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Functional analysis of the superoxide dismutase family in *Aspergillus fumigatus*

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Summary

Reactive oxidant species produced by phagocytes have been reported as being involved in the killing of *Aspergillus fumigatus*. Fungal superoxide dismutases (SODs) that detoxify superoxide anions could be putative virulence factors for this opportunistic pathogen. Four genes encoding putative Sods have been identified in the *A. fumigatus* genome: a cytoplasmic Cu/ZnSOD (AfSod1p), a mitochondrial MnSOD (AfSod2p), a cytoplasmic MnSOD (AfSod3p) and AfSod4 displaying a MnSOD C-terminal domain. During growth, *AfSOD1* and *AfSOD2* were highly expressed in conidia whereas *AfSOD3* was only strongly expressed in mycelium. *AfSOD4* was weakly expressed compared with other *SODs*. The deletion of *AfSOD4* was lethal. Δ*sod1* and Δ*sod2* mutants showed a growth inhibition at high temperature and a hypersensitivity to menadione whereas the *sod3* mutant had only a slight growth delay at high temperature. Multiple mutations had only an additive effect on the phenotype. The triple *sod1/sod2/sod3* mutant was characterized by a delay in conidial germination, a reduced conidial survival during storage overtime, the highest sensitivity to menadione and an increased sensitivity to killing by alveolar macrophage of immunocompetent mice. In spite of these phenotypes, no significant virulence difference was observed between the triple mutant and parental strain in experimental murine aspergillosis models in immunocompromised animals.

Introduction

Reactive oxygen species (ROS) produced by phagocytes have been repeatedly mentioned as playing an essential role in microbial killing (Rada *et al.*, 2008; Huang *et al.*, 2009). Molecules and proteins involved in the resistance to ROS become putative fungal virulence factors. Among ROS, the superoxide ion is the first intermediate of the ROS cascade produced by the NADPH oxidase complex of phagocytes. Accordingly, superoxide dismutases (SODs) that detoxify the extremely reactive superoxide anions have been considered as putative fungal virulence factors.

Function of *SODs* has been investigated in two human yeast pathogens *Candida albicans* and *Cryptococcus neoformans*. In *C. albicans*, four Cu/ZnSODs (*SOD1*, *SOD4*, *SOD5* and *SOD6*) and two MnSODs (*SOD2*, *SOD3*) were identified (Lamarre *et al.*, 2001; Hwang *et al.*, 2003; Martchenko *et al.*, 2004). Mitochondrial Mn-containing SODs were shown to be involved in protection against various stresses in *C. albicans* (Hwang *et al.*, 2003). Among the Cu/ZnSOD, *SOD1* and *SOD5* have been shown to be involved in virulence of *C. albicans* (Hwang *et al.*, 2002; Martchenko *et al.*, 2004; Fradin *et al.*, 2005). Further, *SOD1* is required for the protection of *C. albicans* against oxidative stress produced by macrophages (Hwang *et al.*, 2002). Recently, the GPI-anchored *SOD5* and to lesser extent *SOD4* have also been shown to be involved in the protection of *C. albicans* against oxidative stress produced by macrophage *in vitro* (Frohner *et al.*, 2009). Among the MnSOD, *C. albicans SOD2* is not required for the virulence and the infective impact of *SOD3*, an unusual cytosolic MnSOD lacking the mitochondrial leader peptide has not been explored yet in *C. albicans* (Lamarre *et al.*, 2001). In *C. neoformans*, only two SODs were identified, one cytosolic Cu/ZnSOD (*SOD1*) and one mitochondrial MnSOD (*SOD2*). The analysis of the Δ*sod1* and Δ*sod2* mutants shows that *SOD1* and *SOD2* have distinct roles in *C. neoformans* but that both of them are required for full virulence (Cox *et al.*, 2003; Narasipura *et al.*, 2005).

In *Aspergillus fumigatus*, two SODs were studied previously because of their immunogenic capacity: the Cu/ZnSOD AfSod1p (AFUA_5G09240) is specifically recognized by sera from patients with confirmed *Aspergillus* infections (Hamilton *et al.*, 1995; 1996; Holdom *et al.*, 2000; Centeno-lima *et al.*, 2002; Sarfati *et al.*, 2006) and the MnSOD AfSod3p (Asp f6, AFUA_1G14550) is specifically recognized by IgE from allergic patients (Crameri *et al.*, 1996; Schwienbacher *et al.*, 2005). However, none of these genes were deleted.

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To better understand the function of the four putative SODs (*AfSOD1-4*) identified in the *A. fumigatus* genome, single and multiple deletion mutants were constructed. This study showed that the sod activity is exclusively intracellular in *A. fumigatus* and mostly due to Sod1p and Sod2p although the four *SOD*s are expressed during growth. *sod* mutants were more susceptible to ROS and high temperature than the wild-type strain. Although the conidia of the triple mutant was affected in key biological parameters for the growth of the fungus *in vivo* such as a delay in conidial germination and an increased sensitivity to killing by alveolar macrophage of immunocompetent mice, no significant virulence difference was observed between the triple mutant and parental strain in experimental murine aspergillosis models in immunocompromised animals. Data presented here suggest that the main function of the *A. fumigatus* Sodps is the detoxification of the superoxide anions generated intracellularly by the fungal metabolism.

Results

Analysis of AfSOD1*,* AfSOD2*,* AfSOD3 *and* AfSOD4 *sequences*

BLAST analysis of the *A. fumigatus* genome database (http://www.tigr.org/tdb/e2k1/afu1/) with *C. albicans* and *Saccharomyces cerevisiae* Sod protein sequences identified four paralogues in this species: AFUA_5G09240=*AfSOD1*, AFUA_4G11580=*AfSOD2*, AFUA_1G14550=*AfSOD3*, AFUA_6G07210=*AfSOD4*. The relationship of the *A. fumigatus* homologues with their respective yeast orthologues is shown in Figs S1 and S2. The cDNA corresponding to *AfSOD1* gene was previously cloned and sequenced by Holdom *et al.* (2000). This cDNA encoded a Cu/ZnSOD (AAD42060) with 76% identity to *C. albicans* Sod1p and 68% identity to *S. cerevisiaie* Sod1p (Holdom *et al*., 2000). The comparison between AFUA_5G09240 and AAD42060 (http://www.ncbi.nlm.nih.gov/) revealed an incorrect annotation in the TIGR published genome. The gene encoding AAD42060 was composed of five exons encoding 4-, 60-, 51-, 33- and 6-amino-acid segments separated by four introns of 451, 53, 65 and 66 bp respectively (Holdom *et al*., 2000) whereas the TIGR gene is composed of five exons encoding 8-, 60-, 51-, 33- and 6-amino-acid segments separated by four introns of 99, 53, 65 and 66 bp respectively. The differences of start codon and the intron 1 size changed the first 9-amino-acid residues of the TIGR predicted Cu/ZnSOD from MQGVTPPVI to MVKAV. The three other SOD genes (AFUA_4G11580, AFUA_1G14550 and AFUA_6G07210), encoded putative MnSODs and were named *AfSOD2 AfSOD3* and *AfSOD4* respectively, and their sequence alignment with other fungal MnSODs is shown in Fig. S2. In the TIGR genome database, *AfSOD2* was a 690-bp-long gene composed of four exons encoding amino acid segments separated by three introns of 174, 53 and 69 bp respectively. The AfSod2p predicted sequence showed significant identities with other fungal MnSODs (e.g. 51% identity with the *C. albicans* Sod2p and 52% identity with the *S. cerevisiae* Sod2p, Fig. S2). Moreover, AfSod2p contains the consensus sequences typical of the MnSOD family (Carlioz *et al*., 1988; Parker and Blake, 1988; Borgstahl *et al*., 1992; Fig. S2). As reported for other MnSOD, a putative mitochondrial targeting signal (the first 35 aa) was present at the N-terminus of AfSod2p (TargetP 1.1 Server; http://www.cbs.dtu.dk/services/TargetP/; Emanuelsson *et al*., 2000). The cDNA coding for AfSod3p has been cloned and sequenced (Crameri *et al*., 1996). This cDNA was identical to the one predicted by TIGR. The *AfSOD3* 633-bp-long open reading frame (ORF) is interrupted by two introns of 48 and 67 bp and encodes a predicted protein of 210 aa. Significant identities were observed with sequences of other fungal MnSod present in the GenBank database (e.g. 47% identity with the *C. albicans* Sod3p, 40% identity with the *C. albicans* Sod2p and 42% identity with the *S. cerevisiae* Sod2p, Fig. S2). Interestingly, the protein alignment revealed that Sod3p was shorter at its N-terminus than other fungal Mn mitochondrial SODs. We failed to detect a putative mitochondrial targeting signal using TargetP 1.1 software mentioned above, suggesting that this protein was cytoplasmic. Both AfSod3p and AfSod2p displayed the amino acid consensus sequences characteristic of MnSODs and the two pfam domains 00081 and 02777 at the C- and N-termini respectively. The fourth gene (AFUA_6G07210) called AfSod4p was annotated by TIGR as a SOD encoding gene. The *AfSOD4* 1179-bp-long ORF was interrupted by three introns of 65, 82 and 66 bp and encoded a protein of 321 aa. Sequencing of the cDNA confirmed that the *SOD4* gene only contained the three introns predicted in the TIGR sequence. AfSod4p had a pfam02777 domain at the C-terminus but did not contain the typical Mn SOD N-terminus pfam00081 domain. *SOD4* was highly conserved in all filamentous ascomycetes sequenced to date.

AfSOD1*,* AfSOD2*,* AfSOD3 *and* AfSOD4 *are expressed during growth*

The expression of *AfSOD1*, *AfSOD2*, *AfSOD3* and *AfSOD4* was investigated by real-time RT-PCR at different growth time points in *A. fumigatus akuB* strain (Fig. 1). The *AfSOD1* expression was maximal in conidia (0 h) and in germinated conidia (8 h, Fig. 1). *AfSOD2* was strongly expressed in conidia and during later growth time points (20- and 30-h-old mycelia, Fig. 1). *AfSOD3* was only strongly expressed during the later time points (20- and

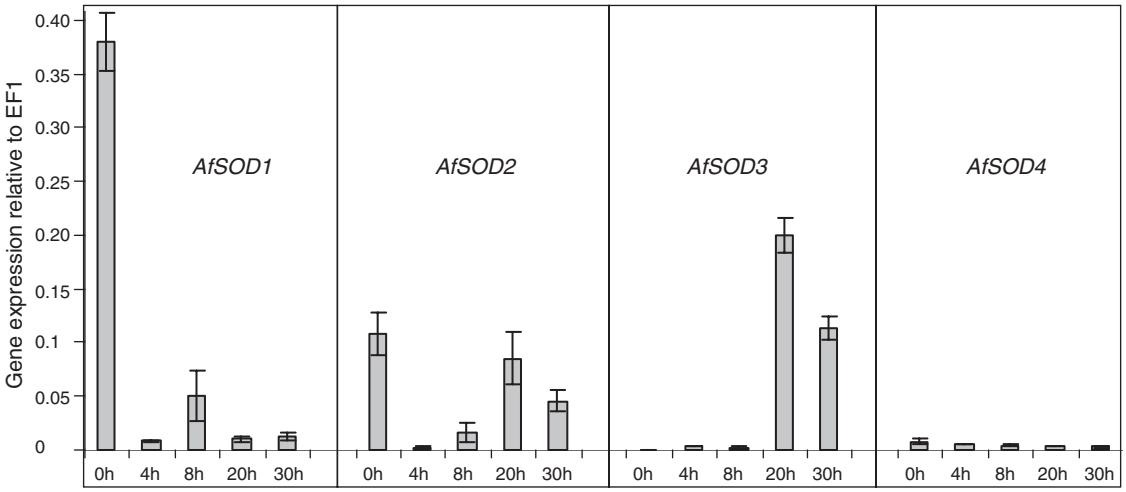

Fig. 1. Expression of the *AfSOD1*, *AfSOD2, AfSOD3* and *AfSOD4* genes during growth. Gene expression during growth was determined by real-time RT-PCR in *akuB* resting conidia (0 h), swollen conidia (4 h), germinated conidia (8 h) and mycelia (20 h and 30 h). Cultures were incubated in YPD broth at 37°C. The expression ratios were normalized using EF1α gene, according to the ΔΔCt method. Data are mean ± standard error from three independent experiments.

30-h-old mycelia, Fig. 1). *AfSOD4* was weakly expressed at all time points (Fig. 1). Spliced *AfSOD4* transcripts identified during the sequencing of the cDNA confirmed that the low level of expression of *SOD4* was indeed real (data not shown).

SOD deletions showed that SOD4 is an essential gene

To analyse the functions of *AfSOD1* to *AfSOD4* in *A. fumigatus*, mutants were constructed by the replacement of each gene by a hygromycin-resistance cassette in an *akuB* strain background. A correct and unique integration event of corresponding drug-resistance gene was seen at the expected locus for *SOD1-3*, as verified by PCR and Southern blot hybridization (see Figs S3, S4 and S5 for Δ*sod1*, Δ*sod2* and Δ*sod3* mutants respectively). In contrast, no Δ*sod4*::*HPH* deletion mutant were obtained by conidial electroporation despite several attempts suggesting this gene was essential. To check for the essentiality of *SOD4*, the technique of heterokaryon rescue developed for *Aspergillus nidulans* (Osmani *et al*., 2006) was applied to *A. fumigatus*. Heterozygous diploid strains *AfSOD4*/Δ*sod4*::*HPH* were constructed. PCR and Southern blots showed that each transformant harboured both a wild type and *SOD4*-disrupted alleles (Fig. S6). Monospore haploid conidia produced by the diploid transformant were seeded on a growth medium in presence or absence of hygromycin. Haploidization of these strains was only possible on non-selective medium but not on hygromycin medium showing that inactivation of *AfSOD4* was lethal in *A. fumigatus*.

Sod1 *and* Sod2 *mutants are highly sensitive to superoxide ions*

The sensitivity to menadione that produces intracellular superoxide radicals (Kawamura *et al*., 2006) was investigated for *sod1–sod3* mutants. The Δ*sod2*::*HPH* strain was the most sensitive to menadione and the Δ*sod1*::*HPH* mutant was slightly less sensitive to menadione than Δ*sod2*::*HPH* mutant. The Δ*sod3*::*HPH* mutant displayed a sensitivity similar to *akuB* reference strain (Fig. 2A). Similar results were observed with paraquat (data not shown). These results were in agreement with expression data obtained after the incubation of *akuB*-swollen conidia in presence of menadione (40 μM) for 2 h. Using this experimental set-up, quantitative RT-PCR data showed that *AfSOD1* and *AfSOD2* were upregulated in the presence of menadione (sixfold and threefold respectively, Fig. 2B). In contrast, no significant change in expression was seen under the same conditions for *AfSOD3* and *AfSOD4* genes. Complementation of the *sod1* and *sod2* mutants by a wild-type allele as described in *Experimental procedures* (Fig. S3C) restored a resistance to menadione similar to the parental strain (Fig. 2A). No effect of the pH between 5 and 9 and iron starvation was seen (data not shown).

A. fumigatus Δsod1 *and* Δsod2 *mutants display a marked growth inhibition at elevated temperatures*

Because high temperatures increase fungal metabolism leading to a greater oxygen consumption and ROS production (Davidson *et al*., 1996; Jeong *et al*., 2001; Narasipura *et al*., 2005), the growth of the *akuB* parental strain and the three single *sod* mutants was assessed at differ-

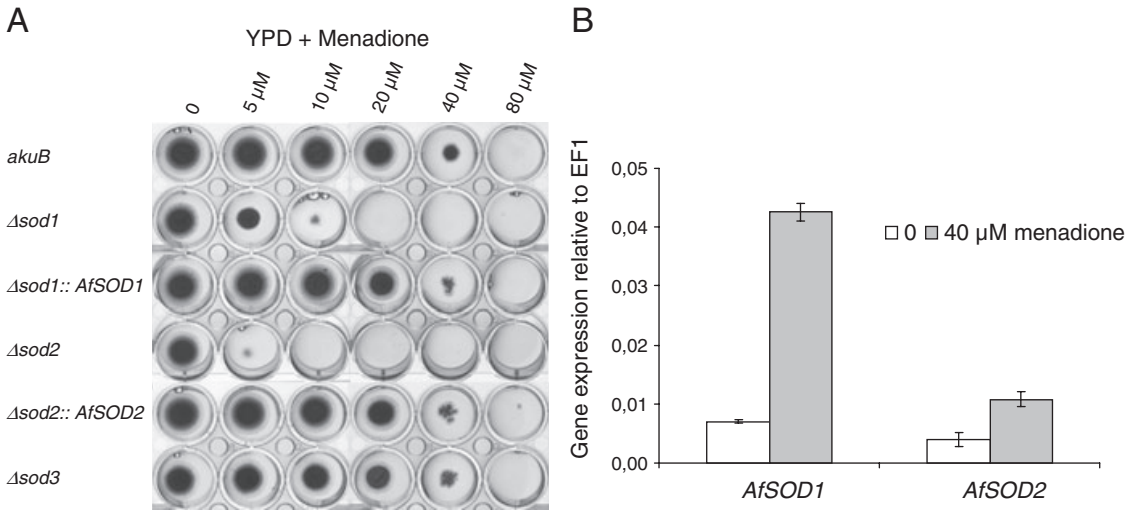

Fig. 2. Sensitivity to menadione of superoxide dismutase deletion mutants of *A. fumigatus*.
A. Sensitivity to menadione of superoxide dismutase single deletion mutants strains in *A. fumigatus* Δ*sod1*, Δ*sod2,* Δ*sod3* and revertant strains (Δ*sod1*::*AfSOD1HIS* and Δ*sod2*::*AfSOD2HIS*). A total of 10^5 conidia of each strain were spotted on YPD agar medium containing either 0, 5, 10, 20, 40, or 80 µM menadione. The plates were incubated at 37°C for 24 h.
B. Upregulation of *AfSOD1* and *AfSOD2* expression by menadione. After an initial 4 h growth of the *akuB* strain in YPD broth at 37°C, menadione (40 µM) was added and cultures were incubated at 37°C for 2 h. The Q-RT-PCR expression ratios were normalized using EF1α gene, according to the ΔΔCt method. Data are mean ± standard error from three independent experiments.

ent temperatures (37°C, 45°C and 50°C) on three different agar media (YPD, RPMI, MM). The growth of *sod* mutants was not affected at 37°C on YPD (Fig. 3A) and RPMI (data not shown). In contrast, the Δ*sod1*::*HPH* and Δ*sod2*::*HPH* mutant growth was inhibited at 45°C and at 50°C on YPD agar media (Fig. 3A). These growth defects were in agreement with expression data obtained when *akuB*-swollen conidia were incubated at 50°C for 2 h. Quantitative RT-PCR data showed that *AfSOD1* and *AfSOD2* were upregulated at 50°C (19-fold and 9-fold respectively, Fig. 3B). Like during menadione stress, no significant changes in expression were seen under the same conditions for *AfSOD3* and *AfSOD4* genes. The reintegration of *AfSOD1* in the Δ*sod1*::*HPH* strain and *AfSOD2* in the Δ*sod2*::*HPH* strain restored the growth defects (data not shown). The Δ*sod3*::*HPH* strain showed a very limited growth inhibition mostly seen at 50°C (Fig. 3A). These results show that *AfSOD1* and *AfSOD2* played a major role in adaptation to mycelial grow at temperature ≥ 45°C.

Multiple sod *mutants displayed the most severe phenotype*

A double deletion mutant strain (Δ*sod1*::*BLE*/Δ*sod3*::*HPH*) and a triple deletion mutant strain (Δ*sod1*::*BLE*/Δ*sod2*::*PTRa*/Δ*sod3*::*HPH*) were constructed. A phleomycin-resistance cassette was used to replace *SOD1* ORF in Δ*sod3*::*HPH* strain and obtain the double mutant strain exempt of cytoplasmic Sod proteins (Δ*sod1*::*BLE*/Δ*sod3*::*HPH*). The triple mutant (Δ*sod1*::*BLE*/Δ*sod2*::*PTRa*/Δ*sod3*::*HPH*) was constructed by replacement of *SOD2* ORF with a pyrithiamine-resistance cassette in the double mutant Δ*sod1*::*BLE*/Δ*sod3*::*HPH* strain. All strains harboured a correct and unique integration event of corresponding drug-resistance gene at the expected locus, as verified by PCR and Southern blot hybridization (Figs S7 and S8). The sensitivity of the double mutant Δ*sod1*::*BLE*/Δ*sod3*::*HPH* to menadione was slightly higher than the single mutant Δ*sod1*::*HPH* (Fig. 4A). The strongest sensitivity to menadione of the triple mutant Δ*sod1*::*BLE*/Δ*sod2*::*PTRa*/Δ*sod3*::*HPH* resulted mainly from the additive effect of the *SOD1* and *SOD2* deletions (Fig. 4A). Similarly, the triple mutant Δ*sod1*::*BLE*/Δ*sod2*::*PTRa*/Δ*sod3*::*HPH* was the most affected at 45°C and 50°C on RPMI agar medium (Fig. 4B) and YPD agar medium (data not shown). The additivity of the phenotype due to the different *SOD* deletions suggested that Sod1p, Sod2p and Sod3p are not involved in the same pathway. As the triple deletion mutant showed the most adverse phenotype, a detailed characterization of the triple Δ*sod1*/Δ*sod2*/Δ*sod3* mutant phenotype was undertaken.

Kinetics of conidial germination is affected in the Δsod1/Δsod2/Δsod3 *mutant*

After 7 h incubation at 37°C on a Sabouraud agar medium the triple mutant displayed a significant germination delay (Fig. 5). After 9.5 h incubation at 37°C, however, 92% and

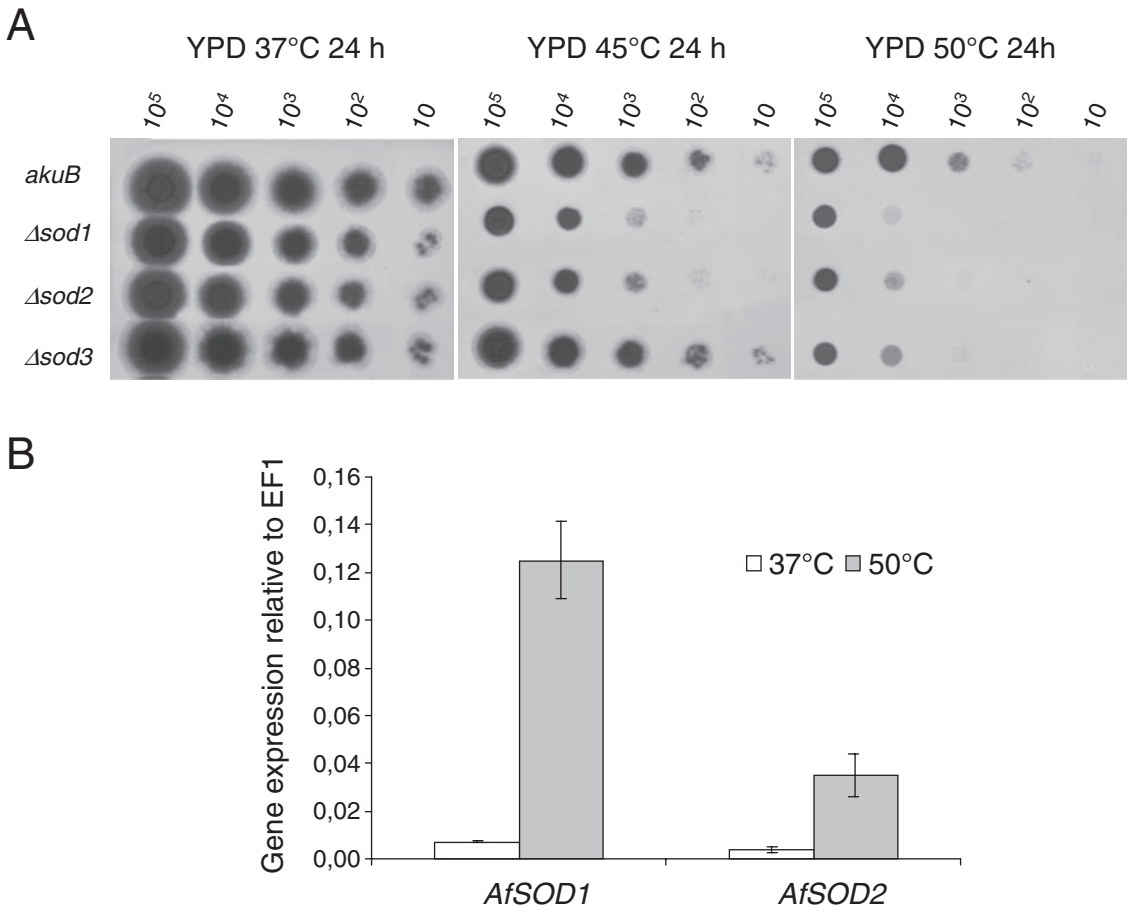

Fig. 3. Growth of SOD deletion mutants in *A. fumigatus* at 37°C, 45°C and 50°C on YPD agar media.
A. The *akuB* parental and the single mutants (Δ*sod1*, Δ*sod2*, Δ*sod3*) were grown in YPD agar medium. Serial 10-fold conidial suspension dilutions were made and 5 μl of each was spotted on YPD agar plates corresponding to 10^5, 10^4, 10^3, 10^2 and 10 conidia per spot (from left to right). Experiments were repeated three times with similar results.
B. Expression of *AfSOD1* and *AfSOD2* after incubation of *akuB* mycelium in YPD broth at 50°C for 2 h. The Q-RT-PCR expression ratios were normalized using EF1α gene, according to the ΔΔCt method. Data are mean ± standard error from three independent experiments.

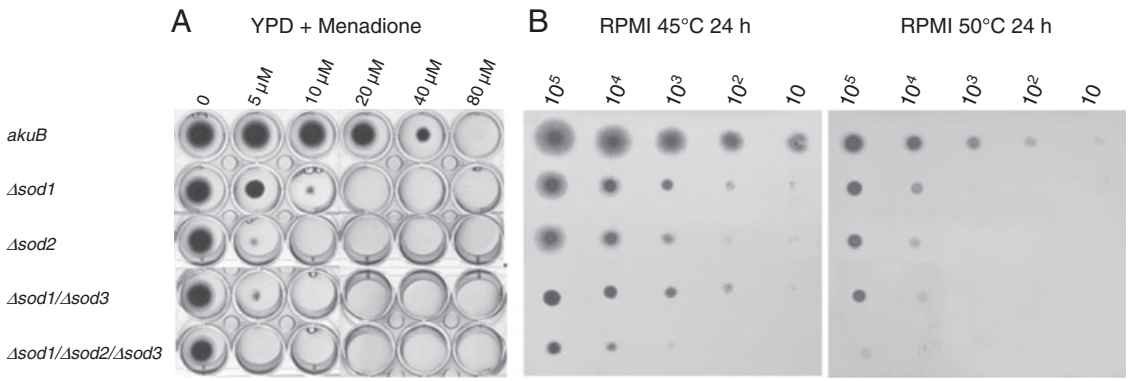

Fig. 4. The multiple *sod* mutants displayed the most severe phenotype.
A. Sensitivity to menadione of double and triple deletion mutants (Δ*sod1*/Δ*sod3*, Δ*sod1*/Δ*sod2*/Δ*sod3*) compared with *sod1* and *sod2* single mutants. Same growth conditions as in Fig. 2A.
B. The double mutant (Δ*sod1*/Δ*sod3*) and the triple mutant (Δ*sod1*/Δ*sod2*/Δ*sod3*) were grown at 45°C and 50°C for 24 h on RPMI agar medium. Conidial dilutions inoculated were as in Fig. 3A.

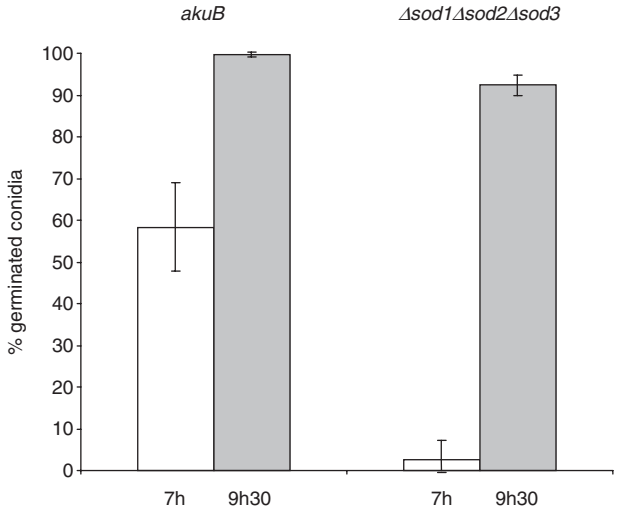

Fig. 5. Germination of Δ*sod1*/Δ*sod2*/Δ*sod3* mutant compared with parental strain (*akuB*). Conidia of parental strain (*akuB*) and triple mutant (Δ*sod1*/Δ*sod2*/Δ*sod3*) were incubated in 2% agar Sabouraud medium for 7 and 9.5 h. Germination percentages were calculated in three independent experiments. One hundred conidia for each strain were counted for germination.

100% of germination was observed for mutant and parental strains respectively (Fig. 5). Similar results were observed on YPD agar medium (data not shown). In contrast, no difference of germination was seen when the conidia were incubated in the same medium but in shake liquid conditions. To investigate if the germination delay of the triple mutant was correlated with an increase in superoxide levels or perturbation of ROS localization during apical growth, superoxide anions were visualized and quantified after staining of the germinating conidia with nitro blue tetrazolium (NBT). No difference in intensity and localization of superoxide anions was observed between the triple mutant and the *akuB* parental strain at all stages of fungal growth (Fig. S9). As observed in *A. nidulans* (Semighini and Harris, 2008), swollen conidia and young germ tubes were homogenously stained by NBT; later on ROS accumulated progressively at the tip of the germ tubes and remained at the tip during mycelial growth. ROS were also quantified in swollen conidia and in germinated conidia stained with NBT after DMSO extraction. No differences were seen in the optical densities read at 415 nm between the *akuB* parental strain and the sod triple mutant.

The conidia of the triple mutant lose viability during storage under aerial conditions

Figure 6 shows that the viability of conidia during storage is affected by the triple *sod* mutation: 85% of conidia of the Δ*sod1*/Δ*sod2*/Δ*sod3* mutant were killed following storage for 1 year at room temperature in tubes containing malt agar medium, whereas only 3% of the *akuB* parental strain conidia were killed under the same storage conditions. After 1 week storage at room temperature in tubes containing malt agar medium, only 8% of the triple mutant conidia were killed whereas no mortality of conidia was observed in the *akuB* parental strain. The dead conidia showed a bright intracellular fluorescence when labelled with fluorescein 5-isothiocyanate (FITC) whereas the living conidia displayed a fluorescence only at the cell wall (Fig. 6). In contrast, no difference in the viability of the conidia between the Sod triple mutant and the *akuB* parental strain was seen when conidia were stored in an aqueous solution containing 0.05% Tween 20 for 1 year.

Virulence of the triple mutant is not affected in an immunocompromised model of invasive aspergillosis

The conidia of the triple mutant were more sensitive to the killing by lung phagocytes of immunocompetent mice than

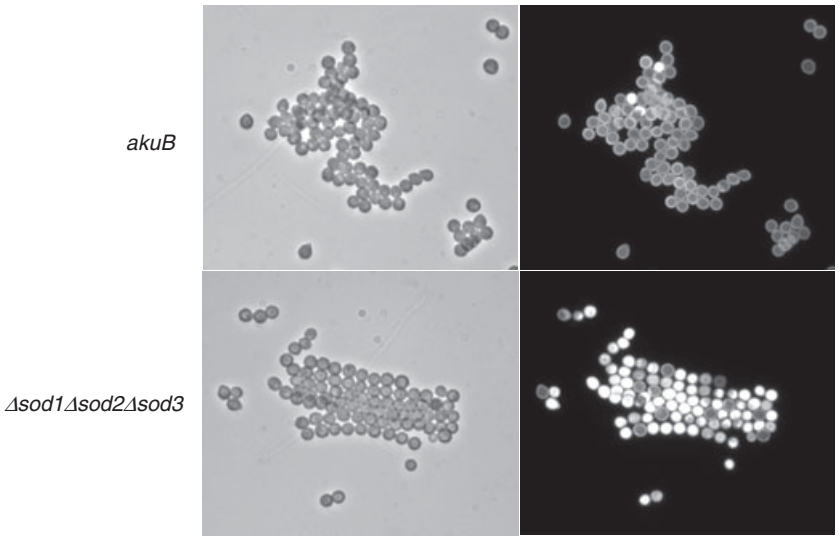

Fig. 6. Viability of 1-year-old conidia of *sod* triple mutant and parental strain (*akuB*). Conidia of parental strain (*akuB*) and triple mutant Δ*sod1*/Δ*sod2*/Δ*sod3* were stored at room temperature for 1 year in tubes containing malt agar medium. Conidia were labelled with FITC. The conidia with a bright intracellular fluorescence are dead whereas the conidia with fluorescent cell wall are alive. Bright-field microscopy images on the left panels, and epifluorescence microscopy on the right panels (×1000).

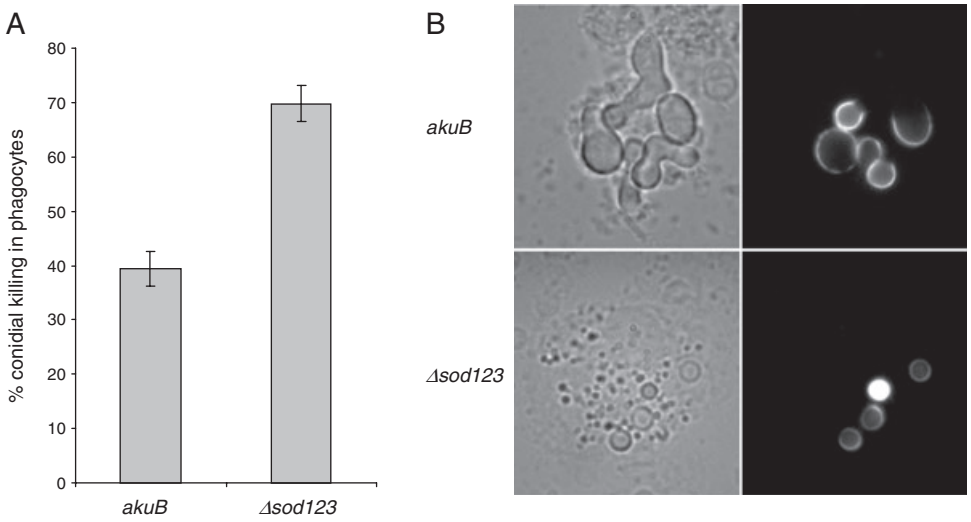

Fig. 7. Conidial germination of sod triple mutant and parental (*akuB*) strains that remained for 36 h in the lung of immunocompetent mice.
A. The percentage of killing was determined as follow: the number of non germinated conidia per 100 counted FITC-labelled conidia, after 5 h incubation in Sabouraud medium at 37°C.
B. An example of the morphology of germinating conidia of the *akuB* wild-type strain and dead conidia of the sod mutant. Right panels are FITC-labelled conidia viewed with an epifluorescent microscope. Note that only the cell wall of the conidial ghost of the parental strain remains fluorescent after germ tube production. In the mutant, conidia did not germinate after 5 and 7 h of incubation in Sabouraud medium. Note that one of the conidia of the triple mutant was dead before inhalation (see Fig. 6). On the upper panels, conidia of the parental strain (*akuB*) and in the lower panel conidia of the triple mutant Δ*sod1*/Δ*sod2*/Δ*sod3*. On the left panels: view by bright-field microscopy (×2000).

the parental strain: 36 h after inhalation, the percentage of dead conidia in the phagocytes were 70 ± 3% and 39 ± 3% for the sod triple mutant and the *akuB* parental strain respectively (Fig. 7). Due to its reduced growth at high temperature, delayed conidial germination and higher sensitivity to reactive oxidants and killing by immunocompetent lung phagocytes, it was expected that the triple mutant would have a reduced virulence in our experimental model of aspergillosis. No significant difference was seen in the survival rate of cohorts of 10 mice infected by wild type and triple mutant (Fig. 8). Development of *A. fumigatus* in the lung of immunocompromised mice results from an inefficient killing response of the lung phagocytes (Philippe *et al.*, 2003). At 36 h (the time where we see a difference in conidial killing in the immunocompetent mice), mycelium was already colonizing the lungs of the immunocompromised mice. The lack of difference in the virulence of the mutant and parental strain resulted only from the immunosuppressed nature of the murine IA model itself. These data questioned the use of immunocompromised models of IA to identify factors favouring the development of *A. fumigatus in vivo.*

SOD activity is exclusively intracellular in A. fumigatus

Substrate non-denaturing gel assays showed the presence of two bands corresponding respectively, to Sod1p for the upper band and Sod2p for the lower band (Fig. 9). Neither Sod3p nor Sod4p gave a positive band representative of a SOD activity. When an equivalent amount of

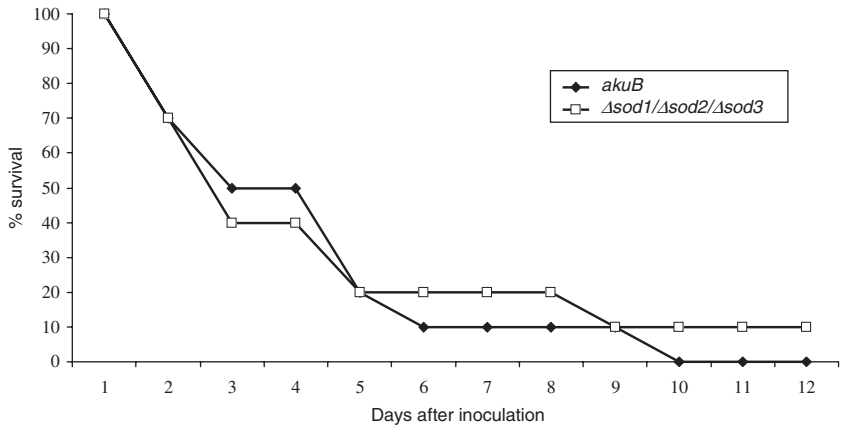

Fig. 8. Virulence of *sod* triple mutant and parental strain (*akuB*) in an experimental murine model of invasive aspergillosis. Survival curve of immunosuppressed mice intranasally inoculated with 10^5 conidia of the parental strain (akuB, ◆) and the triple mutant Δ*sod1*/Δ*sod2*/Δ*sod3* (□). Kaplan Meier analysis showed the lack of significance between the mortality due to the two strains.

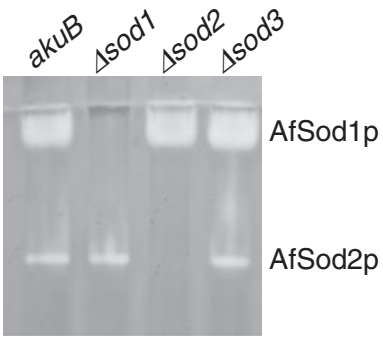

Fig. 9. Intracellular SOD activity shown in non-denaturing polyacrylamide gels. Activity bands of Sod1p and Sod2p were deduced from patterns of activity bands in the parental strain (*akuB*) and in the single SOD deletion mutants (Δ*sod1*, Δ*sod2,* Δ*sod3*); 50 μg of intracellular proteins was loaded per lane; no signals were seen in the cell wall and extracellular fractions(not shown).

protein of the intracellular, cell wall and extracellular fractions were loaded on the gel, SOD activity was only seen in the intracellular extract. As the intracellular protein fraction represented 95–98% of the total cell proteins at any growth stages (from resting conidium to mycelium), these results showed that AfSOD activity was exclusively intracellular and was due exclusively to AfSod1p and AfSod2p. These data were in agreement with the hypersensitivity of Δ*sod1* and Δ*sod2* to menadione that generates intracellular superoxide radicals. In contrast, the triple *sod* mutant was not more sensitive to extracellular superoxide radicals generated by exposing up to 1 mg ml^{-1} riboflavin to light than the parental strain (data not shown). These results indicated that *AfSOD1* and *AfSOD2* played the major role to detoxify intracellular superoxide anions produced by fungal metabolism. As extracellular superoxide ions cannot cross the plasma membrane, this result suggested that none of the intracellular Sodps of *A. fumigatus* were involved in the detoxification of extracellular superoxide anions.

Discussion

This is the first comprehensive study of an SOD family in a filamentous fungus. Data reported in this manuscript showed that the cytoplasmic CuZnAfSod1p and the mitochondrial MnAfSod2p displayed a SOD activity and were able to detoxify superoxide ion generating compounds such as menadione and paraquat. No biochemical proof of the SOD activity of the other two Sodps, Sod3p and Sod4p, has been obtained to date. There are even several arguments suggesting that Sod4p is not a SOD: (i) it is an essential protein and none of the fungal Sodps previously described are essential; (ii) it is the only Sodp that lacks the N-terminus consensus sequence of the Fe/Mn Sodps; (iii) Sod4p has also significant sequence homologies with the *S. cerevisiae* Rsm26 (Yjr101w) encoding a mitochondrial ribosomal protein (Saveanu *et al.*, 2001). In contrast to *A. fumigatus*, in *S. cerevisiae*, the Δ*rsm26* deletion mutant was not lethal and exhibited respiration deficiency and is hypersensitive to H_2O_2 and NaCl (Entian *et al.*, 1999).

Another result that came out from our study is that the entire SOD activity controlled by this family is intracellular in *A. fumigatus*. This result suggests that the function of SODs in *A. fumigatus* is to counteract its own fungal oxidants produced intracellularly rather than to detoxify extracellular ROS. Such role would be especially critical at high temperature where an enhanced activity of the fungal respiratory pathway would lead to an increased production of superoxide anions (Bai *et al.*, 2003). Accordingly, sod mutations have resulted in a higher sensitivity to elevated temperature. In *Aspergillus niger*, when conidiospores were grown at high temperature, the Cu/ZnSOD and catalase activities significantly increased suggesting a role of Cu/ZnSOD and catalase in response to temperature stress (Abrashev *et al.*, 2005). A role in the protection against high temperature has been also associated to *SOD2* of *C. neoformans* because the growth of the Δ*sod2* mutant was totally inhibited at 37°C (Narasipura *et al.*, 2005). This study also showed that in *A. fumigatus*, SODs can have essential metabolic functions controlling conidium dormancy and germination. This is in agreement with the high levels of Sodps present in resting conidia. A better knowledge of the regulation of SOD production may also help understanding their function during fungal growth because their expression seems tightly regulated and correlated to the fungal growth stage. In this respect, the opposite expression profiles of *AfSOD1* and *AfSOD3* is remarkable. The expression of *AfSOD1* (encoding cytoplasmic Cu/ZnSOD) is repressed concomitantly to the induction of *AfSOD3* (encoding cytoplasmic MnSOD) in liquid culture. Interestingly, the same observation has been reported in *C. albicans* with cytoplasmic Cu/ZnSOD *CaSOD1* and cytoplasmic MnSOD *CaSOD3* (Lamarre *et al.*, 2001), suggesting a regulation of *SOD* expression similar in *A. fumigatus* and *C. albicans*. In fungi, ROS play a key role in the spatial regulation of polar growth during the emergence of germ tube and the hyphal growth (for review see Aguirre *et al.*, 2005; Scott and Eaton, 2008). Using the NBT staining method in *A. nidulans*, defects in hyphal polarity observed in different mutant (Δ*atmA*, Δ*prpA* and Δ*noxR* mutants) have been shown to result from a difference of accumulation or localization of superoxide anions (Semighini and Harris, 2008). Using the same method, we showed that the cause of delayed germination observed in the *A. fumigatus* triple sod mutant was not a higher superoxide level in the triple mutant or a perturbation of ROS localization.

There are data suggesting that the upregulation of SODs is a part of the global stress response of the fungus rather than a specific response to oxidants (Sugui *et al*., 2008).

Discrepancies are seen in the role of SODs in the virulence of the different fungal human pathogens. In the case of *A. fumigatus*, our data showed that SODs are not involved in the fungal virulence. In *C. neoformans*, both SODs are required for virulence: SOD1 that is associated to lipid rafts (Siafakas *et al*., 2006) is essential because it controls growth at 37°C. The reason for the involvement of the mitochondrial SOD2 in controlling *C. neoformans* virulence is unknown but it is not associated to reactive oxidant scavenging. Similarly, the slightly higher sensitivity of the triple mutant to the phagocytes of an immunocompetent host does not seem associated to the lack of SOD activity but rather result from the global weakness of the conidia of this mutant. *C. albicans* has six SODs among which three are GPI-anchored, two are cytoplasmic and one mitochondrial (Frohner *et al*., 2009). *C. albicans* lacking the cytoplasmic Sod1p or the cell surface SODs are less virulent because these proteins and especially the surface GPI-anchored Sodps degrade the extracellular ROS produced by the innate immune cells (Hwang *et al*., 2002; Fradin *et al*., 2005; Frohner *et al*., 2009).

The data gathered here question also the role of ROS against *A. fumigatus in vivo* and the nature of the scavengers used by this fungus to combat host-derived reactive oxidant species. Genes encoding enzymes with high ROS-scavenging capacities such as catalases, SODs, glutathione peroxidase and thioredoxin reductase have been shown to be upregulated in hyphae exposed to neutrophils (Sugui *et al*., 2008). Experimental studies performed with phagocytes of human patients suffering from chronic granulomatous disease (CGD), a rare disorder caused by mutations in the NADPH oxidase complex in which phagocytes are defective in generating the reactive oxidant superoxide anion (Almyroudis *et al*., 2005) or their mouse transgenic homologues are less efficient in controlling *A. fumigatus* than normal patients or wild-type mouse. Macrophages from NADPH oxidase-deficient mice (p47phox-/- mice) and CGD patients are indeed unable to kill *A. fumigatus* conidia (Philippe *et al*., 2003; B. Philippe *et al*., unpublished). Neutrophils from CGD patients are unable to kill the hyphal form of *A. fumigatus* (Zarember *et al*., 2007). All these data associated to the high incidence of invasive aspergillosis in the CGD patients suggest that ROS play an essential role in the killing of *A. fumigatus in vivo*. There are, however, an increasing number of studies suggesting an apparent lack of importance of ROS-scavenging mechanisms to control *A. fumigatus* virulence. Such data came out of the analysis of other *A. fumigatus* mutants such as Δ*cat* mutants lacking either conidial or mycelial catalases, Δ*skn7* and Δ*yap1* mutants, where the hypersensitivity to H_2O_2 or/and menadione observed *in vitro* is not correlated with a reduction of fungal virulence in an experimental model of aspergillosis (Paris *et al*., 2003; Lamarre *et al*., 2007; Lessing *et al*., 2007; Qiao *et al*., 2008).

Our working hypothesis was that SODs should have a major impact in virulence because they catalyse directly the dismutation of superoxide anion that is the first precursor of the ROS pathway. Unfortunately, our studies have shown that the Sods are intracellular and do not neutralize extracellular ROS in spite of the high sensitivity of this fungus to intracellular ROS generators. The amount and type of ROS produced during phagocytosis of *A. fumigatus* remain unknown to date. Their identification as well as their capacity to enter the fungal cell is essential to identify the ROS-scavenging pathways with a putative involvement in *A. fumigatus* virulence. Accordingly, other scavenging pathways such as the glutathione and thioredoxin pathways that have never been investigated in *A. fumigatus*, could play a major role in counteracting ROS (Burns *et al*., 2005; Tekaia and Latge, 2005; Kniemeyer *et al*., 2008). In addition to enzymatic protective mechanisms against oxidative stress, *A. fumigatus* conidia contain melanin and mannitol that are able to quench ROS and protect from killing by alveolar macrophages (Jahn *et al*., 2000; Tekaia and Latge, 2005). Originally thought to be exclusive to the conidial surface layer, it is known now that melanin can be produced during mycelial growth (Beauvais *et al*., 2007).

Another explanation of the lack of effect of ROS scavenging is that non-oxidative mechanisms play a major role in *A. fumigatus* killing. Recent data have shown that CGD neutrophils and normal donors inhibit the growth of conidia with equal efficiency (Sugui *et al*., 2008). Among these effectors, it can be cited proteases like cathepsin G and elastase (Tkalcevic *et al*., 2000; Reeves *et al*., 2002), lyzozyme (Diamond *et al*., 1978) antimicrobial peptides like defensins, metabolic antagonists like lactoferrin (Lupetti *et al*., 2005; Zarember *et al*., 2007) and acid pH (Philippe *et al*., 2003). Some of these effectors could be synergistic of the action of ROS. Suppression of the production of the reactive oxygen species in the phagocytes could also occur by a non-scavenging process such as the one mentioned for *Candida* as an alternative strategy to escape the host immune defence mechanisms (Wellington *et al*., 2009). These data showed that in spite of years of studies in the interactions between phagocytes and *A. fumigatus*, the major host factors responsible for the killing of *A. fumigatus in vivo* remain unknown. More work in this direction should be undertaken taking into account the redundancy between the different defence strategies initiated by the host to eliminate *A. fumigatus*.

Experimental procedures

Strains and media

The *A. fumigatus* strain *akuB*^{ku80} (da Silva Ferreira *et al.*, 2006) and the mutant strains used in this study were maintained on 2% malt agar tubes. For the RNA extraction, the fungus was grown in YPD liquid medium at 37°C in shake flasks inoculated with 5×10^8 conidia ml^{-1}. For DNA extraction, mycelium was grown for 16 h at 37°C in a liquid medium containing 3% glucose and 1% yeast extract. For transformation experiments, minimal medium (MM; 1% glucose, 0.092% ammonium tartrate, 0.052% KCl, 0.052% $MgSO_4$ 7 H_2O, 0.152% KH_2PO_4, 1 ml trace element solution l^{-1}, pH 6.8 was used (Cove, 1966). MM containing 250 μg ml^{-1} hygromycin (Sigma) was used for the screening of the single mutants strains (Δ*sod1*::*HPH*, Δ*sod2*::*HPH* and Δ*sod3*::*HPH*), MM containing 30 μg ml^{-1} phleomycin (Invivogen) was used for the selection of the double deletion mutant strains (Δ*sod1*::*BLE*/Δ*sod3*::*HPH*) and Czapek Medium supplemented to 10 μg ml^{-1} pyrithiamine (Sigma) was used for the selection of the triple deletion mutant strains (Δ*sod1*::*BLE*/Δ*sod2*::*PTRa*/Δ*sod3*::*HPH*).

Nucleic acid manipulation

Genomic DNA was extracted as described by Girardin *et al.* (1993). For Southern blot analysis, 25 μg of digested genomic DNA was sized-fractionated on 0.7% agarose, and blotted onto a positively charged nylon membrane (Hybond-N$^+$, Amersham). The *AfSOD4* cDNA was obtained by RT-PCR of RNA from mycelium using Superscript II Reverse Transcriptase (Invitrogen, Cergy Pontoise, France) and using the primers Sod4-F and Sod4-R (Table S1). The *AfSOD4* cDNA was cloned into pCR®-BluntII-TOPO® plasmid (Invitrogen, Carlsbad, CA) and sequenced.

Real-time RT-PCR

Gene expression was quantified by real-time RT-PCR analysis as previously described by Lamarre *et al.* (2009). Total RNA from the wild-type strain (*akuB*) was extracted from resting conidia (0 h) and swollen conidia (4 h), germinated conidia (8 h) and mycelium (20 h, 30 h) obtained from cultures grown in YPD broth at 37°C, 150 r.p.m. To analyse gene expression in the presence of 40 μM menadione or at 50°C, the fungus was grown for 4 h in YPD broth at 37°C and supplemented with menadione (40 μM) or shifted to 50°C and incubated in these conditions for 2 h. Fungal cells were disrupted with 0.5 mm diameter glass beads in 500 μl water satured phenol (pH 5, Prolabo) diluted 1:1 in water using a Fastprep apparatus (3×30 s, power 4.0 at 4°C; BIO 101). Following two additional rounds of phenol and one round of chloroform extractions, RNA was precipitated and cleaned up by DNase treatments: a first DNase treatment was carried out on RNeasy column (Qiagen, Courtaboeuf, France) using DnaseI (Roche, France) and a second one was performed after elution of the RNA from the column with Turbo DNA-free DNAse (Ambion, Courtaboeuf, France). Five micrograms of total RNA was reverse-transcribed using Superscript II Reverse Transcriptase (Invitrogen, Cergy Pontoise, France). Quantitative PCR assays were performed according to Bio-Rad manufacturer's instructions using 96-well optical plates (Thermo Scientific) and an iCycler iQ (170-8740, Biorad). Each run was assayed in triplicates in a total volume of 20 μl containing the DNA template at an appropriate dilution, 1× Absolute SYBR green Fluorescein (Thermo Scientific) and 100 mM of each primer. The primers used for *AfSOD1*, *AfSOD2*, *AfSOD3* and *AfSOD4* were designed using the Beacon Designer 4.0 software and are shown in Table S1. PCR conditions were: 95°C/15 min for one cycle; 95°C/30 s and 55°C/30 s for 40 cycles. Amplification of one single specific target DNA was checked with a melting curve analysis (+0.5°C ramping for 10 s, from 55°C to 95°C). The generated data were then analysed using the Optical Systems v3.1 Software. The expression ratios were normalized to EF1α expression, and calculated according to the ΔΔCt method (Livak and Schmittgen, 2001). 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.

Construction of the AfSOD *deletion and complementation cassettes*

The deletion cassettes used in this work were constructed by PCR fusion (Lamarre *et al.*, 2007). To construct the single mutant strains (Δ*sod1*::*HPH*, Δ*sod2*::*HPH* and Δ*sod3*::*HPH*) and the diploid strain Δ*sod4*::*HPH*/*AfSOD4*, each *SOD* was replaced with the *Escherichia coli HPH* gene (conferring resistance to hygromycin) obtained from the pAN7.1 plasmid (Punt *et al.*, 1987). Hygromycin-resistant transformants were selected for further study. Site-specific recombination was ensured by association with the *HPH* gene of an approximately 0.5 kb upstream and downstream *AfSOD* flanking fragments. The deletion cassette of *AfSOD1* as an example was performed by PCR fusion of three PCR products (PCRa, PCRb and PCRc) shown in Fig. 2B. Primers used for these constructions are reported in Table S1. To disrupt *AfSOD1* in the Δ*sod3*::*HPH* strain for the creation of the double deletion mutant strains (Δ*sod1*::*BLE*/Δ*sod3*::*HPH*), the *AfSOD1* ORF was replaced with *E. coli BLE* gene (conferring resistance to phleomycin) obtained from the pAN8.1 plasmid (Punt *et al.*, 1987). To construct the triple deletion mutant strain (Δ*sod1*::*BLE*/Δ*sod2*::*PTRa*/Δ*sod3*::*HPH*), the *AfSOD2* ORF was replaced with *E. coli PTRA* gene from *Aspergillus oryzae* (conferring resistance to pyrithiamine) carried by plasmid pSK275 (Kubodera *et al.*, 2000; 2002) in the double deletion mutant strain (Δ*sod1*::*BLE*/Δ*sod3*::*HPH*). Site-specific recombination was ensured by association with the *PTRA* gene of an approximately 0.5 kb upstream and downstream *AfSOD2* flanking fragments. *A. fumigatus* transformation experiments were achieved by electroporation with a protocol previously described for *Aspergillus nidulans* (Sanchez and Aguirre, 1996) that was adapted for *A. fumigatus* (Weidner *et al.*, 1998). A total of 2×10^9 conidia were incubated in 125 ml of YG broth (0.5% yeast extract, 2% glucose) for 4 h at 37°C, 350 r.p.m. Swollen conidia were collected and incubated in

12.5 ml of YED broth (1% yeast extract, 1% glucose, 2 mM HEPES pH 8) for 1 h at 30°C, 100 r.p.m. Swollen conidia were collected in 1 ml of 1 M cold sorbitol. Fifty micrograms of electrocompetent conidia and 10 µl of PCR product (1–2 µg) resulting from the PCR fusion were incubated for 15 min on ice. Electroporation was performed at 1 kV, 25 µF and 400 Ω in cuvetts with 0.1 cm electrode gap. After electroporation and incubation at 30°C for 90 min, 100 r.p.m., transformation mixtures were cultured on 20 ml MM agar plates overnight at room temperature. The following day, 10 ml of melted MM (0.6% agarose) containing selective drug was added on plates. The plates were incubated at 37°C for 1 week. To construct the diploid strain Δ*sod4*::*HPH*/*AfSOD4*, the same electroporation protocol was used except that the electric pulse was performed on germinated conidia obtained after 5 h instead of 4 h of growth in YG medium at 37°C.

The *AfSOD1HIS* and *AfSOD2HIS* complementation cassettes were also constructed with the fusion PCR method (Lamarre *et al*., 2007) and allowed the insertion of a 6xhis-tag at the 3′-end of the *AfSOD1* or *AfSOD2* genes. The complementation cassette of the Δ*sod1*::*HPH* mutant as an example was performed by PCR fusion of four PCR products (PCR1, PCR2, PCR3 and PCR4) indicated in Fig. S3C. The resulting major PCR product was gel-purified and used to transform strain Δ*sod1*::*HPH*. The same strategy was performed for the construction of *AfSOD2* reversion cassette. All primers and all strains used in this study are reported in the Tables S1 and S2 respectively.

SOD zymogram

Brian medium (Brian *et al*., 1961) was inoculated with 10^8 conidia per ml. Ungerminated resting conidia and fungal cells were recovered 4 h, 8 h and 20 h after conidial inoculation. The supernatant was dialysed (Sigma, D9652-100FT) against water and concentrated using polyethylene glycol. The fungal cells were disrupted with glass beads (0.5 mm diameter) in FastPrep (3 × 30 s, power 4 at 4°C, MP Biomedicals) in 20 mM pH 7.8 phosphate buffer supplemented with protease inhibitor cocktail tablets (1 tablet in 50 ml, Roche). The cell wall fraction was removed by low speed centrifugation (3000 *g*, 5 min at 4°C) and incubated in the 50 mM NaOAc and 5 mM NaN_3 pH 5.6 solution at 37°C under agitation for 12 h. Protein content of the extracellular, cell wall and intracellular fractions was quantified with the Bradford reagent (Bio-Rad), using bovine serum albumin as a standard. All the fractions were stored at –20°C. To detect SOD activity, 7.5% non-denaturing, non-reducing polyacrylamide gels were used and 30–50 µg of proteins was loaded per well. Electrophoresis was carried out at 120 V at 4°C. SOD activity was visualized as inhibition of the reduction of NBT (Sigma) according to the method of Beauchamp and Fridovich (1971). Following electrophoresis, the gel was washed 2 × 10 min in ice-cold water, soaked in 2.45 mM NBT solution for 20 min in the darkness. This was followed by a further incubation in 2.8×10^{-5} M riboflavin and 0.028 M Temed in 0.036 M potassium phosphate buffer pH 7.8 for 15 min in darkness. Upon illumination, an achromatic band indicating zones of activity appeared in the region of gel where SOD proteins were present.

Phenotypic analysis of mutants

Conidia were obtained from cultures grown on 2% malt agar tubes for 7 days at room temperature, and recovered by vortexing with 0.05% aqueous Tween 20 solution. Homogenous conidial suspensions of each strain were collected following filtration through a 40-µm-pore-size filter (Falcon). For the phenotypic analysis, we tested the sensitivity of the Δ*sod* mutants to oxidative stress conditions induced by menadione (up to 80 µM), paraquat (up to 8 mM). The sensitivity to extracellular superoxide ions of *SOD* mutants was analysed using the method described by Ito-kuwa *et al.* (1999) with the addition of up to 1 mg ml^{-1} riboflavin to the medium in the presence of light. A positive control was performed using an YPD agar plate containing 2.45×10^{-3} M NBT chloride (Sigma) and 0.25 mg ml^{-1} riboflavin in order to confirm the production of superoxide radicals in the medium. Five microlitres of a conidial suspension containing 10^5 conidia was spotted on YPD agar plates and incubated at 37°C for 24–72 h. *A. fumigatus* strains were grown on YPD (yeast extract 10 g l^{-1}, 20 g l^{-1} peptone, 20 g l^{-1} glucose), RPMI (10.26 g l^{-1} RPMI 1640 Sigma without glutamine and $NaHCO_3$, 0.3 g l^{-1} L-glutamine, 0.165 M MOPS, pH 7–7.2) and MM media at 37°C, 45°C and 50°C for 24 h. The percentage germination was quantified microscopically every 30 min from 7 h until 10 h after incubation of 5×10^3 conidia at 37°C in Sabouraud agar medium and YPD agar medium. One-way analysis of variance was performed using the JMP software (Cary, NC). Superoxide anions in the germinated conidia were visualized by light microscopy after incubation of fungal cells with 2.5 mM of NBT chloride for 30 min at room temperature. The NBT is reduced by superoxide anions and it forms a detectable blue formazan precipitate (Munkres, 1990). Superoxide anions after NBT staining of swollen conidia and germinated conidia were quantified after dimethyl sulphoxide extraction of the Formazan and optical density measurement at 415 nm (A. Beauvais, pers. comm.). The effect of the pH was tested YPD medium at three different pH 5 (0.1 M MES), pH 7 (0.1 M MOPS) and pH 9 (0.1 M tris base). Iron starvation was performed with minimum medium without iron (Pontecorvo *et al*., 1953) containing 1% glucose as the carbon source and 20 mM glutamine as the nitrogen source.

Conidial survival and mouse experiments

Conidia of different ages stored either under aerial conditions or in 0.05% Tween 20 aqueous solutions were labelled with 0.1 mg ml^{-1} of FITC (Sigma) in Na_2CO_3 0.1 M pH 9 for 30 min. For mouse experiments, 7-week-old OF1 female mice weighing approximately 22 g (Charles River Laboratory, L'Arbresle, France) were used. Before conidial inhalation, mice were anesthetized with an intramuscular injection of 0.2 ml of a solution containing 10 mg ml^{-1} ketamin (Imalgène® 1000, Merial, France), 1 mg ml^{-1} xylazin (Rompun®, Bayer HealthCare, Germany) per mouse. For conidial survival assays in lungs, 5×10^8 conidia in 30 µl of 0.05% Tween 20 cwere inoculated intranasally per immunocompetent mice, using an automatic pipetting device. After an infection period of 36 h, the mice were euthanized with an intraperitoneal injection of 150 µl of a solution containing 54.7 mg ml^{-1} sodium pento-

barbital (Ceva Santé Animale SA, France). Five mice per fungal strains were used. Broncho alveolar lavage was performed on each lung by the injection and aspiration of eight times 0.5 ml of PBS 1× pH 7.3 (Dulbecco's phosphate-buffered saline solution) into the trachea. To quantify conidial survival, the alveolar macrophages were lysed by a water osmotic shock and homogenate containing conidia was then incubated in an equal volume of 2× Sabouraud (4% glucose-2% mycopeptone) culture medium at 37°C for 5–7 h to take into account the delay in the germination of the mutant strain. The percentage of killing was determined as follow: the number of non-germinated conidia per 100 counted FITC-labelled conidia. Germination was counted under epifluorescence microscope. For virulence assays, mice were immunosuppressed with cortisone acetate (Sigma) and cyclophosphamid monohydrate (Sigma) injected intraperitoneally on days –3 and –1 (112 mg cortisone acetate per kg of mouse and 200 mg cyclophosphamid monohydrate per kg of mouse) before intranasal inoculation of conidia (day 0) for *in vivo* experiments. By means of an automatic pipetting device, each mouse was inoculated intranasally with 10^5 conidia (25 µl per mice of 0.05% Tween 20 conidial suspension at 4×10^6 conidia per ml). Non-infected control immunosuppressed mice only received 25 µl of PBS plus 0.05% Tween. In order to keep immunosuppression, other injection of cyclophosphamid monohydrate is required at days +3, +6, +9 after inoculation. Survival was followed up daily over a period of 14 days.

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