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Niche-Specific Activation of the Oxidative Stress Response by the Pathogenic Fungus *Candida albicans*▽

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Candida albicans **is a major opportunistic pathogen of humans. The pathogenicity of this fungus depends upon its ability to deal effectively with the host defenses and, in particular, the oxidative burst of phagocytic cells. We have explored the activation of the oxidative stress response in *C. albicans* in ex vivo infection models and during systemic infection of a mammalian host. We have generated *C. albicans* strains that contain specific green fluorescent protein (GFP) promoter fusions and hence act as biosensors of environmental oxidative stress at the single-cell level. Having confirmed that *CTA1*-, *TRX1*-, and *TTR1*/*GRX2*-*GFP* reporters respond specifically to oxidative stress, and not to heat shock, nitrosative, or osmotic stresses, we used these reporters to show that individual *C. albicans* cells activate an oxidative stress response following phagocytosis by neutrophils, but not by macrophages. Significantly, only a small proportion of *C. albicans* cells (about 4%) activated an oxidative stress response during systemic infection of the mouse kidney. The response of these cells was generally equivalent to exposure to 0.4 mM hydrogen peroxide in vitro. We conclude that most *C. albicans* cells are exposed to an oxidative stress when they come into contact with neutrophils in the bloodstream of the host but that oxidative killing is no longer a significant threat once an infection has been established in the kidney.**

Candida albicans is a frequent cause of superficial infections, such as vaginitis, diaper rash, and oral thrush. However, *C. albicans* infection can also cause deep-seated, life-threatening systemic infections in severely immunocompromised patients (8, 32). As well as regulating its cell morphology and other cellular virulence factors, *C. albicans* is thought to adapt its metabolism and stress responses to the diverse microenvironments it encounters in the host (7, 28).

Aerobic organisms are constantly exposed to reactive oxygen species that are generated as normal by-products of respiratory metabolism (17). However, oxidative stress responses are thought to be particularly important for pathogenic fungi, because oxidative stress has been predicted to be the stress condition they are most likely to encounter in vivo (for review, see reference 10). Therefore, considerable emphasis has been placed on the oxidative stress response in *C. albicans* (1, 3, 9, 10, 15, 37, 42).

Host phagocytic cells use reactive oxygen species as part of their armory to neutralize foreign microorganisms (16). Reactive oxygen species such as the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (HO) react with lipids, DNA, and proteins, leading to lethal microbial cell damage. To survive these assaults, *C. albicans* expresses enzymes that catalyze the detoxification of reactive oxygen species, such as superoxide dismutase (which converts superoxide into O_2 and H_2O_2) and catalase (which converts the reactive H_2O_2 in neutral H_2O and O_2). *C. albicans* also generates scavengers of reactive oxygen species, such as glutaredoxin (a glutathione-dependent stress protectant) and thioredoxin (to reduce disulfide bonds created by the oxidative stress).

Genome-wide microarray studies have shown that many of these functions are induced at the transcriptional level when *C. albicans* responds to oxidative stresses in vitro (13, 15), e.g., genes involved in H_2O_2 and free radical detoxification (*CTA1* and *SOD2*) and in the thioredoxin (*TSA1*, *TRX1*, and *TTR1*, also known as *GRX2* in *Saccharomyces cerevisiae*) and glutaredoxin (*GPX1* and *GSH1*) systems are transcriptionally induced in response to oxidative stress. Furthermore, the transcription factor Cap1, which is important for oxidative stress tolerance (1, 15, 42), is strongly induced in response to oxidative stress in vitro.

Some oxidative stress functions are induced in *C. albicans* when cells are exposed to human blood or following phagocytosis by granulocytes or neutrophils (19, 20, 35). This observation is consistent with the idea that reactive oxygen species play a significant role in the antimicrobial defenses of host phagocytic cells. However, *C. albicans* only displays a weak oxidative stress response following phagocytosis by macrophages (25). Nevertheless, the significance of oxidative stress responses in the virulence of *C. albicans* has been emphasized by the observation that mutations that inactivate such responses attenuate the virulence of this pathogen (20, 24, 27, 39–41). Therefore, in this study we have used single-cell profiling (4, 5) to examine the extent to which individual *C. albicans* cells activate an oxidative stress response within specific microenvironments relevant to the infection process. We show that while *C. albicans* cells are exposed to a significant oxidative stress following phagocytosis by neutrophils, few fungal cells are exposed to an oxidative stress after exposure to macrophages or once a kidney infection is established. We conclude that an oxidative

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stress response is a niche-specific phenomenon during the establishment and progression of a *C. albicans* systemic infection.

MATERIALS AND METHODS

Strain construction. *C. albicans* strain CAI-4 was used throughout (*ura3::λimm434*/*ura3::λ imm434*) (18). Control plasmids pGFP and pACT1-GFP have been described previously (4). To generate pCTA1-GFP, the *C. albicans CTA1* promoter (−1000 to −1, relative to the start codon) was PCR amplified with the primers CTA1 Xho1 5′ (5′ ATATCTCGAGAAAATCCTGGGTCAC AGAAATATGG 3′) and CTA1 Mlu1 3′ (5′ ATATACGCGTAATAAATAAT TTATATATAAATAGG 3′) and cloned between the XhoI and MluI sites of pGFP. To generate pTTR1-GFP, the *TTR1*/*GRX2* promoter (−1000 to −1) was PCR amplified with the primers TTR1 Xho1 5′ (5′ ATATCTCGAGAACGGT GAGAAAGATCGTGGATTTGGTC 3′) and TTR1 Mlu1 3′ (5′ ATATACGC GTCAAAAGAAAGAAAGAAAAGAAAAAAAAGGGGC 3′) and cloned between the XhoI and MluI sites of pGFP. Similarly, to generate pTRX1-GFP, the *TRX1* promoter region (−1000 to −1) was PCR amplified with the primers TRX1 Xho1 5′ (5′ ATATCTCGAGATGTACTTGAGGAGTAAAGTATAGA AAAACAG 3′) and TRX1 Mlu1 3′ (5′ ATATACGCGTCTTTGAAAAAAAA TATAACTATGTGTTAAGTG 3′) and cloned into pGFP. Plasmids were linearized with StuI and integrated at the *RPS1* locus in the strain CAI-4. This is a standard approach for the analysis of reporter genes in *C. albicans* (4, 5, 29). The genotypes of transformants were confirmed by PCR and Southern blotting, as described previously (4).

Growth and stress conditions. Unless otherwise stated, *C. albicans* was grown in yeast extract-peptone-dextrose (YPD) (36) at 30°C with shaking. Strains were grown overnight to stationary phase, diluted to an optical density at 600 nm of 0.1 in fresh YPD, and regrown to mid-exponential phase (OD at 600 nm, 0.8). For RNA analyses, stresses were applied for a 10-min period to allow comparison with published transcript profiling data. For the green fluorescent protein (GFP) fluorescence experiments, stresses were applied for 2 h to provide sufficient time for the synthesis and folding of GFP (4). Growing cells were heat shocked by transferring them from 23°C to either 37°C or 42°C. Osmotic stress was imposed by addition of NaCl to a final concentration of 0.3 M. Oxidative stress was imposed by addition of H_2O_2 to the concentrations specified. Nitrosative stress was generated as described by Hromatka et al. (22) using buffered YPD with the specified concentrations of (Z)-1-[N-(3-aminopropyl)-N-(3-ammoniopropyl)amino]diazen-1-ium-1,2-diolate (DPTA-NONOate). The yeast-to-hypha morphogenetic transition was induced in YPD by raising the growth temperature to 37°C and adding fetal bovine serum to a final concentration of 10% (vol/vol) or by transferring the cells to RPMI 1640 medium (6, 38).

Northern blotting. Northern analysis was performed as described previously (30). The *GFP* probe was PCR amplified with the primers GFP-R (5′-TTTGT ACAATTCATC) and GFP-F (5′-TCTAAAGGTGAAGAATTATTCACTGG). The PCR product was labeled and detected by the ECL direct nucleic acid labeling and detection system (Amersham Bioscience).

Fluorescence microscopy for in vitro GFP assays. Yeast cells were prepared for fluorescence microscopy as previously described (4, 5). Individual yeast cells and hyphal compartments were defined by phase-contrast microscopy, and then the relative GFP fluorescence intensities generated by these compartments were quantified with Openlab 3.0.9 software (Improvision Ltd., Coventry, United Kingdom). Nonspecific fluorescence was subtracted, and mean fluorescence intensities were calculated for at least 50 individual cells (± standard deviation) (4). GFP fluorescence levels never exceeded the quantitative range of the assay.

Ex vivo infection models. Neutrophils were isolated from human blood (20). Stationary-phase *C. albicans* cells were washed with phosphate-buffered saline and mixed with neutrophils in a 1:1 ratio for 2 h at 37°C. Control *C. albicans* cells were treated with human plasma. Similarly, cultured murine J774A-1 macrophages (34) were mixed with *C. albicans* cells in a 1:1 ratio and analyzed after 3 h. Control *C. albicans* cells were treated in the macrophage growth medium (Dulbecco's modified Eagle's medium [DMEM] containing fetal bovine serum and glutamine). Cells were fixed, mounted, and quantified as previously described (4, 5).

Murine model of systemic candidiasis. Female BALB/c mice were infected with 2×10^4 *C. albicans* cells/g body weight by tail vein injection as described by Barelle and coworkers (5). For the analysis of infections after 1 day, inocula of 2×10^5 cells/g were also used to obtain sufficient fungal cells for single-cell profiling. This increase in inoculum size did not influence the proportion of *C. albicans* cells that displayed GFP fluorescence in the kidney (not shown). Fungal burdens and in vivo kidney sections were analyzed after 1 or 4 days of infection (26). Frozen sections were stained with Calcofluor white to identify fungal cells (21). GFP fluorescence intensities for individual *C. albicans* cells were determined as described previously (5). Regions of sections displaying high levels of yellow autofluorescence were not quantified. Images were generated at 461 nm (Calcofluor white staining), 516 nm (GFP), and 573 nm (rhodamine, as a control for GFP specificity). GFP and rhodamine images were overlaid using Adobe Photoshop 7.0 software (Adobe Systems, Inc.) to highlight the GFP-specific signals. For histopathology, 5-μm paraffin wax-embedded kidney sections were stained with periodic acid-Schiff's reagent and then poststained with hematoxylin to reveal host cell nuclei in addition to fungal cells.

Name	C. db	Contig4	orf19	Function
ACT1	CA5255	Contig4-3061_0012	orf19.5007	Actin
CTA1	CA3011	Contig4-2906_0020	orf19.6229	Catalase A
TTR1	CA4919	Contig4-2977_0001	orf19.6059	Glutaredoxin
TRX1	CA6010	Contig4-3108_0068	orf19.7611	Thioredoxin

REF.	13	13	a	13	13	a	13	13	a	22		
Condition + Timing	HS	HS	HS+	OS	OS	OS+	XS	XS	XS+	NO	NO	NO
	10	30	30	10	30	30	10	30	30	10	40	70
ACT1	1.3	1.2	1.3	1.3	1.3	1.1	0.7	0.7	1.0	1.8	0.9	0.9
CTA1	0.9	0.8	1.1	4.3	5.3	1.2	1.8	3.2	3.7	5.1	0.8	0.8
TTR1	1.4	1.3	1.5	0.9	1.7	1.1	5.5	5.8	7.1	1.7	1.3	1.0
TRX1	1.2	0.9	1.5			1.0	4.5	6.9	2.2	4.3	1.3	1.1

REF.	20			14			14			31		
Condition + Timing	Blood	PMN	MNC	30°C	30°C	30°C	37°C	37°C	37°C	FBS	FBS	FBS
	30	30	30	10	60	180	10	60	180	30	60	360
ACT1	1.2	1.1	1.0	0.9	0.9	1.2	1.0	1.1	1.0	1.5	0.8	0.8
CTA1	3.9	4.0	1.6	0.9	0.9	1.0	1.0	0.8	0.9	0.8	1.2	1.0
TTR1	2.0	2.7	0.9	0.6	0.5	0.5	0.6	0.7	0.5	1.3	1.1	1.3
TRX1	4.3	4.6	1.2	0.9	0.7	0.8	0.7	0.8	0.8		1.6	

FIG. 1. Transcript profiling data for selected oxidative stress genes. Gene annotations and names are given at the top of the figure, along with the data sources. Data from reference a are a personal communication from C. Vaughan and D. Sullivan (unpublished data). Values indicate the expression ratio for each condition relative to the control (before the perturbation): HS (heat shock from 25°C to 37°C) at 10 and 30 min; HS+ (strong heat shock from 25°C to 42°C) at 30 min; OS (osmotic stress of 0.3 M NaCl) at 10 and 30 min; OS+ (strong osmotic stress of 1 M NaCl) at 30 min; XS (oxidative stress of 0.4 mM H_2O_2) at 10 and 30 min; XS+ (strong oxidative stress of 5 mM H_2O_2) at 30 min; NO (nitrosative stress of 1 mM DPTA-NONOate) at 10, 40, and 70 min; blood (exposure to human blood) at 30 min; PMN (exposure to polymorphonuclear cells, mainly neutrophils) at 30 min; MNC (exposure to mononuclear cells, mainly macrophages) at 30 min; 30°C (resumption of growth from stationary phase at 30°C) for 10, 60, and 180 min; 37°C (resumption of growth from stationary phase at 37°C) for 10, 60, and 180 min; FBS (switch to 37°C plus 10% fetal bovine serum to trigger hyphae formation) at 30, 60, and 360 min.

In silico analysis of transcript profiling data. Published transcript profiling data sets were analyzed with Genespring software (Silicon Genetics) to identify *C. albicans* genes that are induced in response to oxidative stress (13–15, 20, 22, 31).

RESULTS

Identification of candidate oxidative stress biomarkers. To identify *C. albicans* genes that might be suitable for use as oxidative stress biomarkers, we took advantage of the growing number of transcript profiling data sets available for this pathogen. We focused especially on those experiments that have examined the global transcriptional responses of *C. albicans* to stresses, morphogenesis, and host-pathogen interac-

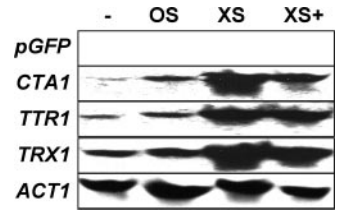

FIG. 2. Northern analysis of GFP reporters. Northern analysis was performed to test expression of the *CTA1*-, *TTR1*/*GRX2*-, and *TRX1*-*GFP* reporters as well as the pGFP and *ACT1*-*GFP* controls. The five *C. albicans* reporter strains were exposed to no stress (-), an osmotic stress (OS; 0.3 M NaCl), a mild oxidative stress (XS; 0.4 mM H_2O_2), or a strong oxidative stress (XS+; 5 mM H_2O_2). Membranes were probed with a *GFP* probe (see Materials and Methods).

tions (13–15, 20, 22, 31; C. Vaughan and D. Sullivan, personal communication). First, from the circa 6,300 genes present on the *C. albicans* microarray we identified 331 genes that are significantly induced in response to an oxidative stress. From this subset we selected 80 genes that are not induced in response to other conditions and which, therefore, appear to be relatively specific for the oxidative stress response. From this subset of *C. albicans* genes we selected three genes with well-defined functions in oxidative stress adaptation (Fig. 1): *CTA1* (encoding a catalase), *TTR1*/*GRX2* (encoding a glutaredoxin), and *TRX1* (encoding a thioredoxin). These genes display relatively strong induction by both mild and strong oxidative stresses, and their transcripts appear to be only transiently induced by nitrosative stress (Fig. 1). The *CTA1*, *TTR1*/*GRX2*, and *TRX1* genes are also activated when *C. albicans* cells are exposed to human blood, probably through the interaction with neutrophil cells (Fig. 1) (19, 20). With the possible exception of *TTR1*/*GRX2*, these genes are not transcriptionally regulated, as cells resume growth or during serum-induced morphogenesis (Fig. 1). Therefore, based on available transcript profiling data, the *TTR1*/*GRX2* and *TRX1* genes appear to respond specifically to oxidative stress. Although the *CTA1* transcript is induced in response to a moderate osmotic stress as well as to oxidative stress (Fig. 1) (15), this promoter was also selected for further analysis. In addition, the *ACT1* promoter was chosen as a positive control because this gene appears to be expressed at roughly similar levels under the conditions analyzed (Fig. 1).

Construction and validation of GFP reporters. The *CTA1*, *TTR1*/*GRX2*, and *TRX1* promoter regions (from −1000 to +1 with respect to the translation initiation codon) were fused to the *GFP* open reading frame in the plasmid pGFP, and each fusion was integrated into the *C. albicans* genome at the *RPS1*

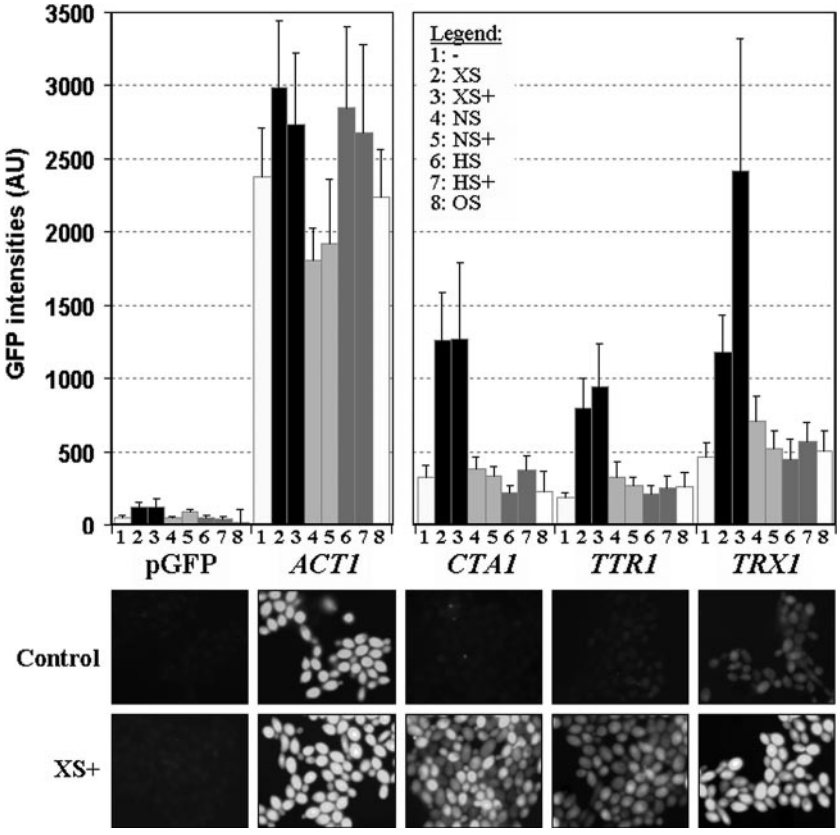

FIG. 3. Responses of the *CTA1*-, *TTR1*/*GRX2*-, and *TRX1*-*GFP* reporters to stresses in vitro. The expression of these three oxidative stress reporters was compared with the pGFP and *ACT1*-*GFP* controls under a range of stress conditions in vitro: -, no stress; XS, mild oxidative stress (0.4 mM H_2O_2); XS+, strong oxidative stress (5 mM H_2O_2); NS, mild nitrosative stress (1 mM DPTA-NONOate); NS+, strong nitrosative stress (5 mM DPTA-NONOate); HS, mild heat shock (25°C to 37°C); HS+, strong heat shock (25°C to 42°C); OS, osmotic stress (0.3 M NaCl). GFP intensities were determined as described in Materials and Methods. Fluorescence micrographs of the *C. albicans* transformants exposed to 0 (control) or 5 mM H_2O_2 (XS+) are shown.

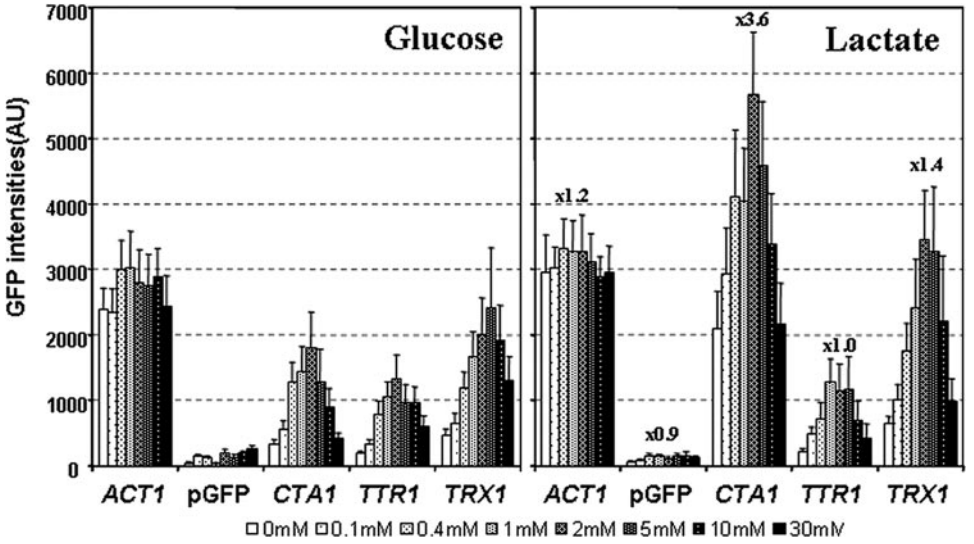

FIG. 4. Dose responses for the oxidative stress reporters. The expression of the *CTA1*-, *TTR1*/*GRX2*-, and *TRX1-GFP* reporters was compared to the pGFP and *ACT1-GFP* controls following exposure to a range of H_2O_2 concentrations. The *C. albicans* strains were grown in glucose (YPD; left panel) or lactate (YPL; right panel). Numbers in the right panel indicate the fold change in GFP fluorescence observed during growth on lactate compared with glucose.

locus to generate a set of *GFP*-marked strains (see Materials and Methods). Northern blot analyses compared the expression pattern of each *GFP* mRNA with the transcript profiling data (Fig. 2). The *TTR1*/*GRX2*- and *TRX1-GFP* mRNAs were induced in response to oxidative stresses, but not after exposure to the osmotic stress. The *CTA1-GFP* mRNA was strongly induced in response to oxidative stress and to a minor extent by osmotic stress. The *ACT1-GFP* mRNA was expressed constitutively under these conditions (Fig. 2). The Northern analyses are consistent with the transcript profiling data for this set of genes.

Next, we examined the green fluorescence generated by these *CTA1*-, *TTR1*/*GRX2*-, and *TRX1-GFP* strains following exposure to various stresses in vitro by quantitative fluorescence microscopy. Once again, the pGFP and *ACT1-GFP* strains were used as negative and positive controls, respectively. As expected, cells containing the promoterless pGFP plasmid generated no significant GFP fluorescence. In contrast, *ACT1-GFP* cells expressed relatively high levels of fluorescence under all of the conditions examined (Fig. 3). The *CTA1*-, *TTR1*/*GRX2*-, and *TRX1-GFP* fusions were induced in response to both high and low oxidative stresses (Fig. 3, lanes 2 and 3; 5.0 and 0.4 mM H_2O_2, respectively). Significantly, the fluorescence levels generated by these reporters were not elevated in response to nitrosative, osmotic, or heat stresses (relative to unstressed control cells) (Fig. 3). Therefore, the short-term induction of the *CTA1* and *TRX1* mRNAs in response to nitrosative stress was not reflected in a significant increase in GFP protein levels (Fig. 1 and 3), and the low level of induction of the *CTA1-GFP* mRNA in response to osmotic stress did not lead to a significant increase in GFP fluorescence (Fig. 2 and 3). These results suggested that the *CTA1*-, *TTR1*/*GRX2*-, and *TRX1-GFP* reporters could be used specifically to monitor the exposure of *C. albicans* cells to oxidative stress.

Oxidative dose responses for the GFP reporters. We challenged the GFP-marked strains with a range of H_2O_2 concentrations to generate dose-response curves for the three reporters. The *CTA1*-, *TTR1*/*GRX2*-, and *TRX1-GFP* strains displayed fluorescence responses that were roughly proportionate to the dose of H_2O_2 (Fig. 4A). The *CTA1*- and *TTR1*/*GRX2-GFP* reporters reached their maximum expression level at a dose of 2 mM H_2O_2, whereas the maximum response of *TRX1-GFP* cells was observed at 5 mM H_2O_2.

Reactive oxygen species are produced to a greater extent under respiratory conditions (17). Hence, we tested whether the *CTA1*-, *TTR1*/*GRX2*-, and *TRX1-GFP* reporters are differentially regulated under fermentative and nonfermentative conditions. The previous dose-response curves were obtained during growth on glucose (YPD) (Fig. 4A). Therefore, we repeated the dose-response curves with *C. albicans* cells grown on lactate (YPL) (Fig. 4B). The levels of green fluorescence generated by *TTR1*/*GRX2-GFP* and *TRX1-GFP* cells were not greatly affected by carbon source. There was a slight increase in *TRX1-GFP* expression levels during growth on lactate, but in general *TTR1*/*GRX2-GFP* and *TRX1-GFP* cells displayed similar dose-response curves whether they were grown on lactate or glucose. In contrast, the expression of the *CTA1-GFP* reporter was significantly up-regulated on lactate. Lactate-grown *CTA1-GFP* cells still displayed a response that was roughly proportional to the dose of H_2O_2, but the fluorescence levels were elevated approximately threefold compared with glucose-grown cells (Fig. 4). The data suggest that the *C. albicans CTA1* promoter responds to both oxidative stress and to carbon source. This observation has been confirmed recently by expression profiling of *C. albicans* cells (A. Rodaki, B. Enjalbert, and A. Brown, unpublished data). Taken together, our data indicate that in principle, the combination of *CTA1*-, *TTR1*/*GRX2*-, and *TRX1-GFP* reporters could be used as oxidative stress biomarkers to titrate the approximate level of oxidative stress in particular microenvironments.

Expression of oxidative stress biomarkers in ex vivo infection models. Transcript profiling experiments performed on *C.*

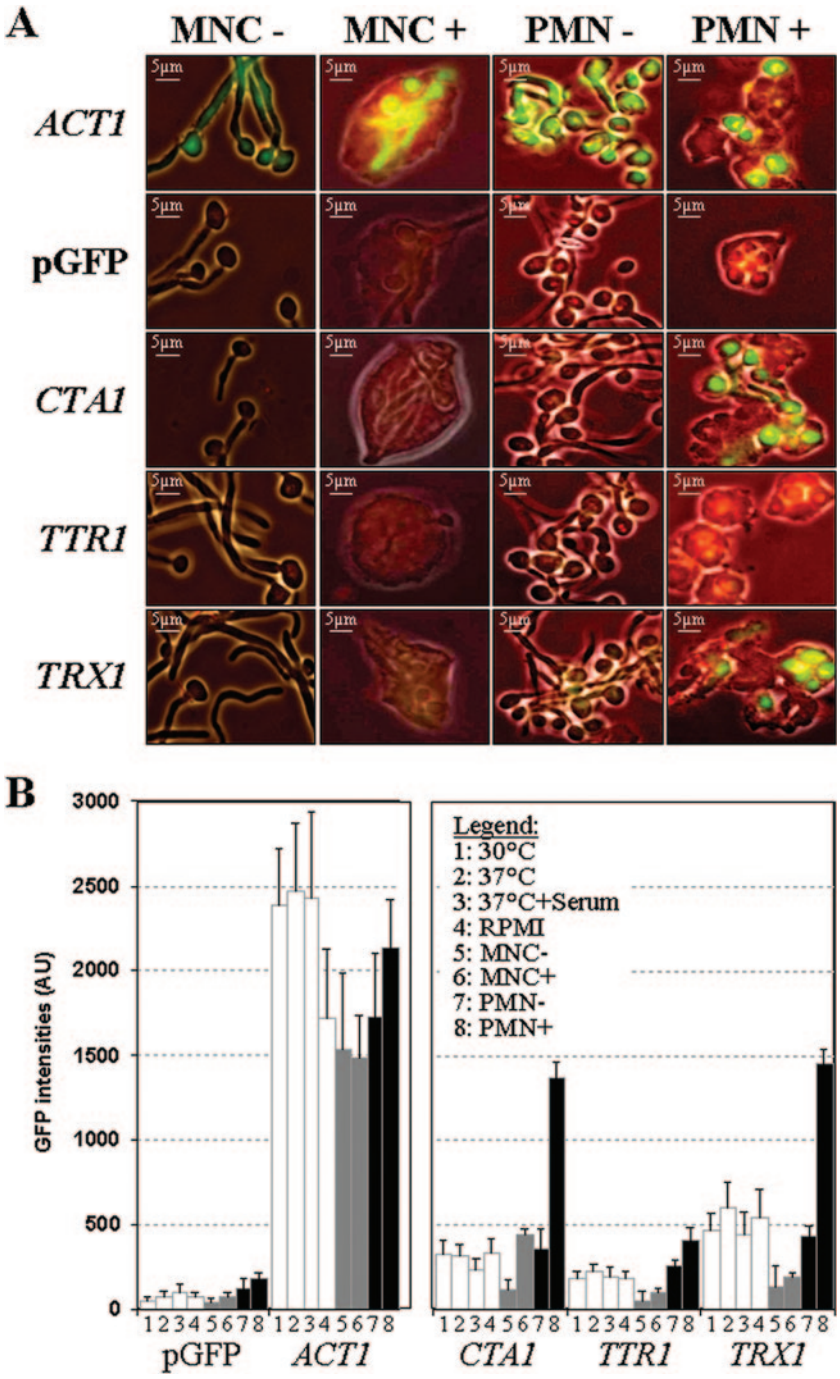

FIG. 5. Responses of oxidative stress reporters in ex vivo models. The expression of the *CTA1*-, *TTR1*/*GRX2*-, and *TRX1-GFP* reporters was compared to the pGFP and *ACT1-GFP* controls before and after phagocytosis by macrophages or neutrophils as described in the text (see Materials and Methods). (A) GFP fluorescence generated by the *C. albicans* reporter strains was measured in cells inoculated for 3 h in DMEM with no macrophages (MNC-), DMEM containing macrophages (MNC+), plasma lacking neutrophils (PMN-), or plasma containing neutrophils (PMN+). (B) GFP intensities were quantified as described in the text (see Materials and Methods) in the above cells and under other types of control conditions: 1, 30°C, mid-exponential yeast cells; 2, 37°C, hyphae cells in YPD at 37°C; 3, 37°C plus serum, hyphae in YPD at 37°C with fetal bovine serum; 4, RPMI, hyphae in RMPI 1640 at 37°C; 5, MNC-, hyphae in DMEM at 37°C; 6, MNC+, cells in macrophages; 7, PMN-, cells in plasma; 8, PMN+, cells in neutrophils.

albicans cells exposed to immune cells have suggested that the *CTA1*, *TTR1*/*GRX2*, and *TRX1* genes are induced following phagocytosis by neutrophils (20, 25) but, with the possible exception of *CTA1* (25), these genes are not induced following phagocytosis by macrophages. These transcript profiling experiments have provided information about the behavior of the fungal cell population as a whole, but our GFP reporters provided the opportunity to examine the behavior of individual

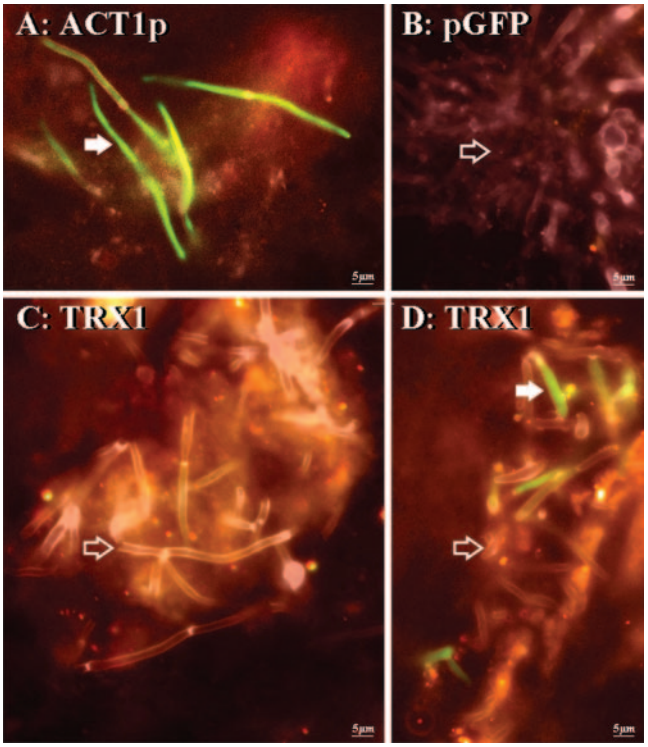

FIG. 6. Expression of GFP reporters in *C. albicans* cells infecting mouse kidney. Collages of three images are presented: Calcofluor white staining of *C. albicans* cells (white), GFP fluorescence (green), and nonspecific background fluorescence (red). (A) *ACT1-GFP* (positive control) after 1 day of infection. (B) pGFP (negative control) after 4 days of infection. (C and D) Two images of *TRX1-GFP* after 1 day of infection. Arrows illustrate examples of GFP-positive cells (filled arrows) and GFP-negative cells (open arrows).

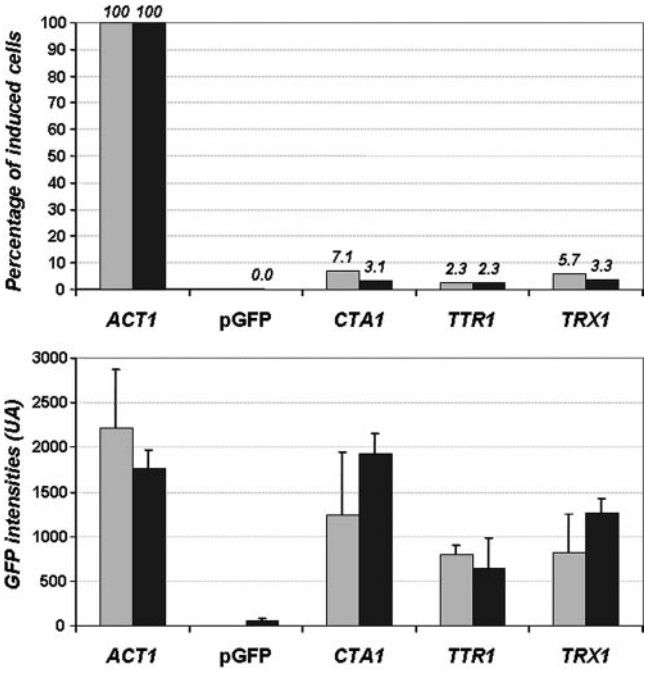

FIG. 7. Expression of oxidative stress reporters in *C. albicans* cells infecting mouse kidney. The *CTA1*-, *TTR1*/*GRX2*-, and *TRX1-GFP* reporter strains and the pGFP and *ACT1-GFP* controls were used to infect mice by tail vein injection (see Materials and Methods). Kidney sections were prepared, and the fungal cells were detected and quantified. Data are from animals infected for 4 days with the standard inoculum (dark gray bars) and 1 day with the elevated inoculum (light gray bars). (A) Proportion of fungal cells displaying GFP fluorescence. (B) Quantification of GFP fluorescence intensity in those fungal cells that were induced.

cells within the population. Also, transcript profiling experiments have not provided much information on the strength of the oxidative stress perceived by *C. albicans* following phagocytosis by host immune cells. Therefore, we used the *CTA1*-, *TTR1*/*GRX2*-, and *TRX1-GFP* reporters to analyze the behavior of *C. albicans* cells following phagocytosis and to titrate the level of oxidative stress after phagocytosis. No significant GFP fluorescence was observed following the engulfment of *CTA1*-, *TTR1*/*GRX2*-, or *TRX1-GFP* cells by macrophages, although significant fluorescence was observed for the positive *ACT1-GFP* control (Fig. 5). However, following phagocytosis by neutrophils, the *CTA1*- and *TRX1-GFP* reporters in most *C. albicans* cells were strongly induced, and the *TTR1*/*GRX2-GFP* reporter was moderately induced. These results are consistent with the transcript profiling data of Fradin and coworkers (20) (Fig. 1). Quantification of the fluorescence levels in phagocytosed *C. albicans* cells suggested that they were exposed to an oxidative stress that is equivalent to about 0.4 mM H_2O_2.

Exposure to oxidative stress during disseminated infection. Having used the *CTA1*-, *TTR1*/*GRX2*-, and *TRX1-GFP* reporters successfully in vitro and in ex vivo infection models, we investigated the behavior of these reporters in the mouse model of systemic candidiasis. Mice were infected with the *C. albicans* reporter strains by intravenous injection, and their kidneys were examined after 1 or 4 days. Two inoculum sizes were used (see Materials and Methods). With a standard inoculum (2×10^4 *C. albicans* cells/g) after 4 days sufficient fungal loads had built up in the kidneys of infected animals to provide adequate population sizes for GFP analysis (1.4×10^6 to 8.2×10^6 CFU/g kidney tissue). Both standard and 10-fold-increased inocula were compared to facilitate the analysis after 1 day of infection. After 1 day the standard inoculum generated fungal burdens of between 2.6×10^5 and 5.3×10^5 CFU/g kidney tissue, whereas the higher inoculum generated burdens of 3.5×10^6 to 5.3×10^6 CFU/g kidney tissue.

Tissue sections were stained with Calcofluor white to detect fungal cells within the kidney tissue (Fig. 6). A small proportion of *C. albicans* cells were dispersed in the kidneys, but even after 1 day most were present in foci of infection. *C. albicans* cells carrying the negative pGFP control displayed no GFP fluorescence (Fig. 6A), whereas all cells with the *ACT1-GFP* reporter displayed significant levels of fluorescence, as expected (Fig. 6B) (4, 5). Only about 4% of *C. albicans* cells carrying an oxidative stress reporter displayed GFP fluorescence. This was the case 1 and 4 days after infection (Fig. 7A). Furthermore, the inoculum size did not influence the proportion of GFP-fluorescing cells in the kidney. For example, only 5 of 113 *CTA1-GFP*-containing cells examined after 1 day of infection expressed GFP. Induced cells were generally clus-

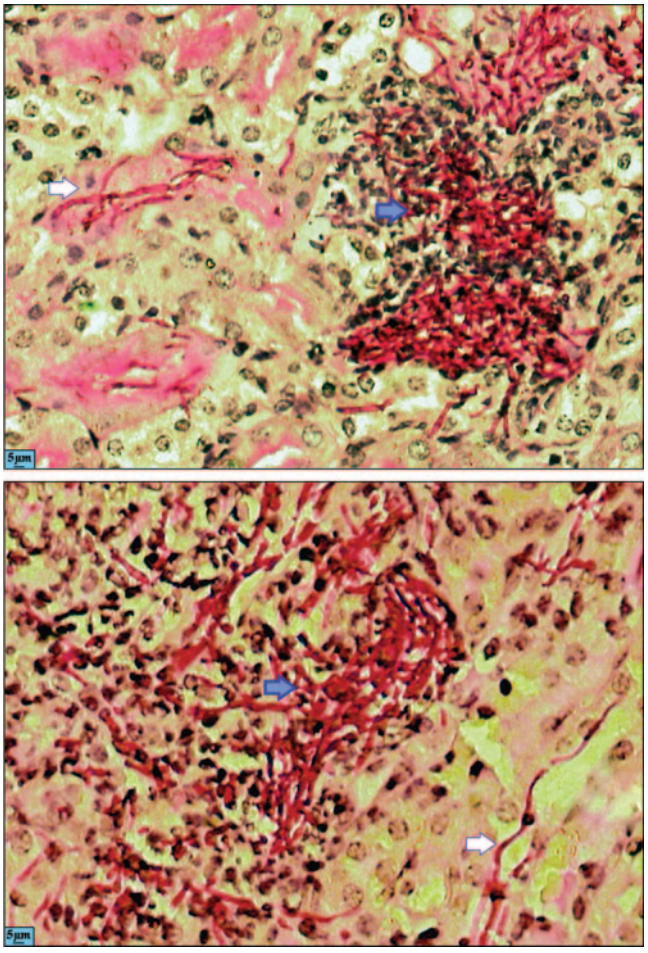

FIG. 8. Visualization of fungal and host cells in kidney sections from animals infected for 1 (upper panel) and 4 (lower panel) days, based on periodic acid-Schiff and hematoxylin stain. At both times a majority of fungal hyphae (strong pink) were surrounded by infiltrates of PMNs (dark staining; blue arrows), but examples of hyphae without surrounding infiltrates were also observed (white arrows).

tered together in groups (Fig. 6D), suggesting that *C. albicans* cells are exposed to a significant external oxidative stress only within specific microenvironments in the kidney. Quantification of these GFP signals suggested that the fungal cells in these microenvironments were exposed to oxidative stresses that were equivalent to about 0.4 mM H_2O_2. There was no significant difference in the strength of the oxidative stress response between the early and late infection time points (Fig. 7B). Similar observations were made for the *CTA1*-, *TTR1*/*GRX2*-, and *TRX1-GFP* reporters. The data indicate that, once infection has been established in the kidney, *C. albicans* cells are rarely exposed to a significant external oxidative stress.

Histopathological examination of infected kidneys revealed obvious polymorphonuclear leukocyte (PMN) infiltrates 1 day and 4 days after *C. albicans* challenge (Fig. 8). Foci of fungal growth were obvious at both times, but the sizes of the lesions on day 4 were larger than on day 1. The majority of the fungal foci (>60%) were associated with PMN infiltrates.

DISCUSSION

The ability to respond appropriately to environmental stress has been shown to be an important factor in the virulence of *C. albicans*. Mutations that disrupt stress signaling attenuate the virulence of this pathogen (2). Furthermore, *C. albicans* virulence is attenuated by the inactivation of catalase (*CAT1*) or superoxide dismutases (*SOD1* and *SOD5*) (20, 24, 27, 39, 40). These observations suggest that oxidative stress responses in particular are important for the establishment of systemic infections. Therefore, we have investigated the extent to which *C. albicans* cells are exposed to oxidative stresses in specific niches associated with the establishment and progression of systemic disease.

Our approach was to use single-cell profiling to examine the behavior of individual fungal cells in these niches. This approach exploits the specific regulatory profiles of particular promoters, which are then fused to the synthetic, codon-optimized yEGFP gene (11). These *GFP* fusions are then used to report the activation of particular molecular responses in *C. albicans* (4, 5). In this study we selected three *C. albicans* promoters which, on the basis of genome-wide expression profiling, respond relatively specifically to oxidative stress: *CTA1*, *TRX1*, and *TTR1*/*GRX2* (Fig. 1). Our analyses of the *TRX1*- and *TTR1*/*GRX2-GFP* reporter strains under a range of experimental conditions in vitro confirmed that they respond specifically to oxidative stresses (Fig. 2 and 3). The *CTA1-GFP* reporter also responded to oxidative stress but was also activated during growth on lactate, indicating that this gene is regulated by carbon source as well as oxidative stress (Fig. 3). This reporter was not activated strongly in response to osmotic stress (Fig. 2 and 3), unlike the wild-type *CTA1* transcript (Fig. 1), suggesting that the wild-type transcript might be regulated at the posttranscriptional level under these conditions. Despite its regulation by carbon source, the *CTA1-GFP* reporter could be used in combination with the *TRX1*- and *TTR1*/*GRX2-GFP* reporters to define the proportion of *C. albicans* cells that are exposed to an environmental oxidative stress. Furthermore, the activation of all three reporters was proportional to the level of oxidative stress, at least up to about 2 to 5 mM H_2O_2 (Fig. 4). This allowed us to estimate the relative strength of the environmental oxidative stress to which the *C. albicans* cells were exposed.

Having defined the specificity and amplitude of the *CTA1*-, *TRX1*-, and *TTR1*/*GRX2-GFP* reporter strains, we examined them in well-established ex vivo neutrophil and macrophage models. We chose these models because they are thought to reflect initial contact between *C. albicans* and host immune cells in the bloodstream and tissues early in a systemic infection. None of the reporter strains was activated following phagocytosis by macrophages (Fig. 5). The expression profiling performed by Lorenz et al. (25) suggests that *C. albicans CTA1* mRNA levels are increased following phagocytosis by macrophages, whereas the transcript profiling data set of Fradin and coworkers (20) suggests that this mRNA is not induced inside macrophages. Our single-cell profiling data are consistent with the latter conclusion. Our data also reinforce the view that neither *TRX1* nor *TTR1*/*GRX2* is induced in macrophages (20, 25). Taken together, the data confirm that *C. albicans* cells are

not exposed to a significant oxidative stress after phagocytosis by macrophages.

In contrast *C. albicans* cells are exposed to an oxidative stress when they are engulfed by neutrophils (Fig. 5). All three oxidative stress reporters were activated under these circumstances, and again, this was entirely consistent with the work of Fradin et al. (20). As part of their genome-wide expression profiling, they reported that the *CTA1*, *TRX1*, and *TTR1*/*GRX2* mRNAs are all induced in response to neutrophils. By quantifying the degree of activation of the *CTA1*-, *TRX1*-, and *TTR1*/*GRX2-GFP* reporter strains, we were able to show that the behavior of the population of phagocytosed cells is relatively homogeneous and that *C. albicans* is exposed to a relatively mild oxidative stress under these conditions (Fig. 5). This stress, which is equivalent to approximately 0.4 mM H_2O_2, is insufficient to activate the stress-activated protein kinase Hog1 (37). Although *C. albicans* cells were exposed to a relatively mild oxidative stress, few were able to survive phagocytosis by neutrophils. This is in contrast to phagocytosis by macrophages, which the *C. albicans* cells were generally able to survive in vitro. This is consistent with the idea that neutrophils use a combination of defense mechanisms to control *C. albicans* (16). The stringency of the environment within the neutrophil (20) or the additional exposure to nitrosative stress (22) might contribute to the anti-*Candida* activity of neutrophils. Our *CTA1*-, *TRX1*-, and *TTR1*/*GRX2-GFP* reporter strains were not triggered by a nitrosative stress (Fig. 3), indicating that the reporters are able to differentiate between these two defense mechanisms.

The relative importance of neutrophils and macrophages in defense against experimental *C. albicans* infections has been examined directly in the past. Carrageenan is a polysaccharide that is toxic for macrophages but which also stimulates a neutrophil leukocytosis. It has been shown to reduce the susceptibility of mice to intravenous *C. albicans* challenge, indicating that the increase in neutrophils is of more importance than the decrease in macrophages (23). With filter chambers implanted intraperitoneally in mice, Cutler and Poor (12) found neutrophils were more strongly candidacidal than macrophages, an observation subsequently confirmed in tests with human peritoneal neutrophils and macrophages (33).

Analysis of the oxidative stress reporter strains in the mouse model of systemic infection indicated that approximately 95% of *C. albicans* cells infecting the kidney are not exposed to a significant oxidative stress (Fig. 7). This was the case early (1 day) and late (4 days) in the infection process. Furthermore, this was not affected by inoculum size. Clearly, oxidative stress is not a constant threat to this pathogen during systemic infection. Histopathology showed that many fungal cells were apparently exposed to a PMN-based attack in infected kidneys (Fig. 8). However, unlike the situation ex vivo, in which PMNs definitely engulf *C. albicans* cells and induce an oxidative stress response, only a small proportion of cells was exposed to an oxidative stress in the kidney (Fig. 6D), with most foci of infection displaying no oxidative stress induction (Fig. 6C). The histopathology revealed few PMNs in direct contact with the *C. albicans* cells, which might account for this difference.

Hence, we conclude that *C. albicans* activates an oxidative stress response in a niche-specific manner during disease establishment and progression. The fungal cells are more likely to be exposed to an oxidative stress early in the establishment of a systemic infection, when they come into direct contact with neutrophils circulating in the bloodstream. At this stage, the ability to adapt appropriately to this oxidative stress might promote the survival of the pathogen and the subsequent development of a deep-seated infection. However, once an infection has been established in the kidney, PMNs surround the fungi but do not stimulate oxidative stress adaptation.

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REFERENCES

1. **Alarco, A. M., and M. Raymond.** 1999. The bZip transcription factor Cap1p is involved in multidrug resistance and oxidative stress response in *Candida albicans*. J. Bacteriol. **181:**700–708.
2. **Alonso-Monge, R., F. Navarro-Garcia, G. Molero, R. Diez-Orejas, M. Gustin, J. Pla, M. Sanchez, and C. Nombela.** 1999. Role of the mitogen-activated protein kinase Hog1p in morphogenesis and virulence of *Candida albicans*. J. Bacteriol. **181:**3058–3068.
3. **Alonso-Monge, R., F. Navarro-Garcia, E. Roman, A. I. Negredo, B. Eisman, C. Nombela, and J. Pla.** 2003. The Hog1 mitogen-activated protein kinase is essential in the oxidative stress response and chlamydospore formation in *Candida albicans*. Eukaryot. Cell **2:**351–361.
4. **Barelle, C. J., C. L. Manson, D. M. MacCallum, F. C. Odds, N. A. R. Gow, and A. J. P. Brown.** 2004. GFP as a quantitative reporter of gene regulation in *Candida albicans*. Yeast **21:**333–340.
5. **Barelle, C. J., C. L. Priest, D. M. MacCallum, N. A. R. Gow, F. C. Odds, and A. J. P. Brown.** 2006. Niche-specific regulation of central metabolic pathways in a fungal pathogen. Cell Microbiol. **8:**961–971.
6. **Beno, D. W., A. G. Stover, and H. L. Mathews.** 1995. Growth inhibition of *Candida albicans* hyphae by $CD8^+$ lymphocytes. J. Immunol. **154:**5273–5281.
7. **Brown, A. J. P.** 2005. Fungal genomics, Mycota XIII, p. 185–203. Springer-Verlag, Heidelberg, Germany.
8. **Calderone, R. A.** 2002. *Candida* and candidiasis. ASM Press, Washington, DC.
9. **Chauhan, N., D. Inglis, E. Roman, J. Pla, D. Li, J. A. Calera, and R. Calderone.** 2003. *Candida albicans* response regulator gene *SSK1* regulates a subset of genes whose functions are associated with cell wall biosynthesis and adaptation to oxidative stress. Eukaryot. Cell **2:**1018–1024.
10. **Chauhan, N., J. P. Latge, and R. Calderone.** 2006. Signalling and oxidant adaptation in *Candida albicans* and *Aspergillus fumigatus*. Nat. Rev. Microbiol. **4:**435–444.
11. **Cormack, B., G. Bertram, M. Egerton, N. A. R. Gow, S. Falkow, and A. J. P. Brown.** 1997. Yeast enhanced green fluorescent protein (yEGFP): a reporter of gene expression in *Candida albicans*. Microbiology **143:**303–311.
12. **Cutler, J. E., and A. H. Poor.** 1981. Effect of mouse phagocytes on *Candida albicans* in in vivo chambers. Infect. Immun. **31:**1110–1116.
13. **Enjalbert, B., A. Nantel, and M. Whiteway.** 2003. Stress-induced gene expression in *Candida albicans*: absence of a general stress response. Mol. Biol. Cell **14:**1460–1467.
14. **Enjalbert, B., and M. Whiteway.** 2005. Release from quorum-sensing molecules triggers hyphal formation during *Candida albicans* resumption of growth. Eukaryot. Cell **4:**1203–1210.
15. **Enjalbert, B., D. A. Smith, M. J. Cornell, I. Alam, S. Nicholls, A. J. P. Brown, and J. Quinn.** 2006. Role of the Hog1 stress-activated protein kinase in the global transcriptional response to stress in the fungal pathogen *Candida albicans*. Mol. Biol. Cell **17:**1018–1032.
16. **Fang, F. C.** 2004. Antimicrobial reactive oxygen and nitrogen species: concepts and controversies. Nat. Rev. Microbiol. **2:**820–832.
17. **Finkel, T., and N. J. Holbrook.** 2000. Oxidants, oxidative stress and the biology of ageing. Nature **408:**239–247.
18. **Fonzi, W. A., and M. Y. Irwin.** 1993. Isogenic strain construction and gene mapping in *Candida albicans*. Genetics **134:**717–728.
19. **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.
20. **Fradin, C., P. De Groot, D. M. MacCallum, M. Schaller, F. Klis, F. C. Odds, and B. Hube.** 2005. Granulocytes govern the transcriptional response, mor-

phology and proliferation of *Candida albicans* in human blood. Mol. Microbiol. **56:**397–415.
21. **Gow, N. A. R., and G. W. Gooday.** 1982. Growth kinetics and morphology of colonies of the filamentous form of *Candida albicans*. J. Gen. Microbiol. **128:**2187–2194.
22. **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.
23. **Hurtrel, B., and P. H. Lagrange.** 1981. Comparative effects of carrageenan on systemic candidiasis and listeriosis in mice. Clin. Exp. Immunol. **44:**355–358.
24. **Hwang, C. S., G. E. Rhie, J. H. Oh, W. K. Huh, H. S. Yim, and S. O. Kang.** 2002. Copper- and zinc-containing superoxide dismutase (Cu/ZnSOD) is required for the protection of *Candida albicans* against oxidative stresses and the expression of its full virulence. Microbiology **148:**3705–3713.
25. **Lorenz, M. C., J. A. Bender, and G. R. Fink.** 2004. Transcriptional response of *Candida albicans* upon internalization by macrophages. Eukaryot. Cell **3:**1076–1087.
26. **MacCallum, D. M., and F. C. Odds.** 2005. Temporal events in the intravenous challenge model for experimental *Candida albicans* infections in female mice. Mycoses **48:**151–161.
27. **Martchenko, M., A. M. Alarco, D. Harcus, and M. Whiteway.** 2004. Superoxide dismutases in *Candida albicans*: transcriptional regulation and functional characterization of the hyphal-induced *SOD5* gene. Mol. Biol. Cell **15:**456–467.
28. **Mavor, A. L., S. Thewes, and B. Hube.** 2005. Systemic fungal infections caused by *Candida* species: epidemiology, infection process and virulence attributes. Curr. Drug Targets **6:**863–874.
29. **Murad, A. M. A., P. R. Lee, I. D. Broadbent, C. J. Barelle, and A. J. P. Brown.** 2000. CIp10, an efficient and convenient integrating vector for *Candida albicans*. Yeast **16:**325–327.
30. **Murad, A. M. A., P. Leng, M. Straffon, J. Wishart, S. Macaskill, D. MacCallum, N. Schnell, D. Talibi, D. Marechal, F. Tekaia, C. d'Enfert, C. Gaillardin, F. C. Odds, and A. J. P. Brown.** 2001. *NRG1* represses yeast-hypha morphogenesis and hypha-specific gene expression in *Candida albicans*. EMBO J. **20:**4742–4752.
31. **Nantel, A., D. Dignard, C. Bachewich, D. Harcus, A. Marcil, A.-P. Bouin, C. W. Sensen, H., Hogues, M. van het Hoog, P. Gordon, T. Rigby, F. Benoit, D. C. Tessier, D. Y. Thomas, and M. Whiteway.** 2002. Transcript profiling of *Candida albicans* cells undergoing the yeast-to-hyphal transition. Mol. Biol. Cell **13:**3452–3465.
32. **Odds, F. C.** 1988. *Candida* and candidosis, 2nd ed. Bailliere Tindall, London, England.
33. **Peterson, P. K., D. Lee, H. J. Suh, M. Devalon, R. D. Nelson, and W. F. Keane.** 1986. Intracellular survival of *Candida albicans* in peritoneal macrophages from chronic peritoneal dialysis patients. Am. J. Kidney Dis. **7:**146–152.
34. **Ralph, P., J. Prichard, and M. Cohn.** 1975. Reticulum cell sarcoma: an effector cell in antibody-dependent cell-mediated immunity. J. Immunol. **114:**898–905.
35. **Rubin-Bejerano, I., I. Fraser, P. Grisafil, and G. R. Fink.** 2003. Phagocytosis by neutrophils induces an amino acid deprivation response in *Saccharomyces cerevisiae* and *Candida albicans*. Proc. Natl. Acad. Sci. USA **100:**11007–11012.
36. **Sherman, F.** 1991. Getting started with yeast. Methods Enzymol. **194:**3–21.
37. **Smith, D. A., S. Nicholls, B. A. Morgan, A. J. P. Brown, and J. Quinn.** 2004. A conserved stress-activated protein kinase regulates a core stress response in the human pathogen *Candida albicans*. Mol. Biol. Cell **15:**4179–4190.
38. **Swoboda, R. K., G. Bertram, S. Delbruck, J. F. Ernst, N. A. R. Gow, G. W. Gooday, and A. J. P. Brown.** 1994. Fluctuations in glycolytic mRNA levels during the yeast-to-hyphal transition in *Candida albicans* reflect underlying changes in growth rather than a response to cellular dimorphism. Mol. Microbiol. **13:**663–672.
39. **Van Dijck, P., L. De Rop, K. Szlufcik, E. Van Ael, and J. M. Thevelein.** 2002. Disruption of the *Candida albicans TPS2* gene encoding trehalose-6-phosphate phosphatase decreases infectivity without affecting hypha formation. Infect. Immun. **70:**1772–1782.
40. **Wysong, D. R., L. Christin, A. M. Sugar, P. W. Robbins, and R. D. Diamond.** 1998. Cloning and sequencing of a *Candida albicans* catalase gene and effects of disruption of this gene. Infect. Immun. **66:**1953–1961.
41. **Zaragoza, O., M. A. Blasquez, and C. Gancedo.** 1998. Disruption of the *Candida albicans TPS1* gene encoding trehalose-6-phosphate synthase impairs formation of hyphae and decreases virulence. J. Bacteriol. **180:**3809–3815.
42. **Zhang, X., M. De Micheli, S. T. Coleman, D. Sanglard, and W. S. Moye-Rowley.** 2000. Analysis of the oxidative stress regulation of the *Candida albicans* transcription factor, Cap1p. Mol. Microbiol. **36:**618–629.

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