating with the hemocytes regardless of whether they were in a yeast or filamenting form (Fig. 4A). The *C. albicans* strains that were previously observed to be able to form hyphae in hemolymph were capable of forming hyphae when associated with hemocytes. Although the *flo8/flo8* and *tec1/tec1* were less virulent than the other strains, there was no correlation between virulence and hemocytes association (Fig. 4B). The scatter plots were divided into three regions and we determined the percentage of cells within each region. Region 3 (R3) depicts hemocytes, region 1 (R1) depicts *C. albicans* cells and region 5 (R5) indicates associations between hemocytes and *C. albicans* (Fig. 4B). The average percentage in R5 for three replicates for each mutant strain was compared to the appropriate reference strain and expressed as a percentage of cells in region 5 relative to the reference strain (Fig. 4C).

4. Discussion

G. mellonella provides a facile model to further the study of *C. albicans*. Brennan and colleagues (2002) established that the *G. mellonella* model was applicable for examining the genetic components of pathogenicity for *C. albicans*. We expanded on their study by examining the role of *BCR1*, *FLO8*, *KEM1*, *SUV3* and *TEC1* in virulence using the *G. mellonella* infection model. These genes are required for filamentation as it relates to biofilm development. We find that *FLO8* and *TEC1* are associated with pathogenicity. Under *in vitro* conditions the strains included in this study show similar association with *G. mellonella* hemocytes. There were defects in filamentation associated with the *flo8/flo8*, *flo8/flo8 TDH3-TEC1* and *suv3/suv3* strains to the degree that these strains were unable to form hyphae. By contrast the *flo8/flo8 TDH3-TEC1* and *suv3/suv3* strains did produce filaments in *G. mellonella* tissue that was

isolated post infection. Although *flo8/flo8 TDH3-TEC1* forms filaments, this formation is not sufficient for virulence at the level of a wild type infection. Similarly, the *tec1/tec1* strain also produced filaments but was hindered in killing larvae.

Infection of *G. mellonella* with the various mutants used in this study revealed that the *tec1/tec1* and *flo8/flo8* mutants were defective in virulence, thus implicating an aspect of hyphal morphogenesis in pathogenicity. Although both *TEC1* and *FLO8* were required for virulence they exhibited differing morphologies. The *flo8/flo8* strain was deficient in filament production *in vitro* or when associated with *G. mellonella* tissue structures however the *tec1/tec1* strain was observed to form filaments associated with *G. mellonella* internal structures and under *in vitro* conditions. These contrasting filament morphologies indicate that filamentation alone is not enough to kill *G. mellonella* since *tec1/tec1* produced filaments that do not result in virulence.

Like Tec1, Bcr1 is also a transcription factors but unlike *TEC1*, *BCR1* is not required for virulence. The *bcr1/bcr1* mutant, defective in expression of many hyphal-specific genes but not in hyphal morphogenesis [9,10], is fully virulent. Thus our findings argue that Bcr1-independent aspects of filamentation, including Bcr1-independent genes as well as morphogenesis *per se*, are critical for virulence. The difference in pathogenicity between the two mutant strains argues for a Bcr1-independent and Tec1-dependent mechanism of virulence.

Pathogenicity in the *G. mellonella* infection model was attributed to factors that are Kem1, Flo8 and Tec1-dependent but Bcr1 and Suv3-independent. Our findings with the *G. mellonella* model agree with previous assessments of *FLO8* and *TEC1* that found both were involved in *C. albicans* pathogenicity in mammalian infection models [11,18]. Observations previously reported with a mouse model of infection showed that a *tec1/tec1* strain was able to filament in a localized infection but exhibited a reduction in virulence in a systemic infection of candidiasis [18]. Although both *FLO8* and *TEC1* are important to virulence, overexpression of *TEC1* in the *flo8/flo8* mutant strain did not restore the virulence phenotype, the capacity to form a biofilm *in vitro* or filament *in vitro*. The strains were kept in *in vitro* conditions for two h and perhaps with a longer incubation filamentation could have been achieved by *flo8/flo8 TDH3-TEC1* but we did not extend the time because the time allotted was sufficient for the wild-type and reference strains to form filaments. *TEC1* overexpression did however restore the capacity to form filaments during infection of *G. mellonella* since filaments were observed in the tissue structures isolated from infected *G. mellonella*. Thus Flo8-dependent genes that respond to Tec1 expression for the restoration of filaments could potentially be dependent upon the conditions in which *TEC1* is expressed.

The recovery of filament formation in the *G. mellonella* tissue by overexpressing *TEC1* in the *flo8/flo8* mutant suggests that Tec1 or Tec1-dependent genes control a similar set of genes as Flo8 in filament formation. Since the recovery of filamentation within *G. mellonella* does not lead to biofilm development or filamentation *in vitro*, Tec1 does not support the full recovery of genes activated by Flo8 but perhaps a subset of genes controlled by Flo8. Also, the inability for *TEC1* overexpression to fully recover the virulence aspect of the *flo8/flo8* phenotype suggests that Tec1 does not influence the virulence factors that are Flo8-dependent. Thus, it is reasonable to assume that there are Tec1-dependent and Flo8-dependent virulence factors, and that the Flo8-dependent virulence factors are Tec1-independent.

The other strain that showed differential filamentation between *in vivo* and *in vitro* conditions was *suv3/suv3*. Although the *suv3/suv3* strain retained the pathogenicity phenotype and formed filaments *in vivo*, it too did not form filaments in our *in vitro* conditions. Our findings indicate that pathogenicity and *in vivo* filamentation are Suv3-independent but filamentation *in vitro* is Suv3-dependent. Thus there is potentially a genetic difference in filamentation between *in*

vitro and *in vivo* conditions. It cannot be discounted that host factors could contribute to filamentation and that these factors were present in the *in vivo* conditions but absent in our *in vitro* experiments or that filamentation was delayed for the *su3/su3* strain within the *in vitro* conditions. This same reasoning can be applied to the *flo8/flo8 TDH3-TEC1* strain that failed to produce filaments in *in vitro* conditions.

The *in vivo* formation of filaments by the virulence deficient strains *tec1/tec1* and *flo8/flo8 TDH3-TEC1* does not necessarily mean that filaments are completely without a role in pathogenicity. Others have shown that filaments provide a means of escaping host defenses in order to establish an infection. In a study of mammalian cell phagocytosis of *C. albicans*, the formation of filaments by yeast cells contributed to phagocytic cell death [19]. We were not able to establish a correlation between association with hemocytes and pathogenicity for the strains examined in this study.

In conclusion, *G. mellonella* was a useful model in evaluating the role of filamentation in *C. albicans* pathogenicity. We determined that the hyphae formation gene *KEM1* [20], hyphal formation regulator gene *TEC1*, which is important for biofilm formation and hyphal formation, as well as virulence in a mouse model [18], and the hyphal-development gene *FLO8* [11] are important for *C. albicans* virulence in *G. mellonella*. Our findings argue that a subset of filamentation properties are required for virulence, and we anticipate that a more refined comparison of the targets of Tec1, Flo8 and Bcr1 will provide mechanistic insight into *C. albicans* virulence traits.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

FBS	fetal bovine serum
FITC	fluorescein-5-isothiocyanate
IPS	insect physiological saline
PAS	Periodic Acid Schiff
PBS	phosphate buffered saline

Fig. 1. The effects of hyphal defects on *C. albicans* pathogenicity in a *G. mellonella* infection model

A Kaplan-Meier plot of *G. mellonella* survival after injection with 10^6 cfu/larva of *C. albicans* demonstrated which mutant strains were defective in pathogenicity. **A.** Strains *bcr1/bcr1* and *tec1/tec1* were compared to the DAY185 reference strain. The hyphal defective mutant *tec1/tec1* demonstrated slower larvae killing compared to the DAY185 reference strain ($P < 0.01$). The *TEC1* complement strain was also evaluated for pathogenicity and found to be virulent. **B.** Larvae infected with *kem1/kem1* or *suv3/suv3* were compared to infections with the reference strain DAY286. The *kem1/kem1* mutant resulted in slower killing rate compared to the DAY286. Infections with the *suv3/suv3* mutant did not adversely affect pathogenicity. **C.** The hyphal defective mutant *flo8/flo8* was compared to the wild-type strain CAN14 and exhibited a slower rate of killing. Injections with PBS were included as a control.

Fig. 2. PAS staining of fungal cells in *G. mellonella* tissue

G. mellonella were infected with 10^6 cfus/larvae and maintained at 37°C for 48 h. Tissue was collected from larvae, formalin fixed and embedded in paraffin. Sections were stained with PAS. Hyphae were observed in *G. mellonella* tissue infected with each of the mutant strains except for the *flo8/flo8* strain. Infection with the *flo8/flo8* strain remained in the yeast form. Arrows indicate the location of yeast cells. Scale bar, 20 μ m (bottom right corner).

Fig. 3. *In vitro* filamentation

C. albicans strains were incubated for two h with hemolymph and Grace's media at 37°C to induce filamentation. *C. albicans* were labeled with FITC. Scale bar, 16 μm (bottom right corner).

Fig. 4. *C. albicans* association with *G. mellonella* hemocytes

A. *C. albicans* were observed to bind to hemocytes when incubated in hemolymph and Grace's media for 2 h at 37°C. *C. albicans* were labeled with FITC. Red arrows indicate *G. mellonella* hemocytes and blue arrows indicate *C. albicans* cells. **B.** *G. mellonella* hemocytes were combined in suspension with FITC-labeled fungal cells and analyzed by FACS for association between hemocytes and fungal cells. Y-axis is side scatter (SSC). X-axis is FITC fluorescence. Hemocytes were submitted to FACS to obtain a characteristic plot for hemocytes in suspension without the addition of fungal cells. A characteristic scatter plot was also generated for fungal cells without hemocytes. Hemocytes were then combined with fungal cells to examine the shift in the plot due to associations between hemocytes and fungal cells. The gated regions of scatter plots represent hemocytes (R3), *C. albicans* (R1) and *C. albicans* associating with hemocytes (R5). **C.** An average of three independent FACS analyses were used to compare the association between *C. albicans* and hemocytes (R5) for each of the *C. albicans* mutant strains to the association between *C. albicans* and hemocytes (R5) for the appropriate reference strain. Therefore the *flo8/flo8* and *flo8/flo8* + *TEC1* strains were both compared to the CAN14 strain; the *bcr1/bcr1* and *tec1/tec1* strains were both compared to strain DAY185; and the *suv3/suv3* and *kem1/kem1* strains were both compared to strain DAY286.

C. albicans strains

Table 1

Strain	Description	Genotype		Reference or source
CAN14	SC5314	Wild type strain SC5314		[21]
BWP17	Parent strain	<u>ura3::λimm434</u>	<u>arg4::hisG</u>	[22]
		<u>ura3::λimm434</u>	<u>his1::hisG</u>	
DAY185	Arg+ Ura+ His+ Reference strain	<u>ura3::λimm434</u>	<u>ARG4::URA3::arg4::hisG</u>	[23]
		<u>ura3::λimm434</u>	<u>his1::hisG</u>	
DAY286	Arg+ Ura+ His- Reference strain	<u>ura3::λimm434</u>	<u>ARG4::URA3::arg4::hisG</u>	[24]
		<u>ura3::λimm434</u>	<u>his1::hisG</u>	
CJN702	<i>bcrl/bcrl</i>	<u>ura3::λimm434</u>	<u>arg4::hisG</u>	[9,17]
		<u>ura3::λimm434</u>	<u>his1::hisG::pHIS1</u>	
GKO443	<i>suw3/suw3</i>	<u>ura3::λimm434</u>	<u>arg4::hisG</u>	[10]
		<u>ura3::λimm434</u>	<u>his1::hisG</u>	
MLR74	<i>kem1/kem1</i>	<u>ura3::λimm434</u>	<u>arg4::hisG</u>	[10]
		<u>ura3::λimm434</u>	<u>his1::hisG</u>	
CJN896	<i>tec1/tec1</i>	<u>ura3::λimm434</u>	<u>arg4::hisG</u>	[17]
		<u>ura3::λimm434</u>	<u>his1::hisG::pHIS1</u>	
CJN1023	<i>tec1/tec1 + pTEC1</i>	<u>ura3::λimm434</u>	<u>arg4::hisG</u>	[9,17]
		<u>ura3::λimm434</u>	<u>his1::hisG::pHIS1-TEC1</u>	
CA2	<i>flo8/flo8</i>	<u>ura3::λimm434</u>	<u>arg4::hisG</u>	[11]
		<u>ura3::λimm434</u>	<u>his1::hisG</u>	
BFI31	<i>flo8/flo8 TDH3-TEC1</i>	<u>ura3::λimm434</u>	<u>flo8::hisG-URA3-hisG</u>	This study
		<u>ura3::λimm434</u>	<u>TDH3-TEC1::NATI</u>	
		<u>ura3::λimm434</u>	<u>TEC1</u>	