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Pseudohyphal Regulation by the Transcription Factor Rfg1p in *Candida albicans*▽

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The opportunistic human fungal pathogen *Candida albicans* is a major cause of nosocomial infections. One of the fundamental features of *C. albicans* pathogenesis is the yeast-to-hypha transition. Hypha formation is controlled positively by transcription factors such as Efg1p and Cph1p, which are required for hyphal growth, and negatively by Tup1p, Rfg1p, and Nrg1p. Previous work by our group has shown that modulating *NRG1* gene expression, hence altering morphology, is intimately linked to the capacity of *C. albicans* to cause disease. To further dissect these virulence mechanisms, we employed the same strategy to analyze the role of Rfg1p in filamentation and virulence. Studies using a *tet-RFG1* strain revealed that *RFG1* overexpression does not inhibit hypha formation *in vitro* or in the mouse model of hematogenously disseminated candidiasis. Interestingly, *RFG1* overexpression drives formation of pseudohyphae under yeast growth conditions—a phenotype similar to that of *C. albicans* strains with mutations in one of several mitotic regulatory genes. Complementation assays and real-time PCR analysis indicate that, although the morphology of the *tet-RFG1* strain resembles that of the mitotic regulator mutants, Rfg1p overexpression does not impact expression of these genes.

The opportunistic fungal pathogen *Candida albicans* is an important cause of human infection, especially in immunocompromised patients such as transplant recipients, chemotherapy patients, and those with HIV/AIDS. Mortality rates from *C. albicans* systemic infections range from 30 to 50% (44, 45). The ability of this fungus to reversibly convert between yeast, pseudohyphal, and true hyphal morphologies has been tied to the ability of this species to cause disease both in humans and in a murine model of disseminated candidiasis. The capacity to filament is particularly important for pathology in an infection; mutant strains that are locked either in the filamentous form (8, 9, 30) or in the yeast form (27, 38, 40) of growth show reduced virulence in the murine model of systemic candidiasis. Further, filamentous cells predominate in tissues recovered from patients succumbing to candidiasis and exposure to serum at normal body temperatures (37°C) induces the fungus to switch from the yeast to the hyphal form.

Genetic analysis has revealed that hypha formation in *C. albicans* is controlled by a number of transcription factors, including Efg1p and Cph1p, which can stimulate filamentation and the transcription of hypha-specific genes (6). These transcription factors induce hypha formation in response to environmental signals transduced by different signaling pathways, including the Cph1p-mediated mitogen-activated protein kinase (MAPK) and Efg1p-mediated cyclic AMP/protein kinase A pathways (7). The activity of these transcription factors is essential for both hypha formation and virulence in *C. albicans*, although under some growth conditions Efg1p is also required to repress hypha formation (43).

Several negative regulators of hypha formation have also been identified, including the DNA binding proteins Nrg1p and Rfg1p and the global transcriptional repressor Tup1p, but the signals and pathways that regulate the activity of these proteins remain poorly understood. The DNA binding protein Nrg1p is a key repressor of hypha formation and acts with Tup1p to suppress hyphal growth and the expression of many hypha-specific genes (10, 17, 23, 28, 30). *NRG1* transcription is elevated in yeast cells, and *NRG1* mRNA levels must fall for cells to progress from yeast to hyphal forms (10, 28, 30). The proteins Nrg1p, Rfg1p, and Tup1p were originally characterized as repressors of filamentation because strains lacking any one of these proteins grow as either filamentous pseudohyphae or hyphae under yeast growth conditions (9, 10, 22, 24, 30). The *nrg1*Δ strain forms wrinkled colonies on yeast extract-peptone-dextrose (YPD) plates, and these colonies consist of a mix of yeast and pseudohyphal cells (10, 30). The *tup1*Δ null strain, however, grows exclusively as pseudohyphae (9). In a previous study, we constructed a *C. albicans* strain in which expression of *NRG1* could be manipulated through the addition or omission of the tetracycline analogue doxycycline (DOX) from the growth medium or drinking water of an infected animal (38). Analysis of this *tet-NRG1* strain revealed that overexpression of *NRG1* not only inhibited filament formation under every hypha-inducing condition tested (14) but

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TABLE 1. Strains used in this study

Strain	Genotype	Reference
SC5314	Wild type	18
CAF2-1	*URA3*/*ura3*::λ*imm434*	16
THE1	*ade2*::*hisG*/*ade2*::*hisG ura3*::λ*imm434*/*ura3*::λ*imm434 ENO1*/*eno1*::*ENO1-tetR*-Sc*HAP4*AD-3×HA-*ADE2*	32
PMC-X7	THE1 with *RFG1*/*rfg1*::*URA3-tetO-RFG1*	This study
PMC-X8	THE1 with *RFG1*/*rfg1*::*URA3-tetO-RFG1*	This study
PMC-X7+empty vector	PMC-X7 with RP10::*CIpSATSA*	This study
PMC-X7+*CLB4*	PMC-X7 with RP10::*CIpSATSACLB4*	This study
YJB6854	*ura3*Δ::λ*imm434*/*ura3*Δ::λ*imm434 his1*::*hisG*/*his1*::*hisG ARG4*::*URA3*::*arg4*::*hisG*/*arg4*::*hisG clb4*::*ARG4*/*clb4*::*HIS1*	4

also rendered the fungus avirulent in the murine model of disseminated candidiasis (38). These studies provide compelling evidence linking morphogenetic changes to the ability of *C. albicans* to cause disease.

The negative regulator Rfg1p also plays an important role in regulating filamentation. This DNA binding protein was originally characterized as a repressor of filamentation because strains lacking Rfg1p form wrinkled colonies on YPD plates under yeast growth conditions with these colonies containing a mixture of yeast and filamentous cells (22, 24). Rfg1p, like Nrg1p, binds specific DNA sequences upstream of several genes and interacts with Tup1p to repress transcription at those sites. Although Rfg1p bears similarity to the *Saccharomyces cerevisiae* protein Rox1p, which is involved in repressing hypoxic genes, Rfg1p is not involved in regulating this process in *C. albicans* (22, 24), an important part of *C. albicans* transcriptional rewiring. Interestingly, exogenous *C. albicans RFG1* expressed in *S. cerevisiae* is able to repress filamentous growth in response to nitrogen starvation conditions (22), reinforcing the observation that, although there have been changes in the regulation of filamentation between these two fungal species, some conservation in the machinery used to achieve filamentous growth remains. Microarray and Northern analyses have helped to define the regulatory targets of the Tup1p, Nrg1p, and Rfg1p repressors, and it appears that about half of Tup1p repression occurs through Nrg1p and Rfg1p together or independently (23). Although Nrg1p and Rfg1p both regulate the expression of several hypha-specific genes, such as *ALS3*, *ECE1*, and *HWP1*, their regulons do not completely overlap (22, 23), indicating a distinct function for each protein in the control of filamentation. This is reinforced by the observation that colonies formed by the double *rfg1*Δ *nrg1*Δ null strain are more wrinkled and have a higher proportion of filamentous cells than does either single mutant strain. Furthermore, the fact that the double mutant does not reach the 100% pseudohyphal phenotype of the *tup1*Δ null strain indicates the involvement of further Tup1p binding partners in the regulation of filamentation (22). The mechanism of Rfg1p regulation and activity remains poorly characterized. A putative Rfg1p binding site has been identified upstream of the hypha-specific genes *HWP1* and *RBP1*, but the hypha-specific cell wall protein-encoding gene *ALS3*, which is also regulated by Rfg1p, lacks this site (1). It is important to note that, although the primary role of Rfg1p appears to be the repression of filamentous growth under yeast conditions, there is a requirement for Rfg1p to properly form hyphae when *C. albicans* is grown under nutrient-limiting conditions (22).

To further explore the role of filamentation in *C. albicans* pathogenesis, we constructed a *tet-RFG1* strain in which the gene encoding this reported repressor protein was placed under the control of a tetracycline-regulatable promoter and analyzed for its ability to filament and cause disease during *RFG1* overexpression. Since exogenous expression of *C. albicans RFG1* was able to repress filamentous growth in *S. cerevisiae*, we believed that overexpressing *RFG1* directly in *C. albicans*, its native species, might have a similar effect. Here we describe a novel role for Rfg1p in the regulation of pseudohyphal growth and examine the characteristics of this new regulatable *tet-RFG1* strain both *in vitro* and in two different infection models.

MATERIALS AND METHODS

Strains and media. The yeast strains and the plasmids used in this study are listed in Tables 1 and 2, respectively. Strains were routinely maintained as −80°C frozen stocks and grown on yeast extract-peptone-dextrose (YPD) medium whereas selections for uracil prototrophy were performed on minimal SD plates lacking uridine (39). Expression from the *tet* promoter was abolished by the addition of 20 μg ml^{-1} doxycycline to the growth medium. For filamentation assays in liquid media, strains were grown overnight in YPD plus doxycycline at 30°C, washed in sterile phosphate-buffered saline (PBS), and diluted 1:20 into fresh media such as RPMI 1640 supplemented with L-glutamine and buffered with morpholinepropanesulfonic acid (Angus Buffers and Chemicals) and incubated with shaking at 37°C. For filamentation assays on solid media, strains were grown as for liquid filamentation assays, washed in sterile PBS, and counted using a hemocytometer. For growth on solid Spider medium (26), cells were resuspended to a final concentration of 5×10^6 cells ml^{-1}. Aliquots of 2 μl (containing approximately 10,000 cells) were spotted onto Spider plates, which were subsequently incubated at 37°C for 7 days. For growth on synthetic low ammonia dextrose (SLAD) medium (19), approximately 200 cells were spread onto SLAD plates and were then incubated at 30°C for 5 days. For embedded growth, approximately 200 cells were mixed with molten YPD agar and poured onto YPD plates, which were subsequently incubated at 30°C for 3 days. Colonies

TABLE 2. Plasmids used in this study

Name	Reference
p97CAU1	32
p97RFG1AB	This study
pA83	36
CIp10	29
CIpSAT	This study
CIpSATSA	This study
CIpSATSA*CLB4*	This study

TABLE 3. Oligonucleotides used for cloning in this study

Name	Sequence[a]	Reference
RFGA.FOR	5′-CACACATAGGTACCCCAATACAC-3′	This study
RFGA.REV	5′-CACTTTAAACAGATAAACTCGAGGATATG-3′	This study
RFGB.FOR	5′-CCACTAGTATTTATTTGCAAACCATCACACC-3′	This study
RFGB.REV	5′-GTCTACCCAATCCGCGGGAAACTTC-3′	This study
SAT.FOR	5′-GGGCCCGAGCTCGCATGCCAGCGTC-3′	This study
SAT.REV	5′-GGACTAGTGATTTCTAGAAGGACCAC-3′	This study
ACTprom.FOR	5′-GGGCGAATTGGTACCGACGTCGC-3′	This study
ACTprom.REV	5′-CATACCCTCGAGTTTGAATGATTATATTT-3′	This study
CLB4.FOR	5′-CCAGAGGTTCACAAGCTTATGCGCGATC-3′	This study
CLB4.REV	5′-GGGAATTCAATGATGATGGTGGTGGTGACTCTGGGACATTATGTGACG-3′	This study

[a] Underlined sequences indicate introduced restriction enzyme sites.

were examined and photographed using a GL9-280 Stereo Zoom microscope (Jenco) equipped with a digital camera. All morphological tests were performed in at least biological triplicate. For growth curves, overnight cultures were diluted 1:30 in fresh YPD with and without doxycycline and grown with shaking at 30°C. Aliquots were removed at the times indicated, and the optical density at 600 nm (OD_{600}) was measured. All plasmid manipulations were performed with *Escherichia coli* strain DH5α with selection on Luria-Bertani plates containing 100 μg/ml ampicillin when necessary.

Fluorescence microscopy. *C. albicans* cells were stained using calcofluor white (Sigma), and nuclei were revealed through the use of Vectashield mounting medium with DAPI (4′,6-diamidino-2-phenylindole; Vector Labs). A DMR epifluorescence microscope (Leica) was used to visualize fluorescence and capture cell images.

Strain construction and transcriptional analysis. The two independently isolated *tet-RFG1* strains PMC-X7 and PMC-X8 were constructed as follows: first, two regions spanning positions −1400 to −499 (RFGA) and positions −21 to +779 (RFGB) relative to the ATG start codon of the *RFG1* open reading frame (GenBank accession no. 3642570) were PCR amplified by using the primer pairs RFGA.FOR with RFGA.REV and RFGB.FOR with RFGB.REV (Table 3). These amplification products were digested with KpnI and XhoI or SpeI and SacII at the sites engineered into the primers and ligated sequentially between the KpnI and XhoI or SpeI and SacII sites in the proximal and distal cloning regions of the p97CAU1 plasmid (32) to form plasmid p97RFG1AB. The entire 3.8-kb promoter-replacing construct was then liberated from this plasmid as a KpnI-SacII fragment and transformed into the TR transactivator gene-containing *C. albicans* strain THE1 (32) using a modified polyethylene glycol-lithium acetate transformation method. Genomic DNA was prepared from several of the Ura^+ transformants obtained using the MasterPure yeast DNA extraction kit (Epicentre Biotechnologies), digested with SpeI, transferred to a positively charged Nytran membrane (Whatman), and subjected to Southern blot analysis as previously described (13). The RFGB PCR product (described above) was labeled with [^{32}P]dCTP using Ready-To-Go DNA labeling beads (GE Healthcare) and used as a probe.

To verify DOX regulation of the modified *tet-RFG1* allele, the wild-type (CAF2-1) and modified strains were grown for 3 h in RPMI 1640 at 37°C and total RNA was isolated using a previously published bead beater protocol (34) and then separated through formaldehyde-containing agarose gels. Transfer and hybridization followed the method described above for the Southern blot assay, again using the RFGB PCR product as a probe.

The strain PMC-X7+*CLB4* was constructed as follows: plasmid CIpSAT was constructed by replacing the *URA3* gene in CIp10 with the *SAT1* gene, encoding a protein conferring resistance to nourseothricin. *SAT1*, along with its *ACT1* promoter, was amplified from plasmid pA83 (36) using primers SAT.FOR and SAT.REV. The amplification product was digested with SacI and XbaI at the sites engineered into the primers and ligated between the SacI and XbaI sites in CIp10 (29), thereby replacing the *URA3* gene. The sequence upstream of the *C. albicans ACT1* gene was amplified from plasmid pA83 using primers ACTprom.FOR and ACTprom.REV. The amplification product was digested with KpnI and XhoI at the sites engineered into the primers and ligated between the KpnI and XhoI sites in CIpSAT, producing CIpSATSA. The *CLB4* coding sequence was amplified from genomic DNA using primers CLB4.FOR and CLB4.REV. These amplification products were digested with HindIII and EcoRI at the sites engineered into the primers and ligated between the HindIII and EcoRI sites in CIpSATSA to produce plasmid CIpSATSA-*CLB4*. Finally, this plasmid was linearized by digestion with StuI and transformed into the *tet-RFG1* strain PMC-X7 using a modified electroporation transformation method (25). Nourseothricin-resistant transformants were selected on YPD agar plates containing 200 μg ml^{-1} nourseothricin (Werner Bioagents, Jena, Germany) as described previously (36). Correct insertion into the *RP10* locus was confirmed by PCR.

Murine virulence assay. For injection, cultures of *tet-RFG1* strain PMC-X7 were grown overnight at 25°C in YPD with doxycycline. Cells were harvested by centrifugation and washed three times in sterile pyrogen-free saline. The cells were counted using a hemocytometer, and appropriate dilutions were made so that the required dosage of cells could be injected in a final volume of 200 μl into the lateral tail veins of 6- to 8-week-old female BALB/c mice that had been placed on either 5% sucrose or 5% sucrose containing 2 mg of doxycycline ml^{-1} 3 days prior to infection. Confirmation of the number and viability of cells present in the infecting inocula was performed by plate count. Groups of eight mice were used for each condition. All experiments were performed in accordance with institutional regulations in place at the University of Texas at San Antonio. Mice were allowed a 1-week acclimatization period before experiments were started. Days on which the animals died were recorded; severely moribund animals were humanely sacrificed to minimize suffering and recorded as having died the following day.

Histopathology. Kidneys excised from deceased or sacrificed mice were fixed in 10% buffered formalin and stored at 4°C until required. After kidneys were embedded in paraffin, tissue slices were removed and stained with Grocott-Gomori methenamine-silver prior to microscopic evaluation (21). The human oral tissue culture models were fixed in 10% buffered formalin and stored at 4°C until required. Tissue slices from paraffin wax-embedded tissue models were stained with Mayer's hematoxylin prior to microscopic evaluation.

Infection of a tissue-engineered 3-dimensional OEMS with *C. albicans*. A commercially available human oral epithelium model system (OEMS) (Skinethic Laboratory, Nice, France) was used to investigate the interaction of the mucosal epithelium with *C. albicans*. Models were maintained according to the manufacturer's instructions. The *tet-RFG1* strain PMC-X7 and wild-type CAF2-1 strain were grown overnight as described above, and cells were washed, counted, and resuspended to a final concentration of 5×10^7 CFU/ml. An aliquot of 100 μl of cell suspension was added to each of the models while negative controls were inoculated with PBS. The models were then incubated for 24 or 48 h at 37°C in a 5% CO_2 humidified incubator and were fixed in 10% buffered formalin.

Quantitative PCR. RNA was isolated from *C. albicans* cells using the MasterPure yeast RNA extraction kit (Epicentre Biotechnologies). RNA was treated with amplification-grade DNase I (Invitrogen) and used for cDNA synthesis with the cMaster RT kit (Eppendorf). The primer sets (Table 4) were used in conjunction with SYBR green PCR master mix (Applied Biosystems) and Twin.tec real-time 96-well PCR plates (Eppendorf) in an ABI 7300 real-time PCR system (Applied Biosystems). Dissociation curves were analyzed for all reactions to verify single peaks/products. Expression levels were analyzed using ABI 7300 system SDS software (Applied Biosystems).

RESULTS

Regulatable strain construction. We previously described the construction of an engineered strain in which one copy of *NRG1* was placed under the control of a tetracycline-regulatable promoter (38) and showed that regulation of one allele

TABLE 4. Oligonucleotides used for quantitative real-time PCR

Name	Sequence	Reference
ACT1-S	5′-ATGTGTAAAGCCGGTTTTGCCG-3′	41
ACT1-A	5′-CCATATCGTCCCAGTTGGAAAC-3′	41
CLB4_FOR	5′-CACAAACAAACACCAATCATCACA-3′	This study
CLB4_REV	5′-ATCGGTTTTCTTTTGCTCTATTTG-3′	This study
RFG1 forward	5′-AACCCTGAAGTTTCCCGAGAA-3′	33
RFG1 reverse	5′-CAGCAAGATTATTCCAATGTTCCTT-3′	33
FKH2_FOR	5′-GCAAACTCGCTCCAATCAAA-3′	This study
FKH2_REV	5′-TGCTTGCGTAATCATTGTCG-3′	This study
HSL1_FOR	5′-GATTGCCGATTTTGGTATGG-3′	This study
HSL1_REV	5′-TGGTGAAGCATAATGAGGAGAA-3′	This study
GIN4_FOR	5′-TTATGCTGCTCCAGAAATCGTT-3′	This study
GIN4_REV	5′-ACCCCACAAGACCAAACATCA-3′	This study
ALS3_FOR	5′-CAACTTGGGTTATTGAAACAAAAACA-3′	31
ALS3_REV	5′-AGAAACAGAAACCCAAGAACAACCT-5′	31
ECE1_FOR	5′-CCAGAAATTGTTGCTCGTGTTG-3′	3
ECE1_REV	5′-CAGGACGCCATCAAAAACG-3′	3
HWP1_FOR	5′-TCAGCCTGATGACAATCCTC-3′	This study
HWP1_REV	5′-GCTGGAGTTGTTGGCTTTTC-3′	This study

was sufficient to control the phenotype of the strain. A similar strategy was used here to construct a strain regulating *RFG1*. A small region of the sequence upstream of one allele of *RFG1* (position −499 to −21 relative to the ATG start codon) was replaced with the bacterially derived *tetO* promoter sequence by integration into the genome of the transactivator-containing *C. albicans* strain THE1. Correct integration of this promoter-modifying fragment at the *RFG1* locus was confirmed for two independent transformants, PMC-X7 and PMC-X8, by Southern blot analysis (Fig. 1A). To verify that *RFG1* expression could be modulated by doxycycline in the transformed strains, RNA was extracted from the wild-type CAF2-1 and the modified strains grown in RPMI 1640 in the presence or absence of doxycycline. Northern blot analysis revealed that the modified *tet-RFG1* allele produced a smaller transcript than did the wild-type copy (Fig. 2B), presumably as a consequence of integration of the *tetO* promoter very close to the ATG start codon producing a shorter 5′ untranslated leader sequence. This allowed the comparison of transcription from the modified *tet-RFG1* allele as well as from the wild-type allele. As predicted, transcription from the modified allele was completely dependent on the presence or absence of doxycycline, and transcription from the remaining unaltered copy of *RFG1* did not appear to be affected by the presence of doxycycline (Fig. 2B). Preliminary analysis of the two independently isolated *tet-RFG1* strains determined that they were phenotypically indistinguishable, and therefore, all of the subsequent experiments were performed only with the PMC-X7 isolate.

***RFG1* overexpression does not inhibit hypha formation under liquid-inducing conditions.** To determine whether overexpression of the repressor *RFG1* would have an inhibitory affect on hypha formation similar to that of *NRG1*, the *tet-RFG1* strain PMC-X7 was grown in medium known to induce hypha formation in *C. albicans*, in both the presence and the absence of doxycycline. Growth in cell culture medium RPMI 1640 at 37°C strongly stimulates hyphal formation in wild-type strains, and this medium is used for *in vitro* biofilm formation on polystyrene surfaces (35). As expected, the wild-type CAF2-1 strain formed hyphae when grown in RPMI 1640 at 37°C irrespective of the presence of doxycycline (Fig. 2A). We had predicted that overexpressing *RFG1* would inhibit formation of hyphae under inducing conditions, but unexpectedly, the *tet-RFG1* strain was still able to respond normally in the absence of doxycycline (Fig. 2A). To assess whether *RFG1* overexpression might block hypha formation only in response to a specific environmental stimulus, the strains were grown in additional hypha-inducing liquid media. The *tet-RFG1* strain formed hyphae normally, irrespective of the presence of doxycycline, when grown in YPD plus 10% serum at 37°C or in M199 at pH 8 (data not shown), despite expression of *RFG1* from the modified allele in the absence of doxycycline (data not shown). This indicates that increased *RFG1* transcription is not sufficient to inhibit hypha formation induced by serum or by the Rim101p-mediated pH induction pathway.

***RFG1* overexpression does not inhibit filament formation on solid hypha-inducing media.** Growth on solid Spider medium is a strong stimulator of hyphal growth in *C. albicans* (26), and although Rfg1p is required to block hypha formation when strains are grown on YPD plates, a strain lacking Rfg1p was shown to be deficient in hypha formation on Spider medium, although an apparent growth defect made this phenotype difficult to assess (22). When wild-type CAF2-1 is grown on Spider plates at 37°C, highly wrinkled colonies are formed, indicating the presence of hyphal cells (Fig. 3). Overexpressing *RFG1* did not appear to affect that ability of the *tet-RFG1*

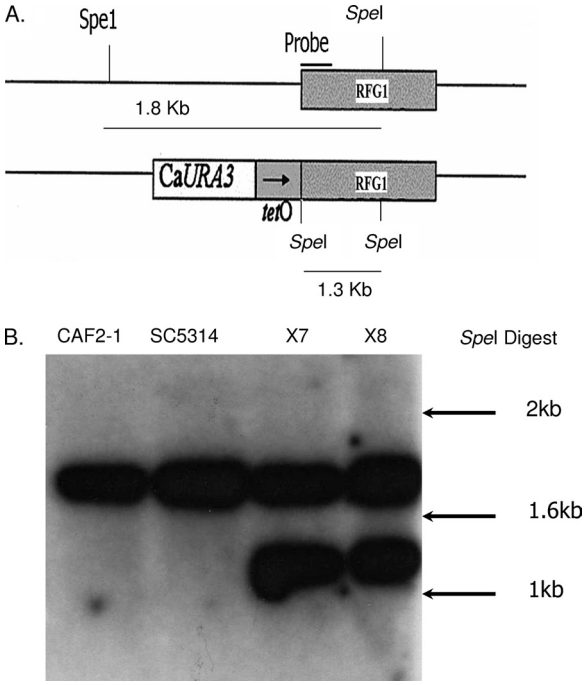

FIG. 1. Construction of the *tet-RFG1 C. albicans* strain. (A) The *tet-RFG1* strain was produced through integration of an *in vitro*-produced DNA construct designed to replace the promoter region (−499 to −21) of *RFG1* with the bacterially derived *tetO* sequence. (B) Appropriate integration of this construct at the *RFG1* locus was confirmed via Southern blot analysis (PMC-X7 and PMC-X8 represent two independent transformants). Genomic DNA was isolated and digested with SpeI and transferred to a Nytran membrane. In the wild-type control strains CAF2-1 and SC5314, the probe hybridized to a single band of approximately 1.8 kbp representing the endogenous allele. In the transformed strains the probe hybridized to an additional band of approximately 1.3 kbp representing the modified allele. Reference sizes are indicated by arrows.

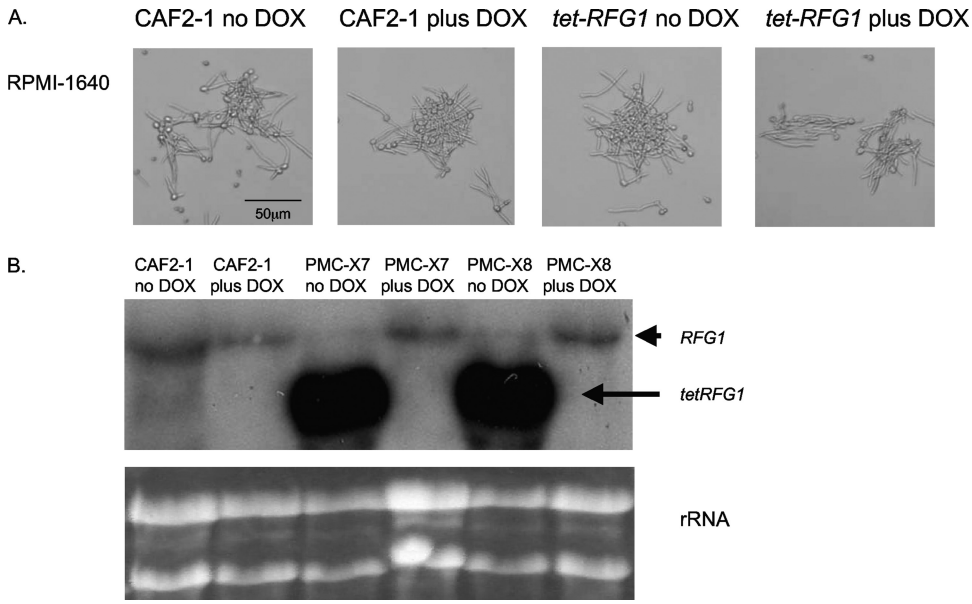

FIG. 2. *RFG1* overexpression does not inhibit hypha formation under inducing conditions. (A) The effect of *RFG1* overexpression was assessed by growing the *tet-RFG1* strain in RPMI 1640 at 37°C, in the absence or presence of doxycycline. Both the wild-type CAF2-1 and the modified *tet-RFG1* strain formed hyphae. (B) *RFG1* transcription in these cultures was examined by Northern blot analysis. RNA was isolated and transferred to a Nytran membrane. The probe was the same as that used for the Southern blot assay. The larger endogenous *RFG1* transcript and the smaller *tet-RFG1* transcript are indicated by arrows. Ethidium bromide-stained rRNA bands are shown to confirm equal loading of all samples. The *tet-RFG1* transcript is absent from the control and from the transformants grown in the presence of doxycycline, indicating doxycycline-dependent regulation of the modified allele.

strain to form hyphae, since wrinkled colonies were formed in both the presence and the absence of doxycycline. Microscopic examination of cells removed from these colonies revealed that they consisted primarily of hyphal cells (data not shown). Thus, alterations in *RFG1* expression did not enhance or impair the ability to form hyphae on solid Spider medium.

C. albicans cells also form hyphae when grown embedded in solid medium at 30°C in response to low oxygen levels and increased physical pressure (11, 43). These signals are transduced through the transcriptional regulators Czf1p, which is required for hyphal growth under embedded conditions, and Efg1p, which normally promotes hypha formation but appears to play a role in the repression of hypha-specific genes under low-oxygen conditions (20). The CAF2-1 cells embedded in the solid medium began to form hyphae after 48 h growth, and by 72 h, all of the cells had formed hyphal colonies (Fig. 3). The *tet-RFG1* strain was able to form hyphae normally in the presence or absence of doxycycline, indicating that under embedded conditions *RFG1* overexpression does not block the signals transduced through Efg1p and Czf1p.

Overexpression of *RFG1* promotes pseudohyphal growth. Although overexpressing *RFG1* does not inhibit hypha forma-

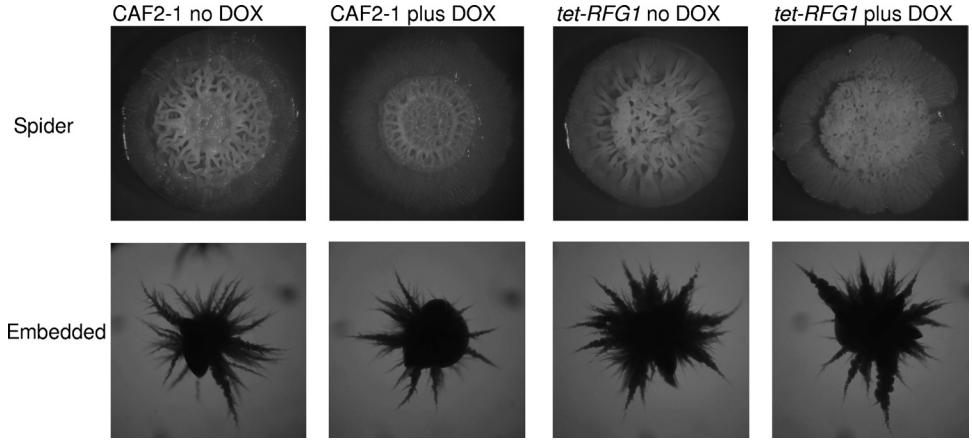

FIG. 3. *RFG1* overexpression does not inhibit hypha formation on solid inducing media. For Spider medium, cells were spotted on Spider plates with and without doxycycline and incubated at 37°C for 7 days (upper panels). For embedded conditions, approximately 200 cells were mixed with molten YPD agar, poured onto YPD plates, and incubated at 30°C for 3 days. The wild-type CAF2-1 and the *tet-RFG1* strain formed wrinkled colonies on Spider plates and filamentous colonies embedded in YPD irrespective of the presence or absence of doxycycline.

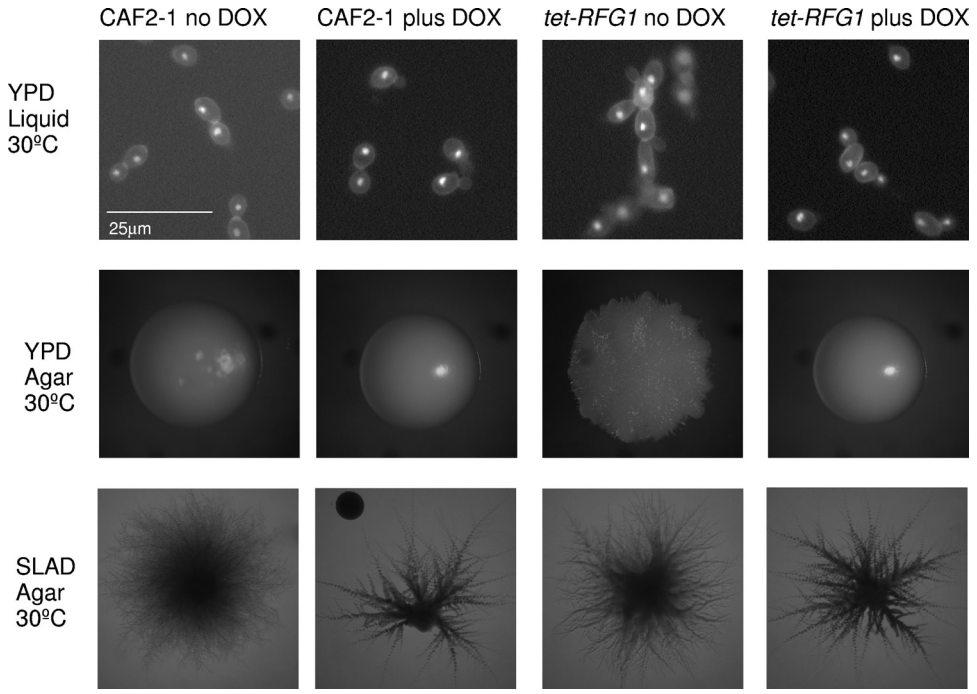

FIG. 4. *RFG1* overexpression promotes pseudohyphal growth. Strains were grown under standard yeast growth conditions, in liquid YPD (top panels) and on solid YPD agar (middle panels) in the presence of absence of doxycycline. The wild-type CAF2-1 strain grows as round yeast cells in liquid medium and forms smooth colonies on solid medium whereas the *tet-RFG1* strain forms chains of elongated pseudohyphal cells in liquid medium and forms wrinkled colonies when *RFG1* is overexpressed. Both strains form pseudohyphae when grown on SLAD medium (bottom panels), although the observed frequency of pseudohyphal growth was higher when *RFG1* was overexpressed.

tion, it does influence morphology under yeast growth conditions. When grown in YPD at 30°C in the presence of doxycycline, the *tet-RFG1* strain resembles the wild-type strain CAF2-1 and the culture consists entirely of free-floating yeast cells. In the absence of doxycycline (elevated *RFG1* expression), however, the *tet-RFG1* cells grew exclusively as pseudohyphae (Fig. 4). Visualization of these cells after staining with calcofluor white and DAPI confirmed that these cells were not hyphal but pseudohyphal (Fig. 4). This same phenotype was observed on solid YPD plates, where CAF2-1 formed smooth round colonies, as did the *tet-RFG1* strain grown in the presence of doxycycline. When *RFG1* levels were elevated, however, the strain formed a wrinkled colony, indicating the presence of filamentous cells (Fig. 4). Microscopic examination revealed that these cells are pseudohyphae rather than hyphae (Fig. 4). Nitrogen-limiting SLAD medium is frequently used to induce pseudohyphal formation in *C. albicans*, and when strains are incubated on SLAD plates at 30°C, a fraction (less than 5%) of colonies formed by the wild-type CAF2-1 or *tet-RFG1* strain in the presence of doxycycline exhibit a smooth center surrounded by a halo of pseudohyphal growth. In the absence of doxycycline, however, greater than 50% of the colonies formed by the *tet-RFG1* strain displayed this pseudohyphal morphology (Fig. 4). Although elevated *RFG1* levels were unable to block the formation of hyphae, we were able to promote pseudohyphal growth under yeast growth conditions by manipulating *RFG1* expression. Previous studies described Rfg1p as a repressor of hyphal growth that is required to block hypha formation under yeast growth conditions (22, 24), while also being necessary for forming hyphae under nutrient-limiting conditions (22). However, Rfg1p may play another, perhaps more significant, role in regulating *C. albicans* morphology since we have now demonstrated that overexpression of *RFG1* can actually drive pseudohyphal growth under yeast conditions.

Virulence is not affected by modulating *RFG1* expression. Having demonstrated that *RFG1* overexpression alters *C. albicans* morphology *in vitro*, we examined its effect on virulence in the murine model of disseminated candidiasis. In this model, strains that are locked in the yeast form or in the hyphal form of growth are avirulent. A strain lacking Rfg1p was previously shown to be avirulent in this disease model (22). Using the tetracycline-regulated promoter system, we are able to manipulate expression of the *tet*-regulated gene within an animal host by the addition or omission of doxycycline from the drinking water (32). Approximately 3.5×10^5 *C. albicans* yeast cells of the *tet-RFG1* strain were injected into mice that had been placed on either 5% sucrose or 5% sucrose containing 2 mg ml^{-1} of doxycycline 3 days prior to infection. All the mice, irrespective of the treatment, succumbed to the infection, and there was no significant difference between the virulence of the *tet-RFG1* strain in the presence of doxycycline and the virulence in its absence (Fig. 5). We had hypothesized that, similar to our previous results with *NRG1*, if *RFG1* overexpression was able to block filamentation in the host, this might render the strain avirulent. However, elevated *RFG1* expression neither impaired nor enhanced virulence, since there was no difference between the survival curves produced in the presence and those produced in the absence of doxycycline.

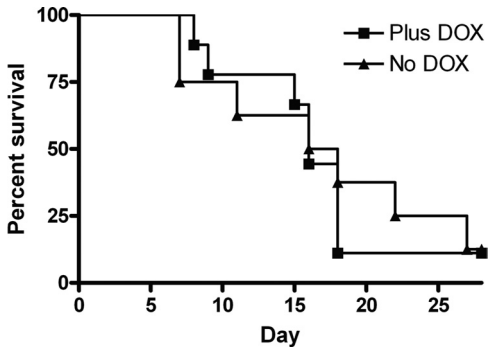

FIG. 5. The virulence of the *tet-RFG1* strain is unaffected by the presence or absence of doxycycline. Approximately 3.5×10^5 *C. albicans* yeast cells of the *tet-RFG1* strain PMC-X7 were injected into mice that had been placed on either 5% sucrose (No DOX) or 5% sucrose containing 2 mg/ml of doxycycline (Plus DOX). There was no difference in the survival curves produced under the two conditions.

Histopathology of *tet-RFG1* strain-infected tissues. To address the possibility that the modified strain had not affected virulence but had nonetheless altered the morphology of the fungal cells in the animal host, we performed a histological analysis on kidneys recovered from mice that had succumbed to the infection both in the presence and in the absence of doxycycline. Tissues from mice that had died early (7 or 8 days) or died later (16 or 18 days) after infection were examined. This examination revealed the presence of extensive lesions containing mainly hyphal fungal cells irrespective of the presence or absence of doxycycline (Fig. 6). The mice that survived longer showed more extensive mycelial lesions but were otherwise identical to the mice that died earlier (data not shown).

Overexpression of *RFG1* in a reconstituted human oral tissue culture model. To further examine the influence of *RFG1* overexpression on pathogenesis, we used the *tet-RFG1* strain to infect a human oral tissue culture model. Overexpressing *RFG1* did not appear to have an effect on the ability of the strain to cause damage or to invade the host tissue, as assessed by lactate dehydrogenase (LDH) release (data not shown) and by histopathological examination of sections from the infected models (Fig. 7).

Rfg1p does not promote pseudohyphal growth via repression of pseudohypha-associated cell cycle regulators. Several mutant *C. albicans* strains display a morphology similar to that of the *tet-RFG1* strain in the absence of doxycycline, forming pseudohyphae under yeast growth conditions. These include strains in which one of several cell cycle regulators of the G_2/M transition has been deleted. This suggested that *RFG1* overexpression could be perturbing the cell cycle by suppressing one of these regulators and thus leading to the observed pseudohyphal phenotype. As a simple test to determine whether overexpressing *RFG1* was affecting cell cycle progression, we therefore compared the growth rates of the *tet-RFG1* strain in the presence and absence of doxycycline. There was no discernible difference between the two cultures (Fig. 8A), suggesting that the pseudohyphal growth phenotype of the *tet-RFG1* strain grown in the absence of doxycycline is not simply a consequence of *RFG1*-mediated cell cycle suppression. However, it is known in *S. cerevisiae* that, when one part of the cell cycle is lengthened, other phases can be shortened to compensate, and thus, the overall length of the cell cycle remains the same (2, 37). For instance, cells of the *CLN3-2* strain, which carries a stabilized allele of the G_1 cyclin *CLN3*, have an accelerated entry into Start but do not show a reduced doubling time because the S and G_2 phases are lengthened (2). Through such a compensation mechanism, Rfg1p could still be acting by repressing expression of cell cycle regulatory genes without affecting the overall growth rate. One of the proteins governing cell cycle progression, the Fkh2p transcription factor, is required for expression of the G_2/M transition regulator *CLB4*, and the *fkh2*Δ null strain is constitutively pseudohyphal but remains able to respond to hypha-inducing signals (5). Gin4p is a kinase is required for septin ring formation, and the *gin4*Δ null strain is also constitutively pseudohyphal but, in this case, unable to form hyphae (46). Deletion of the *HSL1* gene, which encodes a septin-associated mitotic kinase, produces a strain which when grown under yeast conditions forms pseudohyphae at low cell density and grows as yeast cells at high density. These yeast cells are able to respond to hypha-inducing stimuli and can form hyphae (42, 46). Quantitative real-time PCR was used to examine whether the striking constitutive pseudohyphal morphology observed as a result of *RFG1* overexpression was possibly due to transcriptional repression of one or more of these regulators. The *tet-RFG1* strain was grown overnight in the presence of doxycycline, and cells were washed three times in sterile PBS before being diluted 1:20 into fresh YPD in

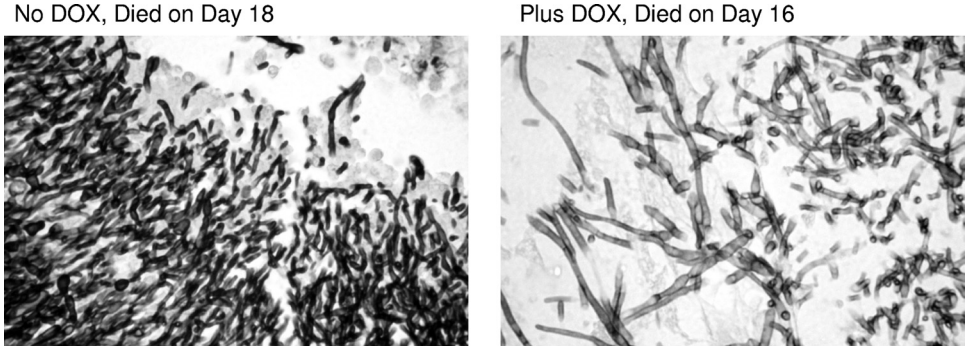

FIG. 6. Histopathology of *tet-RFG1* strain-infected tissues. Examination of kidneys retrieved from mice infected with the *tet-RFG1* strain revealed the presence of extensive lesions containing mainly hyphal cells irrespective of the presence or absence of doxycycline.

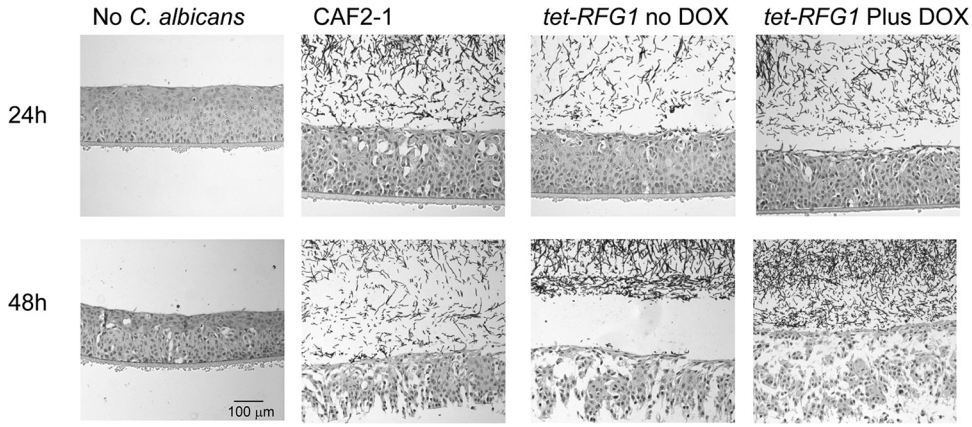

FIG. 7. Histopathology of the human oral mucosal model. *C. albicans* strains were incubated with the oral mucosal model for 24 h or 48 h, and the tissue was fixed, stained, and examined microscopically. Both the wild-type CAF2-1 and *tet-RFG1* strains produce filaments and cause extensive damage to the tissues. The *tet-RFG1* strain is able to damage the tissues to the same extent, even in the presence of elevated *RFG1* transcription (no DOX).

the presence or absence of doxycycline and grown for 6 h at 30°C. While *RFG1* expression was elevated in the absence of doxycycline, expression of the other regulatory genes showed no marked change between the two conditions (Fig. 8B). Although the expression of the mitotic regulatory genes varies during the cell cycle, making it more difficult to track such temporal transcriptional changes with certainty in an asynchronous culture, our results suggest a lack of involve-

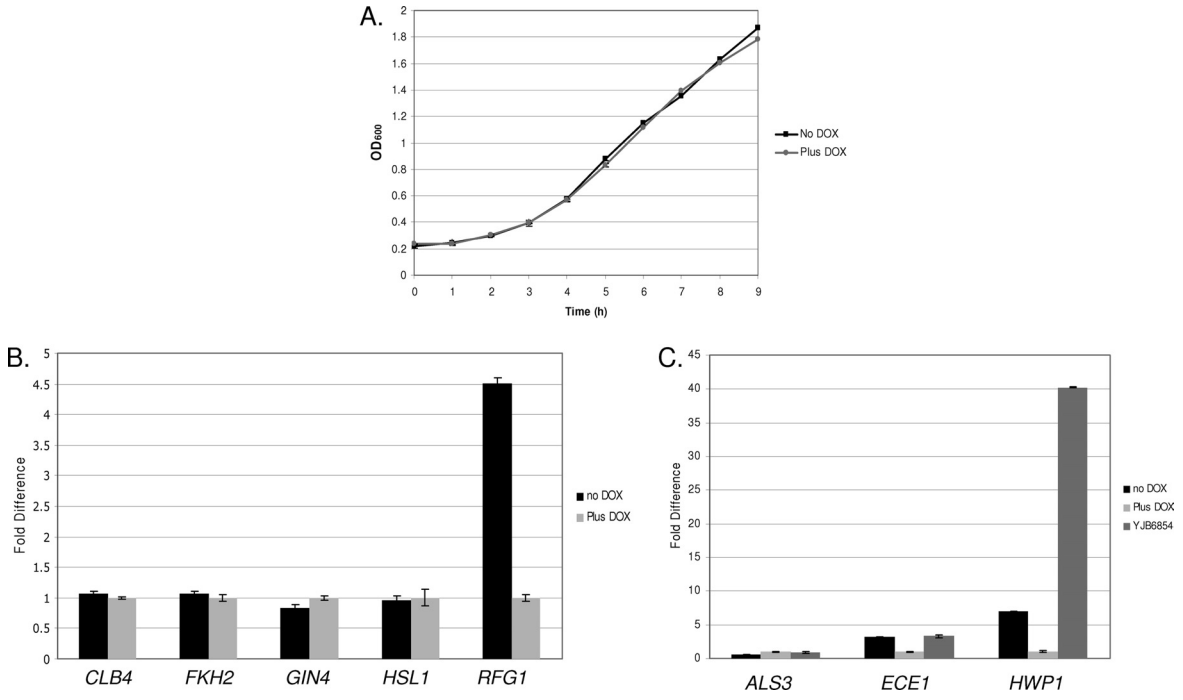

FIG. 8. (A) Growth curves of the *tet-RFG1* strain. The *tet-RFG1* strain was grown overnight in the presence of doxycycline, and cells were washed before being diluted 1:30 in fresh YPD or YPD plus DOX. Duplicate cultures were grown with shaking at 30°C, samples were removed at the times indicated, and the OD_{600} was measured. The strain grew at identical rates regardless of the presence or absence of doxycycline. (B) Quantification of mitotic regulatory gene transcription in the presence of elevated *RFG1*. The *tet-RFG1* strain was grown overnight in the presence of doxycycline, and cells were washed before being diluted 1:20 into fresh YPD and grown for 6 h at 30°C in the presence or absence of doxycycline. At this point the culture grown in the presence of doxycycline consisted entirely of yeast cells, while the culture grown in the absence of doxycycline was approximately 80% pseudohyphal. RNA was isolated from cells harvested from these cultures, and transcript levels were quantified through real-time PCR analysis. Levels were normalized by *ACT1* and are expressed relative to the control *tet-RFG1* plus doxycycline culture (therefore, its results are 1.0). While *RFG1* levels are elevated approximately 4.5-fold in the *tet-RFG1* strain grown in the absence of doxycycline, there are only minor differences in transcript levels of the other genes examined. Error bars represent the standard deviations of the threshold cycle (ΔC_T) values. (C) Quantification of filament-specific gene transcription in the context of elevated *RFG1* expression. The *tet-RFG1* and the *clb4*Δ null (YJB6854) strains were grown, and transcript levels were analyzed as described for panel B. Transcription of these genes followed the same pattern in both the *tet-RFG1* strain grown in the absence of doxycycline and the *clb4*Δ null strain.

ment of these genes in the *RFG1*-mediated pseudohyphal phenotype.

Another protein involved in cell cycle progression, and thought to operate at the G_2/M transition, is the B-type cyclin Clb4p (4). The *clb4*Δ null strain, like the *tet-RFG1* strain, forms pseudohyphae under yeast growth conditions but is still able to form hyphae when grown in the presence of serum at 37°C (4). To confirm that the filaments produced by the *tet-RFG1* strain are pseudohyphae and to determine any further similarities to the *clb4*Δ strain, we compared the expression of several filament-specific genes in two strains grown under yeast conditions. While no pseudohypha-specific transcripts have yet been described, expression of the genes encoding two components of the hyphal wall, *ECE1* and *HWP1*, has previously been shown to be increased in pseudohyphal cells compared to yeast cells (12). Expression of these genes was elevated in both the *clb4*Δ strain and the *tet-RFG1* strain grown in the absence of doxycycline (Fig. 8C) compared to that in the *tet-RFG1* plus doxycycline yeast culture control. Furthermore, transcription of *ALS3*, which is hypha specific (15), was not elevated in the *tet-RFG1* strain filaments, indicating that they are not true hyphae (Fig. 8C) and confirming our microscopic examination (Fig. 4). Although *HWP1* was more highly expressed in the *clb4*Δ strain than in the *tet-RFG1* strain, the similarities in the overall patterns of gene expression and morphology between the two strains suggested that Rfg1p might repress *CLB4* expression. To test this, we integrated an additional extragenic copy of the *CLB4* gene under the control of the *ACT1* promoter into the *tet-RFG1* strain. Overexpression of *CLB4* has previously been demonstrated to slow hypha formation under inducing conditions (4). We therefore tested the functionality of our constitutive *CLB4* gene by monitoring the ability of this strain (PMC-X7+*CLB4*) to filament following inoculation into RPMI 1640 and growth at 37°C. Samples were examined at various time points, and the proportions of yeast and hyphal cells were determined microscopically. The *CLB4*-containing strain showed delayed hypha formation compared to the PMC-X7+empty vector control strain (Fig. 9A), indicating that our constitutive *CLB4* construct was producing functional Clb4p. To test the ability of *CLB4* to rescue the pseudohyphal phenotype, the strains were grown overnight in YPD plus doxycycline (to maintain yeast growth), washed, and resuspended in fresh YPD in the presence or absence of doxycycline. These cultures were incubated at 30°C, and cells were examined microscopically at various time points. We observed no marked difference in the rate at which the PMC-X7+*CLB4* or control strain formed pseudohyphae (Fig. 9B), indicating that Rfg1p is not promoting pseudohypha formation by repressing *CLB4* transcription.

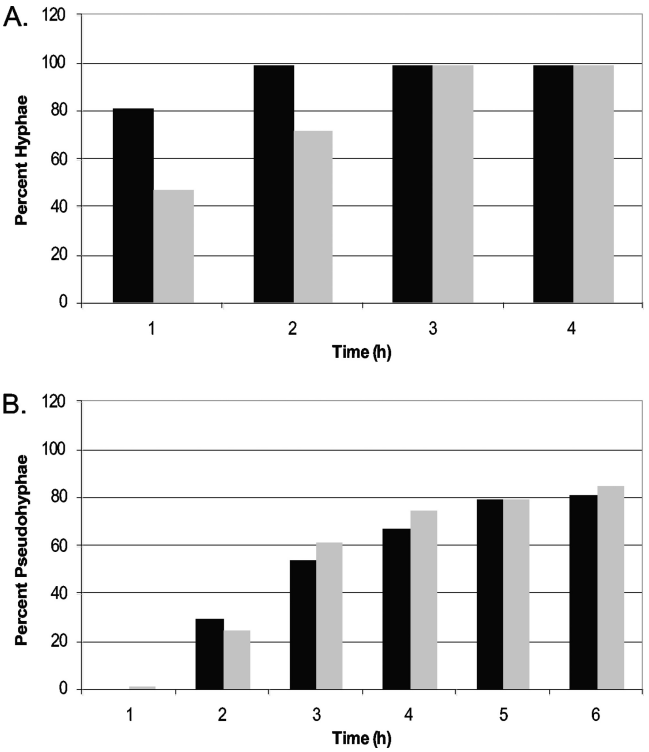

FIG. 9. *CLB4* overexpression does not rescue the pseudohyphal phenotype. (A) The *tet-RFG1*+empty vector control (black bars) and *tet-RFG1*+*CLB4* (gray bars) strains were grown in RPMI 1640 at 37°C and examined at the indicated time points, and the proportion of hyphal cells was determined microscopically. Note the lag in hypha formation observed in the *tet-RFG1*+*CLB4* strain. (B) The same strains were grown in YPD at 30°C and examined at the indicated time points, and the proportion of pseudohyphal cells was determined microscopically. This figure presents data from a single experiment that was repeated three times with the same results.

DISCUSSION

Here we describe the construction of a novel regulatable *tet-RFG1* strain and its characterization both *in vitro* and *in vivo*. In the absence of doxycycline, this strain produces elevated levels of *RFG1* but, contrary to expectation, is able to filament normally when grown in liquid media or on plates incubated under hypha-inducing conditions. When tested in the murine model of disseminated candidiasis, *RFG1* overexpression did not affect the ability to cause disease or to form filaments within infected organs. The *tet-RFG1* strain was likewise able to successfully infect and damage a tissue culture model of the human oral mucosa as efficiently as a wild-type strain.

More surprisingly, overexpression of *RFG1* was able to promote the formation of pseudohyphae when the strain was grown under yeast conditions, indicating an unexpected and novel role for this protein. Microscopic examination and transcriptional analysis confirmed that these cells were pseudohyphal, not hyphal.

Mutations affecting the cell cycle, particularly the G_2/M transition, can result in a pseudohyphal morphology in *C. albicans*. The resemblance of the cells from a culture where *RFG1* was overexpressed to those deleted for several genes involved in cell cycle progression suggested that elevated *RFG1* levels may be affecting the G_2/M transition. Interestingly, although the cell morphology was altered, the overall growth rate of the cells remained unchanged with or without *RFG1* overexpression. In an attempt to determine the mechanism(s) by which *RFG1* overexpression causes this pseudohyphal phenotype, we examined the expression of several cell cycle regulatory genes encoding proteins whose absence is known to produce a similar pseudohyphal phenotype. The

expression of these genes did not appear to vary significantly in the presence or absence of elevated *RFG1* transcription. Furthermore, we attempted to restore a wild-type phenotype through the constitutive expression of one of these genes, *CLB4*, but this was insufficient to inhibit the pseudohyphal morphology.

While a great deal is known about the signaling pathways and genes involved in *C. albicans* hyphal development, our understanding of the process of pseudohypha formation is more rudimentary. The observation that defects in several apparently unrelated genes can lead to pseudohypha formation under yeast growth conditions and additionally that some of these mutants can form hyphae (*tet-RFG1*, *clb4*Δ, and *nrg1*Δ mutants) while others cannot (*tup1*Δ and *gin4*Δ mutants) suggests that multiple pathways or mechanisms are involved in this process. We have attempted to link our novel *RFG1*-induced pseudohyphal phenotype to those caused by mutations in several known genes in an attempt to clarify this picture. These experimental results did not reveal a direct connection between *RFG1* and any of the genes that we tested but reinforced the multifactorial nature of pseudohypha formation in *C. albicans*.

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