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The protein phosphatase PhzA of *A. fumigatus* is involved in oxidative stress tolerance and fungal virulence

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Abstract

Protein phosphatases Z that are unique to the fungal kingdom have been associated to resistance to high salt concentration, cell wall integrity, cell cycle regulation, and oxidative stress in fungi. In *Aspergillus fumigatus* it was shown that *PHZA* is under the control of the transcription factor Skn7 and is only involved in the control of the oxidative stress. Accordingly, the Δ*phzA* mutant showed a defect in virulence in an experimental model of corneal infection in immunocompetent animals and that the impact on susceptibility to cell wall drugs is only secondary.

Keywords

Oxidative stress; Phosphatase; Virulence; *Aspergillus fumigatus*; Corneal infection

1. Introduction

Protein phosphatases Z (Balcells et al., 1997) are found exclusively in yeast and filamentous fungi (Arino, 2002). These phosphatases are composed of a highly conserved COOH-terminal catalytic domain similar to the type 1 S/T phosphatases while the unstructured NH2-terminal regulatory domain contains a N-myristoylation site and a Arg/Ser rich motif that are unique to these fungal proteins (Arino, 2002; Leiter et al., 2012; Posas et al., 1993). Ppz proteins have been extensively characterized in *Saccharomyces cerevisiae*, *Schizzosaccharomces pombe* and *Candida albicans* and shown to be involved in salt tolerance, cell wall integrity, cell cycle regulation, and oxidative stress tolerance (Balcells et al., 1997; Kovacs et al., 2010; Posas et al., 1993, 1995). However, although the *Neurospora crassa* or *Aspergillus nidulans* and *Aspergillus fumigatus PPZ* are able to complement Δ*ppz*

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Appendix A. Supplementary material: Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fgb.2014.02.009.

deletion mutants of *S. cerevisiae*, Ppz proteins of filamentous fungi were shown to be exclusively involved in resistance to oxidative stress (Leiter et al., 2012; Vissi et al., 2001). In contrast to yeasts, the Δ*ppzA* deletion mutant obtained in *A. nidulans* was dispensable for the salt stress response and cell integrity signaling.

Due to the essential role of reactive oxygen species (ROS) in the killing of *A. fumigatus* following infection of the lung (Ben-Ami et al., 2010) and the cornea (Leal et al., 2012), it was of interest to construct a Δ*ppz* mutant of *A. fumigatus* to investigate the role of this protein in the virulence of this opportunistic fungal pathogen. Even though a Δ*ppz* mutant was constructed in *A. nidulans* it was worth constructing an *A. fumigatus* mutant since it has been repeatedly shown in the past that the deletion of orthologous genes in *A. fumigatus* and *A. nidulans* often give different growth phenotypes in the respective mutants (Jimenez-Ortigosa et al., 2012; Takeshita et al., 2005). Moreover, it was attractive to test the effect of cell wall inhibitors on these mutants since the Δ*ppz1* mutants of *S. cerevisiae* have shown an exquisite sensitivity to caspofungin (Parsons et al., 2006) and a cooperative effect with neutrophils, which are the major ROS producers in fungal infections.

Using a Δ*phzA* mutant of *A. fumigatus* we were able to show that the primary function of the Ppz protein was to protect against oxidative stress and that the impact on susceptibility to cell wall drugs was only secondary. *PHZA* was shown to be under the control of the transcription factor skn7 and is involved in the fungal virulence.

2. Material and methods

2.1. Strains and culture conditions

A. fumigatus strains were grown at 37 °C in either minimal medium AMMC (Cove, 1966) containing 1% glucose and 5 mM ammonium tartrate, Sabouraud medium or 2% Malt agar. Conidia were obtained from agar media plates after 6 days of growth at 37 °C, using 0.05% aqueous solutions of Tween 20.

2.2. Development of Δ*phzA* knock-out and reverting strains

The Δ*phzA* deletion mutant was constructed in CEA17 Δ*akuB*^{KU80} background using the β-rec/six site-specific recombination system as described earlier (Hartmann et al., 2010; Jimenez-Ortigosa et al., 2012). The *PHZA* replacement cassette containing the recyclable marker module flanked by 5′ and 3′ homologous regions was generated and cloned in the pUC19 vector. The CEA17ΔakuB^{KU80} parental strain was transformed with the *PHZA* replacement cassette by electroporation. Transformants obtained were verified by PCR and southern blot analysis (Supplemental Fig. 1). Complementation of the Δ*phzA* mutant was obtained by reintroduction in the mutant of the WT copy of the gene flanked by the hygromycin resistance cassette and a 3′ flanking region as described in the supplemental Fig. 2. After cultivation of the Δ*phzA* mutant on minimal medium containing 10% xylose to induce the excision of the deletion cassette, the complementation cassette obtained by cloning was transformed into the resulting excised Δ*phzA* mutant. The presence of the WT copy of the gene at the *PHZA* locus was confirmed Southern Blot analysis.

2.3. Phenotypic analysis of Δ*phzA* deletion strain

The radial growth of the Δ*phzA* mutant, and Δ*phzA*::*PHZA* reconstituted strains were measured on malt agar and minimal plates after 48 h of incubation at 37 °C or 45 °C. Conidia were then harvested to estimate the conidiation rate by counting with haemocytometer. Conidial germination was analyzed kinetically on sabouraud agar medium for up to 9 h. Sensitivity of Δ*phzA* mutant to various stresses were tested: pH 5 to pH 9, 10–50 μg/ml caffeine, 0.4–1.5 M NaCl, 10–30 mM LiCl, 12–100 μg/ml caspofungin, 12–75 μg/ml congo red, 10–50 μg/ml calcofluor white 0.001–0.005% SDS, 1.2–2 mM diamide, 2–10 mM menadione, 0.6–3 mM H_2O_2 and 0.4–1.25 mM tBOOH on minimal medium agar plates. Plates were spotted with conidial suspension calibrated at 1×10^5 condia/ml and grown for 72 h at 37 °C. To analyze the expression of *PHZA* in the presence or absence of 4 mM of t-BOOH in the WT, Δ*yap1* Δ*skn7* and Δ*phzA*::*PHZA* strains, fungus was grown for 16 h in AMMC liquid medium at 37 °C and supplemented with or without t-BOOH (4 mM) for 1 h. Total RNA were extracted and DNAse treated; two 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. Each run was assayed in triplicates in a total volume of 25 μl containing the DNA template at an appropriate dilution, 1× AbsoluteSYBR green Fluorescein (Thermo Scientific) and 100 nM of each primer. The primers used for the amplification of *EF1α* as internal control and *PHZA* are listed in the supplemental Table 1. 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 expression ratios were normalized to *EF1α* expression, and calculated according to the ΔΔ*Ct* method (Livak and Schmittgen, 2001). Three independent biological replicates were performed.

2.4. Sensitivity to ROS neutrophils

Whole-blood samples collected from four healthy donors after written consent were obtained from Hôpital Pitié Salpêtrière through the Etablissement Français du Sang (EFS), Paris, France, after approval for the use of this material by the ethics committees of INSERM and the EFS: convention 12/EFS/079. Human PMNs were isolated as previously described (François et al., 2003) and growth inhibition of the Δ*phzA* and WT mutants after contact with PMN was investigated using 96-well plate (Greiner Bio One, Cell Star). Each well received 100 μl of RPMI 1640 medium supplemented with 4% (v/v) fetal calf serum (FCS) containing 5×10^5 conidia and 1×10^5 PMNs. *A. fumigatus* was also cultured alone or with Amphotericin B (2 μg/ml, Sigma) as positive and negative control respectively. After 4 h of incubation at 37 °C in a 5% CO_2 incubator culture, PMNs were lysed with cold sterile water. Conidia were then collected, plated on Sabouraud agar medium and incubated further for 16 h at 37 °C for the counting of the colony forming unit to calculate percentage of viability.

2.5. Pathogenicity of the Δ*phzA* deletion mutant

For infection assays, OF1 Male mice (26–28 g and 7 weeks old) were immunosuppressed with cortisone acetate (Sigma) and cyclophosphamid monohydrate (Sigma) injected

intraperitoneally on day –3 and –1 (112 mg cortisone acetate per kg of mouse and 200 mg cyclophosphamide monohydrate per kg of mouse) before intranasal inoculation of conidia (day 0) for *in vivo* experiments. Each mouse was inoculated intranasally with either 10^6 conidia (or 4×10^7 conidia resuspended in 1 ml of water plus 0.05% Tween 20). After 2 days post-infection, mice were euthanized by asphyxiation (CO_2). Infected lung DNAs were extracted by using phenol–chloroform method and resuspended in RNase/DNase free water. Q-PCR method was used to estimate the proportion of the wild type (WT) *versus* Δ*phzA* mutant strains in the infected lung. The abundance of the two strains was assessed by using specific PCR primers amplifying *PHZA* and *HPH* genes for the WT and the Δ*phzA* mutant strains respectively. Efficiency standard curves and specificities of primers were tested with serial DNA dilutions of each strain and non-infected lung by using cycling conditions below: 95 °C/15 min for one cycle; 95 °C/30 s and 60 °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). Mean values of triplicate qPCR analysis of the same DNA extracts from three different mice are presented as average and correspond to the quantity of fungal DNA of each strains present in 100 ng of infected lung DNA.

2.6. Murine model of corneal infection

The murine model of fungal keratitis has been described (Leal et al., 2012). Parental and mutant *A. fumigatus* strains were cultured in 2% Malt agar with 6% potassium chloride in 25 cm^2 tissue culture flasks. Dormant conidia were disrupted with a bacterial L-loop and harvested in 5 ml PBS. Pure conidial suspensions were obtained by passing the culture suspension through PBS-soaked sterile gauges placed at the tip of a 10 ml syringe. Conidia were quantified using a hemocytometer and a stock was made at a final concentration of 2×10^4 conidia/μl in PBS. Mice were anaesthetized by intraperitoneal (IP) injection of 0.6% Tribromoethanol, 1.2% tert-butyl alcohol, and PBS. Corneal epithelium was abraded with a 30 gauge needle. Through the abrasion was inserted a 33-gauge Hamilton needle and a 2 μl injection containing 4×10^4 conidia was released into the corneal stroma. Mice were examined daily under a stereomicroscope for corneal opacification, ulceration, and perforation. At set time points, animals were euthanized by CO_2 asphyxiation, and eyes were either placed in 10% formalin and embedded in paraffin and sectioned at 5 μm intervals, or excised and placed in 1 ml of sterile saline and homogenized for quantitative culture. MetaMorph imaging software was used to quantify the percent area of opacity and the integrated corneal opacity as described in Leal et al., 2012. All animals were bred under specific pathogen-free conditions and maintained according to institutional guidelines.

2.7. Statistical analysis

At least three biological replicates were performed per experiment. The statistical significance of the results was evaluated by a one-way variance analysis and means comparison by Student's *t*-test ($p < 0.05$) using the JMP software (SAS Institute, Cary, NC, USA). In the figures and table 1, values followed by different stars (*) are significantly different with the *p*-value at least $p < 0.01$.

3. Results and discussion

3.1. Phenotypic characterization of the Δ*phzA* mutant strain

The genome of *A. fumigatus*, *A. nidulans* and *C. albicans* contains a single putative *PPZ* gene, whereas the yeast *S. cerevisiae* and *S. pombe* contain two *PPZ* genes (Leiter et al., 2012). The *PHZA* gene of *A. fumigatus* (AFUA_2G03950) has a predicted amino acid sequence composed of three motifs characteristic of the Ppz phosphatase family (Supplemental Fig. 2): at the N terminus, a myristoylation motif that allows its membrane localization; a short Arg/Ser rich motif which was described to be important for salt tolerance but not for cell wall integrity in *Debaryomyces hansenii* and *S. cerevisiae* and (iii) the C-terminal catalytic region (about 300 aa) of PhzA similar to PP1 subfamily (Minhas et al., 2012).

The Δ*phzA* deletion mutant was constructed as described in Supplemental Fig. 1. The Δ*phzA* strain did not show any difference in growth rate with the WT and the reconstituted strains after incubation for 48 h at 37 °C or under heat shock at 45 °C on malt or minimal medium agar plates (Fig. 1A). However, the colonies of the Δ*phzA* deletion mutant were fluffy at 45 °C associated to a slight reduction of conidiation in the mutant as compared to the WT strain (Table 1). One week old conidia from the Δ*phzA* mutant were viable and able to germinate on solid agar medium with a rate similar to the parental strain (Fig. 1B).

In yeast, Ppz proteins were described as involved in pH homeostasis, salt tolerance, cell wall integrity, and oxidative stress (Clotet et al., 1996; Leiter et al., 2012; Posas et al., 1993, 1995). The susceptibility of the Δ*phzA* strain to different pH, KCl, NaCl, and LiCl or SDS and caffeine was identical to the parental strain (Supplemental Fig. 3). No growth difference were seen between the Δ*phzA* mutant and WT strains in presence of calcofluor white and caspofungin, while a slight sensitivity to congo red for the mutant was observed (Supplemental Fig. 4).

In contrast, the Δ*phzA* mutant showed an increased susceptibility to reactive oxidants such as menadione, H_2O_2 and ter-butyl peroxide (t-BOOH) (Fig. 2). The combination of congo red and calcofluor white compounds with the t-BOOH increased the susceptibility of the Δ*phzA* mutant to oxidative stress (Fig. 3). In contrast, the combination of caspofungin, a specific inhibitor of β-1.3 glucan synthesis that has a very low MIC in the Δppz mutant in *S. cerevisiae* (Parsons et al., 2006), in combination with t-BOOH did not increase the susceptibility of the mutant strain to oxidative stress. PPZs from Aspergillus and Neurospora were able to complement the Δppz1 deletion mutant of *S. cerevisiae* and *S. pombe* regarding to salt tolerance and sensitivity to caffeine supporting the assumption that *PHZA* is a true functional ortholog and is able to perform the same function in filamentous fungi and yeast (Leiter et al., 2012; Vissi et al., 2001). The results obtained in *A. fumigatus* suggested that the main function of *PhzA* is to be involved in tolerance to oxidative stress and that the sensitivity of the Δ*phzA* mutant to these cell wall inhibitors is only a secondary down-stream effect of the deletion. The CW perturbations and especially changes in permeability due to the Δppz deletion are more important in yeast than Aspergillus. Alterations of the ROS metabolism and especially dysfunction of the mitochondria has been linked to cell wall defects (Qu et al., 2012; Singh et al., 2012).

The transcription factors Skn7 and Yap1 play a major role in regulating the response to the oxidative stress *in vitro* and *in vivo* (Lamarre et al., 2007; Qiao et al., 2008). A cooperative role between Skn7 and Yap1 in the transcriptional regulation of genes in response to oxidative stress has been shown. Thus, deletion mutants of the Skn7 and Yap1 in *A. fumigatus* as well as *S. cerevisiae* were described to be highly sensitive to reactive oxidants, in particular t-BOOH and H_2O_2 (Lamarre et al., 2007; Qiao et al., 2008). To determine if the expression of the *PHZA* gene was increased during oxidative stress and if it was under the control of Yap1 or Skn7, PHZA expression was measured in Δ*yap1* and Δ*skn7* mutants and parental strains before and after oxidative stress induced by t-BOOH (Fig. 4). Quantitative RT-PCR data showed that the expression of *PHZA* was increased 16-fold in the presence of t-BOOH in the WT, reverting, and Δ*yap1* strains. In contrast, the expression level of *PHZA* was 4-fold reduced in the Δ*strain in terms of sensitivity to ROS mirrored skn7* mutant in the presence of t-BOOH compared to the WT. These results suggested that Skn7 regulate the *PHZA* gene during oxidative stress. Moreover, the phenotype of the Δ*phzA* strain in terms of sensitivity to ROS mirrored completely the Δ*skn7* mutant (Lamarre et al., 2007). This result suggested that *PHZA* is the first gene identified as under the exclusive control of Skn7. Earlier studies have suggested that *DNM1* and *OLA1* were under the strict control of Skn7 in *S. cerevisiae* but this result was invalidated later when it was shown that Yap1 and Skn7 interact together during oxidative stress (Mulford and Fassler, 2011).

3.2. Δ*phzA* sensitivity to human neutrophils and fungal virulence on different experimental models

To test whether ROIs are involved in the killing of conidia by human PMN, conidia of the WT strain, the Δ*phzA* and Δ*skn7* mutant strains were co-incubated for 4 h with freshly isolated human PMN (Fig. 5). After 4 h of co-incubation, the counting of the CFU (colony forming unit) showed that the Δ*phzA* and Δ*skn7* mutant strains displayed higher sensitivity to human PMN than the parental strain. Indeed, whereas ∼50% of the WT conidia were still alive after 4 h of co-incubation, only ∼25% of the Δ*phzA* and Δ*skn7* mutant conidia were alive after the same incubation time.

The virulence of the Δ*phzA* mutant strain was investigated in two different murine models of infection. Firstly, the virulence of the Δ*phzA* mutant was estimated in a mixed-infection with the WT and reconstituted strains in an immuno-compromised murine model of pulmonary aspergillosis. Immunosuppression was performed by treatment of mice with corticoids and cyclophosphamide producing global repression of the immune response (Latge, 2001). The quantification of the DNA proportion of each strain in the infected lung tissue of three mice was measured by qPCR analysis. After mixed infections of Δ*phzA* with WT or Δ*phzA*: :*PHZA* with WT, no significant difference was found in the quantity of DNA estimated between strains in infected lungs of the immune-compromised mice since the *PHZA/HPH* ratio obtained was 0.92 ± 0.13 for the WT/Δ*phzA* and 0.96 ± 0.17 for the WT/Δ*phzA*: :*PHZA* mixed infections. This result suggested that in an immunocompromised murine model of aspergillosis the mutant and wild type strain have the same pathogenicity.

The virulence of the Δ*phzA* mutant strain was also examined in a well-established immuno-competent murine model of corneal infection (Fig. 6). Conidia from WT, Δ*phzA* and

reconstituted strains were injected into the corneal stroma of mice, and corneal disease and fungal survival were examined (as described by Leal et al., 2012). As shown in Fig. 6A, B, corneas infected with WT conidia developed severe opacification after 24 h, which is consistent with prior studies. In contrast, corneas infected with the Δ*phzA* mutant had significantly less corneal opacity than the WT and reconstituted strains. To quantify fungal growth/survival, infected eyes were homogenized and CFU was examined. We found significantly less CFU in corneas infected with the Δ*phzA* mutant when compared with corneas infected with either the WT or the reconstituted strain (Fig. 6C). Histological examination shows multiple hyphae in the mice corneal stroma infected with WT or reconstituted strain, but few hyphae in corneas infected with the Δ*phzA* mutant (Fig. 6D). Together, these data indicate that PhzA is a virulence factor in this model of *Aspergillus* infection.

These results pinpoint again the critical choice of the experimental aspergillosis model to investigate the virulence of mutants of *A. fumigatus*. In heavily immunocompromised animals (after cyclophosphamide and cortisone treatment), none of the mutants that display a ROS sensitivity defect consecutive to the deletion of superoxide dismutase, catalase, or the transcription factors Yap1 and Skn7 and the phosphatase PhzA showed a difference in virulence compared to the parental strain (Lamarre et al., 2007; Lambou et al., 2010; Lessing et al., 2007; Paris et al., 2003). These results suggested that in immunosuppressed murine models, the amount of ROS produced is too low to see a virulence defect in ROS scavenging mutants compared to wild type. In contrast, assays in an immunocompetent host such as models of fungal keratitis or cutaneous aspergillosis, mutants with a defect in ROS scavenging enzymes were less virulent than the parental strain (Ben-Ami et al., 2010; Leal et al., 2012).

Even if Ppz phosphatases are well conserved in evolutionarily distantly related fungi, it appears clearly that Ppz proteins from filamentous fungi do not display the same function than in yeast. While Ppz phosphatases from yeasts play an important role in the salt/stress tolerance and cell wall integrity pathways, these functions were not controlled by Ppz in filamentous fungi such as *Aspergillus nidulans* and *A. fumigatus*. *PPZ* genes from these two species are however able to complement the Δ*ppz* mutants from *S. cerevisiae*. This result also suggested that the genes under the control of PhzA and its upstream regulator Skn7 are different in yeast and moulds. However, like *C. albicans*, PhzA is important for the fungal virulence of *A. fumigatus* in immuno-competent mice model, demonstrating the essential role of PhzA in the resistance against reactive oxygen intermediates produced by the immune system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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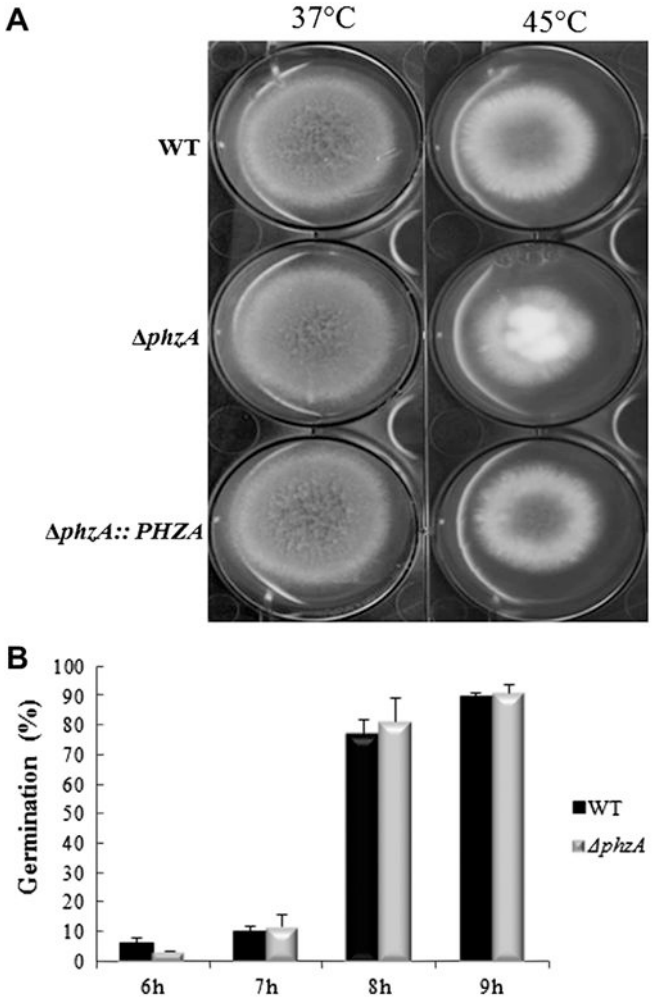

Fig. 1.

Fungal growth and kinetic of germination of the Δ*phzA* mutant strain. The fungal growth of the Δ*phzA* Δ*phzA*: :*PHZA* and the WT strains was estimated after 48 h at 37 °C or 45 °C on malt agar medium (A). Kinetic of germination of the Δ*phzA* and the WT strains were performed on sabouraud agar medium (B).

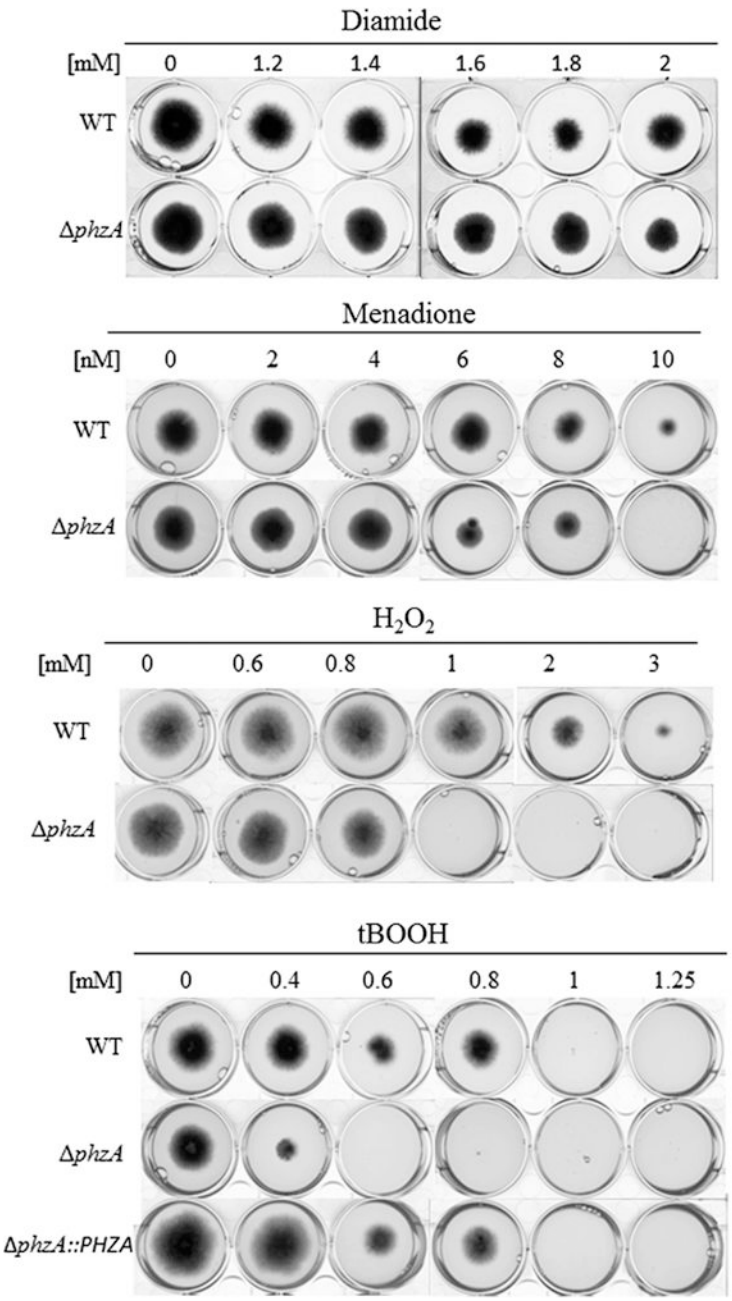

Fig. 2.

Oxidative stress sensitivity of the Δ*phzA* mutant strain. The sensitivity of the different strain to diamide (1.2–2 mM), menadione (2–10 nM), H_2O_2 (0.6–3 mM) and tBOOH (0.4–1.25 nM) were observed after 72 h at 37 °C on minimal medium.

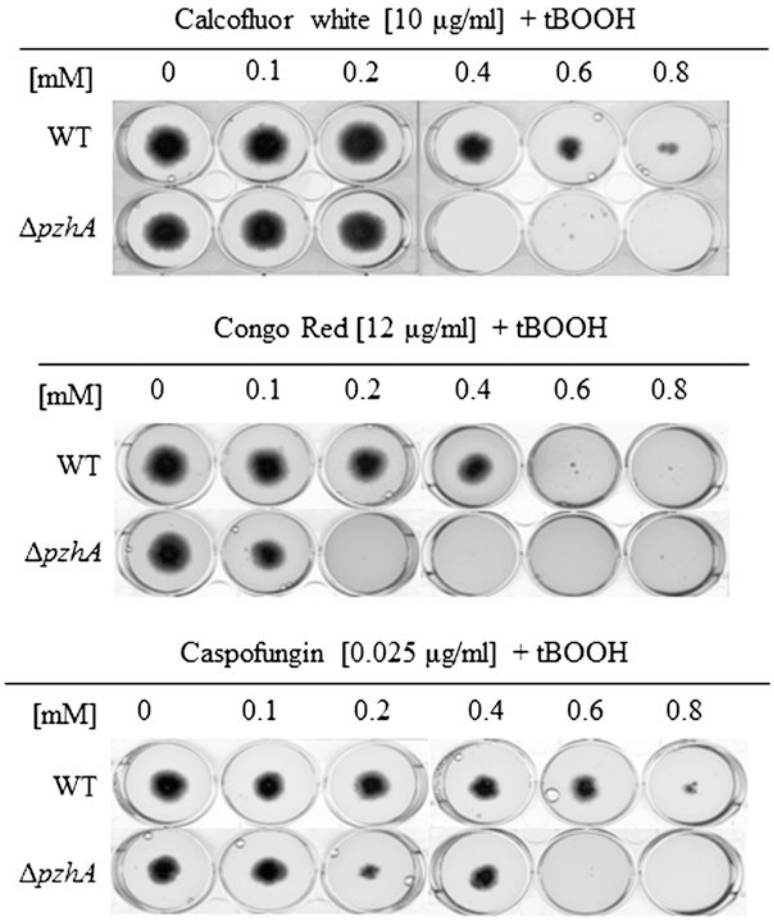

Fig. 3.

Sensitivity of the Δ*phzA* mutant to oxidative stress in combination with cell wall disturbing compounds. The sensitivity of the different strains was observed in presence oxidative stress in combination with cell wall disturbing compounds disturbing compound after 72 h of incubation at 37 °C on minimal medium.

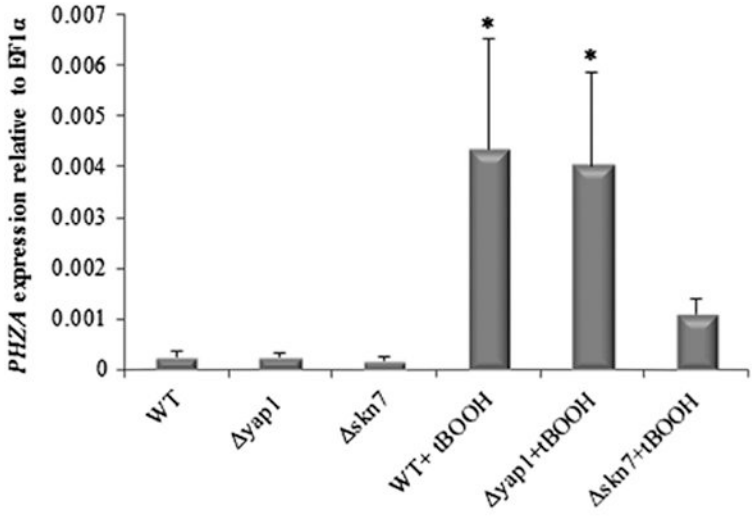

Fig. 4.

Expression levels of *PHZA* in WT, Δ*yap1* and Δ*skn7* strains in presence of t-BOOH. The expression level of *PHZA* in the different strains was estimated after 16 h of culture at 37 °C and after induction of oxidative stress by additional incubation of 1 h at 37 °C in presence of 0.4 mM of t-BOOH. The expression of *PHZA* was related to the *EF1α* as internal control and experiments were done in biological triplicates.

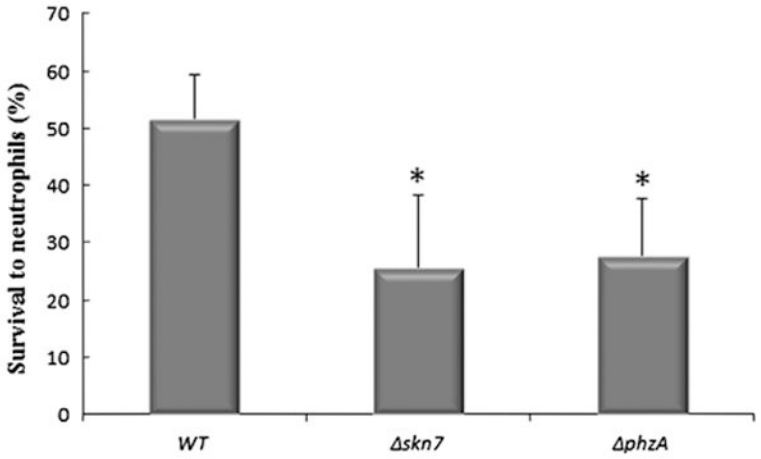

Fig. 5.

Conidial killing of Δ*phzA* and Δ*skn7* mutant strains by human neutrophils. After 4 h of co-incubation with PMN, the percentage of viability of the parental strain, Δ*phzA* and Δ*skn7* mutant strains were established. Viable conidia were quantified by plating after osmotic lysis of PMN and the percentage of viable WT or mutant conidia was calculated in relation to controls without PMN.

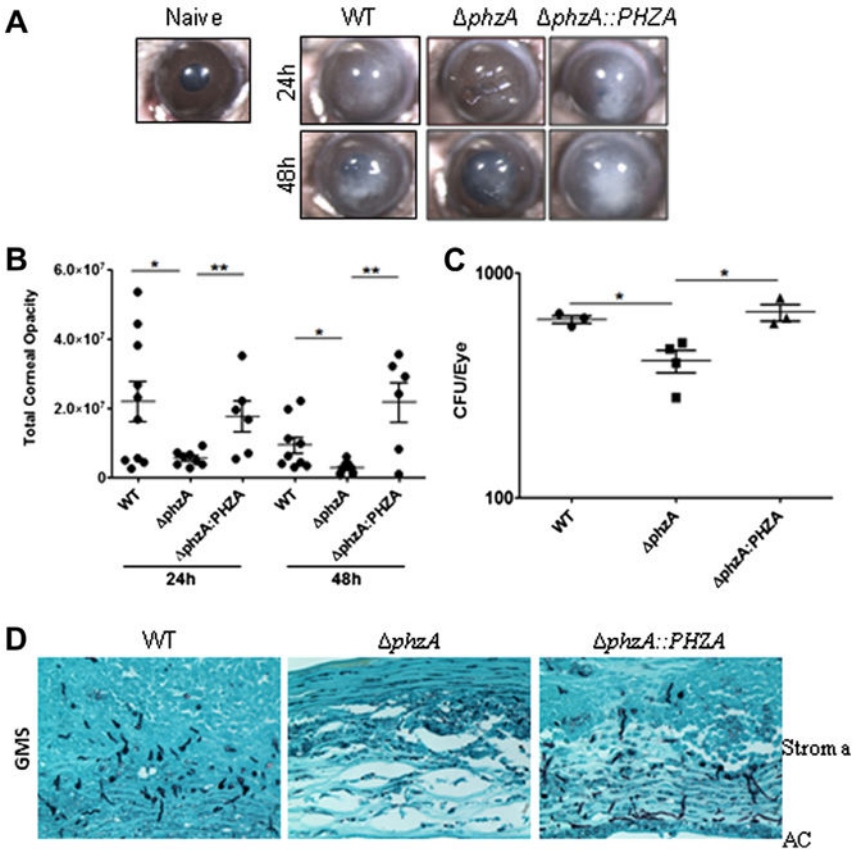

Fig. 6.

Pathogenicity of the Δ*phzA* mutant in mice corneal infection model. C57BL/6 mice infected with conidia isolated from the WT, Δ*phzA* and reconstituted strains. (A) Representative corneas 24 h and 48 h after infection; a transparent cornea of a naïve mouse is shown for comparison. (B) Quantification of corneal opacification by image analysis. (C) CFU 48 h after infection. (D) Representative GMS strains of 5 μm sections of corneas 48 h post-infection showing hyphae in the corneal stroma. (B and C: data points represent individual corneas). Experiments were repeated twice with similar results.

Table 1

Conidiation rate of the Δ*phzA* mutant. The conidiation rate of the different strains was measured by counting after 48 h of incubation at 37 °C or 45 °C on malt agar medium.

Malt medium 48 h	**37 °C**	**45 °C**
WT	$3.82 \times 10^6 \pm 0.10$	$1.87 \times 10^6 \pm 0.11$
Δ*phzA*	$3.77 \times 10^6 \pm 0.13$	$0.44 \times 10^6 \pm 0.16$*
Δ*phzA*: :PHZA	$3.43 \times 10^6 \pm 0.52$	$1.45 \times 10^6 \pm 0.19$

*The definition has been done in the mat et meth section