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Calcineurin Is Essential for Virulence in *Candida albicans*

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Calcineurin is a conserved Ca^{2+}-calmodulin-activated, serine/threonine-specific protein phosphatase that regulates a variety of physiological processes, e.g., cell cycle progression, polarized growth, and adaptation to salt and alkaline pH stresses. In the pathogenic yeast *Cryptococcus neoformans*, calcineurin is also essential for growth at 37°C and virulence. To investigate whether calcineurin plays a role in the virulence of *Candida albicans*, the major fungal pathogen of humans, we constructed *C. albicans* mutants in which both alleles of the *CMP1* gene, encoding the calcineurin catalytic subunit, were deleted. The *C. albicans* Δ*cmp1* mutants displayed hypersensitivity to elevated Na^+, Li^+, and Mn^{2+} concentrations and to alkaline pH, phenotypes that have been described after calcineurin inactivation in the related yeast *Saccharomyces cerevisiae*. Unlike *S. cerevisiae* calcineurin mutants, which exhibit reduced susceptibility to high Ca^{2+} concentrations, growth of *C. albicans* was inhibited in the presence of 300 mM $CaCl_2$ after the deletion of *CMP1*, demonstrating that there are also differences in calcineurin-mediated cellular responses between these two yeast species. In contrast to *C. neoformans*, inactivation of calcineurin did not cause temperature sensitivity in *C. albicans*. In addition, hyphal growth, an important virulence attribute of *C. albicans*, was not impaired in the Δ*cmp1* mutants under a variety of inducing conditions. Nevertheless, the virulence of the mutants was strongly attenuated in a mouse model of systemic candidiasis, demonstrating that calcineurin signaling is essential for virulence in *C. albicans*.

Calcineurin is a widely conserved Ca^{2+}-calmodulin-activated, serine/threonine-specific protein phosphatase that is required for signal transduction, e.g., during the activation of T cells (45). Calcineurin signaling is inhibited by the immunosuppressive drugs cyclosporine A (CsA) and FK506, which diffuse into the cells and bind to the immunophilins cyclophilin and FKBP12, respectively. The resulting cyclophilin-CsA and FKBP12-FK506 complexes then inhibit calcineurin (28). CsA and FK506 also exhibit antifungal activity by inhibiting calcineurin through the same mechanism as in T cells (3, 5).

Calcineurin consists of a catalytic A subunit and a Ca^{2+}-binding regulatory B subunit, both of which are required for enzymatic function. In the yeast *Saccharomyces cerevisiae*, the catalytic subunit is encoded by the two redundant genes *CNA1* (*CMP1*) and *CNA2* (*CMP2*), and the regulatory subunit is encoded by the *CNB1* gene (10, 11, 23, 29). Mutant analyses have demonstrated that calcineurin is not essential for viability of wild-type cells, but it is required for recovery from pheromone-induced growth arrest, cell wall biosynthesis, and adaptation to high salt concentrations and alkaline conditions (10, 28, 34, 36, 40, 41). These responses are mediated by the calcineurin-dependent dephosphorylation and nuclear localization of the transcription factor Crz1p/Tcn1p, which regulates the expression of genes involved in cell wall biosynthesis and cation homeostasis (33, 35, 48, 49).

In the pathogenic yeast *Cryptococcus neoformans*, calcineurin is essential for growth at 37°C but not at ambient temperature (43). Consequently, *C. neoformans cna1* and *cnb1* mutants, in which the gene encoding the catalytic or regulatory subunit of calcineurin is inactivated, are avirulent in animal models of cryptococcosis (8, 16, 43). In addition, calcineurin is also required for hyphal elongation during mating and haploid fruiting in *C. neoformans* (6), similar to its role in the filamentous fungus *Neurospora crassa*, in which calcineurin is essential for hyphal growth and morphology (44).

In contrast to *C. neoformans*, which is found in the environment and accidentally infects humans by inhalation, the opportunistic fungal pathogen *Candida albicans* is a member of the microflora in healthy humans. Therefore, growth at 37°C is intrinsic to the normal lifestyle of *C. albicans*. In immunocompromised patients, *C. albicans* can develop from a commensal into an infectious organism, and a variety of virulence-associated characteristics contribute to its pathogenic potential (42). *C. albicans* can switch between yeast and filamentous growth forms, and the ability to form hyphae during an infection is believed to be an important virulence factor (30).

Because of its essentiality at body temperature and its role in hyphal formation and virulence in *C. neoformans*, we were interested in whether calcineurin plays a similar role in *C. albicans*. The *C. albicans* genome sequence contains a single gene, *CMP1*, encoding the calcineurin catalytic subunit (http://www-sequence.stanford.edu/group/candida/). To investigate the role of calcineurin signaling in this pathogenic yeast, we constructed *cmp1* null mutants and analyzed the effect of *CMP1* deletion on the growth, morphogenesis, and virulence of *C. albicans*.

MATERIALS AND METHODS

Strains and growth conditions. The *C. albicans* strains used in this study are listed in Table 1. The strains were maintained on SD agar plates (6.7 g of yeast nitrogen base without amino acids [YNB; Bio 101, Vista, Calif.], 20 g of glucose, 0.77 g of complete supplement medium without uracil [CSM-URA; Bio 101], and

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TABLE 1. *C. albicans* strains used in this study

Strain	Parent	Genotype[a]	Reference
SC5314		Wild-type strain	18
CAI4	SC5314	Δ*ura3*::*imm434*/Δ*ura3*::*imm434*	15
CMP1M1A	CAI4	*CMP1*/Δ*cmp1*::*URA3-FLIP*[b]	This study
CMP1M1B	CAI4	*CMP1*/Δ*cmp1*::*URA3-FLIP*	This study
CMP1M2A	CMP1M1A	*CMP1*/Δ*cmp1*::*FRT*	This study
CMP1M2B	CMP1M1B	*CMP1*/Δ*cmp1*::*FRT*	This study
CMP1M3A	CMP1M2A	Δ*cmp1*::*URA3-FLIP*/Δ*cmp1*::*FRT*	This study
CMP1M3B	CMP1M2B	Δ*cmp1*::*URA3-FLIP*/Δ*cmp1*::*FRT*	This study
CMP1M4A	CMP1M3A	Δ*cmp1*::*FRT*/Δ*cmp1*::*FRT*	This study
CMP1M4B	CMP1M3B	Δ*cmp1*::*FRT*/Δ*cmp1*::*FRT*	This study
CMP1M5A	CMP1M4A	Δ*cmp1*::*FRT*/Δ*cmp1*::*URA3*	This study
CMP1M5B	CMP1M4B	Δ*cmp1*::*FRT*/Δ*cmp1*::*URA3*	This study
CMP1MK1A	CMP1M4A	Δ*cmp1*::*FRT*/*CMP1-URA3*	This study
CMP1MK1B	CMP1M4B	Δ*cmp1*::*FRT*/*CMP1-URA3*	This study

[a] Apart from the indicated features, all strains have the genotype of their parent.
[b] *URA3-FLIP*, *URA3* flipper cassette.

15 g of agar per liter). The strains were routinely grown in YPD liquid medium (20 g of peptone, 10 g of yeast extract, 20 g of glucose per liter) at 30°C. To support the growth of uridine-auxotrophic strains, 100 μg of uridine ml^{-1} was added to the media. *ura3*-negative derivatives of strains containing the *URA3* flipper were isolated after induction of *FLP* expression by overnight growth in YCB-BSA (23.4 g of yeast carbon base, 2 g of yeast extract, 4 g of bovine serum albumin per liter, pH 4.0) containing 100 μg of uridine ml^{-1} and screening for smaller colonies after growth of the cells on SD agar plates containing 10 μg of uridine ml^{-1}.

Plasmid construction. A *CMP1* deletion construct was generated in the following way. A *Kpn*I-*Xho*I fragment containing *CMP1* upstream sequences from positions −1058 to +77 with respect to the start codon was amplified from genomic DNA of *C. albicans* strain CAI4 with the primer pair CMP1 (5′-GCT AGACGTGAggTACCAACGGGTGG-3′) and CMP2 (5′-CAGTTCATTACCT cgaGTGGTTCGTCTATG-3′) (the lowercase letters represent nucleotide exchanges introduced to create the underlined *Kpn*I and *Xho*I restriction sites). A *Sac*II-*Sac*I *CMP1* downstream fragment from positions +1594 to +2316 was amplified with the primers CMP3 (5′-AATTAccgCGGGGATCATTACCCAA AGGTTC-3′) and CMP4 (5′-TTCATgaGCTCATTAATATATTGAATCAAT AAG-3′). The *CMP1* upstream and downstream fragments were cloned on both sides of the *URA3* flipper cassette of plasmid pSFU1 (39), resulting in pCMP1M2, in which the *CMP1* coding region from positions +78 to +1593 (245 bp before the stop codon) is replaced by the *URA3* flipper (see Fig. 2A).

For reintegration of an intact *CMP1* copy into one of the inactivated *CMP1* alleles of homozygous *cmp1* deletion mutants, a *Pst*I-*Sac*II fragment containing *CMP1* downstream sequences from positions +1825 to +2316 was first amplified with the primers CMP5 (5′-GAAGATTATCTgcAGTTAAACTTTC-3′) and CMP6 (5′-TGGTTCcgcgGCTCATTAATATATTGAATCAATAAG-3′) and cloned behind the *URA3* selection marker of plasmid pGFP26 (38), from which the *Pst*I site in the polylinker had been removed, resulting in pCMP1M3. A fragment containing the complete *CMP1* open reading frame and upstream sequences was then amplified with the primers CMP1 and CMP8 (5′-CAGGG GCAAAAGgatccTTAACTTTGAGAT-3′) and digested at an *Xba*I site at position −528 and at the *Bam*HI site introduced behind the stop codon. The *CMP1* fragment was fused to a *Bgl*II-*Xho*I fragment containing the transcription termination sequence of the *ACT1* gene, which was obtained by PCR with the primers ACT16 (5′-TTCTAAGATctAAATTCTGGAAATCTGG-3′) and ACT21 (5′-at atactcgagGACATTTTATGATGGAATGAATGGG-3′), and cloned into the *Xba*I/*Sal*I-digested pCMP1M3, resulting in pCMP1K1 (see Fig. 2B, top). The control construct pCMP1M4, which served for integration of only the *URA3* selection marker, contains *CMP1* upstream sequences amplified with the primers CMP1 and CMP7 (5′-CGTTGAACAGTggaTCCTGACATGATG-3′) and digested at the *Xba*I site at position −528 and at the *Bam*HI site introduced at position +7 but not the *CMP1* open reading frame in front of the *ACT1* transcription termination sequence (see Fig. 2B, bottom).

***C. albicans* transformation.** C. *albicans* strains were transformed by electroporation (22) with the following gel-purified linear DNA fragments (see Fig. 2): the *Kpn*I-*Sac*I fragment from pCMP1M2 to delete the *CMP1* wild-type alleles, the *Xba*I-*Sac*II fragment from pCMP1K1 to reintroduce an intact *CMP1* copy into one of the destroyed *CMP1* alleles, and the *Xba*I-*Sac*II fragment from pCMP1M4 to integrate only the *URA3* marker but not the *CMP1* open reading frame in the same way. Uridine prototrophic transformants were selected on SD agar plates without uridine.

Isolation of genomic DNA and Southern hybridization. Genomic DNA from *C. albicans* was isolated as described previously (37). Ten micrograms of DNA was digested with *Bgl*II, separated on a 1% agarose gel, and, after ethidium bromide staining, transferred by vacuum blotting onto a nylon membrane and fixed by UV cross-linking. The gel-purified *Kpn*I-*Xho*I *CMP1* upstream fragment from pCMP1M2 and an internal *Sac*I-*Hin*dIII fragment from the *CMP1* coding region (positions +983 to +1365) were used as probes. Probe labeling, hybridization, washing, and signal detection were performed with the ECL labeling and detection kit provided by Amersham (Braunschweig, Germany) according to the instructions of the manufacturer.

Phenotypic assays. For susceptibility tests, serial 10-fold dilutions of yeast suspensions were spotted on YPD plates and YPD plates containing 1.8 or 1.0 M NaCl, 10 mM LiCl, 0.3 M $MnSO_4$, or 0.3 M $CaCl_2$ and incubated at 30°C. In addition, YPD plates buffered with 155 mM Tris-HCl at pH 8.0 were used. For temperature sensitivity tests, growth on YPD plates was monitored at 30, 37, and 43°C.

Filamentous growth was tested at 37°C on agar plates containing Lee's medium (26), synthetic low-ammonium dextrose (SLAD) medium (19), or 10% fetal calf serum (FCS). To analyze hyphal growth in liquid media, cells from a YPD overnight culture were inoculated into Lee's medium buffered at pH 7.0 with 50 mM sodium citrate, Spider medium (27), SLAD medium, or RPMI 1640 medium with 10% FCS and incubated at 37°C.

Virulence tests. Eight- to 10-week-old female BALB/c mice (Charles River Breeding Laboratories, Sulzfeld, Germany) ($n = 5$ to 8 for each group) were infected with 5×10^5 *C. albicans* cells by intravenous injection as described previously (25, 46). Survival curves were calculated according to the Kaplan-Meier method using the PRISM program (GraphPad Software, San Diego, Calif.) and compared using the log-rank test. A P value of <0.05 was considered significant. For microscopical examination, *C. albicans* cells were extracted from potassium hydroxide-solubilized kidneys and stained with calcofluor white (Sigma) as described previously (25, 46). For enumeration of CFU, kidneys were homogenized in phosphate-buffered saline, and replicates of serial dilutions were plated on rich solid media. The kidney fungal burden was calculated as CFU per milligram (wet weight) of kidney.

Sequence analysis. The overall similarity of the CaCmp1 protein with the ScCna1 and ScCna2 proteins was determined using the program GAP from the Genetics Computer Group, Madison, Wis. Multiple alignment of the proteins was performed with the CLUSTALW program (http://www.ebi.ac.uk/clustalw/).

RESULTS

Identification of the *CMP1* gene encoding the calcineurin catalytic subunit of *C. albicans*. A BLAST search identified an open reading frame (6.3161) in the *C. albicans* genome sequence encoding a protein with high similarity to ScCna1p (69% similarity; 61% identity) and ScCna2p (69% similarity; 59% identity), the two calcineurin catalytic subunits of *S. cerevisiae*. The gene is located on contig 6-2213 and has been annotated as *CMP1* by the Stanford Genome Technology Center (http://www-sequence.stanford.edu/group/candida/). The *CMP1* open reading frame is 1,830 bp in length and encodes a protein of 609 amino acids with a theoretical molecular mass of 69.9 kDa. An alignment of CaCmp1p with ScCna1p and ScCna2p is shown in Fig. 1. The presence of the highly conserved calcineurin B and calmodulin binding domains in CaCmp1p indicates that calcineurin may also be regulated by these proteins in *C. albicans*. No other gene with comparable homology is present in the *C. albicans* genome sequence, suggesting that *C. albicans* possesses only one catalytic subunit for calcineurin, as has been found for *C. neoformans* (43).

Generation of *C. albicans cmp1* deletion mutants. To analyze the role of calcineurin in *C. albicans*, we inactivated the *CMP1* gene using the *URA3*-flipping strategy, which is based on the repeated use of the *URA3* selection marker for integrative transformation and its subsequent excision from the genome

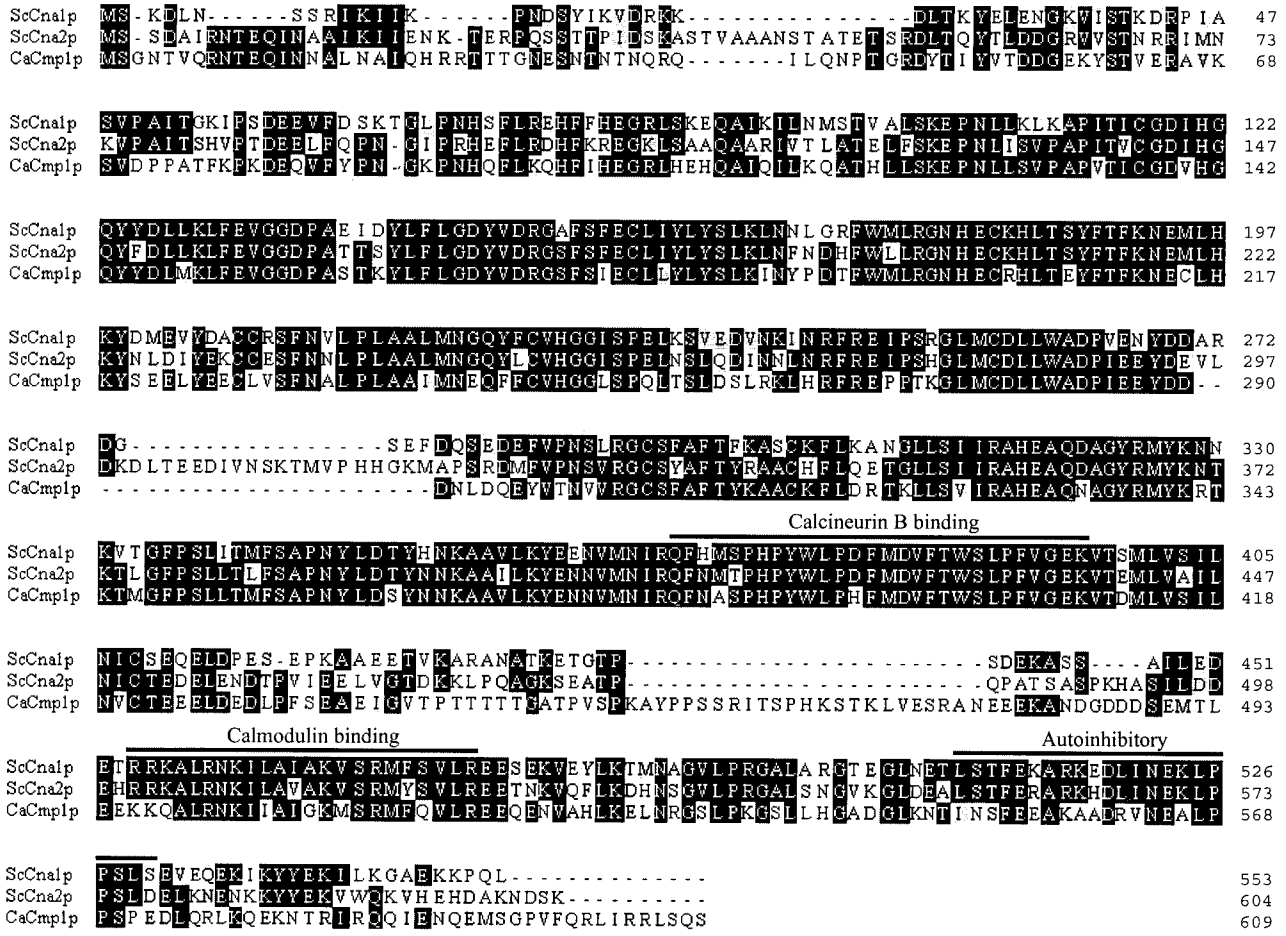

FIG. 1. Alignment of the calcineurin catalytic subunits Cna1p and Cna2p from *S. cerevisiae* (Sc) and Cmp1p from *C. albicans* (Ca). The amino acid sequences of the proteins are displayed in standard single-letter code using the BOXSHADE program (http://bioweb.pasteur.fr/seqanal/interfaces/boxshade.html). Identical residues are on solid backgrounds, and similar residues are on shaded backgrounds. Amino acid positions are shown on the right. The highly conserved calcineurin B binding, calmodulin binding, and autoinhibitory domains are indicated by labeled bars, and their delineation is according to the work of Odom et al. (43).

by the site-specific recombinase FLP (39). The *ura3*-negative strain CAI4 was transformed with a deletion cassette in which almost all of the *CMP1* coding sequence had been replaced by the *URA3* flipper (see Materials and Methods) (Fig. 2A). From two transformants in which the deletion cassette had been correctly inserted in one of the *CMP1* alleles (strains CMP1M1A and CMP1M1B [Fig. 2C, lanes 2 and 3]), the *URA3* flipper was excised by FLP-mediated recombination, resulting in the uridine-auxotrophic strains CMP1M2A and CMP1M2B (Fig. 2C, lanes 4 and 5). When these strains were transformed again with the same deletion cassette, integration was successfully targeted to the remaining wild-type *CMP1* allele in several transformants of both parent strains, demonstrating that *CMP1* is not an essential gene in *C. albicans*. The *URA3* flipper was excised from the two independent homozygous *cmp1* mutants CMP1M3A and CMP1M3B (Fig. 2C, lanes 6 and 7), generating the uridine-auxotrophic derivatives CMP1M4A and CMP1M4B (Fig. 2C, lanes 8 and 9). These strains served as hosts for reintroduction of a complete copy of the *CMP1* gene or the *URA3* selection marker alone into one of the inactivated Δ*cmp1* alleles (see Materials and Methods) (Fig. 2B). This resulted in the generation of the two independent, prototrophic, homozygous *cmp1* mutants CMP1M5A and CMP1M5B (Fig. 2C, lanes 12 and 13) and the corresponding complemented strains, CMP1MK1A and CMP1MK1B (Fig. 2C, lanes 10 and 11).

When the blot shown in Fig. 2C was rehybridized with a probe from the *CMP1* coding region, there also was only one hybridizing band in the parental strain, CAI4, which disappeared after two rounds of allelic replacement in the homozygous Δ*cmp1* mutants (Fig. 2D), providing additional evidence that *C. albicans* possesses only one gene encoding the calcineurin catalytic subunit.

Deletion of *CMP1* causes hypersensitivity to ionic and alkaline pH stresses. We first tested whether deletion of *CMP1* in *C. albicans* resulted in phenotypes that have been described for calcineurin mutants of *S. cerevisiae*. As shown in Fig. 3, the Δ*cmp1* mutants exhibited increased sensitivity to cationic stress, as they grew more slowly than the wild-type strain SC5314 in the presence of increased concentrations of sodium, lithium, or manganese. In addition, deletion of *CMP1* resulted in enhanced susceptibility to alkaline conditions, since

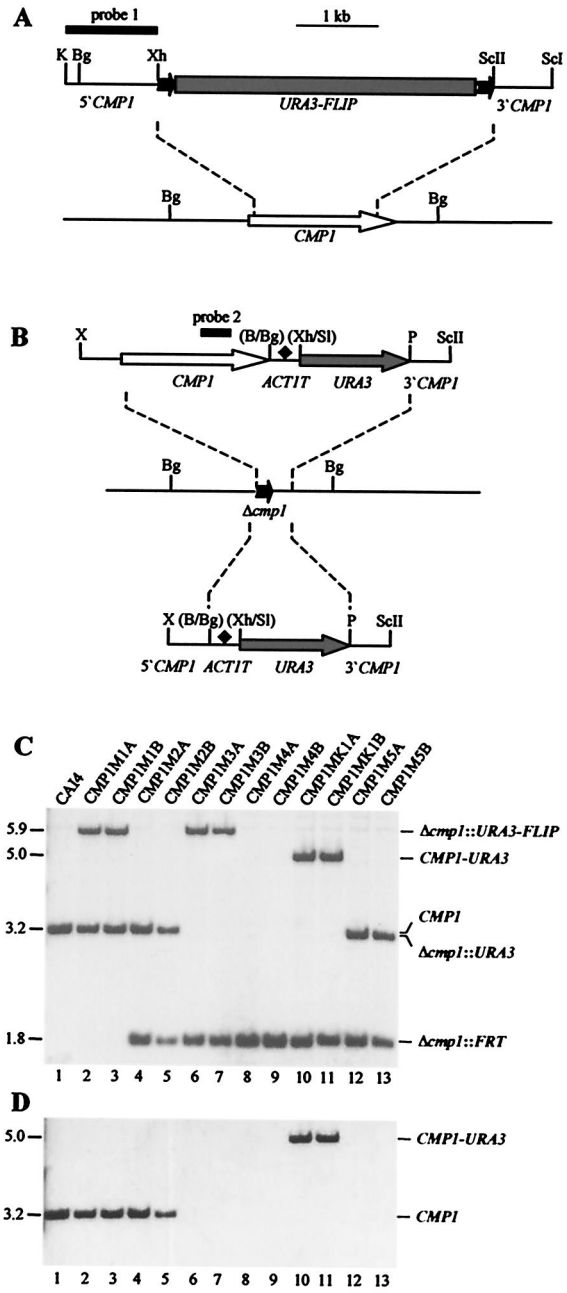

FIG. 2. Construction of *C. albicans cmp1* deletion mutants and complemented strains. (A) Structure of the deletion cassette from plasmid pCMP1M2 (top) and genomic structure of the *CMP1* locus in the parent strain, CAI4 (bottom). The *CMP1* coding region is represented by the open arrow, and the upstream and downstream sequences are represented by solid lines. Details of the *URA3* flipper (shaded rectangle bordered by *FRT* sites [solid arrows]) have been presented elsewhere (39). The 34-bp *FRT* sites are not drawn to scale. The DNA fragment used for Southern hybridization analysis of the mutants is represented by the thick bar (probe 1). (B) Structures of the DNA fragments from pCMP1K1 (top) and pCMP1M4 (bottom), which were used for reintegration of an intact *CMP1* copy (open arrow) or only the *URA3* marker (shaded arrows), respectively, into one of the inactivated *cmp1* alleles (middle). The *ACT1* transcription termination sequence (*ACT1T*) is indicated by the solid diamond. Only relevant restriction sites are given. B, *Bam*HI; Bg, *Bgl*II; K, *Kpn*I; P, *Pst*I; ScI, *Sac*I; ScII, *Sac*II; Sl, *Sal*I; X, *Xba*I; Xh, *Xho*I. The restriction sites shown in parentheses were destroyed by the cloning procedure. The *CMP1* internal fragment used for Southern hybridization analysis of the strains is indicated by the thick bar (probe 2). (C) Southern hybridization of *Bgl*II-digested genomic DNAs of the parent strain, CAI4, and mutant derivatives with the *CMP1* probe 1. The sizes of the hybridizing fragments (in kilobases) are given on the left side of the blot, and their identities are indicated on the right. (D) Rehybridization of the same blot with a *CMP1* internal fragment (probe 2).

growth of the mutants was reduced at pH 8.0. These results provided further evidence that *CMP1* indeed encodes the *C. albicans* orthologue of the calcineurin catalytic subunit.

In *S. cerevisiae*, inactivation of calcineurin results in reduced susceptibility to high Ca^{2+} concentrations. Interestingly, deletion of the *CMP1* gene in *C. albicans* caused the opposite phenotype. Whereas the presence of 300 mM $CaCl_2$ was well tolerated by the wild-type strain, growth of the Δ*cmp1* mutants was inhibited under these conditions, demonstrating that there are also differences between the calcineurin-mediated responses to certain stress conditions in the two species. All these phenotypes were observed in both *URA3*$^+$ and *ura3* Δ*cmp1* mutants, and they were complemented by reintroduction of an intact *CMP1* copy.

***CMP1* is not required for growth at elevated temperatures and hyphal formation in *C. albicans*.** In the pathogenic yeast *C. neoformans*, inactivation of calcineurin results in the inability of the mutants to grow at 37°C. However, in *C. albicans*, deletion of *CMP1* did not cause such a temperature-sensitive phenotype, since the Δ*cmp1* mutants grew as well as the wild-type and complemented strains at 30 or 37°C (Fig. 4). We noted slightly reduced growth of both the mutants and the complemented strains at 43°C in comparison with the wild-type strain SC5314 (Fig. 4, upper row). This phenotype apparently was caused by the genetic background of the CAI4 strain, from which the mutants were constructed, and not by the transformation procedure, since no growth difference was observed in a direct comparison of the *ura3* Δ*cmp1* mutants CMP1M4A and -B and their parental strain, CAI4 (Fig. 4, lower row).

Since *C. neoformans* calcineurin mutants are defective in filamentous growth during mating and haploid fruiting, we tested whether deletion of *CMP1* also affected hyphal formation in *C. albicans*. However, in several solid and liquid media known to induce hyphal growth in *C. albicans*, the Δ*cmp1* mutants formed hyphae as efficiently as the control strains (Fig. 5 and 6). Interestingly, under some conditions, hyphal growth was even increased in the Δ*cmp1* mutants. After 2 days of growth on Lee's agar plates, the mutants formed wrinkled colonies, whereas those of the wild-type and complemented strains were smooth at this time point (Fig. 5). However, after prolonged growth on these plates, the filamentations of mutants and control strains were similar. The enhanced hyphal formation of the Δ*cmp1* mutants was also observed in liquid Lee's medium, in which the mutants aggregated more strongly and settled to the bottom of the culture tubes more rapidly than the control strains (Fig. 6B). These results demonstrated that calcineurin signaling is not required for hyphal formation in *C. albicans* under the conditions tested.

Calcineurin is essential for virulence of *C. albicans*. Calcineurin mutants of *C. neoformans* are avirulent (8, 16, 43). Although calcineurin signaling is involved in many different cellular reactions, the avirulent phenotype of *C. neoformans*

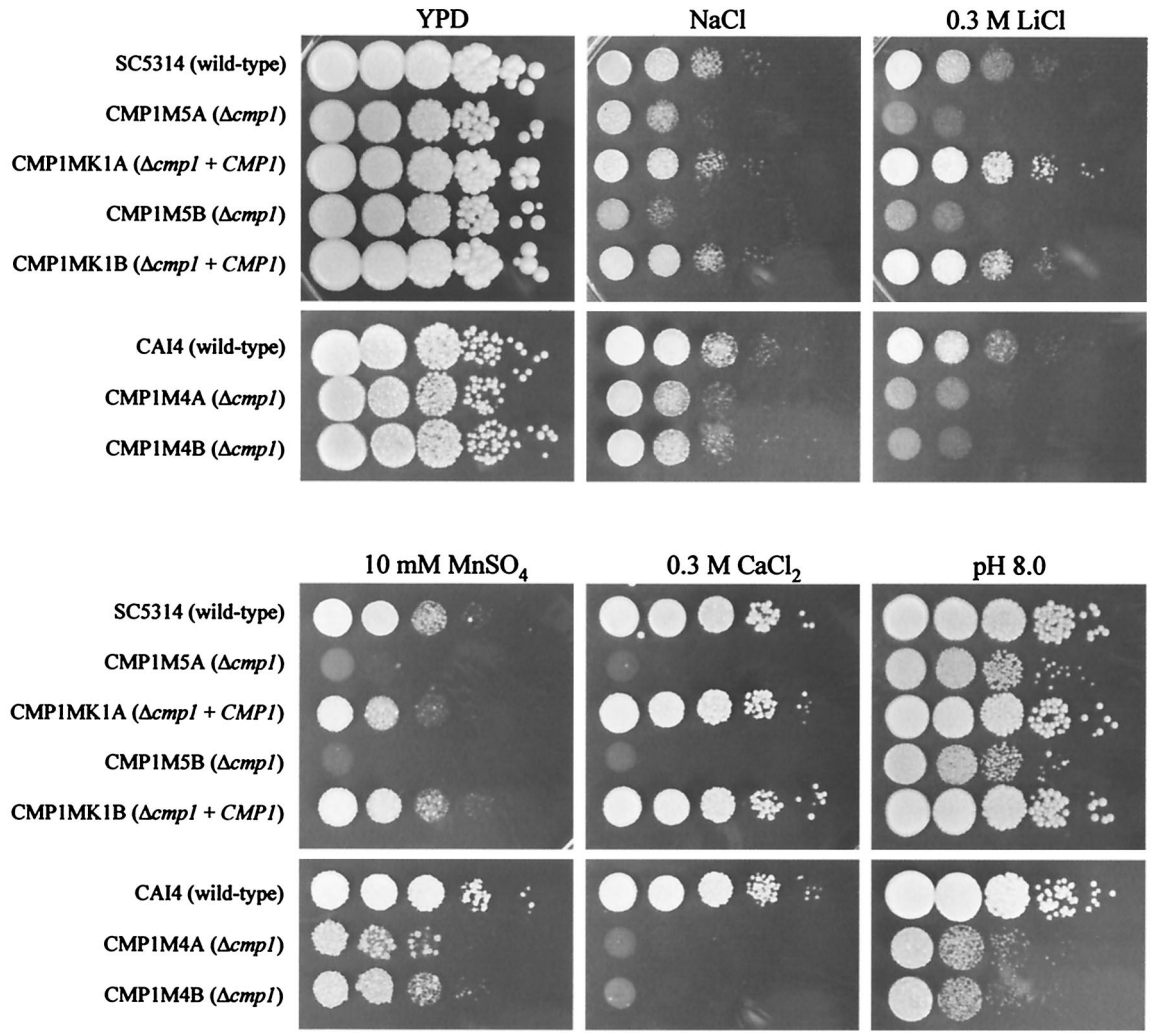

FIG. 3. Deletion of *CMP1* causes hypersensitivity to cationic and alkaline stresses. Serial 10-fold dilutions of the indicated strains were spotted on a YPD plate or YPD plates containing NaCl (1.8 M for *URA3*$^+$ strains and 1.0 M for *ura3* mutant strains), 0.3 M LiCl, 10 mM $MnSO_4$, or 0.3 M $CaCl_2$ and incubated at 30°C for 2 days. Alkaline pH sensitivity was tested on YPD plates buffered at pH 8.0.

calcineurin mutants is not surprising because of their inability to grow at body temperature. Since the *C. albicans* Δ*cmp1* mutants grew well at 37°C, it was important to test whether calcineurin signaling is necessary for the capacity of *C. albicans* to infect a mammalian host.

To address the role of calcineurin in *C. albicans* virulence, mice were intravenously injected either with one of the two independent Δ*cmp1* mutants (CMP1M5A or -B) or with the respective complemented strain (CMP1MK1A or -B). As illustrated in Fig. 7A, all of the mice succumbed to CMP1MK1A or -B infection within 17 days, while the clinical course of Δ*cmp1* mutant infection was much less severe: during follow-up, all animals infected with the Δ*cmp1* mutants survived beyond day 63 postinfection (p.i.) (mutant versus complemented strains, $P \leq 0.026$). The kidneys of CMP1MK1B-infected animals presented with increasing signs of hyperemia (day 6 p.i.), followed by complete tissue destruction (day 8 p.i.) (Fig. 7B), and both hyphae and mycelia were readily detected after calcofluor white staining (data not shown). In contrast, the kidneys of Δ*cmp1* mutant-infected mice showed no sign of disease (days 6 and 14 p.i.) (Fig. 7B), and upon microscopic examination of KOH-solubilized organs, neither *C. albicans* hyphae nor blastoconidia were detectable. Accordingly, enumeration of *C. albicans* CFU in infected kidneys showed that the Δ*cmp1* mutant was cleared very rapidly after infection by the host immune defense. As illustrated in Fig. 7C, we could recover only a few mutant cells from infected kidneys on day 7 and none on day 11, whereas increasing numbers of CFU were obtained from the kidneys of mice infected with the complemented strain CMP1MK1B, which outnumbered those of the mutant by several orders of magnitude. These results demonstrate that calcineurin is essential for virulence in *C. albicans*.

DISCUSSION

Our analysis of *C. albicans* Δ*cmp1* mutants demonstrates similar requirements for calcineurin signaling for adaptation to certain stress conditions, e.g., cationic stress and alkaline pH,

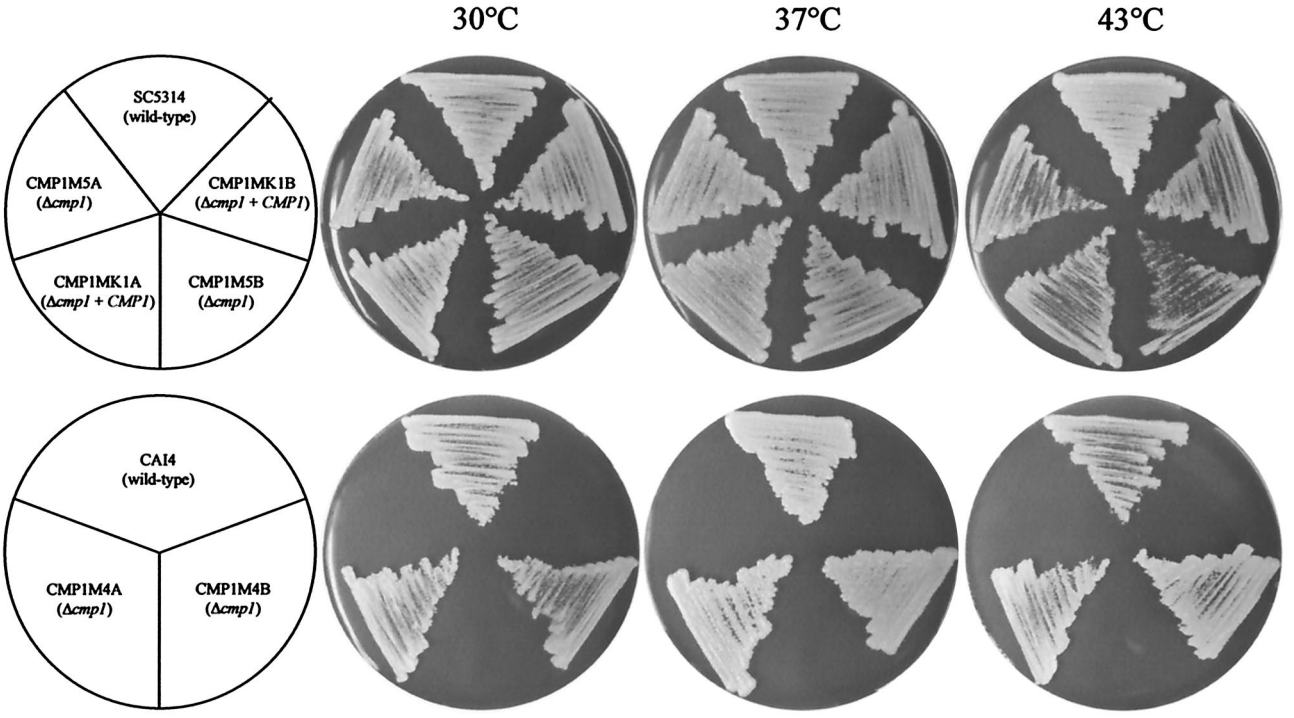

FIG. 4. Deletion of *CMP1* does not cause temperature sensitivity in *C. albicans*. The indicated strains were streaked on YPD plates and grown for 2 days at 30 or 37°C or for 7 days at 43°C. The upper row shows the growth of *URA3* strains, and the lower row shows the growth of *ura3* strains, on uridine-supplemented plates.

in *C. albicans* and *S. cerevisiae*. However, there are also some differences between the calcineurin-mediated cellular responses in these two yeast species. High Ca^{2+} concentrations are not tolerated by wild-type *S. cerevisiae* cells. This phenotype seems to be due to inadequate activation of calcineurin, since calcineurin mutation in *S. cerevisiae* results in resistance to high Ca^{2+} concentrations (9). In contrast, *C. albicans* tolerated high Ca^{2+} concentrations well, but growth under these conditions was inhibited after deletion of *CMP1*. Therefore, calcineurin seems to be required for adaptation of *C. albicans* to high Ca^{2+} concentrations. Opposite effects of calcineurin inactivation have also been described for *S. cerevisiae* and *C. neoformans*. Whereas calcineurin mutants of *S. cerevisiae* show increased sensitivity to Mn^{2+}, inactivation of calcineurin resulted in Mn^{2+} tolerance in *C. neoformans* (14, 43). Therefore, the roles of calcineurin in the adaptation to specific stress conditions are not identical in different yeast species.

Inhibitors of ergosterol biosynthesis stimulate Ca^{2+} influx and activate calcium signaling pathways that are essential for cell survival in *S. cerevisiae* (2). Disturbance of calcium signaling by calcium chelators or by pharmacological or genetic inhibition of calcineurin increases the sensitivity of yeast cells to azoles and terbinafine, antifungal agents that inhibit ergosterol biosynthesis (12). In line with this, fluconazole, a normally fungistatic drug, kills *C. albicans* cells in the presence of the calcineurin inhibitor cyclosporine (32), and the combination of fluconazole and cyclosporine is effective in eliminating *C. albicans* from infected animals (31). During the progress of our study, Cruz et al. reported that *C. albicans* calcineurin mutants, which were obtained by deleting the *CNB1* gene encoding the regulatory subunit of calcineurin, were hypersensitive to membrane stress caused by the presence of sodium dodecyl sulfate or ergosterol biosynthesis inhibitors (7). We confirmed that the growth of our *C. albicans* Δ*cmp1* mutants was also inhibited by the presence of sodium dodecyl sulfate or fluconazole and that the mutants were rapidly killed in the presence of these agents (data not shown).

Of particular interest for us was the role of calcineurin in the pathogenicity of *C. albicans*, since calcineurin mutants of *C. neoformans* have been shown to be avirulent. In fact, as we demonstrate here, calcineurin is essential for virulence in *C. albicans* as well. However, in contrast to *C. neoformans*, calcineurin is not required for growth of *C. albicans* at 37°C. Therefore, the virulence defect of the Δ*cmp1* mutants cannot be explained simply by temperature sensitivity. Another important characteristic of *C. albicans* related to its pathogenic potential is the ability to switch between yeast and hyphal growth forms. Since filamentous growth is abolished after inactivation of calcineurin in *C. neoformans*, we tested if deletion of *CMP1* would also affect hyphal formation in *C. albicans*. Hyphal growth in *C. albicans* is stimulated by different environmental signals, e.g., neutral pH, elevated temperature, nutrient starvation, or the presence of serum (13), and calcium has been implicated as a second messenger in the induction of hyphal formation by environmental signals (20). Interestingly, calcineurin was recently shown to be involved in the transcriptional response to alkaline pH in *S. cerevisiae* (47). Our results demonstrate that hyphal induction by various environmental signals was not affected by inactivation of calcineurin. Deletion of *CMP1* even resulted in an increased stimulation of hyphal

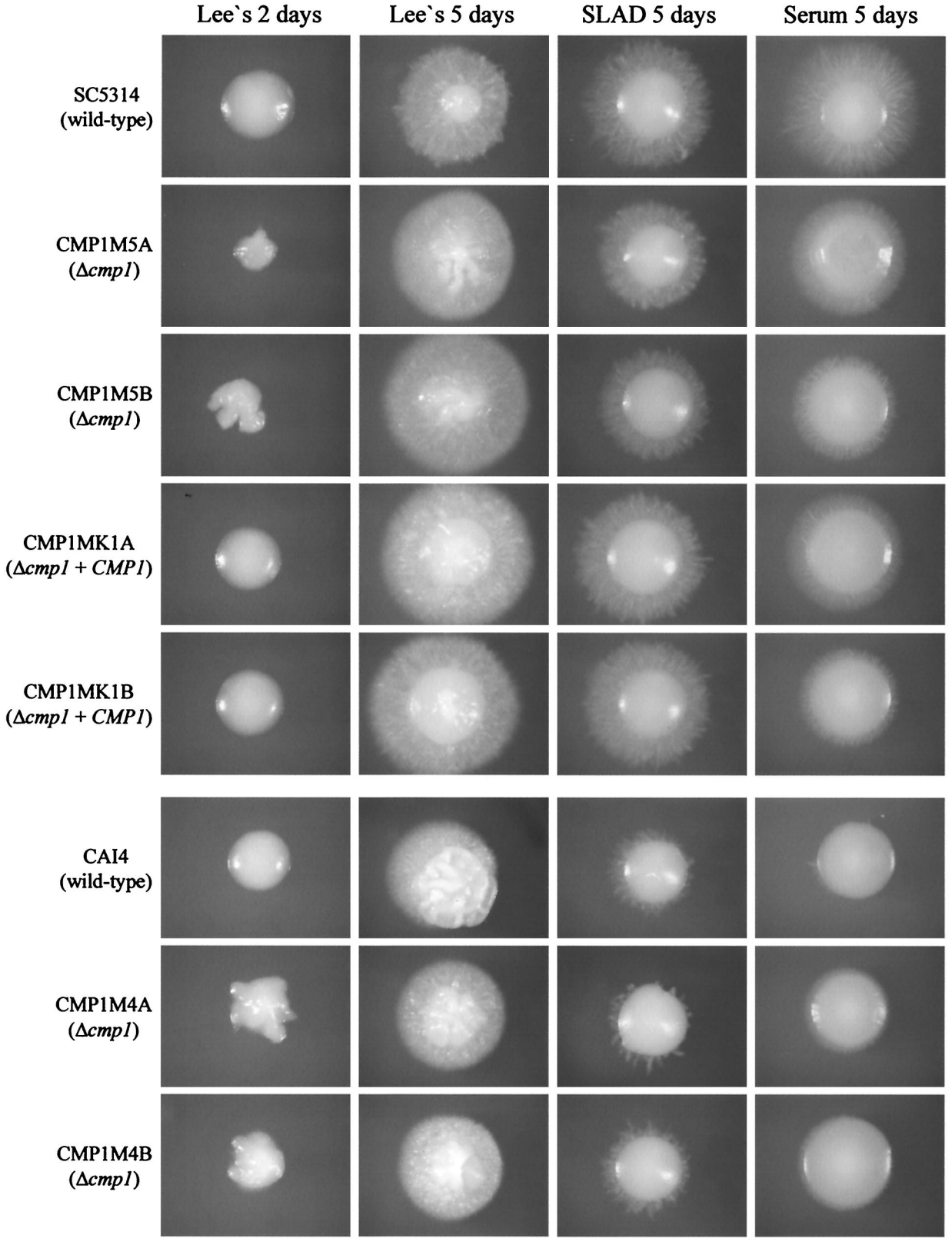

FIG. 5. Filamentous growth of *C. albicans* Δ*cmp1* mutants and control strains on hypha-inducing solid media. The colonies were photographed after 2 or 5 days as indicated.

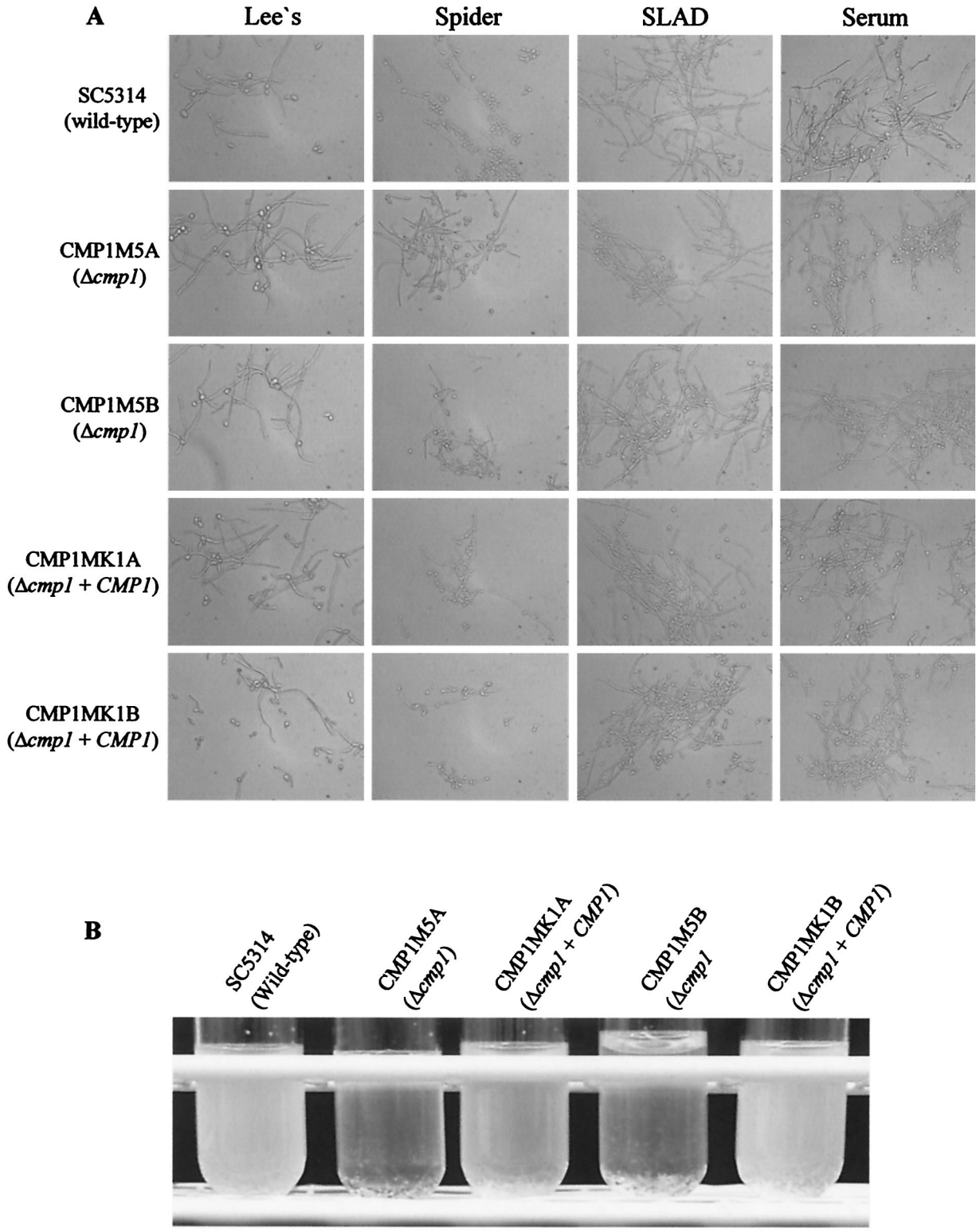

FIG. 6. Hyphal growth of *C. albicans* Δ*cmp1* mutants and control strains in liquid media at 37°C. (A) Microscopic appearance of the indicated strains after 7 h in Lee's medium (pH 7.0), 7 h in Spider medium, 8 h in SLAD medium, or 4 h in RPMI medium with 10% FCS (serum). (B) Aggregation phenotypes of Δ*cmp1* mutants after overnight growth in Lee's medium (pH 7.0). The cultures were briefly shaken and allowed to settle for 1 min before the photograph was taken.

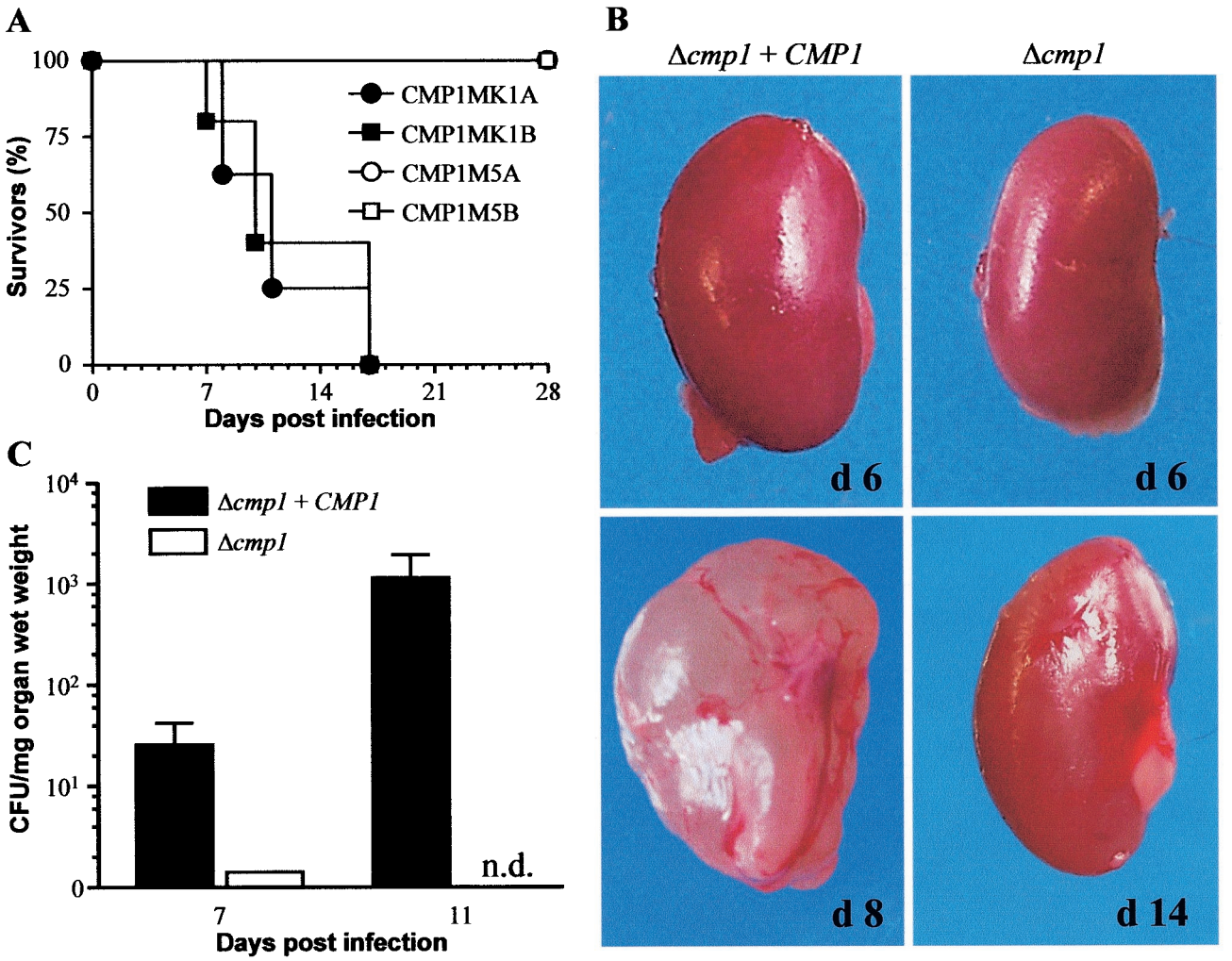

FIG. 7. *C. albicans* Δ*cmp1* mutants are avirulent. (A) Survival curves of mice intravenously infected with 5×10^5 cells of the Δ*cmp1* mutant strains CMP1M5A and -B or the respective complemented strains, CMP1MK1A and -B. (B) Macrographs of kidneys from animals infected with strains CMP1MK1B (Δ*cmp1* + *CMP1*) (left) or CMP1M5B (Δ*cmp1*) (right). The duration of infection is indicated for each frame (d, day p.i.). (C) Fungal organ burden for kidneys. Shown are average numbers of CFU (+ standard deviations) after infection with 5×10^5 cells of either the Δ*cmp1* mutant strain CMP1M5B (open bars) or the complemented strain CMP1MK1B (solid bars) calculated for two or three pairs of kidneys. n.d., not detectable.

formation under certain conditions. However, it is possible that some physical or biochemical properties of hyphae are altered in the Δ*cmp1* mutants compared with the wild type, and this may have impacts on the fungus-host interaction. We note that Sanglard and colleagues recently also reported disruption of the calcineurin catalytic subunit in *C. albicans* (D. Sanglard, F. Ischer, O. Marchetti, and J. Bille, Abstr. 41st Intersci. Conf. Antimicrob. Agents Chemother., abstr. J-1845, 2001) and found reduced virulence of the mutants in a rat model of *Candida* endocarditis, which corroborates our findings of virulence attenuation in a mouse model of systemic candidiasis.

It has recently become evident that the use of the *URA3* marker for the genetic engineering of *C. albicans* can cause problems in interpreting mutant phenotypes. The expression level of an ectopically inserted *URA3* gene depends on the integration locus (24), and since uridine auxotrophy renders *C. albicans* avirulent (4, 21), this may affect virulence and virulence-related traits (1). Sundstrom and colleagues have recently shown that mutant phenotypes unrelated to inactivation of the target gene are obtained when the *URA3* marker is inserted at a different genomic site or in a different way in the homozygous mutants than in the complemented strains (50). To avoid such problems, we paid particular attention to strain construction in our study. In both mutants and complemented strains, the *URA3* marker was inserted at the same genomic site in exactly the same way. To shield the *URA3* gene from the possible influence of upstream sequences, the *ACT1* termination sequence was inserted in front of the *URA3* marker. The only difference between mutants and complemented strains is the presence or the absence of the target gene open reading frame. In addition, in all in vitro phenotypic assays, we compared not only the *URA3*$^+$ Δ*cmp1* mutants with the *URA3*$^+$ control strains, but also the *ura3* Δ*cmp1* mutants with the *ura3* parent, CAI4. A phenotypic difference between mutants and

control strains that is caused by differences in *URA3* expression should not be observed in the latter comparison. All the phenotypic effects of *CMP1* deletion were observed in both comparisons, demonstrating that they were specifically caused by the inactivation of calcineurin. For the virulence tests in mice, we included only those strains that allow optimal comparison, i.e., strains with and without a functional *CMP1* copy, all of which contain one copy of the *URA3* gene integrated in the same way. A comparison of the mutants with the wild-type strain SC5314 is less reliable, since the latter contains two copies of the *URA3* gene and because the *URA3* deletion in strain CAI4 also affects the flanking genes (17). Two independently constructed pairs of mutants and complemented strains, all derived at the same time from the same parent strain, produced identical results, providing convincing evidence that loss of virulence in the mutants was due to deletion of the *CMP1* gene.

The virulence defect of Δ*cmp1* mutants may be explained by an inability of the *C. albicans* cells to adapt to stressful environmental conditions within the host. The fact that calcineurin is essential for virulence not only in *C. neoformans* (8, 16, 43) but also in *C. albicans* suggests that calcineurin represents an attractive target for novel antimycotic agents. Cruz et al. (7) proposed that nonimmunosuppressive analogues of the calcineurin inhibitors FK506 and CsA may be useful in combination with azoles to treat *Candida* infections. Although such agents did not inhibit the growth of *C. albicans* in standard media in vitro, and the growth of calcineurin mutants is not significantly affected under these conditions, the virulence defect of Δ*cmp1* mutants indicates that inhibition of calcineurin by itself might be sufficient to block an infection.

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REFERENCES

1. **Bain, J. M., C. Stubberfield, and N. A. Gow.** 2001. Ura-status-dependent adhesion of *Candida albicans* mutants. FEMS Microbiol Lett. **204:**323–328.
2. **Bonilla, M., K. K. Nastase, and K. W. Cunningham.** 2002. Essential role of calcineurin in response to endoplasmic reticulum stress. EMBO J. **21:**2343–2353.
3. **Breuder, T., C. S. Hemenway, N. R. Movva, M. E. Cardenas, and J. Heitman.** 1994. Calcineurin is essential in cyclosporin A- and FK506-sensitive yeast strains. Proc. Natl. Acad. Sci. USA **91:**5372–5376.
4. **Cole, M. F., W. H. Bowen, X. J. Zhao, and R. L. Cihlar.** 1995. Avirulence of *Candida albicans* auxotrophic mutants in a rat model of oropharyngeal candidiasis. FEMS Microbiol Lett. **126:**177–180.
5. **Cruz, M. C., M. Del Poeta, P. Wang, R. Wenger, G. Zenke, V. F. Quesniaux, N. R. Movva, J. R. Perfect, M. E. Cardenas, and J. Heitman.** 2000. Immunosuppressive and nonimmunosuppressive cyclosporine analogs are toxic to the opportunistic fungal pathogen *Cryptococcus neoformans* via cyclophilin-dependent inhibition of calcineurin. Antimicrob. Agents Chemother. **44:**143–149.
6. **Cruz, M. C., D. S. Fox, and J. Heitman.** 2001. Calcineurin is required for hyphal elongation during mating and haploid fruiting in *Cryptococcus neoformans.* EMBO J. **20:**1020–1032.
7. **Cruz, M. C., A. L. Goldstein, J. R. Blankenship, M. Del Poeta, D. Davis, M. E. Cardenas, J. R. Perfect, J. H. McCusker, and J. Heitman.** 2002. Calcineurin is essential for survival during membrane stress in *Candida albicans.* EMBO J. **21:**546–559.
8. **Cruz, M. C., R. A. Sia, M. Olson, G. M. Cox, and J. Heitman.** 2000. Comparison of the roles of calcineurin in physiology and virulence in serotype D and serotype A strains of *Cryptococcus neoformans.* Infect. Immun. **68:**982–985.
9. **Cunningham, K. W., and G. R. Fink.** 1994. Calcineurin-dependent growth control in *Saccharomyces cerevisiae* mutants lacking *PMC1*, a homolog of plasma membrane Ca2+ ATPases. J. Cell Biol. **124:**351–363.
10. **Cyert, M. S., R. Kunisawa, D. Kaim, and J. Thorner.** 1991. Yeast has homologs (*CNA1* and *CNA2* gene products) of mammalian calcineurin, a calmodulin-regulated phosphoprotein phosphatase. Proc. Natl. Acad. Sci. USA **88:**7376–7380.
11. **Cyert, M. S., and J. Thorner.** 1992. Regulatory subunit (*CNB1* gene product) of yeast Ca2+/calmodulin-dependent phosphoprotein phosphatases is required for adaptation to pheromone. Mol. Cell. Biol. **12:**3460–3469.
12. **Edlind, T., L. Smith, K. Henry, S. Katiyar, and J. Nickels.** 2002. Antifungal activity in *Saccharomyces cerevisiae* is modulated by calcium signalling. Mol. Microbiol. **46:**257–268.
13. **Ernst, J. F.** 2000. Transcription factors in *Candida albicans*—environmental control of morphogenesis. Microbiology **146:**1763–1774.
14. **Farcasanu, I. C., D. Hirata, E. Tsuchiya, F. Nishiyama, and T. Miyakawa.** 1995. Protein phosphatase 2B of *Saccharomyces cerevisiae* is required for tolerance to manganese, in blocking the entry of ions into the cells. Eur. J. Biochem. **232:**712–717.
15. **Fonzi, W. A., and M. Y. Irwin.** 1993. Isogenic strain construction and gene mapping in *Candida albicans.* Genetics **134:**717–728.
16. **Fox, D. S., M. C. Cruz, R. A. Sia, H. Ke, G. M. Cox, M. E. Cardenas, and J. Heitman.** 2001. Calcineurin regulatory subunit is essential for virulence and mediates interactions with FKBP12-FK506 in *Cryptococcus neoformans.* Mol. Microbiol. **39:**835–849.
17. **Garcia, M. G., J. E. O'Connor, L. L. Garcia, S. I. Martinez, E. Herrero, and L. del Castillo Agudo.** 2001. Isolation of a *Candida albicans* gene, tightly linked to *URA3*, coding for a putative transcription factor that suppresses a *Saccharomyces cerevisiae aft1* mutation. Yeast **18:**301–311.
18. **Gillum, A. M., E. Y. Tsay, and D. R. Kirsch.** 1984. Isolation of the *Candida albicans* gene for orotidine-5′-phosphate decarboxylase by complementation of *S. cerevisiae ura3* and *E. coli pyrF* mutations. Mol. Gen. Genet. **198:**179–182.
19. **Gimeno, C. J., P. O. Ljungdahl, C. A. Styles, and G. R. Fink.** 1992. Unipolar cell divisions in the yeast *S. cerevisiae* lead to filamentous growth: regulation by starvation and RAS. Cell **68:**1077–1090.
20. **Gow, N. A.** 1994. Growth and guidance of the fungal hypha. Microbiology **140:**3193–3205.
21. **Kirsch, D. R., and R. R. Whitney.** 1991. Pathogenicity of *Candida albicans* auxotrophic mutants in experimental infections. Infect. Immun. **59:**3297–3300.
22. **Köhler, G. A., T. C. White, and N. Agabian.** 1997. Overexpression of a cloned IMP dehydrogenase gene of *Candida albicans* confers resistance to the specific inhibitor mycophenolic acid. J. Bacteriol. **179:**2331–2338.
23. **Kuno, T., H. Tanaka, H. Mukai, C. D. Chang, K. Hiraga, T. Miyakawa, and C. Tanaka.** 1991. cDNA cloning of a calcineurin B homolog in *Saccharomyces cerevisiae.* Biochem. Biophys. Res. Commun. **180:**1159–1163.
24. **Lay, J., L. K. Henry, J. Clifford, Y. Koltin, C. E. Bulawa, and J. M. Becker.** 1998. Altered expression of selectable marker *URA3* in gene-disrupted *Candida albicans* strains complicates interpretation of virulence studies. Infect. Immun. **66:**5301–5306.
25. **Leberer, E., D. Harcus, D. Dignard, L. Johnson, S. Ushinsky, D. Y. Thomas, and K. Schröppel.** 2001. Ras links cellular morphogenesis to virulence by regulation of the MAP kinase and cAMP signalling pathways in the pathogenic fungus *Candida albicans.* Mol. Microbiol. **42:**673–687.
26. **Lee, K. L., H. R. Buckley, and C. C. Campbell.** 1975. An amino acid liquid synthetic medium for the development of mycelial and yeast forms of *Candida albicans.* Sabouraudia **13:**148–153.
27. **Liu, H., J. Köhler, and G. R. Fink.** 1994. Suppression of hyphal formation in *Candida albicans* by mutation of a *STE12* homolog. Science **266:**1723–1726.
28. **Liu, J., J. D. Farmer, Jr., W. S. Lane, J. Friedman, I. Weissman, and S. L. Schreiber.** 1991. Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. Cell **66:**807–815.
29. **Liu, Y., S. Ishii, M. Tokai, H. Tsutsumi, O. Ohki, R. Akada, K. Tanaka, E. Tsuchiya, S. Fukui, and T. Miyakawa.** 1991. The *Saccharomyces cerevisiae* genes (*CMP1* and *CMP2*) encoding calmodulin-binding proteins homologous to the catalytic subunit of mammalian protein phosphatase 2B. Mol. Gen. Genet. **227:**52–59.
30. **Lo, H. J., J. R. Köhler, B. DiDomenico, D. Loebenberg, A. Cacciapuoti, and G. R. Fink.** 1997. Nonfilamentous *C. albicans* mutants are avirulent. Cell **90:**939–949.
31. **Marchetti, O., J. M. Entenza, D. Sanglard, J. Bille, M. P. Glauser, and P. Moreillon.** 2000. Fluconazole plus cyclosporine: a fungicidal combination effective against experimental endocarditis due to *Candida albicans.* Antimicrob. Agents Chemother. **44:**2932–2938.

32. **Marchetti, O., P. Moreillon, M. P. Glauser, J. Bille, and D. Sanglard.** 2000. Potent synergism of the combination of fluconazole and cyclosporine in *Candida albicans.* Antimicrob. Agents Chemother. **44:**2373–2381.
33. **Matheos, D. P., T. J. Kingsbury, U. S. Ahsan, and K. W. Cunningham.** 1997. Tcn1p/Crz1p, a calcineurin-dependent transcription factor that differentially regulates gene expression in *Saccharomyces cerevisiae.* Genes Dev. **11:**3445–3458.
34. **Mazur, P., N. Morin, W. Baginsky, M. el-Sherbeini, J. A. Clemas, J. B. Nielsen, and F. Foor.** 1995. Differential expression and function of two homologous subunits of yeast 1,3-β-D-glucan synthase. Mol. Cell. Biol. **15:** 5671–5681.
35. **Mendizabal, I., G. Rios, J. M. Mulet, R. Serrano, and I. F. de Larrinoa.** 1998. Yeast putative transcription factors involved in salt tolerance. FEBS Lett. **425:**323–328.
36. **Mendoza, I., F. Rubio, A. Rodriguez-Navarro, and J. M. Pardo.** 1994. The protein phosphatase calcineurin is essential for NaCl tolerance of *Saccharomyces cerevisiae.* J. Biol. Chem. **269:**8792–8796.
37. **Millon, L., A. Manteaux, G. Reboux, C. Drobacheff, M. Monod, T. Barale, and Y. Michel-Briand.** 1994. Fluconazole-resistant recurrent oral candidiasis in human immunodeficiency virus-positive patients: persistence of *Candida albicans* strains with the same genotype. J. Clin. Microbiol. **32:**1115–1118.
38. **Morschhäuser, J., S. Michel, and J. Hacker.** 1998. Expression of a chromosomally integrated, single-copy *GFP* gene in *Candida albicans*, and its use as a reporter of gene regulation. Mol. Gen. Genet. **257:**412–420.
39. **Morschhäuser, J., S. Michel, and P. Staib.** 1999. Sequential gene disruption in *Candida albicans* by FLP-mediated site-specific recombination. Mol. Microbiol. **32:**547–556.
40. **Moser, M. J., J. R. Geiser, and T. N. Davis.** 1996. $Ca2^{+}$-calmodulin promotes survival of pheromone-induced growth arrest by activation of calcineurin and $Ca2^{+}$-calmodulin-dependent protein kinase. Mol. Cell. Biol. **16:**4824–4831.
41. **Nakamura, T., Y. Liu, D. Hirata, H. Namba, S. Harada, T. Hirokawa, and T. Miyakawa.** 1993. Protein phosphatase type 2B (calcineurin)-mediated, FK506-sensitive regulation of intracellular ions in yeast is an important determinant for adaptation to high salt stress conditions. EMBO J. **12:**4063–4071.
42. **Navarro-Garcia, F., M. Sanchez, C. Nombela, and J. Pla.** 2001. Virulence genes in the pathogenic yeast *Candida albicans.* FEMS Microbiol. Rev. **25:** 245–268.
43. **Odom, A., S. Muir, E. Lim, D. L. Toffaletti, J. Perfect, and J. Heitman.** 1997. Calcineurin is required for virulence of *Cryptococcus neoformans.* EMBO J. **16:**2576–2589.
44. **Prokisch, H., O. Yarden, M. Dieminger, M. Tropschug, and I. B. Barthelmess.** 1997. Impairment of calcineurin function in *Neurospora crassa* reveals its essential role in hyphal growth, morphology and maintenance of the apical Ca2+ gradient. Mol. Gen. Genet. **256:**104–114.
45. **Schreiber, S. L., and G. R. Crabtree.** 1992. The mechanism of action of cyclosporin A and FK506. Immunol. Today **13:**136–142.
46. **Schweizer, A., S. Rupp, B. N. Taylor, M. Röllinghoff, and K. Schröppel.** 2000. The TEA/ATTS transcription factor CaTec1p regulates hyphal development and virulence in *Candida albicans.* Mol. Microbiol. **38:**435–445.
47. **Serrano, R., A. Ruiz, D. Bernal, J. R. Chambers, and J. Ariño.** 2002. The transcriptional response to alkaline pH in *Saccharomyces cerevisiae*: evidence for calcium-mediated signalling. Mol. Microbiol. **46:**1319–1333.
48. **Stathopoulos, A. M., and M. S. Cyert.** 1997. Calcineurin acts through the *CRZ1*/*TCN1*-encoded transcription factor to regulate gene expression in yeast. Genes Dev. **11:**3432–3444.
49. **Stathopoulos-Gerontides, A., J. J. Guo, and M. S. Cyert.** 1999. Yeast calcineurin regulates nuclear localization of the Crz1p transcription factor through dephosphorylation. Genes Dev. **13:**798–803.
50. **Sundstrom, P., J. E. Cutler, and J. F. Staab.** 2002. Reevaluation of the role of *HWP1* in systemic candidiasis by use of *Candida albicans* strains with selectable marker *URA3* targeted to the *ENO1* locus. Infect. Immun. **70:** 3281–3283.

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