INFECTION AND IMMUNITY, June 2003, p. 3551–3562 Vol. 71, No. 6
0019-9567/03/$08.00+0 DOI: 10.1128/IAI.71.6.3551–3562.2003

Catalases of *Aspergillus fumigatus*

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Received 11 November 2002/Returned for modification 23 December 2002/Accepted 20 February 2003

Upon infection of a host, the pathogenic fungus *Aspergillus fumigatus* is attacked by the reactive oxygen species produced by phagocytic cells. Detoxification of hydrogen peroxide by catalases was proposed as a way to overcome this host response. *A. fumigatus* produces three active catalases; one is produced by conidia, and two are produced by mycelia. The mycelial catalase Cat1p was studied previously. Here we characterized the two other catalases, their genes, and the phenotypes of gene-disrupted mutants. CatAp, a spore-specific monofunctional catalase, is resistant to heat, metal ions, and detergent. This enzyme is a dimeric protein with 84.5-kDa subunits. The 749-amino-acid polypeptide exhibits high levels of similarity to the *Aspergillus nidulans* CatA catalase and to bacterial catalase HPII of *Escherichia coli*. In spite of increased sensitivity to H_2O_2, killing of Δ*catA* conidia by alveolar macrophages and virulence in animals were similar to the killing of conidia by alveolar macrophages and virulence in animals observed for the wild type. In contrast to the Cat1p and CatAp catalases, the mycelial Cat2p enzyme is a bifunctional catalase-peroxidase and is sensitive to heat, metal ions, and detergent. This enzyme, an 82-kDa monomer, is homologous to catalase-peroxidases of several fungi and bacteria. Surprisingly, mycelium of the double Δ*cat1*Δ*cat2* mutant with no catalase activity exhibited only slightly increased sensitivity to H_2O_2 and was as sensitive to killing by polymorphonuclear neutrophils as mycelium of the wild-type strain. However, this mutant exhibited delayed infection in the rat model of aspergillosis compared to infection by the wild-type strain. These results indicate that conidial catalase is not a virulence factor and that mycelial catalases transiently protect the fungus from the host.

The opportunistic fungal pathogen *Aspergillus fumigatus* is responsible for a variety of respiratory diseases in humans, such as allergic bronchopulmonary aspergillosis, aspergilloma, and invasive aspergillosis (10). This fungus is an airborne saprophyte that is inhaled by every human. Alveolar macrophages and polymorphonuclear cells, cellular components of the innate defense of the lung, cooperate to control and eliminate the fungus in the airways. Macrophages eliminate conidia, and protection against the hyphal form is mediated by polymorphonuclear cells (41). Reactive oxygen species (ROS) produced by alveolar macrophages play an essential role in the killing of *A. fumigatus* conidia (38a). Moreover, in vitro studies of neutrophil function have shown that hydrogen peroxide effectively kills fungal hyphae (12) and that neutrophil-mediated damage is blocked by addition of a commercial catalase (13). Accordingly, catalase, which is a good scavenger of H_2O_2, was considered to be a putative virulence factor of *A. fumigatus* that could counteract the oxidative defense reactions of the host phagocytes (20). No conidial catalase has been identified previously in *A. fumigatus.* In *Aspergillus nidulans*, however, a conidial catalase, CatAp, was described by Navarro et al. (35), and a *CATA* disrupted mutant produced spores that were sensitive to H_2O_2, in contrast to the wild-type, resistant conidia. Previous studies have shown that the mycelium of *A. fumigatus* produces two mycelial catalases, one that is monofunctional and one that is a bifunctional catalase-peroxidase (21). Only one of these catalases has been studied previously, and disruption of the gene encoding it resulted in a phenotype whose mycelial sensitivity to H_2O_2 and polymorphonuclear killing was not modified compared to the properties of the wild-type strain (3).

The purpose of the present work was to investigate the role of the entire panel of conidial and mycelial catalases of *A. fumigatus* in the pathogenicity of the fungus. To do this, catalases of conidia and hyphae were isolated, their genes were cloned and disrupted, and the resistance to H_2O_2 and phagocytes of single- and multiple-catalase-negative mutants was investigated. In addition, the infectivities of the conidial and mycelial mutants in a rat model were assessed. This model of experimental infection was selected because the histological features observed in rats treated with low doses of immunosuppressive drugs resemble those observed in patients with invasive aspergillosis (43).

MATERIALS AND METHODS

***Aspergillus* strains and culture conditions.** *A. fumigatus* strains used in this study are shown in Table 1. G10 and Δ*cat1*-28 were used as the recipient strains for transformation. Cultures of *A. fumigatus* grown in Sabouraud liquid medium (2% glucose, 1% Mycopeptone; Biokar, Beauvais, France) were used for DNA extraction. For detection of mycelial catalase activity, the strains were grown in 1% yeast extract (Difco, Detroit, Mich.) liquid medium at 37°C in flasks shaken at 150 rpm for 3 days. For transformation experiments, minimal medium with 5 mM ammonium tartrate as a nitrogen source was used (8). Conidia were harvested after 1 week of growth at 25°C on 2% malt extract agar. The conidial

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TABLE 1. Strains used in this study

Strain	Genotype	Source
G10		Monod et al.[a]
Δ*catA*-01	Δ*CATA*::*phleoR*	This study
Δ*catA*-02	Δ*CATA*::*phleoR*	This study
Δ*cat1*-28	Δ*CAT1*::*hph*	Calera et al.[b]
Δ*cat2*-3	Δ*CAT2*::*phleoR*	This study
Δ*cat1* Δ*cat2*-18	Δ*CAT1*::*hph* Δ*CAT2*::*phleoR*	This study
Δ*cat1* Δ*cat2*-19	Δ*CAT1*::*hph* Δ*CAT2*::*phleoR*	This study
Δ*catA* Δ*cat1*-21	Δ*CATA*::*phleoR* Δ*CAT1*::*hph*	This study
Δ*catA* Δ*cat1*-39	Δ*CATA*::*phleoR* Δ*CAT1*::*hph*	This study

[a] See reference 34.
[b] See reference 3.

germination rate was monitored with a light microscope after 8 h of incubation at 37°C on Sabouraud agar.

Protein analysis. Intracellular protein extracts were prepared by mechanical disruption of mycelium or conidia in 10 mM Tris-HCl (pH 7.5) as previously described (37). Native polyacrylamide gel electrophoresis (PAGE) was performed as previously described by Lopez-Medrano et al. (29), whereas sodium dodecyl sulfate (SDS)-PAGE was performed with the discontinuous buffer system of Laemmli (28). Catalase and peroxidase activities were detected on native gels by using the ferricyanide negative stain described by Wayne and Diaz (47) and a diaminobenzidine stain (45), respectively. Immunoblotting was performed by the method of Towbin et al. (44). Glycosylation of catalases was detected on Western blots by using a concanavalin A (ConA)-peroxidase conjugate (15).

To inhibit catalase activities, heavy metals ($CuSO_4$, $HgCl_2$, and $ZnSO_4$ [5 mM each]) or 5% SDS was incubated with *A. fumigatus* fractions for 1 h at 37°C, while preparations were incubated with 40 mM dithiothreitol (DTT) overnight at 4°C before electrophoresis (21). Heat sensitivity was assayed by incubating extracts at 45°C for 1 h, at 65°C for 10 min, or at 80°C for 10 min before electrophoresis.

Internal peptides were obtained after endolysin digestion of proteins purified by electroelution of an SDS-PAGE gel. Internal peptide sequencing was performed by J. d'Alayer (Institut Pasteur) with an Applied Biosystems 470 gas-phase sequencer; previously described procedures were used (1).

Purification of CatAp and Cat2p. CatAp was purified from the protein extract fraction of conidia from strain Δ*cat1*Δ*cat2*-18 lacking the two mycelial catalases by using the following two steps. (i) First, gel filtration chromatography was performed on a Superdex 200 HR 10/30 column (Amersham, Orsay, France) in 120 mM NaCl–10 mM Tris-HCl (pH 8.4) by using a flow rate of 0.4 ml/min. (ii) Then a Mono Q HR 5/5 column (Amersham) was used for anion-exchange purification of the catalase-positive gel filtration fractions. Samples were loaded in 10 mM Tris-HCl (pH 8.4) and eluted with a linear NaCl gradient (0 to 350 mM) in 30 min by using a flow rate of 0.8 ml/min. The catalase activity was monitored in each chromatographic fraction by examining the release of O_2 bubbles when 2 to 20 μl of the fraction was added to 1 ml of phosphate-buffered saline containing 0.1 M H_2O_2.

For Cat2p purification strain Δ*catA*Δ*cat1*-39, a double mutant lacking the other mycelial catalase and the conidial catalase, was grown in 1% yeast extract liquid medium. The protein extract fraction of the mycelium supplemented with a protease inhibitor cocktail (Complete; 1 tablet/50 ml; Roche, Mannheim, Germany) was used as the starting material and was subjected to three successive high-performance liquid chromatography steps, as follows. (i) Gel filtration chromatography was performed on a Superdex 200 HR 10/30 column (Amersham) in 150 mM NaCl–5 mM Tris-HCl (pH 7.4) by using a flow rate of 0.4 ml/min. (ii) Affinity chromatography of the catalase-positive fractions on ConA-Sepharose (Amersham) was performed by using the same buffer containing 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 1 mM $CaCl_2$ as the loading buffer and 20 mM acetate buffer (pH 6.0) as the eluting buffer. (iii) The unbound ConA-Sepharose fraction, dialyzed in 10 mM Tris-HCl (pH 8.4), was loaded onto a Mono Q HR 5/5 column (Amersham) and eluted with a linear NaCl gradient (0 to 350 mM) in 30 min. The catalase activity in each chromatographic fraction was monitored as described above. Molecular sizes were determined by gel filtration by using thyroglobulin (669 kDa), ferritin (440 kDa), human immunoglobulin G (160 kDa), and transferrin (81 kDa) as the standards.

Cloning, plasmid construction, sequencing, and sequence comparison. To clone the *CATA* gene, a 807-bp *Nhe*I fragment of the *A. nidulans CATA* gene (pCAN5) (35) was used to screen the cosmid library of *A. fumigatus* provided by P. Borgia (2). To clone the *CAT2* gene, the cosmid genomic library was screened by using a degenerate oligonucleotide probe as described by Monod (33).

Restriction enzyme fragments of hybridizing cosmid clones were ligated into pBluescript SK(+) vector DNA (Stratagene). *Escherichia coli* DH5α was used for plasmid propagation. Both strands of the insert DNA were sequenced by the dideoxy chain termination method (40) by using a SequiTherm cycle sequencing kit (Epicentre Technologies, Madison, Wis.) for the *CATA* gene and by using a Big Dye terminator sequencing ready reaction kit (ABI) (performed at ESGS, Evry, France) for the *CAT2* gene. The identity and location of two introns in *CATA* were initially determined by sequence analysis of the entire open reading frame (ORF) and flanking regions of *CATA*. The sequence indicated that there were reading frame shifts at the start of each presumed intron and was consistent with commonly conserved fungal nucleotide sequences at the 5′ and 3′ ends of the putative introns (46). The presence of the introns was also verified by PCR amplification of cDNA by using homologous primers based on sequences in regions of the ORF flanking the locations of the presumed introns. Sequence analysis of *CAT2* did not reveal any frame shifts, which suggested that there were no introns. The absence of introns was confirmed by reverse transcription-PCR amplification of DNase-treated mRNA performed with sets of primers designed to amplify all regions of the gene. The PCR products were subcloned into pCR2.1 (TOPO TA cloning kit; Invitrogen, Carlsbad, Calif.), and this was followed by sequence analysis of the inserts.

Disruption of *CATA* and *CAT2*. The hybridizing 2.8-kb *Eco*57I fragment containing the *CATA* ORF was cloned into pBluescript to obtain pDW07. The deletion construct pΔ*CATA* was obtained by replacing a 862-bp *Van*91I fragment of pDW07 with a 2.3-kb *Kpn*I fragment of pID624 derived from pUT737 (Cayla, Toulouse, France) containing the phleomycin cassette (Fig. 1). The 4.2-kb *Eco*RI-*Cla*I fragment of pΔ*CATA* was used to transform protoplasts of *A. fumigatus* G10 and Δ*cat1*-28 to phleomycin resistance, as previously described (24).

The deletion construct pΔ*CAT2* was obtained by the following three-step process (Fig. 2). (i) A 1-kb fragment of the *CAT2* gene that contained the promoter region up to four amino acids after the start codon was amplified by PCR and inserted into pBluescript cut with *Eco*RI and *Sal*I, resulting in pP-ATG. (ii) The *Eco*RI-*Xba*I phleomycin cassette of pAN8-1 (31) was then added to pP-ATG cut with *Eco*RI and *Xba*I to obtain pP-ATG-Phleo. (iii) A 1-kb fragment of the *CAT2* gene located after the active site was amplified and introduced into p P-ATG-Phleo cut with *Xba*I and *Sac*II, resulting in pΔ*CAT2*. The 5.5-kb *Kpn*I-*Sac*II fragment of pΔ*CAT2* containing the disrupted *CAT2* gene was used for transformation. The gene replacement was verified by Southern analysis.

Nucleic acid isolation, blotting, and hybridization and DNA manipulation. *A. fumigatus* chromosomal DNA was isolated by the procedure of Girardin et al. (17). Agarose gel electrophoresis of restricted DNA, Southern blotting, and subcloning of genomic DNA fragments into plasmids were performed by using standard protocols (39).

Experimental rat model of aspergillosis. Sprague-Dawley rats were immunosuppressed and infected by the method of Shibuya et al. (43). Briefly, 5-week-old Sprague-Dawley rats (SD-1Crj) were subcutaneously injected with 20 mg of ampicillin per kg, 25 mg of cyclophosphamide per kg, and 25 mg of predonisolone per kg on days −7, −5, −3, −1, 3, 5, and 10. To prepare agarose beads containing conidia, 0.5 ml of a conidial suspension (10^7 conidia/ml) was added to 2.5 ml of melted 2% agarose in phosphate-buffered saline and kept at 48°C. This 3-ml agarose-conidium mixture was added to 15 ml of warm heavy mineral oil and vigorously stirred with a magnetic spin bar. A control without conidia was also prepared. The oil-agarose mixture was rapidly cooled while it was stirred for approximately 5 min; during this time, agarose droplets solidified into beads. These beads were washed twice in 0.25% Tween 80 in phosphate-buffered saline to remove the mineral oil. Beads that were 50 to 100 μm in diameter were selected by differential filtration on nylon mesh, washed, and suspended in phosphate-buffered saline. At zero time, 0.1 ml of the suspension was injected intracheally into the left main bronchus of anesthetized rats. Infected animals were killed on days 5 and 13 after inoculation. Previous studies (43) had shown that these two times were sufficient to analyze early and late pulmonary infection stages. The lungs were fixed with 10% formalin, and paraffin-embedded sections were stained with either hematoxylin and eosin, periodic acid-Schiff (PAS)–Elastica, or Grocott's methenamine silver.

Alveolar macrophage killing assay. Male outbred Swiss OF1 mice (Iffa Credo, Saint Germain sur l'Arbresle, France) that were 6 to 8 weeks old and weighed 32 to 34 g were intranasally infected with 4×10^6 fluorescein isothiocyanate- or Calcofluor-labeled conidia of each strain (38a). Twenty-four hours after infection, alveolar macrophages were recovered from bronchoalveolar lavages by 5 min of centrifugation at 400 × *g*. Each cell pellet was suspended in 0.2 ml of water to lyse the alveolar macrophages and then incubated for 6 to 8 h at 37°C

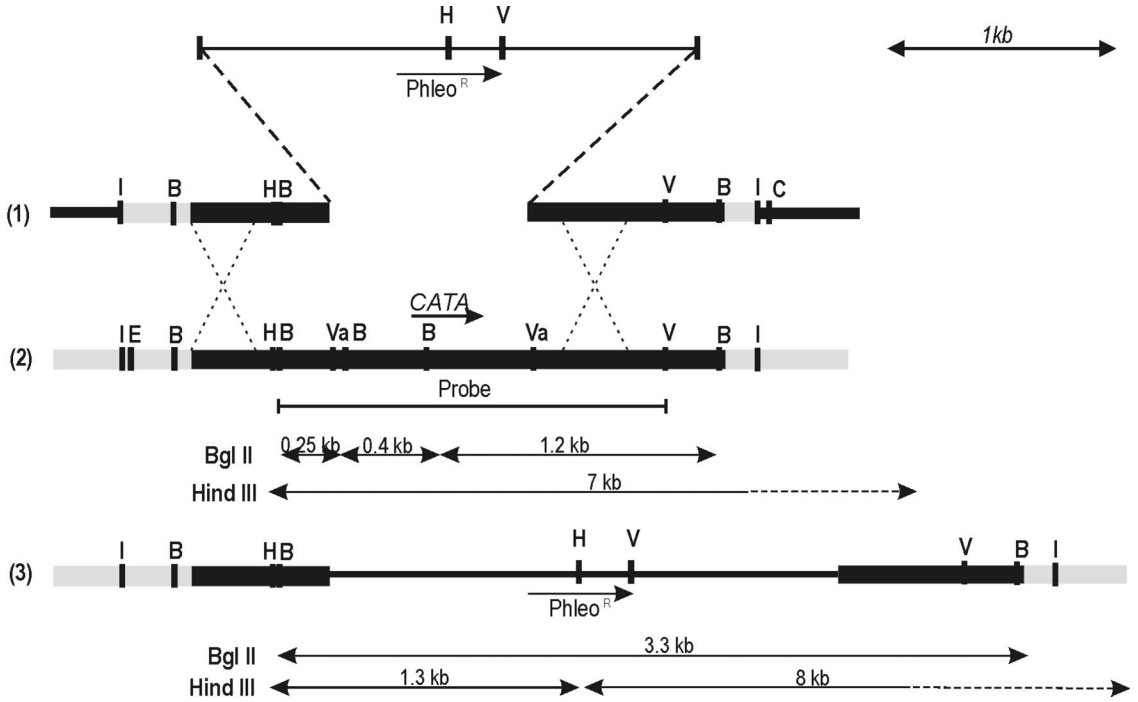

FIG. 1. Disruption of the *CATA* gene. (Line 1) Plasmid pΔ*CATA* was constructed by replacement of the 862-bp *Van*91I fragment of the *CATA* ORF with the 2.3-kb phleomycin resistance cassette (see Materials and Methods). A linear 4.2-kb *Eco*RI-*Cla*I fragment from the resulting construct was used to transform the G10 and Δ*cat1*-28 strains. (Line 2) Genomic DNA of the recipient strains ($CatA^+$). (Line 3) Genomic DNA of Δ*catA* transformants. B, *Bgl*II; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; I, *Eco*57I; V, *Eco*RV; Va, *Van*91I. The thin line represents the phleomycin cassette; the thick line represents the vector; and the gray box represents the *A. fumigatus* DNA flanking the coding region of the *CATA* gene (black box). The expected sizes of restriction enzyme fragments are indicated.

after addition of 200 μl of 2× Sabouraud medium containing 0.1% chloramphenicol to induce germination of the living conidia. The percentage of killing was determined by the number of nongerminated spores per 100 fluorescein isothiocyanate-labeled conidia, as estimated with a fluorescent light microscope. The experiment was performed in triplicate.

PMNL killing assay. For preparation of polymorphonuclear leukocytes (PMNLs), heparinized venous blood from healthy volunteers was sedimented in 3% dextran (Pharmacia); this was followed by centrifugation on Ficoll-Hypaque (Sigma) and lymphocyte separation medium (Gibco BRL, Cergy-Pontoise, France) and hypotonic lysis of residual erythrocytes (14). After separation, PMNLs were washed and suspended in Hanks' balanced salt solution (Gibco) without calcium and magnesium to a concentration of 5×10^6 PMNLs/ml.

The relative susceptibility of germinating hyphae to killing by PMNLs was determined by using the 2,3-bis-(2-methoxy-4-nitro-5-sulfenyl)-(2H)-tetrazolium-5-carboxanilide (tetrazolium dye) assay essentially as previously described (32). *A. fumigatus* conidia were suspended in YNB medium (Difco) and incubated for 16 to 18 h at 37°C, which resulted in germination of more than 90% of the conidia. Then the YNB medium was replaced with Hanks' balanced salt solution, and PMNLs were added at a conidium/PMNL ratio of 20:1. After incubation for 2 h at 37°C in the presence of 5% CO_2, the PMNLs were lysed, and after an additional 1 h of incubation with 2,3-bis-(2-methoxy-4-nitro-5-sulfenyl)-(2H)-tetrazolium-5-carboxanilide, the optical density at 450 nm was determined. Antihyphal activity was calculated by determining the percentage of hyphal damage, as follows: $(1 - X/C) \times 100$, where X is the optical density of experimental wells and C is the optical density of control wells with only hyphae. Each experiment was performed in triplicate.

Analysis of fungal damage by exogenous H_2O_2. The relative sensitivity of conidia to killing by H_2O_2 was assayed essentially as described by Navarro et al. (35). Conidia (10^6 conidia/ml) were incubated at 37°C for 30 min with various H_2O_2 concentrations, and then the conidia were plated on malt extract agar and colonies were counted.

The hydrogen peroxide sensitivity of mycelium was estimated by using a modification of the protocol of Kawasaki et al. (27). Conidia were inoculated onto 2% malt extract agar in enzyme-linked immunosorbent assay plates and grown for 22 h at 25°C to obtain hyphae. The plates were overlaid with various concentrations of H_2O_2 for 10 min, washed twice with water, and incubated at 25°C for an additional 24 h. The MIC was recorded.

Nucleotide accession numbers. The nucleotide sequence data for *CATA* and *CAT2* reported in this paper have been deposited in the GenBank database under accession numbers U87630 and AY125354, respectively.

RESULTS

Conidial and mycelial catalase activities of *A. fumigatus*. A catalase activity zymogram (Fig. 3A) showed that one catalase (CatAp) was present in conidia of *A. fumigatus*, while two catalases (Cat1p and Cat2p) were found in the mycelium.

The conidial catalase, CatAp, was purified after two chromatographic steps. As estimated by gel filtration, the relative molecular mass of the native enzyme was 180 kDa. As determined by SDS-PAGE, the enzyme fraction yielded only one band at 80 to 85 kDa which did not bind to ConA. This suggests that CatAp is a homodimer consisting of nonglycosylated subunits (data not shown). The catalase activity of CatAp was resistant to heat (10 min, 80°C) and was not sensitive to heavy metals at a concentration of 5 mM, to overnight incubation in 40 mM DTT at 4°C, or to incubation for 30 min in 5% SDS at 37°C (data not shown).

The mycelial Cat1p enzyme was characterized previously (3). In addition to catalase activity, mycelial catalase Cat2p has peroxidase activity (Fig. 3B). The bifunctional enzyme Cat2p was purified by a combination of gel filtration, affinity chromatography on ConA-Sepharose, and anion-exchange chromatography. The enzyme had a relative molecular mass of 85 kDa

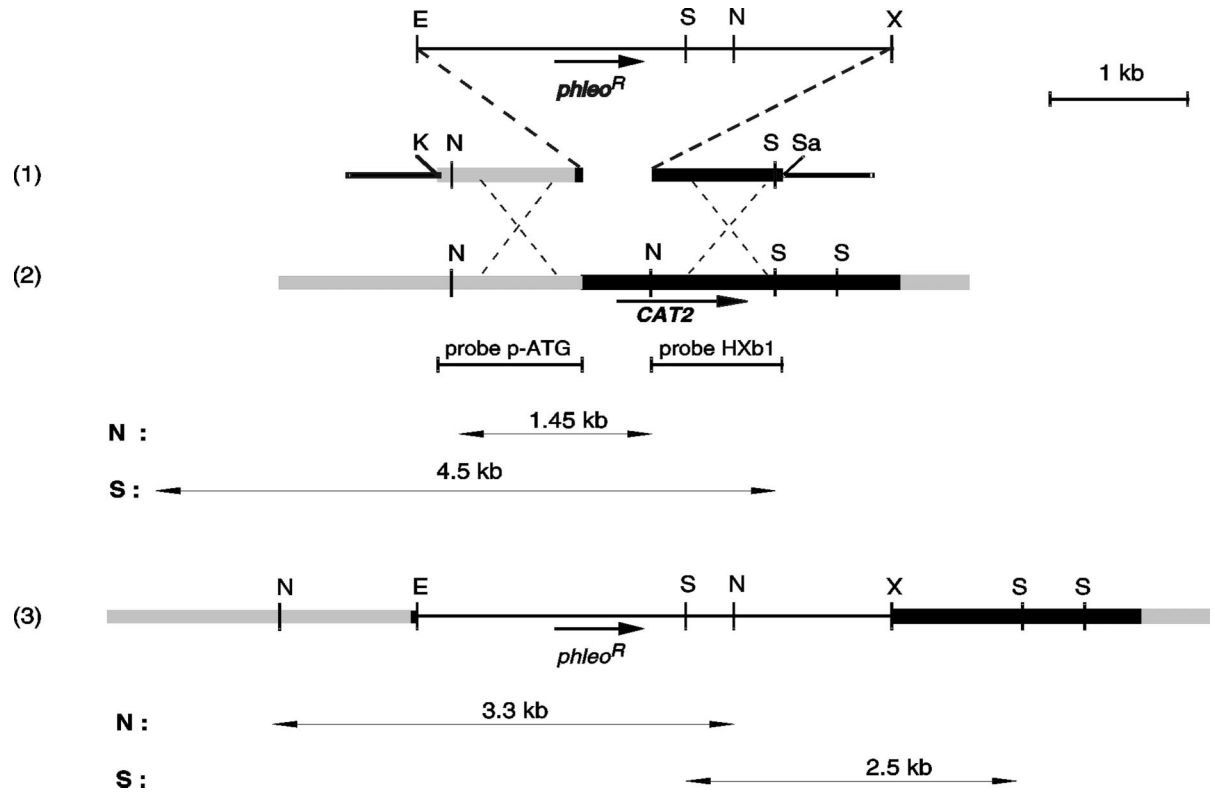

FIG. 2. Disruption of the *CAT2* gene. (Line 1) Plasmid pΔ*CAT2* was constructed to delete a fragment containing the amino acids involved in the catalytic mechanism (R_{93}, W_{96}, H_{97}) of type I peroxidases (36) (see Materials and Methods). This fragment was replaced with a 3.4-kb fragment containing the phleomycin resistance gene (see Materials and Methods). A linear 5.4-kb *Kpn*I-SacII fragment from the resulting construct was used to transform the G10 and Δ*cat1*-28 strains. (Line 2) Genomic DNA of the recipient strain ($Cat2^+$). (Line 3) Genomic DNA of Δ*CAT2* transformants. E, *Eco*RI; K, *Kpn*I; N, *Nco*I; S, *Sal*I; Sa, *Sac*II; X, *Xba*I. For further explanation see the legend to Fig. 1.

as determined by gel filtration and of approximately 80 to 85 kDa as determined by SDS-PAGE, indicating that the peroxidase is a monomer (data not shown). Cat2p enzymatic activity was sensitive to heat (10 min, 80°C), to heavy metals (5 mM, 1 h), and to 30 min of incubation in 5% SDS at 37°C, but it was not sensitive to overnight incubation in 40 mM DTT at 4°C.

Cloning and characterization of the conidial catalase, CatAp. The *CATA* gene was cloned by using the 807-bp *Nhe*I fragment of the *A. nidulans CATA* gene to screen an *A. fumigatus* genomic cosmid library. Four positive clones were found, and restriction enzyme analysis followed by Southern blotting resulted in identification of a 7.5-kb *Pst*I fragment in four of the clones. This fragment contained a 2,355-bp ORF coding for a 749-amino-acid protein interrupted by two putative introns. The predicted molecular mass, 84.5 kDa, was in agreement with the M_r of 80,000 to 85,000 estimated by SDS-PAGE after high-performance liquid chromatography purification. Likewise, ConA-peroxidase blots demonstrated that this enzyme is not glycosylated. The deduced protein sequence was compared with the sequences of other catalases of filamentous fungi (Fig. 4). The highest level of homology was found with *A. nidulans* CatAp (79% identity). *A. fumigatus* CatAp exhibited higher levels of homology with the bacterial catalase CATE HPII of *E.coli* (44% identity) and the fungal catalase (M antigen) of *Histoplasma capsulatum* (41%) than with the mycelial monofunctional catalase of *A. fumigatus*, Cat1p (37% identity).

Cloning and characterization of the mycelial catalase-peroxidase, Cat2p. An internal amino acid sequence of one peptide of Cat2p (HKPTMLTTDLSLR) was selected to design the oligonucleotide probe to screen the *A. fumigatus* genomic cosmid library. Only one hybridizing clone (clone 61D9) was found which contained a fragment with homology to bacterial catalase-peroxidases. The complete sequence of the catalase-peroxidase gene was obtained on cosmid clone 61D9. The nucleotide sequence revealed a 2,224-bp ORF which predicted a 739-amino-acid protein. The absence of introns was confirmed by reverse transcription-PCR analysis of different fragments covering the entire sequence of the gene. A signal peptide sequence which predicted a nonsecreted enzyme was not identified. The predicted molecular mass (82 kDa) was consistent with the SDS-PAGE and gel filtration size data (80 to 85 and 85 kDa, respectively). The deduced Cat2p enzyme was homologous to catalase-peroxidases of several filamentous fungi and bacteria (Fig. 5). Cat2p contained conserved amino acid residues essential for the catalytic site (R_{93}, W_{96}, H_{97}) (36) (Fig. 5). The highest levels of homology were found with the catalase-peroxidases of *Penicillium marneffei* (78% identity), *A. nidulans* (74% identity), and *Neurospora crassa* (72% identity). It is interesting that there is a short sequence in Cat2p between

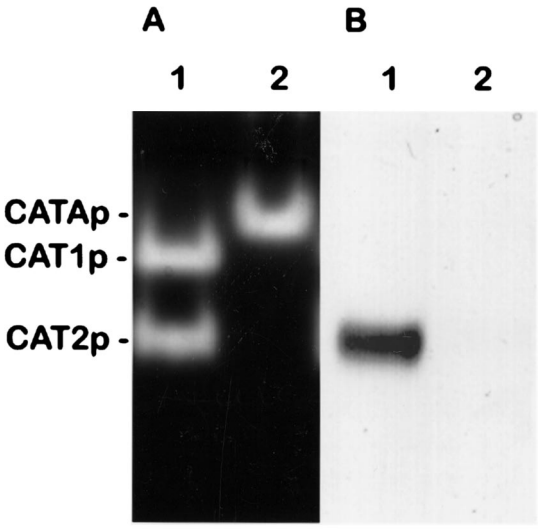

FIG. 3. Native PAGE of protein extracts of mycelium (lane 1) and conidia (lane 2) of *A. fumigatus* strain G10 stained for catalase (A) (ferricyanide negative stain) and peroxidase (B) (diaminobenzidine stain).

amino acids 205 and 226 that is not present in the *A. nidulans* and bacterial enzymes but is present in the *N. crassa* enzyme (Fig. 5). The levels of homology with bacterial catalase-peroxidases were 70, 69, and 66% for the *Caulobacter crescentus*, *Streptomyces reticuli*, and *Bacillus stearothermophilus* enzymes, respectively. Fungal EST from *Mycosphaerella graminicola*, *Fusarium graminearum*, *Leptosphaeria maculans*, *Magnaporthe grisea*, and *Aspergillus niger* coding for presumed peroxidases showed homology with different parts of the sequence (data not shown).

Disruption of *CATA* and *CAT2* genes. For construction of the replacement vector pΔ*CATA*, a 862-bp *Van*91I fragment from the central part of *CATA* was replaced by the phleomycin resistance cassette to produce a nonfunctional allele. The 4.1-kb *Eco*RI-*Cla*I fragment of pΔ*CATA* containing the *CATA*::*phleoR* construct was used to transform strain G10 to obtain a *catA*$^-$ mutant and to transform mutant Δ*cat1*-28 to create a double *catA*$^-$*cat1*$^-$ mutant (Fig. 1). Southern blot analysis of *A. fumigatus* transformants showed that the wild-type *CATA* gene was replaced with the disrupted gene in four transformants, Δ*catA*-01, Δ*catA*-02, Δ*catA*Δ*cat1*-21, and Δ*catA*Δ*cat1*-39 (Fig. 6).

A three-step strategy was used for construction of the replacement vector pΔ*CAT2*, resulting in replacement of a 420-bp fragment of the *CAT2* ORF containing the active site by the phleomycin cassette. The 5.5-kb *Kpn*I-*Sac*II fragment of pΔ*CAT2* was used to transform recipient strain G10 to obtain a *cat2*$^-$ mutant and to transform recipient strain Δ*cat1*-28 to create a double *cat1*$^-$*cat2*$^-$ mutant (Fig. 2). Southern blot analysis of *A. fumigatus* transformants showed that the wild-type *CAT2* gene was replaced with the disrupted gene in three transformants, Δ*cat2*-3, Δ*cat1*Δ*cat2*-18, and Δ*cat1*Δ*cat2*-19 (Fig. 7).

Protein extracts of conidia and mycelia from strain G10 and transformants Δ*catA*-01, Δ*cat1*-28, Δ*cat2*-3, Δ*cat1*Δ*cat2*-18, and Δ*catA*Δ*cat1*-39 were analyzed for catalase activity (Fig. 8), and the results confirmed phenotypically the Southern blot data for the different mutants. The slowly migrating catalase band was absent only in conidial extracts from the Δ*catA* transformants, and the fast-migrating band was absent in the mycelial extracts of the Δ*cat2*-3 and Δ*cat1*Δ*cat2*-18 transformants. All single and double mycelial catalase mutants produced the CatAp band.

Phenotypic analysis of conidial catalase-negative mutants. Survival of conidia after treatment with H_2O_2 was also tested. About 0.1% of the Δ*catA*-01 or Δ*catA*Δ*cat1*-39 conidia survived a 30-min treatment with 15 mM H_2O_2, whereas 100% of the G10 and Δ*cat1*-28 conidia survived the same treatment (Fig. 9). Thus, the Δ*catA*-01 and Δ*catA*Δ*cat1*-39 conidia were more sensitive to H_2O_2 treatment than the parental G10 and Δ*cat1*-28 conidia were.

In spite of the increased sensitivity to H_2O_2, the killing of Δ*catA*-01 conidia by alveolar macrophages of immunocompetent mice was similar to the killing of G10 conidia (Fig. 10). In the rat model of infection, there was no difference in the histopathological lesions of lungs of rats infected with G10 or Δ*catA*-01 (data not shown).

These results indicated that the Δ*catA*-01 mutant was as virulent as G10 and suggested that the conidial catalase, CatAp, does not protect conidia from the oxidative burst of alveolar macrophages and that H_2O_2 is not involved in the killing of conidia in immunosuppressed mice.

Phenotypic analysis of mycelial catalase-negative mutants. The MIC of H_2O_2 for mycelia of the double mutants Δ*cat1*Δ*cat2*-18 and Δ*cat1*Δ*cat2*-19 was 2.5 mM, while the MIC for other strains (G10, Δ*cat1*-28, Δ*cat2*-3, Δ*catA*-01) was 5 mM. Since only the double mutant exhibited slightly increased sensitivity to H_2O_2 and since previous studies have shown that the behaviors of the Δ*cat1*-28 and wild-type parental strains are similar in in vivo studies (3), experiments in which sensitivity to PMNLs and experimental infection were examined were performed only with Δ*cat1*Δ*cat2*-18.

Mycelium of the Δ*cat1*Δ*cat2*-18 mutant was as sensitive to PMNL killing as the wild-type G10 strain mycelium was (79.7% $\pm$ 0.6% and 85.3% $\pm$ 3.1%, respectively), as determined by Student's test. In the rat model of infection, there were significant differences in the histological features of rat lungs infected with G10 and Δ*cat1*Δ*cat2*-18 conidia on days 5 and 13 after infection. On day 5 after infection, the lungs of rats infected with G10 contained numerous large (diameter, 1 mm), confluent lesions throughout tissue sections (Fig. 11a) that were characterized by a prominent infiltrate of PMNLs and by caryolysis (Fig. 12a) associated with extensive hyphal elongation in the necrotic tissue (Fig. 12c). In contrast, the lungs of rats infected with Δ*cat1*Δ*cat2*-18 conidia contained small (diameter, 0.5 mm), scattered nodules (Fig. 11b) which consisted of PMNLs and macrophages (Fig. 12b), as well as restricted hyphal growth from the inoculum (Fig. 12d). On day 13 after infection the lung lesions appeared to be more severe than those observed on day 5 after infection with both parental and mutant strains, but the histopathological virulence differences between the two strains on day 5 were still present on day 13. Lesions seen with the mutant on day 13 (Fig. 11d) were similar to lesions seen with the wild-type strain on day 5 (Fig. 11a). In contrast, the extremely severe lesions characterized by

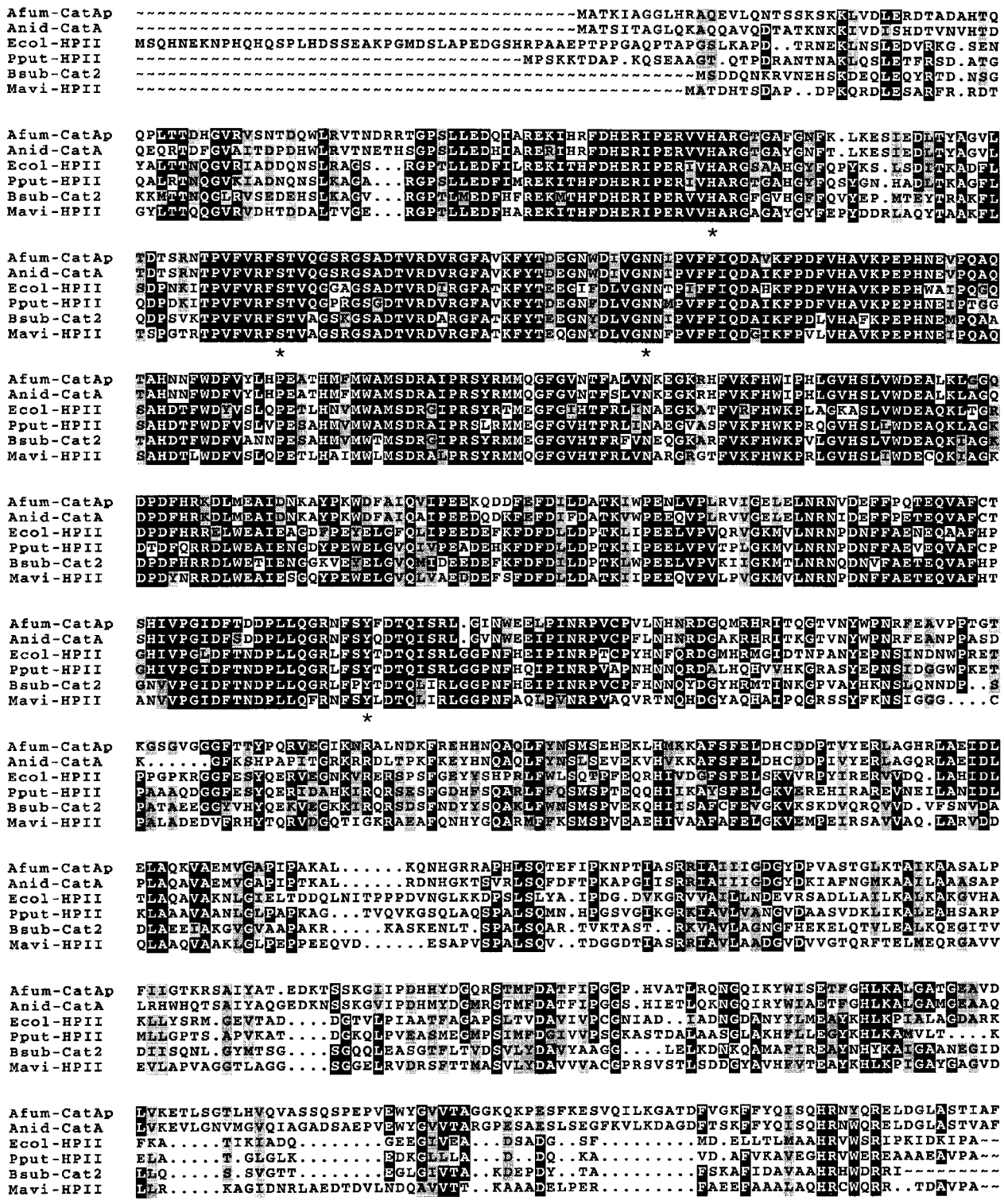

FIG. 4. Amino acid sequence alignment of *A. fumigatus* conidial catalase CatAp and catalase homologs. The protein sequences used were the sequences of *A. fumigatus* CatAp (Afum-CatAp), *A. nidulans* CatAp (Anid-CatA) (accession no. P55305), *E.coli* (Ecol-HPII) (accession no. P21179), *Pseudomonas putida* (Pput-HPII) (accession no. P95539), *Bacillus subtilis* (Bsub-Cat2) (accession no. P42234), and *Mycobacterium avium* (Mavi-HPII) (accession no. P50979). The alignment was constructed by using the GCG Pileup and Boxhade programs (11). Identical residues are indicated by a black background, and similar residues are shaded. Gaps introduced to optimize the alignment are indicated by dots. Conserved amino acids that form part of the active and heme coordination sites are indicated below the sequence by asterisks.

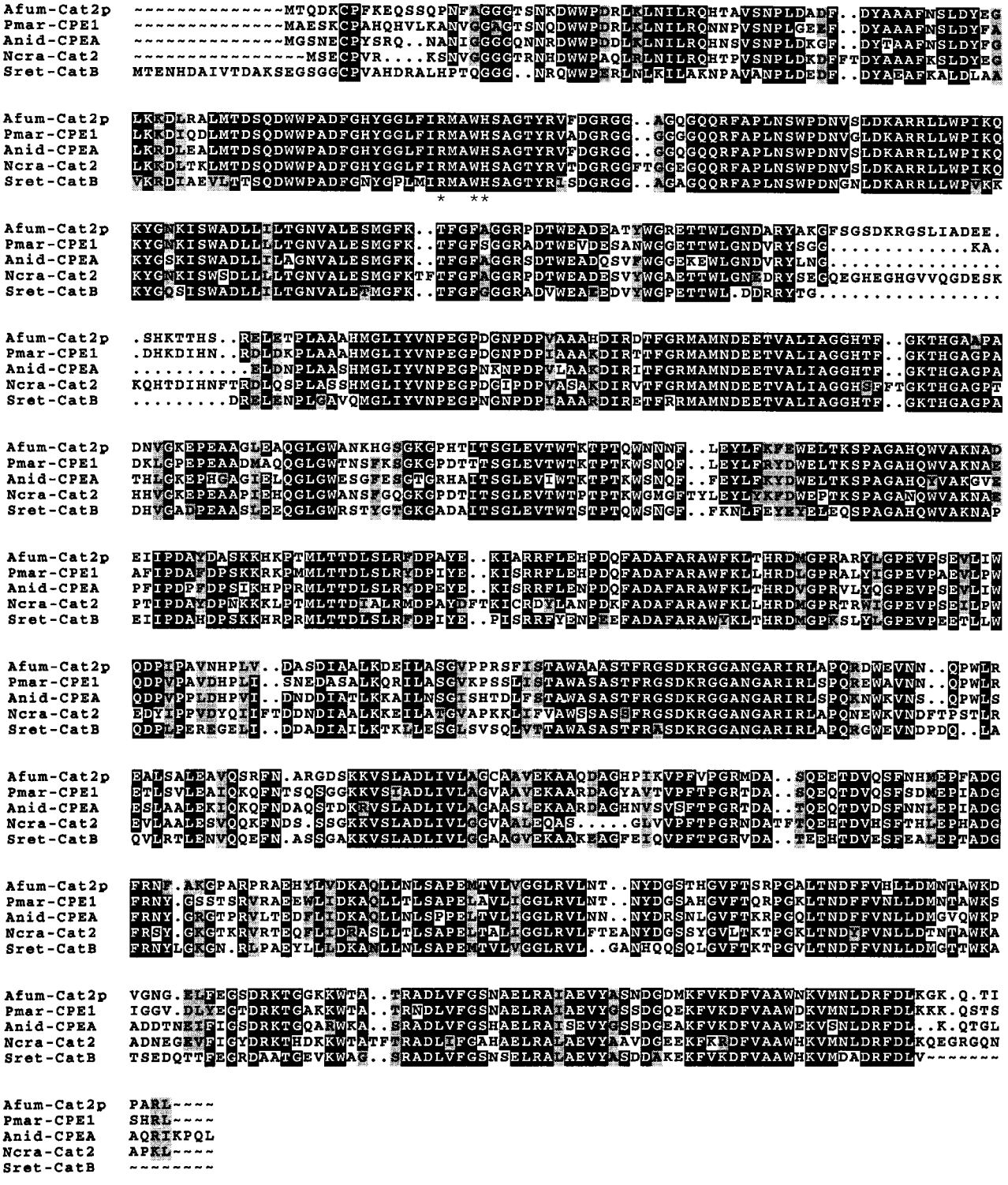

FIG. 5. Amino acid sequence alignment of *A. fumigatus* catalase-peroxidase Cat2p and catalase-peroxidase homologues. Cat2p was aligned with catalase-peroxidases from *P. marneffei* (Pmar-CPE1) (accession no. AF537129), *A. nidulans* (Anid-CPEA) (accession no. CAC59821), *N. crassa* (Ncra-Cat2) (accession no. AF459787), and *S.reticuli* (Sret-CatB) (accession no. 087864). See the legend to Fig. 4 for more information.

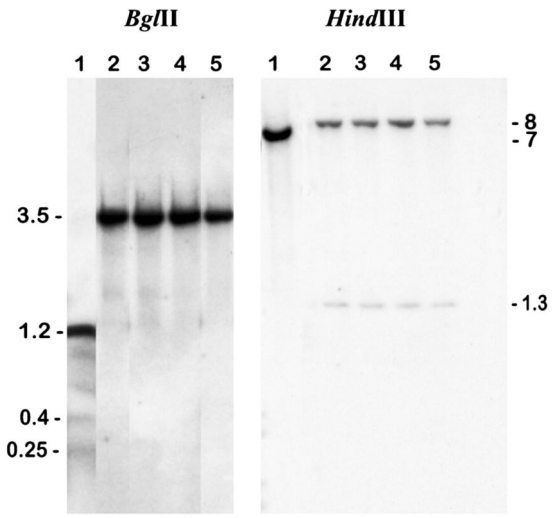

FIG. 6. Southern hybridization of *Bgl*II- and *Hin*dIII-digested genomic DNA of the G10 parental strain (lane 1) and of transformants Δ*catA*-01 (lane 2), Δ*catA*-02 (lane 3), Δ*catA*Δ*cat1*-21 (lane 4), and Δ*catA*Δ*cat1*-39 (lane 5). The 1.7-kb *Hin*dIII-*Eco*RV fragment from pΔ*CATA* was used as the probe. Sizes (in kilobases) are indicated on the left and on the right.

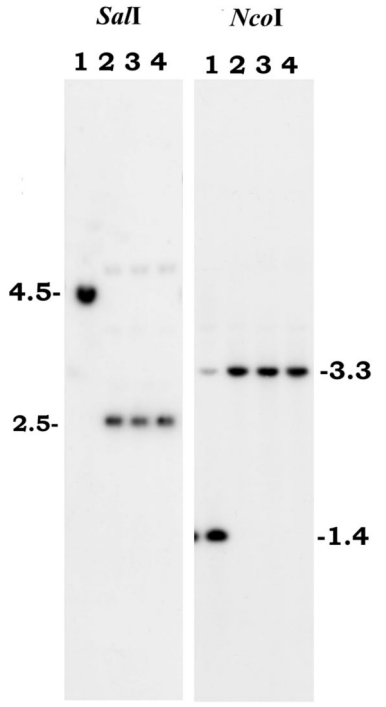

FIG. 7. Southern hybridization of *Nco*I- and *Sal*I-digested genomic DNA of the wild-type G10 strain (lane 1) and of transformants Δ*cat2*-3 (lane 2), Δ*cat1*Δ*cat2*-18 (lane 3), and Δ*cat1*Δ*cat2*-19 (lane 4) probed with the P-ATG fragment (*Nco*I) or the HXb1 fragment (*Sal*I) of the *CAT2* gene. Sizes (in kilobases) are indicated on the left and on the right.

necrosis and emboli in both arteries and veins were seen only with the parental strain (Fig. 11c).

DISCUSSION

The results presented here show that *A. fumigatus* expresses three active catalases, one that is present in the conidia and two that are present in the mycelium, which are encoded by three separate structural genes *CATA*, *CAT1*, and *CAT2*.

Analysis of the conidial catalase, CatAp. CatAp is the only catalase present in resting conidia and is not present in hyphae. This unglycosylated catalase is very resistant to heat, denaturing agents, and metal ions. CatAp was found to be a dimer, like catalases KpA of *Klebsiella pneumoniae* and Cat-2 of *N. crassa* (7, 18), whereas most large-subunit monofunctional catalases are tetrameric (36). The significance of the dimeric structure is not known.

The deduced CatAp polypeptide exhibits the highest homology with the conidial CatAp catalase of *A. nidulans* (79% identity) and is more homologous to HPII of *E. coli* (44% identity) than to Cat1p of *A. fumigatus* (40% identity). This divergence between CatAp and Cat1p was confirmed by a phylogenetic analysis based on the peptide sequences of 210 catalases, which showed that CatAp and Cat1p belong to different subclasses of the large-subunit monofunctional catalases (25).

Role of the conidial catalase, CatAp. Like conidia of an *A. nidulans* Δ*CATA* strain, conidia of the Δ*CATA* strain of *A. fumigatus* were more sensitive to H_2O_2 than the parental conidia were. Although Δ*catA* conidia were killed at lower doses of H_2O_2 than conidia of the G10 parental strain, the killing of G10 conidia by murine alveolar macrophages and the killing of Δ*catA* conidia by such macrophages were identical. Thus, while the conidial catalase, CatAp, protects the spores against the deleterious effects of hydrogen peroxide in vitro, it does not play a role in protecting conidia against the oxidative burst of macrophages that is known to play an essential role in the killing of conidia. This confirms recent data from our laboratory suggesting that the main ROS

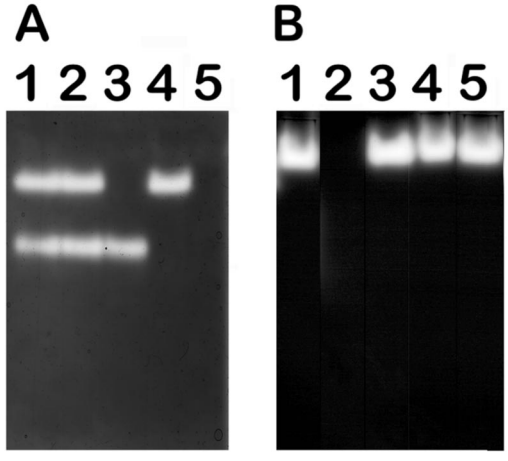

FIG. 8. Native PAGE of mycelial extracts (A) and conidial extracts (B) of the recipient G10 strain and transformants stained for catalase (ferricyanide negative stain). Lane 1, strain G10 (*catA*$^+$*cat1*$^+$*cat2*$^+$); lane 2, Δ*catA*-01 transformant (*catA*$^-$*cat1*$^+$*cat2*$^+$); lane 3, Δ*cat1*-28 transformant (*catA*$^+$*cat1*$^-$*cat2*$^+$); lane 4, Δ*cat2*-3 transformant (*catA*$^+$ *cat1*$^+$*cat2*$^-$); lane 5, Δ*cat1*Δ*cat2*-18 transformant (*catA*$^+$*cat1*$^-$*cat2*$^-$).

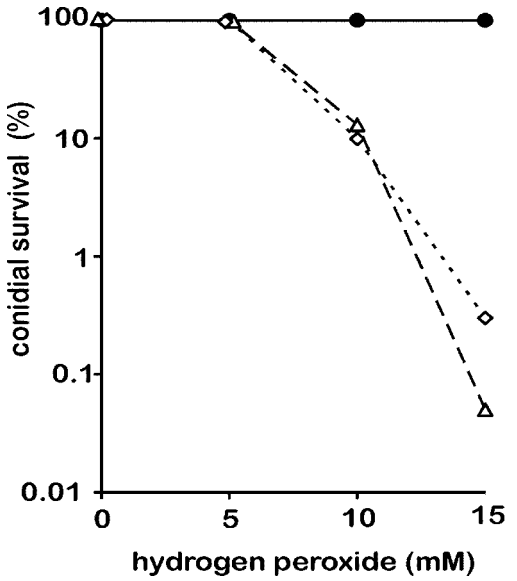

FIG. 9. Effect of hydrogen peroxide on the survival of *A. fumigatus* conidia. Conidia (10^6conidia/ml) were incubated at 37°C for 30 min at each concentration of H_2O_2. The number of surviving conidia was determined by plating on malt extract agar. Symbols: ●, strain G10; △, Δ*catA*-01; ◇, Δ*catA*Δ*cat1*-39. Δ*cat1*-28 conidia were not killed like G10 conidia.

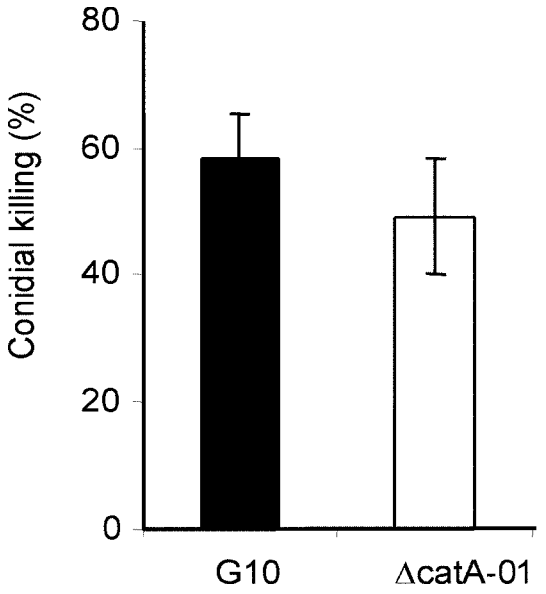

FIG. 10. Estimated in vivo conidial killing by murine alveolar macrophages recovered from mice infected intranasally with 10^5 conidia of wild-type strain G10 and of transformants Δ*catA*-01. The values are means of triplicate determinations. The error bars indicate standard errors.

which plays a role in conidial killing by macrophages is not H_2O_2 (38a). Another possible explanation is that H_2O_2 is scavenged by melanin, as suggested by Jahn et al. (23), and that this process may mask the effect of the presence or absence of the conidial catalase. To test this hypothesis, we constructed a *catA*$^-$ mutant in a *pks*$^-$ background to produce conidia that were white and lacked catalase activity (*pks*$^-$Δ*catA*). The killing of *pks*$^-$Δ*catA* conidia by alveolar macrophages was similar to the killing of parental white conidia (*pks*$^-$) (unpublished results). This result supports the hypothesis that an ROS other than H_2O_2 is important in the killing of conidia by macrophages.

Analysis of mycelial catalase Cat2p. Mycelial catalase Cat2p has peroxidase activity and high electrophoretic mobility, is not glycosylated, and is very sensitive to heat, in contrast to the Cat1p enzyme. Thus, Cat2p corresponds to the fast catalase-peroxidase described by Hearn et al. (21). Cat2p was found to be monomeric. This is surprising since most microbial catalase-peroxidases are active as either dimers or tetramers (36) and only two catalase-peroxidases from halophilic bacteria were found to be monomeric (5, 16).

The *CAT2* gene has no intron, a result that is atypical for the *A. fumigatus* ORFs sequenced so far. The absence of an intron was, however, also observed in the fungal catalase-peroxidase genes sequenced so far (38, 42). The deduced Cat2p polypeptide exhibits high levels of amino acid identity with fungal and bacterial catalase-peroxidases but no sequence similarity with the monofunctional catalases of *A. fumigatus* and *A. nidulans* (3, 26).

Role of the mycelial catalases. Mycelia from Δ*cat1*-28 and Δ*cat2*-3 single mutants were as sensitive to H_2O_2 as the wild-type strain was. These results are in agreement with the similarity of the virulence of the single mycelial catalase mutant and the wild-type strain of *A. nidulans* and *A. fumigatus* in immunosuppressed mice (3, 6). Deletion of both the *CAT1* and *CAT2* genes led to slightly higher H_2O_2 sensitivity of the mycelium and to slower development of the mutant in the lungs of immunosuppressed rats. Thus, both catalases are needed to scavenge deleterious peroxide in vitro and in the rat model of infection. However, the mycelial catalases are not sufficient to protect against the oxidative burst by immunocompetent human PMNLs in vitro. This suggests that mycelial catalases provide only partial resistance to PMNLs. One hypothesis to explain the residual resistance of *A. fumigatus* to H_2O_2 is the presence of an additional catalase(s) that may be specifically expressed during infection. Four other catalase genes (two *CAT1* and two *CAT2* homologs) have indeed been found in the *A. fumigatus* genome sequence in The Institute for Genomic Research database (http://www.tigr.org.) (started after completion of this study). However, this possibility is unlikely since neither catalase nor peroxidase activities could be detected in in vitro induction assays; when the *cat1*$^-$*cat2*$^-$ mutant was grown in vitro in the presence of subinhibitory concentrations of H_2O_2 (0.1 to 1 mM), no additional catalase was seen in our substrate gel assays (data not shown). Another hypothesis is that H_2O_2 is not the primary ROS involved in hyphal killing and that other enzymes, such as superoxide dismutase, may be more efficient than catalases in protecting *A. fumigatus* mycelial growth against another ROS. Indeed, antigenic extracellular superoxide dismutases have been identified in *A. fumigatus* (9, 22) and could play an essential role in protection against ROS. In addition, it is known that in yeast the tripeptide glutathione and the small protein thioredoxin are reductants that protect the cells efficiently when they are exposed to elevated concentrations of superoxide, hydrogen peroxide, and hydroxyl radicals. Yeast homolog genes involved in the biosynthesis and oxidoreduction of glutathione and thioredoxin have

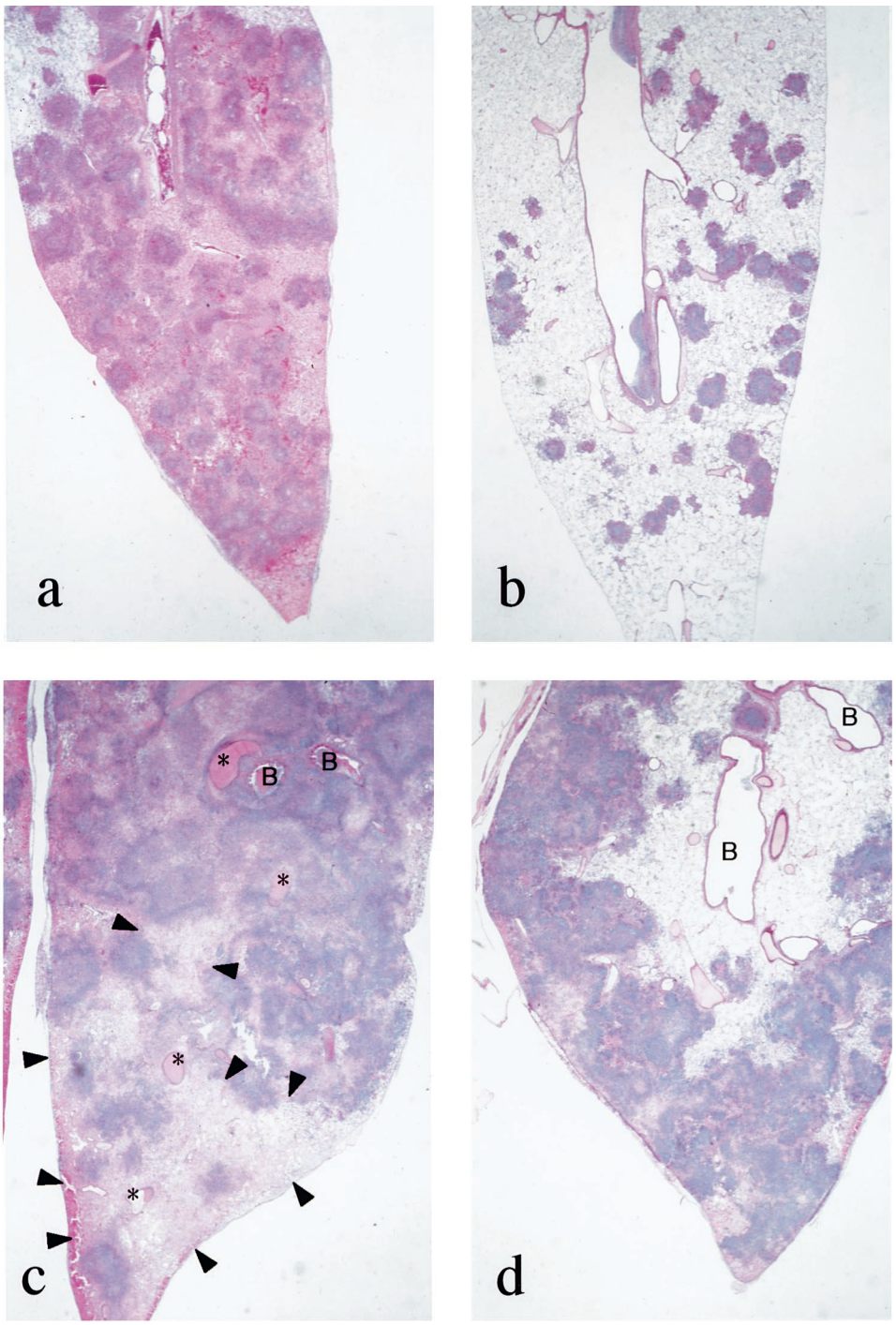

FIG. 11. Sequential changes in the pulmonary lesions of rats infected with *A. fumigatus* strain G10 (a and c) and the Δ*cat1*Δ*cat2*-18 mutant (b and d) on day 5 after infection (a and b) and on day 13 after infection (c and d). On day 5 after infection there were large fused nodules associated with congestive edema when *A. fumigatus* strain G10 was used (a) and there were scattered smaller nodules without edema when the Δ*cat1*Δ*cat2*-18 mutant was used (b). On day 13 after infection widespread necrosis (arrowheads) associated with a fuzzy appearance and numerous fungal emboli (asterisks) were observed when strain G10 was used (c); only enlarged and fused nodules were observed when the Δ*cat1*Δ*cat2*-18 mutant was used (d). The sections were stained with Elastica-PAS stain. Magnification, ×2. B, bronchia.

been found in the *A. fumigatus* database at The Institute for Genomic Research. They could play a role in the resistance of *A. fumigatus* to oxidative stress and killing by phagocytes, as documented in bacteria and yeast (4, 19, 30).

In conclusion, our study with single and double mutants indicated that *A. fumigatus* conidial and mycelial catalases protect the fungus against hydrogen peroxide in vitro. However, while the conidial catalase, CatAp, is not a virulence factor, both mycelial catalases, Cat1p and Cat2p, are involved in the degradation of hydrogen peroxide in vitro and transiently pro-

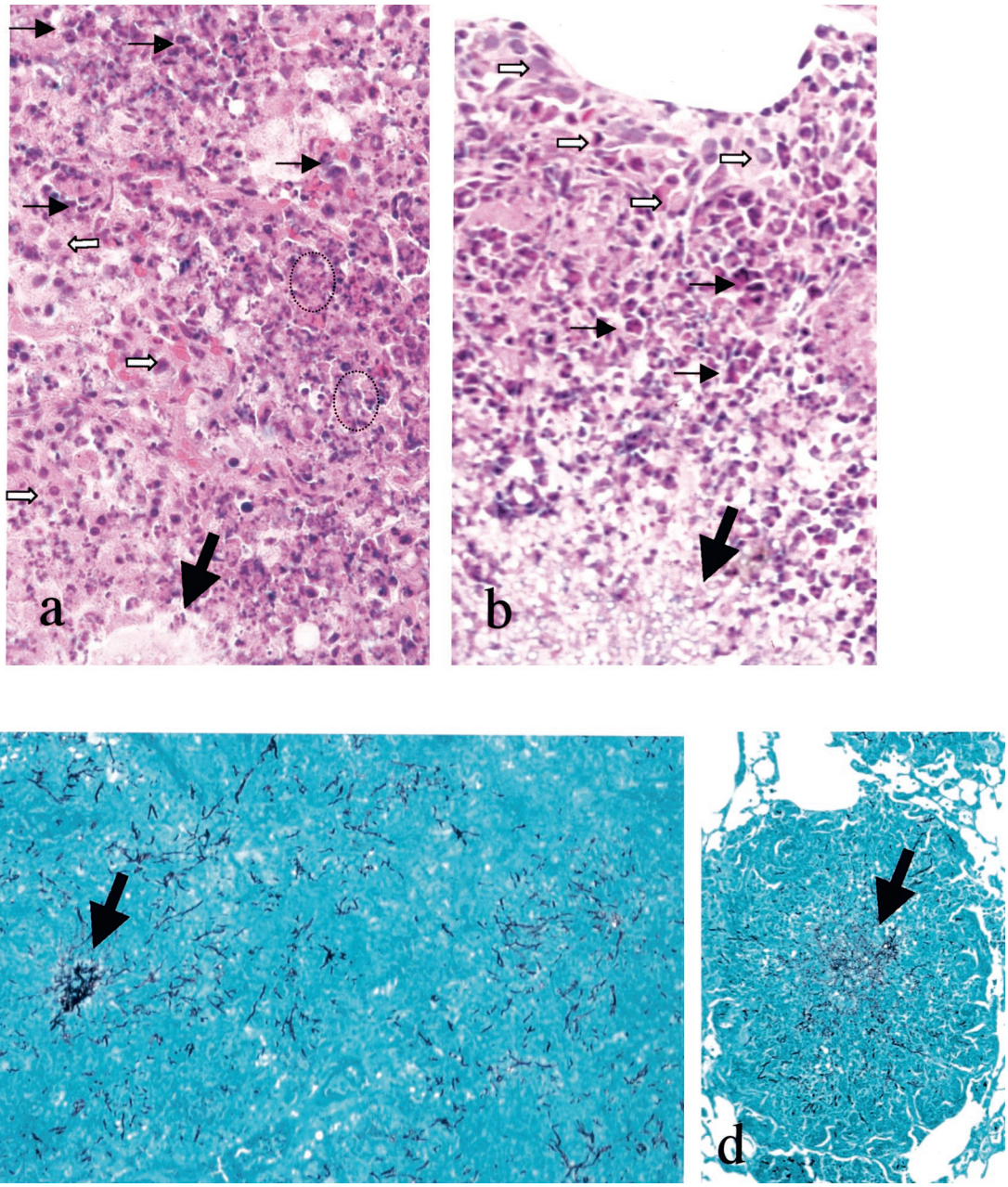

FIG. 12. Details of the lesions in rats on day 5 after infection with *A. fumigatus* G10 (a and c) or with Δ*cat1*Δ*cat2*-18 (b and d). An inoculum bead is indicated by a large arrow. The sections were stained with hematoxylin and eosin (a and b) or with Grocott's stain (c and d). (a) Section of a nodule showing a dense infiltrate of PMNLs (small arrows), nuclear debris (circle), and a limited number of macrophages (open arrows). (b) Small nodule with inner PMNLs (small arrows) and outer macrophages (open arrows). (c and d) Hyphal growth is more important in the fused nodules produced by the wild type (c) than in the small nodules associated with the mutant (d).

tected the fungus against the oxidative burst in our experimental rat model. Nevertheless, other oxidases are needed to overcome the host response. Since ROS have been shown to be essential for the killing of *A. fumigatus* in vitro, our in vivo results suggest that in addition to H_2O_2, another ROS is needed for killing. Analysis of other oxidases required to overcome the oxidative burst in vivo may lead to identification of the molecules that are essential for the killing of *A. fumigatus* by phagocytes.

ACKNOWLEDGMENTS

Plasmids pAN8-1, pID624, and pCAN5 were kindly provided by P. J. Punt (TNO Medical Biological Laboratory, Rijswijk, The Netherlands), J. Brown (Department of infectious Diseases, Hammersmith Hospital, London, United Kingdom), and W. E. Timberlake (Myco Pharmaceuticals Inc., Cambridge, Mass.) respectively. We thank P. T. Borgia (Department of Medical Microbiology and Immunology, Southern Illinois University School of Medicine, Springfield) for providing the *A. fumigatus* genomic cosmid library. We are grateful to K. Uchida (Teikyo University, Tokyo, Japan), M. Takaoka (ICAM Inc.,

Tokyo, Japan), and A. Beauvais for their help during this work. C. Dotigny (Institut Pasteur) is acknowledged for her technical assistance. We are also indebted to R. Calderone for suggestions and critical reading of the manuscript.

REFERENCES

1. **Beauvais, A., M. Monod, J. P. Debeaupuis, M. Diaquin, H. Kobayashi, and J. P. Latgé.** 1997. Biochemical and antigenic characterization of a new dipeptidyl-peptidase isolated from *Aspergillus fumigatus*. J. Biol. Chem. **272:** 6238–6244.
2. **Borgia, P. T., C. L. Dodge, L. E. Eagleton, and T. H. Adams.** 1994. Bidirectional gene transfer between *Aspergillus fumigatus* and *Aspergillus nidulans*. FEMS Microbiol. Lett. **122:**227–232.
3. **Calera, J. A., S. Paris, M. Monod, A. J. Hamilton, J. P. Debeaupuis, M. Diaquin, R. Lopez-Medrano, F. Leal, and J. P. Latgé.** 1997. Cloning and disruption of the antigenic catalase gene of *Aspergillus fumigatus*. Infect. Immun. **65:**4718–4724.
4. **Carmel-Harel, O., and G. Storz.** 2000. Roles of the glutathione and thioredoxin-dependent reduction systems in the *Escherichia coli* and *Saccharomyces cerevisiae* responses to oxidative stress. Annu. Rev. Microbiol. **54:**439–461.
5. **Cendrin, F., H. M. Jouve, J. Gaillard, P. Thibault, and G. Zaccai.** 1994. Purification and properties of a halophilic catalase-peroxidase from *Haloarcula marismortui*. Biochim. Biophys. Acta **1209:**1–9.
6. **Chang, Y. C., B. H. Segal, S. M. Holland, G. F. Miller, and K. J. Kwon-Chung.** 1998. Virulence of catalase-deficient *Aspergillus nidulans* in $p47^{phox\text{-}I^-}$ mice. Implications for fungal pathogenicity and host defense in chronic granulomatous disease. J. Clin. Investig. **101:**1843–1850.
7. **Chary, P., and D. O. Natvig.** 1989. Evidence for three differentially regulated catalase genes in *Neurospora crassa*: effects of oxidative stress, heat shock, and development. J. Bacteriol. **171:**2646–2652.
8. **Cove, D. J.** 1966. The induction and repression of nitrate reductase in the fungus *Aspergillus nidulans*. Biochim. Biophys. Acta **113:**51–56.
9. **Crameri, R., A. Faith, S. Hemmann, R. Jaussi, C. Ismail, G. Menz, and K. Blaser.** 1996. Humoral and cell-mediated autoimmunity in allergy to *Aspergillus fumigatus*. J. Exp. Med. **184:**265–270.
10. **Denning, D. W.** 1998. Invasive aspergillosis. Clin. Infect. Dis. **26:**781–805.
11. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
12. **Diamond, R. D., and R. A. Clark.** 1982. Damage to *Aspergillus fumigatus* and *Rhizopus oryzae* hyphae by oxidative and non-oxidative microbicidal products of human neutrophils in vitro. Infect. Immun. **38:**487–495.
13. **Diamond, R. D., R. Krzesicki, B. Epstein, and W. Jao.** 1978. Damage to hyphal forms of fungi by human leukocytes *in vitro*. A possible host defense mechanism in aspergillosis and mucormycosis. Am. J. Pathol. **91:**313–323.
14. **Diamond, R. D., C. A. Lyman, and D. R. Wysong.** 1991. Disparate effects of interferon-gamma and tumor necrosis factor α on early neutrophil respiratory burst and fungicidal responses to *Candida albicans* hyphae *in vitro*. J. Clin. Investig. **87:**711–720.
15. **Fontaine, T., R. P. Hartland, A. Beauvais, M. Diaquin, and J. P. Latgé.** 1997. Purification and characterization of an endo-β-1,3-glucanase from *Aspergillus fumigatus.* Eur. J. Biochem. **243:**315–321.
16. **Fukumori, Y., T. Fujiwara, Y. Okada-Takahashi, Y. Mukohata, and T. Yamanaka.** 1985. Purification and properties of a peroxidase from *Halobacterium halobium* L-33. J. Biochem. **98:**1055–1061.
17. **Girardin, H., J. P. Latgé, T. Srikantha, B. Morrow, and D. R. Soll.** 1993. Development of DNA probes for fingerprinting *Aspergillus fumigatus.* J. Clin. Microbiol. **31:**1547–1554.
18. **Goldberg, I., and A. Hochman.** 1989. Purification and characterization of a novel type of catalase from the bacterium *Klebsiella pneumoniae*. Biochim. Biophys. Acta **991:**330–336.
19. **Grant, C. M.** 2001. Role of the glutathion/glutaredoxin and thioredoxin systems in yeast growth and response to stress conditions. Mol. Microbiol. **39:**533–541.
20. **Hamilton, A. J., and M. D. Holdom.** 1999. Antioxidant systems in the pathogenic fungi of man and their role in virulence. Med. Mycol. **37:**375–389.
21. **Hearn, V. M., E. V. Wilson, and D. W. R. Mackenzie.** 1992. Analysis of *Aspergillus fumigatus* catalases possessing antigenic activity. J. Med. Microbiol. **36:**61–67.
22. **Holdom, M. D., B. Lechenne, R. J. Hay, A. J. Hamilton, and M. Monod.** 2000. Production and characterization of recombinant *Aspergillus fumigatus* Cu,Zn superoxide dismutase and its recognition by immune human sera. J. Clin. Microbiol. **38:**558–562.
23. **Jahn, B., A. Koch, A. Schmidt, G. Wanner, H. Gehringer, S. Bhakdi, and A. A. Brakhage.** 1997. Isolation and characterization of a pigmentless-conidium mutant of *Aspergillus fumigatus* with altered conidial surface and reduced virulence. Infect. Immun. **65:**5110–5117.
24. **Jaton-Ogay, K., S. Paris, M. Huerre, M. Quadroni, R. Falchetto, G. Togni, J. P. Latgé, and M. Monod.** 1994. Cloning and disruption of the gene encoding an extracellular metalloprotease of *Aspergillus fumigatus*. Mol. Microbiol. **14:**917–928.
25. **Johnson, C. H., M. G. Klotz, J. L. York, V. Kruft, and J. E. McEwen.** 2002. Redundancy, phylogeny and differential expression of *Histoplasma capsulatum* catalases. Microbiology **148:**1129–1142.
26. **Kawasaki, L., and J. Aguirre.** 2001. Multiple catalase genes are differentially regulated in *Aspergillus nidulans*. J. Bacteriol. **183:**1424–1440.
27. **Kawasaki, L., D. Wysong, R. Diamond, and J. Aguirre.** 1997. Two divergent catalase genes are differentially regulated during *Aspergillus nidulans* development and oxidative stress. J. Bacteriol. **179:**3284–3292.
28. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
29. **Lopez-Medrano, R., M. C. Ovejero, J. A. Calera, P. Puente, and F. Leal.** 1995. An immunodominant 90-kilodalton *Aspergillus fumigatus* antigen is the subunit of a catalase. Infect. Immun. **63:**4774–4780.
30. **Manca, C., S. Paul, C. E. Barry, V. H. Freedman, and G. Kaplan.** 1999. *Mycobacterium tuberculosis* catalase and peroxidase activities and resistance to oxidative killing in human monocytes in vitro. Infect. Immun. **67:**74–79.
31. **Mattern, I. E., P. J. Punt, and C. A. M. J. J. Van den Hondel.** 1988. A vector of *Aspergillus* transformation conferring phleomycin resistance. Fung. Genet. Newsl. **35:**25.
32. **Meshulam, T., S. M. Levitz, L. Christin, and R. D. Diamond.** 1995. A simplified new assay for assessment of fungal cell damage with the tetrazolium dye, (2,3)-bis-(2-methoxy-4-nitro-5-sulphenyl)-(2H)- tetrazolium-5-carboxanilide (XTT). J. Infect. Dis. **172:**1153–1156.
33. **Monod, M.** 1994. Isolation of *Aspergillus fumigatus* genes using oligonucleotide probes, p. 33–40. *In* B. Maresca and G. S. Kobayashi (ed.), Molecular biology of pathogenic fungi. A laboratory manual. Telos Press, New York, N.Y.
34. **Monod, M., S. Paris, J. Sarfati, K. Jaton-Ogay, P. Ave, and J. P. Latgé.** 1993. Virulence of alkaline protease-deficient mutants of *Aspergillus fumigatus*. FEMS Microbiol. Lett. **106:**39–46.
35. **Navarro, R. E., M. A. Stringer, W. Hansberg, W. E. Timberlake, and J. Aguirre.** 1996. *cat*A, a new *Aspergillus nidulans* gene encoding a developmentally regulated catalase. Curr. Genet. **29:**352–359.
36. **Nicholls, P., I. Fita, and P. C. Loewen.** 2001. Enzymology and structure of catalases. Adv. Inorg. Chem. **51:**51–106.
37. **Paris, S., C. Fitting, J. P. Latgé, D. Herman, M. T. Guinnepain, and B. David.** 1990. Comparison of conidial and mycelial allergens of *Alternaria alternata*. Int. Arch. Allergy Appl. Immunol. **92:**1–8.
38. **Peraza, L., and W. Hansberg.** 2002. *Neurospora crassa* catalases, singlet oxygen and cell differentiation. Biol. Chem. **383:**569–575.

38a. **Philippe, B., O. Ibrahim-Granet, M. C. Prevost, M. A. Gougorot-Pocidalo, M. Sanchez Perez, A. Van der Meeren, and J.-P. Latgé.** 2003. Killing of *Aspergillus fumigatus* by the alveolar macrophages is mediated by reactive oxidant intermediates. Infect. Immun. **71:**3034–3042.

39. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
40. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
41. **Schaffner, A., H. Douglas, and A. I. Braude.** 1982. Selective protection against conidia by mononuclear and against mycelia by polymorphonuclear phagocytes in resistance to *Aspergillus*. J. Clin. Investig. **69:**617–631.
42. **Scherer, M., H. Wei, R. Liese, and R. Fisher.** 2002. *Aspergillus nidulans* catalase-peroxidase gene (*cpeA*) is transcriptionally induced during sexual development through the transcription factor stuA. Eukaryot. Cell **5:**725–735.
43. **Shibuya, K., M. Takaoka, K. Uchida, M. Wakayama, H. Yamaguchi, K. Takahashi, S. Paris, J. P. Latgé, and S. Naoe.** 1999. Histopathology of experimental invasive pulmonary aspergillosis in rats: pathological comparison of pulmonary lesions induced by specific virulent factor deficient mutants. Microb. Pathog. **27:**123–131.
44. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
45. **Tsang, V. C. W., J. M. Peralta, and A. R. Simons.** 1983. Enzyme-linked immunoelectrotransfer blot techniques (ETTB) for studying the specificities of antigens and antibodies separated by gel electrophoresis. Methods Enzymol. **92:**377–391.
46. **Ward, M.** 1991. *Aspergillus nidulans* and other filamentous fungi as genetic systems, p. 455–496. *In* U. N. Streips and R. E. Yasbin (ed.), Modern microbial genetics, vol. XIV. Wiley-Liss, New York, N.Y.
47. **Wayne, L. G., and G. A. Diaz.** 1986. A double staining method for differentiating between two classes of mycobacterial catalase in polyacrylamide electrophoresis gels. Anal. Biochem. **157:**89–92.

Editor: T. R. Kozel