

Inducible Defense Mechanism against Nitric Oxide in *Candida albicans*†

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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 candidacidal activity of macrophages (4, 15, 43). Despite the increasing understanding of host immune defenses mounted against this opportunistic pathogen, the means by which *C. albicans* resists NO or other microbicidal agents is not well understood.

Microbes protect themselves against NO toxicity by using enzymes that convert NO to less toxic molecules. Flavohemoglobin, an NO dioxygenase (NOD) that converts NO to nitrate (26, 29, 55), is found in bacteria and yeasts (7, 58). This enzyme is encoded by a single gene in several different organisms: for example, by *hmp* in *Escherichia coli* (49) and by *ScYHB1* in *Saccharomyces cerevisiae* (57). Flavohemoglobin is necessary for virulence of a plant pathogen, the bacterium *Erwinia chry-*

santhemii (18). *hmp*-negative bacteria are more easily inhibited by NO-releasing compounds and NO (9, 21, 26, 38), and expression of *hmp* is strongly induced by NO (8, 42). *Hmp* induction by NO is mediated by a derepression mechanism in which NO inactivates a metal-binding transcription factor, Fnr (10, 42) or Fur (8, 11). In the yeast *S. cerevisiae*, deletion of *ScYHB1* abolishes NO-consuming activity and leads to increased growth inhibition upon exposure to an NO-generating compound. In contrast to bacteria, *ScYHB1* expression is not induced by NO (35). The fungal pathogen *Cryptococcus neoformans* has a single flavohemoglobin gene, *FHB1*; deletion of this gene blocks NO consumption and attenuates virulence (12).

We searched the recently completed DNA sequence of the *C. albicans* genome and identified three structural homologues of *ScYHB1*, which we call *CaYHB1*, *CaYHB4*, and *CaYHB5*. Each gene was individually deleted and tested for an NO defense function. Here we report that one of these genes, *CaYHB1*, plays a central role in protection of *Candida* against NO.

MATERIALS AND METHODS

Strains and media. *S. cerevisiae* wild-type strain BY4742, along with its isogenic *yhb1Δ*:KanMX derivative, strain 15887, were obtained from Research Genetics/Invitrogen (Table 1). *C. albicans* strain RM1000 (*his1Δ*/*his1Δ* *ura3Δ*/*ura3Δ*) (Table 1) was provided by Jesus Pla (Universidad Complutense de Madrid, Madrid, Spain). Strains were routinely grown in YEPD medium (1% yeast extract, 2% Bacto Peptone, 2% glucose), with 100- μ g/ml uridine added for the growth of Ura⁻ *Candida* strains.

Construction of *Candida* deletion mutants. Both copies of the entire open reading frame (ORF) of *CaYHB1* were deleted in the diploid *Candida* strain RM1000 (Table 1), using the *URA3-dpl200* cassette from pDDB57 (52) to delete

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† Supplemental material for this article may be found at <http://ec.asm.org/>.

TABLE 1. Yeast strains used in this study

Strain type	Genotype	Source ^b
<i>S. cerevisiae</i>		
BY4742	<i>MATα his3Δ leu2Δ lys2Δ ura3Δ</i>	Res. Genetics
15887	<i>MATα his3Δ leu2Δ lys2Δ ura3Δ yhb1Δ::KanMX</i>	Res. Genetics
<i>C. albicans</i>		
CAF2 ^a	<i>ura3Δ::imm434/URA3</i>	Fonzi and Irwin (19)
RM1000	<i>ura3Δ::imm⁴³⁴/ura3Δ::imm⁴³⁴ his1Δ::hisG/his1Δ::hisG</i>	Alonso-Monge et al. (1)
<i>yhb1Δ/YHB1^c</i>	<i>ura3Δ::imm⁴³⁴/ura3Δ::imm⁴³⁴ his1Δ::hisG/his1Δ::hisG yhb1Δ::dpl200/YHB1⁺</i>	This study
<i>yhb1Δ/YHB1^c ura3Δ/URA3^c</i>	<i>ura3Δ::imm⁴³⁴/URA3⁺ his1Δ::hisG/his1Δ::hisG yhb1Δ::dpl200/YHB1⁺</i>	This study
<i>yhb1Δ/yhb1Δ His⁺ Ura⁺</i>	<i>ura3Δ::imm⁴³⁴/ura3Δ::imm⁴³⁴ his1Δ::hisG/his1Δ::hisG yhb1Δ::HIS1/yhb1Δ::URA3</i>	This study
<i>yhb1Δ/yhb1Δ</i>	<i>ura3Δ::imm⁴³⁴/ura3Δ::imm⁴³⁴ his1Δ::hisG/his1Δ::hisG yhb1Δ::HIS1/yhb1Δ::dpl200</i>	This study
<i>yhb1Δ/yhb1Δ ura3Δ/URA3^c</i>	<i>ura3Δ::imm⁴³⁴/URA3⁺ his1Δ::hisG/his1Δ::hisG yhb1Δ::HIS1/yhb1Δ::dpl200</i>	This study
<i>yhb4Δ/yhb4Δ</i>	<i>ura3Δ::imm⁴³⁴/ura3Δ::imm⁴³⁴ his1Δ::hisG/his1Δ::hisG yhb4Δ::HIS1/yhb4Δ::dpl200</i>	This study
<i>yhb5Δ/yhb5Δ</i>	<i>ura3Δ::imm⁴³⁴/ura3Δ::imm⁴³⁴ his1Δ::hisG/his1Δ::hisG yhb5Δ::HIS1/yhb5Δ::dpl200</i>	This study

^a 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.

^b Res. Genetics, Research Genetics/Invitrogen.

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 *URA3-dpl200* and *HIS1* cassettes. YHB1-2 contains a 61-base sequence homologous to DNA flanking *CaYHB1* on the 3' end of the ORF plus 20 nt complementary to the opposite side of the *URA3-dpl200* and *HIS1* cassettes. In the first round of transformations, deletion cassettes containing *URA3-dpl200* were transformed into RM1000 by a modified lithium acetate method (27). Ura⁺ transformants were selected on YNB/Ura dropout medium (yeast nitrogen base without amino acids [Difco] plus 2% glucose and supplemented with amino acids, but lacking uridine). Deletion transformations were then repeated 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/URA3-1, YHB1-4/URA3-2, YHB1-3/HIS1-1, and YHB1-4/HIS1-2 and a negative PCR with primer pair YHB1-5/YHB1-6 (data not shown). The resulting *cayhb1Δ::HIS1/cayhb1Δ::URA3-dpl200* strain was then selected on 5-fluoroorotic acid-containing medium to select against *URA3* (5). PCR on genomic DNA from Ura⁻ strains was used to identify a *cayhb1Δ::HIS1/cayhb1Δ::dpl200* strain in which the *URA3* gene has been removed by recombination between direct repeats of the flanking *dpl-200* DNA on the *URA3-dpl200* cassette. Final confirmation of *cayhb1Δ/cayhb1Δ* was shown by the absence of a detectable *CaYHB1* mRNA by Northern blot (data not shown). Similar procedures were used to generate the *cayhb4Δ/cayhb4Δ* and *cayhb5Δ/cayhb5Δ* deletion mutants (Table 1), except that *CaYHB4* mRNA could not be detected even in RM1000.

Complementation of the *CaYHB1* deletion. Reintroduction of *CaYHB1* was accomplished through two methods. In the first approach, a complementation plasmid was constructed by using the autonomously replicating plasmid pABSKII (*CaARS2 CaURA3*), generously provided by B. B. Magee (Department of Genetics, University of Minnesota, Minneapolis). A 3.2-kb region containing the entire *CaYHB1* ORF, plus about 1,500 bp upstream and 470 bp downstream of *CaYHB1*, was amplified from RM1000 genomic DNA through PCR with primers YHB1-9 and YHB1-10 (see Table S1 in the supplemental material). The resulting product was digested with *SacI* and *SpeI* and ligated into pABSKII that had been cut with *SacI* and *SpeI*. The resultant plasmid (pABSKII *CaYHB1*) or empty pABSKII was transformed into the *cayhb1Δ::HIS1/cayhb1Δ::dpl200* strain; transformants were selected on YNB/Ura dropout medium. For the second reintroduction approach, a 2-kb *CaYHB1*-containing fragment (including 468 bp upstream and 411 bp downstream of the gene) was created by PCR from RM1000 genomic DNA with primers YHB1-3 and YHB1-4. This PCR product was transformed into the *cayhb1Δ::HIS1/cayhb1Δ::URA3-dpl200* strain, and transformants were selected on 5-fluoroorotic acid-containing medium. A *cayhb1Δ::dpl200/CaYHB1* strain was obtained, and reintegration of *CaYHB1* was confirmed by positive PCRs with YHB1-3/YHB1-4, YHB1-9/YHB1-10, YHB1-

3/YHB1-10, and YHB1-4/YHB1-9 and by negative PCRs with YHB1-3/HIS1-1 and YHB1-4/HIS1-2. All transformations were achieved through the lithium acetate method described above.

Cell NO consumption and extract NOD activity measurements. Fresh cultures were initiated from overnight cultures in YEPD medium plus uridine at $A_{600} = 0.1$, with *S. cerevisiae* grown at 30°C and *C. albicans* grown at 37°C. Cells were grown in air to an A_{600} of 0.2 and were then transferred to an atmosphere containing 960 ppm of NO in 21% O₂ balanced with N₂ or were maintained under air. Maximal gas equilibrium was achieved by shaking sealed 50-ml Erlenmeyer flasks containing a <1:5 ratio of culture volume to flask volume at 275 rpm. Gases were mixed and delivered to the culture flasks from three-way proportioners (Cole-Parmer Instrument Co.) at a rate of 30 ml per min, with gas mixtures passed through a trap containing sodium hydroxide pellets to remove NO₂ and higher oxides of nitrogen formed prior to entering the sealed flasks (24). Cells were exposed for 15, 30, and 60 min for mRNA isolation or for 60 min for NO consumption and NOD activity measurements.

For measurements of NO metabolism by whole cells, culture aliquots were centrifuged, supernatants were aspirated, and cell pellets were washed twice. NO consumption measurements were made with a 2-ml ISO-NOP NO electrode (World Precision Instruments, Sarasota, Fla.) in a 2-ml reaction mixture containing minimal salts glucose medium, cycloheximide, and 2 μM NO in a 37°C, water-thermostatted, glass-stoppered, and magnetically stirred glass reaction vessel (23, 26). For measurements of NOD activity, cell extracts were prepared by harvesting cells by centrifugation, lysing them by addition of Y-PER-S reagent (Pierce), sonicating them, and clarifying them by centrifugation. Clarified extracts were transferred to new tubes and stored at -80°C until assayed. Extract NOD activity measurements were made with the NO electrode in a 2-ml reaction mixture containing 100 mM potassium phosphate (pH 7.0), 0.3 mM EDTA, 100 μM NADH, 0.5-mg/ml Cu,ZnSOD, 1 μM flavin adenine dinucleotide (FAD), and 2 μM NO (22, 23, 25, 26). To avoid interference produced by nonenzymatic decomposition of NO by extracts, rates were determined following a second addition of 2 μM NO. Rates of NO metabolism were determined at 1 μM NO and were corrected for background rates of NO decomposition under otherwise identical conditions. The protein concentration was determined from the A_{280} of cell extracts by applying a value of 1 mg per ml of protein for an absorbance of 1.

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 (ICN 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 pAU95 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 PshAI 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.

Growth inhibition assays. Overnight cultures in YEPD medium plus uridine were diluted to $A_{600} = 0.025$, grown for 4 h at 30°C, and then inoculated with either a small volume of freshly made solution containing an NO-generating compound or of control solution. When nitrite was added to log-phase *Candida* cultures in YEPD medium, the initial pH was between 5.5 and 5.8. After 4 h at 30°C, A_{600} values were recorded and growth during the 4-h period was determined by subtracting the A_{600} values at $t = 0$ from final A_{600} values.

Virulence assays. The *C. albicans* strains used were all Ura⁺ His⁺, with the *URA3* gene present at its normal chromosomal location to avoid any effects on virulence due to misexpression of this gene (3, 6, 46). All animal experiments were performed in accordance with institutional regulations in an Association for Assessment and Accreditation of Laboratory Animal Care-certified facility under the National Institutes of Health guidelines for the care and use of laboratory animals. Female BALB/c mice, 6 to 7 weeks old, obtained from the National Cancer Institute, were housed in groups in bioclean hoods and provided with water and food ad libitum. Eight mice were used for each experimental group. Mice were allowed a 1-week acclimatization period before experiments were started. Cells from the different *Candida* strains were grown overnight at 37°C in YEPD medium, harvested by centrifugation, washed twice, resuspended in pyrogen-free saline, and counted with a hemocytometer. Each mouse received an intravenous challenge of 0.2 ml containing 5×10^5 cells of the corresponding strain. Confirmation of the number and viability of cells present in the infecting inocula was performed by plate count. Mice were monitored for survival daily after infection, and the days of death were recorded. Survival data and differences between groups were analyzed by using the Kaplan-Meier and log rank tests. $P < 0.05$ was considered statistically significant.

RESULTS

Flavoheмоglobin 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* flavoheмоglobin 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* flavoheмоglobins and the *S. cerevisiae* flavoheмоglobin are as follows: CaYhb1, 31%; CaYhb4, 25%; and CaYhb5, 29% (Fig. 1). The X-ray structures of two bacterial flavoheмоglobins 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* flavoheмоglobin extends over the whole protein length: i.e., all three domains, rather than being clustered in just one domain.

The three flavoheмоglobins 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 NO₃⁻ (22, 26, 47). To reduce the ferric ion 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* flavoheмоglobin (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* flavoheмоglobin 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* flavoheмоglobins to each other than to three flavoheмоglobins shown to have an NOD function. The analysis also shows that the flavoheмоglobin 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 flavoheмоglobin 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 ($\leq 2 \mu\text{M}$ 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 flavoheмоglobin 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 ($\leq 2 \mu\text{M}$ 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

A

CaYhb1 : M T V E ----- Y E T K Q L T P A Q K I I L D T V P I E E A G E T L I Q K F Y Q R M I G N Y D E V K P F F N T D Q K L L R Q P K I L A F A L L N Y A K N I E D L T P L D F V K Q T V V K H I G L Q V L P H E P Y T V G T C L I : 111
 CaYhb4 : M P I D I N T K A K C Y F D I R P L T K G E R K I I K D S I P I E Y L D T Q F G E K F F K R L I K Q F P Q Y K P Y F N E T H I K L I L R Q P R S F H C L L E F A R N I D D I R P M K D F I M R I A S K I V A R Q V S P D Q Y R V F G Q V L V : 119
 CaYhb5 : M T V A S A S I I N N Y F E S K P L T P E H T Q I I I D S V P I E H L D V Q L T E K F Y K R L L K Q N P E F K P F F N E T H Q K L L R Q P R I M I H F L I Q Y A K N I Q D I L P M D F I K K T A S K H V G I Q V K P E H Y P K I G Q V L I : 119
 CnYhb : M S A A ----- K Q L F K I V P L T P T E I N F L Q S L A P V V K E H G W T V T S T M Y K Y M F Q T Y P E V R S Y F N M T N Q K T G R Q P K V L A F S L Y Q Y I L H I N D I T P I S G F W N Q T V L I K H C G L G T K P D Q Y P V V G E S L V : 114
 ScYhb1 : ----- M L A E K T R S I I K A T V P V L E Q Q C T V I I R T F Y K N M L T E H T E L L N F N R I N Q V G A Q P N A L A T T V L A A A K N I D D L S V L M D H V K T G H K H R A I Q T K P E H P T V G E Y L L : 103
 EcHmp : ----- M L D A Q T L A T V K A T I P L L V E T G E K L I A H F Y D R M E T H N P E L K E I F N M S N Q R N G D Q R E A L F N A L A A Y A S N I E N L P A L L P A V E K I A Q K H T S F Q L K P E Q N I V L G E H L L : 103
 AeFhb : ----- M L T Q K T R D I V K A T A P V L A E H G Y D I I K C F Y Q R M F A H P E L K N V F N M A H Q E Q C Q Q Q A L A R A V Y A Y A E N I E D P N S L M V L K N T A N K H A S I G V K P E Q Y T I V G E H L L : 103

Y L H Y

CaYhb1 : Q T M V E L L P P E I A N K D F L E A M I T A Y G N L A K L L I D L E A A E Y A Q K P ----- H R M F K D F K V T R I V Q E C K D V K S V Y F T P V D K D ----- L L P I P K P E R G Q Y L C F E N K I P G ----- E E F E I ----- S R E Y S V S E F P K : 221
 CaYhb4 : E V M M D Q F P K E F V D Q E F V E A M T M A F R I L A N I L I N I E V R E Y Q D K P ----- H Y G F K A F R V T R I Q P E C V G T K S F F I T P V D G ----- T P V P I P K R G Q Y L C M E N K F P G ----- E K Q E R ----- I R E Y T I S E F P K : 227
 CaYhb5 : N V I I N L F P K Q L V H D E F I E A M I L A Y Q N L A N L L I K L E S E Q Y V E K P ----- H Y G F K Q F K V T R I Q R E C S D V K S Y I T P V D G ----- S P I P K P K R G Q Y L C M R W L L P G ----- E K H E I ----- T R E Y S I S E Y P K : 227
 CnYhb : Q A F K M V L G ----- E A A D E H F V E V F K K A Y G N L A Q I L I D A E A S V Y K T I L A ----- M E E F K D F R V T K L V K E A E D V T S V Y L T P V D G ----- F K I K P I T P G E Y T S F R M D I H N P D I T D I Q P R E Y S I S Q V K : 223
 ScYhb1 : K A L K E V L G D A A T P E ----- I I N A M G E A Y Q A L A D I F I T V E K K M Y E E A L ----- H P G M K P F D I T A K E Y V A S D I V E F T V K P K F G S G I E L S L P I T P G Q Y L T V N T H P I R Q E N Q V D A L R H Y S I C S A S T : 216
 EcHmp : A T L D E M F S ----- P G Q E ----- V I D A M G K A Y G V L A N V F I N R E A E L Y N E N A S K A G G M E G T R D F R I V A K I P R S A L I I S F E L P V D G G ----- A V A E Y R P G Q Y L G V M L K P E G F P ----- H Q E I R Q Y S I L T R K P D : 213
 AeFhb : A A L K E V L G N A A T D D ----- I I S A M A Q A Y G N L A D V L M G M E S E L Y E R S A E Q P C G M K W R T F V I R E K R P E S D V I T S F I L E P A D G C ----- P V V N F E P G Q Y T S V A I D V P A L G ----- L Q Q I R Q Y S I S L M P N : 215

E R Y S

CaYhb1 : E N E Y R I S V R I V P G G K ----- I S G Y I H N N L K V G D I L K V A P P A G N F V Y D P ----- A T D K E ----- I I F V A G G I G I T P L L S M I E R A L E E ----- G K N V K L L Y S N R S A E T R A F G N L F K E Y S K F G D K F Q A I : 328
 CaYhb4 : N N E Y R I T V R Y I P G G K ----- V I T Y I H E K L A V G D I V Q V S P P C G D C Y Y R ----- S S K R N ----- M V M I A G G N G V V A L A P M I E K G L A E ----- C R K V K F L Y S N R S T E T R S F G Q F F R D L K Q Y Y G D Q L E V V : 333
 CaYhb5 : N N E Y R I T V R Y I P G G K ----- V S N Y I H N N I N V G D I V Y S G P P C G D C V Y E ----- S S L K N ----- L V F L A G G N G V T A L L P M I E A G I T E ----- G R Q V K L L Y S N R S T D S R S F G K L F Q S Y K L Q Y G D R F Q V V : 333
 CnYhb : E N E Y R I S V R D I ----- G T ----- V S Y I N K K I Q V G D I V P V H A P V G T M K Y D S ----- I S K K G V A V L A G G I G I T P M I P I T E H A L K D ----- G K D V E L Y S N R S Y Q S E P F R E F F S N L E K E N N G K F K L N : 329
 ScYhb1 : K N G L R F A V K M E A A R E N F P A G L V S E Y L H K D A K V G D E I K L S A P A G D F A I N K L E I H Q N E V P L V L S S C V G V T P L L A M L E E Q V K C N P N R I Y M I Q S S Y D E K Q A F K K H V D E L L A E C A N V D K I I : 335
 EcHmp : G K G Y R I A V K R E E G G C ----- V S N W L H N H A N V G D V V K L V A P A G D F F M A ----- V A D D T P V I L I S A G V G Q T P M I A M L D T L A K A C H T A Q V N N F H A A E N G D V H A F A D E V K E L G Q S L P R F T A H T : 322
 AeFhb : G R Y Y R I S V K R E G G G P P P G ----- Y V S N L L L H D H V N V G D Q V K L A A P Y G S F H I D ----- V D A K T P I V L I S G G V G L T E M V S M L K V A L Q A ----- P P R Q V V F V H I G A R N S A V H A M R D R L I R E A A K I Y E N L D L F V : 328

S

CaYhb1 : E Y F S E D N F T D D K I V I D K A F N R K L T T D D L D F T A P ----- E H D V Y L V G P R E F M K D I K E H L G K K N V P ----- V K L E Y F G P Y D P ----- : 398
 CaYhb4 : E Y L S R R R V I D P ----- I D K Y Y R R S I T V E D I D F T V P ----- E D D V Y L I G P R T Y M K M I E D Y L K G R G V N ----- V T L D Y F G P Q E V ----- : 400
 CaYhb5 : E F L S R G R T I D P ----- I D K F Y R R S T L F L E D L D F T V P ----- E D D V Y L I G P R T Y M K M I E D Y L K D R N I T ----- V K L D Y F G P R E I ----- : 400
 CnYhb : N Y T S A E ----- N Q K I Q V K D L E H I N P D E Y D Y V I L L G P V A Y M H E F K T Y L V G K G V S D ----- I K M E F F G P T D P D C ----- : 390
 ScYhb1 : V H T ----- D T E P L I N A A F L K ----- E K S P A ----- H A D V Y I C C S L A F M Q A M I G H L K E L E H R D D M I H Y E F F G P K M S T V Q V ----- : 399
 EcHmp : W Y R Q P S E A D R A K Q D F S E G I M D L S K L E G A F S D P ----- T M Q F Y L C G E V G F M Q F T A K Q L V D L G V K Q E N I H Y E C F G P H K V L ----- : 396
 AeFhb : F Y D Q L P E D V G R D Y D Y P L G V D V K Q L E K S I L L P ----- D A D Y Y I C G E I P F M R M Q H D A L K N L G I E A R I H Y E V F G P D L F A E ----- : 403

B

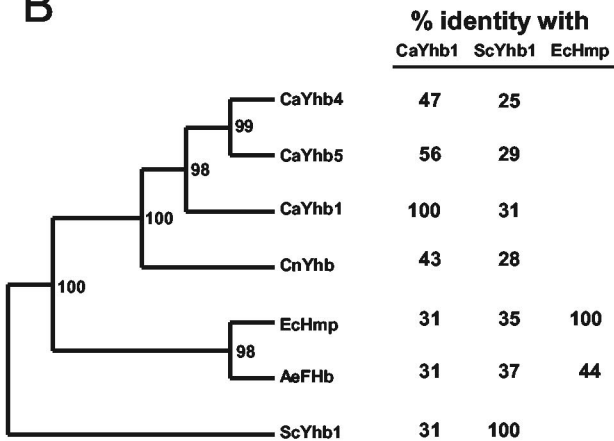


FIG. 1. *C. albicans* contains three predicted flavohemoglobins with similarity to flavohemoglobins from other microbes. (A) Alignment of flavohemoglobins from several microbial genes. CaYhb1, -4, and -5 are the three predicted flavohemoglobins from *C. albicans*. CnYhb is the flavohemoglobin from *Candida norvegensis* (34), also known as *Pichia norvegensis*. ScYhb1 is from *Saccharomyces cerevisiae*. EcHmp is from *Escherichia coli*, and AeFhb is from *Alcaligenes eutrophus*. Amino acids important for binding the coenzymes heme and FAD in the X-ray structure of EcHmp (31) and AeFhb (16) are shown above the aligned sequences. Based on the alignment with the *E. coli* Hmp sequence (31), the globin domain comprises about the first third of the polypeptide chain, the FAD domain starts after the large arrow, and the NAD(P) domain is after the small arrowhead. Alignments were created with ClustalX and displayed with GeneDoc. (B) Phylogenetic tree of a subset of the microbial flavohemoglobins. The tree is based on an alignment (A) of the sum of the amino acids in these flavohemoglobins and is unrooted. Numbers adjoining the branches indicate bootstrap values (percentages from 500 replicates) based on results of the unweighted pair group method with arithmetic mean (40) calculated by using the PHYLIP program suite (J. Felsenstein; <http://evolution.genetics.washington.edu/phylip.html>). The different flavohemoglobins are shown together with the percent identity between select pairs.

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). *YHB1* 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

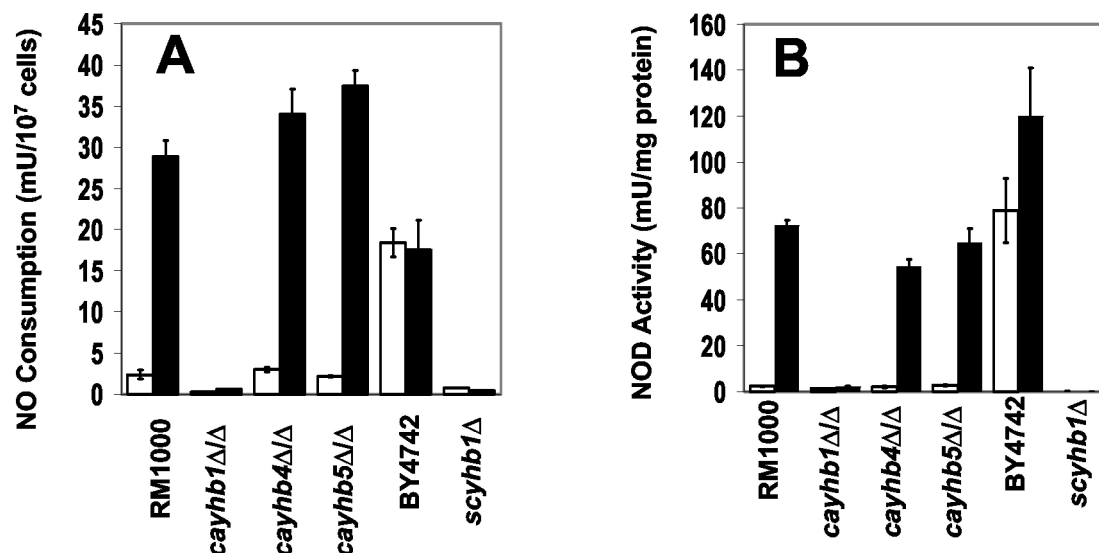


FIG. 2. Flavo-hemoglobin gene *YHB1* is required for inducible NO metabolism in yeast. (A) NO consumption in yeast exposed either to air only (open bars) or to 960 ppm of gaseous NO in an air mixture (solid bars) for 60 min. (B) NOD activities in naïve and NO-exposed cells. Cells were harvested, extracts were prepared, and NOD activities were measured as described in Materials and Methods. *scyhb1Δ* indicates strain 15887, and *cayhbΔ/Δ* refers to *cayhbΔ::HIS1/cayhbΔ::dpl200* (Table 1). One milliunit is equivalent to 1 nmol per min. Error bars represent the standard deviation of three independent trials.

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 \pm standard deviation), respectively. Together these results show that nitrite induction of flavo-hemoglobin 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 flavo-hemoglobin 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* flavo-hemoglobins 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

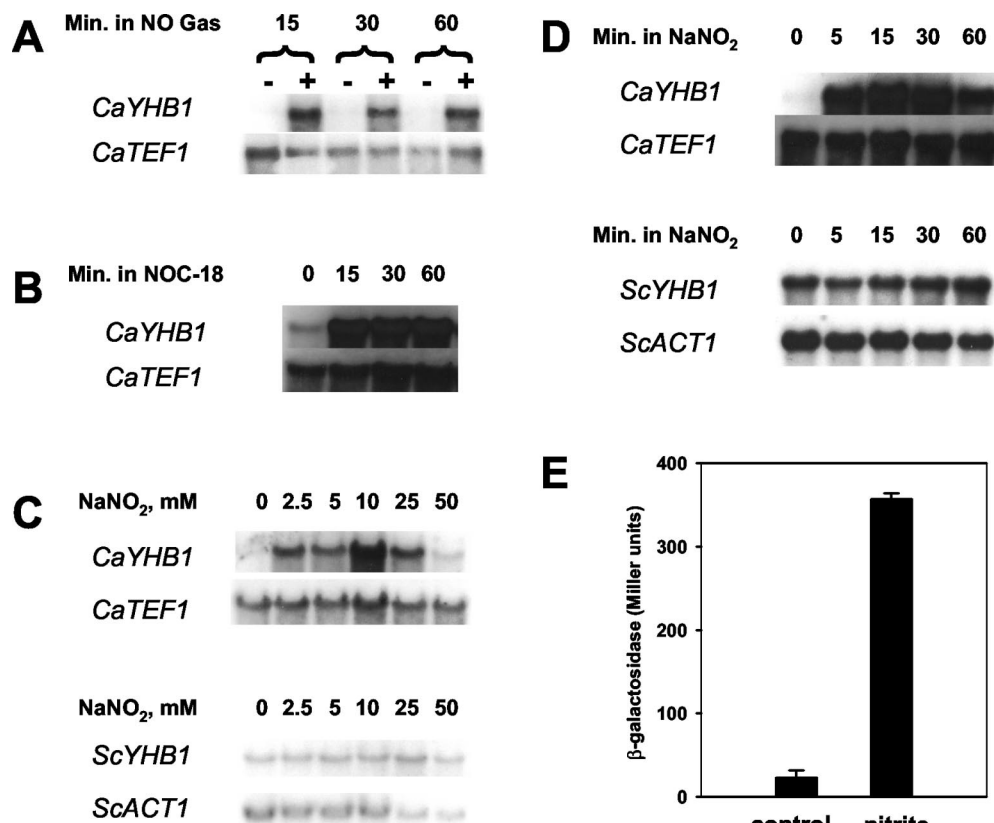


FIG. 3. *CaYHB1* mRNA is induced by NO in *C. albicans*. (A) *CaYHB1* is induced by exposure to 960 ppm of NO gas ($\leq 2 \mu\text{M}$ in solution). Naïve and NO-exposed cultures were grown as described in Materials and Methods, and cells were harvested at 15, 30, and 60 min, followed by mRNA isolation. (B) Induction of *CaYHB1* mRNA by the NO-releasing compound NOC-18. A total of 3 mM NOC-18 was added to exponentially growing yeast (37°C), and cells were collected at 0, 15, 30, and 60 min for RNA extraction. (C) Induction of *YHB1* in *C. albicans* and *S. cerevisiae* by nitrite. Various concentrations of sodium nitrite (NaNO_2) were added to log-phase cultures of the *S. cerevisiae* strain BY4742 (grown at 30°C) or the *C. albicans* strain RM1000 (grown at 37°C). After 30 min of treatment, cells were collected and mRNA was extracted. (D) A total of 25 mM NaNO_2 was added to exponential cultures of BY4742 (30°C) or RM1000 (37°C), and after 0, 5, 15, 30, and 60 min, cells were harvested for mRNA isolation. *ACT1* was probed as a loading control for *Saccharomyces* RNA; *CaTEF1* was the control for *Candida* samples. (E) Induction of *CaYHB1-lacZ* by nitrite. A *C. albicans* strain containing a *CaYHB1-lacZ* reporter gene plasmid, integrated into a chromosome downstream of the *HWPI* gene (see Materials and Methods), was grown in rich YEPD medium with or without 10 mM nitrite at 37°C for 1 h, and β -galactosidase activity in cell extracts was measured as described previously (2).

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-

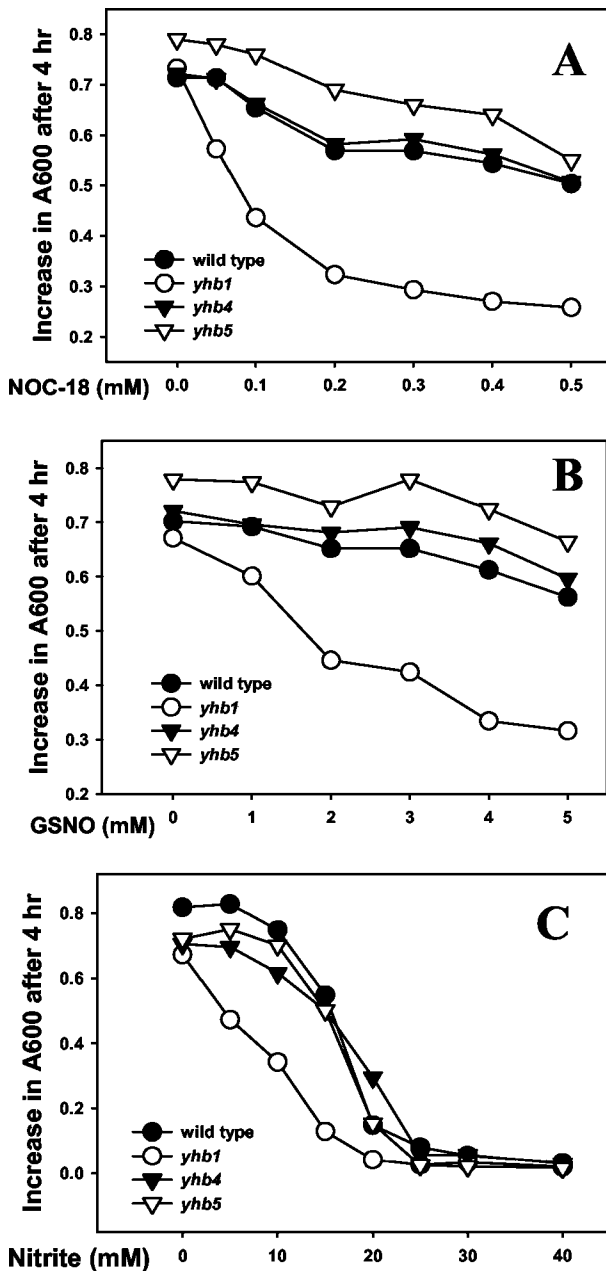


FIG. 4. Deletion of *CaYHB1* increases sensitivity to NO-generating compounds and to nitrite. Growth of wild-type (RM1000; solid circles), *cayhb1Δ/cayhb1Δ* (open circles), *cayhb4Δ/cayhb4Δ* (solid triangles), or *cayhb5Δ/cayhb5Δ* (open triangles) yeast cultures was measured following treatment of log-phase cultures with control solvent or NOC-18 (A), *S*-nitrosoglutathione (GSNO) (B), or sodium nitrite (NaNO₂) (C) at the indicated concentrations. After 4 h of treatment, growth was calculated from the change in turbidity at 600 nm ($A_{600}^{t=4} - A_{600}^{t=0}$). Data are from representative experiments that were repeated at least two times with similar results.

sponse. 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 endo-

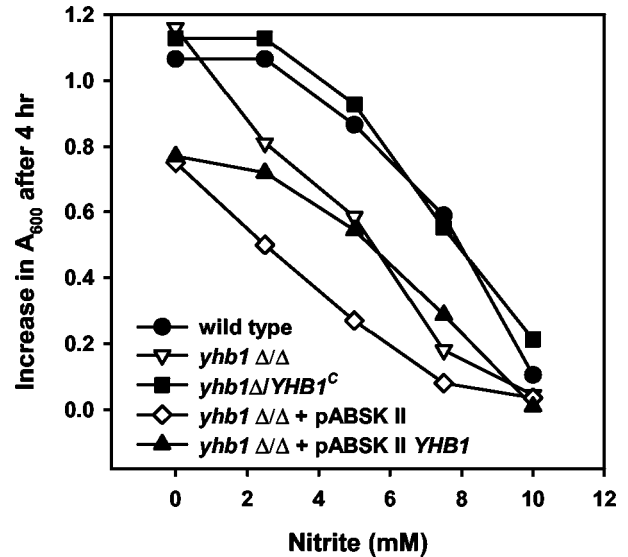


FIG. 5. Reinsertion of *CaYHB1* complements sensitivity of the *cayhb1Δ/cayhb1Δ* strain to sodium nitrite. Growth of cultures of the wild-type strain (RM1000; solid circles) or mutant strains of the types *cayhb1Δ::HIS1/cayhb1Δ::dpl200* (open triangles), *cayhb1Δ::dpl200/CaYHB1* (solid squares), *cayhb1Δ::HIS1/cayhb1Δ::dpl200* transformed with empty plasmid pABSII (open diamonds), or *cayhb1Δ::HIS1/cayhb1Δ::dpl200* transformed with complementation plasmid pABSII *CaYHB1* (solid triangles) was determined following treatment with the indicated concentrations of sodium nitrite for 4 h. Growth was determined from the change in turbidity at 600 nm. The RM1000, *cayhb1Δ/Δ*, and *cayhb1Δ/+* strains were each grown in YEPD medium supplemented with uridine, while the strains containing plasmid pABSII or pABSII *CaYHB1* were grown in YEPD medium without uridine to maintain selection for the URA3-containing plasmid. The exact reason for slower growth of the latter cultures is not known, but it may reflect a low activity of the plasmid *URA3* gene. Results are representative of two or more experiments.

thelia (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

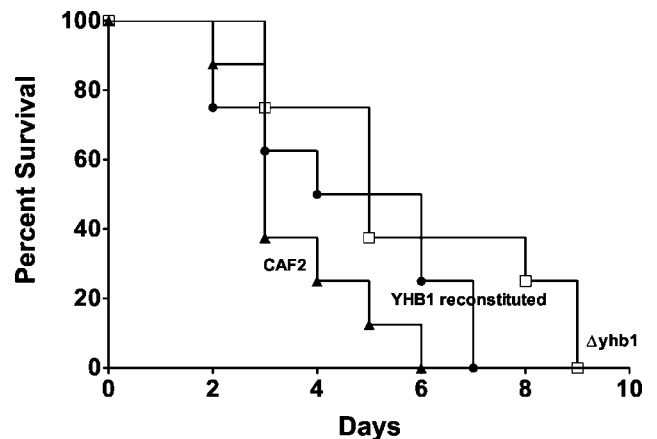


FIG. 6. *C. albicans* strains lacking *YHB1* show attenuated virulence. The His⁺ Ura⁺ *C. albicans* strains used were the CAF2, $\Delta yhb1$ (*yhb1Δ/yhb1Δ ura3Δ/URA3^c*), and YHB1 reconstituted (*yhb1Δ/YHB1^c ura3Δ/URA3^c*) strains shown. Tail vein injection with *Candida* was performed on day 0.

(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 will 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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