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Candida albicans Hyphal Formation and Virulence Assessed Using a *Caenorhabditis elegans* Infection Model[▽]

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Candida albicans **colonizes the human gastrointestinal tract and can cause life-threatening systemic infection in susceptible hosts. We study here *C. albicans* virulence determinants using the nematode *Caenorhabditis elegans* in a pathogenesis system that models candidiasis. The yeast form of *C. albicans* is ingested into the *C. elegans* digestive tract. In liquid media, the yeast cells then undergo morphological change to form hyphae, which results in aggressive tissue destruction and death of the nematode. Several lines of evidence demonstrate that hyphal formation is critical for *C. albicans* pathogenesis in *C. elegans*. First, two yeast species unable to form hyphae (*Debaryomyces hansenii* and *Candida lusitaniae*) were less virulent than *C. albicans* in the *C. elegans* assay. Second, three *C. albicans* mutant strains compromised in their ability to form hyphae (*efg1*Δ/*efg1*Δ, *flo8*Δ/*flo8*Δ, and *cph1*Δ/*cph1*Δ *efg1*Δ/*efg1*Δ) were dramatically attenuated for virulence. Third, the conditional *tet-NRG1* strain, which enables the external manipulation of morphogenesis in vivo, was more virulent toward *C. elegans* when the assay was conducted under conditions that permit hyphal growth. Finally, we demonstrate the utility of the *C. elegans* assay in a screen for *C. albicans* virulence determinants, which identified several genes important for both hyphal formation in vivo and the killing of *C. elegans*, including the recently described *CAS5* and *ADA2* genes. These studies in a *C. elegans*-*C. albicans* infection model provide insights into the virulence mechanisms of an important human pathogen.**

Candida albicans is the most common human fungal pathogen; however, our knowledge of its virulence mechanisms is incomplete, and our best antifungal agents are often ineffective in treating severe candidiasis (3). Infections with *Candida* species account for 70 to 90% of all invasive mycoses (32) and can be associated with devastating consequences, particularly in intensive care units where mortality rates reach 40% (24, 34). The drug resistance of pathogenic fungi exacerbates this problem and often limits therapeutic options (35). The identification of virulence pathways that can be targeted with novel antifungal therapies is urgently needed (31, 38, 46).

One approach to understand the genetic mechanisms of virulence is to use invertebrates, such as the nematode *Caenorhabditis elegans*, as model hosts (43). Studies of *C. elegans* infection with *Pseudomonas aeruginosa* and *Cryptococcus neoformans*, for example, have led to the identification of evolutionarily conserved mechanisms of host immunity and microbial virulence (1, 21, 50). However, efforts to design an accurate nonmammalian model of *C. albicans* pathogenesis have been stymied, in part because it has been difficult to capture the role of *Candida* dimorphism in these systems.

Morphogenesis in *C. albicans* is intricately related to pathogenesis and thus has been intensively studied. *C. albicans* hyphae are important for tissue destruction and host invasion (3). As such, *C. albicans* mutants and non-*albicans Candida* species that are unable to form true hyphae are attenuated for virulence (3, 37). However, *C. albicans* yeast cells also have virulence attributes (4, 33) that are likely involved in dissemination of the fungus through the bloodstream, and the establishment of infection at distant sites. To date, genetic screens to identify the determinants of *Candida* morphology have been conducted in vitro. Determining the role of these genes in virulence has traditionally involved separate and often laborious studies in mammals. Therefore, an expedient system to study morphogenesis of *C. albicans* in vivo and accurately model pathogenesis would offer many important advantages.

Here, we study *C. albicans* pathogenesis using the invertebrate host *C. elegans*. *C. albicans* yeast cells are ingested into the gastrointestinal tract. In liquid media, the yeast cells form hyphae, which results in an aggressive infection that ultimately kills the nematode. Fungal hyphae destroy worm tissues and pierce the collagenous cuticle of the animal, a phenotype that is easily visible using a dissecting microscope. By studying mutants and genetically engineered *C. albicans* strains, we show that hyphal formation is required for full virulence in this system. Finally, we illustrate the utility of the *C. elegans*-*C. albicans* infection assay in a screen for genes involved in *Candida* morphogenesis and virulence.

MATERIALS AND METHODS

Strains and media. The fungal strains used in the present study are listed in Table 1. Yeast strains were grown in liquid yeast extract-peptone-dextrose (BD) broth or on brain heart infusion (BHI; BD) agar containing 45 μg of kanamycin/ml at 30°C. To determine hyphal formation in vitro, *C. albicans* strains were grown in Spider medium for 20 h at 37°C (36) and photographed using Nomarski optics on a Zeiss AxioImager microscope.

The *C. elegans glp-4*; *sek-1* strain was used for all experiments as described previously (6, 40, 45). In brief, the rationale for using the *glp-4*; *sek-1* mutant nematodes instead of wild-type animals is that wild-type *C. elegans* produces many offspring which confounds killing assays, both because it is difficult to determine progeny from adults and because larvae often hatch inside the nematode, leading to death of the worm by a mechanism not directly related to

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TABLE 1. Fungal strains used in this study[a]

Strain	Genotype	Source or reference
C. albicans DAY185	*ura3*Δ::λ*imm434*/*ura3*Δ::λ*imm434 ARG4*::*URA3*::*arg4*::*hisG*/*arg4*::*hisG his1*::*hisG*::*pHIS*/*his1*::*hisG*	15
C. albicans SC5314	Clinical isolate	22
D. hansenii NCYC 2574	Reference strain	NCYC[b]
C. lusitaniae 6856-2	Clinical isolate	52
C. albicans Can16	*ura3*Δ::λ*imm434*/*ura3*Δ::λ*imm434 cph1*Δ::*hisG*/*cph1*Δ::*hisG-URA3-hisG*	37
C. albicans Can33	*ura3*Δ::λ*imm434*/*ura3*Δ::λ*imm434 efg1*Δ::*hisG*/*efg1*Δ::*hisG-URA3-hisG*	37
C. albicans Can34	*ura3*Δ::λ*imm434*/*ura3*Δ::λ*imm434 cph1*Δ::*hisG*/*cph1*Δ::*hisG efg1*Δ::*hisG*/*efg1*Δ::*hisG-URA3-hisG*	37
C. albicans CCF3	*ura3*Δ::λ*imm434*/*ura3*Δ::λ*imm434 flo8*Δ::*hisG*/*flo8*Δ::*hisG-URA3-hisG*	10
C. albicans SSY50-B	*ura3*Δ::λ*imm434*/*tet-NRG1*/*URA3*	47
C. albicans VIC1151	*ada2*Δ::*ARG4*/*ada2*Δ::*URA3 pHIS1*::*his1*::*hisG*/*his1*::*hisG*	9
C. albicans VIC1197	*ada2*Δ::*ARG4*/*ada2*Δ::*URA3 pADA2*::*HIS1*::*his1*::*hisG*/*his1*::*hisG*	9
C. albicans VIC1152	*cas5*Δ::*ARG4*/*cas5*Δ::*URA3 pHIS1*::*his1*::*hisG*/*his1*::*hisG*	9
C. albicans VIC1190	*cas5*Δ::*ARG4*/*cas5*Δ::*URA3 pCAS5*::*HIS1*::*his1*::*hisG*/*his1*::*hisG*	9

[a] The strains in the transcription factor mutant library were reported by Nobile and Mitchell (44).
[b] NCYC, National Collection of Yeast Cultures.

pathogen exposure (so-called matricidal killing). *C. elegans glp-4* mutant animals are unable to produce gonads or progeny at 25°C and thus are suited for these studies; however, sterile animals are long-lived compared to wild-type animals (39). *C. elegans SEK-1* encodes a conserved mitogen-activated protein (MAP) kinase kinase involved in the innate immune response, and *sek-1* animals are relatively immunocompromised (28). Worms were propagated on nematode growth medium on lawns of *Escherichia coli* OP50 by using standard methods (8).

***C. albicans*-*C. elegans* liquid medium pathogenesis assay.** A previously described protocol for infecting *C. elegans* with *C. albicans* in a liquid medium pathogenesis assay (6) was modified for these studies. Freshly grown *C. albicans* cells were inoculated into 1 ml of yeast extract-peptone-dextrose and allowed to grow overnight in a roller drum at 30°C. The following day, 100 μl of yeast was spread into a square lawn on a 10-cm plate containing BHI agar and kanamycin (45 μg/ml), followed by incubation for approximately 20 h at 30°C.

Synchronized adult *C. elegans glp-4*; *sek-1* nematodes grown at 25°C were carefully washed from plates containing their normal food source (*E. coli* OP50 strain) using sterile M9 buffer. Approximately 400 to 500 washed animals were then added to the center of the *C. albicans* lawns. The plates were incubated at 25°C for 4 h. Worms were then carefully washed into a 15-ml conical tube using 6-ml of sterile M9. Great care was taken to minimize the transfer of yeast into the conical tube. Worms were washed three or four times with sterile M9. Sixty to seventy worms were then pipetted into a single well of a six-well tissue culture plate (Corning, Inc.) containing 2 ml of liquid medium (80% M9, 20% BHI) and kanamycin (45 μg/ml). Kanamycin was added to prevent growth of any residual *E. coli* OP50 strain carried on the nematode during the experiment. For control experiments using only *E. coli* OP50, kanamycin was omitted from the liquid medium. Worms were scored daily into one of three categories: alive, dead with hyphae piercing the cuticle, and dead without hyphae piercing the cuticle. Worms were considered to be dead if they did not move in response to mechanical stimulation with a pick. Dead worms were removed from the assay. Microscopy of nematodes was performed by using Nomarski optics on a Zeiss AxioImager microscope.

For studies of the conditional *tet-NRG1 C. albicans* strain, nematodes were infected with this strain (SSY50-B; Table 1) on solid medium for 4 h as described above. Infected nematodes were then transferred to liquid medium with or without doxycycline (20 μg/ml). Because doxycycline rapidly oxidizes in light, the experiments were conducted in the dark.

***C. albicans*-*C. elegans* screen for novel virulence determinants.** We studied 83 transcription factor mutants in the *C. albicans*-*C. elegans* liquid medium assay. The mutant library was constructed using a split-marker transposon insertion strategy as previously described (44). For each mutant, we first evaluated the percentage of worms with visible *C. albicans* hyphae 60 h after infection in liquid medium. We then studied the virulence of mutants that demonstrated reduced hyphal formation in a *C. elegans* survival assay. Mutants attenuated for both in vivo hyphal formation and their ability to kill *C. elegans* are examined here. Of note, the homozygous insertion mutants from the transcription factor library are auxotrophic for histidine. However, the histidine auxotrophy was not responsible for the altered virulence toward *C. elegans* because we found no difference in killing or hyphal formation when we compared *C. albicans* DAY286 (16), a histidine auxotroph, to the prototrophic *C. albicans* strain DAY185 in a *C. elegans* pathogenesis assay. Furthermore, the insertion mutants not identified in our screen had phenotypes similar to *C. albicans* DAY185.

Statistical analyses. *C. elegans* survival was examined by using the Kaplan-Meier method and differences were determined by using the log-rank test (STATA 6; STATA, College Station, TX). Differences in the number of worms with *C. albicans* hyphal formation were determined by using the Student *t* test. Each *C. elegans* pathogenesis assay presented here is representative experiment of at least three independent biologic replicates. A P value of <0.05 in all replicate experiments was considered statistically significant.

RESULTS

***C. albicans* hyphal formation is a key virulence determinant in the *C. elegans* killing assay.** Our laboratory has previously shown that *C. albicans* is pathogenic toward *C. elegans* (6). In the original infection assay, which was designed to identify novel antifungal compounds, yeast cells were ingested by nematodes on solid medium and, after transfer to liquid medium, a minority of worms died with true hyphae piercing through the body of the worm (6). We were intrigued by this observation and sought to develop further a *C. elegans* infection assay for the detailed study of *C. albicans* pathogenesis.

We found that several variables, including the age of the worms and the pathogen exposure time, affected the degree of *C. albicans* hyphal formation within infected nematodes. Specifically, when we exposed adult animals, rather than nematodes in the fourth larval stage (L4), to pathogen for 4 h (rather than 2 h) prior to liquid medium transfer, we noted that a majority of infected worms displayed hyphae after 60 to 70 h of incubation in liquid medium (data not shown). By increasing the number of infected worms with *C. albicans* hyphae piercing their cuticles, it was possible to make statistically significant comparisons of *C. albicans* mutant strains.

We began our studies of *C. albicans* pathogenesis in the liquid medium assay by examining nematode infection with the *C. albicans* laboratory reference strain DAY185 and the *C. albicans* clinical isolate SC5314 (Fig. 1A). We found that more than half of the worms infected with these strains died within the first 48 h (Fig. 1A) and interestingly every worm that was killed during this period had visible hyphae piercing the cuticle (Fig. 1B and data not shown). This rapid initial decline in worm survival was followed by a second phase, in which worms

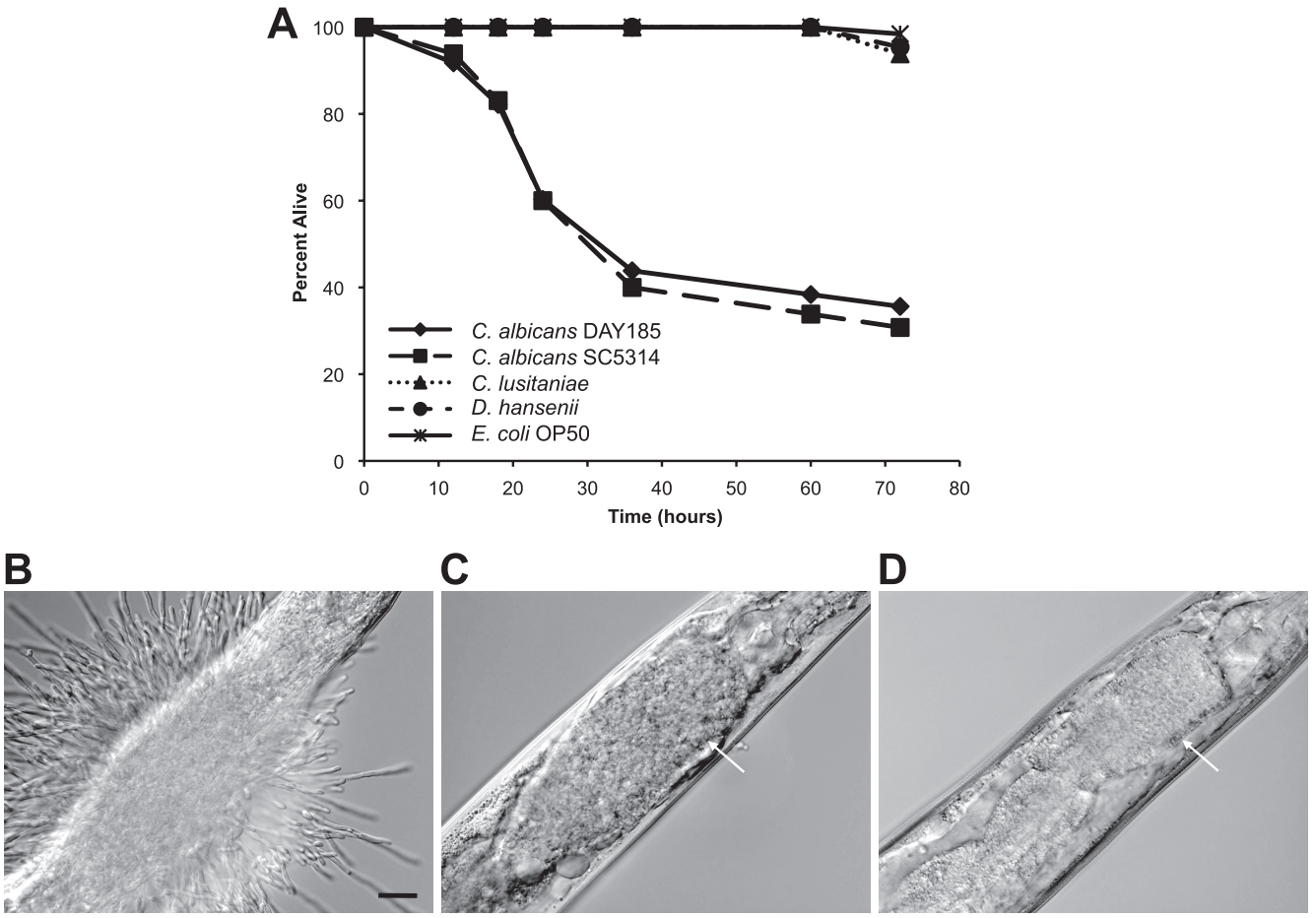

FIG. 1. *C. albicans* hypha-mediated killing of *C. elegans*. (A) The *C. albicans* reference strain DAY185 and the *C. albicans* clinical isolate SC5314 were more pathogenic to *C. elegans* in a liquid medium killing assay than two yeast species not capable of hyphal growth (*D. hansenii* and *C. lusitaniae*) and the normal nematode food source, *E. coli* OP50 during the first 72 h of infection ($P < 0.001$ for the control strains compared to *C. albicans* DAY185 or *C. albicans* SC5314. *P* value was not significant for *C. albicans* DAY185 compared to *C. albicans* SC5314). These data are from a single experiment representative of three independent biologic replicates. Corresponding microscopy images of nematodes infected with *C. albicans* DAY185 (B), *D. hansenii* (C), and *C. lusitaniae* (D) are shown. Hyphae are seen piercing the cuticle of the *C. albicans* DAY185-infected worm, whereas only yeast are seen within the intestine of the *D. hansenii* and *C. lusitaniae* infected worms (white arrows point to the intestinal lumen). Hyphal formation within *C. albicans* SC5314-infected nematodes had a similar appearance to panel B. The nematodes pictured in panels B, C, and D were alive at the time they were photographed. The scale bar in Fig. 1B represents 20 μm.

died more slowly (Fig. 1A). In these latter animals, we never observed any hyphae either in the intestine or piercing the cuticle among hundreds of infected nematodes examined. We considered the possibility that the development of hyphae in this assay was occurring postmortem and thus was a marker of aggressive infection, rather than a determinant of pathogenesis. We therefore photographed animals at various stages of infection with *C. albicans* DAY185. Hyphae start to accumulate within the intestines of live animals 12 to 16 h after infection and these animals die within 48 h (Fig. 1B and data not shown). In the studies presented here, we wanted to characterize *C. albicans* hyphal-mediated killing of the nematode and therefore, we focused our experiments on the first 72 h after infection.

To understand the role of *Candida* dimorphism in worm infection, we studied two fungal species closely related to *C. albicans* (*Debaryomyces hansenii* [*Candida famata*] and *C. lusitaniae*) that are thought to be incapable of hyphal formation. We observed a dramatic difference in *C. elegans* killing after infection with *C. albicans* DAY185 and *C. albicans* SC5314 compared to the two control organisms (Fig. 1A). *D. hansenii* is a hemiascomycetous yeast that is closely related to *C. albicans* (19) but is an uncommon human pathogen (17, 18, 51). As seen in Fig. 1C, no hyphae were observed in *C. elegans* after infection with *D. hansenii.* The organism was still capable of killing the nematode; however, the killing was slow and involved pathogenic distention of the worm intestine (Fig. 1C and data not shown). These features suggest *C. elegans* killing by *D. hansenii* occurred through a yeast-dependent process.

C. lusitaniae is also a relatively rare human pathogen that does not readily employ hyphal formation as a virulence mechanism in mammals (2, 25). During nematode infection with the *C. lusitaniae* clinical isolate 6856-2, only yeast cells were observed within the worm intestine (Fig. 1D), and killing was significantly slower compared to the *C. albicans* clinical isolate SC5314 (Fig. 1A). Consistent with these data, *C. lusitaniae* was

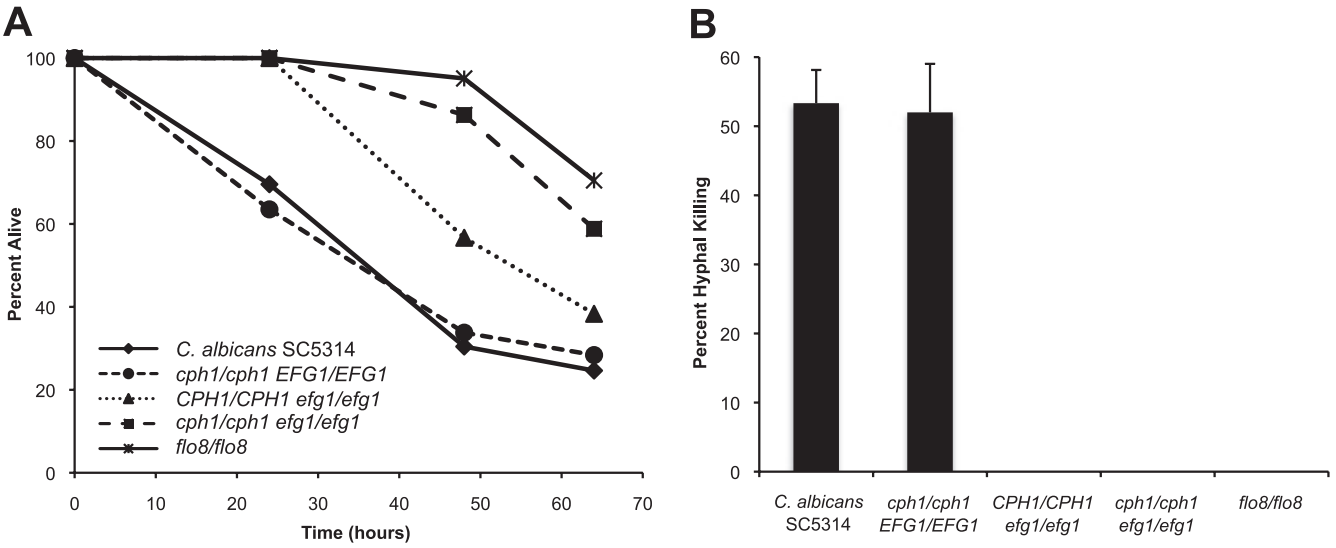

FIG. 2. *C. albicans* hyphal mutants have reduced virulence potential toward *C. elegans.* (A) Killing of *C. elegans* by the *C. albicans flo8*Δ/*flo8*Δ and *efg1*Δ/*efg1*Δ mutants and the double mutant *cph1*Δ/*cph1*Δ *efg1*Δ/*efg1*Δ was attenuated compared to the wild-type strain *C. albicans* SC5314 during the first 70 h of infection ($P < 0.001$ for *flo8*Δ/*flo8*Δ and *cph1*Δ/*cph1*Δ *efg1*Δ/*efg1*Δ versus the wild type, $P = 0.012$ for *efg1*Δ/*efg1*Δ versus the wild type). These data are from a single experiment representative of three independent biologic replicates. (B) These *C. albicans* mutants were unable to form hyphae in vivo during *C. elegans* infection ($P < 0.001$ for *flo8*Δ/*flo8*Δ, *efg1*Δ/*efg1*Δ, and *cph1*Δ/*cph1*Δ *efg1*Δ/*efg1*Δ mutants compared to the wild type). The *cph1*Δ/*cph1*Δ mutant, however, was able to kill the worm and form hyphae at rates similar to the control strain (the *P* values were not significant for both comparisons). The error bars represent the standard errors of the mean for three independent biologic replicates.

also less pathogenic than *C. albicans* in a murine model of fungal infection (2). Taken together, these data suggest that *Candida* hyphal formation is a key virulence determinant toward *C. elegans*, leading to an early, rapid decline in worm survival.

Conservation of *C. albicans* virulence determinants toward mammals and *C. elegans*. *C. albicans* is an opportunist that can grow in a myriad of microenvironments partly because of its ability to respond specifically to environmental cues and adapt by altering its morphology (3, 23). Several conserved signal transduction cascades regulate this response. Specifically, a cyclic AMP-mediated cascade utilizes the transcription factors Efg1p and Flo8p to control hyphal formation in response to a variety of environmental stimuli. Accordingly, *C. albicans efg1*Δ/*efg1*Δ and *flo8*Δ/*flo8*Δ mutants have hyphal formation defects and are attenuated for virulence in animal models of candidiasis (10, 37). Likewise, when we infected adult *C. elegans* animals with the *efg1*Δ/*efg1*Δ and *flo8*Δ/*flo8*Δ mutants, we saw a marked attenuation of virulence compared to the isogenic wild-type *C. albicans* strain SC5314 (Fig. 2A). Furthermore, worms that were infected with these mutants did not develop any hyphae throughout the course of the experiment (Fig. 2B).

We also examined the role of the *C. albicans* MAP kinase signal transduction cascade in virulence toward *C. elegans* by studying a mutant of the *CPH1* gene (36). This pathway coordinates dimorphism in *C. albicans*, but homozygous mutation of the *CPH1* gene results only in a modest hyphal formation defect in vitro and does not affect the virulence potential of *C. albicans* toward a mammal (37). Similarly, we found that the *cph1*Δ/*cph1*Δ mutant was able to make hyphae within *C. elegans* and remained fully virulent in our assay (Fig. 2). Disruption of both the cyclic AMP-mediated signaling pathway and the MAP kinase cascade in a *cph1*Δ/*cph1*Δ *efg1*Δ/*efg1*Δ double mutant, however, resulted in dramatic attenuation of both in vivo hyphal formation and virulence toward *C. elegans* (Fig. 2), findings that are consistent with studies of this double mutant strain in a murine model of hematogenously disseminated candidiasis (37) and a murine model of gastrointestinal colonization and candidemia (29). As observed for *D. hansenii* and *C. lusitaniae*, the *flo8*Δ/*flo8*Δ, *efg1*Δ/*efg1*Δ and *cph1*Δ/*cph1*Δ *efg1*Δ/*efg1*Δ mutant strains eventually killed the worms by a yeast-mediated process (data not shown).

For additional confirmation that *C. albicans* hyphal formation contributes to virulence in the nematode, we used a genetic tool that allows the external manipulation of morphogenesis. Saville et al. engineered a *C. albicans* strain in which one copy of the *NRG1* gene (a hyphal growth regulator) is under the control of a doxycycline-regulatable promoter (47). In the presence of doxycycline, this strain can form hyphae. Worms were infected with this modified strain and then transferred to liquid medium with or without doxycycline. As was observed in murine studies of this strain (47), we found that the presence of doxycycline in the media was sufficient to allow hyphal formation and manipulate the virulence potential of the *C. albicans* (Fig. 3). A total of 60% of infected nematodes died after 66 h in liquid medium containing doxycycline compared to just 11% mortality in the control well ($P < 0.001$) (Fig. 3). Doxycycline was not toxic to the worms and did not alter the ability of the *C. albicans* wild-type strain DAY185 to kill the worm (data not shown).

A *C. elegans*-*C. albicans* assay can be used to screen a library of *C. albicans* mutants for genes involved in hyphal formation in vivo. Given the importance of *C. albicans* hyphal formation in mammalian and nematode pathogenesis, we sought to determine whether this simple model could be used to identify

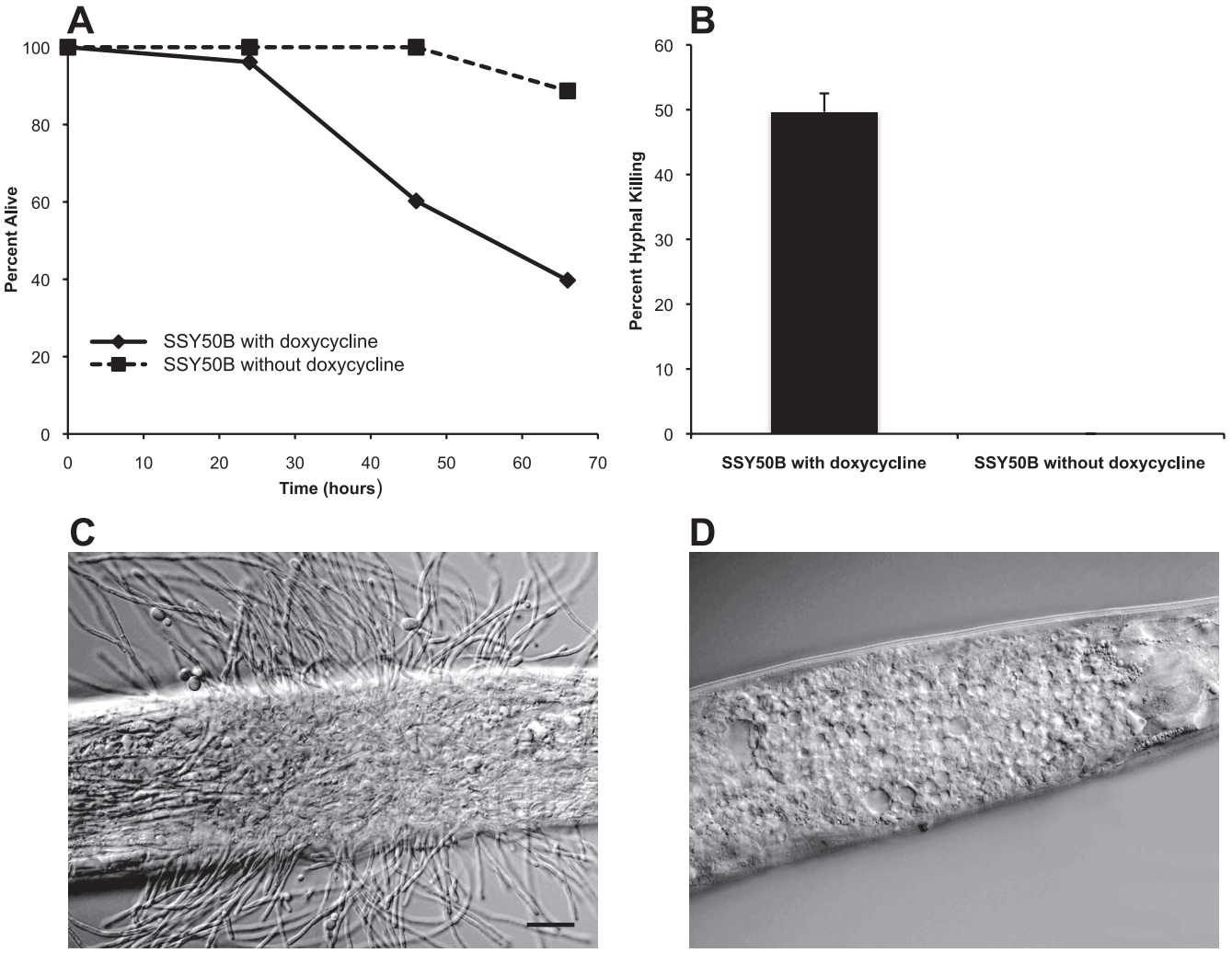

FIG. 3. Hyphal formation in a genetically modified *C. albicans* strain drives virulence. Nematodes were infected with the conditional *tet-NRG1* strain and transferred to liquid medium either with or without doxycycline (20 μg/ml). Doxycycline increased the killing of nematodes infected with the *tet-NRG1* strain (A) by permitting hyphal formation (B) ($P < 0.001$ for both comparisons). The data in panel A are from a single experiment representative of three independent biologic replicates. The error bars in panel B represent the standard errors of the mean for three independent biologic replicates. Worms infected with the *tet-NRG1* strain in liquid media supplemented with doxycycline (C) and without doxycycline (D) are shown. The scale bar in panel C represents 20 μm.

Candida virulence determinants. A *C. albicans* mutant library containing homozygous mutations in 83 transcription factors (44) was screened for mutant clones attenuated both in their ability to form hyphae in vivo and kill *C. elegans*. We identified five mutants. Interestingly, two of the genes we recovered in this screen (*RIM101* and *NRG1*) have previously described roles in hyphal development and are required for the full virulence potential of *C. albicans* in a murine model of candidiasis (Table 2). The identification of these genes from our screen provides internal validation for this approach.

TABLE 2. Genes identified in the *C. elegans-C. albicans* survey of transcription factor function whose molecular role has been previously studied

Library strain[a]	Disrupted gene	Description (reference)	Virulence role in a murine model (reference)
CJN267	*RIM101*	Required for alkaline-induced hyphal growth (15, 20)	Yes (14)
CJN322	*NRG1*	Transcriptional repressor of hyphal genes that acts with *TUP1* (5, 41)	Yes (41)
CJN432	*CAS5*	Acts with Ada2p to promote cell wall integrity (9)	Yes (12)
CJN863	*ADA2*/*CAS3*	Transcriptional coactivator involved in cell wall integrity, metabolic processes and stress responses (9, 48)	Yes (48)

[a] Nobile and Mitchell (44).

We identified two other genes in our screen (*CAS5* and *ADA2*) without defined roles in hyphal formation but whose molecular function has been previously studied (9, 48). Interestingly, these genes are also important for mammalian pathogenesis (Table 2). In addition, our screen recovered *C. albicans* strain CJN878, which carries a homozygous insertion in *ORF19.2458*. However, testing of several independent *orf19.2458*::*UAU1*/*orf19.2458*::*URA3* insertion mutants failed to confirm the phenotype. Strain CJN878 may therefore have a secondary mutation that affects hyphal growth in the *C. elegans* pathogenesis assay.

Thus, our screen successfully identified four of the five genes in the transcription factor library that have previously described roles in mammalian virulence (*RIM101*, *NRG1*, *ADA2*, and *CAS5*). We did not identify the *tec1*::*UAU1*/*tec1*::*URA3* insertion mutant, but it is interesting that this strain was also fully virulent in a *Drosophila melanogaster* model of *C. albicans* pathogenesis (12).

Ada2p and Cas5p are important for *C. albicans* hyphal development in vivo and for pathogenesis toward *C. elegans*. Identification of the *ADA2*/*CAS3* (hereafter referred to as *ADA2*) and *CAS5* genes in our screen of *C. albicans* transcription factors required for *C. elegans* killing generates interesting hypotheses regarding their contributions to *C. albicans* pathogenesis. *ADA2* and *CAS5* are each important for the cell wall damage response and were previously identified in a screen for mutants hypersusceptible to the antifungal agent caspofungin (9). *ADA2* encodes a transcriptional coactivator, which functions as part of the conserved Spt5-Ada-Gcn5 acetyltransferase (SAGA) coactivator complex that regulates numerous metabolic activities in eukaryotes by coordinating posttranslational modification of histones (48). Cas5p is a transcription factor that is the functional equivalent of the *Saccharomyces cerevisiae* protein Rlm1 (9).

We confirmed the findings from our screen by studying a prototrophic *ada2*Δ/*ada2*Δ deletion mutant and a reconstituted strain. The *ada2*Δ/*ada2*Δ mutant strain was significantly attenuated in its virulence toward *C. elegans* (Fig. 4A) and had similar killing kinetics to mutant strains containing lesions in two genes, *RIM101* or *NRG1*, which have well-defined roles in hyphal development (data not shown). We also observed a striking reduction in the ability of the *C. albicans ada2*Δ/*ada2*Δ mutant to produce hyphae in vivo (Fig. 4B and C) and found that this defect was completely restored with *ADA2* complementation of the mutant gene (Fig. 4B and D). The *ada2*Δ/*ada2*Δ mutant eventually killed the worm, but through a separate pathogenic process since we never observed any hyphae within *ada2*Δ/*ada2*Δ mutant-infected nematodes (Fig. 4C and data not shown).

Thus far, an association between *ADA2* and hyphal development has not been described. To confirm further these in vivo observations, we incubated the *ada2*Δ/*ada2*Δ mutant and its reconstituted strain under hypha-inducing conditions in vitro (Spider media at 37°C). We observed that the *ada2*Δ/*ada2*Δ strain is unable to produce hyphae under these conditions (Fig. 4F) compared to its parent strain (Fig. 4E). This phenotype was complemented by reintroduction of the wild-type gene (Fig. 4G). These observations indicate that the genetic network controlling hyphal growth in the *ada2*Δ/*ada2*Δ strain is compromised and suggest that Ada2p also coordinates the transcription of genes involved in *Candida* dimorphism. This hypothesis is strengthened by recent results reported by Sellam et al. (48), who identified 200 genes regulated by Ada2p, 49 of which have roles in *C. albicans* morphogenesis.

We also found that a *C. albicans* strain with homozygous disruption of *CAS5* was attenuated for virulence and did not form hyphae in vivo as readily as *C. albicans* DAY185. We confirmed these observations by studying a *cas5*Δ/*cas5*Δ deletion mutant and a reconstituted strain (Fig. 4A and B). Interestingly, the *cas5*Δ/*cas5*Δ strain was able to make hyphae in a small percentage of worms (~8%, $P < 0.001$ compared to *C. albicans* DAY185) (Fig. 4B), and their appearance was similar to the wild-type control (data not shown). Furthermore, the *cas5*Δ/*cas5*Δ mutant made hyphae equivalent to a wild-type control strain when cultured in vitro (Spider medium at 37°C) (data not shown). These findings argue that the *cas5*Δ/*cas5*Δ mutant still contains the genetic architecture to program wild-type hyphal formation and suggests that there must be a different mechanism to account for the observed in vivo hyphal defects.

DISCUSSION

Nonmammalian infection models have been remarkably useful in the study of host-pathogen interactions (42). We therefore developed a system for the study of *C. albicans* pathogenesis using the nematode *C. elegans*. We show that *C. albicans* hyphal formation is required to efficiently kill the worm and is a phenotype that predicts virulence in murine models of candidiasis. We also demonstrate the utility of this system in a screen of *C. albicans* transcription factor mutants, which identified both established and novel virulence determinants related to *C. albicans* hyphal formation.

An advantage of the C. *elegans*-*C. albicans* pathogenesis system is that it reflects several stages of mammalian infection. In our assay, *C. albicans* yeast cells are ingested into the digestive tract of the nematode. *C. albicans* hyphae then aggressively penetrate through host tissues and ultimately pierce the collagenous cuticle of the worm. Likewise, humans carry *C. albicans* in the gastrointestinal tract and from this location the fungus can disseminate in a susceptible host. In patients with candidiasis, hyphae are often seen at areas of tissue invasion and thus are thought to be involved in dissemination of disease (3, 37, 47). It is also interesting that these assays are conducted at a temperature that does not normally induce hyphal formation (25°C). Thus, studies of *C. albicans* morphogenesis in *C. elegans* also enable the analysis of hyphal growth triggers that are independent of temperature. We hypothesize that environmental factors within the nematode gastrointestinal tract, which may have mammalian counterparts, induce hyphal development in *C. albicans*.

Invertebrate models of candidiasis using *D. melanogaster* and larvae of the greater wax moth *Galleria mellonella* have been developed, and their utility has been demonstrated (7, 11). The *C. elegans*-*C. albicans* system, however, offers several advantages over these models. First, hyphal formation in nematodes can be easily visualized under a dissecting microscope, allowing direct and expedient evaluation of *Candida* dimorphism in vivo. Furthermore, the transparency of the worm allows examination of fungal cells within the intestine at all

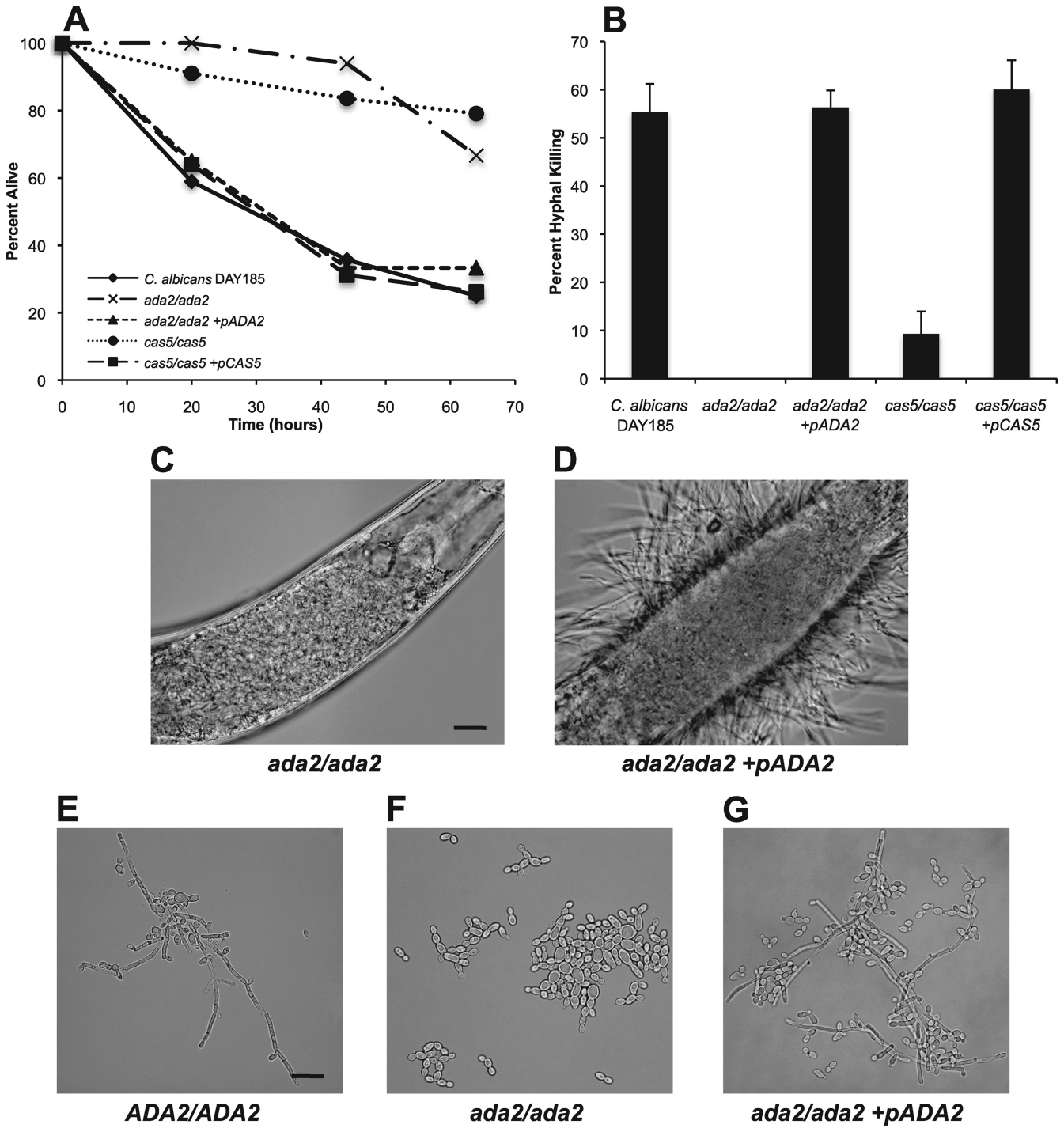

FIG. 4. *ADA2* and *CAS5* are required for the full virulence potential of *C. albicans* in the *C. elegans* infection model. Compared to infection with *C. albicans* DAY185, the *ada2*Δ/*ada2*Δ and *cas5*Δ/*cas5*Δ mutant strains were attenuated for virulence (A) and in vivo hyphal formation (B) in a *C. elegans* assay ($P < 0.01$ for both mutant strains compared to *C. albicans* DAY185). The virulence and hyphal formation defects of the *ada2*Δ/*ada2*Δ and the *cas5*Δ/*cas5*Δ mutants were restored to wild-type levels in corresponding reconstitution strains (the *P* values were not significant for both reconstitution strains compared to the wild type). The data in panel A are from a single experiment representative of three independent biologic replicates. The error bars in panel B represent the standard errors of the mean for three independent biologic replicates. Nematodes infected with the *ada2*Δ/*ada2*Δ mutant (C) and the *ada2*Δ/*ada2*Δ +*pADA2* reconstitution strain (D) are shown to demonstrate the marked hyphal formation defect of the *ada2*Δ/*ada2*Δ mutant in vivo. Compared to the *C. albicans* reference strain DAY185 (indicated as *ADA2*/*ADA2*) (E), the *ada2*Δ/*ada2*Δ mutant (F) also showed attenuated hyphal formation in vitro (Spider medium for 20 h at 37°C). This phenotype was restored in the *ada2*Δ/*ada2*Δ+*pADA2* reconstitution strain (G). The scale bars given in panels C and E represent 20 μm.

stages of infection. Although dimorphism is important for *Candida* pathogenesis in the *D. melanogaster* system, direct visualization of hyphae is not possible. Lastly, the portal of entry for *Candida* infection in the nematode is through the gastrointestinal tract, which is a physiologically accurate representation of human infection. In both fly and larva models, yeast must be injected into the animal to establish infection. However, the innate immune system of *D. melanogaster* is sophisticated and thus studies in both systems will likely be highly complementary.

The *C. elegans*-*C. albicans* infection model is an important new tool that is complementary to existing assays. Nobile et al. created a library of 83 mutants, each with a homozygous insertion in a transcription factor, and tested the strains for their ability to form biofilm on a silicone pad (44). That study found that two genes, *TEC1* and *BCR1*, are required for wild-type in vitro biofilm formation. Interestingly, our screen of this transcription factor mutant library in a *C. elegans*-*C. albicans* liquid medium infection assay identified five mutants that were not found to be defective in biofilm formation in vitro. In addition, neither the *tec1*::*UAU1*/*tec1*::*URA3* nor the *bcr1*::*UAU1*/*bcr1*::*URA3* insertion mutant strains (44) had virulence or hyphal-formation defects in the *C. elegans* assay (data not shown).

The identification of *ADA2* in our screen for *C. albicans* virulence determinants offers a provocative link between chromatin remodeling via the SAGA coactivator complex and *C. albicans* hyphal formation. We found that strains carrying a mutation in the *ADA2* gene were hypovirulent in our *C. elegans*-*C. albicans* assay (Fig. 4A). Phenotypic analyses of the *ada2*Δ/*ada2*Δ deletion mutant in vivo and under hyphal-inducing conditions in vitro suggest that the hyphal formation program in this mutant strain is compromised (Fig. 4). Ada2p functions as a key component of the SAGA coactivator complex, a conserved transcription regulatory system involved in histone modification. In *S. cerevisiae*, the SAGA system modulates the expression of ca. 10% of the genome and specifically affects the transcription of genes upregulated in response to several types of stress (26). The function of this conserved system in *C. albicans* biology and pathogenesis is now being elucidated.

Sellam et al. determined the genome-wide occupancy of Ada2p by using chromatin immunoprecipitation and found that this protein is recruited to the promoters of 200 genes involved in many different metabolic processes and stress responses (48). Interestingly, our analysis of these data revealed that 49 of these 200 genes have a role in *Candida* morphogenesis. Seventeen Ada2p-regulated genes are required for wild-type filamentous growth, and twenty-two of these genes are also controlled by transcription factors that are regulators of *C. albicans* dimorphism. In addition, several Ada2p-regulated genes have particularly prominent roles in morphogenesis. For example, the transcription factor *RIM101* (15); a key regulator of hyphal formation, Hsp90 (49); the MAP kinase kinase and hyphal growth regulator HST7 (30); and Swi1, a component of the Swi/Snf chromatin remodeling complex (analogous to the SAGA coactivator complex), were all identified to be controlled by Ada2p (48). Taken together, the data from our study and from Sellam et al. suggest that activation of a transcriptional network governing dimorphism in *C. albicans* involves the coordination of chromatin remodeling.

The phenotype of the *cas5*Δ/*cas5*Δ mutant in our *C. elegans* assay informs hypotheses regarding *Candida* virulence mechanisms toward both nematodes and mammals. *C. elegans* uses two principle defenses against ingested pathogens, a muscular pharyngeal grinder organ and secreted antimicrobial peptides (13, 27). We speculate that defects in the fungal cell wall damage response of the *cas5*Δ/*cas5*Δ strain increases yeast susceptibility to these defenses. Consistent with this hypothesis, the *cas5*Δ/*cas5*Δ mutant strain is able to make wild-type appearing hyphae but only in a small percentage infected worms (Fig. 4B). We also did not observe any defects in hyphal formation in the *cas5*Δ/*cas5*Δ mutant strain in an in vitro assay. Thus, we suggest that the hyphal formation program in the *cas5*Δ/*cas5*Δ mutant is intact and that these yeasts are more easily cleared from the *C. elegans* digestive tract. It is notable that the *cas5*Δ/*cas5*Δ mutant is also hypovirulent in a murine model of candidiasis and that sections of kidneys from animals infected with the mutant strain demonstrate very few hyphae (12). Taken together with the data from our study, it seems likely that *C. albicans* are exposed to analogous host defenses or mechanical forces during mammalian infection, which accounts for the virulence defect of the *cas5*Δ/*cas5*Δ mutant in the murine model. Thus, the *C. elegans*-*C. albicans* system offers a simple assay to study the ability of mutant strains to form hyphae in vivo, a phenotype that can predict pathogenicity in mammals.

In summary, we describe and validate a system for the identification and characterization of *C. albicans* virulence determinants. We demonstrate the utility of this model in a screen of transcription factor mutants and show that the *C. elegans*-*C. albicans* assay can be used to study the virulence mechanisms of an important human pathogen.

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