



The Vacuolar Ca²⁺ ATPase Pump Pmc1p Is Required for *Candida albicans* Pathogenesis

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ABSTRACT Calcium is a critically important secondary messenger of intracellular signal transduction in eukaryotes but must be maintained at low levels in the cytoplasm of resting cells to avoid toxicity. This is achieved by several pumps that actively transport excess cytoplasmic Ca²⁺ out of the cell across the plasma membrane and into other intracellular compartments. In fungi, the vacuole serves as the major storage site for excess Ca²⁺, with two systems actively transporting cytoplasmic calcium ions into the vacuole. The H⁺/Ca²⁺ exchanger, Vcx1p, harnesses the proton-motive force across the vacuolar membrane (generated by the V-ATPase) to drive Ca²⁺ transport, while the P-type ATPase Pmc1p uses ATP hydrolysis to translocate Ca²⁺ into the vacuole. Ca²⁺-dependent signaling is required for the prevalent human fungal pathogen *Candida albicans* to endure exposure to the azole antifungals and to cause disease within the mammalian host. The purpose of this study was to determine if the Pmc1p or Vcx1p Ca²⁺ pumps are required for *C. albicans* pathogenicity and if these pumps impact antifungal resistance. Our results indicate that Pmc1p is required by *C. albicans* to transition from yeast to hyphal growth, to form biofilms *in vitro*, and to cause disease in a mouse model of disseminated infection. Moreover, loss of Pmc1p function appears to enhance *C. albicans* azole tolerance in a temperature-dependent manner.

IMPORTANCE Maintenance of Ca²⁺ homeostasis is important for fungal cells to respond to a multitude of stresses, as well as antifungal treatment, and for virulence in animal models. Here, we demonstrate that a P-type ATPase, Pmc1p, is required for *Candida albicans* to respond to a variety of stresses, affects azole susceptibility, and is required to sustain tissue invasive hyphal growth and to cause disease in a mouse model of disseminated infection. Defining the mechanisms responsible for maintaining proper Ca²⁺ homeostasis in this important human pathogen can ultimately provide opportunities to devise new chemotherapeutic interventions that dysregulate intracellular signaling and induce Ca²⁺ toxicity.

KEYWORDS *Candida albicans*, Pmc1p, Vcx1p, calcium, pathogenesis, vacuole

The fungal vacuole is an acidic intracellular compartment that plays a central role in the maintenance of cellular homeostasis. This includes facilitating the degradation of macromolecules, the storage of crucial metabolites, and the sequestration of potentially toxic substances (1, 2). The vacuole also serves as the major storage site for metal ions, including calcium, a critically important secondary messenger of intracellular signal transduction in eukaryotes. To fulfill its role as a second messenger, Ca²⁺ must be maintained at low levels in the cytoplasm of resting cells (3). This is achieved by several pumps that actively transport excess cytoplasmic Ca²⁺ out of the cell across the plasma membrane or into intracellular compartments, including the endoplasmic

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reticulum and Golgi apparatus (4). In yeast, more than 90% of the intracellular Ca^{2+} is sequestered in the vacuole (4). Upon appropriate stimulation, Ca^{2+} ions are rapidly released into the cytoplasm from extracellular sources as well as intracellular stores to activate Ca^{2+} -dependent proteins such as calmodulin (5, 6). Resting state equilibrium is subsequently restored through calcium efflux, resulting in transient cytoplasmic Ca^{2+} fluxes that relay the extracellular signal. Failure to remove excess intracellular Ca^{2+} from the cytoplasm or to restore presignal equilibrium dysregulates intracellular signaling and can lead to cell death (7, 8). As such, the mechanisms responsible for Ca^{2+} detoxification are of critical importance to eukaryotic cell viability.

In fungi, two systems are responsible for sequestering cytoplasmic Ca^{2+} ions into the vacuole, namely, the $\text{H}^+/\text{Ca}^{2+}$ exchanger Vcx1p (9–11), which uses the proton gradient across the vacuolar membrane (generated by the V-ATPase) to drive Ca^{2+} transport, and the P-type ATPase Pmc1p (11, 12). Both Pmc1p and Vcx1p are required for *Cryptococcus neoformans* to colonize lung or brain tissue in a mouse model of infection (13–15). While *Aspergillus fumigatus*, a major cause of pulmonary as well as disseminated infections of humans, has three Pmc1p homologues (*PMCA*, *PMCB*, and *PMCC*), with *PMCC* seemingly essential for viability and *PMCA* required for virulence in a neutropenic mouse model of invasive pulmonary aspergillosis (16). Ca^{2+} -dependent signaling, particularly through the calcineurin signaling pathway, is also required for *Candida albicans* to tolerate the azole antifungals (17, 18). However, deletion of the *PMC1* gene has been reported to result in fluconazole resistance in *C. albicans* (18). The purpose of this study was to determine if Pmc1p or Vcx1p is required for *C. albicans* pathogenicity and how these pumps impact antifungal resistance.

RESULTS

Pmc1p is required for *Candida albicans* stress tolerance. To determine if Pmc1p or Vcx1p is required for *C. albicans* pathogenesis, we constructed *pmc1Δ/Δ* and *vcx1Δ/Δ* mutants using a PCR-based approach (19). Complemented strains were made by reintroducing a wild-type *PMC1* or *VCX1* allele into the *pmc1Δ/Δ* or *vcx1Δ/Δ* mutant, respectively, using an integrating vector that fully restores the *IRO1-URA3* locus. We initially examined phenotypes that have been associated with loss of Pmc1p or Vcx1p function in fungi (10–12, 15, 16, 18). Each of the described phenotypes was verified using at least two independently derived clones for each genotype. While the *pmc1Δ/Δ* and *vcx1Δ/Δ* mutants grew to a similar extent as the wild-type control strain on yeast extract-peptone-dextrose (YPD) agar plates, the *pmc1Δ/Δ* mutant was severely impaired by high concentrations of CaCl_2 (Fig. 1A; see also Fig. S1 in the supplemental material). The *pmc1Δ/Δ* mutant was more resistant than the wild type to LiCl and CdSO_4 but hypersensitive to the membrane stressor SDS (Fig. 1A). Reintroduction of *PMC1* into the *pmc1Δ/Δ* mutant only partially restored wild-type phenotypes with respect to LiCl, CaCl_2 , and CdSO_4 sensitivity, suggesting that *PMC1* may be haploinsufficient. The *PMC1* reconstituted strain also remained completely sensitive to SDS at the concentration tested (0.05%), suggesting that the capacity of *C. albicans* to survive plasma membrane stress is especially sensitive to Pmc1p deficiencies. The growth of the *vcx1Δ/Δ* mutant, on the other hand, was unaffected by CaCl_2 , LiCl, or CdSO_4 supplements (Fig. 1A). The *vcx1Δ/Δ* mutant was, however, sensitive to SDS, albeit to a lesser extent than the *pmc1Δ/Δ* mutant. These results confirm the importance of *C. albicans* Pmc1p for Ca^{2+} homeostasis and resistance to ionic as well as membrane stress.

Pmc1p influences *Candida albicans* antifungal susceptibility. A previous study reported that a *C. albicans pmc1Δ/Δ* mutant was resistant to the antifungal drug fluconazole (18). Similarly, we found our *pmc1Δ/Δ* mutant to be more resistant than the wild type to fluconazole when grown at 30°C on YPD agar supplemented with the drug (Fig. 1A) or using Etest strips (see Fig. S2). However, when tested in liquid RPMI medium at 35°C according to the standards of the CLSI protocol (20), or on RPMI agar with Etest strips (Fig. S2), the *pmc1Δ/Δ* mutant was as susceptible as the wild type. This indicated that the fluconazole resistance phenotype of the *pmc1Δ/Δ* mutant was dependent on the medium or some other growth condition. To determine how temperature influ-

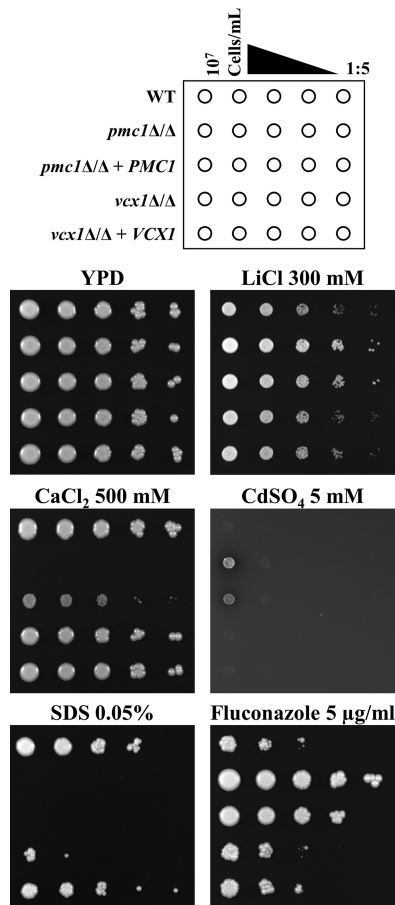


FIG 1 *Candida albicans pmc1Δ/Δ* mutant is hypersensitive to ionic and membrane stress. Wild-type (GP1), *pmc1Δ/Δ*, *vcx1Δ/Δ*, and revertant strains of *C. albicans* were suspended at 1×10^7 cells/ml in sterile deionized water, and serial 1:5 dilutions were prepared. Cell suspensions were then applied to YPD agar plates or YPD agar supplemented with the indicated concentrations of LiCl, CaCl₂, CdSO₄, SDS, or fluconazole using a sterile multipronged applicator. Plates were incubated at 30°C for 48 to 96 h and then imaged.

ences the *pmc1Δ/Δ* mutants' susceptibility to fluconazole, we compared the sensitivity of our *C. albicans* strains on YPD agar using spot dilution assays, following incubation at either 30, 35, 37, or 42°C. This revealed that while the *pmc1Δ/Δ* mutant is more resistant to fluconazole than the wild type at 30°C, it was not significantly different at 35 or 37°C and, paradoxically, was more sensitive at 42°C (see Fig. S3). Thus, the effect of Pmc1p on *C. albicans* sensitivity to fluconazole was temperature dependent. Notably, the *vcx1Δ/Δ* mutants' sensitivity to fluconazole was indistinguishable from that of the wild type under all conditions tested.

Interestingly, the *pmc1Δ/Δ* mutant was slightly more sensitive than the wild type to the morpholine antifungal amorolfine (see Fig. S4). Amorolfine inhibits both C-8 sterol isomerase (Erg2p) and C-14 sterol reductase (Erg24p), both of which act downstream of Erg11p in the ergosterol biosynthetic pathway (21). Again, the susceptibility of the *vcx1Δ/Δ* mutant to amorolfine was indistinguishable from that of the wild-type control (data not shown).

Loss of Pmc1p impairs *Candida albicans* hyphal formation. The ability to form hyphae is important for *C. albicans* pathogenicity (22, 23). We therefore examined the *pmc1Δ/Δ* and *vcx1Δ/Δ* mutants' capacity to form hyphae. The *pmc1Δ/Δ* mutant's ability to form hyphae was severely impaired on M199 or 10% fetal bovine serum (FBS) agar (Fig. 2A), and it remained as yeast cells in liquid FBS (Fig. 3). While a significant fraction of *pmc1Δ/Δ* cells produced short filaments in liquid M199 (Fig. 3), these were substan-

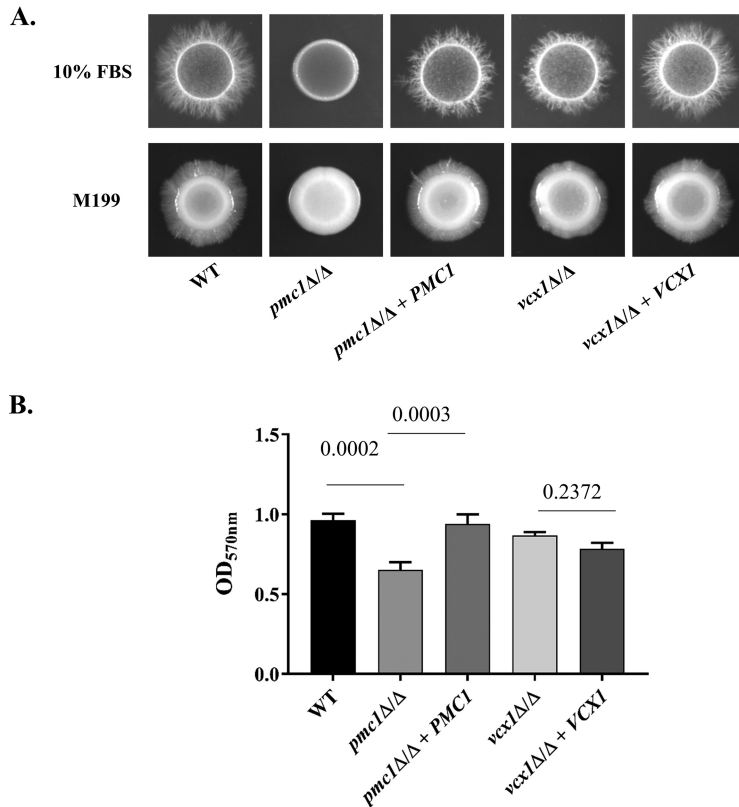


FIG 2 *Candida albicans* *pmc1Δ/Δ* mutant is defective in hyphal growth and biofilm formation. The abilities of the wild-type (GP1), *pmc1Δ/Δ* and *vcx1Δ/Δ* mutants, and isogenic control strains to form hyphae and biofilms were compared. (A) Each strain was resuspended at 1×10^7 cells/ml in sterile deionized water, and $2.5 \mu\text{l}$ was spotted onto either M199 or 10% FBS agar plates. The resulting colonies were imaged after 96 h of incubation at 37°C . (B) Each strain was suspended at 1×10^6 cells/ml in RPMI medium (pH 7.0), and $200 \mu\text{l}$ was dispensed into the wells of a flat-bottomed 96-well plate. After incubating at 37°C for 24 h, the plate was rinsed with PBS, and biofilm formation was detected using a crystal violet staining procedure. Crystal violet was subsequently eluted with 95% ethanol, and resolubilized dye was quantified by measuring the OD₅₇₀ in a microplate reader. The means and standard errors of the means from three biological replicates are shown. The mean of each group was compared using a two-way analysis of variance (ANOVA), and *P* values of relevant comparisons are indicated.

tially shorter than for the wild type, and many cells remained in the yeast form under these conditions. In contrast, the *vcx1Δ/Δ* mutant exhibited no detectable defects in hyphal growth under any of these conditions, indicating that Vcx1p is not required for hyphal growth in *C. albicans*.

Since the *pmc1Δ/Δ* mutant was hypersensitive to high levels of Ca^{2+} and unable to form normal hyphae in FBS, we next evaluated if the defects in hyphal formation were due to the high levels of Ca^{2+} found in serum (3.5 to 4 mM) (24). This was tested using the Ca^{2+} chelator EGTA to sequester free Ca^{2+} in the FBS. The addition of 5 mM EGTA to the FBS largely restored the ability of the *pmc1Δ/Δ* mutant to form filaments (Fig. 4). Interestingly, the ability of the wild type and the complemented control strain to form hyphae was reduced by the addition of EGTA to the FBS. These results establish that the calcium concentrations within host tissues and fluids, as well as the calcium detoxification functions performed by Pmc1p, are an essential determinant of fungal morphogenesis and therefore *C. albicans* pathogenicity.

Finally, since hyphal growth is intimately linked to the capacity of *C. albicans* to form biofilms (25), we compared the *pmc1Δ/Δ*, *vcx1Δ/Δ*, and wild-type control strains abilities to form biofilms using a simple *in vitro* assay. Again, the *pmc1Δ/Δ* mutant was significantly impaired in its capacity to form biofilms, while the *vcx1Δ/Δ* mutant was able to form biofilms to the same extent as the wild-type control and complemented strains (Fig. 2B).

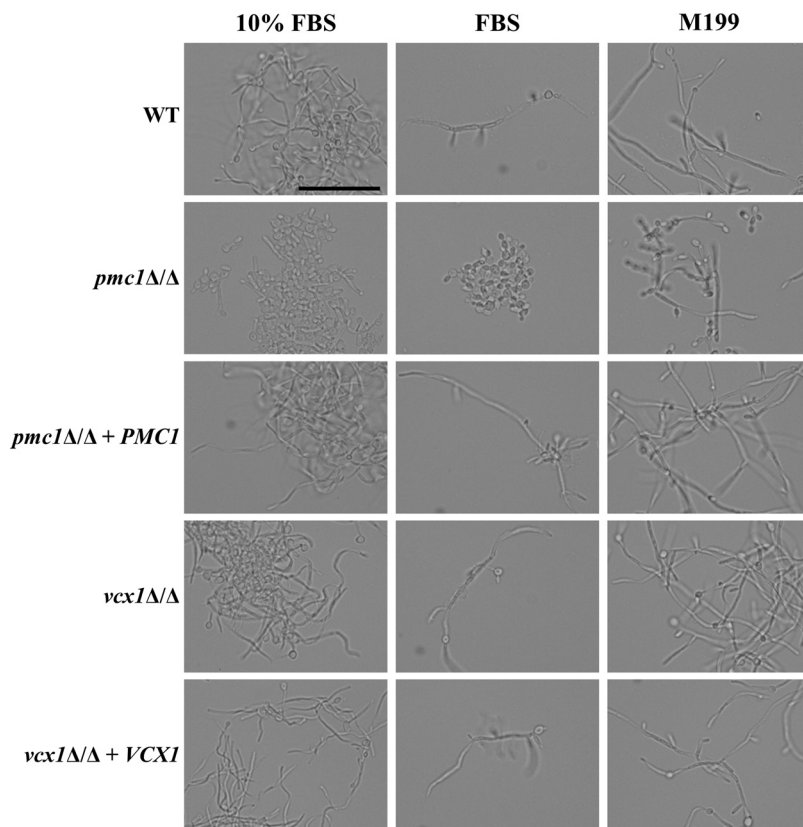


FIG 3 *Candida albicans* *pmc1Δ/Δ* mutant is defective in hyphal formation. Wild-type (GP1), *pmc1Δ/Δ*, *vcx1Δ/Δ*, and revertant strains of *C. albicans* were subcultured at $\sim 1 \times 10^6$ cells/ml in 10% FBS, 100% FBS, or M199 and incubated at 37°C with shaking. Samples were taken after 6 h of incubation and fixed with formalin. Cell morphologies were observed by light microscopy using a 40 \times objective. Bar, 50 μ m.

Pmc1p is required for *Candida albicans* virulence in a mouse model of disseminated infection. To determine if either Pmc1p or Vcx1p is required for *C. albicans* to cause disease within its mammalian host, we compared the virulence of the *pmc1Δ/Δ* and *vcx1Δ/Δ* mutants with that of the wild type in a mouse model of disseminated infection (Fig. 5). All mice infected with wild-type *C. albicans* succumbed within 7 days of infection. However, all 7 of the mice infected with the *pmc1Δ/Δ* mutant survived the duration of the experiment (14 days), with 3 having undetectable levels of fungal colonization within their kidneys and the remainder having relatively low levels (ranging from 3.32×10^3 to 2.34×10^4 CFU/g of kidney). In contrast, the *vcx1Δ/Δ* mutant was as virulent as the wild-type and revertant control strains, as determined by the comparable survival times of mice infected with each strain. These data indicate that Pmc1p, but not Vcx1p, is required for *C. albicans* pathogenicity following dissemination through the bloodstream (Fig. 5).

DISCUSSION

In this study, we explored the contributions of the Pmc1p Ca^{2+} pump and the Vcx1p Ca^{2+} exchanger to stress response and pathogenesis of *C. albicans*. It is evident from these results that the inability of the *pmc1Δ/Δ* mutant to properly remove Ca^{2+} from the cytoplasm into the vacuole renders the excess Ca^{2+} toxic to the mutant in a Ca^{2+} -rich medium.

Another interesting finding was the impairment of hyphal formation in the *pmc1Δ/Δ* mutant in M199 medium and FBS. This suggests that the regulation of Ca^{2+} fluxes by Pmc1p into the vacuole is important for the yeast-to-hyphae transition and follows the findings of previous work indicating that Ca^{2+} release from the fungal vacuole into the cytoplasm via the Ca^{2+} channel Yvc1p is important for hyphal formation (26). This

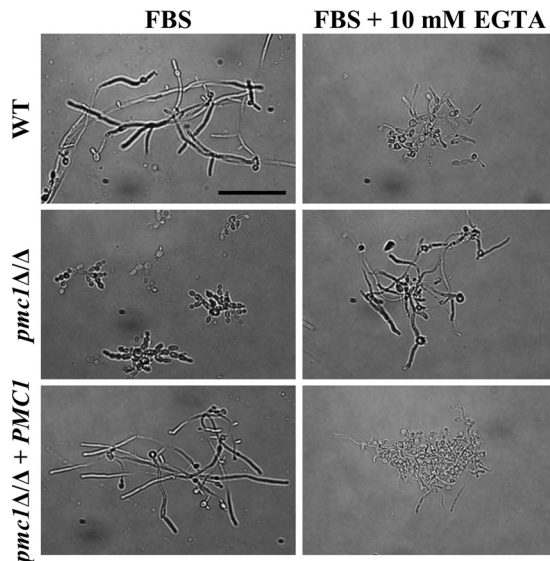


FIG 4 The *Candida albicans* *pmc1*Δ/Δ mutant's hyphal growth defect is calcium dependent. Wild-type (GP1), *pmc1*Δ/Δ, and revertant strains of *C. albicans* were subcultured in FBS or FBS plus 10 mM EGTA at $\sim 1 \times 10^6$ cells/ml and incubated at 37°C with shaking. Samples were taken after 6 h of incubation, and cells were fixed with formalin. Cell morphologies were subsequently observed by light microscopy using a 40× objective. Bar, 50 μm.

highlights the importance of Ca^{2+} uptake into and release from the fungal vacuole in morphogenesis. Interestingly, removal of free Ca^{2+} from the FBS allowed the *pmc1*Δ/Δ mutant to form filaments. Thus, while the concentration of calcium in FBS (~ 3.5 to 4 mM) (24) was substantially lower than that used to test the calcium toxicity herein, it was nonetheless sufficient to impair the mutant's capacity to form hyphae, a characteristic that is intimately associated with the ability of *C. albicans* to cause disease (22, 23). Our results further suggest that the *pmc1*Δ/Δ mutant does not have a mechanical defect in its ability to form hyphae, but rather its inability to remove excess Ca^{2+} from the cytoplasm interferes with the requisite signaling events. It is likely that the dysregulation of Ca^{2+} -based signaling also underlies the wide range of physiological and stress-related phenotypes of the *pmc1*Δ/Δ mutant.

C. albicans mutants deficient in calcineurin signaling were previously reported as exquisitely sensitive to Ca^{2+} levels in serum (18, 24) and had severe defects in the colonization of kidney tissue in a mouse model of disseminated infection (18, 27). This makes sense, since Pmc1p is a downstream effector of calcineurin signaling (18, 28); therefore, a loss of Pmc1p would be expected to produce a similar phenotypic profile

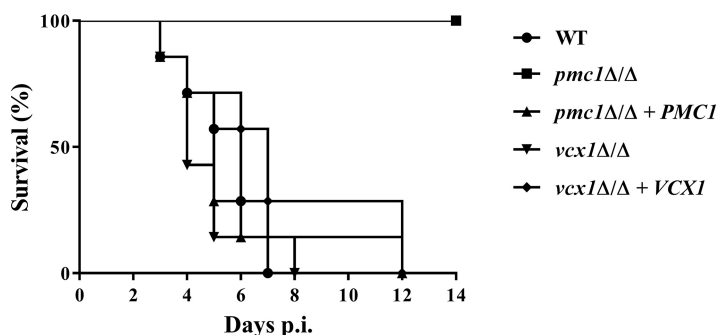


FIG 5 *Candida albicans* *pmc1*Δ/Δ mutant is avirulent in a mouse model of disseminated candidiasis. Groups of BALB/c mice ($N = 7$) were inoculated with $\sim 5 \times 10^5$ CFU of either wild-type (GP1), *pmc1*Δ/Δ, *vcx1*Δ/Δ, or revertant strains via lateral tail vein injections. The mice were then monitored 3 times daily for 14 days, and those showing signs of distress were humanely euthanized. The survival of each group was compared using the log rank test ($P < 0.0001$).

to that resulting from calcineurin dysfunction. Collectively, the phenotypic deficiencies of the *pmc1Δ/Δ* mutant resulting from its reduced capacity to sequester excess Ca^{2+} into the vacuole likely underlie its inability to colonize or invade mammalian tissue in the mouse model of disseminated infection. Interestingly, loss of Vcx1p function had little consequence on *C. albicans* physiology *in vitro* or pathogenicity *in vivo*. Given that Pmc1p is a low-capacity high-affinity Ca^{2+} transporter, while Vcx1p is a high-capacity low-affinity transporter, our results suggest that restoring a low cytoplasmic concentration of Ca^{2+} following a signaling event may be more important with respect to avoiding toxicity than the rate at which Ca^{2+} is initially removed.

While most Pmc1p homologs are not essential for fungal viability *in vitro*, their function appears to be essential for the survival of *C. albicans*, *C. neoformans* and *A. fumigatus* *in vivo*, i.e., within mammalian tissue (13, 15, 16). Furthermore, even partially suppressing the expression of either of two Pmc1p orthologues that localize to intracellular acidocalcisomes is sufficient to cause gross morphological abnormalities and severely inhibit the growth of *Trypanosoma brucei* (29), the causative agent of African sleeping sickness. Collectively, these data underscore the critical importance of vacuolar Ca^{2+} uptake by the high-affinity Pmc1p transporter in promoting the survival of infectious eukaryotes within the mammalian host. Given the severity of the pathogenesis defects that result from loss of Pmc1p activity in each of these human pathogens, and in particular, the consequences upon fungal colonization of mammalian tissue, Pmc1p could provide a potentially efficacious target for antifungal therapy. Alternative approaches that prevent the sequestration of Ca^{2+} within the fungal vacuole, or else mobilize intravacuolar calcium, leading to toxicity, may also be viable therapeutic strategies. Whether Pmc1p itself is vulnerable to small-molecule inhibition and if inhibitors with sufficient fungal selective activity can be derived to provide the basis of a viable antimicrobial pharmacotherapy remain to be determined.

Pmc1p is most closely related to the PMCA-type Ca^{2+} pumps of mammals but has several important distinctions (11, 30). First, fungal Pmc1p proteins localize to the vacuolar membrane rather than the plasma membrane, and thus sequester excess calcium into an intracellular store rather than out of the cell (11, 30). Second, while fungal Pmc1p has been classified as a type 2B P-type ATPase, which are typically characterized by a cytoplasmic autoregulatory domain, fungal Pmc1p proteins completely lack this domain. In mammals, the autoinhibitory domain of all four PMCA pumps is found within an extended cytoplasmic C-terminal domain, shown by crystallography to wrap around and block the catalytic core of the protein (31, 32). Intracellular Ca^{2+} fluxes activate PMCA following Ca^{2+} -calmodulin binding to specific sequences in its C terminus that release autoinhibition. Curiously, fungal Pmc proteins completely lack extended N or C termini or the calmodulin binding sites that form the basis of the autoregulatory domains found in higher eukaryotes (11, 31–34). Thus, key structural determinants, as well as the molecular mechanisms by which the activity of these pumps are regulated in infectious fungi, are fundamentally different from those found in their mammalian host.

Previous reports, which were confirmed in this study, found that *C. albicans pmc1Δ/Δ* mutants are resistant to fluconazole on agar plates (18), while their susceptibility is not detectably different from that of the wild type when the standard CLSI broth microdilution protocol is used (20). Here, we determined that the *pmc1Δ/Δ* mutant's reduced susceptibility to fluconazole was temperature dependent and therefore may relate to a form of azole tolerance known as "trailing growth" (35) rather than outright azole resistance. Trailing growth is observed with a significant proportion of *C. albicans* isolates and manifests as significant residual growth in the presence of the azole that becomes apparent after 48 h of incubation. In its extreme form, trailing growth can be mistaken for true azole resistance; however, patients and experimental animals infected with trailing isolates generally respond well to treatment with the azoles (36, 37). The effect of temperature on azole tolerance was also observed previously in an endosomal trafficking mutant lacking the Rab GTPase Vps21p. A *C. albicans vps21Δ/Δ* mutant displayed enhanced growth in the presence of fluconazole at

35°C but not at 30°C (38). This enhanced growth in the presence of fluconazole also resembled the trailing growth phenomenon and appeared to be dependent on elevated Ca^{2+} signaling (20, 38). Together, these findings further emphasize the importance of temperature and exogenous Ca^{2+} levels, as well as intracellular Ca^{2+} homeostasis, as determinants of the capacity of *C. albicans* to survive following exposure to the azole antifungals.

MATERIALS AND METHODS

Growth conditions. *C. albicans* was routinely grown on YPD medium (1% yeast extract, 2% peptone, 2% dextrose) at 30°C, supplemented with uridine (50 $\mu\text{g}/\text{ml}$) when necessary. Transformant selection was carried out on minimal YNB medium (6.75 g/liter yeast nitrogen base without amino acids, 2% dextrose, 2% Bacto agar), supplemented with the appropriate auxotrophic requirements as described for *Saccharomyces cerevisiae* (39) or 50 $\mu\text{g}/\text{ml}$ uridine.

Plasmid construction. All oligonucleotides used in this study are listed in Table S1 in the supplemental material.

The *PMC1* open reading frame (ORF) with 761 bp of 5'- and 434 nt of 3'-untranslated-region (UTR) sequences was amplified from SC5314 genomic DNA (gDNA) with primer pair PMC1AMPF-KpnI and PMC1AMPR-SacI and then cloned between the KpnI and SacI restriction sites of pLUX (43) to produce plasmid pLUXPMC1.

For construction of the plasmid pLUXVCX1, the *VCX1* ORF with 747 bp of 5'- and 324 bp of 3'-UTR sequences was amplified using primer pair VCX1AMPF-KpnI and VCX1AMPR-SacI and cloned between the KpnI and SacI restriction sites of pLUX.

Candida albicans strains. All strains used in this study are listed in Table S2. Transformation of *C. albicans* with DNA constructs was performed using the lithium acetate method (40). Gene deletion strains were constructed by the PCR-based approach described by Wilson et al. (19), using the *ura3 Δ / Δ his1 Δ / Δ arg4 Δ / Δ* strain BWP17, kindly provided by Aaron Mitchell (Carnegie Mellon University).

The *pmc1 Δ / Δ* strain was constructed as follows. *PMC1* deletion cassettes were amplified by PCR with primers PMC1DISF and PMC1DISR using pRS-ARG4 Δ SpeI, pGEM-HIS1, or pDDB57 (containing a recyclable *URA3-dp1200* marker) (20, 21) as the template. Each *PMC1* allele was then sequentially deleted from BWP17 using *HIS1* and *ARG4* markers to generate *pmc1 Δ / Δ ura3 Δ / Δ* gene deletion mutants. The correct integration of the deletion cassettes was confirmed at each step by PCR with the following primers sets: ARG4INTF2/PMC1AMPR-SacI and ARG4INTR2/PMC1AMPF-KpnI for *ARG4* integration or HIS1INTR2/PMC1AMPR-SacI and HIS1INTF2/PMC1AMPF-KpnI for *HIS1* integration. The absence of an intact *PMC1* allele was confirmed using primer pair PMC1DETF and PMC1DETR. Isogenic mutant and *PMC1*-reconstituted strains were produced by transforming the *pmc1 Δ / Δ ura3 Δ / Δ* mutant with either pLUX (vector alone) or pLUXPMC1 after digestion with NheI. The correct integration of the pLUX vector fully restores *URA3* and adjacent *IRO1* loci, and this was confirmed by PCR using primer pair LUXINTDETF and LUXINTDETR.

VCX1 deletion cassettes were amplified by PCR with primers VCX1DISF and VCX1DISR using pRS-ARG4 Δ SpeI, pGEM-HIS1, or pDDB57 as the templates. The *vcx1 Δ / Δ ura3 Δ / Δ* gene deletion mutants were produced by sequential deletion of each *VCX1* allele using *HIS1* and *ARG4* markers. Correct integration of deletion cassettes was confirmed at each step by PCR with primers pairs ARG4INTF2/VCX1AMPR-SacI and ARG4INTR2/VCX1AMPF-KpnI (*ARG4* integration), or HIS1INTR2/VCX1AMPR-SacI and HIS1INTF2/VCX1AMPF-KpnI (*HIS1* integration). Lack of an intact *VCX1* allele was confirmed by using primer pair VCX1DETF/VCX1DETR. Isogenic *vcx1 Δ / Δ* mutant and *VCX1*-reconstituted strains were produced by transforming the *vcx1 Δ / Δ ura3 Δ / Δ* mutant with either NheI-digested pLUX (vector alone) or pLUXVCX1. Correct integration of either plasmid was confirmed by PCR using primer pair LUXINTDETF/LUXINTDETR.

Stress resistance and hyphal growth assays. Each *C. albicans* strain was grown overnight in YPD at 30°C. The cells were washed in sterile deionized water, the cell density was adjusted to 10^7 cells/ml, and 1:5 serial dilutions were performed in a 96-well plate. Each cell suspension was then applied to agar plates using a sterile multipronged applicator. Resistance to different stresses was determined on YPD agar containing 5 $\mu\text{g}/\text{ml}$ of fluconazole, 0.05% SDS, 1.5 M NaCl, 300 mM LiCl, 5 mM CdSO_4 , or 500 mM CaCl_2 , with incubation at 30°C for 48 to 96 h. To induce hyphal growth, for each strain, 2.5 μl of a 10^7 cells/ml cell suspension was spotted on M199 or 10% fetal bovine serum (FBS) agar plates, and incubated for 96 h at 37°C.

Biofilm formation assay. Biofilm formation was assessed using a protocol based on that described by O'Toole (41). Each *C. albicans* strain was grown overnight in YPD broth at 30°C. Each culture was then washed two times in sterile phosphate-buffered saline (PBS), the cell density was adjusted to 1×10^6 per ml in RPMI medium (pH 7.0), and 200 μl was dispensed into the wells of a flat-bottomed 96-well plate. After incubation at 37°C for 24 h, each well was rinsed 3 times with sterile PBS and then stained with 0.01% crystal violet for 15 min. Each well was again rinsed 3 times with sterile water, and the dye was eluted with 95% ethanol (200 $\mu\text{l}/\text{well}$); 150 μl of resuspended dye from each well was then transferred to a new flat-bottomed microtiter plate, and the optical density at 570 nm (OD_{570}) was measured using a microplate reader.

Antifungal susceptibility testing. Antifungal susceptibility testing of all the strains included in this study was performed using the broth microdilution method described in the CLSI document M27-A3 (42) in a 96-well plate format. All drugs for susceptibility testing used in this study were diluted in dimethyl sulfoxide (DMSO) to 200 times the final concentration. RPMI 1640 medium (Sigma-Aldrich) was prepared according to the CLSI document; the medium was buffered with morpholinepropanesulfonic acid

(MOPS) and pH adjusted using NaOH and HCl. Plates were incubated at 25°C, 35°C, or 42°C without shaking for 24 or 48 h. The content of each well was carefully resuspended by pipetting up and down before the OD₆₀₀ was measured using a Biotek Cytation 5 plate reader.

Susceptibility testing using fluconazole Etest strips was performed on agar plates with MOPS-buffered RPMI medium at pH 7. Cell suspensions were streaked onto the RPMI plates using sterile cotton applicators. Etest strips were applied on the surface of the agar, and the plates were incubated at 35°C for 24 to 48 h.

Ethics statement. The animals used in this study were housed in American Association for Accreditation of Laboratory Animal Care (AAALAC)-approved facilities at the University of Tennessee Health Science Center (UTHSC). The Institutional Animal Care and Use Committee (IACUC) at UTHSC approved the use of all animals and procedures (IACUC protocol numbers 15-081 and 16-156). Mice were given standard rodent chow and water *ad libitum*. Mice were monitored daily for signs of distress, including noticeable weight loss and lethargy, and for the body condition score. The IACUC at UTHSC uses the Public Health Policy on Humane Care and Use of Laboratory Animals (PHS) and the Guide for the Care and Use of Laboratory Animals as a basis for establishing and maintaining an institutional program for activities involving animals. To ensure high standards for animal welfare, the IACUC at UTHSC remains compliant with all applicable provisions of the Animal Welfare Act (AWAR), guidance from the Office of Laboratory Animal Welfare (OLAW), and the American Veterinary Medical Association Guidelines on Euthanasia.

Mouse model of disseminated candidiasis. *C. albicans* strains were grown overnight in YPD broth at 30°C with shaking. Stationary-phase cultures of *C. albicans* strains were washed twice in sterile, endotoxin-free phosphate-buffered saline (PBS) and resuspended in PBS at 5×10^6 cells/ml. Groups of 6 BALB/c mice per *C. albicans* strain were then inoculated via tail vein injections with 100 μ l of the desired cell suspension ($\sim 5 \times 10^5$ cells). Viable cell counts of each inoculum were confirmed by plating appropriate dilutions on YPD agar plates and counting the colonies formed after 48 h of incubation at 30°C. Mice were then monitored for 14 days postinfection, and those showing signs of distress were humanely euthanized. Animals surviving to the end of the experiment (day 14) were euthanized, and their kidneys were extracted, weighed, and homogenized in PBS. Serial dilutions of kidney homogenates were plated on YPD agar plates containing 50 μ g/ml of chloramphenicol. The CFU/g of kidney tissue was then determined from the number of colonies formed on the plates after 48 h of incubation at 30°C.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/mSphere.00715-18>.

FIG S1, DOCX file, 0.2 MB.

FIG S2, DOCX file, 2.2 MB.

FIG S3, DOCX file, 0.6 MB.

FIG S4, DOCX file, 0.1 MB.

TABLE S1, PDF file, 0.1 MB.

TABLE S2, PDF file, 0.1 MB.

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REFERENCES

- Klionsky DJ, Herman PK, Emr SD. 1990. The fungal vacuole: composition, function, and biogenesis. *Microbiol Rev* 54:266–292.
- Veses V, Richards A, Gow NA. 2008. Vacuoles and fungal biology. *Curr Opin Microbiol* 11:503–510. <https://doi.org/10.1016/j.mib.2008.09.017>.
- Cyert MS, Philpott CC. 2013. Regulation of cation balance in *Saccharomyces cerevisiae*. *Genetics* 193:677–713. <https://doi.org/10.1534/genetics.112.147207>.
- Dunn T, Gable K, Beeler T. 1994. Regulation of cellular Ca²⁺ by yeast vacuoles. *J Biol Chem* 269:7273–7278.
- Bonilla M, Nastase KK, Cunningham KW. 2002. Essential role of calcineurin in response to endoplasmic reticulum stress. *EMBO J* 21:2343–2353. <https://doi.org/10.1093/emboj/21.10.2343>.
- Kraus PR, Heitman J. 2003. Coping with stress: calmodulin and calcineurin in model and pathogenic fungi. *Biochem Biophys Res Commun* 311:1151–1157.
- Marchi S, Patergnani S, Missiroli S, Morciano G, Rimessi A, Wieckowski MR, Giorgi C, Pinton P. 2018. Mitochondrial and endoplasmic reticulum calcium homeostasis and cell death. *Cell Calcium* 69:62–72. <https://doi.org/10.1016/j.ceca.2017.05.003>.
- Pinton P, Giorgi C, Siviero R, Zecchini E, Rizzuto R. 2008. Calcium and apoptosis: ER-mitochondria Ca²⁺ transfer in the control of apoptosis. *Oncogene* 27:6407–6418. <https://doi.org/10.1038/onc.2008.308>.
- Miseta A, Kellermayer R, Aiello DP, Fu L, Bedwell DM. 1999. The vacuolar Ca²⁺/H⁺ exchanger Vcx1p/Hum1p tightly controls cytosolic Ca²⁺ levels in *S. cerevisiae*. *FEBS Lett* 451:132–136.
- Cunningham KW, Fink GR. 1996. Calcineurin inhibits VCX1-dependent H⁺/Ca²⁺ exchange and induces Ca²⁺ ATPases in *Saccharomyces cerevisiae*. *Mol Cell Biol* 16:2226–2237.
- Pittman JK. 2011. Vacuolar Ca²⁺ uptake. *Cell Calcium* 50:139–146. <https://doi.org/10.1016/j.ceca.2011.01.004>.
- Cunningham KW, Fink GR. 1994. Calcineurin-dependent growth control in *Saccharomyces cerevisiae* mutants lacking PMC1, a homolog of plasma membrane Ca²⁺ ATPases. *J Cell Biol* 124:351–363.
- Squizani ED, Oliveira NK, Reuwsaat JCV, Marques BM, Lopes W, Gerber

- AL, Vasconcelos Atr d, Lev S, Djordjevic JT, Schrank A, Vainstein MH, Staats CC, Kmetzsch L. 2018. Cryptococcal dissemination to the central nervous system requires the vacuolar calcium transporter Pmc1. *Cell Microbiol* 20:e12803. <https://doi.org/10.1111/cmi.12803>.
14. Kmetzsch L, Staats CC, Simon E, Fonseca FL, de Oliveira DL, Sobrino L, Rodrigues J, Leal AL, Nimrichter L, Rodrigues ML, Schrank A, Vainstein MH. 2010. The vacuolar Ca²⁺ exchanger Vcx1 is involved in calcineurin-dependent Ca²⁺ tolerance and virulence in *Cryptococcus neoformans*. *Eukaryot Cell* 9:1798–1805. <https://doi.org/10.1128/EC.00114-10>.
 15. Kmetzsch L, Staats CC, Cupertino JB, Fonseca FL, Rodrigues ML, Schrank A, Vainstein MH. 2013. The calcium transporter Pmc1 provides Ca²⁺ tolerance and influences the progression of murine cryptococcal infection. *FEBS J* 280:4853–4864. <https://doi.org/10.1111/febs.12458>.
 16. Dinamarco TM, Freitas FZ, Almeida RS, Brown NA, dos Reis TF, Ramalho LN, Savoldi M, Goldman MH, Bertolini MC, Goldman GH. 2012. Functional characterization of an *Aspergillus fumigatus* calcium transporter (PmcA) that is essential for fungal infection. *PLoS One* 7:e37591. <https://doi.org/10.1371/journal.pone.0037591>.
 17. Cruz MC, Goldstein AL, Blankenship JR, Del Poeta M, Davis D, Cardenas ME, Perfect JR, McCusker JH, Heitman J. 2002. Calcineurin is essential for survival during membrane stress in *Candida albicans*. *EMBO J* 21: 546–559.
 18. Sanglard D, Ischer F, Marchetti O, Entenza J, Bille J. 2003. Calcineurin A of *Candida albicans*: involvement in antifungal tolerance, cell morphogenesis and virulence. *Mol Microbiol* 48:959–976.
 19. Wilson RB, Davis D, Mitchell AP. 1996. Rapid hypothesis testing with *Candida albicans* through gene disruption with short homology regions. *J Bacteriol* 181:1868–1874.
 20. Luna-Tapia A, Tournu H, Peters TL, Palmer GE. 2016. Endosomal trafficking defects can induce calcium dependent azole tolerance in *Candida albicans*. *Antimicrob Agents Chemother* 60:7170–7177. <https://doi.org/10.1128/AAC.01034-16>.
 21. Barrett-Bee K, Dixon G. 1995. Ergosterol biosynthesis inhibition: a target for antifungal agents. *Acta Biochim Pol* 42:465–479.
 22. Saville SP, Lazzell AL, Monteagudo C, Lopez-Ribot JL. 2003. Engineered control of cell morphology *in vivo* reveals distinct roles for yeast and filamentous forms of *Candida albicans* during infection. *Eukaryot Cell* 2:1053–1060.
 23. Lo HJ, Kohler JR, DiDomenico B, Loebenberg D, Cacciapuoti A, Fink GR. 1997. Nonfilamentous *C. albicans* mutants are avirulent. *Cell* 90:939–949.
 24. Blankenship JR, Heitman J. 2005. Calcineurin is required for *Candida albicans* to survive calcium stress in serum. *Infect Immun* 73:5767–5774. <https://doi.org/10.1128/IAI.73.9.5767-5774.2005>.
 25. Nobile CJ, Johnson AD. 2015. *Candida albicans* biofilms and human disease. *Annu Rev Microbiol* 69:71–92. <https://doi.org/10.1146/annurev-micro-091014-104330>.
 26. Yu Q, Wang F, Zhao Q, Chen J, Zhang B, Ding X, Wang H, Yang B, Lu G, Zhang B, Li M. 2014. A novel role of the vacuolar calcium channel Yvc1 in stress response, morphogenesis and pathogenicity of *Candida albicans*. *Int J Med Microbiol* 304:339–350. <https://doi.org/10.1016/j.ijmm.2013.11.022>.
 27. Blankenship JR, Wormley FL, Boyce MK, Schell WA, Filler SG, Perfect JR, Heitman J. 2003. Calcineurin is essential for *Candida albicans* survival in serum and virulence. *Eukaryot Cell* 2:422–430.
 28. Stathopoulos AM, Cyert MS. 1997. Calcineurin acts through the CRZ1/TCN1-encoded transcription factor to regulate gene expression in yeast. *Genes Dev* 11:3432–3444.
 29. Luo S, Rohloff P, Cox J, Uyemura SA, Docampo R. 2004. Trypanosoma brucei plasma membrane-type Ca²⁺-ATPase 1 (TbPMC1) and 2 (Tb-PMC2) genes encode functional Ca²⁺-ATPases localized to the acidocalcisomes and plasma membrane, and essential for Ca²⁺ homeostasis and growth. *J Biol Chem* 279:14427–14439. <https://doi.org/10.1074/jbc.M309978200>.
 30. Cunningham KW. 2011. Acidic calcium stores of *Saccharomyces cerevisiae*. *Cell Calcium* 50:129–138. <https://doi.org/10.1016/j.ceca.2011.01.010>.
 31. Di Leva F, Domi T, Fedrizzi L, Lim D, Carafoli E. 2008. The plasma membrane Ca²⁺ ATPase of animal cells: structure, function and regulation. *Arch Biochem Biophys* 476:65–74. <https://doi.org/10.1016/j.abb.2008.02.026>.
 32. Enyedi A, Vorherr T, James P, McCormick DJ, Filoteo AG, Carafoli E, Penniston JT. 1989. The calmodulin binding domain of the plasma membrane Ca²⁺ pump interacts both with calmodulin and with another part of the pump. *J Biol Chem* 264:12313–12321.
 33. Palmgren MG, Axelsen KB. 1998. Evolution of P-type ATPases. *Biochim Biophys Acta* 1365:37–45.
 34. Hofmann F, James P, Vorherr T, Carafoli E. 1993. The C-terminal domain of the plasma membrane Ca²⁺ pump contains three high affinity Ca²⁺ binding sites. *J Biol Chem* 268:10252–10259.
 35. Arthington-Skaggs BA, Lee-Yang W, Ciblak MA, Frade JP, Brandt ME, Hajjeh RA, Harrison LH, Sofair AN, Warnock DW, Candidemia Active Surveillance Group. 2002. Comparison of visual and spectrophotometric methods of broth microdilution MIC end point determination and evaluation of a sterol quantitation method for *in vitro* susceptibility testing of fluconazole and itraconazole against trailing and nontrailing *Candida* isolates. *Antimicrob Agents Chemother* 46:2477–2481.
 36. Peters BM, Luna-Tapia A, Tournu H, Rybak JM, Rogers PD, Palmer GE. 2017. An azole-tolerant endosomal trafficking mutant of *Candida albicans* is susceptible to azole treatment in a mouse model of vaginal candidiasis. *Antimicrob Agents Chemother* 61:e00084-17. <https://doi.org/10.1128/AAC.00084-17>.
 37. Rex JH, Nelson PW, Paetznick VL, Lozano-Chiu M, Espinel-Ingroff A, Anaisie EJ. 1998. Optimizing the correlation between results of testing *in vitro* and therapeutic outcome *in vivo* for fluconazole by testing critical isolates in a murine model of invasive candidiasis. *Antimicrob Agents Chemother* 42:129–134. <https://doi.org/10.1128/AAC.42.1.129>.
 38. Luna-Tapia A, Kerns ME, Eberle KE, Jursic BS, Palmer GE. 2015. Trafficking through the late endosome significantly impacts *Candida albicans* tolerance of the azole antifungals. *Antimicrob Agents Chemother* 59: 2410–2420. <https://doi.org/10.1128/AAC.04239-14>.
 39. Burke D, Dawson D, Stearns T, Laboratory CSH. 2000. *Methods in yeast genetics: a cold spring harbor laboratory course manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
 40. Gietz D, St Jean A, Woods RA, Schiestl RH. 1992. Improved method for high efficiency transformation of intact yeast cells. *Nucleic Acids Res* 20:1425.
 41. O'Toole GA. 2011. Microtiter dish biofilm formation assay. *J Vis Exp* 30:2437. <https://doi.org/10.3791/2437>.
 42. Clinical and Laboratory Standards Institute. 2008. Reference method for broth dilution antifungal susceptibility testing of yeasts; approved standard, 3rd ed. CLSI document M27-A3. Clinical and Laboratory Standards Institute, Wayne, PA.
 43. Ramon AM, Fonzi WA. 2003. Diverged binding specificity of Rim101p, the *Candida albicans* ortholog of PacC. *Eukaryot Cell* 2:718–728.