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Research Article

Expression and Activity of Catalases Is Differentially Affected by GpaA (Gα) and FlbA (Regulator of G Protein Signaling) in *Aspergillus fumigatus*

Kwang-Soo Shin[1,*] and Jae-Hyuk Yu[2]

[1]*Department of Microbiology and Biotechnology, Daejeon University, Daejeon 300-716, Korea*
[2]*Departments of Bacteriology and Genetics, University of Wisconsin, Madison, WI 53706, USA*

Abstract Vegetative growth signaling of the opportunistic human pathogenic fungus *Aspergillus fumigatus* is mediated by GpaA (Gα). FlbA is a regulator of G protein signaling, which attenuates GpaA-mediated growth signaling in this fungus. The *flbA* deletion (Δ*flbA*) and the constitutively active GpaA (GpaA^{Q204L}) mutants exhibit enhanced proliferation, precocious autolysis, and reduced asexual sporulation. In this study, we demonstrate that both mutants also show enhanced tolerance against H_2O_2 and their radial growth was approximately 1.6 fold higher than that of wild type (WT) in medium with 10 mM H_2O_2. We performed quantitative PCR (qRT-PCR) for examination of mRNA levels of three catalase encoding genes (*catA*, *cat1*, and *cat2*) in WT and the two mutants. According to the results, while levels of spore-specific *catA* mRNA were comparable among the three strains, *cat1* and *cat2* mRNA levels were significantly higher in the two mutants than in WT. In particular, the Δ*flbA* mutant showed significantly enhanced and prolonged expression of *cat1* and precocious expression of *cat2*. In accordance with this result, activity of the Cat1 protein in the Δ*flbA* mutant was higher than that of *gpaA*^{Q204L} and WT strains. For activity of the Cat2 protein, both mutants began to show enhanced activity at 48 and 72 hr of growth compared to WT. These results lead to the conclusion that GpaA activates expression and activity of *cat1* and *cat2*, whereas FlbA plays an antagonistic role in control of catalases, leading to balanced responses to neutralizing the toxicity of reactive oxygen species.

Keywords *Aspergillus fumigatus*, Catalases, FlbA, GpaA, H_2O_2, Regulator of G protein signaling

During biological oxidation processes the toxic reactive oxygen species (ROS) are generated by incomplete reduction of oxygen with reduced electron carriers in all aerobic pathogenic fungi [1, 2]. ROS derived from host defense mechanisms can destroy major cellular components and effectively kill the spore and hyphae of pathogens. To protect these lethal effects, the fungi boosted various antioxidant enzyme activities of superoxide dismutases (SODs), which convert superoxide to hydrogen peroxide (H_2O_2), and catalases, which detoxify H_2O_2 to water and molecular oxygen. FlbA, regulator of G protein signaling protein, regulates vegetative growth signaling negatively, mediated by a heterotrimeric G protein and D*flbA* mutant shows autolysis in *Aspergillus nidulans* [3-7]. Our genetic studies in the opportunistic human pathogen *Aspergillus fumigatus* have demonstrated that the FadA homolog GpaA mediates vegetative proliferation, which is attenuated by FlbA, which in turn enables asexual development (conidiation) to occur [7, 8]. Our recent comparative proteomic studies have suggested that the absence of *flbA* may lead to increased catalase activity [9]. In this study, we further investigate the roles of FlbA and GpaA in differently governing the expression and activity of catalases in *A. fumigatus*.

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***Corresponding author**
E-mail: shinks@dju.kr

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MATERIALS AND METHODS

Fungal strain and culture conditions. *A. fumigatus* AF293 (WT) [10], Δ*flbA*4 (*pyrG1*, Δ*flbA*4::*pyrG*$^+$), and

tJH4.04 (*pyrG1*, *gpaA*^{Q204L}, *pyrG*$^{+}$) strains were used [8]. Glucose minimal medium with 0.1% (w/v) yeast extract (MMY) with supplements was used for general culture of fungal strains [11, 12]. For liquid submerged culture, approximately 5×10^5 conidia/mL were inoculated into liquid MMY and incubated at 37°C, 250 rpm. The susceptibility of WT and mutant strains to varying concentrations of H_2O_2 was tested by performance of plate assays. Drop dilution assay was performed in a series of 10-fold dilutions derived from a starting inoculum of 1.0×10^6 conidia per spot. For the hyphal sensitivity test, mycelial plugs cut from the growing edge of five-day old colonies from MMY agar medium were transferred to plates containing 10 mM H_2O_2 and incubated at 37°C for four days.

Nucleic acid isolation and manipulation. Isolation of total RNA was performed as described previously [13]. Five micrograms (5 µg) of total RNA was reverse-transcribed to cDNA using EcoDryTM Premix (Clontech, Mountain View, CA, USA). Quantitative PCR (qRT-PCR) assays were performed according to the manufacturer's instructions (Qiagen, Valencia, CA, USA) using 96-well optical plates and a Rotor-Gene Q (Qiagen). Each run was assayed in triplicate in a total volume of 20 µL containing the DNA template, 2× qPCR SYBR green Mix (Doctor Protein, Seoul, Korea), and 100 mM of each primer. The primers used for qRT-PCR are shown in Table 1. PCR conditions were: 95°C, 15 min followed by 95°C, 30 sec and 55°C, 30 sec for 40 cycles. Amplification of one single specific target DNA was checked by melting curve analysis (+0.5°C ramping for 10 sec, from 55°C to 95°C). The expression ratios were normalized to EF1α expression, and calculated according to the $^{\Delta\Delta}$Ct method [14]. To verify the absence of genomic DNA contamination, negative controls, in which reverse transcriptase was omitted, were used for each gene set. Three independent biological replicates were performed.

Protein extraction and analysis. For protein extraction, 250 mL flasks containing 100 mL liquid MMY were inoculated with WT and mutant strain conidia and grown at 37°C for two days. After washing with phosphate buffered saline (pH 7.4), mycelia were suspended in lysis buffer (50 mM Tris-HCl pH 7.2, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA) and homogenized using a Mini Bead-Beater (BioSpec Products, Bartlesville, OK, USA). The homogenate was centrifuged in a microcentrifuge for 5 min at 15,000 rpm at 4°C, and the supernatant was used for further analyses. For detection of catalase activity on gels, the mycelial extracts were subjected to non-denaturing polyacrylamide gel electrophoresis, and the ferricyanide-negative stain was used to locate bands containing catalase activity [15]. Peroxidase activity on gels was detected according to the method of Wayne and Diaz [15] with a modification of using *o*-dianisidine instead of diaminobezindine as the substrate.

Table 1. Primers used for qRT-PCR

Primer	Sequence (5′→3′)	Purpose
Oligo275	AGCCACGGCTCTTCTACAAT	*cat1* forward
Oligo276	GTTCTTCACCACGGGAGATT	*cat1* reverse
Oligo277	ATTCCAGAGGAGAAGCAGGA	*catA* forward
Oligo278	GAGCTCTCCAATCACACGAA	*catA* reverse
Oligo281	GGTGCTCAACACCAACTACG	*cat2* forward
Oligo282	ATGTCCAGCAGGTGAACAAA	*cat2* reverse
Oligo346	CCATGTGTGTCGAGTCCTTC	EF-1α forward
Oligo347	GAACGTACAGCAACAGTCTGG	EF-1α reverse

qRT-PCR, quantitative PCR.

RESULTS AND DISCUSSION

Catalase is regarded as a putative virulence factor with the capability of scavenging H_2O_2 produced by host phagocytes. Phylogenetic analyses place the catalases into four distinct clades: clade P (peroxisomal catalases), clade C (cytoplasmic catalases), clade A (conidia-specific catalases), and clade B (secreted catalases) [16]. There are three types of catalases

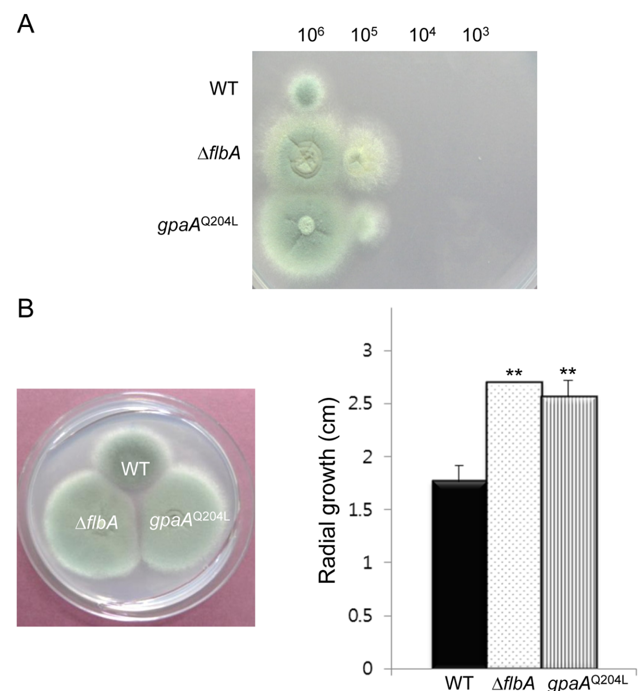

Fig. 1. Differential response of WT, Δ*flbA*, and *gpaA*^{Q204L} strains to H_2O_2. (A) From a series of 10-fold dilutions derived from a starting suspension of 1.0×10^6 conidia of the indicated strains were spotted onto MMY agar plates supplemented with 10 mM H_2O_2. (B) The mycelial plugs cut from the growing edge of five-day colonies from MMY agar medium were transferred to plates containing 10 mM H_2O_2 and incubated at 37°C for four days; radial growth was then measured. Data are expressed as the mean ± standard deviation derived from three independent experiments. Student's *t*-test: $^{*}p < 0.005$; $^{**}p < 0.001$.

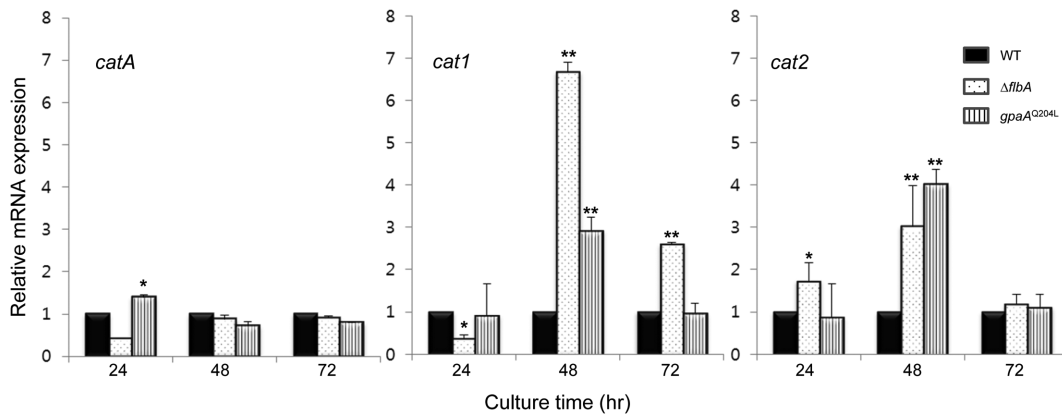

Fig. 2. mRNA levels of the three catalase genes in WT, Δ*flbA*, and *gpaA*^{Q204L} strains. mRNA levels of *catA*, *cat1*, and *cat2* in growing cells of WT, Δ*flbA*, and *gpaA*^{Q204L} strains were determined by quantitative PCR (qRT-PCR). Cultures were incubated in liquid MMY and mRNA levels were normalized using the EF1α gene, according to the $^{\Delta\Delta}$Ct method. Data are expressed as the mean ± standard deviation from three independent experiments. Student's *t*-test: $*p < 0.005$, $**p < 0.001$.

in *A. fumigatus*: CatA (Afu6g03890), produced in conidia; and Cat 1 (Afu3g02270) and Cat2 (Afu8g01670), produced in hyphae [17, 18]. Both the *catA* deletion and *cat1* disrupted mutants showed increased sensitivity to H_2O_2 [17, 18]. The susceptibilities of the Δ*flbA* and *gpaA*^{Q204L} mutants against H_2O_2 were determined in comparison with WT. Drop dilution assay showed that the two mutants were more resistant than WT, where the Δ*flbA* mutant exhibited higher tolerance than the *gpaA*^{Q204L} mutant (Fig. 1A). The radial growth of WT was approximately 62% of that of Δ*flbA* strain, suggesting approximately 1.6 fold enhanced growth by the absence of *flbA* (Fig. 1B). These results led us to hypothesize that expression and activities of catalases are elevated in the Δ*flbA* and *gpaA*^{Q204L} mutants. We performed qRT-PCR for examination of mRNA levels of the three catalases. According to one report, CatAp is the only catalase present in the resting conidia required for conidial resistance to heat, denaturing agents, and metal ions [18]. Accordingly, *catA* mRNA accumulation at low levels was observed in hyphae of all tested strains (Fig. 2). On the contrary, levels of *cat1* mRNA in Δ*flbA* and *gpaA*^{Q204L} strains appeared to be low at 24 hr, highly increased at 48 hr, and then slightly decreased at 72 hr. The highest level of accumulation of *cat1* mRNA was observed in Δ*flbA* strain at 48 hr of growth (Fig. 2). However, the expression pattern of *cat2* mRNA was somewhat different from that of *cat1*. Accumulation of the *cat2* mRNA began to occur at 24 hr and remained at high levels until 48 hr in both mutants and then became indistinguishable among the three strains, including WT (Fig. 2). These results suggest that the absence of FlbA and the activation of GpaA result in enhanced expression of the *cat1* and *cat2* genes. We then performed the zymogram assay for determination of catalase activity; according to our findings, two catalases (Cat1p and Cat2p), while at different levels, were present in all tested strains (Fig. 3A). The Cat1p activity of Δ*flbA* strain was higher than those of *gpaA*^{Q204L} mutant and WT, and the highest activity was

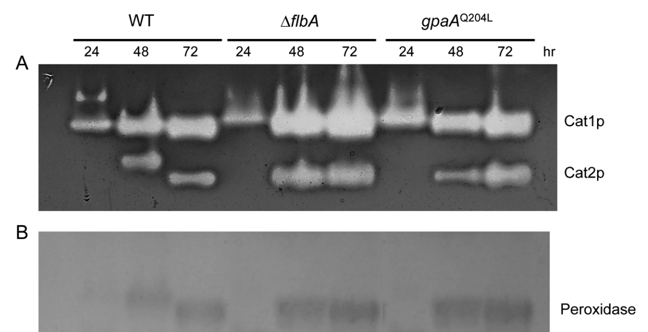

Fig. 3. Native polyacrylamide gel electrophoresis of the mycelial protein extracts of WT, Δ*flbA*, and *gpaA*^{Q204L} strains stained for catalase (A) and peroxidase (B). Equal amount of protein (50 μg) was loaded. Note that the patterns of peroxidase activities were similar to those of Cat2p activities.

observed in Δ*flbA* strain at 72 hr (Fig. 3A). Distinct from CatAp and Cat1p, the Cat2p protein is a bifunctional catalase-peroxidase, conferring resistance to heat, heavy metals, and sodium dodecyl sulfate [18]. In all tested strains, Cat2p activities were not detected at 24 hr, but began to be detectible at 48 hr, and reached the highest level at 72 hr. The patterns of peroxidase activities were similar to those of catalase activity, suggesting that Cat2p has both peroxidase and catalase activities, as reported (Fig. 3B). In our recent study, we found that Δ*flbA* strain exhibits enhanced SOD activity and elevated resistance to menadione and paraquat, suggesting that FlbA-mediated signaling control results in down-regulation of cellular responses associated with detoxification of ROS in *A. fumigatus* [9]. Incorporating the findings of the current study, we propose that GpaA-mediated vegetative growth signaling is involved in increased protection of the fungus from various ROS by increasing the levels of SOD, catalases, and GliT, which are negatively controlled by FlbA. We also propose that the active cellular growth of *A. fumigatus* requires a large amount of energy

generated by respiration leading to increased production of various ROS; thus, the fungus has evolved to couple the enhanced production of defensive cellular enzymes with proliferation.

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