

Redundant Catalases Detoxify Phagocyte Reactive Oxygen and Facilitate *Histoplasma capsulatum* Pathogenesis

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Histoplasma capsulatum is a respiratory pathogen that infects phagocytic cells. The mechanisms allowing *Histoplasma* to overcome toxic reactive oxygen molecules produced by the innate immune system are an integral part of *Histoplasma*'s ability to survive during infection. To probe the contribution of *Histoplasma* catalases in oxidative stress defense, we created and analyzed the virulence defects of mutants lacking CatB and CatP, which are responsible for extracellular and intracellular catalase activities, respectively. Both CatB and CatP protected *Histoplasma* from peroxide challenge *in vitro* and from antimicrobial reactive oxygen produced by human neutrophils and activated macrophages. Optimal protection required both catalases, as the survival of a double mutant lacking both CatB and CatP was lower than that of single-catalase-deficient cells. Although CatB contributed to reactive oxygen species defenses *in vitro*, CatB was dispensable for lung infection and extrapulmonary dissemination *in vivo*. Loss of CatB from a strain also lacking superoxide dismutase (Sod3) did not further reduce the survival of *Histoplasma* yeasts. Nevertheless, some catalase function was required for pathogenesis since simultaneous loss of both CatB and CatP attenuated *Histoplasma* virulence *in vivo*. These results demonstrate that *Histoplasma*'s dual catalases comprise a system that enables *Histoplasma* to efficiently overcome the reactive oxygen produced by the innate immune system.

Reactive oxygen species (ROS) are one of the most effective components of the antimicrobial arsenal produced by the innate immune system. Within mammals, a specialized subset of leukocytes that includes monocytes, polymorphonuclear neutrophils (PMNs), dendritic cells, and macrophages produces superoxide anion through assembly of the NADPH oxidase complex (1). Superoxide gives rise to other reactive oxygen molecules, including hydrogen peroxide, which are capable of damaging numerous macromolecules and killing microbes (2). The persistent bacterial and fungal infections that characterize chronic granulomatous disease, which is caused by genetic deficiency in the NADPH oxidase, underscore the critical importance of ROS in combating microbial infections (3, 4).

In order to survive in the mammalian host, successful pathogens must avoid or neutralize host-derived oxidative stresses. Both enzymatic and nonenzymatic strategies are utilized by microbial pathogens of plants and animals to accomplish this task (5–7). Enzymes such as superoxide dismutase and catalase specifically detoxify the ROS molecules superoxide anion and hydrogen peroxide, respectively (7). In the fungal kingdom, nonenzymatic strategies include production of melanin to absorb ROS or reductants such as thioredoxin to mend oxidative protein damage (8–10). While most organisms express a variety of antioxidant factors to cope with metabolically derived intracellular ROS, microbial pathogens must employ additional, often extracellular, factors to defend against ROS produced by host cells and the host environment (11–18). For microbial pathogens that infect ROS-producing phagocytic cells, the ability to defend against phagocyte-derived ROS becomes even more imperative.

Histoplasma capsulatum is one such intracellular fungal pathogen capable of parasitizing phagocytic immune cells. This fungus is found worldwide, with particular prominence in the United States within the Ohio and Mississippi River valleys. Within this area of endemicity, 80% of the population is estimated to have been exposed to *Histoplasma* (19). Acquisition of *Histoplasma* occurs upon inhalation of mycelium-produced conidia into the

mammalian lung, where the temperature change to 37°C causes conversion of *Histoplasma* into pathogenic yeast cells (20, 21). Yeast cells encounter both neutrophils and alveolar macrophages. However, the innate immune system alone is unable to control *Histoplasma* infections. Following uptake of yeasts by phagocytes, the yeasts proliferate within phagosomes until rupture of the host cell and release of yeasts, which are subsequently taken up by neighboring phagocytes. Both immunocompromised and immunocompetent individuals are susceptible to infection, but in most cases, immunocompetent hosts are able to control yeast growth upon activation of the adaptive immune response, which enhances the antifungal response of phagocytes (22).

The substantial interactions between *Histoplasma* yeasts and host phagocytes make *Histoplasma*'s ability to avoid or detoxify ROS an essential component of its pathogenesis. Although resting macrophages do not generate an oxidative burst in response to *Histoplasma* yeasts, activation of macrophages or opsonization of yeasts triggers ROS production (17, 23, 24). On the other hand, PMNs readily produce ROS upon encounter with *Histoplasma* (17, 25–27). Regardless of the cell type and response, *Histoplasma* yeasts are able to survive the ROS challenge. The molecular mechanisms responsible for this resistance to ROS have largely re-

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TABLE 1 *Histoplasma* strains

Strain ^a	Genotype ^c	Other designation
WU8 ^b	<i>ura5-32Δ</i>	Wild type
OSU15	<i>ura5-32Δ sod3-3Δ::hph/pCR468 (URA5, P_{H2B}-gfp:FLAG)</i>	<i>sod3Δ</i>
OSU16	<i>ura5-32Δ catB-2Δ::hph/pCR468 (URA5, P_{H2B}-gfp:FLAG)</i>	<i>catBΔ</i>
OSU24	<i>ura5-32Δ catB-2Δ::hph</i>	<i>catBΔ</i>
OSU31 ^c	<i>ura5-32Δ catB-3Δ::apt3 sod3-3Δ::hph</i>	<i>catBΔ sod3Δ</i>
OSU45 ^d	<i>ura5-32Δ/pCR468 (URA5, P_{H2B}-gfp:FLAG)</i>	WT
OSU46	<i>ura5-32Δ catB-3Δ::apt3 sod3-3Δ::hph/pCR468 (URA5, P_{H2B}-gfp:FLAG)</i>	<i>catBΔ sod3Δ</i>
OSU51	<i>ura5-32Δ catB-2Δ::hph/pBY08 (URA5, P_{H2B}-CATB:FLAG)</i>	<i>catBΔ/CATB</i>
OSU138	<i>ura5-32Δ catB-2Δ::hph catP-2Δ::apt3</i>	<i>catBΔ catPΔ</i>
OSU149	<i>ura5-32Δ catP-1Δ::apt3</i>	<i>catPΔ</i>
OSU157	<i>ura5-32Δ catP-1Δ::apt3/pCR468 (URA5, P_{H2B}-gfp:FLAG)</i>	<i>catPΔ</i>
OSU158	<i>ura5-32Δ catP-1Δ::apt3/pCR615 (URA5, P_{H2B}-FLAG:CATP)</i>	<i>catPΔ/CATP</i>
OSU159	<i>ura5-32Δ catB-2Δ::hph catP-2Δ::apt3/pCR468 (URA5, P_{H2B}-gfp:FLAG)</i>	<i>catBΔ catPΔ</i>

^a Strains derived from the Panama type strain, G186A (ATCC 26027).

^b Uracil auxotroph derived from G186A (79).

^c Derived from the *sod3Δ* strain (OSU13 [17]).

^d From reference 17.

^e The single colons represent a protein fusion expressed as a transgene.

mained unknown. Recently, we demonstrated that yeasts produce an extracellular superoxide dismutase (Sod3) that protects *Histoplasma* from macrophage- and PMN-generated superoxide and is required for full virulence within a murine infection model (17).

In addition to Sod3, the *Histoplasma* genome encodes three catalase proteins that could potentially assist in combating ROS (28). CatA has been reported to be an 80-kDa catalase expressed by mycelia. CatB is an immunodominant 90-kDa extracellular catalase of *Histoplasma* yeasts also known as M antigen (13, 29–32). The third catalase, CatP, is a 60-kDa catalase (29, 33). CatB has been claimed to be a *Histoplasma* virulence factor, despite the lack of any genetic evidence for this speculation (28, 34). Likewise, no functional studies on the contribution of intracellular catalases have been performed, but these catalases could also contribute to defense against the membrane-permeant peroxide generated by host phagocytes.

In this study, we examined the roles of the extracellular and intracellular catalases in protecting *Histoplasma* from host-derived ROS and determined their contribution to *Histoplasma* virulence. Functional tests were facilitated by generation of *Histoplasma* strains lacking CatB, CatP, or both proteins. We confirm that CatB and CatP are responsible for the extracellular and intracellular catalase activities of *Histoplasma* yeast, respectively. Both proteins can protect *Histoplasma* yeast from hydrogen peroxide *in vitro*, although CatP appears to be more critical. Each catalase provides a modest degree of protection from phagocyte ROS-dependent killing, yet the loss of either protein singly does not attenuate *Histoplasma* survival *in vivo*. Analysis of strains lacking both catalases indicates that CatB and CatP provide redundant functions in defending *Histoplasma* from host-derived ROS during mammalian infection.

MATERIALS AND METHODS

Fungal strains and culture conditions. *Histoplasma capsulatum* strains were derived from the clinical isolate G186AR (ATCC 26027) and are listed in Table 1. *Histoplasma* cultures were grown and maintained in *Histoplasma* macrophage medium (HMM) (35). Liquid cultures were grown with constant aeration (200 rpm) until late exponential growth phase. Uracil auxotrophs were grown in medium supplemented with 100

μg/ml uracil. Yeasts were maintained by growth at 37°C with 5% CO₂–95% air. For monitoring of yeast growth, liquid culture density was determined by treating yeasts with 1 M NaOH and measuring culture turbidity at 595 nm. Mycelia were grown in static liquid cultures at 25°C in 100% air. For *Histoplasma* growth on solid medium, HMM was solidified with 0.6% agarose and supplemented with 25 μM FeSO₄. Yeasts were transformed with linearized plasmids by electroporation, and Ura⁺ transformants were selected on HMM (36). For experiments with phagocytes and murine infections, large clumps of yeasts were first removed from yeast suspensions by centrifugation (50 × g), and yeasts were enumerated by hemacytometer.

***Histoplasma* RNA isolation and quantitative reverse transcription-PCR (RT-PCR).** Wild-type *Histoplasma* yeasts were collected by centrifugation (2,000 × g) and resuspended in Ribozol reagent (Amresco, Inc.). Mycelia were collected from static liquid cultures by filtration onto Whatman no. 5 filter paper and scraping of the retained mycelia into Ribozol. RNA was liberated from *Histoplasma* cells in Ribozol by beating with 0.5-mm-diameter glass beads. RNA was purified by CHCl₃ extraction and alcohol precipitation of the aqueous phase. Genomic DNA was removed by two sequential digestions with Turbofree DNase (Ambion), and the absence of DNA was verified by PCR. Five micrograms of total RNA was reverse transcribed using SuperScript III reverse transcriptase (Invitrogen) and random 15-mer primers.

Quantitative PCR (qPCR) was performed on reverse-transcribed RNAs from yeasts and mycelia. Amplification reaction mixtures were assembled using a SYBR-green-based PCR master mix (Bio-Rad) with reverse-transcribed RNA templates (final dilution, 1:200) and 0.5 μM each gene-specific primer (see Table S1 in the supplemental material). Cycling was performed with a realplex thermocycler (Eppendorf) using the following conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 10 s, 55°C for 15 s, and 72°C for 1 min. *CATA*, *CATB*, and *CATP* transcript abundance in yeasts relative to that in mycelia was determined by the $\Delta\Delta C_T$ method (where C_T is threshold cycle) (37) after normalizing the levels of *CAT* gene expression to the levels of expression of the *RPS15* transcript. To estimate the relative levels of expression of the catalase genes, the normalized C_T (ΔC_T) numbers were used as an indicator of mRNA abundance. A relative copy number (RCN) value for each gene, determined using the equation $2^{-\Delta C_T}$ (38), was used to estimate abundance, and was then compared to the RCN of *ACT1* as a common reference point, which was set at 100%. Statistical analyses were performed on the normalized C_T values using Student's *t* test on the results for RNA from three biological replicate cultures of yeasts and mycelia.

Generation and validation of *Histoplasma* strains with catalase gene deletions. To generate the *catBΔ* or *catPΔ* strains, positive and negative selections were used to enrich for yeasts with a deletion of the *CATB*- or *CATP*-coding region by allelic replacement (39). WU8 uracil-auxotrophic yeasts were transformed with the *URA5* plasmid pBY19 or pEH71. pBY19 and pEH71 contain a hygromycin resistance gene (*hph*) or a G418 resistance gene (*apt3*) flanked by 1.8 kb of sequence upstream and downstream of the *CATB*- or *CATP*-coding sequence, respectively. Multiple *Ura*⁺ transformants were passaged three times in liquid HMM containing uracil and either 150 μg/ml hygromycin or 100 μg/ml G418. Selection for deletion alleles and loss of the *URA5* plasmids was done by plating yeast cultures on HMM containing uracil, 150 μg/ml hygromycin or 100 μg/ml G418, and 1 mg/ml 5-fluorouracil (5-FOA). Hygromycin- or G418-resistant, 5-FOA-resistant colonies were picked and screened for the loss of *CATB* or *CATP* by PCR. Using similar procedures, construction of the *catBΔ catPΔ* and the *catBΔ sod3Δ* double mutant strains was done by disrupting the *CATP* gene in the *catBΔ* background and by disrupting the *CATB* gene in the *sod3Δ* background using plasmids pEH71 and pBY23, respectively. To restore uracil prototrophy, mutant strains were transformed with pCR468, a *URA5* plasmid with a green fluorescent protein (GFP) expression cassette. The *catB* and *catP* deletions were complemented by transforming the mutant yeasts with plasmids pBY08 and pCR615, which express the *CATB* and *CATP* genes from the constitutive *H2B* promoter, respectively.

To confirm the construction of catalase-deficient and catalase-complemented lines, genotypes were initially verified by PCR using genomic DNA isolated from each strain. PCR amplification of the *CATB* gene, *CATP* gene, *hph* cassette, or *apt3* cassette utilized 0.5 μM primers specific for each gene (see Table S1 in the supplemental material). The competence of the *Histoplasma* genomic DNA template for PCR was verified by amplification of the *ACT1* gene. Loss of CatB protein was validated by separation of culture filtrate proteins (5 μg total protein) by SDS-PAGE, followed by silver staining to visualize proteins. The presence or absence of CatP in mutant strains was determined by immunoblotting. Custom antibodies to CatP were generated by immunization of rabbits (Open Biosystems) with *Escherichia coli*-produced full-length *Histoplasma* CatP protein. For production of the immunogen, the *Histoplasma* *CATP* sequence was cloned into a C-terminal 6× His vector (pET28a; EMD Biosciences) and transformed into *E. coli*. The 6× His-tagged CatP protein was purified from *E. coli* lysates by immobilized cobalt affinity chromatography. For immunoblotting, 10 μg of *Histoplasma* total cellular lysate proteins was separated by SDS-PAGE and transferred to nitrocellulose. Immunoblotting was performed with the anti-CatP immune serum. No *Histoplasma* cellular proteins were detected by preimmune serum (data not shown).

Assay of extracellular and intracellular catalase activity. *Histoplasma* yeast cultures were grown in liquid HMM at 37°C with aeration until they reached late exponential/early stationary growth phase. Three biological replicate yeast cultures were used, and the relative number of yeast cells was normalized by comparison of optical densities. For extracellular catalase activity, cell-free supernatant and yeast cell fractions were separated by centrifugation (16,000 × g). For yeast cell lysates, cultures were pelleted, washed twice with phosphate-buffered saline (PBS), and suspended in PBS. Cytoplasmic protein was released by bead beating yeasts with 0.5-mm-diameter glass beads. Protein concentrations were determined using the Bradford method with bovine serum albumin protein as the standard.

Catalase activity assays for extracellular and intracellular samples were performed by spectrophotometrically measuring their ability to reduce the H₂O₂ concentration over time. For the soluble extracellular fractions, normalized amounts of clarified culture supernatants were brought to 200 μl with 50 mM Na-phosphate buffer (pH 7.2), before the addition of 800 μl of 10 mM H₂O₂ in 50 mM Na-phosphate buffer (pH 7.2). Hydrogen peroxide destruction was measured at 240 nm with correction at 595 nm every 30 s for 5 min and compared to that in a control containing buffer

and HMM only. For cell-associated catalase activity, H₂O₂ destruction was determined after incubation of yeasts with a buffered H₂O₂ solution and removal of yeast cells by passage through a 0.22-μm-pore-size filter Spin-X column (Corning) before spectrophotometric measurement at 240 nm. Because of the additional manipulations before reading, all steps and incubations were carried out at 4°C to slow enzymatic destruction of H₂O₂. For intracellular catalase activity determination, cellular lysates (12 μg of total cytosolic protein) were added to the Na-phosphate-buffered H₂O₂ solution, and the H₂O₂ destruction was measured by taking spectrophotometric readings every 30 s for 5 min. For relative comparison of soluble extracellular to cell-associated extracellular activities, the decrease in H₂O₂ concentration was determined for supernatant and yeast samples incubated at room temperature for 5 min, and the results were normalized to the H₂O₂ decrease in the absence of CatB using samples from the *catBΔ* mutant. Statistical analyses were performed on three biological replicates for each strain using Student's *t* test and data points at 5 min.

Determination of *in vitro* sensitivity to hydrogen peroxide. Sensitivity to H₂O₂ *in vitro* was determined by application of H₂O₂-saturated filter disks to growing *Histoplasma* yeasts. *Histoplasma* yeasts (1 × 10⁷) were spread on 10-cm-diameter HMM plates. After inoculation of plates, sterile filter disks treated with 20 μl of 0, 75, 150, or 300 mM H₂O₂ in PBS were applied and the plates were incubated at 37°C with 5% CO₂-95% air to allow *Histoplasma* growth. After 6 days, the plates were photographed and the areas lacking yeast growth were measured using ImageJ software (<http://rsbweb.nih.gov/ij/>) after binary thresholding of the images to define the areas of clearing around the filter disks. Statistical analyses on the area of the clearing zones were performed for three biological replicates for each strain using Student's *t* test.

Phagocyte isolation and infection with *Histoplasma* yeasts. PMNs were isolated from the blood of healthy human donors through venipuncture as previously described (40, 41). Briefly, 10 ml of blood was collected into syringes containing 250 U heparin and diluted into 10 ml 0.9% saline solution. PMNs were harvested by density sedimentation (40 min at 400 × g) after overlaying onto 4.5 ml Ficoll-Paque PLUS (GE Healthcare). Erythrocytes were removed by resuspension of cells in 6 ml 0.9% saline, followed by addition of 6 ml of 3% Dextran (500 kDa) and incubation at 4°C for 20 min to allow the heavier red blood cells to settle. The upper PMN-containing layer was removed, and cells were collected by centrifugation (15 min at 800 × g). Residual erythrocytes were lysed by the addition of 10 ml cold H₂O for 20 s before the addition of 10 ml of 1.8% saline solution to restore osmotic balance.

For infection of PMNs, PMNs were collected by centrifugation (10 min at 800 × g) and washed in Hanks balanced salt solution (lacking Ca²⁺ and Mg²⁺) before enumeration by hemacytometer. Viability of cells was verified to be greater than 98% by trypan blue staining. Autologous serum was prepared from separate blood samples utilizing coagulation and centrifugation (460 × g). PMNs were seeded at 2 × 10⁵ cells per well of a 96-well plate in Dulbecco modified Eagle medium plus 10% autologous serum, 2 × 10⁴ *Histoplasma* yeast cells were added to each PMN-containing well, and the cells were coincubated for 4 h at 37°C with 5% CO₂-95% air. To determine yeast viability, culture medium was removed and the PMNs were lysed by addition of cold H₂O and scraping of the wells with a pipette tip. Lysates were serially diluted and plated on solid HMM to determine viable CFU numbers. Relative survival of yeasts was calculated by comparing infected wells to wells containing yeast only. Statistical analyses were performed using Student's *t* test on three biological replicate tests.

For infection of human monocyte-derived macrophages, peripheral blood was collected from healthy human donors by venipuncture and macrophages were differentiated from monocytes (42). One hundred milliliters of blood was collected, and four 20-ml fractions were each diluted into 15 ml of 0.9% saline before they were added to 14 ml of Ficoll-Paque PLUS (GE Healthcare). Buffy coats were collected into conical tubes following density sedimentation (40 min at 400 × g), and volumes were brought to 50 ml with cold PBS. Cells were pelleted by centrifugation

(15 min at $460 \times g$ and 4°C), the four fractions were resuspended in RPMI 1640 and pooled, and cells were enumerated by hemacytometer. Peripheral blood cells in RPMI 1640 with 20% autologous serum were added to Teflon dishes (Savilex) at 2×10^6 cells/ml and incubated at 37°C with 5% CO_2 -95% air for 5 days to allow differentiation of monocytes into macrophages.

For infection of macrophages, cells were collected after 5 days by washing of the Teflon dishes and recovery of the cells by centrifugation (10 min at $130 \times g$ and 4°C). Cell suspensions were adjusted to 4×10^6 cells/ml in RPMI 1640 with 10% autologous serum after enumeration, and $200 \mu\text{l}$ (8×10^4 macrophages) was added to the wells of a 96-well plate and incubated at 37°C with 5% CO_2 -95% air. After 2 h, nonadherent cells were removed by washing the wells three times with PBS. For cytokine activation, macrophage monolayers were first treated with 1,000 U/ml gamma interferon (IFN- γ) in RPMI 1640 with 10% autologous serum for 2 days. Infection of macrophages with yeasts was done by addition of 2×10^3 yeasts to each monolayer and incubation for 4 h at 37°C with 5% CO_2 -95% air. Macrophages were lysed by adding cold H_2O and scraping the wells with a pipette tip. Lysates were diluted and plated on solid HMM to enumerate the number of viable *Histoplasma* CFU. Survival was determined by comparing the number of CFU from infected wells to the number from wells containing yeast in the absence of macrophages. Statistical analyses were performed using Student's *t* test on three biological replicate tests.

Determination of fungal burdens in murine tissue following respiratory infection. C57BL/6 male mice (NCI) were infected intranasally with 2×10^4 yeast cells suspended in HMM. *Histoplasma* yeast cells were enumerated by hemacytometer following centrifugal removal of yeast aggregates ($50 \times g$). The actual number of CFU in the inoculum delivered was computed by plating serial dilutions of the inoculum suspensions on solid medium. At 4-day incremental time points following infection, lung tissue and spleen tissue were collected from infected mice and homogenized in 5 ml or 3 ml HMM, respectively. Serial dilutions of the homogenates were plated on solid HMM to determine the number of viable fungal CFU in each organ. Statistical analyses were performed on four replicate infections at each time point using Student's *t* test.

All animal experiments were performed in compliance with the National Research Council's Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at Ohio State University (protocol 2007A0241). Human cells were obtained from healthy volunteers after obtaining written informed consent and HIPAA authorization. Human subjects research was approved by the Biomedical Sciences Institutional Review Board at Ohio State University (protocol 2008H0242).

RESULTS

Pathogenic-phase *Histoplasma* cells express *CATB* and *CATP*.

To identify catalases with the potential to protect *Histoplasma* from oxidative stress related to infection, we determined which catalase genes were expressed by pathogenic-phase cells. The *Histoplasma* genome encodes three catalase proteins: CatB, CatP, and CatA (28). We previously showed that *CATB* expression is enriched in pathogenic-phase cells. This pathogenic-phase-restricted expression suggests that its role is linked to pathogenesis (43). We used quantitative RT-PCR to determine the *CATB*, *CATP*, and *CATA* expression profile in the pathogenic yeast cells and in the avirulent mycelia. In contrast to *CATB*, *CATP* and *CATA* did not show phase-dependent regulation (Fig. 1A). *CATA* expression was barely detectable by RT-PCR. Since the preceding analysis indicated only the relative enrichment in the yeast or mycelial phase but did not show to what degree the genes were expressed in yeasts, we compared the catalase mRNA levels to the level of the actin (*ACT1*) housekeeping gene. Both the *CATB* gene and the *CATP* gene were expressed at reasonably detectable levels

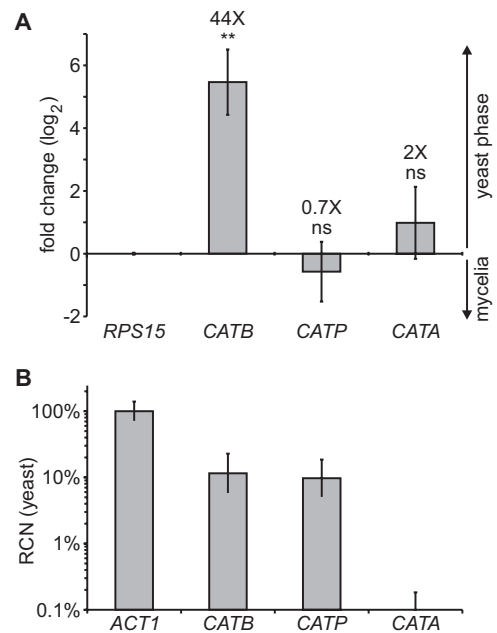


FIG 1 *CATB* and *CATP* are the major *Histoplasma* catalase genes expressed, but only *CATB* shows pathogenic-phase regulation. (A) Yeast- and mycelium-phase expression profiles for the extracellular catalase *CATB* gene and the *CATP* and *CATA* catalase genes. Relative expression of each catalase gene was determined by quantitative PCR of reverse-transcribed RNA from yeasts and mycelia after normalization to the level of expression of the *RPS15* gene. Data represent the log (base 2) value of the fold change in expression of the *CATB*, *CATP*, and *CATA* genes in yeasts compared to mycelia. The fold change (yeast-phase compared to mycelium-phase expression) is indicated by the values above the respective columns. Error bars represent standard deviations of three biological replicate RNA samples. Asterisks represent significant differences between yeast and mycelial phases determined by Student's *t* test (ns, not significant; **, $P < 0.01$). (B) Estimation of the relative level of *CATB*, *CATP*, and *CATA* expression in yeasts. Expression levels were approximated by use of the RCN value, which was calculated from the *RPS15*-normalized cycle thresholds for each gene and compared to the value for *RPS15*-normalized actin (*ACT1*), set at 100%.

in yeast cells (Fig. 1B). However, yeasts expressed comparatively little *CATA*, implying that CatA does not contribute to ROS defenses of pathogenic yeast cells.

CatB and CatP represent the predominant extracellular and intracellular catalase activities of *Histoplasma* yeasts. To facilitate functional characterization of *Histoplasma* catalases, we created *Histoplasma* strains which lacked CatB and CatP. Deletion alleles of *CATB* and *CATP* were created by replacing the coding region with a hygromycin (*hph*) or a G418 (*apt3*) resistance gene, respectively (Table 1). For each deletion strain, a gene-complemented line was created by transforming the mutants with a linear plasmid that expressed either *CATB* or *CATP* from a constitutive promoter. Gene deletions and complementation lines were confirmed by PCR using gene-specific or deletion-allele-specific primers (see Fig. S1 in the supplemental material). To address potential functional redundancy between CatB and CatP, a double mutant strain with deletions of both the *CATB* and *CATP* genes was also generated. The catalase-deletion strains grew at rates similar to the rate for wild-type *Histoplasma in vitro* in rich growth medium (data not shown).

The loss of catalase proteins was verified by visualizing CatB and CatP proteins in the mutant and complemented strains. Anal-

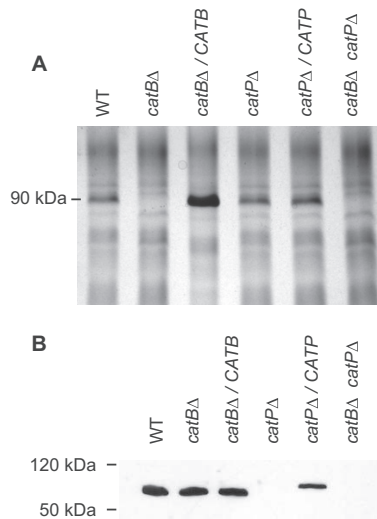


FIG 2 Deletion of the *CATB* and *CATP* genes specifically results in loss of extracellular CatB and intracellular CatP, respectively. (A) Analysis of extracellular CatB production. Total culture filtrate proteins from wild type, the catalase mutants, and their corresponding complemented strains were separated by SDS-PAGE, and proteins were visualized by silver staining to monitor production of the 90-kDa CatB protein. (B) Analysis of intracellular CatP production. Cellular lysates were prepared from wild type, the catalase mutants, and their corresponding complemented strains. Evidence of CatP protein in lysates was shown by immunoblotting of lysate proteins with a custom antibody to *Histoplasma* CatP. WT, wild type (OSU45); *catBΔ*, *catB* mutant (OSU16); *catBΔ*/*CATB*, complemented *catB* mutant (OSU51); *catPΔ*, *catP* mutant (OSU157); *catPΔ*/*CATP*, complemented *catP* mutant (OSU158); *catBΔ catPΔ*, *catB catP* double mutant (OSU159).

ysis of extracellular proteins by silver staining following electrophoretic separation of yeast culture filtrates through polyacrylamide showed loss of a 90-kDa protein specifically in single and double mutant strains with the *catB* deletion allele (Fig. 2A). The identity of this 90-kDa band as CatB was further confirmed by the increased abundance of the protein band by overexpression of the *CATB* gene from a strong, constitutive promoter in the *CATB*-complemented line (*catBΔ*/*CATB*). Due to the complexity of the intracellular protein population, yeast cellular lysates were probed for the presence of the CatP protein by immunoblotting with CatP-specific antisera (Fig. 2B). The 60-kDa CatP protein was absent from lysates derived from the *catPΔ* strain and the *catBΔ catPΔ* double mutant, whereas CatP was easily detected in lysates from the wild type and the *catBΔ* mutant. Complementation of the CatP deficiency by expression of the *CATP* gene (*catPΔ*/*CATP*) restored the presence of the CatP protein in yeast cells. The slightly slower migration of CatP in the complemented line was due to the addition of a FLAG epitope to CatP. Thus, mutant strains were depleted of their respective catalase. Furthermore, compensatory production of catalase enzymes was not apparent, as the levels of CatP in the *catBΔ* mutant and of CatB in the *catPΔ* mutant were not noticeably changed from those in the wild-type strain. *CATA* was also not upregulated in the CatB- and CatP-deficient yeasts (data not shown).

To determine if CatB was the source of extracellular catalase activity produced by *Histoplasma* yeast, we examined the H₂O₂-degrading capacity of extracellular fractions of the CatB-deficient mutants. Cell-free culture supernatants from wild-type *Histoplasma* yeast readily destroyed hydrogen peroxide, as determined

by the decrease in the absorbance at 240 nm of an H₂O₂-containing solution (Fig. 3A). However, yeast culture supernatants collected from the *catBΔ* and *catBΔ catPΔ* mutants showed virtually no ability to degrade H₂O₂. Catalase activity was restored by complementing the *catB* deletion strain with a copy of the *CATB* gene. Importantly, loss of CatP did not impact the extracellular catalase activity, as the catalase activity in culture supernatants from the *catPΔ* mutant was not statistically different from that in supernatants from the wild type.

We used the *catBΔ* mutant to test if CatB associated with the yeast cell surface also contributes to *Histoplasma* yeasts' ability to break down exogenous H₂O₂. Previous studies showed that in addition to soluble CatB protein, wild-type yeasts also have CatB associated with the yeast cells (32). Consistent with the cell association of CatB, washed, intact *Histoplasma* yeasts could destroy H₂O₂ (Fig. 3B). This activity was dependent on production of the CatB protein, as *catBΔ* and *catBΔ catPΔ* mutant yeast cells did not degrade exogenous H₂O₂. Additionally, the extracellular catalase activity associated with cells was not affected by loss of CatP. Complementation of CatB deficiency with *CATB* restored peroxide destruction, confirming that CatB is the source of the extracellular catalase activity. The cell-associated catalase kinetic assays were performed at 4°C due to the manipulations required to remove yeast cells before spectrophotometric readings. To determine the relative amount of catalase activity associated with cells compared to that present in the culture filtrate, endpoint assays (5 min) were performed at room temperature with equivalent amounts of yeasts from which supernatant samples were derived. Comparison of the H₂O₂ degradation rates between wild type and the *catBΔ* mutant yeast and culture filtrates showed that (i) all of the extracellular catalase activity is produced by CatB and (ii) roughly half of the total extracellular catalase activity is found in the cell-free supernatant and half is cell associated (data not shown).

In contrast to the CatB-dependent extracellular catalase activity, CatP was responsible for all the intracellular catalase activity of *Histoplasma* yeast. Previously, Howard showed that cellular lysates of *Histoplasma* yeasts are capable of degrading hydrogen peroxide (44). To determine if CatP produces the observed intracellular catalase activity, CatP-deficient yeast cells were lysed and the clarified lysates were examined for the ability to destroy hydrogen peroxide (Fig. 3C). Lysates from wild-type yeast and from *catBΔ* yeast readily degraded hydrogen peroxide *in vitro*. However, cell lysates from the *catPΔ* mutant strain were completely unable to degrade peroxide, unless a plasmid copy of *CATP* was expressed. The CatP activity in the complemented line was only about one-third of that in wild type (Fig. 3C). Lysates from the *catBΔ catPΔ* double mutant were indistinguishable from those from the *catPΔ* single mutant and from buffer alone. These data indicate that CatP is the source of intracellular hydrogen peroxide-degrading activity and that CatB does not contribute to the intracellular activity. Furthermore, the lack of extracellular catalase activity in CatB-deficient strains and the lack of intracellular catalase activity in CatP-deficient strains further prove that CatA does not contribute to the catalase activity of *Histoplasma* yeast cells, consistent with its lack of expression (Fig. 1).

CatB and CatP protect *Histoplasma* from H₂O₂ *in vitro*. To determine if CatB and/or CatP can defend *Histoplasma* yeasts against peroxide *in vitro*, CatB- and CatP-deficient yeasts were tested for their resistance to hydrogen peroxide using a disk diffu-

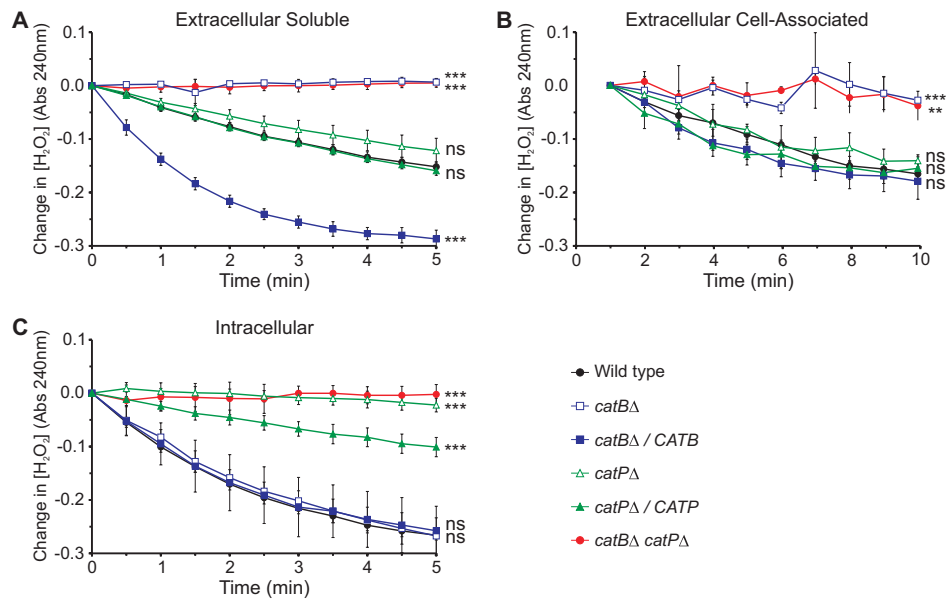


FIG 3 CatB and CatP are specifically responsible for extracellular and intracellular catalase activity, respectively. (A) Extracellular cell-free (soluble) catalase activity. Culture filtrates harvested from wild-type and catalase-deficient yeasts were tested for catalase activity by monitoring the ability of the culture filtrate proteins to destroy H₂O₂. Relative H₂O₂ destruction was measured as the decrease in absorbance (Abs) at 240 nm over time at 25°C. (B) Extracellular catalase activity associated with yeast cells. The ability of yeasts to eliminate H₂O₂ was monitored by incubation of washed yeast cells with H₂O₂ and quantitation of the H₂O₂ remaining by determination of the absorbance at 240 nm. At 1-min intervals, yeasts were removed by centrifugation through 0.45- μ m-pore-size filters and the absorbance of the clarified solution was determined. The assay was performed at 4°C to slow the enzyme kinetics to allow experimental manipulations. (C) Intracellular catalase activity of yeast cells. Cellular lysates were tested for catalase activity by addition of total yeast lysates to 10 mM H₂O₂ and monitoring the decrease in H₂O₂ by determination of the absorbance at 240 nm over time at 25°C. Error bars represent standard deviations from three biological replicate samples. Asterisks represent significant differences from the wild type strain determined by Student's *t* test (ns, not significant; **, *P* < 0.01; ***, *P* < 0.001). WT, wild type (OSU45); *catBΔ*, *catB* mutant (OSU16); *catBΔ*/*CATB*, complemented *catB* mutant (OSU51); *catPΔ*, *catP* mutant (OSU157); *catPΔ*/*CATP*, complemented *catP* mutant (OSU158); *catBΔ catPΔ*, *catB catP* double mutant (OSU159).

sion assay. Increasing sensitivity of *Histoplasma* yeasts was scored as the increased area around filter disks saturated with hydrogen peroxide in which there was negligible yeast growth (Fig. 4A). Wild-type *Histoplasma* yeasts expressing both CatB and CatP catalases showed the lowest sensitivity to hydrogen peroxide *in vitro*. Loss of CatB slightly increased the sensitivity of *catBΔ* mutant yeast, and H₂O₂ resistance was restored upon complementation of the *catBΔ* mutant with *CATB*. Loss of CatP was the most detrimental to yeast, as the *catPΔ* mutant had the largest area of clearing. The loss of both CatB and CatP catalases resulted in peroxide sensitivity nearly identical to that of the single *catPΔ* mutant, indicating that, although CatB can provide some protection, the major source of *in vitro* peroxide defense is the CatP catalase. Complementation of the CatP deficiency largely restored yeast resistance to hydrogen peroxide. Interestingly, zones of clearing for strains lacking CatP were completely clear of yeast growth, but regardless of the size of the clearing zone, strains with CatP activity had microcolonies of surviving yeast (Fig. 4B). Gradation in the concentration of hydrogen peroxide applied to the disks led to a dose response in the area cleared of yeast growth around the disk (data not shown). The ratio of the area cleared around the disk between mutant strains and the wild type was relatively constant across the range of hydrogen peroxide concentrations tested (data not shown).

CatB and CatP are both required to protect *Histoplasma* from phagocytes. To functionally test the role of CatB and CatP catalases in defending *Histoplasma* from oxidative killing by PMNs, CatB- and CatP-deficient yeast cells were coincubated with

human PMNs and yeast viability was determined. Previous reports indicated that *Histoplasma* yeasts are able to resist oxidative killing by phagocytic cells, including PMNs (17, 25, 26). When added to human PMNs in culture, 54% of the wild-type yeasts were viable after 4 h (Fig. 5A). Loss of CatB modestly impaired *Histoplasma*'s ability to survive these fungicidal cells; survival was decreased to 80% of that of wild type (43% versus 54%). Likewise, the loss of CatP diminished *Histoplasma*'s ability to survive PMN challenge, although to a greater extent than loss of CatB; loss of CatP decreased survival to 69% of that of wild type (37% versus 54%). Complementation of either catalase deficiency restored yeast survival to that of wild type. Loss of both CatB and CatP activities caused a considerable survival defect to 26% of wild-type yeast survival (14% versus 54%). This suggests that CatB and CatP act redundantly to optimally defend *Histoplasma* against peroxide produced by human PMNs. The decreased survival of the *catBΔ*, the *catPΔ*, and the *catBΔ catPΔ* mutants compared to the survival of wild-type yeast was reversed by addition of the phagocyte oxidase inhibitor diphenylene iodonium (DPI), indicating that the enhanced sensitivity of the mutant strains is due to an inability to resist PMN-produced reactive oxygen (Fig. 5A).

Since the primary host cell for *Histoplasma* yeast during mammalian infection is the macrophage, we determined if CatB and/or CatP was required for *Histoplasma*'s ability to survive and grow within these phagocytes. For these experiments, human monocyte-derived macrophages were stimulated with IFN- γ to enhance the phagocyte oxidative burst response to *Histoplasma* yeast, since resting human macrophages are unable to induce oxidative killing

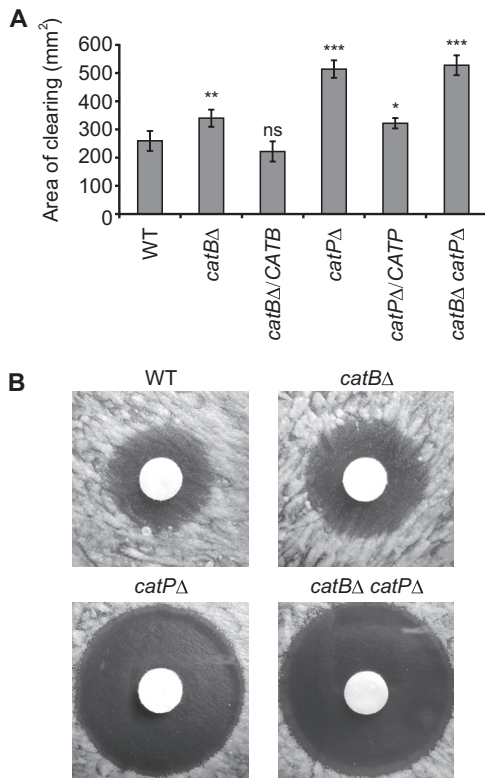


FIG 4 CatB and CatP provide protection to yeasts from H₂O₂ *in vitro*. Resistance of wild-type and catalase-deficient yeasts to killing by H₂O₂. H₂O₂ killing of yeast was determined by a filter disk diffusion assay on solid medium using filters with 300 mM H₂O₂. (A) Quantitation of the sensitivity to H₂O₂. The zone of clearing around the disks was determined as the total area lacking yeast cell growth. Data presented are the average areas of clearing for three replicate tests, with error bars representing standard deviations. Asterisks represent significant differences from the wild-type strain determined by Student's *t* test (ns, not significant; *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001). (B) Images of *Histoplasma* yeast growth around H₂O₂-saturated disks showing the presence of microcolonies of surviving yeasts in zones of clearing for wild type and for CatB-deficient strains but not for strains lacking CatP (*catPΔ* or *catBΔ catPΔ* strains). WT, wild type (OSU45); *catBΔ*, *catB* mutant (OSU16); *catBΔ/CATB*, complemented *catB* mutant (OSU51); *catPΔ*, *catP* mutant (OSU157); *catPΔ/CATP*, complemented *catP* mutant (OSU158); *catBΔ catPΔ*, *catB catP* double mutant (OSU159).

of yeast (17, 45–47). The CatB-deficient *catBΔ* strain had no defect in survival compared to wild type after infection of macrophages (Fig. 5B). Loss of CatP in the *catPΔ* mutant yeasts modestly reduced survival to 79% of wild-type survival (63% versus 80%). Resistance to macrophage killing was restored by expression of the *CATP* gene in the complemented mutant. However, loss of both CatB and CatP substantially decreased yeast survival following infection of macrophages; survival of *catBΔ catPΔ* mutant yeast was 50% of wild-type yeast survival (40% versus 80%). As with PMNs, killing of catalase-deficient yeasts by macrophages was prevented by treatment of macrophages with DPI, indicating that killing of catalase-deficient yeasts was predominantly ROS mediated. These results indicate that CatB and CatP serve redundant functions with respect to defense against peroxide stress and that either is largely sufficient to protect *Histoplasma* yeast from killing by macrophages.

As *Histoplasma*'s extracellular superoxide dismutase (Sod3) also contributes to the resistance of *Histoplasma* to killing by host

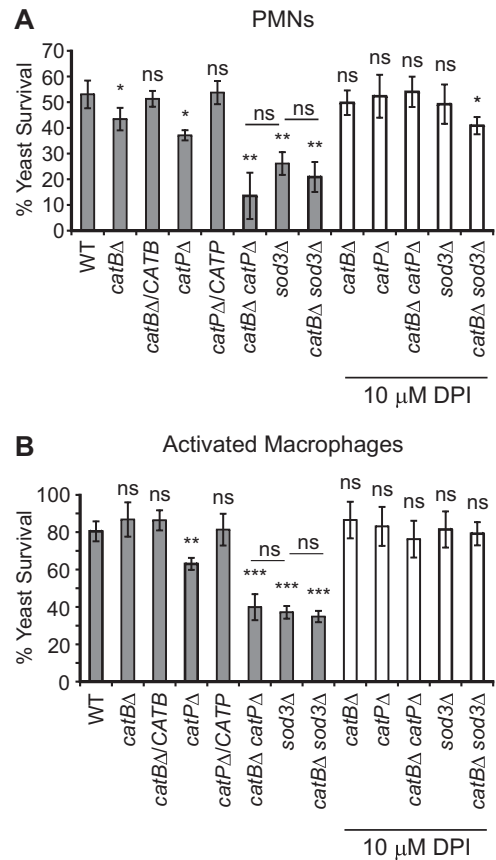


FIG 5 CatP and CatB can provide protection to *Histoplasma* yeasts against phagocyte-produced reactive oxygen. (A) Survival of *Histoplasma* yeasts after coincubation with human PMNs. Wild-type and catalase- and/or superoxide dismutase-deficient yeasts were added to PMNs isolated from peripheral human blood. Relative yeast survival (mean ± standard deviation) was determined by enumeration of the number of viable CFU after 4 h. In some experiments, DPI was added 24 h prior to yeast addition to inactivate the phagocyte NADPH oxidase. (B) Survival of *Histoplasma* yeast after infection of activated human macrophages. Wild-type, catalase, and superoxide dismutase (Sod3) mutants were added to IFN-γ-activated macrophages derived from human peripheral blood monocytes. Relative yeast survival (mean ± standard deviation) was determined by enumeration of viable CFU after 4 h. In some experiments, DPI was added 24 h prior to yeast addition to inactivate the phagocyte NADPH oxidase. Asterisks represent significant differences from survival of the wild-type strain determined by Student's *t* test of three replicates (ns, not significant; *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001). Additionally, statistical significance was determined for the Sod3-deficient mutant compared to the double mutant lacking both the extracellular superoxide dismutase and the extracellular catalase. WT, wild type (OSU45); *catBΔ*, *catB* mutant (OSU16); *catBΔ/CATB*, complemented *catB* mutant (OSU51); *catPΔ*, *catP* mutant (OSU157); *catPΔ/CATP*, complemented *catP* mutant (OSU158); *catBΔ catPΔ*, *catB catP* double mutant (OSU159); *sod3Δ*, *sod3* mutant (OSU15); *catBΔ sod3Δ*, *catB sod3* double mutant (OSU46).

ROS (17), we tested whether loss of the extracellular catalase, CatB, further increased the sensitivity of yeast to PMNs and activated macrophages in the absence of extracellular Sod3. Both CatB and Sod3, as extracellular enzymes, could contribute to protection of yeast from extracellular ROS, and a double *catBΔ sod3Δ* mutant was constructed to test this. Compared to the survival of the *sod3Δ* mutant strain, the *catBΔ sod3Δ* mutant survival after coincubation with PMNs (Fig. 5A) or with activated human macrophages (Fig. 5B) was not further reduced. Thus, there was no

additive defect if CatB was depleted from a strain already lacking Sod3. DPI treatment of PMNs and macrophages alleviated the sensitivity of the *sod3Δ* and *catBΔ sod3Δ* mutants to phagocyte killing, confirming that the effects of catalase and superoxide dismutase deficiency on yeast viability result from the lack of defenses against PMN- and macrophage-produced ROS.

CatB is not required *in vivo* for *Histoplasma* virulence. To determine if the pathogenic-phase-regulated, extracellular CatB catalase is necessary for full *Histoplasma* virulence *in vivo*, CatB-deficient yeasts were tested for their ability to establish respiratory infection and disseminate to the spleen. Following inhalation of a sublethal inoculum, wild-type *Histoplasma* yeasts survived and replicated within lung tissue, reaching a maximum fungal burden (33-fold above the inoculum) at between 8 and 12 days postinfection (Fig. 6A). Extrapulmonary dissemination of the infection to the spleen occurred beginning at day 4 postinfection and reached a peak fungal burden on day 8 (Fig. 6B). Subsequent activation of cell-mediated immunity caused a decline of the fungal burden in the lungs and spleen after day 8 postinfection. Despite speculations that CatB is a virulence factor, the loss of CatB, surprisingly, did not impair *Histoplasma* yeasts' ability to infect the lung (Fig. 6A) or to disseminate (Fig. 6B). The rate of clearing of CatB-deficient yeasts from the lung was slightly increased from the rate of clearing of wild-type yeasts, although this was only barely at statistical significance and did not manifest in a repeat experiment (Fig. 7A). Thus, CatB appears to be dispensable for murine lung infection.

As Sod3 provides substantial *in vivo* defense against phagocyte-derived ROS (17), we tested whether loss of CatB function in the ROS-sensitive *sod3Δ* strain would reveal an important CatB function *in vivo*. Similar to results with cultured phagocyte infections, *sod3Δ* mutant yeasts were attenuated in their ability to survive in the lung, showing a decline in fungal burden from the inoculum at day 4 and only a net 1.7-fold overall increase by day 8 (Fig. 6A). Dissemination of the *sod3Δ* mutant was practically absent (Fig. 6B). The *catBΔ sod3Δ* double mutant lacking both extracellular superoxide dismutase and catalase functions was similarly attenuated (Fig. 6A and B). In fact, the defect in lung infection and dissemination was nearly identical between the *sod3Δ* single mutant and the *catBΔ sod3Δ* double mutant at all time points, indicating that the additional loss of CatB in the Sod3-deficient strain does not further impair *Histoplasma* virulence *in vivo*.

Redundant CatB and CatP functions are required for full *Histoplasma* virulence. The unanticipated finding that CatB alone is not required for *Histoplasma* virulence could be explained by redundant ROS-protective functions provided by the intracellular CatP catalase. To determine if CatP and CatB act redundantly, we compared the infection kinetics of *catBΔ* or *catPΔ* single mutant strains with that of the double mutant lacking both catalases. Neither the loss of CatP alone nor the loss of CatB alone negatively affected the virulence of *Histoplasma*, as the mutant strains established similar fungal burdens as wild type in the lung (Fig. 7A). These results of an independent test of the *catBΔ* mutant were highly comparable to those obtained previously (Fig. 6A). Rates of clearance from the lung for singly CatP- and CatB-deficient strains were not significantly different from those for wild type. Thus, CatP and CatB individually are not critical to *Histoplasma* survival and growth within the lung. A similar lack of attenuation was apparent from fungal burdens in spleen (Fig. 7B).

In contrast to the results for the single *catBΔ* and *catPΔ* mu-

tants, the loss of both CatB and CatP catalases significantly reduced lung infection and dissemination of *Histoplasma* yeast (Fig. 7A and B). Net survival and replication of the *catBΔ catPΔ* double mutant strain in lungs were 6.2- to 16-fold lower than those of wild type before the yeasts were cleared by day 16 postinfection. This defect of *Histoplasma* yeasts lacking both catalases was similar to the infection kinetics of Sod3-deficient yeasts, although the Sod3-deficient strain was more attenuated (Fig. 6A). A similar trend in the fungal burdens was observed in spleens when the *catBΔ catPΔ* double mutant was compared to the *catBΔ* and *catPΔ* single mutants (Fig. 7B). Thus, CatB and CatP can functionally compensate for each other during infection and together provide for greater *Histoplasma* yeast survival *in vivo*.

DISCUSSION

The ability to avoid or detoxify antimicrobial ROS produced by phagocytes in response to infection is a prerequisite for microbial pathogens, particularly those pathogens that successfully survive within ROS-generating phagocytes. The optimal strategy is to prevent the production of reactive oxygen compounds. To accomplish this, *Histoplasma* yeasts minimize exposure of immunostimulatory cell wall β -glucans by concealing them beneath an α -linked glucan polysaccharide (48). Other fungal pathogens use different mechanisms to achieve the same end with various degrees of success. For example, *Cryptococcus* yeasts produce a polysaccharide capsule that hides β -glucan and mannan pathogen-associated molecular patterns (49, 50), and *Aspergillus* conidial β -glucans are not revealed until germination (51, 52). However, avoidance of detection is not perfect, and entry into host cells, particularly by phagocytosis, can be accompanied by ROS production. Thus, before uptake into phagocytes, intracellular fungal pathogens must have preformed antioxidant systems to destroy phagocyte-derived ROS to ensure fungal survival. In this study, we demonstrate that CatB and CatP comprise an effective, redundant approach that enables *Histoplasma* yeasts to optimally contend with peroxide stress as a component of host-derived ROS.

Although the *Histoplasma* genome has three catalase genes, only CatB and CatP are expressed by *Histoplasma* yeasts. Lack of *CATA* expression in yeasts by quantitative RT-PCR is consistent with a previous analysis of the *CAT* genes by Northern blotting (28). However, Johnson et al. reported *CATA* expression by mycelia, which we did not observe (28). This difference is due to the different genetic backgrounds used: *CATA* expression is 10- to 20-fold higher in G217B (the strain used by Johnson et al. [28]) than in G186A (the strain characterized in this study) by quantitative RT-PCR (data not shown). Regardless of the strain, *CATA* is not significantly expressed by pathogenic *Histoplasma* yeasts. By phylogenetic analysis, CatP belongs to the peroxisomal class of catalase enzymes (28). Our results show that CatP accounts specifically for the intracellular catalase activity of *Histoplasma* yeasts, consistent with this prediction. CatB belongs to the B clade of catalases, which are found only in Ascomycetes. Opposite from the findings for CatP, CatB is responsible for all the extracellular catalase activity of *Histoplasma* yeasts, consistent with its secretion (43) and cell wall association (32). *In vitro*, roughly half of the CatB activity is secreted into the surrounding medium and half remains associated with the yeast cell. The mechanism retaining CatB on the cell surface is unknown, and no predicted attachment motifs (e.g., glycosylphosphatidylinositol anchors) are evident from the primary amino acid sequence. Virtually all extracellular

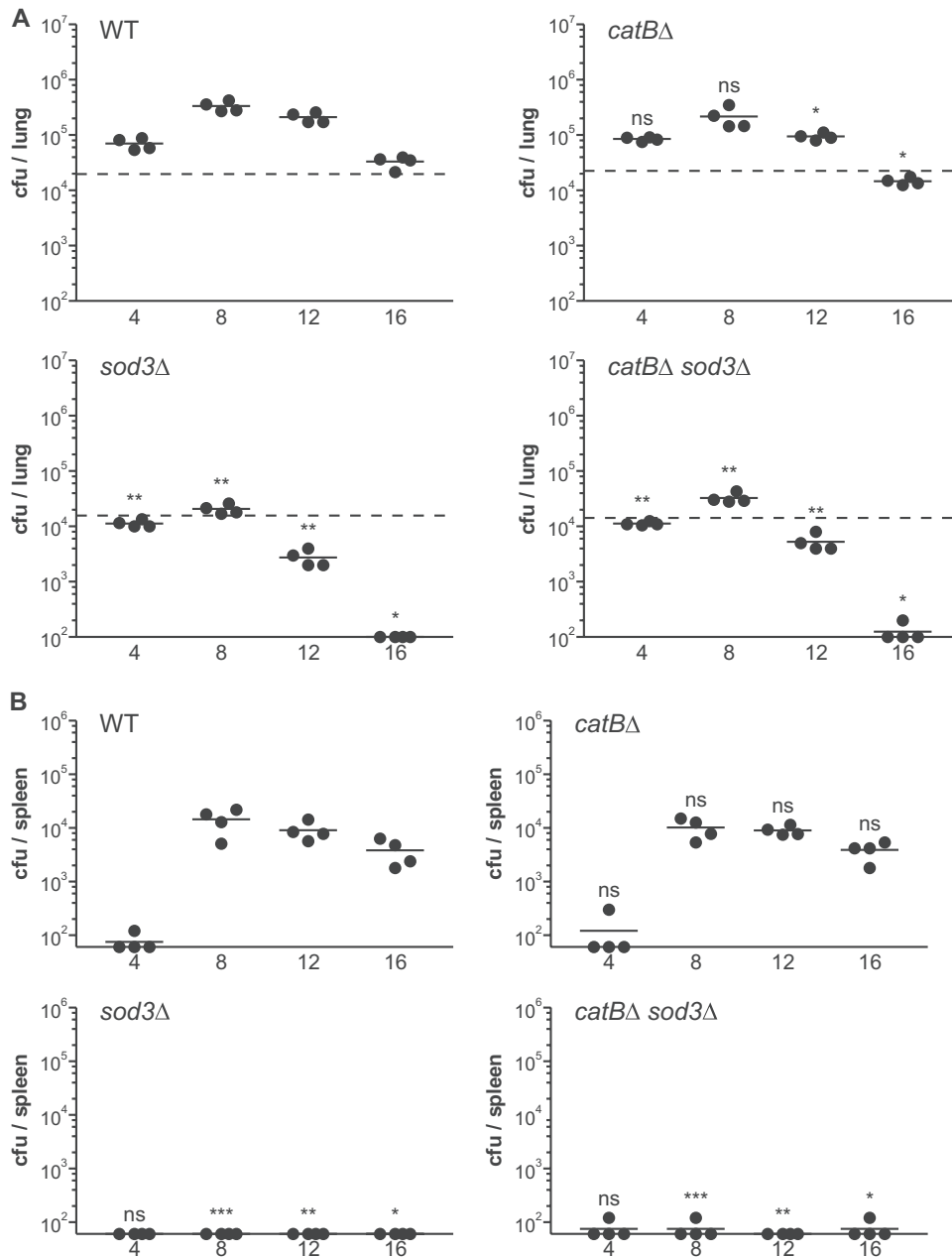


FIG 6 Sod3 deficiency, but not CatB deficiency, attenuates *Histoplasma* virulence in mice. (A) Pulmonary fungal burden kinetics after sublethal lung infection by *Histoplasma* yeast. Wild-type C57BL/6 mice were infected intranasally with 2×10^4 wild-type, CatB-deficient, Sod3-deficient, or doubly CatB- and Sod3-deficient yeasts. At 4-day intervals postinfection, fungal burdens were determined by quantitative plating of lung homogenates for determination of the number of *Histoplasma* CFU. The lower limit of detection was 10^2 CFU. Dashed horizontal lines indicate the actual number of CFU delivered in the inoculum. (B) Dissemination kinetics of *Histoplasma* yeasts after sublethal lung infection. Wild-type C57BL/6 mice were infected intranasally with 2×10^4 wild-type, CatB-deficient, Sod3-deficient, or CatB- and Sod3-deficient yeasts. At 4-day intervals postinfection, splenic fungal burdens were determined by quantitative plating of spleen homogenates for determination of the number of *Histoplasma* CFU. The lower limit of detection was 60 CFU. No yeasts had disseminated from the lung to the spleen at day 0. Each data point represents the CFU counts per organ from an individual animal ($n = 4$). Horizontal bars represent the mean fungal burden. Asterisks indicate statistically significant differences from wild type determined by Student's *t* test (ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). WT, wild type (OSU45); *catB*Δ, *catB* mutant (OSU16); *sod3*Δ, *sod3* mutant (OSU15); *catB*Δ *sod3*Δ, *catB sod3* double mutant (OSU46).

and intracellular catalase activities are eliminated by deletion of the *CATB* and *CATP* genes, respectively, indicating that CatB and CatP, but not CatA, account for the catalase activities of *Histoplasma* yeasts.

The extracellular location of CatB and its restricted expression by pathogenic-phase yeasts imply that its purpose is to combat

exogenous peroxide stress produced by the host. Both *Cryptococcus* and *Candida albicans* lack extracellular catalase activity (53, 54). *Aspergillus fumigatus*, like *Histoplasma*, produces a glycosylated catalase (Cat1) with a signal sequence sufficient to direct its secretion (55). Loss of CatB function increases the sensitivity of *Histoplasma* yeasts to H₂O₂ *in vitro*, showing that H₂O₂ can be

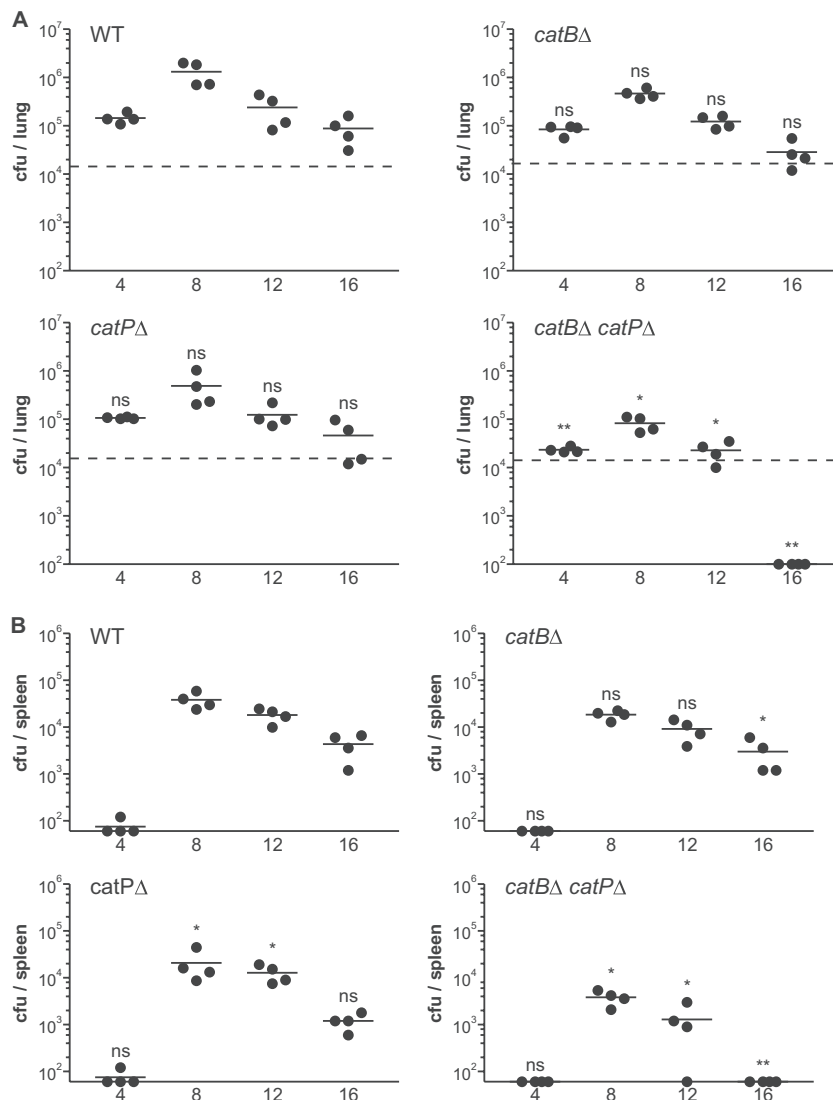


FIG 7 CatB and CatP provide redundant catalase functions necessary for *Histoplasma* virulence *in vivo*. (A) Pulmonary fungal burden kinetics after sublethal lung infection by *Histoplasma* yeast. Wild-type C57BL/6 mice were infected intranasally with 2×10^4 wild-type, CatB-deficient, CatP-deficient, or double mutant yeasts lacking both catalases. At 4-day intervals postinfection, fungal burdens were determined by quantitative plating of lung homogenates for determination of the number of *Histoplasma* CFU. The lower limit of detection was 10^2 CFU. Dashed horizontal lines indicate the actual number of CFU delivered in the inoculum. (B) Dissemination kinetics of *Histoplasma* yeast after sublethal lung infection. Wild-type C57BL/6 mice were infected intranasally with 2×10^4 wild-type, CatB-deficient, CatP-deficient, or double catalase mutant yeasts. At 4-day intervals postinfection, splenic fungal burdens were determined by quantitative plating of spleen homogenates for determination of the number of *Histoplasma* CFU. The lower limit of detection was 60 CFU. No yeasts had disseminated from the lung to the spleen at day 0. Each data point represents CFU counts per organ from an individual animal ($n = 4$). Horizontal bars represent the mean fungal burden. Asterisks indicate statistically significant differences from wild type determined by Student's *t* test (ns, not significant; *, $P < 0.05$; **, $P < 0.01$). WT, wild type (OSU45); *catB*Δ, *catB* mutant (OSU16); *catP*Δ, *catP* mutant (OSU157); *catB*Δ *catP*Δ, *catB catP* double mutant (OSU159).

antimicrobial to *Histoplasma* yeasts and that CatB can detoxify this ROS molecule. However, loss of CatB has no deleterious consequences for *Histoplasma* virulence *in vivo* and only minor effects on yeast survival against cultured phagocytes. This discrepancy between *in vitro* and *in vivo* phenotypes for catalase mutants is not without precedent; the loss of catalase proteins does not attenuate virulence for bacterial pathogens such as *Neisseria gonorrhoeae* (56), *Salmonella enterica* serovar Typhimurium (57), or *Haemophilus influenzae* (58). A similar situation exists for fungal pathogens; elimination of the *Aspergillus fumigatus* conidial catalase (CatA) increases conidial sensitivity to H₂O₂ *in vitro* but does not

impact conidial killing *in vivo* (59). Similarly, *Cryptococcus* cells lacking multiple catalases are completely virulent in a mouse inhalation model (54). Normal virulence is also manifest for *Candida glabrata* yeasts lacking the Cta1 catalase (60) and *Magnaporthe oryzae* cells lacking the CpxB catalase (61).

One possibility for this discrepancy is that the H₂O₂ concentration produced by phagocytes *in vivo* may differ substantially from fungicidal levels *in vitro*. Alternatively, reactive oxygen compounds other than peroxide may be more relevant to fungicidal mechanisms *in vivo*. In *Histoplasma*, loss of the Sod3 superoxide dismutase increases the sensitivity of yeasts to ROS-mediated

phagocyte killing and attenuates virulence *in vivo* (17). In this study, we show that additional loss of CatB from Sod3-deficient yeasts does not further increase yeast sensitivity to phagocyte ROS *in vitro* or virulence *in vivo* (Fig. 6). As peroxide is derived from superoxide by either enzymatically catalyzed or spontaneous dismutation, the attenuation of Sod3-deficient yeasts indicates that the CatB (and CatP) catalase(s) is not sufficient to protect *Histoplasma* from host ROS. This confirms that although superoxide leads to production of peroxide, superoxide is a major fungicidal immune defense molecule that *Histoplasma* must combat and does so specifically through Sod3. Nevertheless, *Histoplasma* virulence requires protection from peroxide stress as well, since yeasts lacking both CatB and CatP (i.e., the *catBΔ catPΔ* mutant) are attenuated, even though they express Sod3. The similarity of the *catBΔ sod3Δ* mutant's survival to the *catBΔ* single mutant's survival indicates that *Histoplasma* can still eliminate peroxide stress in the absence of CatB, despite elevated ROS. This suggests that *Histoplasma* has additional peroxide detoxification mechanisms (e.g., CatP).

A second factor contributing to the contrasting requirement for catalases *in vitro* and *in vivo* is the existence of redundant or compensatory peroxide-detoxifying mechanisms. Similar to *Aspergillus* and *Cryptococcus*, *Histoplasma*'s multiple catalases create possible redundancy. Our results indicate that intracellular CatP can supply sufficient peroxide-destroying defense functions in the absence of extracellular CatB. The loss of CatP results in a more severe defect in response to peroxide *in vitro* and more severe survival defects when *Histoplasma* is cocultured with phagocytic cells than loss of CatB. This suggests that CatP provides more catalase-based ROS defense. However, like CatB-deficient yeasts, CatP-deficient yeasts are fully virulent *in vivo*. Only loss of both catalases attenuates virulence, consistent with CatB and CatP serving redundant defense functions during infection. It is unlikely that *Histoplasma* CatA contributes to yeast ROS defenses, as CatA is not expressed by yeasts. Furthermore, the lack of single mutant phenotypes is not explained by compensatory upregulation of other catalases; CatB and CatP production is not altered in the *catPΔ* and *catBΔ* single mutants, respectively (Fig. 2). In *Aspergillus*, Cat1 and Cat2 mycelial catalases are redundant, with the double mutant showing a 2-fold increase in sensitivity to H₂O₂ compared to that of single catalase mutants (59). In contrast to the catalase redundancy of *Aspergillus*, *Cryptococcus*, and *Histoplasma*, *Candida albicans* has only a single intracellular catalase (Cat1), and loss of its function eliminates all catalase activity, impairs survival of *Candida* against PMNs, and attenuates virulence in a candidemia model (53, 62). The lack of attenuation of most fungal catalase mutants is often ascribed to noncatalase enzymes that reduce intracellular ROS (e.g., peroxiredoxins [63], glutaredoxins [64], cytochrome *c* peroxidase [65], alternative oxidase [66, 67]) and/or ROS scavengers (e.g., melanin [7], mannitol [68], capsule [69], and trehalose [70, 71]). The current findings revealed by *Histoplasma* yeasts lacking both CatB and CatP and the data for *Candida albicans* mutants lacking Cat1 highlight the critical role of catalases in pathogenesis for these fungi, whereas other fungal pathogens likely rely on other non-catalase-based mechanisms to combat host ROS.

In some fungal systems, oxidative stress increases the expression of catalases and other antioxidant systems (62, 70, 72–74). This response is often mediated by AP1-class transcription factors, and loss of AP1 leads to enhanced sensitivity of fungi to

peroxide and can attenuate virulence (60, 74–77). However, the rapid lethal action of host-derived ROS leaves little time for adaptive responses. Since *Histoplasma* is predominantly associated with phagocytes, the synthesis of catalases by yeasts before interaction with ROS-producing host cells before the host-pathogen interaction makes intuitive sense. Consistent with this, exogenous peroxide does not upregulate CatB and CatP expression (28). The only evidence for *Histoplasma* CatB regulation is increased *CATB* expression when iron concentrations increase, presumably to prevent the formation of hydroxyl radicals by destroying the predecessor molecule, hydrogen peroxide (78).

The redundant functions of *Histoplasma* CatB and CatP provide an optimal system for protection against host-derived ROS. However, the roles of CatB and CatP go beyond simple redundancy in detoxifying peroxide. Extracellular phagocyte-produced ROS would first encounter and be detoxified by extracellular Sod3 and CatB defense factors. Unlike short-lived superoxide anion, stable hydrogen peroxide that escapes destruction by CatB is membrane permeant and can diffuse into the yeast cell and affect intracellular targets. However, the *Histoplasma* intracellular CatP catalase comprises a second line of defense, degrading this now intracellular peroxide challenge and thereby protecting *Histoplasma* yeast from cellular damage. The appearance of microcolonies of yeasts that escape H₂O₂-mediated destruction in the disk diffusion assay lends support to this model. Only microcolonies of wild-type and CatB-deficient yeasts appear in the zones of clearing because intracellular CatP enables some yeasts to survive by continual detoxification of peroxide that diffuses into cells. On the other hand, microcolonies never appeared with either of the CatP-deficient strains; although CatB largely detoxifies the extracellular peroxide stress, peroxide that diffuses into cells is not eliminated without CatP. Consequently, no yeasts that lack CatP escape killing in the clearing zone around the peroxide-saturated disk. Thus, this spatial separation of individual catalases, while singly not required to protect *Histoplasma* *in vivo*, nonetheless (i) enables *Histoplasma* to efficiently detoxify the majority of ROS before it can cause cellular damage and (ii) provides a backup system to degrade any ROS that eludes the primary extracellular defense system. As a consequence, *Histoplasma*'s dual catalases enable yeasts to readily survive infection in the presence of ROS-producing phagocytes, thereby facilitating *Histoplasma* pathogenesis.

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