Calcineurin Controls Drug Tolerance, Hyphal Growth, and Virulence in Candida dubliniensis

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Candida dubliniensis is an emerging pathogenic yeast species closely related to Candida albicans and frequently found colonizing or infecting the oral cavities of HIV/AIDS patients. Drug resistance during C. dubliniensis infection is common and constitutes a significant therapeutic challenge. The calcineurin inhibitor FK506 exhibits synergistic fungicidal activity with azoles or echinocandins in the fungal pathogens C. albicans, Cryptococcus neoformans, and Aspergillus fumigatus. In this study, we show that calcineurin is required for cell wall integrity and wild-type tolerance of C. dubliniensis to azoles and echinocandins; hence, these drugs are candidates for combination therapy with calcineurin inhibitors. In contrast to C. albicans, in which the roles of calcineurin and Crz1 in hyphal growth are unclear, here we show that calcineurin and Crz1 play a clearly demonstrable role in hyphal growth in response to nutrient limitation in C. dubliniensis. We further demonstrate that thigmotropism is controlled by Crz1, but not calcineurin, in C. dubliniensis. Similar to C. albicans, C. dubliniensis calcineurin enhances survival in serum. C. dubliniensis calcineurin and crz1/crz1 mutants exhibit attenuated virulence in a murine systemic infection model, likely attributable to defects in cell wall integrity, hyphal growth, and serum survival. Furthermore, we show that C. dubliniensis calcineurin mutants are unable to establish murine ocular infection or form biofilms in a rat denture model. That calcineurin is required for drug tolerance and virulence makes fungus-specific calcineurin inhibitors attractive candidates for combination therapy with azoles or echinocandins against emerging C. dubliniensis infections.

Although Candida albicans is the most prevalent species causing candidiasis, >40% of Candida infections are now caused by evolutionarily diverged non-albicans Candida species (NACS). Candida dubliniensis, an emerging NACS that occurs globally, was first described as a separate species in 1995 (80), and its complete genome was recently sequenced (41). C. dubliniensis is the closest relative of the important human fungal pathogen C. albicans and commonly isolated from the oral cavities of patients with AIDS or individuals who are human immunodeficiency virus (HIV) positive and is occasionally found in the oral microflora of healthy individuals (78). Clinically, C. dubliniensis causes 2 to 7% of candidemia cases (40, 79), and it has been suggested that the gastrointestinal tract is a source of the C. dubliniensis in candidemia patients (13). Moreover, C. dubliniensis is now ranked as either the second or third most frequently isolated Candida species from patients with HIV/AIDS (6, 82). Interestingly, in addition to humans as the source, C. dubliniensis can be isolated from nonhuman sources, including ticks that parasitize seabirds (56) and the excrement of seabirds (49). Most avian C. dubliniensis isolates are genetically distinct from human isolates, but one avian isolate (AV7) has been shown to be indistinguishable from a human isolate by multilocus sequence typing (49), suggesting that transmission may occur between birds and humans.

C. dubliniensis isolates are susceptible to azole antifungal agents. However, C. dubliniensis can rapidly develop azole resistance during clinical therapy (52, 64). Chunchanur et al. recently reported that ~23% of C. dubliniensis isolates from HIV-infected patients were resistant to fluconazole (22). Moreover, ERG11 mutations in C. dubliniensis isolated from HIV-infected individuals contribute to decreased susceptibility to fluconazole (64). Thus, new therapies that involve novel or combination drug treatments are needed. The calcineurin inhibitors tacrolimus (FK506) and cyclosporine A (CsA) target calcineurin through the intracellular receptor FK506 binding protein 12 (FKBP12) or cyclophilin A (CyA), respectively. Calcineurin is a eukaryotic calmodulin-dependent serine/threonine protein phosphatase. It forms a heterodimer protein consisting of the catalytic A (Cna1) and regulatory B (Cnb1) subunits, which are

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highly conserved between yeasts and mammals (3). In response to stress, the transcription factor Crz1 is dephosphorylated by calcineurin and then migrates to the nucleus to regulate expression of genes encoding cell wall biosynthetic enzymes and proteins involved in ion homeostasis (42, 72, 75, 76). Calcineurin is required for azole and echinocandin tolerance in C. albicans (61, 70, 84), C. neoformans (28, 45), and A. fumigatus (74); thus, the combination of a calcineurin inhibitor with either class of antifungal drug results in synergistic fungicidal activity.

The ability to undergo dimorphic transitions is integral to the virulence of C. albicans. The C. albicans ability to produce yeast cells is critical for dissemination, whereas the ability to form hyphae underlies survival and escape from macrophages and the ability to penetrate and invade tissues (15). Mutants locked in either the yeast (cphl efg1) (47) or hyphal (tup1) form (14) exhibit attenuated virulence in murine systemic infection models. The role of the calcineurin pathway in hyphal growth of C. albicans is unclear. Two groups, including our own, found no role for calcineurin or Crz1 in hyphal growth (5, 62), while one group presented evidence interpreted to suggest a role for calcineurin and Crz1 in hyphal growth on filament-inducing media (42, 68). These differing results might be due to different genetic backgrounds of the strains or experimental protocols. C. dubliniensis is the only NACS capable of producing true hyphae, although morphogenesis is typically less robust than C. albicans on most filament-inducing media, which could explain its attenuated virulence in a murine systemic infection model compared with C. albicans (77). Thus, it is of interest to investigate the roles of calcineurin in hyphal growth and virulence in C. dubliniensis. In addition, C. albicans calcineurin and Crz1 are required for trophic responses, a phenotype linked to hyphal growth. C. albicans Crz1 is involved in thigmomotropism and galvanotropism while calcineurin is involved in galvanotropism, suggesting that trophic responses are Crz1 dependent (9–11). In addition to hyphal growth, survival to stress, the transcription factor Crz1 is dephosphorylated by calcineurin and then migrates to the nucleus to regulate expression of genes encoding cell wall biosynthetic enzymes and proteins (42, 72, 75, 76).

Materials and Methods

Yeast strains, media, and chemicals. Fusarium strains used in this study are listed in Table 1. The following media were used in this study: YPD (1% yeast extract, 2% peptone, 2% glucose) liquid medium and agar (2%) plates, spider medium (10 g nutrient broth, 10 g mannitol, 4 g K2HPO4, 14 g Bacto agar in 1 liter double-distilled water [ddH2O]; pH was adjusted to 7.2 with H2PO4), serum agar (50% serum, 2% agar), synthetic low-ammonium dextrose [SLAD; 1.7 g yeast nitrogen base without amino acids and without ammonium sulfate, 20 g glucose, 5 ml of 10 mM (NH4)2SO4, 20 g Bacto agar in 1 liter of ddH2O], and filament agar (2% 1.7 g yeast nitrogen base without amino acids and without ammonium sulfate, 5 g glucose, 40 g Bacto agar in 1 liter ddH2O). YPD medium containing 100 μg/ml nourseothricin was used to select transformants. The supplements FK506 (Astellas Pharma Inc.), cyclosporine A (CSA; LC Laboratories), sodium dodecyl sulfate (SDS; Fisher), fetal bovine serum (Invitrogen), calcofluor white (CFW; fluorescent brightener 28; Sigma), Congo red (Sigma), fluconazole (Bedford Laboratories), posaconazole (Sequoia Research Products Ltd.), voriconazole (Sigma), caspofungin (Merck), micafungin (Astellas Pharma Inc.), and anidulafungin (Pfizer Inc.) were added to the media at the concentrations indicated.

Strain construction. Both alleles of the C. dubliniensis CNA1, CNB1, and CRZ1 genes were disrupted with the SAT1 flipper (66). For the CNA1 gene disruption, approximately 1-kb 5′ (amplified with primers JC17/JC58; see Table S1 in the supplemental material) and 3′ (amplified with primers JC59/JC60) flanking regions (NCRs) of the CNA1 open reading frame (ORF) were PCR amplified from genomic DNA of the wild-type strain CD36. The 4.2-kb SAT1 flipper sequence was amplified from plasmid pFS2A (66) with primers JC17/JC58. The three PCR products were treated with CreI and used to remove contaminating primers and deoxynucleotide triphosphates (dNTPs) and then transformed into C. albicans with an overlap PCR strategy. In this approach, the first PCR reaction was performed using primers JC288/JC289 (PCRs 1 and 2 in the supplemental material), which amplify 100 bp closer to the coding region than the primers used for the five-fragment method (see Fig. S2A in the supplemental material). The PCR products were digested with CreI and ligated into KpnI-HindIII-digested pGM175, resulting in the first disruption allele, 5′/CNB1 NCR-SAT1 flipper-3′/CNB1 NCR. The second allele was generated with primers JC100/JC103 (flipper-3′/CNB1 NCR) to generate the disruption allele by overlap PCR using flanking primers JC61/JC62 (~100 bp closer to the CNB1 ORF compared with JC57/JC60, respectively), resulting in an ~6-kb 5′/CNA1 NCR-SAT1 flipper-3′/CNA1 NCR disruption allele. The first allele of the CNA1 gene was disrupted in the wild-type strain CD36 by transformation with 0.2 to 1 μg of gel-purified disruption DNA by electroporation (17). Two independent heterozygous nourseothricin-resistant mutants (YC31 and YC29; Table 1) were obtained from two separate transformations. Liquid YPM (1% yeast extract-2% peptone-2% maltose) medium was used to drive expression of the FLP recombinase under the control of a C. albicans MAL2 promoter (see Fig. S1 in the supplemental material). The SAT1 flipper was then excised, leaving an FLP recombination target (FRT), and used in nourseothricin-sensitive CNA1/cnb1 mutant strains (YC36 and YC73). The second allele of the CNA1 gene was disrupted with the same overlap PCR allele, resulting in nourseothricin-resistant homologous cnb1/cnb1 mutants YC40 and YC94 (Table 1). A similar approach was employed to disrupt the CNB1 and CRZ1 genes, with ~0.7-kb 5′ and 3′ noncoding regions for homologous recombination. To generate the ~5.4-kb cnb1 disruption allele, the overlap PCR DNA products 5′/CNB1 NCR (amplified with primers JC82/JC83), SAT1 flipper (amplified with primers JC17/JC58), and 3′/CNB1 NCR (amplified with primers JC86/JC87) were mixed in a 1:3:1 molar ratio and used to drive expression of the FLP recombinase under the control of a C. albicans MAL2 promoter (see Fig. S1 in the supplemental material). The SAT1 flipper was then excised, leaving an FLP recombination target (FRT), and used in nourseothricin-sensitive cnb1/cnb1 mutants (YC36 and YC73). The second allele of the CNB1 gene was disrupted with the same overlap PCR allele, resulting in cnb1/cnb1 mutants YC40 and YC94 (Table 1). A similar approach was employed to disrupt the CNB1 and CRZ1 genes, with ~0.7-kb 5′ and 3′ noncoding regions for homologous recombination. To generate the ~5.4-kb crz1 disruption allele, the overlap PCR DNA products 5′/CRZ1 NCR-SAT1 flipper-3′/CRZ1 NCR were ligated into the KpnI-HindIII-digested pGM175, resulting in the second disruption allele, and this fragment was used to transform the nourseothricin-sensitive crz1/crz1 mutant strain YC280, resulting in crz1/crz1 mutants YC30 and YC31 (Table 1). A similar approach was employed to disrupt the CRZ1 gene, resulting in crz1/crz1 mutants YC40 and YC94 (Table 1). The CRZ1 gene was disrupted with the SAT1 flipper by overlap PCR using flanking primers JC63/JC64 (~100 bp closer to the CRZ1 ORF compared with JC57/JC60, respectively), resulting in an ~5.5-kb crz1 disruption allele, and this fragment was used to transform the nourseothricin-sensitive crz1/crz1 mutant strain YC280, resulting in crz1/crz1 mutants YC30 and YC31 (Table 1). A similar approach was employed to disrupt the CRZ1 gene, resulting in crz1/crz1 mutants YC30 and YC31 (Table 1). A similar approach was employed to disrupt the CRZ1 gene, resulting in crz1/crz1 mutants YC30 and YC31 (Table 1).
DNA was subjected to Southern blot analysis. The genomic DNAs of the cna1/cna1, cnb1/cnb1, and crz1/crz1 mutants were digested with PpuMI, HpaI, and EcoRV, respectively (see Fig. S1 in the supplemental material). PCR products containing 5'cna1/cna1/CRZ1 (amplified with primers JC57/JC58), 5'cnb1/cnb1/CRZ1 (amplified with primers JC102/JC103), and 5'crz1/crz1/CNA1 (amplified with primers JC82/JC83), and 3' SSCP1M4 (amplified with primers JC80/JC79) were used as probes. Radiolabeled probes were generated using the Rediprime-it kit (Stratagene) and [α-32P]dCTP. PCR mixtures of 25 μl included 5 ng cDNA (in 10 nM reverse primer, 0.125 μl of nuclease-free H2O, and 0.375 μl of ROX dye. Quantitative PCR conditions were the following: 95°C for 10 min (denaturation); 95°C for 15 s and 60°C for 1 min (40 times, cycling stage); and 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s (melting curve). Primers for probes were designed using Primer3 (http://frodo.wi.mit.edu/primer3/) and are

### Table 1. *C. dubliniensis, C. albicans, and C. neoformans* strains used in this study

| Strain            | Genotype                | Background          | Reference |
|-------------------|-------------------------|---------------------|-----------|
| **Candida dubliniensis** |                         |                     |           |
| CD36              | Prototrophic wild type  | Clinical isolate    | 80        |
| YC31              | CNA1/cna1: SAT1-FLP     | CD36                | This study |
| YC36              | CNA1/cna1: FRT          | YC31                | This study |
| YC40^a             | cnb1:: FRT/cnb1:: SAT1-FLP | YC36                | This study |
| YC29              | CNA1/cna1: SAT1-FLP     | CD36                | This study |
| YC73              | CNA1/cna1: FRT          | YC29                | This study |
| YC94^a             | cnb1:: FRT/cnb1:: SAT1-FLP | CD36                | This study |
| YC47              | CNB1/cnb1:: SAT1-FLP    | YC47                | This study |
| YC69              | CNB1/cnb1:: FRT         | YC69                | This study |
| YC87^d             | cnb1:: FRT/cnb1:: SAT1-FLP | YC87                | This study |
| YC41              | CNB1/cnb1:: FRT         | CD36                | This study |
| YC82              | CNB1/cnb1:: FRT         | YC41                | This study |
| YC96^d             | cnb1:: FRT/cnb1:: SAT1-FLP | YC69                | This study |
| YC81              | CRZ1/crz1:: SAT1-FLP    | YC81                | This study |
| YC102             | CRZ1/crz1:: FRT         | YC102               | This study |
| YC107^e             | crz1:: SAT1-FLP/crz1:: SAT1-FLP | YC107               | This study |
| YC80              | CRZ1/crz1:: FRT         | CD36                | This study |
| YC100             | CRZ1/crz1:: FRT         | YC80                | This study |
| YC108^e             | crz1:: FRT/crz1:: SAT1-FLP | YC100               | This study |
| YC280             | crz1:: FRT/crz1:: FRT + CRZ1 | YC280               | This study |
| YC512             | crz1:: FRT/crz1:: FRT + CRZ1 | YC512               | This study |

**Candida albicans**

| Strain            | Genotype                | Background          | Reference |
|-------------------|-------------------------|---------------------|-----------|
| SC5314            | Prototrophic wild type  | Clinical isolate    | 36        |
| SCCMP1M4          | cna1:: FRT/cna1:: FRT   | SC5314              | 5         |
| SCCMP1MK2         | cna1:: FRT/cna1:: FRT + CNA1 | SCCMP1M4            | 5         |
| DAY185            | ura3::ura3 his1::hisG/ his1::hisG: HIS1 | BWP17               | 27        |
| JRB64             | ura3::ura3 arg4:: arg4 his1::cnb1::URA33/cnb1::UAU1 + HIS1 | BWP17               | 8         |
| MCC85             | ura3::ura3 his1::hisG: CNB1-HIS1/ his1::hisG | BWP17              | 26        |
| OCC1.1            | ura3::ura3 his1::hisG: HIS1/ his1::hisG arg4::arg4 his1::cnb1::URA33 | BWP17               | 62        |
| OCC7              | ura3::ura3 his1::hisG: CRZ1-HIS1/ his1::hisG arg4::arg4 his1::cnb1::URA33 | BWP17              | 62        |
| CAF2-1            | ura3::URA3 | SC5314              | 32        |
| DSY2091           | cna1::hisG/cna1::hisG: UR3A: hisG | CAF2-1              | 68        |
| DSY2115           | cna1::hisG/cna1::hisG: LEU2::CNA1::URA3 | CAF2-1              | 68        |
| DSY2195           | crz1::G/crz1::hisG:: UR3A: hisG | CAF2-1              | 42        |
| MKY268            | crz1::hisG/ hisG: LEU2:: CRZ1::URA3 | CAF2-1              | 42        |

**Cryptococcus neoformans**

| Strain | Genotype | Background | Reference |
|--------|----------|------------|-----------|
| H99    | Prototrophic wild type | Clinical isolate | 65        |
| KK1    | cna1:: NAT    | H99        | 44        |

^a Two independent cna1/cna1 mutants.  
^b Two independent cnb1/cnb1 mutants.  
^c Two independent crz1/crz1 mutants.
listed in Table S1 in the supplemental material. An ABI Prism 7900HT machine and StepOne software v2.1 (Applied Biosystems) were used to determine threshold old cycle (ΔΔCT) and relative quantity (RQ). The bar graphs of ACT1-normalized RQ compared with the wild type (CD36) were created with Prism 5.03.

**Disk diffusion assays.** Cells were grown overnight at 30°C and diluted to 1 OD/ml, and then 100 μl (0.1 OD) was spread onto YPD in the absence or presence of FK506 (1 μg/ml) or CsA (100 μg/ml). After 10 min, sterile disks were placed onto the surface of the agar. Ten microliters of 0.1% RQ compared with the wild type (CD36) were created with Prism 5.03.

**OD/ml, and then 100**

**ration, each sample was examined thoroughly by microscopy for analysis of**

**formed by the Department of Pathology at Duke University. After slide preparation**

**day 14, fixed in 10% phosphate-buffered formalin (Fisher), and Gomori methenamine silver (GMS) and hematoxylin and eosin (H&E) stainings were performed by the Department of Pathology at Duke University. After slide preparation, each sample was examined thoroughly by microscopy for analysis of**

**Candida colonization (GMS) and tissue necrosis (H&E). Images were captured using an Olympus Vanox microscope (PhotoPath; Duke University Medical Center).**

**Murine ocular infection model.** Cells were grown in YPD broth overnight at 37°C. Cultures were pelleted by centrifugation (10,000 rpm for 15 min) and washed three times with sterile PBS (pH 7.4). Cells were suspended and diluted in sterile PBS to yield a fungal concentration of 10^6 cells/5 μl. Concentration was determined by using spectrophotometer optical density reading at a 600-nm wavelength and multiplying it with a conversion factor of 1 OD_(600) equivalent to 3 × 10^7 cells/ml. Inoculum concentration was verified by plating on YPD for 48 h at 37°C.

For murine ocular infection, outbred ICR mice (Research Institute for Tropical Medicine, Alabang, Philippines) around 6 to 8 weeks of age (20 to 25 g) were used in the experiment in accordance with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research. An experimental keratomycosis protocol described previously (89) was used for C. dubliniensis with minor modifications and was approved by the University of Perpetual Help Institutional Review Board. Mice were maintained in comfortable cages with a constant supply of food and water, and the cages were periodically sanitized with Sterilum to minimize potential other infections during the course of the observations. Mice were immunocompromised by intraperitoneal injection of cyclophos- phamide (150 mg/kg body weight; Sigma-Aldrich) and treated with 10% NaCl 5 days, 3 days, and 1 day before the inoculation. Prior to inoculation, mice were anesthetized by intramuscular injection of Zoletil 50 (15 mg/kg body weight; Virac, Australia) followed by topical application of proparacaine hydrochloride ophthalmic solution (Alcaine; Alcon-Couvreur, Belgium) to the eyes. Once animals were anesthetized, the right eye was superficially scarified in a grid pattern using a sterile 25-gauge hypodermic needle. A 10 μl solution (10^6 cells) was placed into each eye. Inoculum was distributed uniformly by rubbing the eye with the eyelid. A mock-infection experiment was performed using sterile PBS as control. Disease severity of fungal keratitis was assessed for 8 days with the aid of a dissecting microscope based on the procedure described previously (89). In this procedure, corneal involvement was assessed and scored according to three parameters, namely, (i) area of opacity, (ii) density of opacity, and (iii) surface regularity. Grading of 0 to 4 was assigned on each of these criteria to yield a maximum score of 12.

**Wax moth infection studies.** Wax moths (Galleria mellonella) of the final-instar larval stage (~0.3 g) from Vanderhorst (Ohio) were used (10 per strain) within 7 days from the day of shipment. The larval infection protocol was adapted from previously described methods for C. neoformans (53) with minor modifications. Each larva was infected with 10^5 C. dubliniensis or C. albicans cells in 5 μl PBS by injection into the last pseudopod and incubated at 24°C in a petri dish with wood shavings. Larvae showing signs of severe morbidity, such as change of body color and no response to touch, were sacrificed by cold treatment at -20°C. The number of surviving wax moths was monitored and recorded daily.

**Epithelial cell interactions.** The extent of damage to oral epithelial cells caused by oral candidal cells required to C. albicans was determined by a minor modification of our previous method (63). Briefly, the FaDu oral epithelial cell line (American Type Culture Collection) was grown in 24-well tissue culture plates. The epithelial cells were infected with either 10^5 yeast cells of C. dubliniensis or C. albicans in 5 μl PBS by injection into the last pseudopod and incubated at 24°C in a petri dish with wood shavings. Larvae showing signs of severe morbidity, such as change of body color and no response to touch, were sacrificed by cold treatment at -20°C. The number of surviving wax moths was monitored and recorded daily.

**Murine systemic infection model.** The extent of damage to oral epithelial cells caused by oral candidal cells required to C. albicans was determined by a minor modification of our previous method (63). Briefly, the FaDu oral epithelial cell line (American Type Culture Collection) was grown in 24-well tissue culture plates. The epithelial cells were infected with either 10^5 yeast cells of C. dubliniensis or C. albicans in 5 μl PBS by injection into the last pseudopod and incubated at 24°C in a petri dish with wood shavings. Larvae showing signs of severe morbidity, such as change of body color and no response to touch, were sacrificed by cold treatment at -20°C. The number of surviving wax moths was monitored and recorded daily.

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a day (BID), during the course of the experiment. A 32-gauge stainless steel Babcock orthodontic wire (Miltex) was threaded across the hard palate and secured between cheek teeth (54). Teeth were etched with Uni-Etch 32% semiconductor etchant with benzalkonium chloride (Bisco, Inc.). A metal spatula was placed over the hard palate to create a space for Candida inoculation. Cold cure acrylic temporary crown and bridge material (HP MaxiTemp; Henry Schein) was applied over cheek teeth and wire and was allowed to solidify for 5 min. After removal of the spatula, the hard palate beneath the acrylic device was inoculated with Candida at 10⁸ cells/ml (0.1 ml). Animals were sacrificed after 48 h of denture placement, and devices were processed for scanning electron microscopy (SEM) as previously described (2, 54). Briefly, devices were washed with PBS and placed in fixative (1% [vol/vol] glutaraldehyde and 4% [vol/vol] formaldehyde in PBS) overnight. The samples were rinsed with PBS, treated in 1% osmium tetroxide for 30 min, and rinsed with PBS. The samples were then dehydrated in a series of ethanol washes, and final desiccation was accomplished by critical-point drying (Tousimis, Rockville, MD). Specimens were mounted on aluminum stubs and sputter coated with gold. Dentures were imaged on a JEOL 6100 at 10 kV. The images were processed for display using Adobe Photoshop.

Animals were maintained in accordance with the American Society for Accreditation of Laboratory Animal Care (AAALAC) criteria, and all studies were approved by the Institutional Animal Care and Use Committee (IACUC).

Statistical analysis. Statistical analysis was conducted using Prism 5.03 software (GraphPad, La Jolla, CA), with the exception that SPSS software was used to analyze thigmotropic responses (Dunnnett’s t test). For the mouse and Galleria larval infection studies, Kaplan-Meier survival curves were generated and the log-rank (Mantel-Cox) test was employed to compare significance. The significance of differences in fungal burden, germ tube formation, and real-time RT-PCR was determined using one-way analysis of variance (ANOVA) and Dunnett’s multiple comparison tests. The significance of the capacity of Candida species to cause damage to oral epithelial cells and murine corneas was determined by unpaired t test and Student’s t test, respectively. A P value of <0.05 was considered significant.

RESULTS

Calcineurin mutation confers cell wall integrity defects in C. dubliniensis. The newest class of antifungal drugs in clinical use, the echinocandins, target fungal cell wall synthesis. Therefore, there is increased interest in the study of Candida cell wall integrity (19, 26, 87). Recently, Jackson et al. reported that the genome sequences of C. dubliniensis and C. albicans are highly conserved with considerable synteny, with the exception of 168 species-specific genes which included cell wall-related secreted aspartyl protease and agglutinin-like protein families (41). Calcineurin is required for cell wall integrity in C. albicans (26, 68) and A. fumigatus (73), but it is not known if calcineurin has an analogous role in C. dubliniensis. The C. dubliniensis orthologs of C. albicans CNA1/CMP1 and CNB1 and the calcineurin target CRZ1 genes were identified by reciprocal BLAST searches between the two species and in all cases identified a reciprocal best BLAST hit ortholog as the reciprocal BLAST searches between the two species and in all cases identified a reciprocal best BLAST hit ortholog as the C. dubliniensis CNA1 (CD36_00650), CRZ1 (CD36 85720) genes. C. dubliniensis CNA1, CNB1, and Crz1 share 91%, 100%, and 81% identity, respectively, over the full-length proteins with their corresponding C. albicans orthologs. Calcineurin (Cna1) has the conserved calcineurin B binding, calmodulin-binding, and autoinhibitory regions. Calcineurin B (Cnb1) has four EF-hand Ca²⁺ binding motifs, while Crz1 shares zinc finger domains with the respective ortholog in C. albicans. Two independent calcineurin and crz1/crz1 mutants were generated using the SAT1 flipper cassette and confirmed by PCR and Southern blot analysis. Real-time RT-PCR analysis confirmed loss of expression of the CNA1, CNB1, or CRZ1 gene in the respective null mutant strains (Fig. 1A).

The cell wall integrity of the C. dubliniensis calcineurin (cna1/cna1 and cnb1/cnb1) and crz1/crz1 mutants was assayed by growing them in the presence of SDS, a reagent which compromises cell membrane/wall integrity; calcofluor white (CFW), which destabilizes chitin polymerization; and Congo
C. dubliniensis calcineurin mutants exhibit echinocandin hypersusceptibility. Echinocandins (caspofungin, micafungin, and anidulafungin) are a new class of antifungal drugs that inhibit fungicidal activity with caspofungin (at a nonfungicidal concentration) against Candida isolates from the oral cavities of HIV/AIDS patients (6, 82). A calcineurin inhibitor and fluconazole exhibited synergistic fungicidal activity against C. albicans (26) and C. neoformans (28). To test our hypothesis that calcineurin is required for fluconazole tolerance in C. dubliniensis, we used spot, disk diffusion, and time-kill curve assays. C. dubliniensis cnal/cnal and cnbl/cnbl mutants were hypersusceptible to fluconazole while crz1/crz1 mutants exhibited susceptibility intermediate between the wild type and the calcineurin mutants (Fig. 3A), suggesting that other regulators control fluconazole tolerance in addition to Crz1. This is similar to C. albicans calcineurin and crz1/crz1 mutants, suggesting that azole tolerance governed by the calcineurin pathway has been conserved during evolution of the two Candida species. By disk diffusion assays, we found that pharmacological inhibition of calcineurin phenocopies calcineurin deletion while crz1/crz1 mutants exhibit an interme-
diate effect between the wild type and calcineurin mutants in *C. dubliniensis* (Fig. 3B). This strongly suggests that fluconazole tolerance is controlled by other calcineurin downstream targets in addition to the Crz1 transcription factor. Time-killing curve assays showed that *C. dubliniensis cna1/cna1* and *cnb1/cnb1* mutants initially (3 h) proliferated in the presence of fluconazole (10 μg/ml) but that survival was dramatically decreased over 24 h compared with the wild type (*P < 0.0001*; Fig. 3C). *C. dubliniensis crz1/crz1* mutants exhibited initial growth, but the growth rate dropped significantly at 24 h (*P < 0.05*; Fig. 3C). The synergistic fungicidal effects of fluconazole are therefore strongly linked to the loss of calcineurin activity and are partially mediated by the transcription factor Crz1. Calcineurin mutants also exhibited hypersensitivity to the new-generation azoles, posaconazole and voriconazole, in *C. dubliniensis* and *C. albicans*, while *crz1/crz1* mutants showed differential susceptibility (see Fig. S4 in the supplemental material). This suggests that azole tolerance is at least in part mediated by the transcription factor Crz1. However, *C. dubliniensis Crz2* (CD36_32610, encoding a putative transcriptional regulator) does not play a role in azole tolerance because *crz2/crz2* and *crz2/crz2* *crz1/crz1* mutants did not exhibit hypersensitivity compared with the wild type and *crz1/crz1* mutants, respectively (data not shown).

### Calcineurin and Crz1 control cation homeostasis in *C. dubliniensis*

The roles of calcineurin and Crz1 in *C. dubliniensis* have been elucidated in *C. albicans* (18, 68, 69). Calcineurin activity in the two species. Elevated temperature (≥30°C) and elevated temperature (≥30°C) *crz1/crz1* mutants are hypersensitive to Ca²⁺ compared with the calcineurin mutants in two closely related species (see Fig. S5 in the supplemental material). Interestingly, *crz1/crz1* mutants exhibit intermediate Ca²⁺ sensitivity phenotype compared with wild type and calcineurin mutants at 24°C in both *C. dubliniensis* and *C. albicans* (Fig. S5). *C. dubliniensis cna1/cna1* and *cnb1/cnb1* mutants are also hypersensitive to Mn²⁺ stress (Fig. 4A), whereas *crz1/crz1* mutants exhibit intermediate sensitivity between the calcineurin mutants and wild type, indicating that another regulator(s) in addition to Crz1 contributes to Mn²⁺ homeostasis. Interestingly, *C. dubliniensis* calcineurin mutants are hypersensitive to Na⁺ (1 M), while *C. albicans* calcineurin mutants do not exhibit significant differences compared with their wild-type counterparts (Fig. 4A). In fact, *C. albicans* calcineurin mutants are hypersensitive to a high Na⁺ concentration (2 M; data not shown). Thus, the difference in roles of calcineurin in Na⁺ ion homeostasis between two closely related species (Fig. 4A) is due to the differential Na⁺ sensitivity between the species and not to differences in calcineurin activity in the two species.

The mechanisms of *C. dubliniensis* calcineurin and *crz1/crz1* mutants’ hypersensitivity to mono- or divalent cations might involve defects in cation efflux systems, resulting in cation accumulation in the cytosol. We show here that transcription of *PMCl* encoding a vacuolar Ca²⁺ transporter, *CD36_81200* is regulated by calcineurin and Crz1 in *C. dubliniensis* (Fig. 4B, *P < 0.001*), indicating a mechanism by which Ca²⁺ likely accumulates in the cytosol of calcineurin or *crz1/crz1* mutants. The transcription of *PMCl* was also shown to be regulated by calcineurin and Crz1 in *C. albicans* (42, 68) and in the rice blast pathogen *M. oryzae* (20). However, the transcription of *PMRI* (encoding a Golgi Ca²⁺/Mn²⁺ transporter, CD36_70530), *CCHI* (encoding a voltage-gated Ca²⁺ channel, CD36_01040), and *MIDI* (encoding a Ca²⁺ channel, CD36_53710) was not controlled by calcineurin or Crz1 (Fig. 4B) in *C. dubliniensis*.

Calcineurin but not Crz1 is required for serum survival in *C. dubliniensis*. An essential role for calcineurin, but not Crz1, in serum survival has been demonstrated in *C. albicans* (8), and calcium stress in serum has been elucidated to be the cause of lethality in calcineurin mutants (7). An inability to survive in serum explains, at least in part, why *C. albicans* calcineurin mutants exhibit attenuated virulence in a murine systemic in-
fection model but not in pulmonary or vaginal infection models (5), indicative of niche-specific roles of calcineurin. However, the roles of calcineurin and Crz1 have not yet been investigated in the less virulent species *C. dubliniensis*. Here, we demonstrate that, similar but not identical to *C. albicans* calcineurin mutants, *C. dubliniensis cna1/cna1* and *cnb1/cnb1* mutants were hypersensitive to serum but were in general less sensitive than *C. albicans* calcineurin mutants (Fig. 5A), suggesting an evolutionary divergence between two closely related species. By quantitative measurements, we found that the survival percentage of *C. dubliniensis cna1/cna1* mutants (0.354% ± 0.125%) was 24-fold higher than *C. albicans cna1/cna1* mutants (0.015% ± 0.006%) (*P* = 0.0094) on a 50% serum agar plate (data not shown). However, Crz1 is not required for serum survival in either *Candida* species (Fig. 5 and data not shown). We further characterized germ tube formation of the wild type and *cna1/cna1, cnb1/cnb1*, and *crz1/crz1* mutants in YPD medium ± fluconazole. The data are represented as means ± SDs from triplicate experiments.

FIG. 3. Calcineurin is required for fluconazole tolerance in *C. dubliniensis*. (A) Cells were grown overnight in YPD at 30°C, 5-fold serially diluted, and spotted onto YPD medium ± fluconazole (FL). The plates were incubated at 30°C for 48 h. (B) Disk diffusion assays were used to determine fluconazole susceptibility of wild-type and mutant strains. Cells were grown overnight at 30°C, and 0.1 OD600 (in 100 μl) was spread on the surface of YPD medium ± FK506 or CsA at the concentrations indicated. A disk was placed on the surface of the medium, and fluconazole (1 μg) was added to each disk. The plates were incubated at 30°C for 24 h and photographed. Scale bar = 6 mm. (C) Time-killing curve of wild type and *cna1/cna1, cnb1/cnb1*, and *crz1/crz1* mutants in YPD medium ± fluconazole. The data are represented as means ± SDs from triplicate experiments.
Calcineurin and Crz1 are required for hyphal growth in \textit{C. dubliniensis}. It is unclear if calcineurin is required for hyphal growth in \textit{C. albicans}. Two groups, including our own, found no clear role for calcineurin in hyphal growth (5, 8), while another group suggested that calcineurin and Crz1 may be required for hyphal growth on spider medium (carbon source starvation) (42, 68). Sanglard et al. reported that a \textit{C. albicans} cna1/cna1 mutant also exhibits attenuated hyphal growth on SLAD medium (nitrogen source starvation) (68). Here, we clarify the roles of calcineurin in \textit{C. dubliniensis} hyphal growth in response to carbon or nitrogen limitation. We found that hyphal growth of cna1/cna1 and cnb1/cnb1 mutants and FK506-treated wild type (carbon source starvation) is integral for growth while Crz1 is not. However, the hyphal growth of crz1/crz1 mutants was attenuated on serum agar compared with the wild-type or complemented strains (Fig. 6 and data not shown), suggesting a specific role of Crz1 in regulating hyphal growth in solid serum agar.

We also found that \textit{C. dubliniensis} cna1/cna1 and cnb1/cnb1 mutants and FK506-treated wild type but not crz1/crz1 mutants exhibit reduced germ tube formation in liquid spider medium compared with the wild type (\(P < 0.001\)) (see Fig. S6 in the supplemental material). In contrast, a \textit{C. albicans} cna1/cna1 mutant or FK506-treated wild-type cells exhibit normal germ tube formation in liquid spider medium (Fig. S6), suggesting that calcineurin function in response to nutrient starvation in liquid may be diverged between the two species. O’Connor et al. recently reported that unlike \textit{C. albicans}, \textit{C. dubliniensis} exhibits differential hyphal growth in response to nutrient starvation (57). Interestingly, \textit{C. dubliniensis} Crz1 shows differential responses to hyphal growth in solid and liquid spider media (Fig. 6; see also Fig. S6). In contrast to defects on solid spider medium, \textit{C. dubliniensis} crz1/crz1 mutants exhibit normal germ growth on culture media.

### FIG. 4. Calcineurin and Crz1 control cation homeostasis in \textit{C. dubliniensis}.

A) Cells were grown overnight in YPD at 30°C, 5-fold serially diluted, and spotted onto YPD medium containing CaCl\(_2\), MnSO\(_4\), or NaCl at the concentration indicated. The plates were incubated at 30°C for 48 h. (B) Quantitative real-time PCR was used to assay expression of genes involved in cation homeostasis (PMC1 and PMR1) or calcium channel (CCH1 and MID1) in the wild-type and mutant strains. The fold changes in transcription of each gene were normalized to the endogenous control ACT1. The error bars represent means ± SDs from a triplicate experiment. One representative figure of three independent experiments is shown. Asterisks indicate \(P < 0.001\) compared with the wild type.
channels, which initiates a turning response in the hyphal tip mechanism involving activation of plasma membrane calcium specific topography of penetration sites in the host leaf (1). Contour substrate allows fungi to locate and identify the specific cues. In plant pathogens, sensing encountered in the environment. The growing tip can circumnavigate impenetrable objects, mediated by Crz1 in response to various environmental cues. The hyphae of filamentous fungi generally grow toward the cathode in an applied electric field (galvanotropism) (25). In C. albicans, deletion of CNA1, CNB1, or CRZ1 resulted in the attenuation of the galvanotropic response (11). However, we were unable to test the response of C. dubliniensis using this assay due to its failure to generate hyphae on application of an electric field of 10 V/cm⁻¹, although the cells remained viable in a field applied for 6 h and grew as normal in the medium used when no field was present. One possibility is that the field generated an electrolytic product that inhibited growth in C. dubliniensis but was not fungicidal.

**Deletion of calcineurin and Crz1 attenuates virulence in mice.** C. dubliniensis is generally considered to be a less pathogenic species compared to C. albicans (77). However, it has also been reported that C. dubliniensis can be more virulent than C. albicans in a murine systemic infection model (35, 85). Here, we used C. albicans as the control group to compare its virulence to C. dubliniensis and determined the virulence of calcineurin and crz1/crz1 mutants of C. dubliniensis in a murine systemic infection model. The median animal survival following tail vein infection with 5 × 10⁶ cells is 20 days for C. dubliniensis and 2 days for C. albicans, respectively, showing a dramatic virulence difference (P < 0.0001) between these two Candida species. In C. albicans, cna1/cna1 and cnb1/cnb1 mutants exhibit strongly attenuated virulence (4, 5, 8, 68), while crz1/crz1 mutants have either full (62) or slightly reduced (42) virulence in a murine systemic infection model. Here we showed that C. dubliniensis cna1/cna1 (YC40 and YC94) or cnb1/cnb1 (YC87 and YC96) mutants exhibit strongly attenuated virulence compared with the wild type (P < 0.0001) (Fig. 8A) while crz1/crz1 mutants (YC107 and YC108) exhibit attenuated virulence compared with the wild type (P < 0.002). However, there is no statistically significant difference between calcineurin and crz1/crz1 mutant-infected mice (P > 0.3), indicating that both calcineurin and Crz1 affect the virulence of C. dubliniensis.

To determine colonization ability, we performed kidney fungal burden analysis of animals infected with wild type and the mutants. cna1/cna1 mutants (YC40 and YC94) exhibited 42-fold-reduced fungal burden in the kidneys compared with the wild type (P < 0.01) (Fig. 8B). In contrast, the difference of fungal burden between cnb1/cnb1 mutants (YC87 and YC96) and wild type was less pronounced (P = 0.08) (Fig. 8B). One cnb1/cnb1 mutant (YC96) exhibited a 44-fold-reduced fungal burden compared with wild type (P = 0.02), while another cnb1/cnb1 mutant (YC87) exhibited a 2.8-fold (lower fold change is attributable to a single outlier)-reduced fungal burden compared with wild type (P = 0.2) (Fig. 8B). When the
outlier animal from the YC87 infection is excluded from the analysis, the cnb1/cnb1 mutants (YC87 and YC96) exhibited a 42-fold-reduced fungal burden compared with the wild type \( (P \leq 0.01) \). The fungal burden of mice infected with crz1/crz1 mutants (YC107 and YC108) was 3.4-fold reduced compared with the wild type \( (P \leq 0.09) \) (Fig. 8B). Taken together, mice infected with C. dubliniensis calcineurin and crz1/crz1 mutants exhibited a reduced fungal burden overall compared with the wild type.

In histopathological analysis, GMS-stained tissues revealed that the wild type readily forms hyphae and proliferates extensively in tissues around the renal pelvis, while cells of the cna1/cna1, cnb1/cnb1, and crz1/crz1 mutants were not observed (Fig. 8C), indicating that hyphal growth may be reduced \textit{in vivo} for the calcineurin pathway mutants. In the H&E staining, tissue damage or necrosis was observed only in animals infected with the wild type and not with the calcineurin or crz1/crz1 mutants (Fig. 8C).

Calcineurin mutants are unable to establish murine ocular infection. Candida species were isolated from AIDS patients with corneal infections (keratitis) (38, 39). Candida keratitis caused by \textit{C. albicans} and NACS, including \textit{Candida glabrata} and \textit{Candida parapsilosis} (16, 81), continues to be an important cause of ocular morbidity, including loss of vision. Although \textit{C. dubliniensis} is frequently found in AIDS patients, it is unclear if \textit{C. dubliniensis} has the ability to cause keratitis of patients.

**FIG. 6.** Calcineurin controls colony hyphal growth in \textit{C. dubliniensis}. Cells were grown overnight and washed twice with ddH\textsubscript{2}O. Cells were separated by sonication, counted with a hemocytometer, and then serially diluted to 10\textsuperscript{5} cells/ml. One hundred microliters containing ~100 cells was spread on a variety of filament-inducing media \( \pm \) FK506 (1 \( \mu \)g/ml) and incubated at 37°C for the number of days indicated. The experiments were repeated at least three times, and one representative image is shown. Scale bar = 1 mm.

**FIG. 7.** The thigmotropic response is attenuated in \textit{C. dubliniensis} crz1/crz1 but not calcineurin mutants. (A) The thigmotropic response was determined when the growing tip reoriented against 0.79-\( \mu \)m ridges in the substrate. The turning hyphae are indicated by arrowheads. Scale bar = 25 \( \mu \)m. One representative figure from three independent experiments is shown. (B) Bar graph shows the percentage of total hyphae that reoriented. The error bars represent the means \( \pm \) SDs.
The comparison of virulence between *C. albicans* and *C. dubliniensis* in murine ocular infection has not yet been reported. We here investigate the virulence difference between these two closely related species and test if calcineurin promotes ocular infection in *C. dubliniensis*. Mice infected with an inoculum of $10^6$ cells of *C. albicans* exhibited visible opacity and surface opacity in immunocompetent ICR mice (100%, 15/15). However, *C. dubliniensis* at an inoculum of $10^6$ did not result in persistent manifestation of fungal keratitis in immunocompetent mice, suggesting that *C. dubliniensis* is less virulent in murine ocular infection. An immunocompromised mouse model for fungal keratitis is well established for *C. albicans* (60, 89) but not yet tested for *C. dubliniensis*. We thus administered cyclophosphamide (180 to 220 mg/kg body weight), a potent inhibitor of lymphocyte proliferation, to mice on days 5, 3, and 1 prior to inoculation. All corneas (100%, 15/15) infected with $10^6$ cells of *C. albicans* SC5314 developed fungal keratitis compared to 26.7% (4/15) of those exposed to *C. dubliniensis* CD36 (Fig. 9A). At all time points, keratitis caused by *C. albicans* SC5314 was more severe than that caused by *C. dubliniensis* CD36 (Fig. 9B). The disease score of *C. albicans* keratitis is persistent, while *C. dubliniensis* keratitis has a peak at day 3 but drops subsequently, suggesting that *C. dubliniensis* is not a successful pathogen compared to its closely related species *C. albicans*. This is the first demonstration that compares these two species using a fungal keratitis model.

Corneas infected with *C. dubliniensis* CD36 wild type (26.7%, 4/15) and *crz1/crz1* mutant (22.2%, 4/18), but not calcineurin mutants (*cna1/cna1* and *cnb1/cnb1*), showed visible keratitis (Fig. 9), suggesting that *C. dubliniensis* calcineurin is required for establishing murine ocular infection. The mean keratitis score for the CD36 wild-type strain was similar to the *crz1/crz1* mutant. Keratitis caused by CD36 was moderate grade by day 1 (6.25 ± 0.96) and became more severe by day 3 (8.00 ± 0.82, P = 0.033) with inflammation starting to resolve by day 4 (5.75 ± 1.26) (Fig. 9B). Fungal keratitis resulting from the *crz1/crz1* mutant started to resolve by day 3 and was significantly different by day 6 (P = 0.027) compared with the CD36 strain at day 6 (Fig. 9B), suggesting a difference between CD36 and *crz1/crz1* mutant in developing keratitis at later stages. Compared with the CD36 wild-type and *crz1/crz1* mutant strains, it is clear that *C. dubliniensis* calcineurin mutants are unable to establish murine ocular infection (Fig. 9), supporting our previous finding that *C. albicans* calcineurin mutants exhibit attenuated virulence in a fungal keratitis model (60).

Calcineurin is required for biofilm formation in a rat denture model. In addition to *C. albicans* and *C. glabrata* (23), *C.
that calcineurin is required for hyphal growth in an mutant resulted in only a yeast monolayer (Fig. 10), suggesting components (Fig. 10). In contrast, inoculation of the denture surface consisting of yeast, hyphae, and matrix components of C. dubliniensis strains, exhibiting visible signs of keratitis, were plotted. VOL. 10, 2011 ROLES OF CALCINEURIN PATHWAY IN C. DUBLINIENSIS

Roles of calcineurin and Crz1 in cell wall integrity and drug tolerance. Here we demonstrate that calcineurin and Crz1 control cell wall integrity and drug tolerance in C. dubliniensis, suggesting potential merit for calcineurin inhibitors as novel therapeutic agents. Most antifungal drugs target fungal protein components on either the cell membrane or cell wall. For example, azoles inhibit ergosterol biosynthesis in the cell membrane, and echinocandins inhibit β-1,3-glucan biosynthesis in the cell wall (58). In C. albicans and C. glabrata, calcineurin is required for cell wall integrity (26, 50). The cell membrane or wall defects caused by calcineurin mutation render antifungal azoles fungicidal in C. albicans (26). Here we show that cell membrane/wall integrity is partially mediated by Crz1 in C. dubliniensis; crzl/crz1 mutants exhibit SDS hypersensitivity intermediate between the wild type and calcineurin mutants (Fig. 1B). However, C. dubliniensis Crz1 does not play a clear role in response to cell wall perturbation by CFW and Congo red (Fig. 1B). These lines of evidence suggest that calcineurin plays an important role governing cell wall integrity which might involve other cell wall integrity pathways such as the protein kinase C (PKC) and high-osmolarity glycerol (HOG) signaling pathways.

In C. albicans, cell membrane perturbation by fluconazole can enhance uptake and toxicity of calcineurin inhibitors (26). Conversely, it is possible that cell membrane defects caused by calcineurin mutation result in increasedazole uptake and toxicity, leading to synergistic fungicidal activity. Roles for calcineurin and Crz1 in azole (26, 68) or echinocandin (70) tolerance have been studied in C. albicans. However, data showing interactions between calcineurin and other signaling pathways to regulate drug tolerance are limited. Singh et al. demonstrated that heat shock protein 90 (Hsp90) physically interacts with calcineurin and governs echinocandin resistance in C. albicans, and drug inhibitors of Hsp90 or calcineurin exhibit synergistic fungicidal activity with echinocandins (at a nonfungicidal concentration) (70). Recently, LaFayette et al. showed that PKC signaling regulates azole and echinocandin tolerance via circuits comprised of calcineurin, Hsp90, and Mkc1 in C. albicans (46). It is possible that C. dubliniensis shares these conserved pathways that may function in coordination with the calcineurin pathway to effect cell wall integrity and drug tolerance.

Roles of calcineurin and Crz1 in hyphal growth and contact response orientation. In C. albicans, the roles of calcineurin in hyphal growth are unclear; two groups, including our own, were unable to find a role for calcineurin in hyphal growth (5, 26), while another group reported that a calcineurin mutant (cnal/cnal) exhibited hyphal growth defects on spider and SLAD solid media (68) (Table 3). The contrasting results regarding the roles of the calcineurin pathway in hyphal growth of C. albicans may be due to different C. albicans backgrounds or experimental details. In this study, we aimed to use C. dubliniensis, a species closely related to C. albicans, to investigate the roles of calcineurin (Cna1 and Cnb1) in hyphal growth, a phenotype linked to virulence. We find that calcineu-
rin (Cna1 or Cnb1) is clearly required for hyphal growth in response to either carbon or nitrogen source limitation in \textit{C. dubliniensis} (Fig. 6; Table 3). However, in \textit{C. albicans} we are unable to appreciate a clear role for calcineurin in hyphal growth upon either carbon or nitrogen source starvation. In \textit{C. albicans}, the roles of the transcription factor Crz1 in hyphal growth remain elusive. Karababa et al. reported that Crz1 is required for hyphal growth on spider medium (42), while Noble et al. showed that \textit{crz1/crz1} mutants exhibited no hyphal growth defects on spider medium from a systematic

| Phenotype                      | \textit{C. albicans} | \textit{C. dubliniensis} |
|--------------------------------|----------------------|--------------------------|
| Cell wall integrity           |                      |                          |
| SDS                            | Inviable             | Intermediate\(^a\)       |
| CFW                            | Sensitive            | Wild type                |
| Congo red                      | Wild type            | Wild type                |
| Drug tolerance                 |                      |                          |
| Echinocandin                   | Hypersusceptible     | Hypersusceptible         |
| Azole                          | Hypersusceptible     | Intermediate\(^b\)       |
| Ion homeostasis                |                      |                          |
| Ca\(^{2+}\)                    | Hypersensitive\(^c\) | Hypersensitive           |
| Mn\(^{2+}\)                   | Hypersensitive       | Hypersensitive           |
| Na\(^+\)                      | Hypersensitive\(^d\) | Wild type                |
| Serum survival                 | Hypersensitive       | Wild type                |
| Hyphal growth (solid surface)  |                      |                          |
| Carbon limitation              | Wild type            | Impaired                 |
| Nitrogen limitation            | Wild type            | Impaired                 |
| Tropic responses               |                      |                          |
| Thigmotropism                  | Wild type            | Wild type                |
| Galvanotropism                 | Attenuated           | Attenuated               |
| Virulence (murine systemic infection) | Attenuated           | Attenuated               |

\(^a\) Intermediate phenotype between wild type and calcineurin mutants.
\(^b\) Mutants exhibit either no response or intermediate susceptibility to echinocandins.
\(^c\) These observations were found at 24 and 30°C (0.4 M). Interestingly, \textit{C. albicans} mutants exhibit the wild-type phenotype while \textit{C. dubliniensis} mutants exhibit the sensitive phenotype at 37°C (see Fig. S5 in the supplemental material).
\(^d\) Hypersensitivity at 2 M NaCl.
\(^e\) Hypersensitivity at 1 M NaCl.
\(^f\) Failure to generate hyphae when an electric field was applied.
screen (55). The confounding results may be due to different experimental methods or genetic backgrounds of *C. albicans* strains. However, the calcineurin target CrzA in *A. fumigatus* has been demonstrated to regulate hyphal growth (24), indicative of a potential global role of the calcineurin target Crz1/CrzA in regulating hyphal growth in fungal pathogens. In support of the interpretation that Crz1 is a global regulator of hyphal growth, we find that Crz1 is required for hyphal growth on solid spore and serum media in *C. dubliniensis* (Fig. 6). However, *C. dubliniensis* Crz1 is not required for germ tube formation in either liquid spore or serum medium (Fig. 5; see also Fig. S6 in the supplemental material), indicating a fascinating role for Crz1 in adhering to a solid surface.

Brand et al. demonstrated that hyphal orientation (thigmotropism or galvanotropism) is linked to the calcium signaling and calcineurin pathway in *C. albicans* (9–11). We observed that thigmotropism was reduced in *C. dubliniensis* (CD36) by 25 to 30% compared with *C. albicans* (SC5314) (data not shown). The defective thigmotropism in the *C. dubliniensis crz1/crz1* mutant but not calcineurin mutants is consistent with the findings that thigmotropism is mediated by Crz1 in *C. albicans* (11) (Table 3). In *C. albicans*, loss of the thigmotropic response correlated with reduced tissue penetration and damage of oral epithelial cells in an in vitro assay (12). Thus, the attenuated thigmotropism of *C. dubliniensis crz1/crz1* mutants may partly explain their attenuated virulence in a murine systemic infection model (Fig. 7), but thigmotropism does not appear to contribute to the attenuated virulence of the *C. albicans* and *C. dubliniensis* calcineurin mutants, in which attenuated virulence may simply be due to the essential role of calcineurin for survival in serum in both species.

**Role of calcineurin and Crz1 in serum survival and virulence.** Fungal pathogens require calcineurin for virulence, but the precise role of calcineurin is species dependent (18). The roles of calcineurin in serum survival have been demonstrated in the human fungal pathogens *C. albicans* (7) and *A. fumigatus* (73). In contrast, in *C. neoformans* calcineurin supports growth at mammalian body temperature (37°C) (59). The plant fungal pathogens *M. oryzae* (21) and *Ustilago maydis* (29) have adapted calcineurin for different pathogenic mechanisms involving appressorium formation and filamentous growth, respectively (18). Here we demonstrate that calcineurin is required for serum survival in *C. dubliniensis* (Fig. 5) and, as a consequence, calcineurin mutants (*cnal/cnal* and *cnb1/cnb1*) exhibit attenuated virulence (Fig. 8). The requirement for calcineurin in hyphal growth and cell wall integrity suggests additional mechanisms by which calcineurin promotes successful infection. Strikingly, *C. dubliniensis* Crz1 is required for hyphal growth (Fig. 6) and virulence in a murine systemic infection model but is not required for serum survival (Fig. 5), suggesting that Crz1 and calcineurin may contribute to virulence by both common and distinct pathways. In accord with our observations is the fact that defects in cell wall integrity of *C. albicans* often result in attenuated virulence in murine systemic infection models (19, 46, 51).

Our studies also demonstrate that, similar to *C. albicans* and *Saccharomyces cerevisiae*, *C. dubliniensis* calcineurin and Crz1 are not required for growth at high temperature (see Fig. S3 in the supplemental material). Thus, calcineurin in *C. dubliniensis* does not control virulence through promoting high-temperatur growth, in contrast to the basidiomycete *C. neoformans*, in which calcineurin is essential for survival at host body temperature (59). In addition to temperature sensitivity, a *Schizosaccharomyces pombe* calcineurin mutant (ppb1) exhibits a cold-sensitive phenotype associated with cytokinesis defects (91). It remains largely unknown how calcineurin controls responses to thermal stress in model or pathogenic fungi.

*C. dubliniensis* has been isolated from nonhuman sources such as seabird-associated excrement or ticks, suggesting that the wax moth (*G. mollenella*) might be a candidate virulence model for *C. dubliniensis*. Interestingly, we found that *C. dubliniensis* is as virulent as *C. albicans* in the *G. mollenella* insect model (*P* = 0.32; see Fig. S7 in the supplemental material), in contrast to their marked virulence difference in the murine model. All wax moth larvae were dead by day 3, when injected with 10⁶ *C. dubliniensis* (CD36) yeast cells (*P* < 0.0001, compared with PBS curve) (Fig. S7), indicating that *C. dubliniensis* might be an insect pathogen. However, the roles of calcineurin and Crz1 in virulence in this insect model do not completely phenocopy the murine model (data not shown), suggesting that a specific niche might be required for the *C. dubliniensis* calcineurin pathway to be operative during successful infection.

*C. dubliniensis* is frequently found in the oral cavities of HIV/AIDS patients; however, its role in this specific niche is unclear. To test if *C. dubliniensis* (CD36) grows and causes damage to oral epithelium, we used FaDu oral epithelial cells to analyze cell-host interactions. We found that *C. dubliniensis* exhibits less extensive hyphal growth compared with *C. albicans* (see Fig. S8A in the supplemental material). Spiering et al. showed that *C. dubliniensis* grew as yeast for the duration of the experiment (12 h) in infected reconstituted human oral epithelium (RHE) (71). We used a ⁵¹Cr release assay to determine if *C. dubliniensis* causes cell damage. We found that *C. dubliniensis* causes no damage while *C. albicans* triggers damage within 6 h (Fig. S8B). This suggests that *C. dubliniensis* might have lost virulence determinants that are necessary to colonize oral epithelial cells from *C. albicans*.

Although there are no published clinical reports of keratitis caused by *C. dubliniensis*, it is possible that *C. dubliniensis* could be an emerging and opportunistic pathogen and cause ocular infection when the host immune system is compromised. Our keratitis data support this possibility because *C. dubliniensis* can cause keratitis in an immunocompromised host model. Our lab has demonstrated that CsA and flucona-zole exhibit fungicidal activity against *C. albicans* in a murine ocular infection model (60), suggesting a potential combination therapy for keratitis caused by *Candida* species.

A summary of the phenotypes of the *C. dubliniensis* and *C. albicans* calcineurin and crz1/crz1 mutants is shown in Table 3. The *C. dubliniensis* calcineurin pathway exhibits both conserved and distinct roles compared with *C. albicans*. Taken together, the mechanisms linking calcineurin to *C. dubliniensis* pathogenesis involve serum survival and hyphal growth, whereas the virulence impairment of crz1/crz1 mutants may be attributable to their defect in hyphal growth. However, it is possible that other factors such as cell wall integrity may also contribute to calcineurin and Crz1 effects on pathogenicity. These lines of evidence suggest that calcineurin could be a potential drug target in the emerging NACS *C. dubliniensis*.
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