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Transcriptional Response of *Candida albicans* to Nitric Oxide and the Role of the *YHB1* Gene in Nitrosative Stress and Virulence[D]

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Here, we investigate how *Candida albicans*, the most prevalent human fungal pathogen, protects itself from nitric oxide (•NO), an antimicrobial compound produced by the innate immune system. We show that exposure of *C. albicans* to •NO elicits a reproducible and specific transcriptional response as determined by genome-wide microarray analysis. Many genes are transiently induced or repressed by •NO, whereas a set of nine genes remain at elevated levels during •NO exposure. The most highly induced gene in this latter category is *YHB1*, a flavohemoglobin that detoxifies •NO in *C. albicans* and other microbes. We show that *C. albicans* strains deleted for *YHB1* have two phenotypes in vitro; they are hypersensitive to •NO and they are hyperfilamentous. In a mouse model of disseminated candidiasis, a *YHB1* deleted *C. albicans* strain shows moderately attenuated virulence, but the virulence defect is not suppressed by deletion of the host *NOS2* gene. These results suggest that •NO production is not a prime determinant of virulence in the mouse tail vein model of candidiasis and that the attenuated virulence of a *yhb1*Δ/*yhb1*Δ strain is attributable to a defect other than its reduced ability to detoxify •NO.

INTRODUCTION

Cells of the innate immune system employ several highly effective mechanisms to defend against microbial pathogens. One of the most intriguing is the production of nitric oxide (•NO). At low concentrations, •NO is used as a signaling molecule in both animals and plants to control a diverse set of physiological processes; for example, in mammals it regulates both smooth muscle vasodilation and signaling between nerve cells (Nathan, 1992). At much higher concentrations, •NO is toxic to cells and is used as a defense mechanism against invading pathogens.

•NO is produced from arginine by the enzyme nitric oxide synthase (NOS). In mammals, there are three distinct isoforms of this enzyme, which differ both in their function and expression, and are encoded by separate genes, *NOS1*, *NOS2*, and *NOS3*. The *NOS2* gene (often referred to as *iNOS*) is the only inducible isoform and is predominately expressed in cells of the innate immune system and epithelia upon exposure to microorganisms, resulting in production of toxic levels of •NO (for reviews see Fang, 1997; Nathan, 1997; Nathan and Shiloh, 2000; Chakravortty and Hensel, 2003; Fang, 2004). For example, induction of macrophage *NOS2* by the cytokine IFN-γ or microbial components such as lipopolysaccharide (LPS) leads to •NO production (Xie *et al.*, 1992; Chinen *et al.*, 1999).

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•NO and its immediate derivatives, termed reactive nitrogen intermediates (RNI), react with many different cellular components, and it is widely recognized that DNA and many classes of proteins and lipids are damaged by exposure to •NO or other RNI species (see reviews cited above). •NO is also known to regulate the catalytic activity of various enzymes primarily by interaction with Fe-S clusters, oxidized copper (Cu^{2+}) centers, hemes, and tyrosyl radicals. For example, •NO reversibly binds to the Cu^{2+} of cytochrome *c* oxidase (Cox) thereby shutting down mitochondrial oxidative phosphorylation and cellular respiration (Burney *et al.*, 1999; Carreras *et al.*, 2004). Although high levels of •NO can also damage host cells, its protective value apparently outweighs this incidental damage (Nathan and Shiloh, 2000).

Pathogens have evolved many strategies for combating the adverse affects of •NO; they can detoxify •NO, they can repair •NO-induced damage, and they can modulate host •NO production (see reviews cited above). Prominent among the detoxification enzymes are the flavohemoglobins, an ancient protein family that predates the divergence of bacteria and eukaryotes. Under aerobic conditions, these two-domain proteins can detoxify •NO in a rapid reaction of heme-bound •NO with oxygen to form innocuous nitrate and ferric flavohemoglobin. The enzyme is subsequently reduced back to its ferrous form via electron transfer between NAD(P)H and its flavin-containing FAD oxidoreductase domain. Flavohemoglobins (and closely related proteins) have been suggested, and in many cases definitively shown, to confer resistance to •NO in a wide variety of microbes including *Escherichia coli* (Vasudevan *et al.*, 1991; Gardner *et al.*, 1998; Hausladen *et al.*, 1998), *Salmonella typhimurium* (Crawford and Goldberg, 1998), *Mycobacterium tuberculosis* (Hu *et al.*, 1999; Pathania *et al.*, 2002), *Saccharomyces cerevisiae* (Liu *et al.*, 2000), *Cryptococcus neoformans* (de Jesus-

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

Strain	Genotype	Origin
CAF2-1	*ura3Δ::imm^{434}/URA3, iro1Δ::imm^{434}/IRO1*	Fonzi and Irwin (1993)
RM1	*ura3Δ::imm^{434}/URA3, his1Δ::HisG/HIS1, iro1Δ::imm^{434}/IRO1*	Negredo *et al.* (1997)
RM1000	*ura3Δ::imm^{434}/ura3Δ::imm^{434}, his1Δ::HisG/his1Δ::HisG, iro1Δ::imm^{434}/iro1Δ::imm^{434}*	Negredo *et al.* (1997)
BH79	*ura3::URA3/ura3Δ:: imm^{434}, his1Δ::HisG/his1Δ::HisG, iro1::IRO1/iro1Δ:: imm^{434}, yhb1Δ::HisG/yhb1Δ::HIS1*	This study
BH94	*ura3::URA3/ura3Δ:: imm^{434}, his1Δ::HisG/his1Δ::HisG, iro1::IRO1/iro1Δ:: imm^{434}, yhb1::YHB1/yhb1Δ::HIS1*	This study
BH97	*ura3::URA3/ura3Δ:: imm^{434}, his1Δ::HisG/his1Δ::HisG, iro1::IRO1/iro1Δ:: imm^{434}, yhb1::YHB1/yhb1Δ::HIS1*	This study
BH98	*ura3::URA3/ura3Δ:: imm^{434}, his1Δ::HisG/his1Δ::HisG, iro1::IRO1/iro1Δ:: imm^{434}, yhb1::YHB1/yhb1Δ::HIS1*	This study
BH115	*ura3::URA3/ura3Δ:: imm^{434}, his1::HIS1/his1Δ::HisG, iro1::IRO1/iro1Δ:: imm^{434}, yhb1::HisG/YHB1*	This study
BH117	*ura3::URA3/ura3Δ:: imm^{434}, his1::HIS1/his1Δ::HisG, iro1::IRO1/iro1Δ::imm^{434}*	This study
MMY272	*ura3Δ::imm^{434}/ura3Δ:: imm^{434}, his1Δ::HisG/his1Δ::HisG, iro1Δ::imm^{434}/iro1Δ:: imm^{434}, yhb1::URA3/yhb1::HIS1*	This study

Berrios *et al.*, 2003), and *C. albicans* (Ullmann *et al.*, 2004; for review see Poole and Hughes, 2000).

In this article, we examine the transcriptional response of *C. albicans*, the major fungal pathogen of humans, to ·NO. *C. albicans* causes mucosal infections in healthy individuals and disseminated infections in immunocompromised patients and is capable of colonizing most tissues in the human body. The available evidence suggests that ·NO is likely important in controlling *C. albicans* infections; it has been implicated in the candidacidal activity of both macrophages and saliva, as determined in vitro (Elahi *et al.*, 2001; Netea *et al.*, 2002). Recently, it was reported that a *C. albicans* strain deleted for both copies of a putative flavohemoglobin gene (*YHB1*) is hypersensitive to ·NO killing and reduced for virulence in the tail vein model of disseminated candidiasis (Ullmann *et al.*, 2004).

Here, we describe the genome-wide response of *C. albicans* to ·NO and provide an independent characterization of a strain deleted for *YHB1*, the most highly ·NO-induced gene. We show that *YHB1* is not required for the transcriptional response of *C. albicans* to ·NO; indeed, the transcriptional response is accentuated in a *yhb1Δ/yhb1Δ* strain. Finally, in a mouse tail vein model of systemic infection, we show that the virulence defect of the *yhb1Δ/yhb1Δ* mutant is likely due to a phenotype other than increased sensitivity to ·NO.

MATERIALS AND METHODS

Strains and Media

C. albicans strains used in this study are listed in Table 1 and described in greater detail below. CAF2–1, RM1, and RM1000 have been described previously (Fonzi and Irwin, 1993; Negredo *et al.*, 1997). MMY272, BH79, BH94, BH96, BH98, BH113, and BH117 were all derived from RM1000.

Cultures were grown at 30°C in YEPD unless otherwise noted, and *C. albicans* transformations were performed according to the standard lithium acetate method (Gietz *et al.*, 1995). Transformants were selected on Sabouraud dextrose (SD) medium lacking uracil or histidine (Ura$^-$ or His$^-$), depending on the marker used, and correct integration of disruption fragments was confirmed by PCR. For strains manipulated at the *YHB1* locus, Southern blot analyses were performed with the Nonradioactive Labeling and Detection kit (Boehringer Mannheim, Indianapolis, IN; Supplementary Figure 1A) and pulsed-field gel electrophoresis was used to verify that none of the newly constructed strains had gross chromosomal abnormalities (Supplementary Figure 1B). The *C. albicans URA3* gene was recycled by plating on 5-fluoroorotic acid-containing medium (5-FOA medium) as previously described (Boeke *et al.*, 1984; Alani *et al.*, 1987).

For experiments using DPTA NONOate (see below), *C. albicans* strains were grown in YEPD broth buffered with 80 mM HEPES, pH 7.5.

Nitric Oxide-releasing and -scavenging Chemicals

Dipropylenetriamine NONOate (DPTA NONOate; Cayman Chemicals, Ann Arbor, MI), which releases two molecules of ·NO per amine molecule in a pH-dependent manner (pH 7.0–7.4), was stored at −80°C and resuspended to 0.75 M in 10.0 mM NaOH immediately before use. To activate the DPTA NONOate, the inactive alkaline DPTA NONOate solution was added to buffered YEPD (see above for buffer details) at a final concentration of 1.0 mM DPTA NONOate and ~13.3 nM NaOH unless otherwise noted. For the ·NO scavenging experiment, Carboxy PTIO potassium salt (Carboxy PTIO; Cayman Chemicals), a molecule that scavenges nitric oxide, was solubilized in 1× phosphate-buffered saline (PBS; pH 7.2) and used at a final concentration of 16.0 mM.

Construction of yhb1Δ/yhb1Δ Mutant

All primer sequences are listed in Supplementary Table 3. The *C. albicans YHB1* gene was disrupted by a PCR method (Wilson *et al.*, 1999, 2000) in RM1000 (Negredo *et al.*, 1997), which is auxotrophic for uridine and histidine. Primers 4 and 5 (see Supplementary Table 3 for primer sequences) were used in separate PCR reactions with the templates pGEM-*HIS1* (Wilson *et al.*, 1999)

and pDDB57 (Wilson *et al.*, 2000) to generate the PCR disruption products. Whole-cell PCR, using primers internal to *HIS1* (primers 6 and 7) and *URA3* (primers 8 and 9), and outside the flanking region of homology to the *YHB1* locus (primers 10 and 11), was used to identify a *yhb1*Δ::*URA3*/*yhb1*Δ::*HIS1* isolate (MMY272). The absence of a product using primers internal to the open reading frame (primers 12 and 13) confirmed that the *YHB1* sequence had not relocated to another position in the genome. Ura$^+$ *yhb1*Δ/*yhb1*Δ strains were plated on 5-FOA-containing medium to select for loss of the *URA3* gene. To restore a single copy of *URA3* and *IRO1* to their native loci, Ura$^-$ strains were transformed with pLUBP (Fonzi and Irwin, 1993), linearized at *Pst*I and *Bgl*II which released a 4.9-kb restriction fragment containing the *URA3* and *IRO1* genes, and grown on Ura$^-$ selective medium. Whole-cell PCR, using primers internal to the *URA3-IRO1* sequence (primer 15) and outside the flanking region of homology to the *URA3-IRO1* sequence (primer 14) were used to confirm that the *URA3-IRO1* open reading frames had reintegrated at their endogenous loci. Southern blot analysis (Supplementary Figure 1A) of BH79 (*yhb1*Δ::*HisG*/*yhb1*Δ::*HIS1*) with a probe to the *YHB1* promoter confirmed the absence of both *YHB1* open reading frames, integration of *HIS1* at one *YHB1* allele, and the excision recombination of *URA3* at the other *YHB1* allele (see Table 1 for complete strain genotype).

Reintroduction of Wild-type YHB1 to yhb1Δ/yhb1Δ Mutants

The 1194-base pair *YHB1* open reading frame, 500 base pairs of upstream promoter sequence, and 300 base pairs of 3′ untranslated region (UTR) were amplified by PCR (primers 16 and 17) with Ex Taq polymerase (TaKaRa), yielding a 2060-base pair sequence containing engineered *Bam*HI sites at both ends. The PCR fragment was gel purified on a 0.8% agarose gel before purification with QIAquick gel extraction kit (Qiagen, Chatsworth, CA), digested with the *Bam*HI enzyme, and ligated into *Bam*HI-linearized pBB510 (Braun and Johnson, 2000), a derivative of pMB7 that contains the *HisG-URA3-HisG* cassette (Fonzi and Irwin, 1993). The resulting plasmid was named pBH2, and DNA sequencing of the insert confirmed identity to the *YHB1* gene reported in the *C. albicans* Diploid Assembly 19 database of the Stanford Genome Technology Center (http://www-sequence.stanford.edu/group/candida/). The intermediate *yhb1*Δ/*yhb1*Δ strain (Ura$^-$, Iro1$^-$, His$^+$) described earlier was transformed with pBH2, which had been linearized at *Sac*II and phosphatase treated, and selected for on Ura$^-$ medium. Correct integration of *YHB1-URA3* was confirmed by whole-cell PCR using primers 5′ to the integrated sequence (primer 18) and internal to the *YHB1* open reading frame (primer 19). Integration at the 3′ end of insertion was confirmed by using primers internal to the inserted plasmid sequence (primer 20) and 3′ to the integrated sequence (primer 21). Correct integrants were plated on 5-FOA to select for loss of the *URA3* gene, and the *URA3-IRO1* open reading frames were restored to their endogenous loci and confirmed by PCR as described above. Three addback strains, BH94, BH96, and BH98, were obtained and their sensitivity to $^{\bullet}$NO was assessed by exposing them to DPTA NONOate as described below. Southern blot analysis (Supplementary Figure 1A) with a probe to the *YHB1* promoter confirmed reconstitution of *YHB1* at one allele, and the presence of *HIS1* at the other *YHB1* allele.

Construction of Wild-type URA3 Isogenic Strain

As noted earlier in this report, the *yhb1*Δ/*yhb1*Δ null, *yhb1*Δ/*YHB1* heterozygous, and *yhb1*Δ/*yhb1*::*YHB1* addback strains were derived from RM1000. Because virulence differences can result from differential expression of *URA3* at heterologous loci (Kirsch and Whitney, 1991; Sundstrom *et al.*, 2002; Cheng *et al.*, 2003; Brand *et al.*, 2004), we created our wild-type comparator (BH117) from RM1000, adding back both *URA3* and *HIS1* to their endogenous loci. The *URA3-IRO1* fragment was restored and confirmed by PCR as described above. To restore *HIS1*, the resulting Ura$^+$ strain was transformed with pGEM*HIS* (Wilson *et al.*, 1999), linearized at Nru1. Whole-cell PCR, using primers internal to the *HIS1* sequence (primers 23 and 24) and outside the flanking region of homology to the *HIS1* sequence (primers 22 and 25), was used to confirm that the *HIS1* open reading frame had reintegrated at its endogenous locus.

Construction of C. albicans Microarrays

The *C. albicans* microarrays used in this article have been previously described (Bennett *et al.*, 2003) and include two *YHB1* cDNA features of 1039 and 356 base pairs in length (the entire *YHB1* ORF is 1194 base pairs in length). Because *YHB1* is part of a gene family including *YHB4* and *YHB5* (*YHB1* has 48 and 57% overall identity to *YHB4* and *YHB5*, respectively), and cross-hybridization of large PCR-generated cDNA features could have obscured our data, we designed three 70-base pairs oligomers, each specific to one *YHB* gene. The *YHB1* oligomer has 14.8 and 33.7% identity, respectively, to the *YHB4* and *YHB5* oligomers, and each oligomer is spotted in triplicate on our microarray. See Supplementary Table 3 for primer sequences (primers 1–3).

Culture Growth for Nitric Oxide Microarrays

Ten-milliliter cultures of CAF2–1, RM1, RM1000, and MMY272 (*yhb1*Δ/*yhb1*Δ) were grown overnight, and each was used to inoculate a 1.8-L culture to an OD (600 nm) of 0.15. The RM1, RM1000, and MMY272 cultures were supplemented with 100 mg/L uridine and 5 g/L histidine. When the OD (600 nm) of the cultures reached ~1.0, three zero time-point samples were harvested for each strain. The remainder of each culture was then perturbed with (1) DPTA NONOate and NaOH or (2) NaOH alone (mock treated; see above for details on chemicals). Samples were collected by filtration after 10, 40, 70, and 120 min of perturbation and cell pellets were stored at −80°C.

Culture Growth for Wild-type versus yhb1Δ/yhb1Δ Mutant Microarrays

Ten-milliliter cultures of *yhb1*Δ/*yhb1*Δ (BH79) and its isogenic wild-type comparator (BH117) were grown overnight and used to inoculate two 150-ml cultures each, one for growth at 30°C and the other at 37°C. When the four cultures reached an OD (600 nm) of ~1.0, they were harvested by filtration and pellets were stored at −80°C. The experiment was repeated three times at 30°C and twice at 37°C.

RNA Isolation, Microarray Hybridization, and Data Analysis

Total RNA, poly-A RNA, and cDNA were prepared as previously described (Bennett *et al.*, 2003). cDNAs for individual time-points (coupled to Cy5) were hybridized against a reference pool comprising cDNAs from every time point in the series (coupled to Cy3). Microarray hybridizations were incubated for 18–24 h according to the methods described by Bennett *et al.* (2003). Arrays were scanned on a GenePix 4000 scanner (Axon Instruments, Foster City, CA), and fluorescence signals were assigned to individual features using GENEPIX PRO version 3.0. Data were further processed with NOMAD (available at http://derisilab5.ucsf.edu/NOMAD/nomad-cgi/login.pl). For the nitric oxide time-course experiment, individual microarray fluorescence signals were transformed over the median fluorescence signal of three untreated zero time-point microarrays, and for the wild-type versus *yhb1*Δ/*yhb1*Δ mutant experiment, individual *yhb1*Δ/*yhb1*Δ microarray fluorescence signals were transformed over wild-type fluorescence signals. The primary data are available on the Johnson laboratory website, http://itsa.ucsf.edu/%7Emicro/Faculty/Johnson/johnson_index.html. Pairwise average linkage clustering analysis was performed with the program CLUSTER (available at http://rana.stanford.edu/software) as previously described (Eisen *et al.*, 1998), and data sets were visualized with the program TREEVIEW (available at http://rana.stanford.edu/software).

Quantitative RT-PCR of YHB1, YHB4, YHB5, and PAT1

A 5-ml culture of CAF2–1 was grown overnight at 30°C, diluted to an OD (600 nm) of 0.15, and upon reaching an OD (600 nm) of ~1.0, a zero time-point sample was harvested by centrifugation at room temperature (RT) and frozen at −80°C. The remaining culture was split into four 10-ml cultures and treated with 1) DPTA NONOate and Carboxy PTIO (scavenger), 2) DPTA NONOate alone, 3) Carboxy PTIO (scavenger) alone, or 4) mock-treated with NaOH and PBS (see above for details on chemicals). After 10 and 120 min of perturbation, samples were collected by centrifugation. RNA was extracted as previously described (Miller and Johnson, 2002), linearly reverse-transcribed (Superscript), and cDNA was amplified by quantitative PCR in a DNA Engine Opticon 2 (Bio-Rad, Waltham, MA). Signals from each sample were transformed over the average of three untreated zero time-point samples, and the entire experiment was repeated three times and averaged (see Figure 2). Primer sequences for *YHB1*, *YHB4*, *YHB5*, and *PAT1* (primers 26–33) are listed in Supplementary Table 3.

In Vitro Growth Inhibition Assay

Five-milliliter cultures of BH79, BH94, BH96, BH98, BH115, and BH117 were grown overnight as described above and used to inoculate a 10-ml culture of each respective strain to an OD (600 nm) of 0.15. Cells were allowed to recover for 1 h and then divided into five 1-ml cultures. DPTA NONOate was added to final concentrations of 0.0, 0.5, 1.0, 2.0, and 3.5 mM, respectively. Cultures were grown for 8 h and serially diluted and plated, and after 24 h of growth at 30°C, colony forming units (CFUs) were counted.

Virulence Tests Using the Mouse Tail Vein Injection Model

Virulence assays were performed in 10-wk-old female BALB/c immunocompetent mice (Charles River Laboratories, Wilmington, MA) and in 9-wk-old male C57BL/6 NOS2$^{+/+}$ (Jackson ImmunoResearch Laboratories, West Grove, PA) or NOS2$^{-/-}$ mice (B6.129P2-Nos, Jackson ImmunoResearch Laboratories). Ten or 11 mice were tested per strain. Ten-milliliter cultures of BH117, BH98, and BH79 were grown overnight as described earlier and

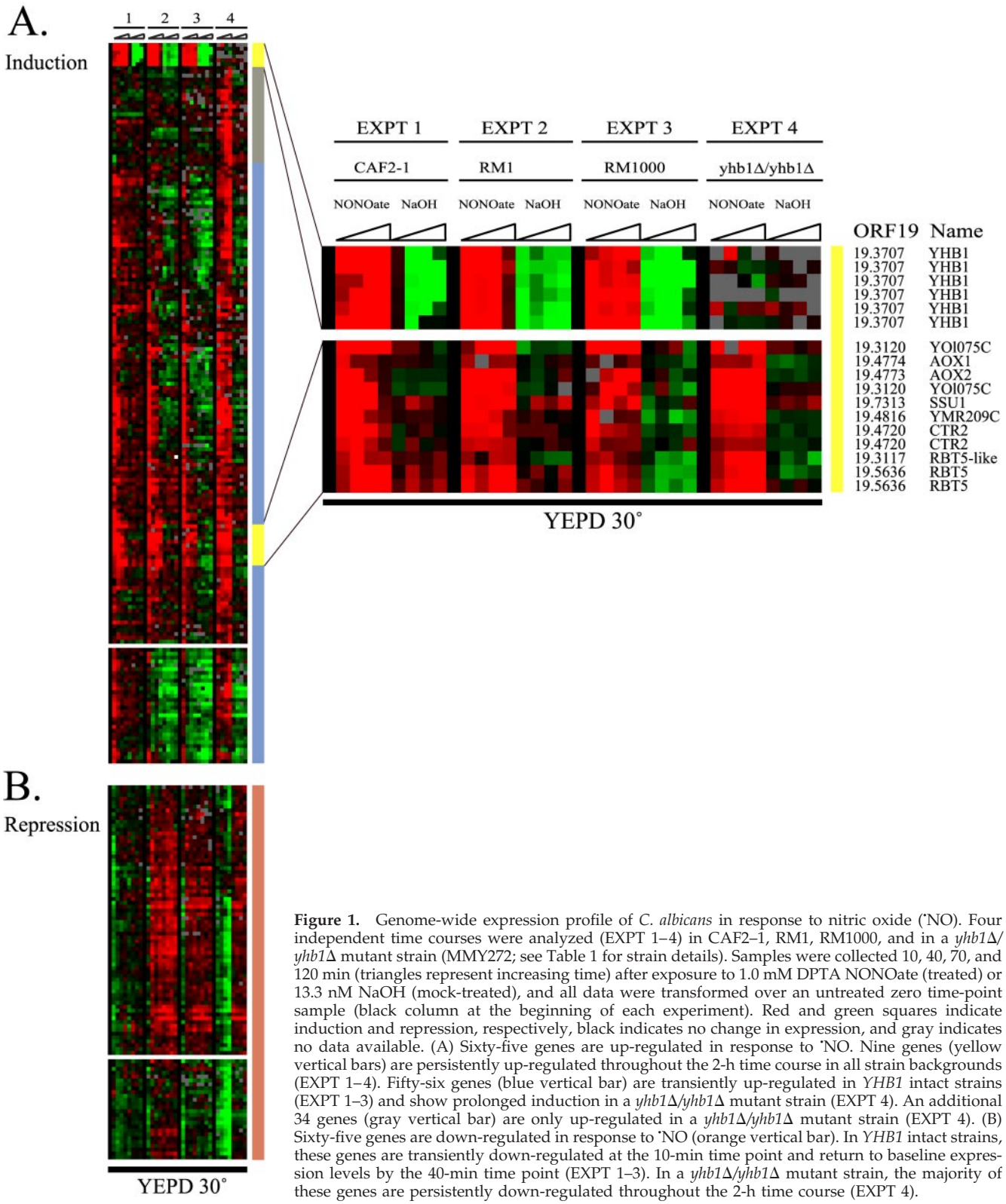

Figure 1. Genome-wide expression profile of *C. albicans* in response to nitric oxide (•NO). Four independent time courses were analyzed (EXPT 1–4) in CAF2–1, RM1, RM1000, and in a *yhb1*Δ/*yhb1*Δ mutant strain (MMY272; see Table 1 for strain details). Samples were collected 10, 40, 70, and 120 min (triangles represent increasing time) after exposure to 1.0 mM DPTA NONOate (treated) or 13.3 nM NaOH (mock-treated), and all data were transformed over an untreated zero time-point sample (black column at the beginning of each experiment). Red and green squares indicate induction and repression, respectively, black indicates no change in expression, and gray indicates no data available. (A) Sixty-five genes are up-regulated in response to •NO. Nine genes (yellow vertical bars) are persistently up-regulated throughout the 2-h time course in all strain backgrounds (EXPT 1–4). Fifty-six genes (blue vertical bar) are transiently up-regulated in *YHB1* intact strains (EXPT 1–3) and show prolonged induction in a *yhb1*Δ/*yhb1*Δ mutant strain (EXPT 4). An additional 34 genes (gray vertical bar) are only up-regulated in a *yhb1*Δ/*yhb1*Δ mutant strain (EXPT 4). (B) Sixty-five genes are down-regulated in response to •NO (orange vertical bar). In *YHB1* intact strains, these genes are transiently down-regulated at the 10-min time point and return to baseline expression levels by the 40-min time point (EXPT 1–3). In a *yhb1*Δ/*yhb1*Δ mutant strain, the majority of these genes are persistently down-regulated throughout the 2-h time course (EXPT 4).

diluted 30-fold into fresh medium. Cultures were allowed to recover for 4–5 h until they reached an OD (600 nm) of ~3.0. They were washed twice with 10-ml of sterile normal saline, and an aliquot was taken for quantification using a hemacytometer. To confirm the cell number, serial dilutions of the inocula were plated onto YEPD at 30°C, and CFUs were counted. In experiment one (see Figure 6A), the tail veins of BALB/c mice were injected with 3×10^5 CFUs in a total volume of 100–200-μl. In experiment two (Figure 6B), C57BL/6 NOS2 $^{+/+}$ or NOS2$^{-/-}$ mice were injected with 5×10^5 CFUs in a total volume of 300–400 μl. The mice were fed ad libitum, evaluated twice daily, and sacrificed when moribund as described by Noble and Johnson (2005). Data were analyzed by the Log Rank test (NCSS software) and $p \leq 0.05$ was considered significant.

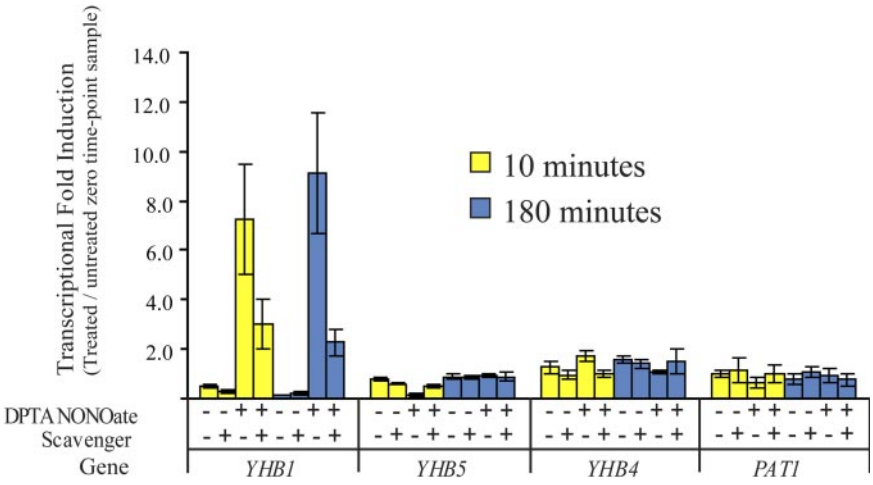

Figure 2. Fold induction of *C. albicans* flavohemoglobin genes (*YHB1, YHB4, YHB5*) in response to nitric oxide (•NO). The *PAT1* transcript was used as a control and reveals no induction by •NO. Samples were collected 10 (yellow) and 180 (blue) min after exposure to 1.0 mM DPTA NONOate, 8.0 mM Carboxy PTIO (Scavenger), or a combination of both chemicals. Addition of a chemical is indicated by a (+) and no-addition indicated by a (−). Three independent quantitative RT-PCR reactions were performed, and in each experiment, the signals were divided by an untreated zero time-point sample. The plot and error bars represent the average of the three experiments.

RESULTS

Genome-wide Expression Profile of C. albicans in Response to Nitric Oxide

To identify *C. albicans*' transcriptional response to nitric oxide (•NO), three different laboratory strains were treated with DPTA NONOate (Caymen Chemicals, Ann Arbor, MI), a chemical that releases •NO in a pH-dependent manner, and the transcriptional profile was monitored over a time course using *C. albicans* genomic DNA microarrays. The microarrays for this analysis contain 11,325 spots representing ~6550 protein-encoding nuclear genes, or 95% of the estimated number of *C. albicans* nuclear genes (Bennett *et al.*, 2003; Jones *et al.*, 2004). Many *C. albicans* genes are represented by more than one microarray spot, thereby allowing independent evaluation of gene expression in a single experiment. Time courses of •NO response were performed with CAF2–1, RM1, and RM1000 (see Table 1 for strain descriptions, and *Materials and Methods* for details on media supplementation of nutritionally auxotrophic strains). In all time courses, log-phase cells (OD 600 nm = 1.0) were treated with DPTA NONOate (1.0 mM), and DPTA NONOate-treated and untreated samples were collected 10, 40, 70, and 120 min after treatment. Changes in gene expression were normalized to an untreated zero-time-point sample.

As shown in Figure 1, the expression levels of ~131 *C. albicans* genes rapidly changed upon exposure to DPTA NONOate and, based on their response, these genes fall into three categories: 1) those transiently induced by •NO (Figure 1A, blue vertical bar), 2) those whose induction persisted throughout the time course (Figure 1A, yellow vertical bar), and 3) those repressed by •NO (Figure 1B, orange vertical bar). A fourth group of genes was induced in a *yhb1*Δ*/yhb1*Δ mutant treated with •NO (Figure 1A, gray vertical bar) and will be discussed later. The 131 •NO induced or repressed genes are graphically displayed in Figure 1 and are listed in Table 2 and Supplementary Table 1.

Genes Up-regulated On Nitric Oxide Exposure

Approximately 65 genes show elevated expression (2- to 25-fold increase) 10 min after the addition of DPTA NONOate and return to baseline expression levels by the 40-min time point (Figure 1A, blue vertical bar). Many (~19) of these genes encode proteins, homologues of which have previously been implicated in protection against oxidative stress. These include 6 glutathione conjugating and modifying enzymes, 10 NADPH oxidoreductases/dehydrogenases, and the catalase gene (*CAT1*; see oxidative stress genes, Table 2B). This response makes conceptual sense because, as a potent inhibitor of mitochondrial oxidative phosphorylation, •NO leads to the reduction of O_2 and to the production of superoxide anion ($O_2^{-}\cdot$), peroxynitrite (ONOO−), and hydrogen peroxide (H_2O_2; Poderoso *et al.*, 1996; Carreras *et al.*, 2004). Many of the oxidative stress protection enzymes require iron as a cofactor, and it is perhaps not surprising that several genes that encode iron uptake systems are also transiently induced (see iron acquisition genes, Table 2B). Other genes induced transiently upon exposure to •NO include three genes involved in sulfur assimilation, two zinc finger transcription factors, at least seven transporters for oligopeptides, drugs, or heavy metals, and various other enzymes whose functional roles are not easily discerned (Table 2B). Twelve proteins transiently induced by •NO are either conserved proteins of unknown function or proteins that lack clear homologues in other genome sequences (Table 2B).

Although the expression of the majority of the genes induced by •NO at the 10-minute time point return to baseline levels of expression by 40 min, a group of nine genes remain highly expressed throughout the 2-h time course and in all strain backgrounds tested (Figure 1A, yellow vertical bar, EXPT 1–4, and Table 2A). We hypothesize that this set of genes is involved directly in combating the effects of •NO and that, once these genes are induced and exert their protective effects, the majority of the •NO-induced genes return to their preinduction levels (see accompanying article by Sarver and DeRisi, 2005). Of the nine persistently induced genes, the most highly expressed is *YHB1* (orf19.3707), which encodes a putative flavohemoglobin, a protein that has been shown to be important for detoxifying •NO in a variety of pathogens including *C. albicans* (Ullmann *et al.*, 2004). Although there are three putative flavohemoglobins in the *C. albicans* genome (*YHB1*, *YHB4*, and *YHB5*), only *YHB1* is induced by DPTA NONOate under the conditions tested here (log phase, 30°C, YEPD). Based on its homology to well-characterized flavohemoglobins, Yhb1 is a two-domain protein with an N-terminal globin heme-binding domain and a C-terminal flavin $FADH_2$-binding domain. Yhb1 likely requires heme as a cofactor, and we note that another persistently induced gene, *RBT5* (orf19.5636), encodes a

Table 2. Nitric oxide-induced genes (two-fold or greater in at least one column)

A. Transcripts persistently induced throughout time course in wild-type and *yhb1*Δ/*yhb1*Δ strains[a]

C. albicans ORF19 No.[b]	*C. albicans* name[c]	*S. cerevisiae* homolog[d]	Description/Function[e]	10 min WT[f,g]	120 min WT[f,g]	10 min *yhb1*Δ/Δ[f]
orf19.3707	*YHB1*	*YHB1*	Flavohemoglobin/nitric oxide dioxygenase	15.8	4.1	1.4
orf19.4773	*AOX2*	*AOX1*	Alternative oxidase	11.6	4.0	6.1
orf19.7313		*SSU1*	Sulfite transporter	7.3	3.9	9.5
orf19.3120		*YOL075C*	Ferric cation import ABC transporter	6.7	5.4	6.4
orf19.4816		*YMR209C*	Unknown function	5.1	1.7	3.1
orf19.4720		*CTR2*	Copper transporter	3.8	2.0	2.3
orf19.5636	*RBT5*		GPI-linked heme acquisition protein	3.1	4.7	2.6
orf19.4774	*AOX1*	*AOX1*	Alternative oxidase	2.6	1.8	2.3
orf19.3117			GPI-linked CFEM domain protein	2.3	2.1	2.4

B. Transcripts induced early in time course, prolonged induction in *yhb1*Δ/*yhb1*Δ strain only[h]

C. albicans ORF19 No.[b]	*C. albicans* name[c]	*S. cerevisiae* homolog[d]	Description/Function[e]	10 min WT[f,g]	120 min WT[f,g]	10 min *yhb1*Δ/Δ[f]
orf19.125	*EBP1*	*OYE2*	NADPH oxidoreductase	25.5	0.8	18.0
orf19.3132		*MSC2*	Zinc transporter	18.1	1.2	64.4
orf19.2693		*URE2*	Glutathione *s*-transferase	16.7	1.2	10.8
orf19.1149		*ETR1*	Mitochondrial 2-enoyl thioester reductase	12.5	1.6	14.4
orf19.4290		*TRR1*	NADPH thioredoxin-disulfide reductase	11.4	1.1	8.7
orf19.2262		*ZTA1*	NADPH quinone oxidoreductase	10.7	1.4	4.2
orf19.3433		*OYE2*	NADPH oxidoreductase	10.4	0.9	3.6
orf19.6398		*JLP1*	Iron dependent sulphonate dioxygenase	10.2	2.8	1.0
orf19.3443		*OYE2*	NADPH oxidoreductase	9.9	1.2	9.9
orf19.3131		*OYE3*	NADPH oxidoreductase	8.0	0.7	6.5
orf19.2601	*HEM1*	*HEM1*	5-Aminolevulinate synthase	6.8	1.7	10.1
orf19.5059	*GSH1*	*GSH1*	Glutamate-cysteine ligase	6.8	2.0	5.2
orf19.7374		*YAF1*	Zinc finger transcription factor	6.2	1.5	4.3
orf19.7042			No good BLAST homology	6.0	1.3	3.0
orf19.6229	*CAT1*	*CTA1*	Catalase	6.0	1.6	6.2
orf19.3395		*YHR048W*	Drug transporter	5.7	1.0	1.4
orf19.3122	*ARR3*	*ARR3*	Arsenite transporter	5.6	1.4	4.4
orf19.5785			No good BLAST homology	5.2	0.9	3.5
orf19.2356		*CRZ1*	Zinc finger transcription factor	5.0	1.6	1.7

(*Continues*)

heme-acquisition protein (Weissman and Kornitzer, 2004). Also persistently induced is orf19.3117 (called orf19.3119 in the *Candida* Genome Database), which encodes a protein with a CFEM motif and is homologous to characterized cell surface heme-binding proteins (Weissman and Kornitzer, 2004). Heme-based ·NO sensors have been described in signal transduction (Gilles-Gonzalez and Gonzalez, 2005), and it is possible that orf19.3117 is involved in *C. albicans* detection of ·NO. Other transcripts persistently induced by ·NO throughout the 2-h time course include *SSU1* (orf19.7313), which is homologous to the *S. cerevisiae SSU1* that mediates sulfite efflux from the cell (Park and Bakalinsky, 2000), and two alternative oxidases (orf19.4774/*AOX1*, orf19.4773/*AOX2*), which accept electrons from the ubiquinone pool of the electron transport chain and reduce molecular oxygen (O_2) to water (Huh and Kang, 1999, 2001). These Aox enzymes prevent pools of O_2 from being reduced to the toxic superoxide anion ($O_2^{-}\cdot$), and in effect, function to prevent ·NO-induced oxidative stress. We also see prolonged induction of *CTR2* (orf19.4720), which encodes a putative copper transporter; *YOL075C* (orf19.3120), which encodes a putative ferric cation transporter; and *YMR209C* (orf19.4816), which encodes a protein of unknown function that contains a GMP kinase domain.

When the genes induced by ·NO in *C. albicans* are compared with those in *S. cerevisiae* and *Histoplasma capsulatum*, some common patterns emerge, although the detailed responses are highly individual. For example, genes encoding catalase and iron acquisition proteins are up-regulated in all three organisms in response to ·NO (see accompanying articles by Nittler *et al.*, 2005; Sarver and DeRisi, 2005). In *C. albicans* and *H. capsulatum*, alternative oxidase enzymes are up-regulated and are likely functioning to prevent the formation of reactive oxygen intermediates (ROI). In *S. cerevisiae* and *C. albicans*, *YHB1* is highly induced by ·NO, yet *H. capsulatum* seems to lack this conserved flavohemoglobin altogether. In both *C. albicans* and *S. cerevisiae*, exposure to ·NO prolonged induction of only a few genes (nine in *C. albicans* to seven in *S. cerevisiae*). Of these latter genes, only two (*YHB1* and *SSU1*) are induced in both species.

Table 2. (Continued)

orf19.5770		*YGL114W*	Oligopeptide transporter	4.9	1.2	4.4
orf19.5635	*PGA7*		Putative GPI-anchored protein	4.6	2.5	1.9
orf19.4370			No good BLAST homology	4.5	1.1	2.9
orf19.5517		*ADH7*	NADPH alcohol dehydrogenase	4.2	1.2	6.1
orf19.7091			No good BLAST homology	4.0	1.0	3.4
orf19.4147		*GLR1*	Glutathione reductase	3.9	1.4	4.2
orf19.6586		*YJR115W*	Conserved ORF/Function unknown	3.6	1.6	1.2
orf19.7417	*TSA1*	*TSA1*	Thiol peroxidase	3.6	1.1	3.7
orf19.5674	*PGA10*	*CSA1*	Heme utilization protein	3.2	1.9	1.2
orf19.6478	*YCF1*	*YCF1*	Glutathione *s*-conjugate transporter	3.1	1.3	2.0
orf19.113			Cadmium-induced CIP1 like protein [i]	3.1	0.9	3.8
orf19.711			No good BLAST homology	3.1	1.0	3.1
orf19.1763		*YNL134C*	NADPH alcohol dehydrogenase	3.0	0.7	3.0
orf19.4757		*NAR1*	Nuclear prelamin A recognition factor	3.0	1.4	2.6
orf19.4907		*YCR061W*	Conserved ORF/Function unknown	2.8	1.0	3.8
orf19.5258			No good BLAST homology	2.7	1.3	1.6
orf19.7214		*YBR056W*	Glucan 1,3-β-glucosidase[j]	2.7	1.4	1.6
orf19.5713		*NDE1*	NADH dehydrogenase	2.7	1.0	3.5
orf19.8434		*SSY1*	Amino acid binding protein	2.7	1.3	2.7
orf19.4802	*FTH1*	*FTH1*	Iron transporter	2.6	1.3	2.4
orf19.7316			Conserved ORF/Function unknown	2.5	0.9	3.6
orf19.6928	*SAP9*	*SAP9*	Secreted aspartyl proteinase	2.5	1.4	3.6
orf19.3432		*YCR023C*	Conserved ORF/Function unknown	2.5	1.1	2.2
orf19.3803		*MNN2*	Mannosyltransferase	2.5	1.2	1.8
orf19.239		*STH1*	ATPase activity/DNA helicase activity	2.5	0.9	4.3
orf19.1343		*DRE2*	Conserved ORF/Function unknown	2.5	1.6	1.8
orf19.4754	*ZWF1*	*ZWF1*	Glucose-6-phosphate dehydrogenase	2.5	1.1	1.6
orf19.2165			No good BLAST homology	2.4	1.1	1.9
orf19.5634	*FRP1*	*FRE5*	Ferric reductase	2.4	1.0	2.6
orf19.1027		*PDR16*	Phosphatidylinositol transporter	2.3	1.3	2.5
orf19.6947		*GTT1*	Glutathione transferase ($4e^{-04}$) [k]	2.3	1.2	1.5
orf19.5604	*MDR1*	*FLR1*	Multidrug efflux pump	2.3	1.2	4.4
orf19.2179	*SIT1*	*ARN1*	Ferrichrome siderophore transporter	2.2	1.0	2.0
orf19.4150		*YBR014C*	Glutaredoxin	2.2	1.0	3.0
orf19.2175		*CPD1*	NADH or NADPH oxidoreductase	2.2	1.4	1.4
orf19.7495		*OYE3*	NADPH dehydrogenase	2.1	1.2	4.9
orf19.2995		*LOT5* ($2e^{-07}$)	Low temperature responsive protein	2.1	1.1	3.5
orf19.3538		*CFL1*	Ferric reducatase	1.9	1.1	2.7

[a] See yellow vertical bars in Figure 1A.

[b] Unless noted, all ORF19 number designations were taken from the *Candida* Genome Database (CGD; http://www.candidagenome.org/).

[c] Gene names taken from CGD for named *C. albicans* genes.

[d] Gene names taken from the *Saccharomyces* Genome Database (SGD) for named *S. cerevisiae* homologs (http://www.yeastgenome.org/).

[e] Unless noted, all gene descriptions and functions taken from published sources, or 1) CGD for named *C. albicans* genes or 2) SGD when no CGD description present and closest homolog is a *S. cerevisiae* gene.

[f] If more than one microarray spot corresponded to an ORF, the average of the fold changes is represented.

[g] Average fluorescence signal of CAF2–1, RM1, and RM1000.

[h] See blue vertical bar in Figure 1A.

[i] *Schizosaccharomyces pombe.*

[j] Similarity to *C. albicans* protein.

[k] BLAST score from *Vibrio vulnificus.*

Functional Processes:

Oxidative stress
Iron acquisition
Membrane transport
Transcription
Respiration
Sulfur metabolism

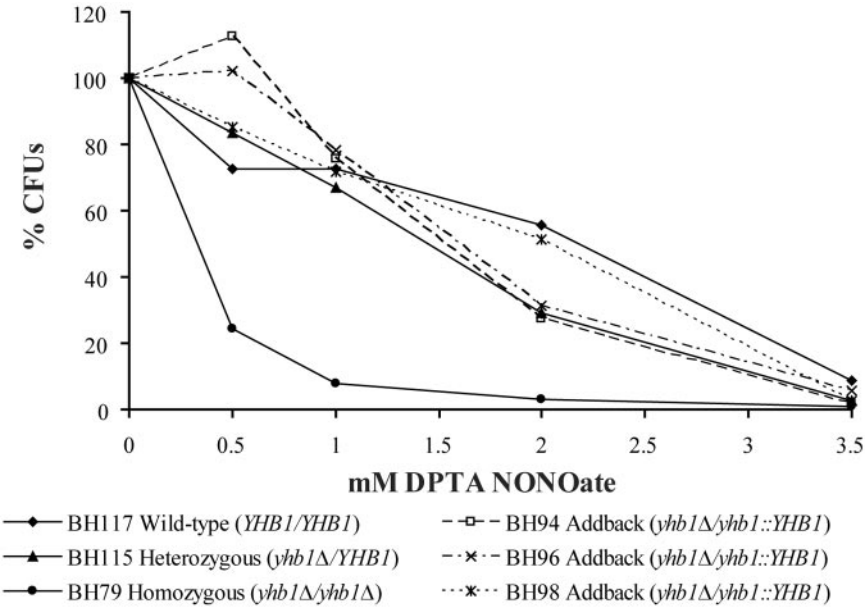

Figure 3. The *yhb1*Δ/*yhb1*Δ mutant is hypersensitive to nitric oxide (·NO) in vitro. *C. albicans* cultures were exposed to five different concentrations of DPTA NONOate (0.0, 0.5, 1.0, 2.0, 3.5 mM) and after 8 h of exposure, were serial diluted and plated. After 24 h of incubation, colony-forming units were counted. Wild-type (BH117), *yhb1*Δ/*YHB1* heterozygous (BH115), and *yhb1*Δ/*yhb1::YHB1* addback (BH94, BH96, BH98) strains showed comparable survival at low concentrations of DPTA NONOate (0.5 and 1.0 mM); the *yhb1*Δ/*yhb1*Δ mutant (BH79, ●), however, is hypersensitive to ·NO at these concentrations. At higher concentrations of DPTA NONOate (3.5 mM), all strains are sensitive.

Genes Down-regulated on Nitric Oxide Exposure

Approximately 65 genes are repressed upon addition of DPTA NONOate and return to normal levels of expression by the 40-min time point (Figure 1B and Supplementary Table 1). Twenty-five of these genes encode proteins of the mitochondrial electron transport chain, many of which contain prosthetic groups that are known to react with ·NO, such as heme, Fe-S, and Cu^{2+} (see respiration genes, Supplementary Table 1). Specifically, ·NO has been shown to reversibly inhibit the cytochrome *c* oxidase enzyme (Cox), to block cellular respiration (Carreras *et al.*, 2004) and to markedly increase the production rate of $O_2^{-}\cdot$ and H_2O_2 (Poderoso *et al.*, 1996). Examples of proteins involved in respiration whose expression was transiently down-regulated by ·NO include 2 subunits of the NADH dehydrogenase complex I, 4 subunits of the succinate dehydrogenase complex II, 12 subunits of the ubiquinone-cytochrome *c* oxidoreductase complex III, including cytochrome *c* itself, and four subunits of the cytochrome oxidase complex IV (see respiration genes, Supplementary Table 1). Also included in the set of ·NO repressed genes are 17 ribosomal proteins, representing about one third of the total number of *C. albicans* annotated ribosomal genes (see ribosomal components, Supplementary Table 1). The repression of ribosomal genes was also observed upon phagocytosis of *C. albicans* by macrophages (Lorenz *et al.*, 2004) and has also been observed in *S. cerevisiae* upon exposure to various stresses and has been characterized as part of the general stress response in that organism (Gasch *et al.*, 2000).

The Induction of YHB1 Is a Result of Nitric Oxide

We further investigated the induction of *YHB1* because, of all the genes persistently induced by ·NO, *YHB1* showed the highest induction ratio and is known to have a role in detoxifying ·NO in many microorganisms. To verify that induction of the *YHB1* transcript results from exposure to the ·NO released by DPTA NONOate and not some other part of the molecule, we added the ·NO scavenging molecule Carboxy PTIO to the induction experiments. For the experiment in Figure 2, DPTA NONOate alone, scavenger alone, or DPTA NONOate and scavenger together (at eightfold molar excess over DPTA NONOate) were added to log phase cultures (OD 600 nm = 1.0) of the *C. albicans* wild-type strain CAF2–1. Samples were collected 10 min and 2 h after treatment and analyzed by quantitative RT-PCR. As shown in Figure 2, the induction of *YHB1* mRNA was substantially blocked upon addition of the scavenger, supporting the conclusion that *YHB1* induction results from exposure to ·NO (or another RNI product) and not the DPTA NONOate backbone. mRNA of *PAT1* (orf19.3792), which is homologous to a topoisomerase II-associated protein in *S. cerevisiae*, was used as a control in this experiment and shows no induction by ·NO. These data confirm a previous report showing that *YHB1* is induced by ·NO, as demonstrated by its induction in response to three different sources of ·NO: gaseous ·NO, DETA NONOate, and sodium nitrite (Ullmann *et al.*, 2004). Figure 2 also confirms that, of the three putative *C. albicans* flavohemoglobins, only the *YHB1* gene is induced by nitric oxide; under the conditions tested, expression of *YHB4* and *YHB5* is detectable, but is not affected by the presence of ·NO.

The C. albicans yhb1Δ/yhb1Δ Mutant Displays Increased Sensitivity to Nitric Oxide In Vitro

To study the biological role of *YHB1*, we deleted both copies of the gene in the lab strain RM1000. We also constructed a heterozygous strain, as well as a *YHB1* addback strain in which a wild-type allele of *YHB1* was reintroduced into the homozygous deletion mutant. All strains are described in the *Materials and Methods* section and listed in Table 1. Parental and mutant cultures were exposed to five different concentrations of DPTA NONOate (0.0, 0.5, 1.0, 2.0, and 3.5 mM) and after 8 h of exposure were serially diluted and plated. After 24 h of incubation at 30°C, CFUs were counted. As shown in Figure 3, the *yhb1*Δ/*yhb1*Δ strain (BH79) was significantly more sensitive to ·NO than a genetically matched wild-type strain (BH117), a heterozygous *yhb1*Δ/*YHB1* strain (BH115), and three different gene addback strains *yhb1*Δ/*yhb1::YHB1* (BH94, BH96, BH98). The results of Figure 3 show that the *YHB1* addback strains, where one copy of *YHB1* has been added back to its endogenous locus, have reacquired the ability to survive nitrosative challenge

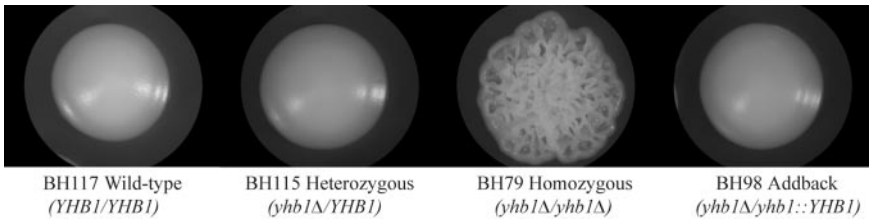

Figure 4. The *yhb1*Δ*/yhb1*Δ mutant is hyperfilamentous under nonfilamenting conditions. Wild-type (BH117), *yhb1*Δ*/YHB1* heterozygous (BH115), *yhb1*Δ*/yhb1*Δ mutant (BH79), and *yhb1*Δ*/yhb1::YHB1* addback (BH98) strains were tested for filamentation defects on YEPD at 37°C.

and that expression of one copy of *YHB1* is sufficient for protection against •NO in vitro. We note that growth of the *yhb1*Δ*/yhb1*Δ strain (BH79) was not significantly sensitive to oxidative stress, as measured by exposure to both hydrogen peroxide (H_2O_2) and the superoxide anion ($O_2^{-}\cdot$) generator menadione (unpublished data). Consistent with this observation, Ullmann *et al.* (2004) reported that *YHB1* is not induced by H_2O_2 or $O_2^{-}\cdot$.

*Genome-wide Expression Profile of the yhb1*Δ*/yhb1*Δ *Mutant in Response to Nitric Oxide*

We next investigated the transcriptional response of the *yhb1*Δ*/yhb1*Δ deletion strain to •NO (Figure 1A, EXPT 4). Although the response of the *yhb1*Δ*/yhb1*Δ strain generally resembles that of wild-type cells, it differs in three important respects: 1) genes that show only transient induction or repression in wild-type cells show prolonged changes in the *yhb1*Δ*/yhb1*Δ strain, 2) genes that are persistently induced in wild-type cells continue to increase in expression throughout the time course in the *yhb1*Δ*/yhb1*Δ strain, whereas they level off in the wild-type strains, and 3) a new set of 34 genes is induced in the *yhb1*Δ*/yhb1*Δ strain. We believe that most of these effects arise because deletion of the *YHB1* gene removes a major source of •NO detoxification; hence the deletion mutants are in essence subject to significantly higher intracellular levels of •NO. These results also indicate that •NO is produced throughout the time course of the experiment and that in wild-type cells the protective effects of *YHB1* are responsible for the rapid return of the transiently induced and repressed genes to their normal levels. The results with the *yhb1*Δ*/yhb1*Δ mutant are also consistent with the observation made in *S. cerevisiae* that the magnitude of an environmental stress is correlated with the time required to transcriptionally adapt to it (Gasch *et al.*, 2000).

The new class of 34 genes encodes proteins required for repair of DNA damage, two transcription factors that may regulate genes involved in protecting against •NO, multiple transporters, iron acquisition proteins, and additional oxidative stress proteins (Figure 1A, gray vertical bar, and Supplementary Table 2). It is possible that these genes are subtly induced in the wild-type time courses and that loss of *YHB1* simply enhances this effect. The induction pattern reveals that *YHB1* itself is not required for either the transient or persistent gene induction produced by •NO. It should be noted that, in the *yhb1*Δ*/yhb1*Δ strain, no induction of the *YHB4* or *YHB5* transcripts was observed by microarray analysis or quantitative RT-PCR (unpublished data), indicating that loss of the Yhb1 protein is not compensated by enhanced expression of the other *YHB* genes.

*The C. albicans yhb1*Δ*/yhb1*Δ *Mutant Is Hyperfilamentous under Nonfilamenting Conditions*

Wild-type (BH117), *yhb1*Δ*/yhb1*Δ (BH79), a strain heterozygous for *YHB1* (BH115), and three *YHB1* addback strains (BH94, BH96, BH98) were tested for a series of additional phenotypes, and a defect in filamentation was observed for the *yhb1*Δ*/yhb1*Δ strain. All strains appeared similar to wild type on YEPD + 10% serum at 37°C and Spider at 30°C, two conditions that strongly induce filamentous growth (unpublished data). However, ~90% of the *yhb1*Δ*/yhb1*Δ (BH79) colonies exhibited filamentous growth on YEPD at 37°C, a condition where the wild type and a heterozygous deletion strain did not form filaments (Figure 4).

We note that only one of the three *YHB1* addback strains tested (BH98) showed complete reversal of the filamentation defect (Figure 4). Using an RT-PCR-based methodology, we determined that, under a variety of conditions (YEPD at 30°C, YEPD at 37°C, and YEPD + •NO at 30°C) the addback strains had consistently lower levels of *YHB1* than either the *YHB1/YHB1* or *yhb1*Δ*/YHB1* strains (unpublished data). Of the three addback strains, BH98 had the highest levels of *YHB1* expression (unpublished data). We therefore chose BH98, an addback strain that did show full complementation of both the filamentation and •NO induced lethality defects, for subsequent analyses in mice.

*Genome-wide Expression Profile of the yhb1*Δ*/yhb1*Δ *Mutant Compared with Wild-type in the Absence of Nitric Oxide*

The hyperfilamentous phenotype of the *yhb1*Δ*/yhb1*Δ strain was further investigated using microarray analysis. We compared the transcriptional profile of the *yhb1*Δ*/yhb1*Δ mutant (BH79) and the otherwise isogenic wild-type strain (BH117) under two conditions, YEPD at 30°C and YEPD at 37°C. Competitive hybridizations were performed on microarrays, and the results are shown in Figure 5. Compared with wild-type cells grown in YEPD at 37°C, eight genes were induced greater than sixfold in the *yhb1*Δ*/yhb1*Δ mutant grown under the same conditions. The majority of these have been shown to be part of the filamentous growth program in *C. albicans* (Nantel *et al.*, 2002; Kadosh and Johnson, 2005), supporting the observation that at least part of the filamentous pathway is inappropriately activated in the *yhb1*Δ*/yhb1*Δ strain.

*The yhb1*Δ*/yhb1*Δ *Mutant Is Attenuated in Virulence*

We utilized a murine model of systemic candidiasis to test whether the *yhb1*Δ*/yhb1*Δ strain has a virulence defect compared with its parental strain and the *YHB1* addback strain. In all strains (BH79, BH98, BH117), *URA3* was restored to its native locus, thereby controlling for complications that can arise from *URA3* position effects (Lay *et al.*, 1998; Cheng *et al.*, 2003; Brand *et al.*, 2004).

Ten immunocompetent female BALB/c mice per strain (BH79, BH98, and BH117) were injected with 3×10^5 *C. albicans* cells. Figure 6A shows that mice infected with the *yhb1*Δ*/yhb1*Δ mutant strain (BH79) lived significantly longer than mice infected with either the *YHB1* addback strain (BH98) or wild-type strain (BH117; $p = 0.0015$ and 0.0002, respectively). At day 28 the experiment was ended and the six remaining mice infected with the *yhb1*Δ*/yhb1*Δ strain (BH79) were sacrificed. Although some of these remaining

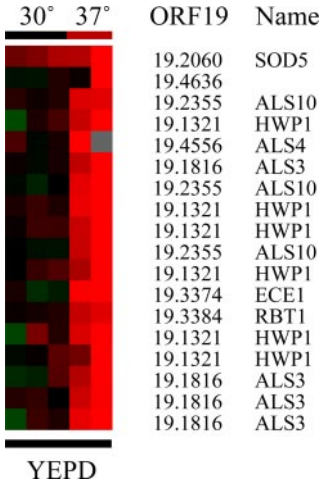

Figure 5. Hyphal-specific genes are induced in the *yhb1*Δ/*yhb1*Δ mutant in YEPD at 37° in the absence of nitric oxide (•NO). Genes induced over sixfold in the *yhb1*Δ/*yhb1*Δ mutant (BH79) are shown (mutant (BH79) transformed over wild type (BH117)). The induction of hyphal-specific transcripts supports the hyperfilamentous plate phenotype of the *yhb1*Δ/*yhb1*Δ mutant (see Figure 4).

mice had initially shown signs of infection, by the end of the experiment all appeared healthy and some had gained weight. Two additional experiments revealed similar trends in virulence (unpublished data); that is, in all three experiments, the *yhb1*Δ/*yhb1*Δ strain showed reduced virulence compared with the wild-type (BH117) and *YHB1* addback (BH98) strains. We note that the virulence defect of the *yhb1*Δ/*yhb1*Δ mutant is moderate compared with those of other published virulence mutants (see, for example Buurman *et al.*, 1998; Braun *et al.*, 2000). Nevertheless, the defect is statistically significant and reproducible. Complementation of the virulence defect by restoration of a single wild-type copy of *YHB1* confirms that the virulence defect of the *yhb1*Δ/*yhb1*Δ knockout strain is dependent on *YHB1*. The doubling times of the *yhb1*Δ/*yhb1*Δ strains grown at 30°C in YEPD medium were comparable to that of wild-type when measured in vitro (unpublished data), suggesting that the virulence attenuation does not result from a nonspecific growth defect. Attenuated virulence of a *yhb1*Δ/*yhb1*Δ mutant relative to wild type was also observed by Ullmann *et al.* (2004) using a set of independently derived strains.

The Virulence Defect of the yhb1Δ/yhb1Δ Mutant in the Tail Vein Model of Systemic Infection May Be Unrelated to Its Increased Sensitivity to Nitric Oxide

To determine whether the attenuated virulence of the *yhb1*Δ/*yhb1*Δ mutant was due to its hypersensitivity to nitrosative stress, we infected male knockout mice deleted for both copies of the *NOS2* gene, which encodes the inducible nitric oxide synthase (iNOS; NOS2; see *Materials and Methods* for experimental details). The increased susceptibility of NOS2$^{-/-}$ mice to numerous pathogens has been extensively documented (for reviews see Nathan and Shiloh, 2000; Fang, 2004). Furthermore, NOS2 is the only isoform that can be induced to yield high levels of •NO for sustained periods in immune cells (Lowenstein and Padalko, 2004). Thus, NOS2$^{-/-}$ mice have been used to determine the importance of •NO production for the control of various pathogens (see cited reviews above). The mice used in this experiment were of the C57BL/6 background, which is different from the BALB/c background used for the virulence experiment described above. Although the great majority of knockout mouse strains have the C57BL/6 genetic background, most published *C. albicans* virulence experiments have been performed in the BALB/c background. Genetically matched NOS2 $^{+/+}$ mice were used as controls.

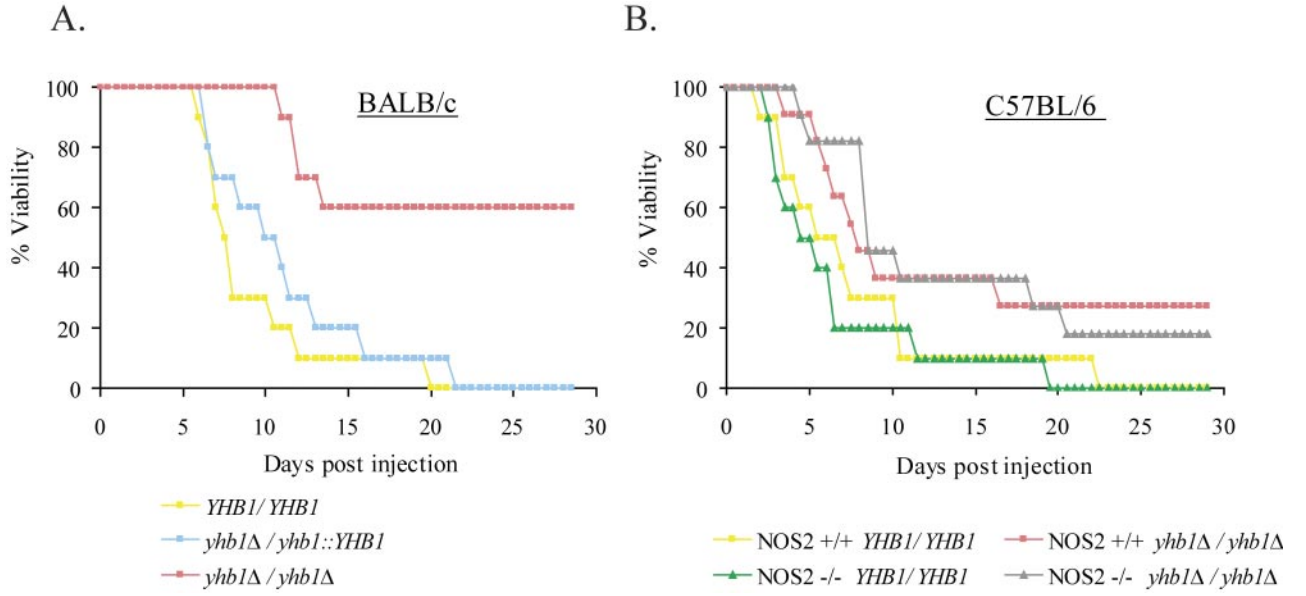

Figure 6. *YHB1* is important for virulence in a mouse tail vein model of systemic candidiasis. (A) Groups of 10 immunocompetent BALB/c mice were tail vein injected with 3×10^5 cells of wild-type (BH117, yellow squares), *yhb1*Δ/*yhb1*Δ mutant (BH79, pink squares), or *yhb1*Δ/*yhb1::YHB1* addback (BH98, blue squares) strains, and survival was monitored. (B) Groups of 10 or 11 congenic C57BL/6 NOS2$^{+/+}$ or NOS2$^{-/-}$ mice were tail vein injected with 5×10^5 cells of wild-type (BH117, yellow squares and green triangles) or *yhb1*Δ/*yhb1*Δ mutant (BH79, pink squares and gray triangles) strains, and survival was monitored.

The results of this experiment are shown in Figure 6B. First, we consider whether NOS2$^{-/-}$ and NOS2$^{+/+}$ mice show any difference in their susceptibility to wild-type *C. albicans*. As shown in Figure 6B (green triangles vs. yellow squares), there is no statistically significant difference between the two mouse strains in their susceptibility to *C. albicans* using the tail vein injection model ($p = 0.5088$, with $p < 0.05$ considered significant). We next consider the effects of deleting the *YHB1* gene from *C. albicans.* Both NOS2$^{+/+}$ and NOS2$^{-/-}$ mice (Figure 6B, pink squares and gray triangles) show a statistically significant increase in survival when infected with the *yhb1*Δ/*yhb1*Δ knockout strain compared with wild-type ($p = 0.0399$ and 0.0166, respectively, for NOS2$^{+/+}$ and NOS2$^{-/-}$). In other words, loss of *YHB1* results in a virulence defect that is not suppressed by removing the primary •NO synthetic machinery from the immune cells of the host. This observation implies that *YHB1* has an important function in virulence, measured by the tail vein model, which is distinct from protection against •NO. Given that other hyperfilamentous strains of *C. albicans* show virulence defects in the mouse tail vein injection model (Braun *et al.,* 2000, 2001; Kadosh and Johnson, 2001; Murad *et al.,* 2001), it is plausible that this property, as opposed to the increased sensitivity to •NO, is responsible for the decreased virulence of the *yhb1*Δ/*yhb1*Δ strain.

DISCUSSION

A major defense mechanism mounted against invading pathogens is the production of nitric oxide (•NO), a free radical that rapidly diffuses across cell membranes and is capable of reacting with a variety of molecules and causing multiple types of cell damage. The relationship between •NO and individual human pathogens is complex and has not been systematically investigated for *C. albicans*. However, available evidence supports the view that •NO is important for the control of *C. albicans* infections. For example, it has been reported that mice deficient in •NO production are hypersensitive to *C. albicans* infections as judged by organ load (Netea *et al.,* 2002). In addition, killing of *C. albicans* by murine saliva and macrophages has been shown, in some cases, to require *NOS2* (Elahi *et al.,* 2001; Netea *et al.,* 2002; Balish *et al.,* 2005).

Although the physiological importance of •NO in control of *C. albicans* requires further investigation, it has been well established that •NO production by the NOS2 enzyme is crucial for the control of other pathogens, including *Mycobacterium tuberculosis*, *Leishmania* spp. (for review see Nathan and Shiloh, 2000), and *Cryptococcus neoformans* (de Jesus-Berrios *et al.,* 2003). In the latter organism, it was shown that deletion of a flavohemoglobin gene (*FHB1*) results in attenuated virulence in wild-type mice that can be suppressed in infections of NOS2$^{-/-}$ mice (de Jesus-Berrios *et al.,* 2003).

Because of the established importance of •NO in the control of numerous other pathogens, we examined the genome-wide response of *C. albicans* to •NO. In this article, we identified 131 genes that are induced or repressed in wild-type *C. albicans* in response to •NO (see Figure 1, Table 2, and Supplementary Table 1). Based on their kinetic profiles, these genes fall into three classes: 1) transiently induced genes, 2) transiently repressed genes, and 3) persistently induced genes. As described in *Results*, we propose that the transiently induced and repressed genes primarily encode proteins involved in counteracting the secondary •NO-induced effects such as oxidative stress, whereas the persistently induced genes encode proteins that specifically protect against •NO. Transiently induced transcripts encode oxidative stress proteins such as glutathione-conjugating and -modifying enzymes, NADPH oxidoreductases/dehydrogenases, catalase, iron acquisition proteins, transcription factors, sulfur assimilation enzymes, transporters of oligopeptides, drugs, and heavy metals, and heme-binding proteins (see *Results* and Table 2B). Transiently repressed transcripts encode subunits of the mitochondrial electron transport chain and ribosomal proteins (see *Results* and Supplementary Table 1). Only nine genes are persistently induced by •NO; these include *YHB1*, two alternative oxidases, two putative cell surface heme-binding proteins, putative transporters for copper, sulfite, and iron, and a conserved protein of unknown function (see Table 2A). As described in the accompanying article (Sarver and DeRisi, 2005), two of these nine genes (*YHB1* and *SSU1,* a putative sulfite transporter) are also persistently induced when *S. cerevisiae* is exposed to •NO.

On deletion of the *YHB1* gene in *C. albicans*, the genome-wide expression profile in response to •NO is more pronounced. That is, the genes that show only transient induction or repression in wild-type strains show prolonged and enhanced changes in expression in the *yhb1*Δ/*yhb1*Δ deletion strain (Figure 1, EXPT 4). In addition, a cluster of 34 new genes showed significant induction only in the *yhb1*Δ/*yhb1*Δ strain (Figure 1, gray vertical bar); these genes encode proteins involved in the repair of DNA damage, as well as additional proteins involved in processes such as oxidative stress, iron acquisition, and transport (Supplementary Table 2). The prolonged and enhanced changes in •NO-induced gene expression observed upon removal of the *YHB1* gene, strongly supports the idea that Yhb1 is indeed functioning to detoxify •NO in *C. albicans*. Consistent with this idea, growth of the *yhb1*Δ/*yhb1*Δ mutant in vitro is much more sensitive to •NO than is wild type (Figure 3). This latter result was also reported by Ullmann *et al.* (2004), using an independently derived *yhb1*Δ/*yhb1*Δ mutant.

Also in agreement with Ullmann *et al.* (2004), we demonstrated that the *yhb1*Δ/*yhb1*Δ strain is moderately attenuated for virulence in BALB/c mice, as assessed by the mouse tail vein model of disseminated candidiasis (Figure 6A). To our surprise, however, the *C. albicans yhb1*Δ/*yhb1*Δ virulence defect was not suppressed in mice deleted for the *NOS2* gene (Figure 6B). This result implies that the virulence defect of the *yhb1*Δ/*yhb1*Δ mutant is not solely due to an inability to detoxify •NO and implicates an additional function as being responsible. One possibility is a role for *YHB1* in the control of filamentous growth. As described in the *Results* section, the *yhb1*Δ/*yhb1*Δ strain is hyperfilamentous, as observed as altered colony morphologies on laboratory media (Figure 4) and as the expression of "filament-specific" genes under conditions where they should be repressed (Figure 5). Several *C. albicans* hyperfilamentous mutants have been shown to have defects in virulence; these include *tup1*Δ/*tup1*Δ and *nrg1*Δ/*nrg1*Δ (both of which are severely hyperfilamentous) (Braun *et al.,* 2000, 2001; Murad *et al.,* 2001), as well as *rfg1*Δ/*rfg1*Δ (which has a mild hyperfilamentous phenotype similar to that of *yhb1*Δ/*yhb1*Δ; Kadosh and Johnson, 2001). It is also possible that the *yhb1*Δ/*yhb1*Δ strain has additional defects that render it less virulent. For example, *YHB1* is also induced in media rich in iron (Lan *et al.,* 2004), upon phagocytosis by macrophages (Lorenz *et al.,* 2004), and by sodium sulfite (our unpublished result). Thus, Yhb1 likely has roles in addition to protecting against nitrosative stress, and at least one of these additional functions must be required for full virulence in the mouse tail vein model of candidiasis.

Another unanticipated result concerns the susceptibility of mice lacking the *NOS2* gene to infection by *C. albicans*. We

found that NOS2$^{-/-}$ mice are not significantly more susceptible than immunocompetent mice to killing by *C. albicans*, as assessed by the tail vein model of infection (Figure 6B). These results indicate that NOS2 production of ·NO has little or no effect on the susceptibility of mice to *C. albicans* infection through this route. By introducing *C. albicans* directly into the venous system, this model bypasses, for example, mucosal and epithelial barriers, and thereby escapes a step where ·NO production may have a significant effect on the outcome of the infection. These observations suggest that the *yhb1*Δ*/yhb1*Δ mutant strain could be a useful tool to identify models of infection that do require ·NO for containing infections caused by *C. albicans* and, by implication, to identify the steps of infection at which ·NO is important for defense against this pathogen. Given the high conservation of the ·NO transcriptional response in *C. albicans* and *S. cerevisiae* (see accompanying article [Sarver and DeRisi, 2005]), it seems likely that both fungi routinely encounter concentrated levels of ·NO in their environment. Further studies employing the *C. albicans yhb1*Δ*/yhb1*Δ mutant strain should illuminate the host microenvironment where ·NO presents a serious threat for *C. albicans*.

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