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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 spore 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 H_2O_2, heat shock, paraquat, or uric acid catabolism but not by osmotic stress. This pattern of regulation and the protective role against H_2O_2 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 H_2O_2 detoxification pathways in *A. nidulans* were indicated by the fact that *catA*/*catB* double mutants were able to grow in substrates whose catabolism generates H_2O_2.

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 (H_2O_2:H_2O_2 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 H_2O_2. On the other hand, it has recently been shown that H_2O_2 can also perform regulatory functions in plants (23) and animals (38). Previous work has implicated reactive oxygen species in induction and control of asexual sporulation in *Neurospora crassa* (20, 41–43) and in general microbial differentiation (19). 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 H_2O_2 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 H_2O_2 to growing hyphae, independently from the presence of catalase A. In addition to its developmental regulation, catalase B is induced by H_2O_2 or H_2O_2-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 H_2O_2 detoxification.

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MATERIALS AND METHODS

Strains, media, and transformation and growth conditions. The *A. nidulans* strains used were FGSC26 (*biA1 veA1* [Fungal Genetics Stock Center]), RMS011 (*pabaA1 yA2 ΔargB::trpCΔB veA1 trpC801* [37]), TOS1 (*pabaA1 yA2 ΔargB::trpCΔB catA::argB veA1 trpC801* [30]), TLK12 (*pabaA1 ya2 ΔcatB veA1* [this work]), TLK24 (*pabaA1 yA2 veA1* [this work]), CLK12 (*biA1 metG1 catA::argB veA1* [this work]), CLK14 (*biA1 metG1 catA::argB ΔcatB veA1* [this work]), CLK15 (*biA1 metG1 ΔcatB veA1* [this work]), and TJA22 [*biA1 brlA(−2900 p/l)::lacZ* (*argB*$^+$/*argB::CAT*) *metG1 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

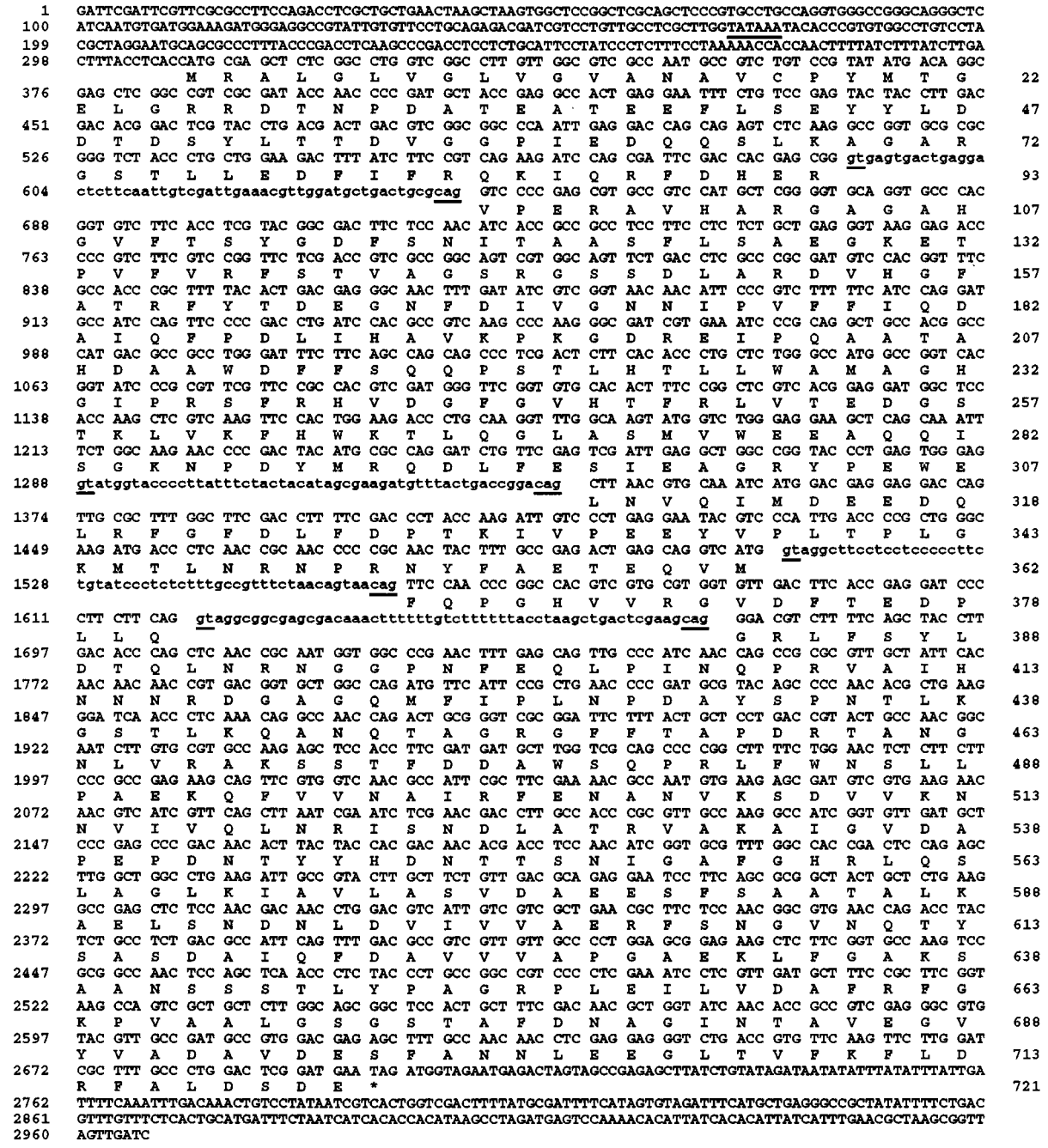

FIG. 1. *catB* nucleotide and predicted polypeptide sequences. A potential TATA box and consensus splice signals are underlined.

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

Catalase induction by different types of stress and H_2O_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 9×10^5 spores/ml were grown for 8 h at 37°C and 300 rpm before the different treatments. Paraquat and H_2O_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_2O_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_2O_2 solutions, determined that 30 h was the appropriate time to carry out the H_2O_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_2O_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 incu-

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CatA        .......... .......... .......... .........m atsiTagLqk AQqavqdtaT knkkivdish dtvnvhTDqe qrTDfGvaIt Dpdhwlrvtn  100
AFU87630    .......... .......... .......... .........m atkiaGgLhr AQEvlqntss kskklvdLer dtadahTqqp LTTDhGvrVs ntdqwlrvtn
CatB        .......... .......... ....mralgl vglvGVAnAv CPYMTGELg. rrD..tnpDa teatEefLse .yYldDTDsY LTTDvGgPIE DQqSL....k
AFCATGENE   .......... .......... ....mrltFi PsliGVAnAv CPYMTGELn. rrDeisdgDa aaatEefLsq .yYlnDnDaF MTsDvGgPIE DQnSL....s
CatR        .......... .......... ...mrhfwLl PavaGIAgAq CPYLsGEMsf tQEqdnagDT ievtEqpidn tlYvnDTgsY MTTDfGtPIs DQtSL....k
HPII        msqhneknph qhqsplhdss eakpgmdsLa PedgshrpAa ePtppGaqpt ApgslkapDT rneklnsLed vrkgsEnyaL tTnggvriaD DQnSL....r
Consensus   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

CatA        ethsGPsLLE DhIaRerIhR FDHERIPERV VHARGtGAyG nFTlkesied lTyAgvLtDt srnTPVFVRF STVQGSRGSA DTvRDVrGFA vkFYTDEGNW  200
AFU87630    drrtGPsLLE DqIaReKIhR FDHERIPERV VHARGtGAfG nFklkesied lTyAgvLtDt srnTPVFVRF STVQGSRGSA DTvRDVrGFA vkFYTDEGNW
CatB        AGaRGsTLLE DFIFRqKIQR FDHERVPERa VHARGAGAhG vFTSYgdFSn ITAAsFLSae gKeTPVFVRF STVaGSRGSs DlaRDVhGFA TrFYTDEGNF
AFCATGENE   AGeRGPTLLE DFIFRqKIQR FDHERVPERa VHARGAGAhG vFTSYgdFSn ITAAsFLake gKqTPVFVRF STVaGSRGSs DlaRDVhGFA TrFYTDEGNF
CatR        AGpRGPTLLE DFIFRqKlQR FDHERVPERV VHARGAGAyG tFkSYadWSn VTAAdFLSan dKeTPmFcRF STVvGfRGSv DTaRDVhGhA crFYTDEGNY
HPII        AGsRGPTLLE DFILReKIth FDHERIPERI VHARGsaAhG yFqpYksLSd ITkAdFLSDp nKiTPVFVRF STVQGgaGSA DTvRDIrGFA TkFYTEEGiF
Consensus   ----G--LLE D-I-R----- FDHER-PER- VHARG--A-G -F-------- -T-A--L--- ---TP-F-RF STV-G--GS- D--RD--G-A --FYT-EG--

CatA        DIVGNNIPVF FIQDAIKFPD FVHAVKPEPH NEVPQAqTAH nnfWDFvylh PEatHmFMWA MSdRaIPRSY RmMqGPGVnT FsLVNkEGKr hFVKFHWiPh  300
AFU87630    DIVGNNIPVF FIQDAVKFPD FVHAVKPEPH NEVPQAqTAH nnfWDFvylh PEatHmFMWA MSdRaIPRSY RmMqGPGVnT FaLVNkEGKr hFVKFHWiPh
CatB        DIVGNNIPVF FIQDAIqFPD LIHAVKPkgd rEIPQAaTAH DaaWDFfSqQ PstLHtLLWA MaghGIPRSF RHvDGPGVHT FRLVteDGst KLVKFHWKtL
AFCATGENE   DIVGNNIPVF FIQDAIlFPD LIHAVKPrgd NEIPQAaTAH DsaWDFfSqQ PstMHtLLWA MSghGIPRSF RHvDGPGVHT FRFVtdDGas KLVKFHWKsL
CatR        DIVGiNfapF FIQDAIqFPD LVHAIKPmPn NEIPQAaTAH tsaWDFfSqQ staLHsaLWl MSgnGIPRSF RHMnGYGVHs FRFVaanGts KvVrtpWKsq
HPII        DlVGNNtPIF FIQDAhKFPD FVHAVKPEPH waIPQgqsAH DtfWDYvSlQ PEtLHnvMWA MSdRGIPRSY RtMEGPGIHT FRLINaEGKa tFVrFHWKPL
Consensus   D-VG-N---F FIQDA--FPD --HA-KP--- ---PQ---AH ---WD----- ----H---W- M----IPRS- R---G-G--- F------G-- --V---W---

CatA        lGVhSLVWDE AlKLaGqdPD FHRkDLmEAI DnkaYPkWDF aIQaipEEDQ dkFeFDiFDa TKVwPEEqVP LrvvGeLELN RNiDeFFpET EQVAFctsHI  400
AFU87630    lGVhSLVWDE AlKLgGqdPD FHRkDLmEAI DnkaYPkWDF aIQVipEEkQ ddFeFDiLDa TKIwPEnLVP LrviGeLELN RNvDeFFpqT EQVAFctsHI
CatB        QGlASMVWEE AQqisGKnPD YmRQDLFEsI EaGrYPeWEL nVQImdEEDQ LRFgFDLFDP TKIVPEEYVP LTplGKMtLN RNPrNYFAET EQVmFQPGHV
AFCATGENE   QGkASMVWEE AQqtsGKnPD FmRQDLhDAI EaGrYPeWEL gVQImdEEDQ LRFgFDLLDP TKIVPEEFVP iTklGKMqLN RNPrNYFAET EQVmFQPGHI
CatR        QGVASLVWDE AQaaaGKnsD YHRQDLYnAm pnGhYPkYEL qaQImdEaDm LRFgFDLLDP TKlVPEEvVP yTplGmMELN aNPtNYFAEv EQagFQPGHV
HPII        aGkASLVWDE AQKLtGrdPD FHRrELWEAI EaGdFPeYEL gfQlipEEDe FkFdFDLLDP TKlIPEELVP vqrvGKMvLN RNPDNFFAEn EQaAFhPGHI
Consensus   -G--S-VW-E A----G---D --R--L---- -----P---- --Q---E--- --F-FD--D- TK--PE--VP ----G---LN -N----F--- EQ--F---H-

CatA        VPGIDFsDDP LLQGRnFSYq DTQiSRL.Gv NWEeiPINRP vCPflNhNRD GAkrhrItkg tvnYwPNrFe anpPasdk.. ....gFkshp apItGrkrRd  500
AFU87630    VPGIDFTDDP LLQGRnFSYF DTQiSRL.Gi NWEelPINRP vCPVlNhNRD GqmrhrItqg tvnYwPNrFe avpPtgtkgs gvgGgFTtyp qrVeGiknRA
CatB        VrGVDFTEDP LLQGRLFSYL DTQlnRnGGP NFEqlPINqP RvaIHNNNRD GAGqMfIPlN PdaYsPNtLk gstlkQANQT aGrGfFTAPd RtanGnlVRA
AFCATGENE   VrGVDFTEDP LLQGRLFSYL DTQlnRhGGP NFEqlPINqP RvPVHNNNRD GAGqMfIPlN PhaYsPktsv ngsPkQANQT vGdGfFTAPg RttsGklVRA
CatR        VPGIDFTDDP LLQGRLFSYL DTQltRhGGP NFEqiPVNRP RkPVHNNNRD GfGqqqIPtN nwaYtPNsMs ngyPmQANQT qGhGfFTAPy RyasGhlVRq
HPII        VPGlDFTnDP LLQGRLFSYt DTQiSRLGGP NFheiPINRP tCPyHNfqRD GmhrMgIdtN PanYePNsin dnwPretppg pkrGgFesyq erVeGnkVRe
Consensus   V-G-DF--DP LLQGR-FSY- DTQ--R--G- N----P-N-P -----N--RD G-----I--- ---Y-P---- ---------- -----F----- ----G---R-

CatA        ltPkFkEyhn QaqLFYNSLs evEKvHVkkA fSFEldhcdd PIVyErlagQ rLaeIDlpLA QaVAemVGap iPtkaLrdnh gkTsvrLsqf d....FtpKa  600
AFU87630    lndkFrEhhn QaqLFYNSMs ehEKlHmkkA fSFEldhcdd PtVyErlagh rLaeIDlELA QkVAemVGap iPakaLkqnh grraphLsqT e....FipKn
CatB        kSstFdDaWS QPRLFWNSLl PAEKQfVVnA irFEnanVkS dVVknnVIvQ .LnrIsnDLA tRVAkaIGVD aPE......P dnTYYHdNtT snIGaFGhrL
AFCATGENE   vSssFeDvWS QPRLFYNSLv PAEKQfVIdA irFEnanVkS PVVknnVIiQ .LnrIDnDLA rRVAraIGVa ePE......P dPTFYHnNKT adVGtFGtKL
CatR        tSPtFnDhWS QPaMFWNSLi PAEqQmVVnA ivFEnskVnS PhVrknVVnQ .LnmVnnnLA vRVArglGlD ePs......P nPTYYtsNKT snVGtFGkpL
HPII        rSPsFgEyYS hPRLFWlSqt PfEqrHIVdg fSFElskVvr PyIrErVVdQ .LahIDltLA QaVAknlGIE ltDdqLnitP pPdvngLkKd pslslYaipd
Consensus   ----F----- ----F--S-- --E------- --FE------ ---------- -L------LA --VA---G-- ---------- ---------- ----------

CatA        pgIisrrIAI iigDgyDkia fngmkaAilA aaSaplrhwh qtsaIYAqGe dknsskgvip DhmYdGmrst mFDAtfIpgG shIEtLqkng qiryWiaeTf  700
AFU87630    ptIasrrIAI iigDgyDpva StglktAiKA asalpfIigt krsaIYA.te dktsskgiip DhhYdGqrst mFDAtfIpgG phVatLrqng qikyWiseTf
CatB        qslaGlkIAV L..aSVDaeE SfsaatALKA eLSndnlDvi vVaErFsnG. .........V nqTYsaSDaI qFDAVVVapG ..aEkLFgak s.......aA
AFCATGENE   kkldGlkVgV L..gSVqhpg SvegastLrd rLkddgVDvv lVaErLADG. .........V DqTYstSDaI qFDAVVVaaG ..aEsLFAas sftggsansA
CatR        lsIeGlqVgf L..aSnshpE SikqgqAMaA qFSaagVDln iVtEaYADG. .........V ntTYalSDaI dFDAlIIadG ..VqsLFAsp alanqmnsTA
HPII        gdVkGrvVAI LlnDeVrsaD llailkALKA kgvhakllys rmgEVtADd. ....gtvlpI aaTFaGapsl tvDAVIVpcG ..niadiAdn gdanY.....
Consensus   ---------- ---------- ---------- ---------- ---------- ---------- ---------- --DA-----G ---------- ----------

CatA        ghlkaLgamG eaaQlvkEvL gnvmgVqiAG adS.....Ae pvEwygvVta rgpesaESLs EgFkvlkdAg dFtskFfyqi sqhrnwqrel dglastvaf*  800
AFU87630    ghlkaLgatG eavdlvkEtL sgtlhVqvAs SqS.....pe pvEwygvVta ggkqkpESFk EsvqilkgAt dFvgkFfyqi sqhrnyqrel dglastiaf*
CatB        nssStLYPaG RPLeILVDaF RFGKPVAAlG SGStAFdNAG IntavEGVYV ada.vdESFA nnLeEgLtvF kFlDRFalDs de*..... ..........
AFCATGENE   sgaSsLYPtG RPLQILIDgF RFGKtVgAlG SGtaALrNAG IatsrDGVYV aqs.vtDdFA nDLkEgLrtF kFlDRFpvDh .......... ..........
CatR        ..tStLYPpa RPFQILVDsF RYGKPVAAvG SGSvALkNAG IdssrsGVYt gssettEkiA kEvlEgLytF rFvDRFalDe .......... ..........
HPII        .......... ....yLmEaY khlKPIAlAG darkfkatik IaDqgEegiV eadsadgSFm DELltlMaAh rvwsRipkid kipa...... ..........
Consensus   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
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FIG. 2. Comparison of *A. nidulans* CatB with highly similar catalases. CatB was aligned with *A. niger* CatR (16), *E. coli* HPII (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 sequence shows identical amino acids in all catalases.

bation at 37°C for 30 h, colonies were overlaid with a solution of H_2O_2, 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 H_2O_2 treatment were counted as survivors.

***catB* cloning, cosmid identity, plasmid construction, sequencing, and sequence comparisons.** A *Pst*I *catA* fragment was removed from pCAN5 (30) and further digested with *Nhe*I 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 *Eco*RI-*Hin*dIII 7.5-kb fragment from cosmid W7C08 was cloned into pBluescript SK+ (Stratagene) to generate pDW1. Finally, a 2.6-kb *Pst*I-*Sal*I 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.

The plasmid pLK2, used to disrupt *catB*, was constructed in two steps. First, the *Bam*HI site present at the polylinker of pDW2 was removed by digestion with *Sma*I-*Not*I, filling in, and blunt-end ligation (31) to generate pLK1. Second, the *catB Bam*HI-*Eco*RV fragment present in pLK1 was substituted with a *Bam*HI-*Nru*I fragment from pDC1 (3), containing the *A. nidulans argB* gene as a selective marker, to generate pLK2.

Hybridization analyses and nucleic acid isolation. Genomic DNA was isolated as described by Timberlake (39). Total RNA was obtained with the RNaid Plus kit (Bio-101, La Jolla, Calif.). RNA was fractionated in formaldehyde-agarose gels, transferred to Hybond-N nylon membranes, and hybridized as described by the manufacturer (Amersham). The *Pst*I-*Sal*I fragment from pDW2 was used as the *catB*-specific probe, and the *Bam*HI-*Nru*I fragment from pDC1 was used as the *argB*-specific probe. Both were labeled with ^{32}P by using the BRL random priming labeling system.

Catalase activity determination. Native polyacrylamide gels were prepared with an 8% resolution gel and a 5.1% stacking gel, and 25 to 40 μg of total protein was loaded. The gels were run at 100 V for 3.5 h in a Miniprotean II Cell (Bio-Rad), and catalase activity was detected as described before (8, 30). Direct catalase activity was determined by measuring initial rates of O_2 production with a micro-Clark electrode. The reaction was started by injecting protein extracts into a sealed chamber filled with 2 ml of 50 mM phosphate buffer, pH 7.2, and 10 mM H_2O_2 (11).

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

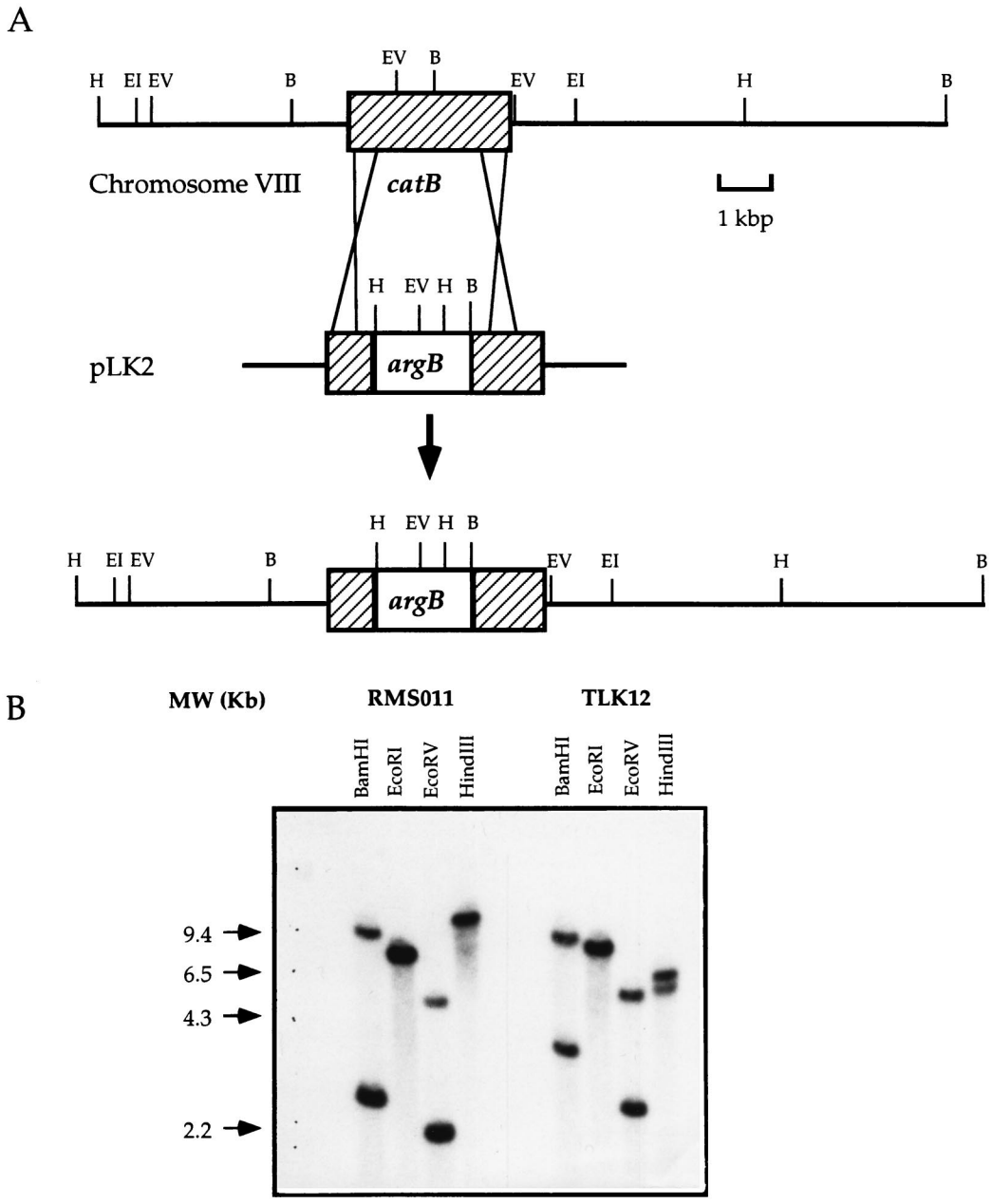

FIG. 3. Disruption of the *catB* gene. (A) Plasmid pLK2 was constructed by removing a central region from *catB* (amino acids 170-376 from putative polypeptide) and replacing it with the *argB* gene as a selectable marker. Linear pLK2 was used to transform the *argB*-deleted strain RMS011 to arginine independence. Restriction sites: B, *Bam*HI; EI, *Eco*RI; EV, *Eco*RV; H, *Hin*dIII. (B) Total DNA from strains RMS011 and TLK12 was digested with the indicated restriction enzymes and used for Southern blot analysis using the *Pst*I-*Sal*I *catB* fragment from pDW2 as a probe. Probing with the *argB* gene (not shown) gave results also consistent with the integration event shown in panel A.

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 *Pst*I-*Sal*I *catA*-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

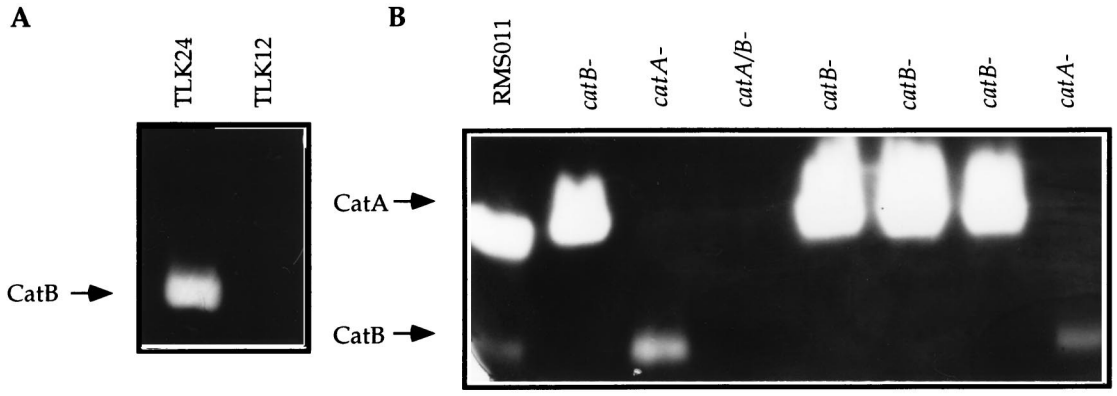

FIG. 4. *catB* disruption eliminates catalase B activity. (A) Protein extracts from 18-h-grown mycelia were prepared from the wild-type strain TLK24 and from *catB*-disrupted strain TLK12. Proteins were fractionated in native polyacrylamide gels and used to determine catalase activity. (B) Conidial protein extracts were prepared from randomly selected progeny from a sexual cross between TLK12 and the *catA* mutant strain CLK12 and processed for catalase activity determination as before. Conidia from strain RMSO11 were included as a reference.

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 GxAxAxCPYxxGE 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), H_2O_2

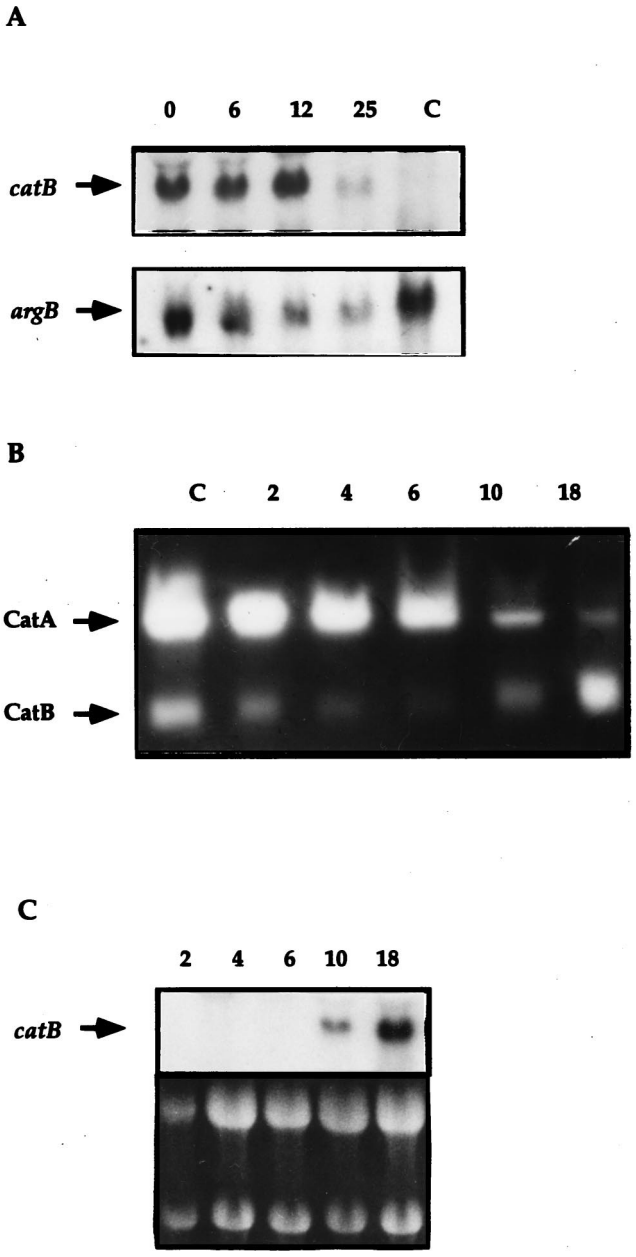

FIG. 5. Developmental regulation of *catB*. (A) Total RNA extracted from 18-h-grown mycelia (0), from mycelia induced to conidiate for the indicated times (hours), or from isolated conidia (C) was fractionated in formaldehyde-agarose gels, transferred to a nylon membrane, and hybridized with the *Pst*I-*Sal*I *catB* fragment from pDW2. The same membrane was hybridized with an *argB*-specific probe as a loading control. The different times of development correspond to the following morphologies: 0 h of development (18 h of growth), growing hyphae; 6 h, conidiophore stalks; 12 h, conidiophores and immature conidia; 25 h, mature conidiophores and conidia. (B) Purified conidia (C) were used to inoculate a liquid culture and harvested at the indicated times (hours). Resulting samples were processed for catalase activity determination as indicated before. The different times of development correspond to the following morphologies: conidia (C), latent ungerminated spores; 2 h, swollen conidia; 4 h, swollen conidia with short germ tubes; 6 h and 10 h, actively growing hyphae; 18 h, prestationary phase of growth. (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.

(0.5 mM), the superoxide-generating compound paraquat (5 mM), and growth in uric acid as the sole nitrogen source were tested. The last treatment was included because uric acid catabolism produces H_2O_2 (33).

Figure 6 shows that uric acid, paraquat, and H_2O_2 were able to induce catalase B 2.1-, 13.2-, and 8.4-fold, respectively, compared with nontreated cultures. In contrast, catalase A was not induced by these treatments (30a). Heat shocks (2 h) of 50 or 42°C (not shown) and osmotic shock treatments failed to induce catalase B. However, when these treatments (42°C, 1 M sorbitol or 100 mM LiCl) were monitored for 3, 4, and 5 h, only the heat shock was able to induce catalase B 3.2-, 2.3-, and 1.8-fold, respectively.

An assay was developed (see Materials and Methods) to test if catalase B was able to provide protection against H_2O_2. We grew otherwise isogenic *catA*, *catB*, and *catA*/*catB* double mutants in petri dishes for 30 h and then covered the resulting colonies with H_2O_2 solutions at different concentrations for 10 min. After the H_2O_2 solutions were removed, incubation was continued and the surviving colonies were counted after 26 h.

Results in Fig. 7 show that virtually all *catB* mutant colonies were killed by 15 to 20 mM H_2O_2, while the *catA* mutant and wild-type strains were practically unaffected by the same H_2O_2 concentrations. In contrast, spores from *catA* mutants were dramatically sensitive to H_2O_2 (30). Other than this sensitivity to H_2O_2, no obvious sporulation phenotype was detected for the *catB* or *catA*/*catB* mutants. These results show that both catalase B and catalase A provide protection against H_2O_2 treatment at different phases of the *A. nidulans* asexual cycle.

Alternative H_2O_2 detoxification pathways in *A. nidulans*. To explore the role of CatA and CatB in conditions where H_2O_2 is physiologically generated, we decided to test the growth response of *catA*, *catB*, and *catA*/*B* mutants in substrates that generate H_2O_2. Uric acid utilization as the sole nitrogen source (33) induced CatB (Fig. 6). Fatty acid catabolism is another condition that generates H_2O_2 through peroxisomal β-oxidation. In fact, the catalase inhibitor 3-aminotriazole has been used to isolate mutants defective in peroxisome assembly in yeast (45) and *A. nidulans* (12). Table 1 shows that all mutants grew as the wild type in every condition tested, except in lauric acid and starch plus lauric acid, where the *catA*/*catB* double mutant was unable to grow, just as wild-type yeast and *A. nidulans* strains are unable to grow in lauric acid only when the catalase inhibitor 3-aminotriazole is present (12, 45). In addition to catalases, most aerobic organisms contain peroxidases, and although no *A. nidulans* peroxidases have been reported, results in Table 1 indicate that unidentified peroxidases can perform part of the catalase function in vivo.

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. 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

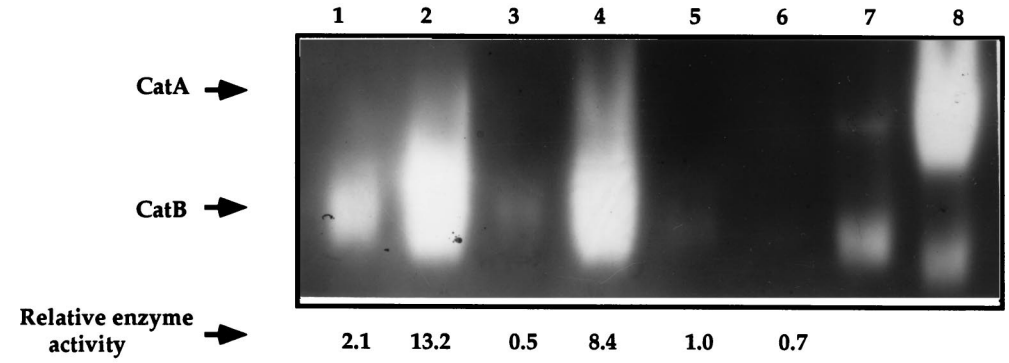

FIG. 6. Nondevelopmental regulation of catalase B. Mycelia from *catA* mutant strain TOS1 grown for 8 h were subject to different types of stress for 2 h (see Materials and Methods). After the different treatments, protein extracts were prepared for catalase activity determination as before. Lane 1, 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_2O_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_2O_2 decomposed/min/mg of protein.

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_2O_2 to growing colonies independently from catalase A, whereas catalase A supplied protection to isolated conidia against H_2O_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

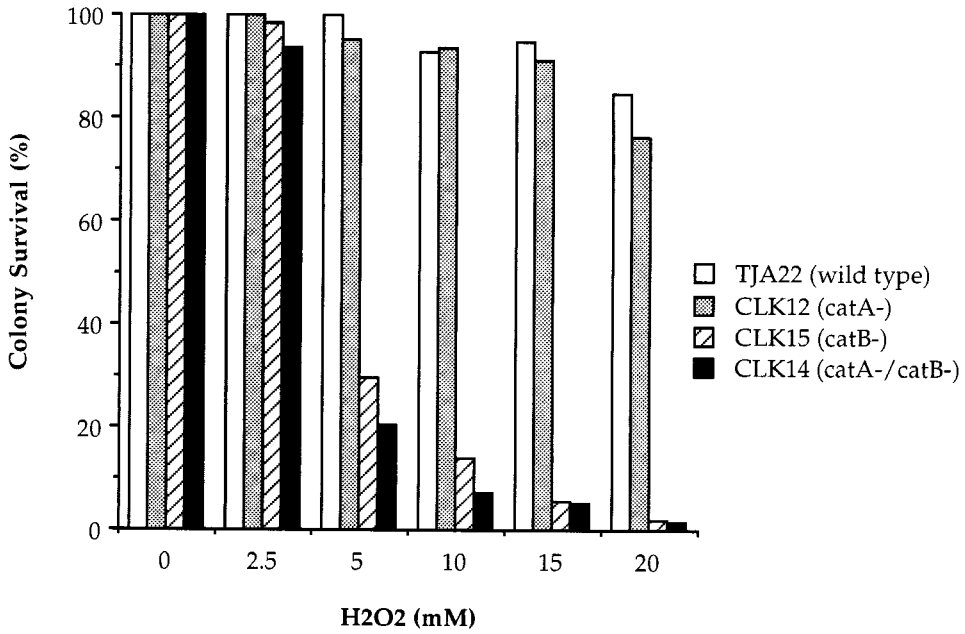

FIG. 7. Growing colonies from *catB* but not *catA* mutants are sensitive to hydrogen peroxide. Spores from strains TJA22, CLK12, CLK14, and CLK15 were inoculated onto petri dishes and grown for 30 h. After this time, colonies were flooded with aqueous solutions of H_2O_2 at the indicated concentrations 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_2O_2 concentrations. A representative experiment is shown.

TABLE 1. Growth response of wild-type strain and catalase mutants in H_2O_2-generating substrates

Substrate	Growth response[a]			
	TJA22 (wild type)	CLK12 (*catA* mutant)	CLK15 (*catB* mutant)	CLK14 (*catA*/*catB* mutant)
Glucose	+++++	+++++	+++++	+++++
Oleic acid	++++	++++	++++	++++
Starch	++++	++++	++++	++++
Lauric acid	+	+	+	−
Lauric acid plus starch	++	++	++	−
Tributyrin	+++	+++	+++	+++
Uric acid	++++	++++	++++	++++

[a] Spores from the indicated strains were used to point inoculate solid glucose-nitrate minimal medium or media in which glucose was replaced by oleic acid (6 mM), lauric acid (0.03%), starch (0.5%), starch plus lauric acid (0.5% and 0.03%, respectively) or tributyrin (1%) as the sole carbon sources. Uric acid (0.8 mg/ml) was 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.

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 spore-associated catalase 2 (encoded by the *katB*/*katE* gene [15]) have been characterized, showing that *katA* expression is induced by H_2O_2 but not by heat shock or salt stress and that *katE* is induced by heat shock, salt, and glucose starvation but not by H_2O_2 (15).

Whereas the cellular localization of CatA and CatB remains to be determined, the hydrophobic region formed by the first 21 or 22 amino acids from CatB, and the deduced catalases from *A. fumigatus* AFCATGENE/AFU87850 and *A. niger* CatR, show a high degree of similarity between them (Fig. 2) and signal peptides present in secretory proteins (16). An *A. fumigatus* catalase with a 90-kDa molecular mass has been identified as an antigen present in 90% of serum samples from patients with aspergilloma (25). If this catalase corresponds to the AFCATGENE/AFU87850 gene, which, in turn, would be a *catB* homolog, it will be interesting to determine a possible developmental regulation for the *A. fumigatus* catalase and its possible roles in dealing with oxidative products derived from neutrophil attack in vitro (14) or during human pathogenesis.

While catalases A and B are necessary for protection against exogenous H_2O_2, alternative pathways for the in vivo detoxification of this compound are indicated by the fact that *catA*/*catB* double mutants were able to grow as the wild type in media that contained uric acid as the sole nitrogen source or fatty acids as the sole carbon source. The presence of peroxidases could explain these results, as well as the lack of any obvious sporulation phenotype in *catA*/*catB* double mutants. Several peroxidases have been reported in fungi (26, 29), but little is known about their role in general antioxidant response or development. We have not detected catalase activity in *catA*/*catB* mutants subjected to many different physiological conditions. However, the existence of more catalases in *A. nidulans* cannot be formally excluded.

The developmental regulation of CatB and its specific induction by H_2O_2 or by conditions that favored intracellular H_2O_2 formation, such as the paraquat and uric acid treatments, suggest that intracellular H_2O_2 concentration could rise during stationary and conidiation phases of the *A. nidulans* life cycle. Our current research is aimed at understanding how the developmental regulation of *catA* and *catB* is achieved and whether the same mechanisms operate during oxidative stress.

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