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A novel family of dehydrin-like proteins is involved in stress response in the human fungal pathogen Aspergillus fumigatus

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ABSTRACT During a search for genes controlling conidial dormancy in Aspergillus fumigatus, two dehydrin-like genes, DprA and DprB, were identified. The deduced proteins had repeated stretches of 23 amino acids that contained a conserved dehydrin-like protein (DPR) motif. Disrupted DprAΔ mutants were hypersensitive to oxidative stress and to phagocytic killing, whereas DprBΔ mutants were impaired in osmotic and pH stress responses. However, no effect was observed on their pathogenicity in our experimental models of invasive aspergillosis. Molecular dissection of the signaling pathways acting upstream showed that expression of DprA was dependent on the stress-activated kinase SakA and the cyclic AMP-protein kinase A (cAMP-PKA) pathways, which activate the bZIP transcription factor AtfA, while expression of DprB was dependent on the SakA mitogen-activated protein kinase (MAPK) pathway, and the zinc finger transcription factor PacC. Fluorescent protein fusions showed that both proteins were associated with peroxisomes and the cytosol. Accordingly, DprA and DprB were important for peroxisome function. Our findings reveal a novel family of stress-protective proteins in A. fumigatus and, potentially, in filamentous ascomycetes.

INTRODUCTION

The human pathogen Aspergillus fumigatus has the remarkable ability to survive extended periods of adverse environmental conditions. This filamentous fungus produces large amounts of dormant asexual spores (conidia). Dormant conidia exhibit resistance to a variety of environmental stresses, including desiccation, extreme temperatures, and osmotic or oxidative stress. When favorable conditions occur, these metabolically inactive cell types will reanimate the cell biochemical and genetic machineries, and alleviate resistance-associated functions. It is expected that the expression of genes involved in the establishment of conidial dormancy would be down-regulated during germination. Indeed, transcriptomics studies initiated at a time the genome was not yet sequenced indicated that around one-fourth of the A. fumigatus genes had transcripts in the dormant conidia, and that the expression of 22% of these genes was down-regulated during germination (Lamarre et al., 2008), leading to the hypothesis that at least some of these genes would be essential for the establishment and control of conidial dormancy. Using this strategy led us to identify Afu4g00860, a gene constitutively transcribed in dormant conidia, the expression of which was down-regulated within 30 min in yeast extract peptone-dextrose (YPD; Lamarre et al., 2008). This gene codes for a protein of unknown function harboring five repeated domains and was called DprA based on the amino acid repeats. Another gene, encoding nine such domains, was also found in the genome of A. fumigatus (DprB: Afu6g12180). This study reports on the functional analysis of DprA and DprB. Phenotype analysis of disruption mutants indicated the involvement of DprA and DprB in specific stress responses. The use of signal transduction mutants in...
monitoring Dpr expression levels suggested convergence of several pathways on the regulation of Dpr genes. Subcellular localization revealed that both proteins were associated with the cytosol and peroxisomes. This study uncovers novel proteins involved in stress protection in *A. fumigatus*.

### RESULTS

**DprA (Afu4g00860) and DprB (Afu6g12180) encode fungal dehydrin-like proteins**

The gene Afu4g00860 was retrieved from an expression profiling study conducted in *A. fumigatus* aiming at the identification of germination-regulated genes (Lamarre et al., 2008). The deduced sequence corresponded to a protein containing 246 amino acids. Scanning of the sequence by prediction software revealed conserved discrimination signatures. The DPR motif is indicated with a red border. Asterisks designate a predicted phosphorylation site conserved in all DPR domains.

| D1A | 25 | GPHESDRPAKVDPRVDSAR | 47 |
| D2A | 57 | GPHESDRIKNDPRVDSAR | 79 |
| D3A | 113 | GPHESRANKLPRVDSAR | 135 |
| D4A | 170 | GPHESERKIGNDPRVDSAR | 192 |
| D5A | 216 | GPHESRANKLPRVDSAR | 238 |
| D1B | 45 | GPHESRATKIVDFPRVDSL | 67 |
| D2B | 111 | GPHESKRFASKPRVDSAR | 133 |
| D3B | 143 | GPHESRANKVDFPRVDSL | 165 |
| D4B | 194 | GPHESKFLKIVDFPRVDSL | 216 |
| D5B | 226 | GPHESRANKVDFPRVDSL | 248 |
| D6B | 273 | GPHESRANKVDFPRVDSL | 295 |
| D7B | 305 | GPHESRANKVDFPRVDSL | 327 |
| D8B | 360 | GPHESRANKVDFPRVDSL | 382 |
| D9B | 403 | GPHESRANKVDFPRVDSL | 425 |

**FIGURE 1:** Alignment of the DPR domains from DprA and DprB. Conserved amino acids are boxed in black (identical) or gray (similar). D1A-D5A designate the five domains from DprA and D1B-D9B refer to the nine domains from DprB, numbered from N- to C-terminal end. Numbers indicate the amino acid positions. The DPR motif is indicated with a red border. Asterisks designate a predicted phosphorylation site conserved in all DPR domains.

**DprA and DprB are down-regulated upon conidial germination**

Expression of DprA and DprB was assessed during conidial germination (Figure 2A). In dormant conidia (time 0), DprA transcripts were ~120 times more abundant than DprB. Both transcript types underwent significant down-regulation with a 1000- and a 350-fold decrease in expression level from 0 to 30 min, respectively. Very weak expression of DprA was detected beyond 30 min, at least up to 24 h. In contrast, DprB transcripts became abundant again after 8 h and reached a twofold increase at 24 h.

To monitor the expression during development, the coding sequences of DprA and DprB were fused to eGfp under the control of their own promoters. DprA-eGfp fluorescence was detected only in dormant conidia (Figure 2B). In agreement with the real-time PCR data, DprB-eGfp fluorescence was observed in dormant conidia. However, in contrast to DprA-eGfp, DprB-eGfp was also observed in the conidiophores (Figure 2C) and in hyphae (unpublished data), indicating DprB was associated with late stages of development, unlike DprA.

**DprA is involved in the oxidative stress response of conidia**

Growth of DprAΔ (but not DprBΔ) mutants was inhibited by oxidative stress generated by hydrogen peroxide or paraquat at concentrations > 2 mM (Figure 3A). Consistent with this, DprA expression was up-regulated upon treatment with 2 mM H2O2 or 2 mM paraquat (Figure 3B). In the absence of stress, no difference was observed between the germination curves of the mutant and control strains (Figure 3C). However, when 2 mM H2O2 was added to the medium, germination of the DprAΔ (and DprAΔ DprBΔ) mutants was impaired (Figure 3D). After 13 h, germination had reached its maximum stage. At that time point, only 30% of the mutant conidia had undergone swelling, compared to 70% for the control strains. This defect in swelling, and subsequently in germination, could be explained by the higher susceptibility of DprAΔ conidia to the fungicidal effect of H2O2. To check the behavior of conidia challenged with host-derived reactive oxidant species, conidial survival was assessed after 36 h in the lungs of immunocompetent mice. Accordingly, the conidia of the DprAΔ and DprAΔ DprBΔ mutants were hypersensitive to killing by the lung phagocytes (Figure 3E). However, no difference was observed in the virulence of the strains in two experimental models of invasive aspergillosis (Figure S2).

**DprB is involved in the osmotic stress response**

When grown in the presence of sorbitol, DprBΔ (in contrast to DprAΔ mutants), displayed an abnormal colony morphology, which could be observed as from 0.5 M sorbitol (Figure 4A). DprBΔ colonies were restricted to forming a “central zone,” in which conidiation would take place, with no “peripheral zone,” in which the colony would normally expand by apical extension of the hyphae. With 1.5 M sorbitol, the DprBΔ colonies had one-half the diameter of the control strain colonies. Microscopic observation of the colony edges showed extensive branching (Figure 4B). The results were similar irrespective of the osmoticum (sorbitol, glycerol, mannitol, NaCl, KCl) or medium (Sabouraud, YPD, minimal medium, malt extract; unpublished data) used. Addition of 1.5 M sorbitol to the medium did not affect the germ tube emergence nor the germination kinetics of the mutant conidia compared with the control strains (unpublished data), indicating that osmostress did not affect the DprBΔ mutants at the conidial stage, but rather at later stages of development. When the wild-type strain was subjected to sorbitol concentrations ranging from 0 to 1 M, DprB transcript levels were up-regulated (Figure 4C). Regulation was dose-dependent, with an expression peak with 0.75 M sorbitol.

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The DprBΔ mutant displays a pH-dependent phenotype that is PacC-related

Growth of the DprBΔ (but not of the DprAΔ) mutant was impaired at pH 7 and pH 9, but not at pH 5 (Figure 5A). At pH 9, after 72 h of growth, the DprBΔ colonies had one-half the diameter of the control strain colonies (unpublished data). Real-time PCR indicated DprB was expressed preferentially at neutral or alkaline pH (Figure 5B), consistent with the phenotype of the DprBΔ mutant. As seen in the assays under osmostress, germination of the conidia was not affected by pH stress (unpublished data). The PacC transcription factor controls pH-regulated genes in Aspergillus spp. (Tilburn et al., 1995). A putative binding site of PacC (GCCAGG) was detected in the promoter of DprB at position −264. The binding of recombinant PacC to the DNA sequence was confirmed (Figures 5C and S3). In Aspergillus spp., PacC acts as both an activator of alkaline-expressed genes and a repressor of acid-expressed genes. Loss-of-function mutations of PacC (PacCΔC) cause an acidity-mimicking phenotype and result in an increased expression of acid-expressed genes, and a reduced expression of alkaline-expressed genes. Gain-of-function, alkalinity-mimicking mutations of PacC (PacC+) result in a phenotype opposite that of acidity-mimicking mutations. DprB expression was checked in the alkalinity-mimicking and in the acidity-mimicking strains (Amich et al., 2009). In agreement with a positive regulation of DprB by PacC, the expression of DprB in the acidity-mimicking strain was similar to that in the wild-type, whereas it was overexpressed in the alkalinity-mimicking strain (Figure 5D).

DprA and DprB act downstream of the SakA MAPK

To gain insight into the signaling cascades involved in the recruitment of Dpr proteins, the expression of Dpr genes was assessed in signal transduction mutants from the mitogen-activated protein kinase (MAPK) pathways (SakAΔ, MpkAΔ, MpkBΔ, and MpkCΔ; Du et al., 2006; Reyes et al., 2006; Valiante et al., 2008), the cyclic AMP (cAMP) signaling pathway (AcyΔ, PkaC1Δ, and PkaRΔ; Liebmann et al., 2003, 2004; Grosse et al., 2008), and the calcium/calcineurin transduction pathway (calcΔ; da Silva Ferreira et al., 2007). In contrast with other signaling mutants, DprA and DprB transcripts were not detected in the SakAΔ mutant (Figure 6, A and B), indicating DprA and DprB acted downstream of the stress-activated kinase (SAK) SakA MAPK cascade. In A. fumigatus, the SakA signaling pathway regulates the response to hyperosmotic and oxidative stress (Du et al., 2006; Reyes et al., 2006). An in silico comparative analysis was undertaken to identify targets of SakA that could link the MAPK to Dpr genes. In Saccharomyces cerevisiae, the SakA homologue Hog1 interacts with 4 transcription factors, Msn2, Msn4, Sko1, and Hot1 (Hohmann et al., 2007). However, A. fumigatus lacked clear orthologues of these proteins (Bahn, 2008), indicating that other sets of transcription factors achieved stress-response regulation downstream of SakA. In Schizosaccharomyces pombe, two bZIP-type transcription factors, Atf1 and Pap1, intervene downstream of the SakA-related MAPK cascade in response to environmental stress signals (Toda et al., 1991; Takeda et al., 1995; Shiozaki and Russell,
Aspergillus spp. possess an Atf1 and a Pap1 homologue, and SakA was shown to interact with AtfA in A. nidulans (Lara-Rojas et al., 2011). Mutant strains for AtfA (Afu3g11330) and Yap1 (Afu6g09930) were constructed, and real-time PCR analysis showed that DprA expression was impaired in the AtfAΔ but not in the Yap1Δ mutant, indicating that DprA acted downstream of AtfA (Figure 6, A and B).

The expression of DprA was also affected in the adenyl cyclase mutant AcyAΔ (Figure 6C). When the AcyAΔ strain was grown on medium supplemented with 25 mM cAMP, the wild-type expression level of DprA was restored, indicating regulation by the cAMP-related pathway. Consistent with this finding, putative cAMP-responsive element (CRE) sequences were found in the promoter of DprA at positions −142 (TGACGTAA) and −23 (GAACGTCA), to which a recombinant AtfA protein was able to bind (Figures 6D and S3).

DprA and DprB are induced by DTT

A major class of stress-protective molecules is represented by molecular chaperones. These molecules are essential for cells to prevent the aggregation of partially unfolded proteins. This requirement is increased when cells experience protein unfolding stresses. Upon treatment with dithiothreitol (DTT), an inducer of the unfolded-protein response, significant up-regulation of DprA and DprB expression was observed (Figure 7), suggesting a potent role of the corresponding proteins as molecular chaperones.

DprA and DprB fused to eGfp are associated with the cytosol and the peroxisomes

To check their subcellular localization, DprA and DprB were fused at their carboxy-terminal end to eGfp, under the control of their native promoters, in the respective mutant strains. The functionality of the constructs was checked by the restoration of the wild-type phenotype (unpublished data). Both DprA-eGfp and DprB-eGfp fusion proteins accumulated in the cytoplasm and in punctuate organelles. The labeled organelles did not stain with the membrane- and endocytosis-selective dye FM4–64 (Supplemental Movie S1; Fischer-Parton et al., 2000). To test the hypothesis that the organelles might be peroxisomes, the DprA- and DprB-eGfp strains were transformed with a plasmid bearing a DsRed-serine-lysine-leucine (DsRed-SKL) fusion typical of the type 1 peroxisomal targeting sequence PTS1 (Ruprich-Robert et al., 2002; Elleuche and Pöggeler, 2008). Colocalization of eGfp and DsRed showed that DprA and DprB were associated with peroxisomes (Figure 8 and Movie S2).

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DprΔ mutants have altered catalase and β-oxidation activities

Peroxisomes contain a large battery of enzymes that are important notably for oxygen species detoxification and β-oxidation of fatty acids. Catalases have a protective role against \( \text{H}_2\text{O}_2 \) and have been shown to be localized in peroxisomes (Schrader and Fahimi, 2006).

A. fumigatus possesses three catalase activities: CatA, which is produced exclusively in conidia, and Cat1 and Cat2, which are produced in the mycelium (Paris et al., 2003). In an attempt to explain the higher sensitivity of \( \text{DprA}\Delta \) conidia to \( \text{H}_2\text{O}_2 \), catalase activity was assessed in conidial protein extracts. As shown by in-gel detection, activity due to the conidial catalase CatA was reduced in the \( \text{DprA}\Delta \) mutant (Figure 9A). This was not the case for the mycelial catalases.
Growth of the DprBΔ mutant was impaired when short-chain fatty acids (butyrate [C4], valerate [C5], hexanoate [C6]) were used as sole carbon sources (Figure 9B), but not with longer-chain fatty acids such as oleic acid (C18), or Tween 20, whose major component is lauric acid (C12; unpublished data). In Aspergillus spp., β-oxidation of short-chain fatty acids with odd-numbered carbons (notably valerate) requires the peroxisomal β-oxidation pathway (Hynes et al., 2008). The two results are in agreement with a peroxisomal association of Dpr proteins.

DISCUSSION

Dpr genes encode specific stress-response proteins

While the DprAΔ mutant was impaired in growth on media supplemented with H2O2 or paraquat, the DprBΔ mutant growth was affected under hyperosmotic conditions or when pH was maintained at 7 or 9. Other stress conditions, e.g., extreme temperatures (storage at −20°C or 4°C, culture at 50°C), drug treatments (with caspofungin, Congo red, calcofluor white, or SDS), or high concentrations of metal ions (Fe2+, Cd2+, Co2+, Zn2+, or Ni2+) had no effect on the development of the mutants (unpublished data), indicating DprA and DprB were involved specifically but differently in oxidative, osmotic, and pH stress.

The protection effect exerted by Dpr proteins against stress was confirmed by overexpression studies of DprA and DprB that resulted in enhanced resistance to oxidative stress, and osmotic and pH stress, respectively (Figure S4). This is in good accordance with studies related to the overexpression of dehydrins and molecular chaperones, in either homologous or heterologous systems (Swire-Clark and Marcotte, 1999; Brini et al., 2007; Montero-Barrientos et al., 2008), although the stresses involved were different.

An excellent correlation was obtained between expression levels under stress and mutant phenotypes, suggesting that Dpr regulation was predominantly transcriptional. Gene expression was modulated transiently within the time frames described previously in filamentous fungi for the regulation of oxidative stress (30–60 min; Li et al., 2008), osmotic stress (30–90 min; Han and Prade, 2002), and the pH response (30–120 min; Galindo et al., 2007), indicating a genetic program involving DprA and DprB was rapidly switched on to reestablish homeostasis. However, posttranslational regulation cannot be excluded, since putative phosphorylation sites were detected (Figures 1 and S1).
Signal transduction pathways involved in Dpr-related stress response

In this study, we showed that several pathways were involved in the regulation of Dpr genes. A hypothetical model is proposed in Figure 10. Common to Dpr gene regulation, the SakA pathway was known to control stress responses, either oxidative- (Nguyen et al., 2000; Du et al., 2006; Hagiwara et al., 2009; Zhang et al., 2009; Balazs et al., 2010) or osmotic-related (Han and Prade, 2002; Liu et al., 2008; Zhang et al., 2009). Together with the cAMP-related pathway, SakA contributes to the oxidative stress response mediated by DprA. The AtfA transcription factor, known to intervene downstream of the cAMP-related pathway (Rehfuss et al., 1991; Neely and Hoffman, 2000), is activated by SakA, most probably upon phosphorylation (Lara-Rojas et al., 2011). The acquisition of DprA-related oxidative stress tolerance in the conidia is consistent with the fact that Pka- and AtfA-mediated oxidative stress tolerance as Aspergillus spp. is acquired specifically in conidia (Kawasaki et al., 2002; Zhao et al., 2006; Hagiwara et al., 2008). Upon osmotic stress, transcription of DprB was dependent on the SakA-related pathway and on a hypothetical osmotic stress regulator, OsrA. The SakAΔ strain was unaffected by pH stress, whereas the PacC mutants were unaffected by osmostress (unpublished data), suggesting the two pathways converged independently on DprB.

The involvement of SakA in two distinct responses raises the question of signaling specificities. In S. cerevisiae, the Fus3- and the Kss1-related MAPK pathways converge on the Ste12 transcription factor to regulate mating or filamentation, respectively, in response to different stimuli. Signaling specificity is achieved by selective ubiquitination and sumoylation of Ste12 and its cofactor Tec1, leading to either degradation or protection of the respective transcription factors (Bruckner et al., 2004; Chou et al., 2008). Cross-activation of AtfA and OsrA in A. fumigatus could be prevented by a similar interplay between ubiquitination and sumoylation. This hypothesis is supported by the presence of two sumoylatable sites on AtfA (positions 399 and 474). Alternatively, dual phosphorylation of SakA by specific phosphatases could lead to the activation of different downstream effectors or OsrA could interact with SakA in the absence of phosphorylation, as shown for Hot1 (Saito and Tatebayashi, 2004).

Possible cellular function of Dpr proteins

Widely distributed in eukaryotes, intrinsically unstructured proteins (IUPs), which lack defined folding and three-dimensional structures, have high intramolecular flexibility (Tompa, 2002; Dyson and Wright, 2005). Dehydrins are IUPs that typically accumulate in plants in response to environmental stimuli with a dehydrative component (notably drought), salinity, and seed maturation (Battaglia et al., 2008). They possess one or several K-segments (EKKGIDIKKEKLPG) that are presumed to interact with key cellular regulators prone to misfolding, which allows their stabilization in a chaperone-like manner (Close, 1996; Kovacs et al., 2008), thus precluding aggregation of proteins fatally damaged by stress. Tompa and Kovacs (2010) proposed that in virtue of their structural plasticity, IUPs may serve as potent chaperones. The possible role of Dpr proteins as molecular chaperones is supported by several lines of evidence: 1) Dpr genes are induced under stress. 2) DprA and DprB expression is up-regulated upon addition of DTT, an inducer of protein misfolding. 3) The flexible structure of Dpr proteins, proline residues, and phosphorylation sites within the DPR domains are compatible with the physicochemical features expected in chaperones (Tompa and Kovacs, 2010). 4) Their association with the cytoplasm and the peroxisomes is consistent with the shuttling of peroxisomal precursors from the cytoplasm, where they are synthesized, to
the peroxisomes, where they are addressed (Götte et al., 1998; Hettema et al., 1998).

The absence of peroxisome-targeting signal (PTS) is in favor of a peripheral association of Dpr proteins with the peroxisomes. Other proteins, notably peroxins, which are known to be associated both with the cytosol and the peroxisomes, participate in peroxisomal protein import and do not exhibit any obvious PTS (Pex1 [Tamura et al., 2006], Pex5 [van der Klei et al., 1995], Pex7 [Marzioch et al., 1994], Pex19 [Sacksteder et al., 2000], Pex18/21 [Purdue et al., 1998]). Such proteins are either “piggybacked” by partners that have a PTS (Glover et al., 1994; Lee et al., 1997; Klein et al., 2002; Islinger et al., 2009) or are cytosolic proteins associated with the outer sides of peroxisomes (Götte et al., 1998; Hettema et al., 1998; Purdue et al., 1998).

Molecular chaperones associated with proteins being imported to the peroxisomes belong predominantly to the heat shock protein Hsp70/Hsp40 family, members of which were first identified due to their specific induction during the cellular response to stress conditions. Requirement of members of the chaperone Hsp70/Hsp40 family for peroxisomal matrix protein import was demonstrated (Walton et al., 1994; Legakis and Terlecky, 2001). Other molecular chaperones involved in peroxisomal protein import include the peroxin Pex19 (Jones et al., 2004; Vizeacoumar et al., 2006) or the ankyrin repeat protein Ankr2A (Shen et al., 2010).

In conclusion, our study uncovered novel proteins involved in the protection against stress. DprA and DprB likely act in the cell as molecular chaperones. Their identification from a conidial dormancy transcript profiling study points out the necessity for the fungus to exhibit a high level of stress resistance during the dormancy period.

**MATERIALS AND METHODS**

**Strains and growth conditions**

The A. fumigatus strains used in this study are listed in Supplemental Table S1. The strain AkuB (da Silva Ferreira et al., 2006) was used as the recipient strain for genetic transformation, unless otherwise specified. All strains were maintained on 2% malt agar slants at 25°C. Conidia were recovered following a 7-d culture at 25°C, in sterile 0.05% Tween 20, and filtered through a 40 μm pore size filter (BD Falcon, le Pont de Claix, France). Recombinant proteins were produced in Escherichia coli BL21 (DE3) or Pichia pastoris GS115.

**In silico analysis**

Blast analyses were submitted to the National Center for Biotechnology Information facility (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Estimation of intrinsic protein disorder was done with PrDOS (http://prdos.hgc.jp/cgi-bin/top.cgi; Ishida and Kinoshita, 2007). Prediction of phosphorylation sites was done with NetPhos software (http://www.cbs.dtu.dk/services/NetPhos/). The hydrophobic cluster analysis (Gaboriaud et al., 1987) plots were generated by the Mobyle Project’s HCA server (http://mobyle.rpbs.univ-paris-diderot.fr/cgi-bin/portal.py?form=HCA). Prediction of sumoylation...
Construction of A. fumigatus deletion, overexpression, and fusion strains

The cassettes used for the genetic transformation of A. fumigatus were generated by fusion PCR, or inverse PCR combined with conventional cloning, as described previously (Karababa et al., 2006; Lamarre et al., 2007). The primers are listed in Tables S2 and S3 and the constructs are schematized in Figures S5–S7. The deletion constructs consisted of a selection marker cassette encompassed by ~1 kb flanking regions of the target gene. Complementation cassettes were made out of 1 kb of the 5’ flanking region, followed by the wild-type coding sequence and the selection marker cassette. The resistance genes to hygromycin (Hph, amplified from pAN7.1; Punt and van den Hondel, 1992) or phleomycin ( Ble, amplified from pAN8.1; Punt and van den Hondel, 1992) were used to construct the cassettes. The double mutant was constructed by deletion of DprA in the DprΔ mutant. For the overexpression of DprA and DprB, the genomic sequences of DprA and DprB encompassed with 1 kb upstream and downstream regions were amplified and cloned into the pPTRII vector (Takara, Saint-Germain-en-Laye, France) in the HindIII and Smal restriction sites, respectively. For the subcellular localization of DprA and DprB, eGfp (Thastrup et al., 1999) was fused to the carboxy-terminal ends and the expression was driven by 1 kb of the gene native promoters. The mutant strains DprΔA and DprΔB were cotransformed with the eGfp fusion cassettes together with pAN8.1 or the pAN7.1 linearized with Ndel or HindIII, respectively. For colocalization experiments with the SKL-DsRed construct, the A. fumigatus strain CBS144–89 was used as the recipient strain for the eGfp fusions. The strains obtained were cotransformed with the plasmid bearing the linearized SKL-DsRed construct (Elleuche and Pöggeler, 2008), together with linearized pAN7.1 or pAN8.1, respectively. Transformation of A. fumigatus was carried out by conidial electroporation, as previously described (Lambou et al., 2010). The transformants were verified by Southern blot analysis and real-time PCR.

Phenotype analyses

Phenotypic tests were carried out mostly in Sabouraud medium (2% glucose, 1% mycopedone; Oxoid, Dardilly, France). When mentioned, YPD, 2% malt extract, or minimal medium (Cove, 1966) were used as alternatives. For pH response assays, Sabouraud medium was amended with 0.1 M Tris, MOPS, or HEPES, according to the desired buffer range. For carbon source assays, ouraud medium was amended with 0.1 M Tris, MOPS, or HEPES, 10 mM ammonium chloride, instead of ammonium tartrate, was added to minimal medium. In-gel catalase activity and germination rates were determined as described previously (Paris et al., 2003; Du et al., 2006).

Production of recombinant proteins and gel-mobility shift assays

The plasmid pGEX-PacC (Tilburn et al., 1995) was used to transform E. coli. The fusion protein was produced and purified on glutathione agarose (Sigma, Saint Quentin Fallavier, France) according to the manufacturer’s instructions. The coding sequence of AtfA was amplified from A. fumigatus AkuB cDNA with the primers listed in Table S4, and cloned into the pKJ113 vector (Borg-von Zeipel et al., 1998) between the Xhol and Notl restriction sites. Following transformation of P. pastoris, the recombinant 6×His-tagged protein was produced and purified on ProBond resin (Invitrogen, Cergy Pontoise, France) as suggested by the manufacturer. The DNA sequences used as probes are listed in Table S4. Probe labeling and gel-mobility shift experiments were carried out as described by Herbert et al. (2002).

Real-time PCR

Total RNA (5 μg) was reverse-transcribed using the Superscript II Reverse Transcriptase kit (Invitrogen, Cergy Pontoise, France). Quantitative PCR assays were performed using 96-well optical plates (Thermo Scientific, Courtaboeuf, France) in an iCycler iQ (170–8470; Bio-Rad, Marnes-la-Coquette, France) according to Bio-Rad’s manufacturer instructions. Each run was assayed in triplicate in a final volume of 20 μL containing the DNA template at an appropriate dilution, 1× ABsolute SYBR green Fluorescein (Thermo Scientific), and 100 mM each primer (Beacon Designer 4.0 software; Premier International Software, Palo Alto, CA; Table S5). The cycling program was 95°C for 15 min, 40 cycles of 95°C for 30 s, and 55°C for 30 s. Amplification of one single specific target DNA was checked with a melting curve analysis at the end of the PCR (+0.5°C ramping for 10 s, from 55°C to 95°C). The generated data were then analyzed with Optical Systems Software 3.1 (Bio-Rad). 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 control templates in which reverse transcriptase was omitted were used for each gene set.

Live-cell imaging

For all microscopy experiments, A. fumigatus was cultured in Lab-Tek 8-well chamber-slides (Nalge Nunc International, Roskilde, France) containing Vogel’s minimal medium N (Vogel, 1964). Wide-field epifluorescence imaging was performed on an inverted Nikon TE2000 microscope equipped with a Hamamatsu Orca ER CCD camera and a pE-2 LED excitation system (CoolLED) as the epifluorescence light source. Exposure times ranged from 200 to 400 ms; z-stacks of optical sections at 0.5 μm steps. Images were processed through 10 iterative deconvolutions using AutoQuant X software (Media Cybernetics, Bethesda, MD). For time-course experiments, images were acquired every 30 s for 10 min. Movies were generated and manipulated using ImageJ software (http://rsbweb.nih.gov/ij/). For membrane staining, germlings were incubated in 10 μM FM4–64 (Molecular Probes, Cergy Pontoise, France) for 30 min at 30°C.

In vivo experiments

Conidial survival assays in mouse lungs were carried out with inoculation of 5 × 10⁸ conidia as described by Lambou et al. (2010). The virulence of the strains was tested in two models of invasive aspergillosis: cyclophosphamide and cortisone acetate-treated mice (Smith et al., 1994) and Galleria mellonella waxworms (Renwick et al., 2006). The virulence in immuno-suppressed mice was as described by Mouyna et al. (2010), except that the inoculation was with 1.5 × 10⁸ conidia, and the cyclophosphamide dosage was 150 mg/kg. For the G. mellonella experiments, waxmoth (about 0.3–0.4 g in body weight) in the final larval stage (R Mous Livebait WOF, Netherlands) was used (10 per strain). 1.5 × 10⁴ spores in phosphate-buffered saline (PBS) per larvae were used for injection into the hemocoel of 10 larvae per strain. The waxmoth larvae were then incubated at 37°C for up to 10 d, and survival was recorded daily.
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