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Cloning and Disruption of the Antigenic Catalase Gene of *Aspergillus fumigatus*

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Aspergillus fumigatus **possesses two catalases (described as fast and slow on the basis of their electrophoretic mobility). The slow catalase has been recognized as a diagnostic antigen for aspergillosis in immunocompetent patients. The antigenic catalase has been purified. The enzyme is a tetrameric protein composed of 90-kDa subunits. The corresponding *cat1* gene was cloned, and sequencing data show that the *cat1* gene codes for a 728-amino-acid polypeptide. A recombinant protein expressed in *Pichia pastoris* is enzymatically active and has biochemical and antigenic properties that are similar to those of the wild-type catalase. Molecular experiments reveal that CAT1 contains a signal peptide and a propeptide of 15 and 12 amino acid residues, respectively. *cat1*-disrupted mutants that were unable to produce the slow catalase were as sensitive to H_2O_2 and polymorphonuclear cells as the wild-type strain. In addition, there was no difference in pathogenicity between the *cat1* mutant and its parental *cat1*$^+$ strain in a murine model of aspergillosis.**

The opportunistic fungus *Aspergillus fumigatus* has been associated with a wide spectrum of diseases in humans, such as allergic bronchopulmonary aspergillosis, aspergilloma, and invasive aspergillosis (22). One of the antigens of *A. fumigatus* which has been reported as being important in the immunodiagnosis of aspergillosis has a catalase enzymatic activity. This activity was originally reported as being associated with precipitins obtained after immunoelectrophoresis of *A. fumigatus* crude extracts in the presence of serum samples from patients with aspergillosis or from hyperimmunized rabbits (43). Several other reports have confirmed the significance of the antigenic catalase in the diagnosis of aspergilloma (13, 27, 38). Hearn et al. (13) have shown the presence of at least two catalases in *A. fumigatus*. They were designated F and S (fast and slow) with respect to their electrophoretic mobility in nondenaturing polyacrylamide gel electrophoresis (PAGE) and differed biochemically from one another. Enzyme band S displayed exclusively catalase activity, whereas the F band had an additional peroxidase function. López-Medrano et al. (26) identified by sodium dodecyl sulfate (SDS)-PAGE an immunodominant 90-kDa antigen from *A. fumigatus* as the subunit of a catalase designated CAT1.

Catalase is an antioxidant metalloenzyme which is virtually ubiquitous among aerobic organisms and protects cells against oxidative damage caused by hydrogen peroxide. It has been documented that oxidative mechanisms are important in the killing of *A. fumigatus* by polymorphonuclear cells and that this fungus is sensitive in vitro to oxygen metabolites (24). A deficiency in catalase activity could make *A. fumigatus* vulnerable to rapid killing by the H_2O_2-generating systems of phagocytes, and consequently, *A. fumigatus* catalase CAT1 is a putative virulence factor.

In this communication we describe the molecular cloning of the antigenic CAT1 catalase gene of *A. fumigatus*, its overexpression in the yeast *Pichia pastoris*, the construction of a mutant with a defective copy of the catalase gene, the susceptibility of the mutant to in vitro killing by H_2O_2 and polymorphonuclear cells, and its virulence in a mouse model.

MATERIALS AND METHODS

Strains and culture conditions. *A. fumigatus* AFN (26) and CBS144-89 (Centraalbureau voor Schimmelcultures, Baarn, The Netherlands) are clinical isolates. Strain G10, a spontaneous nitrate reductase mutant of strain CBS144-89 (30), was chosen as the recipient strain for transformations. *Escherichia coli* LE392 and Y1090 were used for propagation of bacteriophages λEMBL3 and λgt11, respectively. All plasmid subcloning experiments were performed in *E. coli* DH5α. Three media were used to grow *A. fumigatus* at 25°C on shaken flasks or on a fermentor: Sabouraud medium (2% glucose, 1% Mycopeptone Biokar; Prolabo, Beauvais, France), 1% yeast extract medium (Difco, Detroit, Mich.), and Bacto Czapek-Dox broth (Difco).

Electrophoresis and immunoblotting. Nondenaturing electrophoresis and SDS-PAGE were performed as previously described (26) with the discontinuous buffer system (23). Immunoblotting was performed by the method of Towbin et al. (42).

The glycosylation of CAT1 was detected on Western blots by the concanavalin A (ConA)-peroxidase-conjugated technique of Fontaine et al. (6).

Catalase activity. Catalase activity was observed in phosphate-buffered saline (PBS) containing 0.1 M H_2O_2 by the release of O_2 bubbles. For the detection of catalase activity on nondenaturing gels, water-soluble extracts obtained by mycelial disruption as previously described (12) were subjected to nondenaturing PAGE, and the ferricyanide-negative stain of Woodbury et al. (47) was used to locate bands containing catalase activity as described by Wayne and Diaz (46).

Purification of CAT1. The water-soluble fraction of *A. fumigatus* (strain CBS144-89, grown in Sabouraud medium), obtained by the method of Hearn et al. (13), was used as the starting material. A Mono Q HR 5/5 column (Pharmacia, Uppsala, Sweden) was used for the anion-exchange purification step. Samples were loaded in 10 mM Tris-HCl (pH 7.5) and eluted with a linear NaCl gradient (0 to 350 mM in 30 min). Gel filtration chromatography was performed on a Superdex 200 HR 30/10 column (Pharmacia) with 120 mM NaCl–10 mM Tris-HCl, pH 7.5, using a flow rate of 0.4 ml/min. Cation-exchange chromatography was used as the last purification step (Mono S HR 5/5 column [Pharmacia] with 10 mM acetate buffer [pH 4.1] and a 0 to 350 mM linear NaCl gradient in 30 min). Catalase activity was monitored in each chromatographic fraction as described above. Molecular size measurements were performed by gel filtration

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with thyroglobulin (669,000), ferritin (440,000), human immunoglobulin G (160,000), and transferrin (81,000) as the size standards.

Monospecific anti-CAT1 antibodies. Monospecific anti-CAT1 antibodies were purified by immunoaffinity from a 1:500 dilution of anti-p90 rabbit antiserum (26) with a polyvinylidene difluoride (PVDF) slice containing 60 μg of electroblotted pure CAT1 by the method of Sambrook et al. (37). The monospecificity of anti-CAT1 was confirmed by Western blotting of a crude water-soluble extract.

N-terminal amino acid sequence analysis. Two-dimensional gel electrophoresis of the water-soluble extract from mycelium grown in Czapek broth has shown that no contaminant protein comigrated with p90 on one-dimensional SDS-PAGE (26). After electroblotting of 20 nmol of p90 onto a PVDF membrane (Millipore, St. Quentin en Yvelines, France) and staining with Ponceau S red, the polypeptide band was excised and used for N-terminal amino acid sequencing by Eurosequence (Gröningen, The Netherlands). The N terminus of recombinant CAT1 (rCAT1) was sequenced by J. D'Alayer (Institut Pasteur).

DNA manipulations. Hybridization of positively charged nylon membranes (Amersham, Les Ulis, France) with a cDNA labelled with digoxigenin by random priming (Boehringer [Mannheim, Germany] kit and protocol) was carried out at 68°C; blots were washed at high stringency (68°C; 0.1× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] and 0.1% SDS) as described by Sambrook et al. (37). *A. fumigatus* chromosomal DNA was isolated by the procedure of Girardin et al. (10). Recombinant plasmids were constructed by standard techniques (37). *Sal*I restriction fragments of genomic DNA containing the *cat1* gene were subcloned into pBluescript SK+ vector (Stratagene, La Jolla, Calif.). Both strands were sequenced by Genome Express (Grenoble, France).

Screening of the λgt11 cDNA expression library and λEMBL3 genomic library. A previously constructed λgt11 library and λEMBL3 genomic library of *A. fumigatus* CHUV 192-88 was used (18). The immunological screening of the λgt11 library was performed by standard protocols (37). For screening of the λEMBL3 genomic library, recombinant plaques of the genomic library were immobilized on nylon membranes (Zeta-Probe; Bio-Rad) and probed with ^{32}P-labelled oligonucleotides (Amersham) as previously described (18).

Transformation of *A. fumigatus*. The transformation of *A. fumigatus* G10 was performed as previously described (32). After overnight expression of the hygromycin B resistance gene (*hph*), the transformants were selected on medium containing 200 μg of hygromycin B ml^{-1}. The ability of transformants to express CAT1 was tested on 1% yeast extract medium.

Murine aspergillosis model. Parental G10 strain (*cat1*$^+$) and Δ*cat1*-28 mutant (monospore isolates) were inoculated by inhalation into cohorts of 10 Swiss mice at a dosage of 10^4, 10^5, 10^6, or 10^7 conidia per mouse as previously described (17). We observed mortality for 10 days. *Aspergillus* strains were reisolated from the lung tissue of dead mice. Control mice pretreated with cortisone received intranasal injections of saline solution only. Control mice survived at least 2 weeks after challenge. Survival analysis was performed by the stratified log rank test ($P < 0.05$) (StatView program; Abacus Concepts, Inc., Berkeley, Calif.).

Hydrogen peroxide and PMNL killing assays. To measure killing of *A. fumigatus* germinating hyphae, conidia were germinated on 24-well tissue culture plates (Nalge-Nunc International, London, United Kingdom) in 1 ml of Hanks balanced salt solution (HBSS) (without Mg^{2+} and Ca^{2+}) (Life Technologies, Paisley, United Kingdom) containing 20 mM HEPES buffer and 10% normal human AB sera (Sigma, Poole, Dorset, United Kingdom) at a concentration of 5×10^4 conidia/well at 37°C for 15 hours. Wells were aspirated, and either $KMnO_4$, H_2O_2 (Sigma), or polymorphonuclear leukocytes (PMNL) were added.

(i) Assay of H_2O_2-induced hyphal damage. One milliliter of H_2O_2 (Sigma) (10^{-1} to 10^{-6} M in PBS) or $KMnO_4$ (10^{-2} to 10^{-6} M; used as control) was added to each well. Plates were then incubated at 37°C for either 30 min or 2 h, and wells were then aspirated and washed twice in 2 ml of sterile distilled water. A group of wells containing hyphae incubated with sterile PBS only was included on each plate. The relative susceptibilities of germinating hyphae (parental and Δ*cat1*-28 strains) to killing by H_2O_2 and $KMnO_4$ was determined by the 3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide (MTT) assay (25; see below).

(ii) Assay of PMNL-induced damage. To assess the relative susceptibilities of the two strains to immune effector cell killing, PMNL were prepared from venous blood (taken in sodium citrate [Sigma] at a ratio of 1:9 [vol/vol]) from a healthy donor by dextran sedimentation, followed by centrifugation over Lymphoprep (Nycomed Pharma, Oslo, Norway) and two successive washes in HBSS. Hypotonic lysis of remaining erythrocytes was then performed with 0.83% (wt/vol) ammonium chloride in sterile water. PMNL were resuspended in 10 ml of HBSS and counted on a hemocytometer with trypan blue to assess viability. PMNL were added to wells containing germinating conidia as described above at various effector-to-target cell ratios in a final volume of 1 ml of HBSS containing 20 mM HEPES and 10% (vol/vol) homologous human sera. After 2 h of incubation at 37°C in a 5% CO_2 atmosphere, wells were aspirated and PMNL were lysed with 300 μl of 0.5% (wt/vol) sodium deoxycholate (Sigma). Wells were then washed twice in 2 ml of sterile distilled water and treated with MTT as described below.

(iii) MTT assay. The MTT colorimetric assay previously described (25) was used to assess the damage to hyphae. After exposure to either H_2O_2, $KMnO_4$, or PMNL, 1 ml of RPMI 1640 with L-glutamine (Life Technologies) containing 0.5 mg of MTT per ml was added to each well, and plates were incubated at 37°C for 3 h. Wells were then aspirated, and 200 μl of acidified isopropanol (containing 0.05 N HCl) was added to each well. After gentle agitation for 5 min, 150 μl of isopropanol containing the solubilized blue precipitate was transferred to 96-well microtiter plates (Nunc-Nalge). The optical density (OD) of the wells was determined on a microplate reader by using a test wavelength of 570 nm and a reference wavelength of 630 nm, with a blank consisting of a well containing 150 μl of isopropanol only. Results were expressed as percentage of control MTT conversion according to the following formula: MTT conversion = 100 × [(mean OD in the test wells − mean OD in blank wells)/(mean OD in wells containing only hyphae − mean OD in blank wells)].

Construction of the expression plasmids. The plasmid used to express catalase in *P. pastoris* was constructed by cloning a PCR product of the catalase gene (CAT1) in the multiple cloning site of the *E. coli-P. pastoris* shuttle vector pHILS1. PCR was performed with homologous primers based on the DNA sequence of CAT1 and cDNA as the templates: AAT GCT CGA GTA TGT CCC TAT ATG ACC GGC (base 456) and GGT AGA TCT CTA $(GTG)_6$ ATC CAC GGG AAA CCG GTC (antisense, base 2889). The PCR products were purified with the High Pure PCR product purification kit (Boehringer) and were digested by *Xho*I and *Bgl*II restriction enzymes for which a site was previously designed at the 5′ extremity of the primers. Subsequently, the digested PCR products were cloned into the *Xho*I and *Bam*HI sites of the multiple cloning site of pHILS1 by standard protocols (37).

P. pastoris GS115 was transformed by spheroplasting with 10 μg of linearized DNA by *Bgl*II. Yeast transformants were selected as described elsewhere (15a). Portions (10 μl) of supernatant of selected transformant cultures were loaded on SDS-PAGs to identify clones successfully expressing CAT1.

Nucleotide sequence accession number. The nucleotide sequence of the *cat1* gene has been submitted to the GenBank database under accession number U97574.

RESULTS

Purification of CAT1. Catalase-positive fractions from the water-soluble extract of *A. fumigatus* were eluted at 200 to 250 mM NaCl from the Mono Q anion-exchange column. The catalase-containing fractions were collected, pooled, and further purified by gel filtration. On Superdex 200, catalase activity was found in fractions of 300 to 350 kDa. These fractions were pooled, dialyzed, and subjected to cation-exchange chromatography. The catalase-positive fraction bound to the Mono S column and eluted at 220 to 230 mM NaCl as a single sharp peak monitored by UV absorbance at 280 nm. By gel filtration chromatography, the molecular mass of the catalase was estimated to be 330 to 340 kDa. It corresponded to the pure native CAT1; when analyzed by SDS-PAGE, this fraction yielded only one band of 90 kDa, which corresponded to the CAT1 subunit previously identified by López-Medrano et al. (26). This result confirms that CAT1 is a tetrameric protein composed of 90-kDa subunits.

Cloning and characterization of the *A. fumigatus cat1* gene. Monospecific anti-CAT1 antibodies purified by immunoaffinity were used to screen a cDNA expression library constructed from *A. fumigatus*. Out of 50,000 recombinant phage plaques, 15 clones were identified and purified. Restriction analysis of these clones with *Not*I and *Eco*RI enzymes demonstrated that only four clones contained an insert of 2.4 kb, in agreement with the estimated molecular mass of the deglycosylated subunit of CAT1 (83 kDa) (26). Subcloning and nucleotide sequence analysis of this 2.4-kb insert confirmed that it encoded the CAT1 subunit.

From the electroeluted CAT1 subunit, the N-terminal amino acid sequence was determined to be DEISDGDAAAA TEEFLSQYYLND. The peptide fragment AAAATEEFLS QYY was chosen to design the oligonucleotide probe (5′-GCY GCYGCYGCYACYGAGGAGTTCCT[I]TCYCAGTACTA) to screen the *A. fumigatus* genomic library. Out of 60,000 plaques, seven positive clones were identified. Restriction enzyme analysis and Southern blotting of purified bacteriophage DNA showed that four clones had two fragments in common, the 3.8- and 1.7-kb *Sal*I fragments that hybridized with the 2.4-kb *cat1* cDNA probe. These two fragments were subcloned in pBluescript. Sequence analysis revealed that the 3.8- and

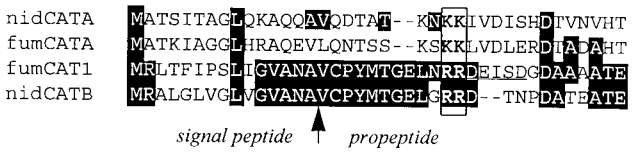

FIG. 1. Comparison of the deduced N-terminal amino acid sequence of *A. fumigatus* CAT1 (fumCAT1) with those of *A. nidulans* CATA (nidCATA) (GenBank accession no. U37803) (29), *A. fumigatus* CATA (fumCATA) (GenBank accession no. U87630), and *A. nidulans* CATB (nidCATB) (GenBank accession no. U80672) (20). The arrow indicates the putative site for signal peptidase cleavage according to the (−3,−1) rule. Amino acid sequence corresponding to the N-terminal sequence of the mature CAT1 is underlined. Residues involved in the putative processing site of the N terminus of the catalases are shown in bold white letters and boxed. Amino acid residues similar to those in CAT1 are shown on a black background. Gaps introduced to optimize alignment are indicated by dashes.

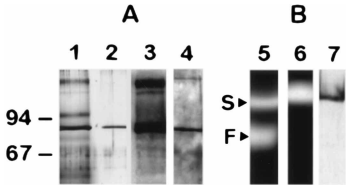

FIG. 2. Analysis of rCAT1. (A) SDS-PAGE (10% polyacrylamide) gel either stained with silver nitrate (lanes 1 and 2) or transferred to nitrocellulose and probed with a pool of sera from aspergilloma patients (lane 3) or developed by the ConA-peroxidase method (lane 4). Lanes 1 and 3, culture filtrate of *P. pastoris* containing the recombinant catalase; lanes 2 and 4, purified recombinant catalase. (B) Nondenaturing PAGE (7.5% polyacrylamide) gel either stained for catalase activity (lanes 5 and 6) or immunoblotted with the monospecific anti-CAT1 antibodies (lane 7). Lane 5, water-soluble extract of G10 strain showing the two catalase bands (S and F); lanes 6 and 7, recombinant catalase migrates at the same rate as the slow catalase. Only a representative part of the gel is shown. Size markers are protein standards (in kilodaltons).

1.7-kb *Sal*I fragments contained the 5′ and 3′ ends of *cat1*, respectively.

The 5′ end of the cloned cDNA starts at bp −55 upstream of the start codon (in position 355), and the *cat1* coding sequence is interrupted by five introns of 66, 49, 85, 56, and 59 bp in positions 696 to 761, 988 to 1036, 1453 to 1537, 1703 to 1758, and 1934 to 1992, respectively. The 5′ untranscribed region of *cat1* contains a putative TATA box and a CAAT motif 118 and 232 bp upstream of the translation start site, respectively. In the 3′ untranslated region, there is an AATAAA-like sequence (14) 129 bp after the TAG stop codon to signal the start of the poly(A) tail.

The predicted molecular mass of the open reading frame of CAT1 is 80,017 Da, and its pI is 5.57. According to the amino acid sequence, it has four potential N-linked glycosylation sites (Asn-X-Ser/Thr) (9). These data are in agreement with the biochemical data, indicating that CAT1 had at least two N-linked oligosaccharidic chains (26).

During the preparation of this manuscript, two sequences coding for *A. fumigatus* catalases appeared in the GenBank data base under accession numbers Y07763 and U87630 that were 99.7% and 99.0% identical to CAT1, respectively, indicating that they code for the same protein. In contrast, a comparison of the deduced amino acid sequence of CAT1 with the known sequences from other mycelial catalases (*A. niger* CATR [7]; *A. nidulans* CATB [20]) and other catalases from spores of *A. nidulans* (CATA [29]) and of nonfungal origin (*E. coli* KatE [45]; bovine liver catalase [39]) showed identities of only 60, 78, 35, 40, and 33%, respectively. Regions of consensus homology among the different catalases seem to exist only in the N-terminal two-thirds of the proteins.

Maturation of CAT1. The N-terminal region of *cat1* has a series of uncharged amino acid residues with a high content of hydrophobic amino acids preceded by a positively charged residue (Arg_2) corresponding to a signal peptide (Fig. 1). However, the N terminus of the mature CAT1 (Asp-Glu-Ile-Ser-Asp) is not preceded by the typical signal for cleavage by the signal peptidase according to the (−3,−1) rule (44). This suggests that CAT1 has both a signal peptide (the first 15 amino acids) and a propeptide (the next 12 amino acids). To study this problem, a cDNA corresponding to a CAT1 polypeptide containing the 12 amino acids of the putative propeptide was cloned in pHILS1. Transformation of *P. pastoris* with this cDNA resulted in the secretion of a rCAT1 in the culture medium. The molecular mass of the recombinant catalase subunits estimated by SDS-PAGE was 90 kDa and was identical to the mass of the subunit of the purified wild-type CAT1. In addition, the N-terminal sequence of rCAT1 obtained with a cDNA containing the prosequence peptide was identical to the N-terminal peptide sequence of the mature wild-type protein (Asp-Glu-Ile-Ser-Asp). This result demonstrated that the presence of the 12-amino-acid propeptide is required for the correct maturation of the CAT1 protein and that the signal cleavage of this peptide is the pair of basic amino acid residues Arg_{26}-Arg_{27}.

The results of gel filtration analysis showed that rCAT1 had a molecular mass of 330 to 340 kDa, confirming it was expressed as a tetrameric polypeptide. Nondenaturing PAGE analysis showed that rCAT1 and wild-type CAT1 migrated in similar ways and had catalase activity (Fig. 2). The positive reactivity of rCAT1 with the ConA-peroxidase conjugate (inhibited by 0.3 M methyl α mannoside) showed that this recombinant protein was glycosylated with mannose residues (Fig. 2). rCAT1 was recognized by patients' sera and by the monospecific anti-CAT1 rabbit antiserum (Fig. 2) and therefore has biochemical and antigenic properties similar to those of the wild-type CAT1.

***cat1* disruption.** In order to study the physiological role of CAT1, the *cat1* gene was disrupted. Plasmid pΔcat1 was designed to function as a one-step gene disruption vector in a transformation experiment and was constructed with pN4 and pucCAT38 (Fig. 3). pΔcat1 containing *cat1*, disrupted by the *E. coli* hygromycin B-resistant gene (*hph*) which also served as a selectable marker gene, was used to confer hygromycin resistance on the *A. fumigatus* G10 strain. Transformation of protoplasts of *A. fumigatus* with the 7-kb *Sma*I fragment of pΔcat1 generated 60 *A. fumigatus* colonies resistant to hygromycin B. Thirty hygromycin B-resistant stable transformants were tested for their ability to produce catalase activity. Of the three catalase-deficient transformants, one (transformant 28) was selected for further study.

Southern blots of *Eco*RI- and *Sal*I-digested genomic DNA of transformant 28, recipient G10 and wild-type strain CBS144-89 were performed with the 0.5-kb *Eco*RV fragment of pCAT38 containing part of the *cat1* gene (Fig. 4). One band of 1.1 kb and one of 8 kb hybridized to this probe in G10 and CBS144-89 DNA digested with *Eco*RI and *Sal*I, respectively. DNA of transformant 28 digested with *Eco*RI yielded two fragments of 1.85 and 2.95 kb, and the same DNA digested by *Sal*I produced two fragments of 9 and 3.3 kb. This pattern is consistent with the correct replacement of *cat1* by the *cat1*Δ::*hph* construct on the chromosome. Western blot of the water-soluble extract of transformant 28 revealed no p90 protein binding to the anti-CAT1 serum

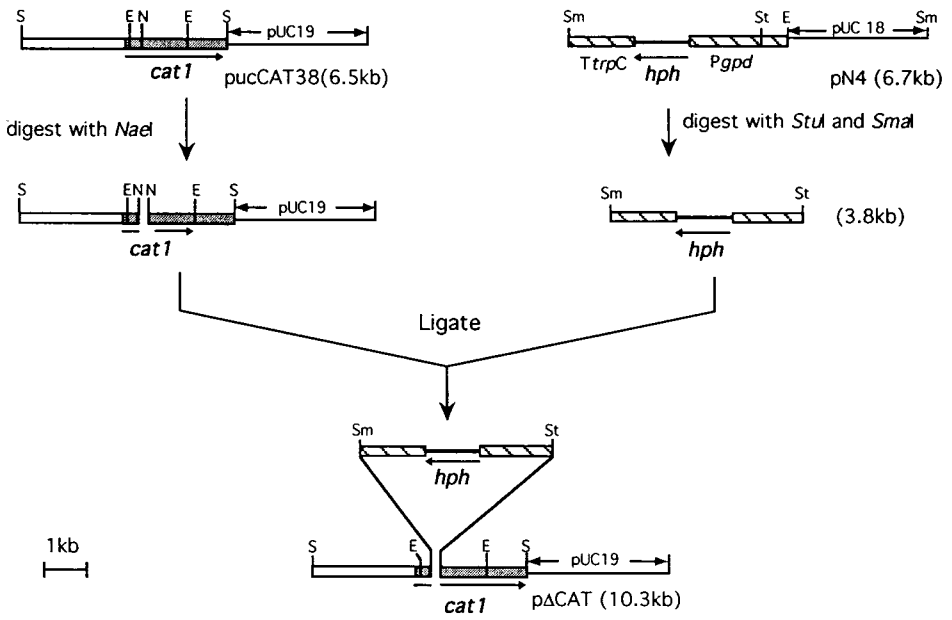

FIG. 3. Construction of plasmid pΔcat1. The plasmid pucCAT38 was obtained by cloning the 3.8-kb *Sal*I fragment of pCAT38, which carries *cat1*, into pUC19. The plasmid pN4 containing the *hph* gene has been described elsewhere (33). Shown is the *A. fumigatus* sequence (white box) flanking the *cat1* gene (grey box). P*gpd* is the 5′ expression signal of the *A. nidulans gpd* gene; T*trpC* is the terminator region of the *A. nidulans trpC* gene. Restriction endonuclease abbreviations: E, *Eco*RI; N, *Nae*I; S, *Sal*I; Sm, *Sma*I; St, *Stu*I.

(data not shown). Consequently, this transformant was a disruption mutant without CAT1 activity and was called the Δ*cat1*-28 strain.

Water-soluble extracts from this transformant were then analyzed for catalase activity on native gels. Figure 5 shows the presence of two catalase components (slow and fast) in CBS144-89 and G10 water-soluble extracts of mycelia grown in 1% yeast extract medium, while the fast catalase band was present only in the Δ*cat1*-28 disruption mutant. The fast catalase band was not recognized by anti-CAT1 antiserum on Western blots (data not shown).

Pathogenicity of *cat1* mutant. Conidia of the Δ*cat1*-28 disruption mutant and the parental G10 strain were administered to cortisone-treated mice. Figure 6 shows an example of the survival data of mice infected with 10^6 conidia per mouse. No significant difference in pathogenicities were seen for mice infected by the parental and mutant strains at four different concentrations of conidia. Survival data analyzed in a stratified log rank test with the concentrations of spores used as strata gave a *P* value of 0.70.

Resistance of *cat1* mutants to H_2O_2 and PMNL. Parental and mutant strains of *A. fumigatus* were exposed to various concentrations of H_2O_2 and $KMnO_4$, and data for 2-h exposure, expressed as percent control of MTT conversion as detailed above are shown in Table 1. There were no apparent differences in the susceptibilities of the parental and mutant strains to killing by either chemical oxidant. Lethal concentrations were 10^{-2} M for $KMnO_4$ and 10^{-1} M for H_2O_2. Parental and mutant strains were also exposed to various concentrations of PMNL to measure their relative susceptibility to killing. As demonstrated in Fig. 7, there were no apparent differences in the susceptibilities of the parental and mutant strains to killing by PMNL over the range of cell concentrations used.

DISCUSSION

Previous biochemical and immunological work has shown that the antigenic *A. fumigatus* CAT1 catalase is not an intracellular protein, since it has been detected in the cell wall, membrane fractions, and culture broth (27a). Moreover, the glycosylation of CAT1 (26) also implies that this protein is translocated to the endoplasmic reticulum and Golgi compartments. From these biochemical data, it was expected that the corresponding catalase gene would possess a signal peptide responsible for the membrane translocation of the polypeptide. The presence of a signal peptide has been confirmed in this study by two observations. The first observation was that the N-terminal sequence of the mature catalase starts at the Asp_{28} residue of the deduced amino acid sequence of its cDNA, while the second was that analysis of the first 15 amino acid residues reveals features characteristic of a signal peptide (16, 34, 44). These features are the presence of the following: (i) a positively charged residue (Arg_2) next to Met_1, (ii) a core of 10 or more hydrophobic amino acid residues, (iii) amino acid residues that can disrupt α and β secondary structures, and (iv) a palindromic sequence (**Val_{12}-Ala_{13}-Asn_{14}-Ala_{15}-Val_{16}**). The (−3,−1) rule (44) predicts a peptide cleavage site by a typical signal peptidase at Ala_{15}. Our study has shown that the signal peptide is flanked by a propeptide which is cleaved by a KEX2-like endopeptidase at the Arg_{26}-Arg_{27} locus. In yeasts, the role of KEX2 in the maturation and processing of proteins has been widely demonstrated (8, 19, 41). The proteolytic activation by a KEX2-like endopeptidase in *Aspergillus* has been previously reported (3). This cleavage occurs also during the expression of a recombinant *Aspergillus* glucoamylase and a neutral protease in *Saccharomyces cerevisiae* (15, 40). In a similar way, the expression vector used in this study was able to cleave the prosequence after Arg_{27}. In the absence of the propeptide, *Pichia* does not secrete rCAT1, which con-

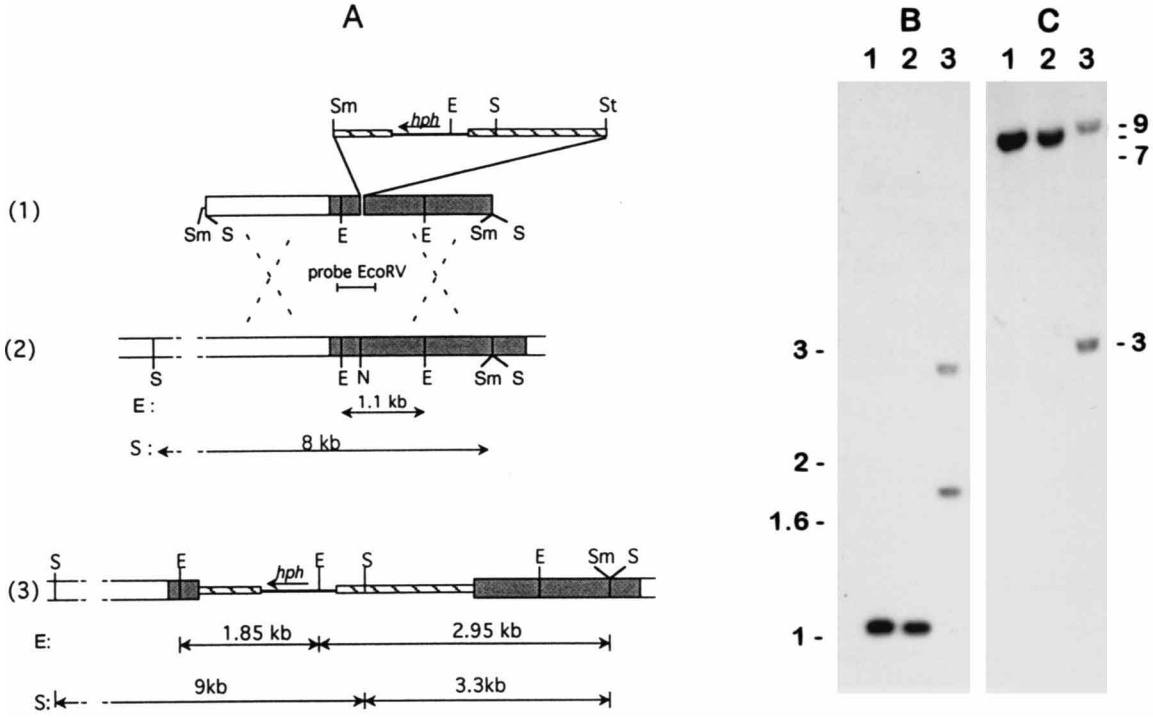

FIG. 4. (A) Disruption of *cat1* in *A. fumigatus*. (Map 1) Map of the *Sma*I fragment of plasmid pΔcat1 containing *cat1* disrupted by the *hph* gene; (Map 2) genomic DNA of the G10 recipient strain (*cat1*$^+$); (Map 3) genomic DNA of *cat1* transformants. *A. fumigatus* sequence (white box) flanking the *cat1* gene (grey box) and *A. nidulans* DNA (hatched bar) are indicated. *Eco*RI and *Sal*I fragments before and after gene disruption are shown below the genomic DNA maps. The diagrammed double-crossover event produces the desired *cat1* mutation. Restriction endonuclease abbreviations: E, *Eco*RI; N, *Nae*I; S, *Sal*I; Sm, *Sma*I; St, *Stu*I. (B and C) Southern blots of genomic DNA from *A. fumigatus* CAT1-producing strains and the non-CAT1-producing transformant. Chromosomal DNA was subjected to electrophoresis after cleavage with *Eco*RI (B) or *Sal*I (C) and transferred to a positively charged nylon Amersham membrane. The filter was hybridized at 65°C with the labelled 0.5-kb *Eco*RV fragment of plasmid pcat38. Lanes 1, CBS144-89; lanes 2, recipient strain G10; lanes 3, Δ*cat1*-28 transformant (*cat1*Δ::*hph*). Sizes are given in kilobases.

firms the requirement of the presence of the propeptide for the correct maturation of the protein through the secretory pathway (data not shown).

Mycelial catalases of *Aspergillus* (CATB [20] and CATR [6]) show significant sequence homologies to CAT1. The only mycelium catalase characterized biochemically (CATR of *A. niger*) is very similar to CAT1: CATR has a signal peptide, is a tetrameric protein with a molecular mass of 385 kDa, and is glycosylated (21). CAT1 also appears to be very closely related to the CATB of *A. nidulans* (20), which is produced in the mycelial phase. Although these proteins are biochemically related, their immunogenicity is different, since anti-CAT1 antibodies do not cross-react with *A. niger* CATR and *A. nidulans* CATB proteins (27a). Interestingly, the developmentally regulated CATA catalase found only in spores of *A. nidulans* (31) and the CATA catalase of *A. fumigatus* (GenBank accession number U87630) present in the mycelium do not have more homology to CAT1 than the *E. coli* HPII catalase or bovine catalase do (39, 45). In spite of differences in degree of homology between all catalases, the three amino acid residues (His_{102}, Ser_{141}, and Asn_{175}) of the catalytic site as well as six of the seven amino acid residues (Val_{101}, Thr_{142}, Phe_{180}, Phe_{188}, Arg_{385}, and Tyr_{389}) involved in the heme binding site (5, 35)

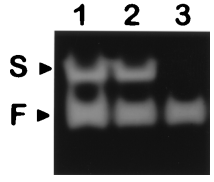

FIG. 5. PAGE of water-soluble fractions stained for catalase. Lane 1, wild-type CBS144-89 (*nia*$^+$ *cat*$^+$); lane 2, recipient G10 (*nia cat*$^+$); lane 3, Δ*cat1*-28 transformant (*nia cat1*Δ::*hph*). The positions of the fast catalase (F) and the slow catalase CAT1 (S) are indicated.

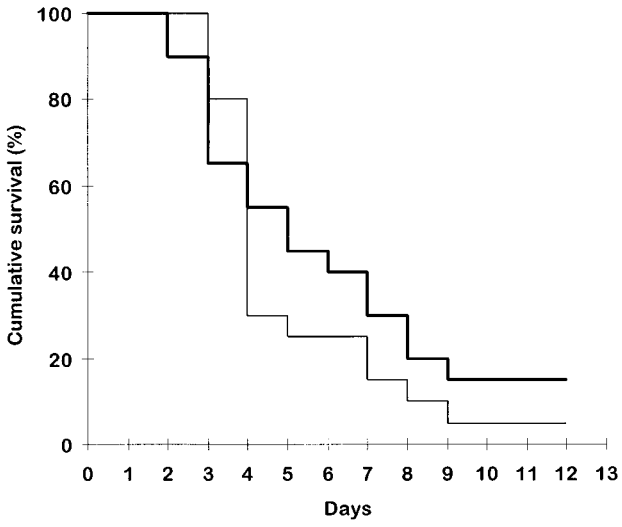

FIG. 6. Survival of mice infected with conidia from the parental G10 strain (thick line) and the Δ*cat1*-28 mutant strain (thin line). Averages of two separate experiments (10 mice per strain per experiment) were used.

TABLE 1. Susceptibilities of parental strain (G10) and catalase-deficient mutant (Δ*cat1*-28) to chemical oxidants

Chemical oxidant and concn (M)	% Control of MTT conversion (mean ± SD)	
	Parental	Mutant
$KMnO_4$ (n = 4)		
10^{-2}	0.0	0.0
10^{-3}	30.0 ± 10.4	36.0 ± 10.6
10^{-4}	79.6 ± 6.8	88.0 ± 8.6
10^{-5}	90.1 ± 6.6	98.1 ± 3.3
10^{-6}	100	100
H_2O_2 (n = 5)		
10^{-1}	0	0
10^{-2}	4.0 ± 2.7	4.5 ± 2.7
10^{-3}	19.5 ± 2.5	25.5 ± 6.6
10^{-4}	63.1 ± 7.1	66.7 ± 3.2
10^{-5}	90.2 ± 3.5	88.8 ± 11.9
10^{-6}	100	100

are conserved among all catalases. In *Aspergillus*, incorporation of heme and formation of the tetrameric structure occurs in the absence of any covalent bond, as demonstrated by the lack of a cysteine residue in the deduced amino acid sequence and the absence of activity of reducing agents.

Reactive oxygen species, and hydrogen peroxide in particular, have been implicated as playing a central role in the pathogenicity of microbial infections. Hence, neutrophils of patients with chronic granulomatous disease do not mount an efficient respiratory burst, and these individuals are susceptible to infections with catalase-positive microorganisms (36). The export of catalase is one of the strategies used by microorganisms to counteract the toxicity of oxidants produced in response to infection, and there is a correlation between the amount of catalase produced and the susceptibility of various microorganisms to phagocyte killing (28). However, we have demonstrated in this study that in *A. fumigatus* the disruption of one of the catalase genes is not sufficient to reduce the invasiveness of the mutant in our experimental model of invasive aspergillosis in mice treated with steroids. This in vivo observation is supported by in vitro experiments which show equal sensitivities to H_2O_2 and PMNL killing by isogenic *cat1*$^+$ and *cat1* mutant strains. Catalases are usually described as poor scavengers of low-level H_2O_2, while peroxidases are considered the main factor in defense against peroxide. Since the fast catalase described by Hearn et al. (13) also has a peroxidase activity, this second enzyme, which has not yet been characterized, may play a major role in the resistance to phagocyte killing. Consequently, only a double mutant with no catalase activity will be of use in assessing the role of this enzyme activity during the infectious process. In addition to the difference in substrate specificity, the two *A. fumigatus* catalases also have different antigenic properties, since anti-CAT1 antibodies did not recognize the fast catalase. Their expression is also differentially regulated, since CAT1 has been found in all media tested whereas the fast catalase was present only after growth in a yeast extract medium and not in Czapek medium (data not shown). In fact, the regulation of the expression of catalase activity, rather than its mere presence, may be a key factor to understanding the roles of the *A. fumigatus* catalases in vivo.

Catalase is another example of a major antigen of *A. fumigatus* which possesses an enzyme activity. This situation seems quite frequent in this fungus, since of the few antigens characterized up to now, several molecules (proteases [2, 29], superoxide dismutase [11], RNase ASPFI [1]) display enzyme activities which may promote fungal growth in vivo, whereas the antigenic activity of such molecules would favor the response of the host against aspergillosis. Such a situation makes a precise understanding of the actual role of enzymatic antigens in the pathogenesis of *Aspergillus* infections difficult. For example, the inhibition of lymphocyte proliferation by the RNase ASPFI in mixed lymphocyte culture was abolished when a mutated protein without any enzymatic activity but with a fully conserved antigenic function was used (4). The construction of mutated proteins by site-directed mutagenesis is a strategy which could be used to quantify the part played by the antigenic or enzymatic activity of such proteins during infection.

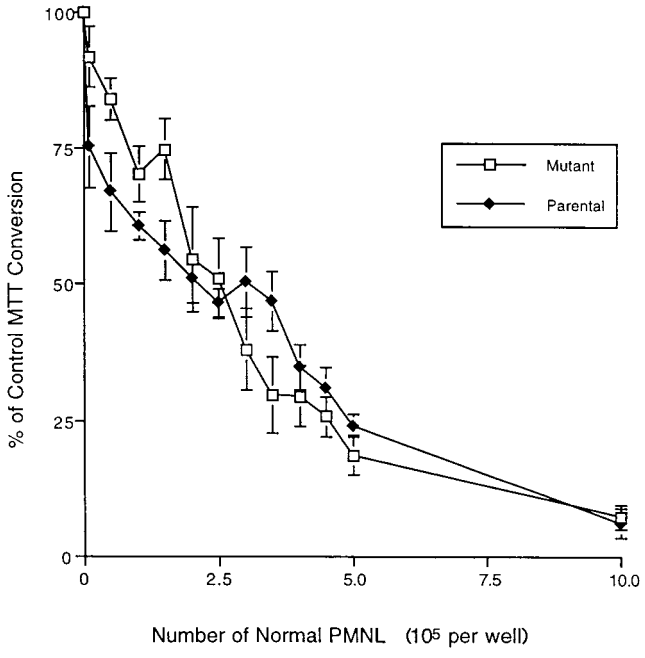

FIG. 7. Survival of *A. fumigatus* parental (G10) and mutant (Δ*cat1*-28) strains (expressed as percent control of MTT conversion) after exposure to human PMNL over a range of cell concentrations. Data are the means from five separate experiments (in triplicate).

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