Two Divergent Catalase Genes Are Differentially Regulated during Aspergillus nidulans Development and Oxidative Stress

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Catalases are ubiquitous hydrogen peroxide-detoxifying enzymes that are central to the cellular antioxidant response. Of two catalase activities detected in the fungus Aspergillus nidulans, the catA gene encodes the spore-specific catalase A (CatA). Here we characterize a second catalase gene, identified after probing a genomic library with catA, and demonstrate that it encodes catalase B. This gene, designated catB, predicts a 721-amino-acid polypeptide (CatB) showing 78% identity to an Aspergillus fumigatus catalase and 61% identity to Aspergillus niger CatR. Notably, similar levels of identity are found when comparing CatB to Escherichia coli catalase HPII (43%), A. nidulans CatA (40%), and the predicted peptide of a presumed catA homolog from A. fumigatus (38%). In contrast, the last two peptides share a 79% identity. The catalase B activity was barely detectable in asexual spores (conidia), disappeared after germination, and started to accumulate 10 h after spor inoculation, throughout growth and conidiation. The catB mRNA was absent from conidia, and its accumulation correlated with catalase activity, suggesting that catB expression is regulated at the transcription level. In contrast, the high CatA activity found in spores was lost gradually during germination and growth. In addition to its developmental regulation, CatB was induced by H2O2, heat shock, paraquat, or uric acid catabolism but not by osmotic stress. This pattern of regulation and the protective role against H2O2 offered by CatA and CatB, at different stages of the A. nidulans life cycle, suggest that catalase gene redundancy performs the function of satisfying catalase demand at the two different stages of metabolic and genetic regulation represented by growing hyphae versus spores. Alternative H2O2 detoxification pathways in A. nidulans were indicated by the fact that catA/catB double mutants were able to grow in substrates whose catabolism generates H2O2.

Toxic reactive oxygen species such as superoxide, hydrogen peroxide, and the hydroxyl radical are produced during normal aerobic metabolism. Virtually all aerobic organisms contain enzymatic and nonenzymatic defense systems against such forms of activated oxygen (18). Catalases (H2O2:H2O2 oxidoreductase, EC 1.11.1.6) are central components of the enzymatic detoxification pathways that prevent the formation of the highly reactive hydroxyl radical by decomposing H2O2. On the other hand, it has recently been shown that H2O2 can also perform regulatory functions in plants (23) and animals (38). The ascomycete fungus Aspergillus nidulans provides an excellent tractable genetic system, with an elaborate asexual reproductive pathway (conidiation), which can be used to approach the study of general antioxidant responses and their possible roles in cell differentiation. Conidiation in A. nidulans can be initiated by exposing nondifferentiated mycelia to air or by nutrient starvation (36, 40). This process involves formation of spore-producing specialized structures called conidiophores, which are composed of several different cell types. Genes that play fundamental roles in this process have been identified and characterized (1, 6, 7, 9, 50; for a review, see reference 40). We have initiated the study of catalase regulation in this fungus during its asexual reproduction with the long-term goal of evaluating the role of reactive oxygen species in gene regulation and sporulation. Our previous work identified two catalase activities in A. nidulans. Catalase B activity is present before induction of conidiation, increases its activity during conidiation, and is barely detectable in isolated spores, whereas catalase A appears at later stages of conidiation and is highly accumulated in spores. Catalase A is encoded by the catA gene, whose expression is induced during conidiation independently from major conidiation regulatory genes and whose mutation renders spores H2O2 sensitive (30). Here we characterize the catalase B encoding gene catB and show that it is developmentally regulated in opposite fashion to catA. Consistent with this, catalase B is able to provide protection against H2O2 to growing hyphae, independently from the presence of catalase A. In addition to its developmental regulation, catalase B is induced by H2O2 or H2O2-generating conditions. We also show that catA/catB double mutants can still grow in some lipids as the sole carbon source or in uric acid as the sole nitrogen source, implying the existence of alternative pathways for H2O2 detoxification.

MATERIALS AND METHODS

Strains, media, and transformation and growth conditions. The A. nidulans strains used were FGSC26 (bca1 veA1 [Fungal Genetics Stock Center]), R8901 ([baba1 a2 ΔargB ΔtrpC801 DargB Δiep1 Δiep2 [this work]], TLK12 ([baba1 a2 ΔbrlA veA1 [this work]], TLK24 ([a2 veA1 [this work]], CLK12 ([bca1 metG1 catA ΔmetG1 ΔmetG1 veA1 [this work]], CLK14 ([bca1 metG1 catA ΔmetG1 ΔmetG1 veA1 [this work]], and TLK22 ([bca1 bca1–Δ2000 p3];ΔcZ ΔargB ΔcatA ΔcatA ΔmetG1 ΔmetG1 veA1 [2]). All strains were grown in supplemented minimal-nitrate medium (21). When fatty acids were used as sole carbon sources, they were added to nitrate medium with agar previously dissolved by heating, emulsified with a blender, and autoclaved. Developmental cultures were induced as described

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before (2). Strain RMS011 was transformed with plasmid pLK2 linearized with PstI by using standard techniques (49).

Catalase induction by different types of stress and H$_2$O$_2$ sensitivity assays. The catA minus strain TOS1 was used to study catalase B induction by different types of stress. Liquid cultures inoculated at a density of $9 \times 10^5$ spores/ml were grown for 8 h at 37°C and 300 rpm before the different treatments. Paraquat and H$_2$O$_2$ were added as concentrated solutions to final concentrations of 5 and 0.5 mM, respectively, the second being added after the first 8 h of growth and then 30, 60, and 90 min later. For growth in uric acid, mycelia were filtered through a nitrocellulose membrane and then transferred to media containing uric acid (0.8 mg/ml) as a sole nitrogen source. Heat shock was performed by shifting an 8-h culture to 42 or 50°C. Cultures were incubated for 2 h and in some cases for 3, 4, and 5 h under indicated conditions. Mycelia were harvested and frozen with liquid nitrogen. Ground lyophilized mycelia were used to prepare protein extracts and to determine catalase activity in native gels (30) or by using a Clark oxygen electrode as reported previously (reference 11 and see below).

A reproducible assay was developed for testing of H$_2$O$_2$ sensitivity in solid media. Such testing in liquid cultures was precluded by the early aggregation of spores during germination and by the formation of mycelial pellets of heterogeneous size at later stages of growth. Preliminary experiments following O$_2$ evolution (bubbling) by individual colonies of a catA mutant, overlaid with 20 mM H$_2$O$_2$ solutions, determined that 30 h was the appropriate time to carry out the H$_2$O$_2$ sensitivity assay. At that time, catalase B activity was readily detectable, colonies were not yet hydrophobic and could be easily covered by the H$_2$O$_2$ solutions, and conidiation had not started, thus preventing catalase A interference when using catA strains.

Conidia from strains TJA22, CLK12, CLK14, and CLK15 were inoculated onto petri dishes to a final density of 200 to 400 colonies per dish. After incubation for 7 to 10 days at 37°C, the plates were flooded with 200 ml of sterile water and incubation was continued for an additional 15 h. The mycelium was then removed from the dishes by shaking vigorously, and 200 ml of medium was added to each dish. After 1 h, 200 ml of 10 mM K$_2$HPO$_4$ was added to each dish, and the cultures were incubated for an additional 15 h. The mycelium was then removed, weighed, and homogenized in liquid nitrogen. The homogenates were then used to determine catalase activity.

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FIG. 1. catB nucleotide and predicted polypeptide sequences. A potential TATA box and consensus splice signals are underlined.
bation at 37°C for 30 h, colonies were overlaid with a solution of H2O2, at concentrations ranging from 0 to 20 mM, for 10 min. After such treatment, the solution was carefully decanted and colonies were incubated further for 26 h at 37°C. Colonies able to resume growth and to sporulate after H2O2 treatment were counted as survivors.

**catB cloning, cosmid identity, plasmid construction, sequencing, and sequence comparisons.**

A PstI catA fragment was removed from pCAN5 (30) and further digested with NheI to obtain an internal catA 800-bp fragment, which was used to probe an A. nidulans chromosome-specific cosmid library (5) under low stringency conditions. Cosmids W7C08, W15C07, and W19D03, previously assigned to chromosome VIII, were identified. A catA-hybridizing EcoRI-HindIII 7.5-kb fragment from cosmid W7C08 was cloned into pBluescript SK+ (Stratagene) to generate pDW1. Finally, a 2.6-kb PstI-SalI fragment from pDW1 was subcloned into pBluescript SK+ to generate pDW2. DNA sequencing was carried out by the dideoxy chain termination method of Sanger et al. (32). Both strands were sequenced by using pDW2 as a template and with specific oligonucleotide primers. Sequence similarity, identity, and catalase alignments were done with the Genetics Computer Group (GCG) programs FASTA, GAP, PILEUP, and PRETTY (version 8.0) by using default parameters (13).

**A. nidulans physical maps were consulted at Internet:** http://fungus.genetics.uga.edu:5080/physical_maps.html.

**Nucleotide sequence accession number.** The GenBank accession number for the sequence shown in Fig. 1 is U80672.

**FIG. 2.** Comparison of A. nidulans CatB with highly similar catalases. CatB was aligned with A. niger CatR (16), E. coli PII (46), A. nidulans CatA (30), and the A. fumigatus catalases deduced from sequences AFU87630 (Wysong et al., GenBank accession number U87630) and AFCATGENE (Takasuka et al., GenBank accession number Y07763); the nearly identical catalase sequence AFU87850 has been reported by Wysong et al., GenBank accession number U87850), using the GCG programs PILEUP and PRETTY (13). Capital letters represent identical or conserved amino acids in at least three of the sequences. Consensus sequences show identical amino acids in all catalases.
RESULTS

Cloning, nucleotide sequence, and comparison of a second *A. nidulans* catalase gene. We have previously shown that the *A. nidulans* *catA* gene encodes a spore-associated catalase. Disruption of *catA* did not affect the activity of a second catalase, designated catalase B, whose activity was detected at 18 h of growth and increased during asexual sporulation (30). To isolate the catalase B corresponding gene, we used a *catA* radiolabeled probe to hybridize against an *A. nidulans* chromosome-specific cosmid library (5). Cosmids W7C08, W19D03, and W15C01 were identified. These cosmids were previously assigned to chromosome VIII, cosmid W15C01 being part of a contig that includes the genes *facB* and *palB* (47) (see Materials and Methods). Restriction analysis of the three cosmids indicated that they represented the same genomic region. A 2.6-kb *PstI-SalI* *catB*-hybridizing fragment from cosmid W7C08 was subcloned and fully sequenced.

Sequence analysis revealed an ATG-initiated open reading frame interrupted by four putative introns, predicting a 721-amino-acid polypeptide ($M_r = 79,143$) with significant similarity to catalases from many different organisms, including *A. nidulans* CatA. We therefore provisionally named this gene.
catB, and the corresponding predicted polypeptide was named CatB (Fig. 1). Alignment of CatB to several catalases (Fig. 2) indicated that CatB shows ~78% overall identity to the catalase deduced from the two nearly identical A. fumigatus sequences AFCATGENE (Takasuka et al., GenBank accession number Y07763) and AFU87850 (Wysong et al., GenBank accession number U87850) and a 61% identity to the Aspergillus niger CatR (16). Whereas in most reported catalases, the highly conserved regions lay in the central portion of the proteins, CatB, the predicted peptide from AFCATGENE/AFU87850, and CatR also share amino acid sequences at their amino- and carboxyl-terminal ends (Fig. 2), such as the sequences GxAXaCpYxxGE and FxFxDRFxxD, respectively (x representing nonidentical amino acids). Comparisons of CatB to CatA (30) and Escherichia coli HPII (46) catalases indicates that CatB is as similar to the other A. nidulans catalase (40% identity) as it is to the prokaryotic catalase HPII (43% identity). In contrast, CatA shares a 79% identity with a second A. fumigatus catalase (Fig. 2) deduced from sequence AFU87630 (Wysong et al., GenBank accession number U87630). Moreover, the position conservation of the only catA intron (30) and the first putative AFU87630 intron strongly suggests that these genes are homologs.

Assuming that the catB intron positions are confirmed experimentally, the precise conservation of intron I position between catB, AFCATGENE, and catR also favors the idea that these three genes are homologs. The six catalases compared here constitute the largest reported known sequences and the highly conserved regions lay in the central portion of the proteins. CatB, the predicted peptide from AFCATGENE/AFU87850, and CatR also share amino acid sequences at their amino- and carboxyl-terminal ends (Fig. 2), such as the sequences GxAXaCpYxxGE and FxFxDRFxxD, respectively (x representing nonidentical amino acids). Comparisons of CatB to CatA (30) and Escherichia coli HPII (46) catalases indicates that CatB is as similar to the other A. nidulans catalase (40% identity) as it is to the prokaryotic catalase HPII (43% identity). In contrast, CatA shares a 79% identity with a second A. fumigatus catalase (Fig. 2) deduced from sequence AFU87630 (Wysong et al., GenBank accession number U87630). Moreover, the position conservation of the only catA intron (30) and the first putative AFU87630 intron strongly suggests that these genes are homologs.

Assuming that the catB intron positions are confirmed experimentally, the precise conservation of intron I position between catB, AFCATGENE, and catR also favors the idea that these three genes are homologs. The six catalases compared here constitute the largest reported known sequences and the ones sharing the highest similarity.

The catB gene encodes catalase B. To find out if the cloned catB gene (Fig. 1) encoded the previously detected catalase B (30), we designed plasmid pLK2 by replacing the most conserved region of the putative catB open reading frame (amino acids 170 to 376 deleted) by the argB gene, and we used this plasmid to transform an A. nidulans argB-deleted strain to arginine independence. Figure 3A shows the event expected upon integration of pLK2 at catB. Figure 3B shows a Southern blot analysis of transformant TLK12, containing a single copy of plasmid pLK2 integrated at catB. The band pattern was fully consistent with the predicted integration event. Protein extracts from 18-h-grown mycelia from this and other transformants were analyzed for catalase activity in native gels. Figure 4A shows that the band of activity corresponding to catalase B was missing in transformant TLK12, demonstrating that catB encodes catalase B. When catB mutant strain TLK12 was crossed to a catA mutant strain, recombination of both mutations was observed (Fig. 4B), in agreement with the different chromosomal locations of the genes.

Catalase B activity is regulated during the A. nidulans life cycle. We reported that catalase B activity was present in cultures approaching the stationary phase of growth (18 h), increased gradually during sporulation, and was barely detectable in asexual spores (30). We investigated if there was correspondence between enzyme activity and the catB mRNA levels during sporulation. A Northern blot analysis using RNA from developmental cultures and isolated spores, and using catB as a probe, is shown in Fig. 5A. argB probing of the same membrane was used as a reference for RNA loading. Normalized catB/argB pixel ratios of digitized images gave values of 1, 1.3, 1.8, 0.7, and 0.08 for 0, 6, 12, and 25 h of conidiation and for purified conidia, respectively. This indicates that, except for the 25-h point, where a slight decrease is detected, the catB mRNA levels correspond with the catalase activity found previously at the same times of development; i.e., catB mRNA is accumulated in 18-h growth samples and during conidiation and is virtually absent from purified spores. This pattern of accumulation implied that catB mRNA had to accumulate sometime between spore germination and 18 h of growth. When catalase activity was monitored during spore germination and growth, the catalase A activity which had accumulated in spores was gradually lost and barely detectable by 10 h. At that time, catalase B activity started to increase, after being virtually lost by 4 h of germination (Fig. 5B). Under these conditions, catalase B activity was also correlated with the catB mRNA levels (Fig. 5C).

These results indicate that catalase B is developmentally regulated during the A. nidulans asexual cycle, in opposite fashion to catalase A during the growth phase. They also indicate that catalase B activity is regulated at the transcript accumulation level.

Nondevelopmental regulation of catalase B and function. To explore if catalase B was also regulated in response to different types of stress, we determined catalase B activity in a catA mutant subjected to different types of stress. Since a slight induction of catalase B was detected between 6 and 10 h of growth (Fig. 5B), an intermediate time of 8 h was selected to evaluate the effects of the different stimuli, so that even low levels of induction would be easily detectable. Heat shock (42 and 50°C), osmotic stress (1 M sorbitol, 100 mM LiCl), H2O2...
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FIG. 5. Developmental regulation of catB. (A) Total RNA extracted from 18-h-grown mycelia (0), from mycelia induced to conidiate for the indicated times (hours), or from isolated conidia (C) was fractionated in formaldehydeagarose gels, transferred to a nylon membrane, and hybridized with the catB specific probe as a loading control. The different times of development correspond to the following morphologies: 0 h, growth; 6 h, mature conidiophores and immature conidia; 12 h, conidiophore stalks; 18 h, latently germinated spores; and conidia. (B) Purified conidia (C) were used to inoculate a liquid culture and harvested at the indicated times (hours). (C) Part of the samples obtained in panel B were used for total RNA extraction and Northern blot analysis as indicated before. The bottom part of the figure shows rRNA bands in the ethidium bromide-stained gel used to prepare the blot.

DISCUSSION

Here we have characterized the catB gene and shown that it encodes a second catalase from A. nidulans. Based on sequence similarity and intron position conservation, it is likely that catB, A. fumigatus AFCATGENE/AFU87850 (accession numbers Y07763 and U87850, respectively), and A. niger catR (16) are homologs. By the same criteria, the A. fumigatus catB, A. nidulans catA, and A. fumigatus AFU87630 (accession number U87630) can be considered homologs. Five of the catalases shown in Fig. 2 are the only catalases from filamentous fungi for which putative primary sequences are reported. Together with Neurospora crassa (8), Penicillium vitale (44), and, notably, E. coli HPII (46) catalases, these constitute a group of enzymes that are much larger than reported catalases from any other sources. The relatively high similarity of the corresponding fungal genes to E. coli katE would be consistent with the proposed migration of
catalase gene sequences from eukaryotes to prokaryotes during evolution (28, 30).

The catalase B activity was shown to be regulated in a developmental fashion along the *A. nidulans* life cycle: it was barely detectable in spores, disappeared after 4 h during germination, and started to accumulate at about 10 h of mycelial growth. During conidiation, it gradually increased but was practically excluded from conidia. Activity was correlated with the *catB* mRNA abundance, indicating that *catB* is regulated, at least in part, at the transcript accumulation level. This pattern of regulation contrasted with the one observed for catalase A: its activity and corresponding mRNA were highly accumulated in spores, but while the mRNA disappeared after 2 h of germination (30a), the enzyme activity remained detectable up to 10 h of growth; it then disappeared until the later stages of conidiation, when conidia were formed. Except at about 10 h of growth, when both enzyme activities were barely detectable, the patterns of activity observed for both catalases during development virtually show that the activity of one enzyme is present while the other is low or absent. This pattern of regulation and the fact that catalase B provided protection against H$_2$O$_2$ to growing colonies independently from catalase A, whereas catalase A supplied protection to isolated conidia against H$_2$O$_2$ (30), suggests that catalase redundancy serves the purpose of protecting the fungus against oxidative stress at different stages of its cell cycle. A potentially similar situation has been reported for the two *A. nidulans* alpha-tubulin genes *tubA* and *tubB*, which, being highly divergent and differentially required at discrete stages of the life cycle, are nevertheless functionally exchangeable, supporting the interpretation that

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**FIG. 6.** Nondevelopmental regulation of catalase B. Mycelia from *catA* mutant strain TOS1 grown for 8 h were subject to different types of stress for 2 h (see Materials and Methods). After the different treatments, protein extracts were prepared for catalase activity determination as before. Lane 1, mycelia shifted to medium containing uric acid (0.8 mg/ml) as the sole nitrogen source; lane 2, paraquat (5 mM) added to the medium; lane 3, culture shifted to 50°C; lane 4, H$_2$O$_2$ (0.5 mM) added to the culture; lanes 5 and 6, strain TOS1 grown continuously for 10 and 8 h, respectively, as controls; lanes 7 and 8, 18-h-grown mycelia from strain FGSC26 and spores from strain RMS011 as catalase B and A references, respectively. Direct catalase activity, determined by O$_2$ evolution, is indicated at the bottom of the figure as relative enzyme activity. A specific activity of 1.0 (lane 5, 10 h of growth) corresponded to 482.6 μmol of H$_2$O$_2$ decomposed/min/mg of protein.

**FIG. 7.** Growing colonies from *catB* but not *catA* mutants are sensitive to hydrogen peroxide. Spores from strains TJA22, CLK12, CLK14, and CLK15 were inoculated onto petri dishes and grown for 30 h. After this time, colonies were flooded with aqueous solutions of H$_2$O$_2$ at the indicated concentrations for 10 min, the solutions were removed, and plates were incubated for 26 more hours. The colonies that were able to resume growth were counted as survivors. Shown are the percentages of treated colonies (200 to 400) that were able to survive the different H$_2$O$_2$ concentrations. A representative experiment is shown.
two functionally redundant polypeptides exist to facilitate altered patterns of expression during development (22).

Although two or more differentially regulated catalase genes within the same organism have been reported (8, 15, 17, 24, 51), their functions are not well understood. In plants, most catalases are organelle associated (17, 51), whereas in yeast, a peroxisomal catalase A, repressed by glucose and induced by fatty acids, and a cytoplasmic catalase T have been characterized (10, 35). Like catalase T (27, 34), CatB was induced by oxidative and heat shock stress and during later stages of growth, but in contrast to catalase T, it was not induced by osmotic stress (34, 48). In Bacillus subtilis, a vegetative catalase 1 (encoded by the katA gene [4, 24]) and a splice-associated catalase 2 (encoded by the katB/katE gene [15]) have been characterized, showing that katA expression is achieved and that katE is induced by heat shock, salt, and glucose starvation but not by H₂O₂ (15).

Whereas the cellular localization of CatA and CatB remains to be determined, the hydrophobic region formed by the first 40 amino acids of CatA and CatB homologs, it will be interesting to determine a possible association with the plasma membrane (30). Furthermore, the amino acids encoded by the catA and catB genes are conserved in all fungi, suggesting that these genes might be involved in common metabolic pathways (31).

H₂O₂ but not by heat shock or salt stress and that exogenous H₂O₂, alternative pathways for the in vivo detoxification of H₂O₂, may be used instead of nitrate as the sole nitrogen source. Growth was evaluated after 48 h at 37°C. Relative growth: ++++, very good; +++, good; +++, low; +, scarce; +, very scarce; −, none.

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