EUKARYOTIC CELL, Feb. 2008, p. 268–278 Vol. 7, No. 2
1535-9778/08/$08.00+0 doi:10.1128/EC.00240-07

CTA4 Transcription Factor Mediates Induction of Nitrosative Stress Response in *Candida albicans*▽†

Wiriya Chiranand,[1] Ian McLeod,[2] Huaijin Zhou,[3] Jed J. Lynn,[1] Luis A. Vega,[1] Hadley Myers,[1] John R. Yates III,[2] Michael C. Lorenz,[3] and Michael C. Gustin[1]*

Department of Biochemistry and Cell Biology, Rice University, Houston, Texas[1]; Proteomic Mass Spectrometry Lab, Department of Cell Biology, Scripps Research Institute, La Jolla, California[2]; and Department of Microbiology and Molecular Genetics, The University of Texas Health Science Center, Houston, Texas[3]

Received 3 July 2007/Accepted 28 November 2007

This work has identified regulatory elements in the major fungal pathogen *Candida albicans* that enable response to nitrosative stress. Nitric oxide (NO) is generated by macrophages of the host immune system and commensal bacteria, and the ability to resist its toxicity is one adaptation that promotes survival of *C. albicans* inside the human body. Exposing *C. albicans* to NO induces upregulation of the flavohemoglobin Yhb1p. This protein confers protection by enzymatically converting NO to harmless nitrate, but it is unknown how *C. albicans* is able to detect NO in its environment and thus initiate this defense only as needed. We analyzed this problem by incrementally mutating the *YHB1* regulatory region to identify a nitric oxide-responsive element (NORE) that is required for NO sensitivity. Five transcription factor candidates of the Zn(II)2-Cys6 family were then isolated from crude whole-cell extracts by using magnetic beads coated with this DNA element. Of the five, only deletion of the *CTA4* gene prevented induction of *YHB1* transcription during nitrosative stress and caused growth sensitivity to the NO donor dipropylenetriamine NONOate; Cta4p associates in vivo with NORE DNA from the *YHB1* regulatory region. Deletion of *CTA4* caused a small but significant decrease in virulence. A *CTA4*-dependent putative sulfite transporter encoded by *SSU1* is also implicated in NO response, but *C. albicans ssu1* mutants were not sensitive to NO, in contrast to findings in *Saccharomyces cerevisiae*. Cta4p is the first protein found to be necessary for initiating NO response in *C. albicans*.

This study analyzes a key defense mechanism that allows *Candida albicans* to tolerate the toxic effects of nitric oxide produced from numerous sources in its mammalian host. *C. albicans* is a dimorphic fungus responsible for a considerable proportion of fungal infections in humans. A normal inhabitant of the digestive and genitourinary systems, it can switch from an innocuous yeast form into an invasive mycelial one. In addition to superficial infections like oral and vaginal thrush that can occur in relatively healthy individuals, patients with compromised immune systems may succumb to entrenched infections that can become deadly.

One process occurring in host mammalian systems and their commensal bacteria that limits the growth of microflora like *C. albicans* is the discharge of nitric oxide (NO), a reactive nitrogen species that is toxic at high concentrations. Consequently, *C. albicans* must tolerate NO generated from dietary nitrates and nitrites by bacteria in the oral cavity (37) or in the gut (45) or from nitrite-treated food moving through the digestive system (18), as well as NO nonenzymatically generated in the stomach, in urine, or on the skin. As an invasive pathogen, it typically encounters low levels of NO synthesized by many mammalian cell types, as well as higher concentrations of NO released by immune cells in host defense.

Like many microorganisms that are able to infect mammalian tissues, *C. albicans* can counter various immune system attacks. We previously reported that the flavohemoglobin Yhb1p in *C. albicans* detoxifies NO (50), a finding that was subsequently confirmed by Hromatka et al. (19). This enzyme acts as a nitric oxide dioxygenase; it neutralizes NO toxicity by incorporating O_2 to produce nitrate (NO_3^-). Flavohemoglobins are part of the ancient family of globin proteins, which also includes the better-known hemoglobins of vertebrate blood cells and the myoglobins of muscle. Flavohemoglobins are characteristically composed of a single polypeptide with a single heme group in an N-terminal domain homologous to vertebrate and plant hemoglobins and a C-terminal domain containing flavin adenine dinucleotide and NADPH binding sites (39).

Homologous flavohemoglobins are predicted to occur in many other microorganisms on the basis of genome sequencing and experiments using PCRs (31). Flavohemoglobin function in NO response has been reported in many bacterial species, such as *Escherichia coli* (38), *Salmonella enterica* serovar Typhimurium (11, 46), *Yersinia pestis* (44), *Bacillus subtilis* (41), and *Erwinia chrysanthemi* (5), as well as the protozoan social amoeba *Dictyostelium discoideum* (20) and the fungi *Saccharomyces cerevisiae* (27), *Cryptococcus neoformans* (10, 12, 33), and *C. albicans* (19, 50). Under hypoxic conditions, the mitochondria respiratory chain of *S. cerevisiae* will use endogenous nitrite instead of oxygen as an electron acceptor, thereby generating nitric oxide internally (9). The Yhb1p flavohemo-

* Corresponding author. Mailing address: Department of Biochemistry and Cell Biology, Rice University, P.O. Box 1892, Houston, TX 77251-1892. Phone: (713) 348-5158. Fax: (713) 348-5154. E-mail: gustin@rice.edu.

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

▽ Published ahead of print on 14 December 2007.

globin redistributes to the mitochondria under hypoxic conditions and consumes nitric oxide there (8). Thus, flavohemoglobins protect against nitric oxide coming from either exogenous or endogenous sources.

Flavohemoglobins are also a central mechanism for pathogenic fungi to counteract nitrosative stress during infection (32). For example, *C. neoformans* cells lacking the *FHB1* flavohemoglobin show defects in NO consumption, increased inhibition of growth by NO, and decreased virulence (12). In *C. albicans*, similar results were observed in cells lacking the *YHB1* flavohemoglobin (19, 50). Studies of mice lacking the inducible nitric oxide synthase (NOS) show that *FHB1*-dependent virulence of *C. neoformans* requires this source of NO (12) while *YHB1*-dependent virulence of *C. albicans* does not (19). One interpretation of this latter result is that the role of *YHB1* in *C. albicans* virulence is independent of NO. However, the inducible NOS-dependent defense against systemic *C. albicans* infection via the tail vein route appears relatively small. Further examination of this question will require virulence assays in which *C. albicans* infection starts in mucosal tissues where NO levels are typically much higher.

Genome scale, inducible responses to NO in pathogenic fungi have been analyzed in *C. neoformans* (10, 33), *Histoplasma capsulatum* (35), and *C. albicans* (19). NO induces a large number of stress response genes in these fungi; the *C. albicans* study identified a gene set specifically induced by NO and not other stresses. Genes of this small set include (19) the *YHB1* flavohemoglobin and *SSU1*, a sulfite transporter whose *S. cerevisiae* homolog is involved in the NO response, thus signifying that *C. albicans* has a means to sense NO in its environment; this signaling pathway has yet to be characterized. The use of NO as a signaling molecule is widespread, and many animals possess NOSs. To date, several mechanisms of NO sensing and response have been elucidated in other organisms. For example, in mammals soluble guanylyl cyclase acts as a NO receptor and stimulates production of cyclic GMP (6). A number of bacterial species utilize various simple pathways in which the NO sensing protein also functions as the transcription factor which induces the nitrosative stress defense mechanism. In *E. coli*, for example, the NorR protein binds NO at its N-terminal domain and regulates expression of a nitric oxide reductase gene by binding DNA with a helix-turn-helix motif in its C terminus (16). However, little is known about fungal nitrosative stress response mechanisms, and homologs of pathway components found in other organisms seem to be absent in fungi. Thus far, a single fungal transcription factor involved in NO sensing has been found—the C2H2 zinc finger factor Fzf1p in *S. cerevisiae* (42).

NO signaling in *C. albicans*, however, appears to be governed differently. We have focused on uncovering components of an NO regulatory pathway in *C. albicans*. In this work, we have not attempted to distinguish between effects from direct action by NO and indirect action by NO-derived products. This study identifies a putative transcription factor that is involved in nitrosative stress response in *C. albicans* but is not homologous to Fzf1p. Cta4p, a previously uncharacterized DNA-binding protein of the Zn(II)2-Cys6 family of fungal transcription factors, is necessary for induction of *YHB1* transcription when *C. albicans* encounters nitrosative stress.

MATERIALS AND METHODS

Strains and culturing. Strains used in this project are listed in Table S2 in the supplemental material. (Strains used in regulatory region analysis are derived from RM1000 [34]; deletion strains and the corresponding complementation strains were constructed from strain SN152 or SC5314.) *C. albicans* strains were routinely grown in either YPD (1% yeast extract, 2% peptone, 2% dextrose) medium for Ura^+ strains or in YPD medium plus uridine (1% yeast extract 2% peptone, 2% dextrose, and 40 μg/ml uridine) for Ura^- strains. YPD medium used with the NO donor dipropylenetriamine NONOate (DPTA NONOate) was adjusted to pH 7.4 and filter sterilized. *C. albicans* transformants were grown on selective synthetic medium (2% dextrose, 6.7% yeast nitrogen base with ammonium sulfate, and appropriate auxotrophic supplements).

Construction of linker scanning regulatory region mutants. The *YHB1* regulatory region-open reading frame (ORF)-*lacZ* constructs were created from the pAU95 plasmid (49). Genomic DNA of *C. albicans* strain RM1000 was amplified by PCR using TripleMaster polymerase (Eppendorf) in HighFi buffer and the primers *YHB1*-5′-F and *YHB1*-ORF-R. Primers used in constructing linker scanning mutant strains are listed in Table S3 in the supplemental material. The reaction parameters were as follows: 94°C for 2 min; then 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 2 min; and finally 72°C for 10 min. The PCR product and the pAU95 plasmid were each digested with Acc65I and PstI, gel purified, and ligated together. The regulatory region of the new pAU95-*YHB1-lacZ* plasmid was sequenced to check sequence fidelity. Since *C. albicans* is diploid, each gene has two alleles. To ensure that the mutant regulatory regions were compared for the same allele (orf19.3707) each time, mutant regulatory regions were created by PCR amplification from pAU95-*YHB1-lacZ.* Substitution mutations were made in two consecutive rounds of PCR. First, two reactions were carried out in parallel to amplify the *YHB1* regulatory region and ORF DNA on either side of the intended mutation, with the substituted nucleotides on a linker segment of the internal primers. Second, the two PCR products were fused and amplified in a third reaction. The reaction parameters were as follows: the fusion reaction was incubated at 94°C for 2 min, which was followed by 2 cycles of 94°C for 45 s, 40°C for 45 s, and 72°C for 2 min. Then, end primers *YHB1*-5′-F and *YHB1*-ORF-R were added, and the amplification reaction was carried out as follows: 94°C for 2 min; 30 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 2 min; and finally 72°C for 10 min. The final PCR product and the vector pAU95-*YHB1-lacZ* were each digested with Acc65I and PstI, and the appropriate fragments were ligated together. Plasmids containing the substitution mutation were identified by a new restriction site (XhoI, PmeI, or MfeI) and confirmed by DNA sequencing. The plasmids were each transformed into strain RM1000 using a lithium acetate method (17). Each plasmid was targeted to the intergenic region downstream of the *HWP1* gene by digesting the reporter gene at the PshAI site within the 3′ untranslated region (UTR) of *HWP1* on the plasmid and then transforming the linearized plasmid. Insertion at the correct location in the genome was verified by PCR amplifying the chromosome-plasmid junctions using two pairs of primers: the pair Msub border F1 and Msub border R1 and the pair Msub border F2 and Msub border R2. Reaction parameters were as follows: 94°C for 2 min; then 35 cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 2 min; and finally 72°C for 8 min.

β-Galactosidase assays. Procedures were modified from Kaiser et al. (22). Overnight cultures were diluted to an optical density at 600 nm (OD_{600}) of 0.08 in 3.0 ml of YPD medium and grown for 3 h at 37°C in a tissue culture rotator. Sodium nitrite ($NaNO_2$) dissolved in water was added to a final concentration of 10 mM, or an equal volume of water alone was added as a control. Cultures were incubated at 37°C for 1 h. Then, cells were pelleted by centrifugation, the supernatant was aspirated off, and 0.8 ml of Z buffer, 80 μl of 0.1% sodium dodecyl sulfate (SDS), and 120 μl of chloroform were added and mixed by vortexing. Samples were preincubated for 5 min at 37°C, and then 56 μl of a 1.355 mg/ml chlorophenol red-β-D-galactopyranoside solution in Z buffer (100 mM sodium phosphate, 10 mM KCl, 1 mM $MgSO_4$, 38 mM β-mercaptoethanol, pH 7.0) was mixed in. When color changed from yellow to dark orange, the reaction was stopped with 0.4 ml of 1 M $NaCO_3$. Cell debris was spun down by centrifugation, and β-galactosidase-catalyzed conversion of chlorophenol red-β-D-galactopyranoside to chlorophenol red was measured by absorbance at 578 nm in 1 ml of supernatant. Activity was expressed as β-galactosidase units, calculated as follows: $OD_{578}/[OD_{600}$ of assayed culture × volume assayed × time]. Each mutation was tested in at least three independent *C. albicans* transformant strains in three independent trials (minimum of nine data points). All trials were performed with wild-type controls, consisting of strains carrying the wild-type *YHB1* regulatory region and ORF joined to the *lacZ* reporter gene. Data were normalized in each trial to the mean activity measurements of induced wild-type controls.

Purification and identification of NORE-binding proteins. Procedures for isolating proteins using NO-responsive element (NORE) DNA-coated Dynabeads and their identification by tandem mass spectrometry are available in Data S4 in the supplemental material.

Gene deletions. Homozygous gene deletion mutants of *CTA4*, *STB5*, *WAR1*, *ZCF29*, *ZCF36*, and *YHB1* were constructed in strain SN152 using methods previously described (36). For primers used in constructing these deletions see Table S3 in the supplemental material. The ORF of each transcription factor gene was replaced with cassettes containing *Candida maltosa LEU2* and *Candida dubliniensis ARG4*, whose 5′ UTRs and ORFs were amplified by PCR from plasmids pSN40 and pSN69. Replacement of the first gene copy with the *C. maltosa LEU2* marker was confirmed by two PCRs using two pairs of primers: (gene) 5′ del check with Universal primer 5 and Universal primer 2 with (gene) 3′ del check, each amplifying a junction of the insert [where "(gene)" is the gene of interest]. Replacement of the second gene by Cd*ARG4* was verified by PCR of each junction again, but this time with primers specific to the Cd*ARG4* gene: (gene) 5′ del check with *ARG4* 3′ check-F (+1261) and *ARG4* 5′ check-R (+131) with (gene) 3′ del check. A negative test by PCR within the ORF DNA with the primers intracheck-F and intracheck-R specific for each gene verified that no copies of the genes remained in the genome.

The *ssu1*Δ/*ssu1*Δ mutant was constructed using the *SAT1*-flipper cassette (40). Briefly, a 300-bp fragment upstream of *SSU1* was amplified by PCR from genomic DNA and cloned between KpnI and XhoI sites on pSFS1. Next, a 300-bp fragment downstream of *SSU1* was amplified and cloned between the SacII and SacI sites on the same vector. The resulting plasmid was digested with KpnI and SacI to release the disruption cassette. The gel-purified disruption cassette was used to transform either SC5314 or the *cta4*Δ/*cta4*Δ mutant strain by electroporation; transformants were selected on YPD plates containing 100 μg/ml nourseothricin. Correct integration was confirmed by PCR. Nourseothricin-sensitive colonies were obtained following growth on yeast carbon base-bovine serum albumin medium to excise the cassette, as described previously (40), and confirmed by PCR. The second allele was disrupted using the same process.

Complementation strains. Complementation plasmids were developed from the plasmid CIp20 (13) (GenBank accession no. DQ015894). *CTA4* or *YHB1* was amplified from SN152 genomic DNA using the primer pair *CTA4* gene UP1 and *CTA4* gene DN1 or the pair *YHB1* gene UP1 and *YHB1* gene DN1. For each gene the region amplified included the putative regulatory region, the ORF, and the 3′ UTR. For primers used in constructing complementation strains, see Table S3 in the supplemental material. The *URA3* marker on the plasmid was replaced with either the *CTA4* gene or the *YHB1* gene by digesting the insert and vector with the restriction enzymes SacI and NheI, gel purifying the DNA fragments, and ligating them together. Complementation plasmids were transformed into the appropriate strain, *cta4*Δ/*cta4*Δ or *yhb1*Δ/*yhb1*Δ, and checked for integration into the desired location at the *RPS1* locus by PCR using primers CIp20-upcheck-F with CIp20-upcheck-R for one junction and CIp20-downcheck-F with CIp20-downcheck-R for the other junction. The *ssu1*Δ/*ssu1*Δ mutants were complemented by replacing the 5′ fragment of *SSU1* in the disruption plasmid with the complete ORF. This cassette was excised and used to transform the mutant strains. Correct integration into the *SSU1* locus was confirmed by PCR.

Growth inhibition assays. Overnight cultures were diluted to an OD_{600} of 0.07 and grown at 30°C for 2 h in YPD medium buffered with 50 mM sodium phosphate, pH 7.4. Cultures were divided into 3-ml aliquots. Freshly prepared DPTA NONOate (a polyamine NO donor with a half-life of 3 h at 37°C at pH 7.0 to 7.4) solutions were dissolved in the same medium and added to each aliquot to final concentrations of 0, 0.1, 0.2, 0.3, 0.4, 0.5, or 1 mM. Cultures were incubated at 30°C for 3.5 h, and cell density was compared by OD_{600} measurements. Data were normalized by setting the cell density measurement in the 0 mM DPTA NONOate condition as equal to 1. Remaining cell density measurements (at OD_{600}), recorded over the range of DPTA NONOate concentrations for each strain, were then expressed as a fraction of the maximum for that strain recorded during that particular trial. Three independent trials were performed on each set of strains.

Northern blotting. Overnight cultures were diluted to an OD_{600} of 0.08 and grown in YPD medium for 4 h at 30°C. DPTA NONOate (0 mM or 0.1 mM) or sodium nitrite (0 mM or 10 mM) was added, and cultures were incubated at 30°C for 15 min. The cultures were chilled in ice water, and cells were harvested by centrifugation. Cell pellets were snap-frozen in dry ice and ethanol and stored at −80°C. Total RNA was isolated by hot phenol extraction (43) except that phenol-chloroform-isoamyl alcohol (125:24:1), pH 4.3, was used for all phenol solutions indicated above. RNA (25 μg per lane) was electrophoresed, blotted onto a Hybond-N+ nylon membrane (GE Healthcare), probed with *YHB1* DNA labeled with [α-^{32}P]dATP, and then stripped in boiling 0.1% SDS and reprobed with *TEF1*. Blots were exposed to a phosphor imaging plate. Images were scanned with a Fujifilm FLA-50001R imager, and contrast was adjusted in some scans across the entire image by using Adobe Photoshop, version 10.0. For sequences of the primers used in PCRs to make probes see Table S3 in the supplemental material.

Construction of strains expressing epitope-tagged proteins. The plasmid pMGC2 was constructed to generate a core module for targeted insertion of DNA encoding nine copies of the Myc epitope into the protein coding region of *CTA4* and other *C. albicans* genes. (For sequences of the primers involved seeTable S3 in the supplemental material.) The *ACT1* 3′ UTR was digested from pAU36 (49) using BamHI and XbaI and ligated into pSN52 (Cd*HIS1*) (36) digested with BamHI and SpeI, producing pMGC1. DNA encoding nine copies of the Myc tag was amplified by PCR from pYM6 (24) using 9×Myc-UP and 9×Myc-DN primers, digested with BglII and HindIII, and ligated into pMGC1 digested with BamHI and HindIII, producing pMGC2. The fusion PCR procedure used for COOH-terminal tagging of Cta4p with Myc_9 is closely similar to that used by Noble and Johnson (36) for high-efficiency deletion of *C. albicans* genes by homologous recombination. The core module (B) containing Myc_9/*ACT1*-3′ UTR/Cd*HIS1* was amplified by PCR from pMGC2 using the linking primers 2 and 5. One DNA segment (A) of ~300 bp within the protein coding region of *CTA4* and just upstream of the *CTA4* stop codon was amplified by PCR from genomic DNA using primers *CTA4*-1 and *CTA4*-3. Another DNA segment (C) of ~300 bp just downstream of the *CTA4* stop codon and within the 3′ UTR of *CTA4* was amplified by PCR using primers *CTA4*-4 and *CTA4*-6. DNA segments A, B, and C were fused and amplified by PCR in one reaction using primers *CTA4*-1b and *CTA4*-6b. These b primers have the same 5′ end as the *CTA4*-1 and *CTA4*-6 primers, respectively, but are 5 to 10 nucleotides longer, allowing greater yield and purity of the final targeting module (A-B-C fusion product). The targeting module was transformed into a *CTA4*/*cta4*Δ::*LEU2* strain using a lithium acetate-based protocol (17), and successful targeting of the core module to *CTA4* was confirmed in transformants by Western blotting and PCR amplification of the 3′ junction (linking primer 2 and *CTA4* 3′ del check) and 5′ junction (primers CTA4 myc upcheck F and *CTA4* myc upcheck R). The Myc_9-tagged *CTA4* gene is fully functional because the DPTA-NONOate sensitivity of the heterozygous transformant was the same as the wild-type (SN152) strain while the growth of a *cta4*Δ/*cta4*Δ strain was inhibited when exposed to the NO-releasing drug.

ChIP assay. Chromatin immunoprecipitation (ChIP) assays were performed as described by Aparicio et al. (3) with some modifications. Briefly, 50-ml cultures were grown in YPD medium to an OD_{600} of ~1.0 to 1.2. Next, 10 mM sodium nitrite was added to induce nitric oxide response in half of the cultures for 15 min. Cells were then cross-linked with 1% formaldehyde for 20 min, washed with Tris-buffered saline, and lysed by bead beating in 1 ml of lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, and 0.1% SDS) with 2 mM phenylmethylsulfonyl fluoride. Chromatin was sheared to an average size of 500 bp by sonication with a Misonix Sonicator 3000 with Cup Horn. The input sample (100 μl) was taken, and DNA was extracted as described below. The remaining sample (900 μl) was incubated for 1.5 h with 50 μl of protein G resin (GenScript) and 20 μl of c-Myc (9E10) antibody (SantaCruz). Beads were washed three times with lysis buffer and eluted in 100 μl of elution buffer (50 mM Tris, pH 7.5, 10 mM EDTA, 1% SDS) at 65°C for 10 min. Cross-linking was reversed by a 2-h incubation with 10 μl of 10 mg/ml proteinase K at 42°C and then for 6 h at 65°C. DNA was purified by phenol-chloroform extraction and ethanol precipitation. PCR was carried out in 50-μl reaction volume using 1/10 of the immunoprecipitated DNA and 1/15 of 10% input DNA. The PCR primers (TAGGTTAAAGCGCTCCGGCATT TTCC and GTTTAATGGTTTTCCCGCTGATGCTCC) were designed to flank the NORE of the *YHB1* regulatory region. Samples were run through 26 PCR cycles and then separated on a 2.5% agarose gel. The gel was loaded with 22 μl of immunoprecipitation sample and 5 μl of input sample.

Reverse transcription-PCR. Overnight cultures were diluted to an OD_{600} of 0.05 and grown at 30°C for 1 h. DPTA NONOate was added to a concentration of 4 mM, and cultures were incubated for 5 h. Cells were collected by centrifugation and washed with ice-cold water. Cell pellets were frozen in dry ice and ethanol and stored at −80°C. Total RNA was isolated by hot phenol extraction (43). First-strand cDNA was synthesized according to the Invitrogen SuperScript II RT protocol. A 12-μl reaction mixture including 100 ng of RNA, 0.5 μg of oligo(dT), and 10 pmol of the deoxynucleoside triphosphates was heated at 65°C for 5 min, chilled on ice, and centrifuged. First-strand buffer and 0.1 M dithiothreitol were added, and the reaction mixture was incubated at 42°C for 2 min. SuperScriptII RT was added, and the reaction mixture was incubated at 42°C for 50 min and heat inactivated at 70°C for 15 min. The cDNA product was diluted

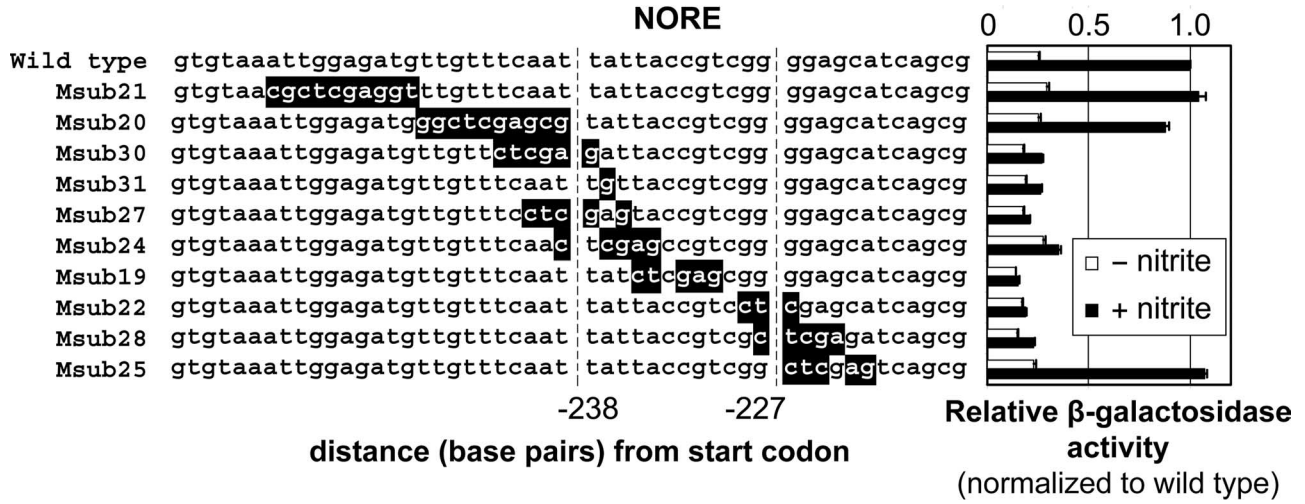

FIG. 1. NORE element position based on data from linker scanning substitution mutations of *YHB1* regulatory region. A close-up of the mutations made closest to the NORE is shown. β-Galactosidase activity (on right) was determined as described in Materials and Methods and normalized to activity of wild-type cells under 10 mM sodium nitrite induction. Substituted nucleotides of mutations Msub19 through Msub31 are highlighted in gray. Each strain was tested in three independent trials with at least three independent transformants. Error bar, ±1 standard error of the mean.

1:10 twice, and 2 μl of each dilution was used as a template in the PCR. Primers (for a list, see Table S3 in the supplemental material) were designed to amplify a ~300-bp internal fragment of *SSU1* or *ACT1* as a control.

In vivo virulence assays. Mouse virulence assays were performed as described previously (28). Adult, female, outbred ICR mice (21 to 25g) were obtained from Harlan. Cultures of *C. albicans* were grown in YPD medium to mid-exponential phase and collected by centrifugation. Cells were washed and resuspended at 1×10^7 cells/ml in phosphate-buffered saline, and mice were infected via tail vein injection with 10^6 *C. albicans* yeast-form cells. The group size was 10 mice per strain. Infected mice were subsequently monitored for signs of infection and euthanized when moribund, according to approved protocols. Survival data were analyzed with Prism 3 (Graphpad Software) using a log rank test. Statistical significance was defined as a *P* value of less than 0.05. All animal assays were conducted in accordance with protocols approved by the University of Texas Health Science Center Animal Welfare Committee.

RESULTS

Identification of a NORE in the *YHB1* regulatory region. NO exposure causes accumulation of *YHB1* mRNA (19, 50), and we investigated this effect to find a DNA-binding transcription factor involved in this signaling pathway. In *S. cerevisiae*, the regulatory region of a gene is typically immediately upstream of the ORF within a kilobase of DNA from the ATG start codon. *C. albicans* belongs to the same fungal family and seems to be similarly configured with compact regulatory regions. We analyzed the regulatory region of the *YHB1* gene to locate a *cis*-regulatory DNA element that could be a cognate binding site for a transcription factor.

Previously, it was found that the 1-kb region preceding the *YHB1* ORF could confer nitrite inducibility on a reporter gene (50). Preliminary evidence with our β-galactosidase reporter system indicated that a 479-bp region upstream of *YHB1* had similar activity levels as the 1-kb upstream sequence (data not shown). It was also found that substituting the *YHB1* 3′ flanking region for the *HWP1* 3′ UTR on the plasmid did not enhance the responsiveness of the *YHB1-lacZ* construct. Smaller 10-bp substitution mutations were developed to analyze the region −481 to −352 bp from the start codon, but these failed to show a loss of nitric oxide induction (see Fig. S5 in the supplemental material). The remaining 352 bp of the regulatory region was analyzed for potential *cis*-acting elements with the program ClusterBuster (15) (http://zlab.bu.edu/cluster-buster/), which compares selected or user-supplied transcription factor binding site matrices against a large background portion of DNA from the chosen organism. ClusterBuster finds possible binding sites based on the concept that regulatory regions are often patterned so that *cis*-acting elements are arranged in tight clusters. When we entered a 13-kb stretch (contig6-2060) of *C. albicans* DNA containing the *YHB1* ORF and selected all prelisted matrices for transcription factors on the page, the program identified one 21-bp segment at −228 to −208 bp from the start codon in our 352-bp area of interest, shown underlined here among DNA in its vicinity: CAATTATTACCGTCGGGGAGCATCAGCGGGAAAAC CATTAAACATGTTTTTT.

Mutants were constructed to test the activity of this region in the β-galactosidase reporter assay. The crucial nucleotides turned out to contain a palindromic sequence at the upstream edge of the 21-bp sequence. Testing more linker scanning substitution mutations—Msub14 through Msub31—with the reporter assay refined the boundaries of the NORE sequence to the 12-bp sequence TATTACCGTCGG essential for nitrite induction, at −238 to −227 bp from the start codon. Results from the most informative mutations of this set are shown in Fig. 1 (for the complete set, see Fig. S6 in the supplemental material). These mutations define the edges of the element and do not elucidate which internal base pairs were critical for induction in response to nitrosative stress. The analysis of the *YHB1* regulatory region for NO-responsive sequences was not exhaustive in the 352 bp upstream and closest to the start codon. Therefore, it remains possible that another *cis*-acting element required for NO-responsiveness is present in the *YHB1* regulatory region.

Five putative transcription factors bind to the NORE in vitro. Isolation of candidate proteins became feasible upon identification of mutations in the regulatory region which prevented nitrosative stress-specific induction of *YHB1* transcription. Transcription factor binding sites are typically 6 to 12 bp long in *S. cerevisiae*, so a 33-bp NORE sequence was conservatively chosen for purifying specific binding proteins by affinity. For comparison we also created another sequence, identical except for a 5-bp substitution mutation also used in the *YHB1* regulatory region mutant Msub19, which had been shown to be uninducible by nitrite in the β-galactosidase reporter assay (Fig. 1). Each sequence was biotinylated at the 5′ end and affixed to streptavidin M-280 Dynabeads (Dynal Biotech).

Our strategy for isolating proteins that bind to the NORE was largely based on an approach described by Kroeger and Abraham (25). The major changes made were using crude whole-cell extracts rather than nuclear extracts, starting the affinity binding purification with a 10-fold higher protein concentration, and visually selecting proteins on an SDS-polyacrylamide gel for mass spectrometry analysis based on their binding specificity. Whole-cell extract containing 20 mg of protein from *C. albicans* strain RM1000 was incubated with the beads in the presence of 10 mM sodium nitrite (an NO donor) and 3 nmol of competitor DNA, identical to the 5-bp substitution mutant DNA used on one set of beads. The beads were washed with buffer, and proteins attached to the affinity beads were then eluted in a high-salt solution. The eluate contents were separated by SDS-polyacrylamide gel electrophoresis and detected by Coomassie staining. A similar silver-stained polyacrylamide gel is shown for illustration (Fig. 2) because the higher contrast allows greater band visibility.

We selected five protein bands that appeared more abundant in the eluate from the wild-type NORE than in the mutant NORE eluate; only three of the five chosen bands are clearly visible in Fig. 2. Each of these five bands showed NORE selective enrichment in at least four independent preparations. We analyzed the protein bands by tandem mass spectrometry mass spectrometry using a linear quadrupole ion trap mass spectrometer (Thermo Electron).

Before mass spectrometry analysis, protein bands were first digested with trypsin, and high-performance liquid chromatography separated the peptide components within each band. Structural data were obtained by fragmenting the eight most intense peaks from each band using collision-induced dissociation. Spectra from a mass spectrometry scan of the resulting fragments were compared against predicted spectra generated by Sequest from sequences in the *Candida* Genome Database (http://www.candidagenome.org/). Five putative transcription factors were identified: Cta4p, Stb5p, War1p, Zcf29p, and Zcf36p. All five belong to the Zn(II)2-Cys6 zinc cluster family of fungal transcription factors, of which the best-studied member is *S. cerevisiae* Gal4p. The full list of proteins identified after DTASelect filtering of the mass spectrometry data is shown in Table S7 in the supplemental material. Because the eluates still contained a complex mixture of proteins and because one-dimensional electrophoresis cannot separate multiple proteins of similar sizes, it cannot be assumed that the proteins isolated and identified here have preferential binding to the NORE DNA.

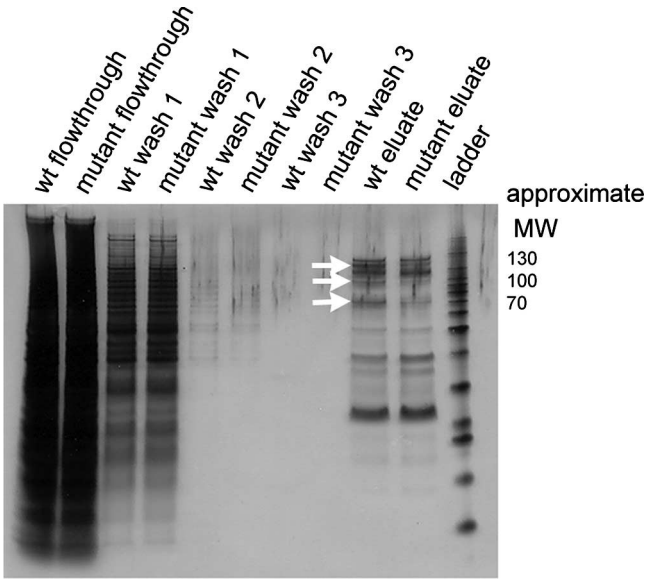

FIG. 2. Differential binding of proteins to wild-type and mutant NORE DNA sequences. Streptavidin-coated magnetic beads were attached to biotinylated DNA containing either the wild type or a mutant NORE and then incubated with 20 mg of whole-cell extract from *C. albicans* induced with 10 mM sodium nitrite. Lanes from left to right, 1 and 2, flowthrough after initial incubation with whole-cell extract and 3 nmol of competitor DNA; 3 and 4, wash 1 with buffer; 5 and 6, wash 2 with buffer and 1 μmol of poly(dI-dC) nonspecific competitor DNA; 7 and 8, wash 3 with buffer; 9 and 10, final high-salt eluate; 11, Benchmark ladder (Invitrogen). Three bands of proteins with greater affinity for wild-type NORE beads than mutant NORE beads are indicated by white arrows. Similar results were obtained in three other independent trials. MW, molecular weight (in thousands).

Growth of the *cta4*Δ/*cta4*Δ deletion mutant is hypersensitive to nitrosative stress. To find out whether any of the candidate proteins are biologically relevant in NO response, deletion strains were constructed for each of the transcription factors identified by mass spectrometry using a gene deletion system designed by Noble and Johnson (36). Both copies of the gene ORFs were removed entirely from the auxotrophic strain SN152 (*his1*Δ/*his1*Δ, *arg4*Δ/*arg4*Δ, and *leu2*Δ/*leu2*Δ) and replaced by homologous recombination with selective markers, *LEU2* and *ARG4*. A *yhb1*Δ/*yhb1*Δ strain was also created with the same markers for comparison. As a control, we created strain SLA1.1 from the SN152 reference strain by inserting the *LEU2* and *ARG4* marker cassettes at the site of the corresponding deleted native genes (i.e., the *ARG4* cassette went into the former location of one copy of *C. albicans ARG4*).

We then investigated whether the homozygous deletion mutants exhibit sensitivity to NO treatment. Previous studies demonstrated that proliferation ability of a *yhb1*Δ/*yhb1*Δ mutant decreased as concentrations of various NO donors increased (19, 50). Removing a transcription factor necessary for inducible *YHB1* expression is predicted to also cause hypersensitivity to NO, provided that the effect is not masked by functional redundancy. The deletion mutants were tested for their ability to grow in the presence of the NO donor DPTA NONOate. The growth rates of all five deletion mutants are similar to SN152 when grown in medium without DPTA NONOate. The five homozygous mutants were subjected to various concentra-

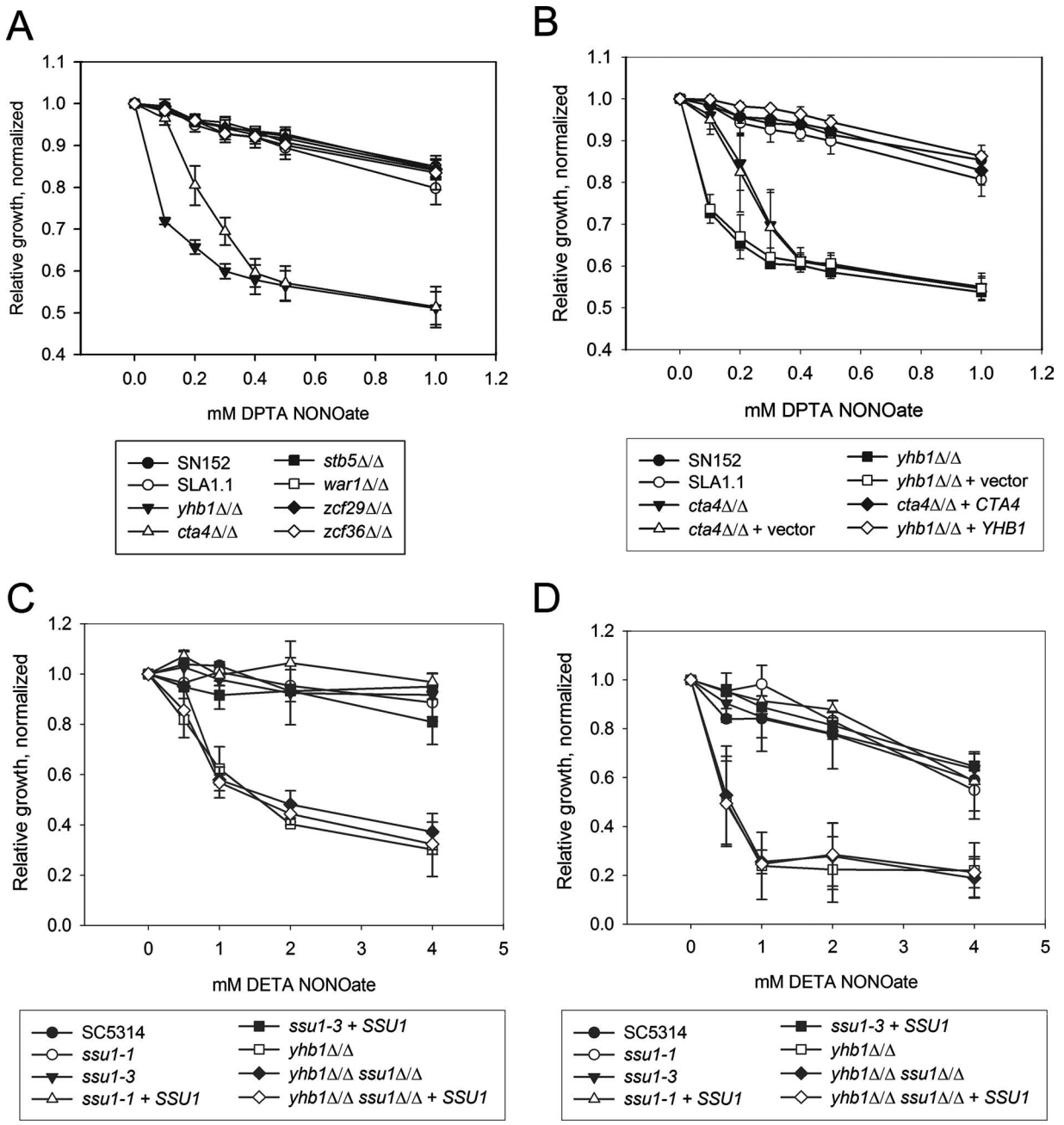

FIG. 3. Growth inhibition of *C. albicans* strains by NONOates. (A) Growth of the *cta4*Δ/*cta4*Δ deletion mutant is hypersensitive to the nitric oxide donor DPTA NONOate. Transcription factor deletion mutants *cta4*Δ/*cta4*Δ (written in the format *cta4*Δ/Δ), *stb5*Δ/*stb5*Δ, *war1*Δ/*war1*Δ, *zcf29*Δ/*zcf29*Δ, and *zcf36*Δ/*zcf36*Δ were tested with parental strain SN152 and isogenic wild-type SLA1.1. Cultures were exposed to DPTA NONOate concentrations of 0, 0.1, 0.2, 0.3, 0.4, 0.5, or 1.0 mM and were incubated at 30°C for 3.5 h. OD_{600} readings were then taken as a measure of cell density. Data have been normalized between trial repetitions by expressing cell density readings for a strain as a fraction of the 0 mM DPTA NONOate reading of that strain in each separate trial. Results are from four independent experiments. Error bar, ±1 standard deviation. Analysis of the data for 0.2 mM DPTA NONOate using one-way analysis of variance with Bonferroni's multiple comparison test shows that *cta4*Δ/Δ is significantly different than *yhb1*Δ/Δ ($P < 0.001$). (B) The *CTA4* and *YHB1* complementation strains recover resistance to the nitric oxide donor DPTA NONOate. Homozygous deletion strains with complementation plasmids (symbolized by *cta4*Δ/Δ + *CTA4* and *yhb1*Δ/Δ + *YHB1*) were tested for DPTA NONOate sensitivity along with the SN152 background strain, deletion strains *cta4*Δ/*cta4*Δ and *yhb1*Δ/*yhb1*Δ, and the deletion strains transformed with the plasmid vector CIp20 used for complementation. The experiment was conducted as described for panel A. Results shown are from three independent experiments. Error bar, ±1 standard deviation. (C) *SSU1* does not affect NO sensitivity in YPD medium. The *ssu1*Δ and *yhb1*Δ *ssu1*Δ, plus the *SSU1*-complemented controls and wild-type strains were grown for 4 h at 30°C in YPD medium with the indicated concentration of DETA NONOate. Cell densities were measured and expressed as a fraction of the control without DETA NONOate, as in panel A. Two independent *ssu1*Δ mutants and complemented strains were tested, and both behaved identically; for simplicity only one mutant is shown. The data are the average of two experiments. (D) *SSU1* does not affect NO sensitivity in synthetic complete medium. The experiment was conducted as in panel C, using synthetic complete medium.

tions of DPTA NONOate for 3.5 h, and cell density was measured by absorbance at 600 nm. For comparison, three other strains were tested simultaneously with the mutants: the parent strain SN152, matched wild-type SLA1.1, and *yhb1*Δ/*yhb1*Δ. Of all the putative transcription factor gene mutants, only the *cta4*Δ/*cta4*Δ mutant showed hypersensitivity to DPTA NONOate (Fig. 3A). The *cta4*Δ/*cta4*Δ phenotype is not as strong as that of *yhb1*Δ/*yhb1*Δ until the DPTA NONOate concentration reaches about 0.5 mM. We tested whether reinsertion of a wild-type copy of *CTA4* or *YHB1* would reverse the NO hypersensitivity phenotype of the *cta4*Δ/*cta4*Δ or *yhb1*Δ/*yhb1*Δ mutants, respectively. One copy of each respective gene was inserted into plasmid CIp20 (13), which was then transformed into the genome at the *RPS1* locus. The resistances of the complementation strains to DPTA NONOate appear similar to the wild-type level at the concentrations tested (Fig. 3B).

The role of *SSU1* in NO resistance in *C. albicans* was not known. In *S. cerevisiae*, *yhb1*Δ mutants are sensitive to NO in rich, undefined (YPD) medium, but not in synthetic dextrose medium. Conversely, *ssu1*Δ strains are resistant in rich medium but sensitive in synthetic medium (42). To test whether *SSU1* participates in NO detoxification in *C. albicans*, we constructed *ssu1*Δ and *yhb1*Δ *ssu1*Δ mutants. Surprisingly, deletion of *SSU1* had no effect on NO sensitivity under growth conditions in either rich (Fig. 3C) or synthetic (Fig. 3D) medium, with growth rates essentially identical to the wild-type or *yhb1*Δ parent strains. The *ssu1*Δ mutant is sensitive to exogenous sulfite (see Fig. S8 in the supplemental material), a phenotype shared with the *S. cerevisiae ssu1*Δ strain. Sulfite resistance is not regulated by *CTA4*: the *cta4*Δ mutant strain behaves identically to wild type in this assay (data not shown). Thus, though we have confirmed that *SSU1* is an NO-inducible gene and is regulated by *CTA4*, it does not have an obvious role in NO detoxification.

Deletion of *CTA4* prevents NO-specific induction of *YHB1* transcription. We tested whether loss of any of the putative transcription factors altered *YHB1* mRNA induction by NO. Only *cta4*Δ/*cta4*Δ appears to have lost the ability to induce the *YHB1* gene when nitrite or DPTA NONOate is present (Fig. 4A and B). The mutants for the other transcription factors have a nitrosative stress response similar to wild type. Reintroducing *CTA4* or *YHB1* to the appropriate deletion strain restored the induced increase in *YHB1* mRNA (Fig. 4C). Transcriptional activity of *YHB1* was reproducibly weaker for both the *CTA4* and *YHB1* complementation strains relative to SN152, possibly because of having only one gene copy present or because of positional effects from inserting the genes at the nonnative locus within the *RPS1* ORF.

The putative sulfite transporter *SSU1* is induced by NO in both *S. cerevisiae* and *C. albicans*. To determine how broad the role of *CTA4* was in the NO response, we examined expression of *SSU1* in wild-type and *cta4*Δ/*cta4*Δ cells by reverse transcription-PCR. *SSU1* was induced in the presence of 2,2′-(hydroxynitrosohydrazono)bis-ethanimine (DETA)-NONOate in the wild-type strain, but this induction was either abolished or reduced, depending on the strain background, in the absence of *CTA4* (see Fig. S9 in the supplemental material). Thus, this gene is also under *CTA4* control.

In vivo binding of Cta4p to the NORE. Zinc cluster transcription factors typically bind directly to promoter DNA sequences of the genes they regulate. Also, the CCGTCGG palindrome within the NORE of *YHB1* is clearly similar to the three nucleotide repeats observed for binding sites of other zinc cluster proteins (47). To examine whether Cta4p binds to *YHB1* in vivo, we first constructed a strain expressing a functional, Myc_9 epitope-tagged Cta4p from the native *CTA4* promoter. Binding of Cta4p to the NORE region of the *YHB1* promoter in cells was then assessed using ChIP, as shown in Fig. 5. To determine whether nitric oxide induces a recruitment of Cta4p to the *YHB1* NORE region, we compared Cta4p binding to this DNA by ChIP assay in control cells versus cells treated with 10 mM sodium nitrite for 15 min. Exposure of *C. albicans* to nitrite as a source of nitric oxide promotes strong induction of *YHB1* mRNA after 15 min (50). Cta4p was bound

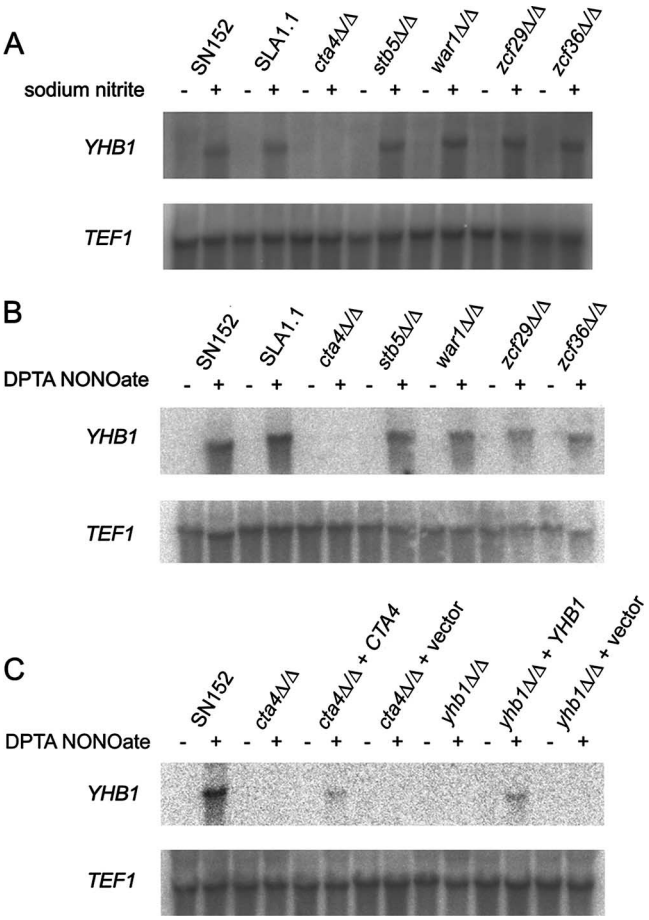

FIG. 4. Lack of *YHB1* RNA accumulation in response to nitrosative stress in the *cta4*Δ/*cta4*Δ mutant. Results of Northern blot assays on total RNA from deletion and complementation strains are shown. Cultures were grown to mid-exponential phase and then treated with nitric oxide donor or control solution for 15 min. Blots were probed with *YHB1* DNA and loading control *TEF1* DNA. (A) Deletion strains treated with 0 mM or 10 mM sodium nitrite. (B) Deletion strains and controls treated with 0 mM or 0.1 mM DPTA NONOate. Homozygous deletion strains are indicated a format where *cta4*Δ/*cta4*Δ is represented by *cta4*Δ/Δ, for example. (C) Complementation strains and controls were treated with 0 mM and 0.1 mM DPTA NONOate. The symbol *cta4*Δ/Δ + *CTA4* indicates the homozygous *CTA4* deletion mutant *cta4*Δ/*cta4*Δ with the *CTA4* complementation plasmid transformed into the genome.

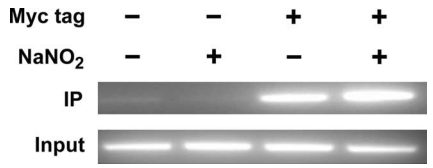

FIG. 5. In vivo binding of Cta4p to the NORE in both the presence and absence of sodium nitrite. Results of ChIP assay using a yeast strain expressing a single copy of Cta4p with a Myc_9 tag at the C terminus are shown. Cells were grown in YPD medium, and immunoprecipitation (IP) was performed in the presence of mouse anti-Myc antibody 9E10 and protein G beads. Genomic DNA was amplified using PCR primers designed to flank the NORE of the *YHB1* regulatory region and give a predicted PCR product of 241 bp. A yeast strain carrying wild-type Cta4p was used as a negative control. Results are representative of three independent experiments.

to *YHB1* to approximately the same extent in control cells and nitrite-treated cells (Fig. 5), indicating that Cta4p is already present on this promoter in the absence of an inducing signal. This mode of regulation is found among several members of the zinc cluster transcription factor family, most notably *S. cerevisiae* Gal4p (48).

Virulence of the *cta4*Δ/*cta4*Δ mutant is attenuated. We tested the *cta4*Δ/*cta4*Δ and *yhb1*Δ/*yhb1*Δ deletion and complementation strains for virulence using a mouse tail vein injection model of systemic candidiasis. Survival rate of the mice injected with *cta4*Δ/*cta4*Δ mutant *C. albicans* was significantly higher ($P = 0.015$) than that of the mice injected with wild-type SC5314. The survival curves show that mice infected with the *cta4*Δ/*cta4*Δ mutant also lasted longer on average than those infected with the *CTA4* complementation strain ($P = 0.027$; mean time to death: with SC5314, 4.7 days; with the *cta4*Δ/*cta4*Δ strain, 6.7 days; with the complemented strain, 5.0 days) (Fig. 6A). Previous reports (19, 50) have shown *yhb1*Δ/*yhb1*Δ mutant strains to be mildly attenuated in virulence as well (mean time to death, 7.1 days); we tested a *yhb1*Δ/*yhb1*Δ strain in the SN152 background and found a reduction in virulence (Fig. 6B), similar in magnitude to the *cta4*Δ/*cta4*Δ strain (despite the similar survival curves, the result with the *yhb1*Δ/*yhb1*Δ strain is not statistically significant as we used fewer mice to confirm this previously published result). There was no significant difference between results with *yhb1*Δ/*yhb1*Δ and *cta4*Δ/*cta4*Δ ($P = 0.60$).

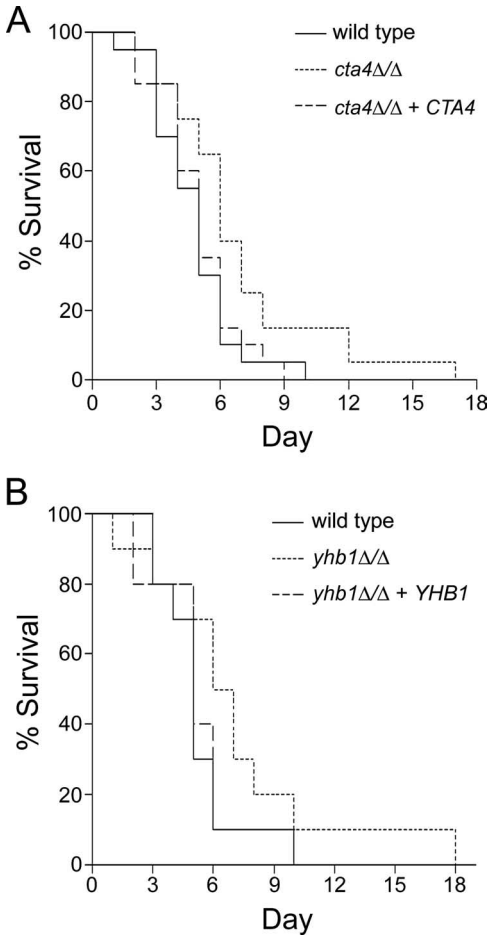

FIG. 6. Virulence of *cta4*Δ/*cta4*Δ and *yhb1*Δ/*yhb1*Δ *C. albicans* mutant strains against the wild type in a murine tail vein injection model. For each *C. albicans* strain tested, 10 adult female ICR mice were injected intravenously with 10^6 mid-exponential-phase yeast cells through the tail vein. (A) The *cta4*Δ/*cta4*Δ homozygous deletion strain is symbolized by *cta4*Δ/Δ. The complementation strain with one copy of *CTA4* is indicated as *cta4*Δ/Δ + *CTA4.* This assay was repeated twice ($n = 20$ mice/strain total), and the data from both experiments are combined in the figure. (B) The *YHB1* strains are denoted as in panel A. This assay was performed once ($n = 10$ mice/group).

DISCUSSION

Identifying a NORE involved in nitrosative stress response establishes a foundation for determining a mechanistic pathway of NO sensing in *C. albicans*. The 12-bp sequence TATTACCGTCGG, located about −230 bp from the *YHB1* translation start site, is presently the only known *cis*-acting element involved in NO regulation in *C. albicans*. Mass spectrometry analysis identified five putative transcription factors that may have had specific affinity for the NORE sequence in vitro.

Several findings show that one of these five factors, *CTA4*, is required for NO signaling in *C. albicans*. When the five putative transcription factor genes were separately deleted, only the *cta4*Δ/*cta4*Δ homozygous deletion mutant manifested a decline in resistance to nitrosative stress. Exposing *cta4*Δ/*cta4*Δ cells to various NO sources decreased the growth rate and drastically reduced induction of *YHB1* gene expression. ChIP results also indicated that the Cta4p protein localizes at the *YHB1* regulatory region in vivo.

Data on *CTA4* outside of this work are sparse. *CTA4* was first isolated in a one-hybrid assay in *S. cerevisiae* during a screen for a *C. albicans trans*-activating regulator of pyruvate decarboxylase gene expression (21). Later, various microarray studies reported that transcription of *CTA4* is upregulated by exposure to nitrosative stress (19) or growth in whole human blood (14) and downregulated upon adherence to polystyrene (30). The closest homolog for Cta4p in *S. cerevisiae* is Oaf1p, an oleate receptor which heterodimerizes with Pip2p to activate peroxisomal β-oxidation genes for breaking down fatty acids (4), but the *C. albicans cta4*Δ mutant is able to utilize

oleic acid as the sole carbon source, indicating that this gene does not regulate β-oxidation (M. Ramirez and M. Lorenz, unpublished observations). The next closest homolog among *S. cerevisiae* proteins is Hap1p, a heme-responsive factor which binds DNA as a homodimer and is involved in oxygen regulation (52).

Cta4p belongs in the Zn(II)2-Cys6 transcription factor family, a group of proteins unique to fungi, whose members bind DNA by means of a binuclear cluster of six cysteine residues that coordinate two zinc atoms. The *C. albicans* genome is predicted to have genes for 77 of these factors (7). The prototype for this class of proteins is the well-studied transcription factor Gal4p in *S. cerevisiae*. The protein structure typically contains three domains: a DNA-binding domain almost always located at the N terminus, a central region believed to regulate the transcriptional activation activity of the factor, and an acidic activation domain at the C terminus (29). Zn(II)2-Cys6 transcription factors function as homodimers, heterodimers, or sometimes monomers. Their cognate DNA binding sites often have CGG pairs that may be oriented as direct, inverted, or everted repeats. The spacing between these repeats varies widely between different proteins and is often a critical factor in recognition by a specific transcription factor (47). The NORE sequence TATTACCGTCGG fits this archetype in that it contains an everted CGG repeat, but it also has the additional palindromic sequence TATTA. It may be comparable to Hap1p binding sites in *S. cerevisiae*, which have similar, conserved TA-rich sequences that interact with the N terminus of Hap1p between the CGG direct repeats (23).

Along with Cta4p, four other putative transcription factors from this zinc binuclear cluster family demonstrated in vitro affinity to NORE-coated magnetic particles: Zcf29p, Zcf36p, War1p, and Stb5p. Presently, we have no further evidence that these four transcription factors are biologically relevant to NO regulation. Their binding to the NORE could merely be an artifact of the artificial binding conditions, or they may have redundant functions in NO regulation that are not apparent when only one gene is eliminated at a time. The functions of the *ZCF29* and *ZCF36* genes, which are also the closest homologs of *CTA4* in the *C. albicans* genome, are currently unknown. *C. albicans WAR1* is known to provide resistance to the weak acid sorbate, a phenotype similar to its homolog in *S. cerevisiae*. Although the function of *STB5* in *C. albicans* has not been investigated, its homolog in haploid *S. cerevisiae* (32.5% amino acid sequence identity) has a variety of functions, including roles in multidrug resistance (1), oxidative stress response (26), and low-temperature response (2).

The process of upregulation of the flavohemoglobin gene *YHB1* during nitrosative stress in *C. albicans* appears to differ from that in *S. cerevisiae*. Liu et al., who first established the NO resistance function of *YHB1* in *S. cerevisiae*, did not detect any increase in *YHB1* enzymatic activity during nitrosative stress (27). Additionally, we previously reported constitutively activated *YHB1* expression in *S. cerevisiae*, regardless of the presence of NO-generating compounds (50). However, Sarver and DeRisi documented induction of *S. cerevisiae YHB1* transcription in a different strain specifically during nitrosative stress by transcription factor Fzf1p, a C2H2 zinc finger factor (42). The closest homolog of *S. cerevisiae FZF1* in *C. albicans* has 19.3% amino acid sequence identity, and it is not known whether it can carry out similar functions. We have found that the analogous NO response in *C. albicans* requires a different factor, Cta4p.

Another difference between *C. albicans* and *S. cerevisiae* is the role of the sulfite transporter *SSU1*. This gene mediates NO resistance under specific environmental conditions in *S. cerevisiae*, notably in synthetic medium. We hypothesized that the *C. albicans SSU1* may have a similar function and that the *yhb1*Δ *ssu1*Δ double mutant would have a more severe phenotype, both in the presence of NO in vitro and in vivo. However, *SSU1* has no role in NO detoxification in *C. albicans* under the conditions tested here, though its induction by NO is at least partly *CTA4* dependent. Why *SSU1* is part of the NO regulon is not immediately apparent.

We also demonstrated that deletion of the *CTA4* gene produces a statistically significant reduction in virulence in a mouse infection model, though the effect was small. Deleting the *YHB1* gene produced a similar mild attenuation of virulence, but this was not statistically significant, possibly because of the smaller number of mice tested. These data, together with earlier reports (19, 50), indicate that increasing susceptibility to NO has a small effect on virulence in the mouse tail vein model of candidiasis.

The mildness of the virulence reduction could indicate that *YHB1* and *CTA4* aid *C. albicans* survival against other NO sources that are not tested by this experiment. Injecting *C. albicans* directly into the bloodstream may be an appropriate model for certain situations, such as infection by contaminated implants, catheters, or needles, but this delivery method circumvents several common infection routes. Since *C. albicans* most often resides as a commensal organism on the mucosal linings of the urogenital and digestive tracts, it usually must infiltrate these barriers first before it can reach the bloodstream and disseminate throughout the body.

The NO in these environments could affect how vital *YHB1* and *CTA4* are to the survival and spread of this pathogen inside a host organism. For example, gastric NO concentrations in the human small intestine following ingestion of dietary nitrates can rise to levels thousands of times higher than in tissues (51). Mouse models in which *C. albicans* is introduced orally may be more informative in testing this. Additionally, the major function of the *CTA4* and *YHB1* genes could be more subtle than direct opposition against the host immune system; they may instead be more relevant in providing *C. albicans* a survival advantage against other microorganisms vying for the same territory. Using more than one infection model will be important in establishing when and where these genes are critical for NO defense.

Our work takes the initial steps to decipher a pathway for NO signaling in *C. albicans*. Since this species is a major cause of fungal infections in humans, learning how it evades normal immune defenses and exploits weakened ones is essential for developing new strategies for treating infection.

ACKNOWLEDGMENTS

We thank Alexander Johnson for supplying plasmids and strains, Aaron Mitchell for providing the *cta4*::*UAU* mutant, and Melissa Ramirez for her assistance with the virulence assays in mice.

This work was supported by the National Science Foundation under grants DGE-0114264, MCB-0091236, and MCB-0520873.

REFERENCES

1. **Akache, B., and B. Turcotte.** 2002. New regulators of drug sensitivity in the family of yeast zinc cluster proteins. J. Biol. Chem. **277:**21254–21260.
2. **Akache, B., K. Wu, and B. Turcotte.** 2001. Phenotypic analysis of genes encoding yeast zinc cluster proteins. Nucleic Acids Res. **29:**2181–2190.
3. **Aparicio, O., J. V. Geisberg, A. Sekinger, A. Yang, Z. Moqtaderi, and K. Struhl.** 2005. Chromatin immunoprecipitation for determining the association of proteins with specific genomic sequences in vivo, p. 1–33. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology. John Wiley and Sons, Inc., New York, NY.
4. **Baumgartner, U., B. Hamilton, M. Piskacek, H. Ruis, and H. Rottensteiner.** 1999. Functional analysis of the Zn(2)Cys(6) transcription factors Oaf1p and Pip2p. Different roles in fatty acid induction of beta-oxidation in *Saccharomyces cerevisiae*. J. Biol. Chem. **274:**22208–22216.
5. **Boccara, M., C. E. Mills, J. Zeier, C. Anzi, C. Lamb, R. K. Poole, and M. Delledonne.** 2005. Flavohaemoglobin HmpX from *Erwinia chrysanthemi* confers nitrosative stress tolerance and affects the plant hypersensitive reaction by intercepting nitric oxide produced by the host. Plant J. **43:**226–237.
6. **Boon, E. M., and M. A. Marletta.** 2005. Ligand discrimination in soluble guanylate cyclase and the H-NOX family of heme sensor proteins. Curr. Opin. Chem. Biol. **9:**441–446.
7. **Braun, B. R., M. van Het Hoog, C. d'Enfert, M. Martchenko, J. Dungan, A. Kuo, D. O. Inglis, M. A. Uhl, H. Hogues, M. Berriman, M. Lorenz, A. Levitin, U. Oberholzer, C. Bachewich, D. Harcus, A. Marcil, D. Dignard, T. Iouk, R. Zito, L. Frangeul, F. Tekaia, K. Rutherford, E. Wang, C. A. Munro, S. Bates, N. A. Gow, L. L. Hoyer, G. Kohler, J. Morschhauser, G. Newport, S. Znaidi, M. Raymond, B. Turcotte, G. Sherlock, M. Costanzo, J. Ihmels, J. Berman, D. Sanglard, N. Agabian, A. P. Mitchell, A. D. Johnson, M. Whiteway, and A. Nantel.** 2005. A human-curated annotation of the *Candida albicans* genome. PLoS Genet. **1:**36–57.
8. **Cassanova, N., K. M. O'Brien, B. T. Stahl, T. McClure, and R. O. Poyton.** 2005. Yeast flavohemoglobin, a nitric oxide oxidoreductase, is located in both the cytosol and the mitochondrial matrix: effects of respiration, anoxia, and the mitochondrial genome on its intracellular level and distribution. J. Biol. Chem. **280:**7645–7653.
9. **Castello, P. R., P. S. David, T. McClure, Z. Crook, and R. O. Poyton.** 2006. Mitochondrial cytochrome oxidase produces nitric oxide under hypoxic conditions: implications for oxygen sensing and hypoxic signaling in eukaryotes. Cell Metab. **3:**277–287.
10. **Chow, E. D., O. W. Liu, S. O'Brien, and H. D. Madhani.** 2007. Exploration of whole-genome responses of the human AIDS-associated yeast pathogen *Cryptococcus neoformans* var. *grubii*: nitric oxide stress and body temperature. Curr. Genet. **52:**137–148.
11. **Crawford, M. J., and D. E. Goldberg.** 1998. Role for the *Salmonella* flavohemoglobin in protection from nitric oxide. J. Biol. Chem. **273:**12543–12547.
12. **de Jesus-Berrios, M., L. Liu, J. C. Nussbaum, G. M. Cox, J. S. Stamler, and J. Heitman.** 2003. Enzymes that counteract nitrosative stress promote fungal virulence. Curr. Biol. **13:**1963–1968.
13. **Dennison, P. M., M. Ramsdale, C. L. Manson, and A. J. Brown.** 2005. Gene disruption in *Candida albicans* using a synthetic, codon-optimised Cre-*loxP* system. Fungal Genet. Biol. **42:**737–748.
14. **Fradin, C., M. Kretschmar, T. Nichterlein, C. Gaillardin, C. d'Enfert, and B. Hube.** 2003. Stage-specific gene expression of *Candida albicans* in human blood. Mol. Microbiol. **47:**1523–1543.
15. **Frith, M. C., M. C. Li, and Z. Weng.** 2003. Cluster-Buster: Finding dense clusters of motifs in DNA sequences. Nucleic Acids Res. **31:**3666–3668.
16. **Gardner, A. M., C. R. Gessner, and P. R. Gardner.** 2003. Regulation of the nitric oxide reduction operon (*norRVW*) in *Escherichia coli.* Role of NorR and sigma54 in the nitric oxide stress response. J. Biol. Chem. **278:**10081–10086.
17. **Gietz, R. D., and R. A. Woods.** 2002. Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. Methods Enzymol. **350:**87–96.
18. **Gladwin, M. T.** 2004. Haldane, hot dogs, halitosis, and hypoxic vasodilation: the emerging biology of the nitrite anion. J. Clin. Investig. **113:**19–21.
19. **Hromatka, B. S., S. M. Noble, and A. D. Johnson.** 2005. Transcriptional response of *Candida albicans* to nitric oxide and the role of the *YHB1* gene in nitrosative stress and virulence. Mol. Biol. Cell **16:**4814–4826.
20. **Iijima, M., H. Shimizu, Y. Tanaka, and H. Urushihara.** 2000. Identification and characterization of two flavohemoglobin genes in *Dictyostelium discoideum*. Cell Struct. Funct. **25:**47–55.
21. **Kaiser, B., T. Munder, H. P. Saluz, W. Kunkel, and R. Eck.** 1999. Identification of a gene encoding the pyruvate decarboxylase gene regulator CaPdc2p from *Candida albicans*. Yeast **15:**585–591.
22. **Kaiser, C., S. Michaelis, and A. Mitchell.** 1994. Methods in yeast genetics: a laboratory manual, p. 169–172. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
23. **King, D. A., L. Zhang, L. Guarente, and R. Marmorstein.** 1999. Structure of a HAP1-DNA complex reveals dramatically asymmetric DNA binding by a homodimeric protein. Nat. Struct. Biol. **6:**64–71.
24. **Knop, M., K. Siegers, G. Pereira, W. Zachariae, B. Winsor, K. Nasmyth, and E. Schiebel.** 1999. Epitope tagging of yeast genes using a PCR-based strategy: more tags and improved practical routines. Yeast **15:**963–972.
25. **Kroeger, K. M., and L. J. Abraham.** 1997. Magnetic bead purification of specific transcription factors using mutant competitor oligonucleotides. Anal. Biochem. **250:**127–129.
26. **Larochelle, M., S. Drouin, F. Robert, and B. Turcotte.** 2006. Oxidative stress-activated zinc cluster protein Stb5 has dual activator/repressor functions required for pentose phosphate pathway regulation and NADPH production. Mol. Cell. Biol. **26:**6690–6701.
27. **Liu, L., M. Zeng, A. Hausladen, J. Heitman, and J. S. Stamler.** 2000. Protection from nitrosative stress by yeast flavohemoglobin. Proc. Natl. Acad. Sci. USA **97:**4672–4676.
28. **Lorenz, M. C., and G. R. Fink.** 2001. The glyoxylate cycle is required for fungal virulence. Nature **412:**83–86.
29. **MacPherson, S., M. Larochelle, and B. Turcotte.** 2006. A fungal family of transcriptional regulators: the zinc cluster proteins. Microbiol. Mol. Biol. Rev. **70:**583–604.
30. **Marchais, V., M. Kempf, P. Licznar, C. Lefrancois, J. P. Bouchara, R. Robert, and J. Cottin.** 2005. DNA array analysis of *Candida albicans* gene expression in response to adherence to polystyrene. FEMS Microbiol. Lett. **245:**25–32.
31. **Membrillo-Hernandez, J., and R. K. Poole.** 1997. Bacterial flavohaemoglobins: a consensus sequence and identification of a discrete enterobacterial group and of further bacterial globins. FEMS Microbiol. Lett. **155:**179–184.
32. **Missall, T. A., J. K. Lodge, and J. E. McEwen.** 2004. Mechanisms of resistance to oxidative and nitrosative stress: implications for fungal survival in mammalian hosts. Eukaryot. Cell **3:**835–846.
33. **Missall, T. A., M. E. Pusateri, M. J. Donlin, K. T. Chambers, J. A. Corbett, and J. K. Lodge.** 2006. Posttranslational, translational, and transcriptional responses to nitric oxide stress in *Cryptococcus neoformans*: implications for virulence. Eukaryot. Cell **5:**518–529.
34. **Negredo, A., L. Monteoliva, C. Gil, J. Pla, and C. Nombela.** 1997. Cloning, analysis and one-step disruption of the *ARG5,6* gene of *Candida albicans*. Microbiology **143:**297–302.
35. **Nittler, M. P., D. Hocking-Murray, C. K. Foo, and A. Sil.** 2005. Identification of *Histoplasma capsulatum* transcripts induced in response to reactive nitrogen species. Mol. Biol. Cell **16:**4792–4813.
36. **Noble, S. M., and A. D. Johnson.** 2005. Strains and strategies for large-scale gene deletion studies of the diploid human fungal pathogen *Candida albicans*. Eukaryot. Cell **4:**298–309.
37. **Palmerini, C. A., R. Palombari, S. Perito, and G. Arienti.** 2003. NO synthesis in human saliva. Free Radic. Res. **37:**29–31.
38. **Poole, R. K., M. F. Anjum, J. Membrillo-Hernandez, S. O. Kim, M. N. Hughes, and V. Stewart.** 1996. Nitric oxide, nitrite, and Fnr regulation of *hmp* (flavohemoglobin) gene expression in *Escherichia coli* K-12. J. Bacteriol. **178:**5487–5492.
39. **Poole, R. K., and M. N. Hughes.** 2000. New functions for the ancient globin family: bacterial responses to nitric oxide and nitrosative stress. Mol. Microbiol. **36:**775–783.
40. **Reuss, O., A. Vik, R. Kolter, and J. Morschhauser.** 2004. The *SAT1* flipper, an optimized tool for gene disruption in *Candida albicans*. Gene **341:**119–127.
41. **Rogstam, A., J. T. Larsson, P. Kjelgaard, and C. von Wachenfeldt.** 2007. Mechanisms of adaptation to nitrosative stress in *Bacillus subtilis*. J. Bacteriol. **189:**3063–3071.
42. **Sarver, A., and J. DeRisi.** 2005. Fzf1p regulates an inducible response to nitrosative stress in *Saccharomyces cerevisiae*. Mol. Biol. Cell **16:**4781–4791.
43. **Schmitt, M. E., T. A. Brown, and B. L. Trumpower.** 1990. A rapid and simple method for preparation of RNA from *Saccharomyces cerevisiae*. Nucleic Acids Res. **18:**3091–3092.
44. **Sebbane, F., N. Lemaitre, D. E. Sturdevant, R. Rebeil, K. Virtaneva, S. F. Porcella, and B. J. Hinnebusch.** 2006. Adaptive response of *Yersinia pestis* to extracellular effectors of innate immunity during bubonic plague. Proc. Natl. Acad. Sci. USA **103:**11766–11771.
45. **Sobko, T., C. I. Reinders, E. Jansson, E. Norin, T. Midtvedt, and J. O. Lundberg.** 2005. Gastrointestinal bacteria generate nitric oxide from nitrate and nitrite. Nitric Oxide **13:**272–278.
46. **Stevanin, T. M., R. K. Poole, E. A. Demoncheaux, and R. C. Read.** 2002. Flavohemoglobin Hmp protects *Salmonella enterica* serovar Typhimurium from nitric oxide-related killing by human macrophages. Infect. Immun. **70:**4399–4405.
47. **Todd, R. B., and A. Andrianopoulos.** 1997. Evolution of a fungal regulatory gene family: the Zn(II)2Cys6 binuclear cluster DNA binding motif. Fungal Genet. Biol. **21:**388–405.

48. **Traven, A., B. Jelicic, and M. Sopta.** 2006. Yeast Gal4: a transcriptional paradigm revisited. EMBO Rep. **7:**496–499.
49. **Uhl, M. A., and A. D. Johnson.** 2001. Development of *Streptococcus thermophilus lacZ* as a reporter gene for *Candida albicans*. Microbiology **147:** 1189–1195.
50. **Ullmann, B. D., H. Myers, W. Chiranand, A. L. Lazzell, Q. Zhao, L. A. Vega, J. L. Lopez-Ribot, P. R. Gardner, and M. C. Gustin.** 2004. Inducible defense mechanism against nitric oxide in *Candida albicans*. Eukaryot. Cell **3:**715–723.
51. **Weitzberg, E., and J. O. Lundberg.** 1998. Nonenzymatic nitric oxide production in humans. Nitric Oxide **2:**1–7.
52. **Zhang, L., A. Hach, and C. Wang.** 1998. Molecular mechanism governing heme signaling in yeast: a higher-order complex mediates heme regulation of the transcriptional activator HAP1. Mol. Cell. Biol. **18:**3819–3828.