The yeast *Candida albicans* is an opportunistic pathogen that threatens patients with compromised immune systems. Immune cell defenses against *C. albicans* are complex but typically involve the production of reactive oxygen species and nitrogen radicals such as nitric oxide (NO) that damage the yeast or inhibit its growth. Whether *Candida* defends itself against NO and the molecules responsible for this defense have yet to be determined. The defense against NO in various bacteria and the yeast *Saccharomyces cerevisiae* involves an NO-scavenging flavohemoglobin. The *C. albicans* genome contains three genes encoding flavohemoglobin-related proteins, *CaYHB1*, *CaYHB4*, and *CaYHB5*. To assess their roles in NO metabolism, we constructed strains lacking each of these genes and demonstrated that just one, *CaYHB1*, is responsible for NO consumption and detoxification. In *C. albicans*, NO metabolic activity and *CaYHB1* mRNA levels are rapidly induced by NO and NO-generating agents. Loss of *CaYHB1* increases the sensitivity of *C. albicans* to NO-mediated growth inhibition. In mice, infections with *Candida* strains lacking *CaYHB1* still resulted in lethality, but virulence was decreased compared to that in wild-type strains. Thus, *C. albicans* possesses a rapid, specific, and highly inducible NO defense mechanism involving one of three putative flavohemoglobin genes.

The dimorphic fungus *Candida albicans* causes infections in immunocompromised hosts and is particularly problematic for AIDS and cancer patients. In healthy individuals, phagocytic immune cells such as macrophages (17), monocytes (37, 45), and neutrophils (45) defend against *Candida* infections by producing several growth inhibitors and cytotoxic compounds, including microbicidal enzymes (41) and reactive oxygen and nitrogen species (50). One potentially powerful weapon against *C. albicans* is nitric oxide (NO). Macrophages produce high concentrations of this free radical via the action of an inducible NO synthase (36), inhibition of which strongly decreases the concentrations of this free radical via the action of an inducible NO synthase (36), inhibition of which strongly decreases the concentration of potentially toxic species such as nitric oxide (NO) that damage the yeast or inhibit its growth. Whether *Candida* defends itself against NO and the molecules responsible for this defense have yet to be determined. The defense against NO in various bacteria and the yeast *Saccharomyces cerevisiae* involves an NO-scavenging flavohemoglobin. The *C. albicans* genome contains three genes encoding flavohemoglobin-related proteins, *CaYHB1*, *CaYHB4*, and *CaYHB5*. To assess their roles in NO metabolism, we constructed strains lacking each of these genes and demonstrated that just one, *CaYHB1*, is responsible for NO consumption and detoxification. In *C. albicans*, NO metabolic activity and *CaYHB1* mRNA levels are rapidly induced by NO and NO-generating agents. Loss of *CaYHB1* increases the sensitivity of *C. albicans* to NO-mediated growth inhibition. In mice, infections with *Candida* strains lacking *CaYHB1* still resulted in lethality, but virulence was decreased compared to that in wild-type strains. Thus, *C. albicans* possesses a rapid, specific, and highly inducible NO defense mechanism involving one of three putative flavohemoglobin genes.
the first copy of the gene and the HIS1 cassette from pGEM-HIS1 to replace the second copy (53). Primers YHB1-1 and YHB1-2 (see Table S1 in the supplemental material) were used to amplify each marker gene cassette by PCR and thereby construct a CaYHB1-specific deletion cassette. YHB1-1 contains a 60-nucleotide (nt) sequence homologous to DNA flanking the 5′ end of the CaYHB1 ORF plus 19 nt complementary to one side of the URAS3-dpl200 and HIS1 cassettes. YHB1-2 contains a 61-base sequence homologous to DNA on the 3′ end of the ORF plus 20 nt complementary to the opposite end of the ORF. The resultant marker gene cassette from pGEM-HIS1 to replace the HIS1 cassette. Final complementation of the Yhb1ΔHis+ Ura+ transformants were selected on YNB/Ura dropout medium (yeast nitrogen base plus amino acids [Difco] plus 2% glucose and supplemented with amino acids, but lacking uridine). Deletion transformations were then performed in repeated rounds in subsequent rounds with HIS1-containing cassettes, followed by selection on YNB/Ura His dropout medium (as described above, but also lacking histidine) until a null mutant was identified. Homozygous deletion of CaYHB1 in a specific His+ Ura+ transformant was confirmed by positive PCRs with primer pairs YHB1-3/URAS-1, YHB1-4/URAS-2, YHB1-3/ HIS1-1, and YHB1-4/HIS1-2 and a negative PCR with primer pair YHB1-3/HIS1-1 and YHB1-4/HIS1-2. All transformations were achieved through the lithium acetate method described above.

**Table 1. Yeast strains used in this study**

| Strain type | Genotype | Source |
|-------------|----------|--------|
| **S. cerevisiae** | **BY4742** | MATa his3Δ leu2Δ lys2Δ ura3Δ | Res. Genetics |
| | **15887** | MATa his3Δ leu2Δ lys2Δ ura3Δ ybh1Δ/kanMX | Res. Genetics |
| **C. albicans** | **CAF2** | ura3Δ::imm434/URA3 | Fonzi and Irwin (19) |
| | **RM1000** | ura3Δ::imm434/ura3Δ::imm444 his1Δ/::hisG his1Δ/::hisG | Alonso-Monge et al. (1) |
| | **ybh1ΔYHB1** | ura3Δ::imm434/ura3Δ::imm444 his1Δ/::hisG his1Δ/::hisG ybh1Δ::dpl200/YHB1** | This study |
| | **ybh1ΔYHB1 ura3Δ/URAS** | ura3Δ::imm434/URA3 his1Δ/::hisG his1Δ/::hisG ybh1Δ::dpl200/YHB1 | This study |
| | **ybh1Δyhb1Δ His+ Ura+** | ura3Δ::imm434/ura3Δ::imm444 his1Δ/::hisG his1Δ/::hisG ybh1Δ::dpl200/YHB1 | This study |
| | **yhb1Δyhb1Δ His+ Ura+** | ura3Δ::imm434/ura3Δ::imm444 his1Δ/::hisG his1Δ/::hisG ybh1Δ::dpl200/YHB1 | This study |
| | **yhb4Δyhb4Δ** | ura3Δ::imm434/ura3Δ::imm444 his1Δ/::hisG his1Δ/::hisG ybh1Δ::dpl200/YHB1 | This study |
| | **yhb5Δyhb5Δ** | ura3Δ::imm434/ura3Δ::imm444 his1Δ/::hisG his1Δ/::hisG ybh1Δ::dpl200/YHB1 | This study |

*CAF2 is from reference (19) and is the progenitor of RM1000 (1). The superscript c denotes a heterozygote at that locus in which one wild-type copy of the relevant gene was inserted by transformation back into its native chromosomal location in a homozygous deletion strain. The wild-type gene copy used for transformation was obtained by PCR from genomic DNA of SC5314, the CAF2 progenitor (19), and included DNA flanking the original deletion on each side. Correct insertions into genomic DNA of the transformants were confirmed by PCR analysis. 

*Res. Genetics, Research Genetics/Invitrogen.

**Northern blot analysis.** Total RNA was isolated from pelleted log-phase culture cells by bead beating with glass beads in the presence of phenol and RNA lysis buffer (0.3 M NaCl, 1 mM EDTA, 10 mM Tris [pH 7.5], 0.2% sodium dodecyl sulfate). Standard electrophoretic techniques, denaturing formaldehyde gels, and blotting procedures were employed (44). DNA probe templates were prepared by PCR from RM1000 genomic DNA by using gene-specific primers (see Table S1 in the supplemental material). These were then radioactively labeled with [α-32P]dATP (ICT Radiochemicals), using the DECAprime II kit (Ambion).

**lacZ reporter gene.** Part of CaYHB1 containing the 1.2-kb ORF plus either 998 bp (presumptive full promoter) or 279 bp (minimal promoter) of DNA upstream of the start codon was amplified by PCR from genomic DNA and inserted...
upstream of Streptococcus thermophilus lacZ in the pA395 plasmid (48), replacing the 0.5-kb-long HWP1 promoter DNA between KpnI and PstI. The resulting CaYHB1-lacZ plasmids were then cut at a unique pShAl site in the HWP1 3' untranslated region downstream of lacZ, and the linearized DNAs were each transformed into the Ura- strain RM1000. For each reporter gene plasmid, β-galactosidase assays were then performed on cell extracts (2) of a Ura- transformed strain that by PCR analysis had correctly integrated the plasmid downstream of the HWP1 locus.

**RESULTS**

**Flavohemoglobin orthologues in C. albicans.** The BLAST program (National Center for Biotechnology Information) was used to search the completed DNA sequence of the *C. albicans* genome (version 6; http://www-sequence.stanford.edu/group/candida) for sequences related to the *Saccharomyces* flavohemoglobin gene ScYHB1. This search yielded three structural homologues, two of which are named YHB1 and YHB5 based on contig annotation (YHB1, contig6-2060, nt 7817 to 9013; and YHB5, contig6-2400, nt 9264 to 10467). We refer to them in this work as *CaYHB1* and *CaYHB5*. The third unannotated putative ORF is more closely related to *CaYHB5* than *CaYHB1* and therefore is referred to as *CaYHB4* (contig6-2518, nt 174143 to 175346). The percentages of sequence identity between the predicted *Candida* flavohemoglobins and the *S. cerevisiae* flavohemoglobin are as follows: *CaYhb1*, 31%; *CaYhb4*, 25%; and *CaYhb5*, 29% (Fig. 1). The X-ray structures of two bacterial flavohemoglobins show three domains: a globin domain, an FAD-binding domain, and an NAD(P)-binding domain (16, 31). The homology between the three *Candida* *CaYhb* proteins and the *E. coli* flavohemoglobin extends over the whole protein length: i.e., all three domains, rather than being clustered in just one domain.

The three flavohemoglobins show sequence differences at key positions thought to be important for NOD activity and function. Catalytic NO dioxygenation is achieved by O2 binding to the ferrous heme and a rapid reaction of NO with the bound O2 to form NO2· (22, 26, 47). To reduce the ferric iron in heme during repetitive cycles of NO dioxygenation, electrons are univalently transferred from FADH2 to heme, which in turn receives electrons from NAD(P)H. Interestingly, the Tyr B10, which is essential for high O2 affinity and efficient NOD function of the *E. coli* flavohemoglobin (22), is conserved in the distal heme pocket in *CaYhb1* and *CaYhb5*, but not in *CaYhb4* (Fig. 1A). *CaYhb4* and *CaYhb5* also lack the unique Leu E11 residue that contacts ferric iron in *E. coli* flavohemoglobin structure (31) and is conserved for NOD activity function (16). Amino acids whose side chains contact FAD (Arg212, Tyr214, Ser215, and Ser238) and amino acids within the proximal region of the heme pocket (His93, Tyr103, and Glu146) are conserved in all isoforms.

The phylogenetic comparison in Fig. 1B shows the closer relatedness of each of the *Candida* flavohemoglobins to each other than to three flavohemoglobins shown to have an NOD function. The analysis also shows that the flavohemoglobin of *Candida norvegensis* is more closely related to those from *C. albicans* than to the others (Fig. 1B).

**Constitutive and inducible NO metabolism in C. albicans versus *S. cerevisiae*.** To investigate the role of the three flavohemoglobin genes in the response of *C. albicans* to NO, we generated mutant strains for each *YHB* gene in which both gene copies were deleted. These mutants were generated by replacing each gene copy sequentially in the *C. albicans* strain RM1000 (his1/his1 ura3/ura3) with a selectable marker (HIS1 or URA3) following the methods of Wilson et al. (52, 53) as detailed in Materials and Methods.

To determine whether the NO consumption rate of *C. albicans* is induced by exposure to NO, RM1000 cells were treated for 1 h with 960 ppm of gaseous NO (≈2 μM in solution) and compared to untreated cells for NO consumption rate and extract NOD activity. After the 1-h exposure of cells to NO, whole-cell NO consumption and cell extract NOD activities are increased by about 13- and 30-fold, respectively (Fig. 2). The inducible and constitutive NO metabolism by *C. albicans* is eliminated by the deletion of *CaYHB1*, but not by deletion of either of the *CaYHB4* or *CaYHB5* homologues. Thus, the *CaYHB1* gene confers the majority of the NO metabolic activity observed under these conditions.

Similar to previously reported results (35), intact *Saccharomyces* cells exhibit NO consumption (Fig. 2A). Furthermore, *S. cerevisiae* cell extracts contain high levels of NOD activity (Fig. 2B). Consistent with a previous report (35), NO consumption and NOD activity of *Saccharomyces* are eliminated in a strain containing a deletion of *ScYHB1*. However, there are clear differences in the NO stress responses of these two yeasts. Basal levels of NO consumption by *C. albicans* cells are about sevenfold lower than those of *S. cerevisiae* cells (Fig. 2A). NO exposure induces only a modest 1.5-fold increase in *S. cerevisiae* cell extract NOD activity and no detectable increase in NO consumption by intact cells (Fig. 2). These small differences between *S. cerevisiae* extract and cell measurements of NO metabolic activity changes may reflect intracellular substrate or cofactor limits on flavohemoglobin catalysis.

**NO induction of *YHB* mRNA in *C. albicans* versus *S. cerevisiae*.** Using a gene-specific probe and RNA from log-phase cultures, Northern blot analysis shows that *CaYHB1* mRNA is strongly induced by exposure of *Candida* to gaseous NO (≈2 μM in solution) (Fig. 3A). The chemical specificity of *CaYHB1* induction was probed by addition of hydrogen peroxide or the superoxide-generating drug plumbagin (32), and only gaseous
NO exposure strongly induces CaYHB1 expression (data not shown). We also tested the effects of an additional NO source on CaYHB1 expression. A large induction of CaYHB1 mRNA is observed with the NO-releasing chemical NOC-18 (Fig. 3B), but not with its other breakdown product, diethylenetriamine (33) (data not shown). Northern blot analysis shows a detectable mRNA for CaYHB5 but not for CaYHB4 (data not shown). CaYHB1 mRNA levels were also analyzed in C. albicans or S. cerevisiae cells treated with sodium nitrite, a nitrosating agent and a physiologically relevant NO donor (14). ScYHB1 exhibits little or no change in mRNA levels after exposure of S. cerevisiae to sodium nitrite. In contrast, there is a strong induction of CaYHB1 mRNA after such treatment (Fig. 3C).

Induction by nitrite is also observed earlier (5 min) in Candida than in Saccharomyces (15 min) (Fig. 3D). There is no detectable increase in either CaYHB4 or CaYHB5 mRNA after treatment of cells with sodium nitrite. Thus, induction of a single flavohemoglobin gene is observed in both yeasts, although the magnitude and rate of the nitrite-induced changes in YHB1 mRNA levels are much greater in Candida than in Saccharomyces. NO-induced increases in mRNA levels could be mediated by either changes in transcription or mRNA degradation. To test for changes in transcription, a strain was constructed that contains a lacZ reporter gene fused downstream of the CaYHB1 promoter (998 bp) and ORF. In this strain, nitrite induced a large increase in β-galactosidase (Fig. 3E). To test
whether the CaYHB1 promoter is required for this induction, a strain was constructed that contains the same reporter gene construct except that the CaYHB1 promoter segment was truncated to just 279 bp upstream of the ORF. In this strain, expression of β-galactosidase in the presence and absence of 10 mM nitrite was 2.3 ± 0.2 and 1.5 ± 0.4 Miller units (mean ± standard deviation), respectively. Together these results show that nitrite induction of flavohemoglobin expression is mediated by an increase in transcription rather than by changes in stability of CaYHB1 mRNA or CaYhb1 protein.

Protection of C. albicans against NO toxicity. To test whether CaYHB1, CaYHB4, or CaYHB5 protects C. albicans from NO toxicity, we compared the CaYHB deletion strains and the parental strain (RM1000) for sensitivity to NO-releasing compounds and nitrite. Growth of the cayhb1Δ/cayhb1Δ, cayhb4Δ/cayhb4Δ, or cayhb5Δ/cayhb5Δ strain is similar to that of the parent strain RM1000 in rich YEPD medium under aerated conditions. Growth of the CaYHB1 deletion strain is potently inhibited by the NO-generating compounds NOC-18 and S-nitrosoglutathione (Fig. 4A and B). In comparison, neither NO-generating compound significantly inhibits the growth of RM1000 or the CaYHB4 or CaYHB5 deletion strain. The results are similar to those previously reported for wild-type C. albicans strains treated with these agents (51). In contrast, sodium nitrite inhibits the growth of all strains tested, but the cayhb1Δ/cayhb1Δ strain consistently shows greater sensitivity to nitrite (Fig. 4C). Furthermore, nitrite resistance was restored by introducing an intact CaYHB1 gene into the cayhb1Δ/cayhb1Δ strain, either by transformation with a plasmid (pABSKII CaYHB1) expressing CaYHB1 or by insertion of the intact CaYHB1 gene into the original locus (Fig. 5). These findings demonstrate that CaYHB1, but not CaYHB4 or CaYHB5, protects C. albicans against NO toxicity.

Analysis of virulence of CaYHB1 deletion mutant C. albicans strain, using a murine model. The effect of CaYHB1 in systemic candidiasis was evaluated by comparing the survival curves of mice given isogenic C. albicans strains, including the homozygous cayhb1/cayhb1, reconstituted CaYHB1/cayhb1, and wild-type (CAF-2) strains in a mouse model of hematogenously disseminated candidiasis. Experiments revealed that, although infections with the cayhb1 mutant still resulted in lethality in this model (all animals died within less than 10 days), its virulence was somewhat attenuated and/or delayed compared to that exhibited by CAF-2 (Fig. 6, median survival times of 5 versus 3 days; \( P = 0.0294 \)). Reintroduction of one copy of the gene at its original locus partially restored virulence and resulted in survival curves that were intermediate between those generated with the homozygous mutant and those generated with CAF-2 (\( P = 0.1651 \) versus CAF-2).

DISCUSSION

In C. albicans, removal of NO is dependent upon the presence of a single flavohemoglobin gene, CaYHB1. CaYHB1 is induced by micromolar NO, confers NOD activity, and protects cells against NO toxicity. Our results also indicate that the three Candida flavohemoglobins are not functionally equivalent. Thus, CaYHB5 mRNA is expressed, but in contrast to CaYHB1, is not induced by exposure to NO or NO-generating agents. As for CaYHB4, we were unable to detect its mRNA under either normal or inducing conditions. Furthermore, neither the cayhb4Δ/cayhb4Δ strain nor the cayhb5Δ/cayhb5Δ strain displays NO sensitivity or growth impairment in aerated YEPD medium. Our preliminary data show that Candida strains lacking CaYHB5 show a very small reduction in virulence (data not shown), although the significance of this finding...
awaits further analysis of the enzymatic function of this flavohemoglobin. These data demonstrate that CaYHB1 is the only flavohemoglobin with a detectable role in NO metabolism and detoxification. Nevertheless, it remains possible that NO metabolic functions of CaYHB4 or CaYHB5 are expressed under specialized growth conditions or during specific stages of differentiation.

Interestingly, the results suggest a low predictive value of phylogenetic analyses for an NOD function. CaYHB1 is more closely related to CaYHB4 and CaYHB5 than ScYHB1 or bacterial flavohemoglobins (Fig. 1B). Yet, CaYHB1, but not CaYHb4 or CaYHb5, functions in NO detoxification. The presence of the active site residues Tyr B10 and Leu E11 appears to be a strong indicator of a NOD function. On the other hand, structurally diverse and distantly related hemoglobins and myoglobins also show NOD activity and function but show different active site structures (13, 54), thus cautioning against an exclusion of an NO detoxification function based solely upon structure analysis. Clearly, evidence for significant NO metabolism or induction of activity in response to NO stress is required to establish an NOD function.

Unlike in other organisms, the inducibility of CaYHB1 observed in C. albicans appears to be a specific response to NO stress. The hmp gene in E. coli bacteria is induced not only by NO stress, but also by paraquat, which produces both superoxide and hydrogen peroxide stress (39). Although C. albicans CaYHB1 transcript levels are increased by NO generators, as well as authentic NO, CaYHB1 mRNA levels do not change upon treatment of Candida with sublethal levels of hydrogen peroxide or the superoxide-generating compound plumbagin (data not shown). These differences between bacterial and Candida flavohemoglobin expression suggest unique regulatory mechanisms. Our data show that transcriptional regulation is responsible for at least part of the NO induction of CaYHB1. To our knowledge, this is the first study to show NO-regulated expression of a physiologically relevant gene target in fungi, so there is very little knowledge so far about potential transcription factors that could mediate this re-
We are currently analyzing the CaYHB1 promoter to identify the cis-acting DNA sequence; the data obtained so far suggest the involvement of multiple DNA elements.

As a commensal organism and opportunistic pathogen, C. albicans is likely to be exposed to NO in a variety of concentrations and locations. For example, NO is produced by endothelia (20), various epithelia (28, 30, 56), and macrophages (50). In the oral cavity, where Candida is commonly found, NO is also produced by bacteria and by abiotic acidification of nitrite (14). Because of its growth-inhibiting (cytostatic) effects we are currently analyzing the CaYHB1 promoter to identify the cis-acting DNA sequence; the data obtained so far suggest the involvement of multiple DNA elements.

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(51), NO released by these sources is likely to limit the domain of Candida. Yet in our experiments on virulence using a murine tail vein injection model, the mutant strain lacking CaYHB1 still shows virulence, although that virulence is less than that of the control wild-type strain or the CaYHB1-complemented mutant. These results are similar to those obtained in the fungal pathogen C. neoformans (12), showing that flavohemoglobin is not absolutely required for virulence when the fungus is directly injected into the bloodstream. However, because it seems likely that NO concentrations may vary widely in different parts of the body, future analysis using other types of virulence-testing models, including mucosal infection routes, will be needed to fully understand the role of CaYHB1 in C. albicans infection in humans.

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