The role of trehalose biosynthesis in *Aspergillus fumigatus* development, stress response and virulence

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Running title: Trehalose in *A. fumigatus*

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ABSTRACT

Aspergillus fumigatus is a pathogenic mold which causes invasive, often fatal, pulmonary disease in immunocompromised individuals. Recently, proteins involved in the biosynthesis of trehalose have been linked with virulence in other pathogenic fungi. We found that trehalose content increased during the developmental life cycle of A. fumigatus, throughout which putative trehalose synthase genes tpsA and tpsB were significantly expressed. The trehalose content of A. fumigatus hyphae also increased after heat shock but not in response to other stressors. This increase in trehalose directly correlated with an increase in expression of tpsB, but not tpsA. However, deletion of both tpsA and tpsB was required to block trehalose accumulation during development and heat shock. The ΔtpsAB double mutant had delayed germination at 37°C, suggesting a developmental defect. At 50°C, the majority of ΔtpsAB spores were found to be non-viable, and those that were viable had severely delayed germination, growth and subsequent sporulation. ΔtpsAB spores were also susceptible to oxidative stress. Surprisingly, the ΔtpsAB double mutant was hypervirulent in a murine model of invasive aspergillosis, and this increased virulence was associated with alterations in the cell wall and resistance to macrophage phagocytosis. Thus, while trehalose biosynthesis is required for a number of biological processes that both promote and inhibit virulence, in A. fumigatus the predominant effect is a reduction in pathogenicity. This finding contrasts sharply with other fungi in which trehalose biosynthesis acts to enhance virulence.
Aspergillus fumigatus is a ubiquitous thermotolerant mold which plays an important role in the recycling of environmental carbon and nitrogen (32, 38). A. fumigatus is also an important opportunistic human pathogen that invades the lungs of immunocompromised patients, causing a progressive pneumonia that can disseminate to the heart, brain and other organs (21). Invasive aspergillosis is a leading cause of death in transplant and leukemic patients, with a mortality rate exceeding 50% despite the best available antifungal therapy (19). This poor response to existing antifungal therapy has led to a great interest in novel therapeutic approaches directed against this organism.

In both the environment and the host, A. fumigatus is exposed to a variety of stressors. While growing in compost, A. fumigatus is commonly found at temperatures exceeding 50°C (5). Similarly, in human tissues, hyphae are subjected to nutrient deprivation and oxidative stress from host immune cells (24, 31). Little is known about the mechanisms by which A. fumigatus survives under these conditions. One possible mechanism by which A. fumigatus adapts to environmental stress is via the biosynthesis of the carbohydrate trehalose, which is involved in mediating the stress response and virulence of other pathogenic fungi such as Candida albicans and Cryptococcus neoformans (1, 22, 30, 48). The trehalose content of these fungal cells may increase up to 50-fold in response to oxidative stress, osmotic stress and heat shock (27).

Trehalose is a non-reducing disaccharide of glucose that is synthesized by bacteria, plants, insects and fungi. In fungi, trehalose functions both as a reserve carbohydrate and as a stress metabolite (37, 39, 40). As a reserve carbohydrate, trehalose is found in vegetative resting cells and spores where it can constitute up to 15% of the dry weight of these structures (46). It is
an important source of energy in fungal development as it is utilized in cell processes such as
glycolysis, sporulation, and germination. In addition, trehalose helps the cell withstand
environmental stress and nutrient limitation. Trehalose molecules protect the cell by preventing
aggregation of denatured proteins and scavenging free radicals (37). Interestingly, trehalose is
absent from mammalian cells, therefore enzymes involved in trehalose biosynthesis have been
considered as potential targets for anti-fungal therapy.

In medically relevant fungi, trehalose is synthesized from glucose. First, the enzyme
hexokinase converts a molecule of glucose into glucose-6-phosphate. Then, trehalose-6-
phosphate synthase (Tps) catalyzes the production of trehalose-6-phosphate from glucose-6-
phosphate and a molecule of UDP-glucose. Finally, a phosphate group is removed from
trehalose-6-phosphate by trehalose-6-phosphate phosphatase (Tpp) to yield trehalose (29, 43).
When trehalose is required for energy, it can be recycled back to glucose through the universal
enzyme trehalase (20). The enzymes involved in fungal trehalose biosynthesis have been well
characterized in S. cerevisiae. These proteins are encoded by four genes: TPS1 which encodes
trehalose-6-phosphate synthase, TPS2 which encodes trehalose-6-phosphate phosphatase, and
TPS3 and TSL1, redundant genes which encode a large regulatory subunit of the synthase
complex (6, 15, 28, 29, 43, 44). All four proteins are tightly regulated both at the genetic and at
the protein level, and form a protein complex to catalyze the synthesis of trehalose (6, 45).

In other fungi, trehalose biosynthesis appears to play similar but not identical roles in
governing growth, stress response and virulence. In the pathogenic yeasts C. albicans and C.
neofomans, the single-copy trehalose-6-phosphate synthase Tps1 is required for normal
oxidative stress response, hyphal development, and has a role in mediating virulence (1, 22, 30,
48). In Aspergillus nidulans, the trehalose-6-phosphate synthase TpsA is necessary for normal
fungal development, thermosensitive growth and response to sub-lethal exposure to oxidative stress (16). In *Aspergillus niger*, two putative trehalose-6-phosphate synthases, TpsA and TpsB have been identified. Inactivation of *tpsA* resulted in a reduction in T6P activity, however the role of TpsA and TpsB in trehalose metabolism has not been examined (47). Importantly, trehalose biosynthesis has not been studied in *A. fumigatus* which is the most important filamentous fungus causing human disease.

We undertook the study of the role of trehalose biosynthesis in *A. fumigatus* development, stress response and virulence. We found that trehalose content increased in *A. fumigatus* hyphae throughout development and when actively-growing hyphae were exposed to heat shock, but not to other environmental stresses. Increases in trehalose content directly correlated with elevated expression levels of two putative trehalose-6-phosphate synthase genes *tpsA* and *tpsB*. Disruption of both *tpsA* and *tpsB* was required to prevent trehalose biosynthesis.

Abolition of trehalose biosynthesis via disruption of both *tpsA* and *tpsB* affected conidial germination, thermotolerance, and high level oxidative stress response *in vitro*. Surprisingly, disruption of *tpsA* and *tpsB* resulted in a strain that was hypervirulent by several measures in a murine model of invasive aspergillosis.

**MATERIALS AND METHODS**

**Strains and growth conditions.** Molecular manipulations were done using the *A. fumigatus* strain Af293 (a generous gift from P. Magee, University of Minnesota). Mutant Af293 strains ΔtpsA, ΔtpsB and ΔtpsAB, as well as complemented strains ΔtpsA::tpsA, ΔtpsB::tpsB and
ΔtpsAB::tpsA were used in this study. The identities of genes deleted in the *A. fumigatus* mutant strains used in this study are Afu6g12950 for *tpsA*, Afu2g04010 for *tpsB*, Afu4g03190 for *tpsC*, and Afu5g14300 for *tpsD*. For most experiments, strains were grown on YEPD agar (1% yeast extract, 2% peptone, 2% dextrose, 1.5% agar) at 37°C. Some experiments required the use of *Aspergillus* minimal medium containing trace elements (Na$_2$B$_4$O$_7$•10H$_2$O, CuSO$_4$•5H$_2$O, FePO$_4$•4H$_2$O, MnSO$_4$•H$_2$O, Na$_2$MoO$_4$•2H$_2$O), salts (KCl, MgSO$_4$•7H$_2$O, KH$_2$PO$_4$), 1% glucose and NaNO$_3$ adjusted to pH 6.5. Media used in specific experiments included *Aspergillus* minimal medium containing 0.5% glucose (*Aspergillus* minimal glucose medium), *Aspergillus* minimal glucose medium supplemented with 0.5% trehalose, and *Aspergillus* minimal glucose medium supplemented with 1M sorbitol. To obtain hyphae, conidia were inoculated at 5×10$^5$ cells/ml into liquid YEPD medium and allowed to germinate and grow in a 37°C incubator shaking at 200rpm. Hyphae were isolated as described previously (25). When developmental time courses were performed, the organisms were collected after 8h, 12h, 18h and 24h of incubation to sample various stages in the *A. fumigatus* life cycle. To test the effect of environmental stressors on hyphae, strains were grown for 12h at 37°C in liquid YEPD, and then subjected for 1h to oxidative shock with 100mM H$_2$O$_2$, osmotic shock with 0.5M NaCl, or heat shock at 50°C. For the effects of stress on conidia, suspensions of 5×10$^5$ conidia/ml were grown in *Aspergillus* minimal glucose medium at 37°C for 5.5h. Samples were either exposed to 1.5mM H$_2$O$_2$ to test prolonged exposure to sub-lethal oxidative stress, or 100mM H$_2$O$_2$ for 10 minutes to test severe oxidative shock.

**Determination of fungal trehalose content.** An enzymatic assay was used to determine trehalose content of the organisms. Trehalose was extracted from 30 mg of hyphae or 10$^7$ conidia
in water by heating at 95-98°C for 3.5h. Samples were then treated with 1M acetic acid and 0.2M sodium acetate. Following centrifugation, supernatants were treated with 0.05U/ml of trehalase from porcine kidney (Sigma) or left untreated as a control. Supernatants were incubated overnight at 37°C to allow for the conversion of trehalose to glucose. Glucose concentration of all samples was assayed using the Glucose (GO) Assay Kit (Sigma) following the manufacturer’s instructions. The glucose concentration of all test samples was calculated from a standard curve after subtraction of untreated control sample values. The trehalose content of these samples was then determined from glucose concentration.

Real-time RT-PCR. Total RNA was extracted from hyphae using the Nucleospin RNA Plant Mini Kit (Machery-Nagel). The RNA was reverse transcribed into cDNA using the M-MuLV reverse transcription kit (Fermentas). After reverse transcription, quantitative real-time PCR was performed as previously described (17). Expression of all genes was normalized to the endogenous house-keeping gene TEF1 (10, 17, 34, 41). The primers for real-time PCR are listed in Table 1.

Strain construction. The genomic sequences and flanking regions of the putative tps genes were retrieved from GenBank®. Analysis of sequences, including homology, was done using DNASAN software. To obtain gene disruption constructs for single ΔtpsA and ΔtpsB null mutants, the split marker strategy for molecular cloning was used to increase the rate of targeted homologous integration (9, 34). In this strategy, two incomplete but overlapping fragments of the hygromycin resistance cassette (hph) fused to ~2 kb of flanking sequences of the tpsA and tpsB genes were generated. These constructs were obtained by fusion PCR as previously described.
(41), using the Expand High Fidelity PCR system (Roche). The primer sets F1-F2 and F3-F4 for each tps gene was used to amplify flanking sequences from total Af293 DNA and the M13F-M13R primer set was used to amplify hph from the pAN-71 plasmid for a first round of PCR, then constructs were generated using primer sets F1-YG and F4-HY in a second round of PCR using products from the first round of PCR as templates (Table 1). The resulting constructs were then integrated into the A. fumigatus genome at the tpsA or the tpsB locus by protoplast transformation, replacing the entire protein coding regions of these genes with an intact hph.

Constructs for the complementation of ΔtpsA and ΔtpsB mutants with tpsA or tpsB were generated using standard molecular cloning techniques. These constructs contained an intact copy of tpsA or tpsB including promoter and terminator sequences for complementation, and the phleomycin resistance gene (ble) as a selection marker. High fidelity PCR was used to amplify the tpsA and tpsB genes including upstream and downstream sequences using F1-F4 primer sets from total Af293 DNA, and ble was amplified using Prom Ble-Term Ble primer sets from the p402 plasmid. Restriction sites were added to these primers where applicable (Table 1). The ΔtpsA complementation cassette was constructed by cloning the tpsA gene within plasmid p402 at the HindIII and SpeI restriction sites. This construct was linearized with ClaI to direct integration of tpsA into its native locus. Likewise, the ΔtpsB complementation cassette was constructed by cloning of the tpsB and ble genes at the NotI restriction sites of the pGEM-T plasmid (Promega). This construct was linearized with SacI to direct integration of tpsB into its native locus.

To create a double disruption of both tpsA and tpsB, tpsA was disrupted in the ΔtpsB mutant background using phleomycin as the second selection marker. The tpsA disruption cassette was constructed within the plasmid p402 in which ~2kb of 5’ and 3’ flanking sequences
of tpsA were cloned into the HindIII-SpeI and XhoI-XbaI sites respectively. Flanking sequences were amplified by high fidelity PCR using F1-F2 and F3-F4 primer sets with generated restriction sites HindIII, SpeI, XhoI and XbaI respectively (Table 1). Finally, to construct strains of ΔtpsAB complemented with tpsA, an intact tpsA gene including 1-2 kb of flanking sequences was amplified by high fidelity PCR from whole genomic Af293 DNA using F1-F4 primer sets, and these products were used directly for fungal transformation.

A. fumigatus was transformed by spheroplasting as described previously (7, 41). Transformants were selected on hygromycin or phleomycin containing plates where applicable. Since the ΔtpsAB mutant was resistant to both phleomycin and hygromycin, selection of ΔtpsAB strains complemented with tpsA was performed using growth at 50°C. Successful integration of all constructs was confirmed at the DNA level by PCR and RNA expression levels of the tpsA gene was confirmed using real-time RT-PCR.

**In vitro characterization of tps mutants.** The size of conidia was assessed using flow cytometry. For preparation, 10^6 freshly harvested conidia were killed with 3% formaldehyde for 1 hour, washed 3 times with PBS+0.1% Tween 80, and resuspended in 300µL of PBS+0.1% Tween 80. Samples were then analyzed with a FACScalibur analyzer (BD biosciences). The estimated volume of mutant conidia was compared to wild-type conidia using forward scatter (FSC).

Germination time courses were performed using 6-well plates in which 5 ml of medium in each well was inoculated with 10^5 conidia and incubated at 37°C. To assess germination, conidia were monitored microscopically and 100 cells of each strain were counted every hour. Germination was scored based on the detection of visible germ tubes elongating from conidia.
Growth and development of each strain was assessed by spot inoculating 10-fold serial dilutions of conidia onto agar plates in triplicate and incubating at 37°C and at 50°C for 2-5 days. Conidial viability of each strain at 50°C as compared to 37°C was determined by quantitative culture. The percent viability at 50°C was determined by expressing the colony forming units at 50°C as a percent of 37°C counts for the same strain. The viability of swollen conidia exposed to oxidative stress was also assessed by quantitative culture and compared with untreated controls.

**Transmission electron microscopy.** *A. fumigatus* conidia were grown on YPD agar (Difco) plates at 37°C and harvested at day 6 with PBS + 0.1% Tween 80 before fixing. Hyphae were obtained by incubating conidia for 24 h in 24-well culture plates containing serum free RPMI 1640 w/o phenol red. All specimens were fixed overnight at 4°C with 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer. Samples were then washed three times with 0.1 M sodium cacodylate buffer, and post-fixed with 1% osmium tetroxide and 1.5% potassium ferrocyanide in ddH$_2$O for 45 min at 4°C. Washed conidia and hyphae were then dehydrated with increasing concentrations of acetone or ethanol (30%, 50%, 70%, 80%, 90%, 100%), respectively, followed by embedding in Epon 812. Ultrathin sections were cut using an ultramicrotome (Reichert-Jung ultra cut E), stained with uranyl acetate and lead citrate, and viewed under FEI Tecnai T12 transmission electron microscope.

**Macrophage phagocytosis assays.** RAW264.7 cells were seeded at a density of $5 \times 10^6$ cells in a 6-well plate (3 ml RPMI-10% FBS, pen/strep) and allowed to adhere for 24h. Cells were then infected with conidia at a multiplicity of infection (M.O.I.) of 10:1. The plates were centrifuged at 800 x g for 5 min to bring the conidia to the bottom of the wells. After 4h of co-incubation at

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37ºC, 5% CO₂, cells and conidia were scraped from the wells and fixed with 4% paraformaldehyde for 30 min. The cell pellets were washed twice, and resuspended in PBS+0.1% Tween 80. Samples were then analyzed with a FACScalibur flow cytometer (BD Biosciences, Mississauga, ON). Analysis was done using the FlowJo v8.8.6 program (Tree Star, Ashland, OR).

Antifungal susceptibility testing. The susceptibilities of all strains to amphotericin B, voriconazole and caspofungin were assessed using a broth microdilution method based on the recommendations in the CLSI (NCCLS) document M38-A (26). To assess metabolic activity in each drug well quantitatively, an XTT metabolic assay was performed (2-4). Relative inhibition was compared to control wells for each strain in which no drug was added. For the susceptibility to calcofluor, serial dilutions of conidia of each strain were plated on Aspergillus minimal media with or without calcofluor and incubated at 37ºC overnight.

In vivo characterization of tps mutants. The virulence of tps mutants was tested in a murine intranasal model of invasive aspergillosis (36). In this model, male BALB/c mice (Taconic Labs, Germantown, NY, USA) were immunosuppressed with cortisone acetate (Sigma-Aldrich) as previously described (17). Groups of 11 mice were infected with each A. fumigatus strain using an aerosol chamber. Three mice were used for verification of fungal inocula and the remaining eight were monitored for survival. To monitor for toxicity of the immunosuppression, eight additional mice were immunosuppressed but uninfected. All procedures involving mice were approved by the Institutional Animal Use and Care Committee, according to the National Institutes of Health guidelines for animal housing and care.
In separate experiments, 8 to 10 mice were immunosuppressed and then infected with each strain as described above. After 3 days of infection, the mice were sacrificed, after which their lungs were harvested and homogenized as previously described (13). The pulmonary galactomannan content was measured using the Platelia® Aspergillus kit (Bio-Rad). A standard curve was established using serial dilutions of a pool of lung homogenates from twelve immunosuppressed mice heavily infected with the Af293 wild-type strain for 6 and 8 days (35). The lung myeloperoxidase content, which is a measure of phagocyte accumulation, was determined using an enzyme immunoassay (Cell Sciences) (35). To avoid the healthy survivor bias, galactomannan and myeloperoxidase studies were performed on the third day of infection at which point at least 90% of infected animals remained alive.

Statistical analysis. For experiments comparing two groups, single-factor ANOVA was performed. Survival curves were analyzed using the log-rank test. When comparing non-parametric groups, the Wilcoxon rank sum test was performed. A P value of <0.05 was defined as statistically significant.

RESULTS

Identification of genes encoding trehalose-6-phosphate synthase in A. fumigatus. Analysis of the available whole genome sequence of A. fumigatus identified four putative genes that were annotated as encoding trehalose-6-phosphate synthases based on homology to other fungi (Afu6g12950, Afu2g04010, Afu4g03190, and Afu5g14300). At the protein level, Afu6g12950
was most significantly homologous to *A. niger* TpsA and *A. nidulans* TpsA respectively. We therefore named this protein TpsA. Similarly, Afu2g04010 was most significantly homologous to *A. niger* TpsB. We therefore named this protein TpsB. Afu4g03190 and Afu5g14300, TpsC and TpsD respectively, were not closely related to any of the trehalose synthases in other yeasts and fungi. At the DNA level, *tpsA* and *tpsB* as well as *tpsC* and *tpsD* were highly homologous, with the areas of sequence divergence interspersed throughout the entire gene.

**Trehalose content and expression of tps genes during *A. fumigatus* development and in response to stress.** In *A. nidulans* trehalose content is low in actively growing hyphae, accumulates during or shortly before conidiation and remains high in conidia (11). To test if trehalose content was similar in *A. fumigatus*, we measured the trehalose content and trehalose synthase gene expression in wild-type *A. fumigatus* during development. Germlings and immature hyphae had low trehalose content. However, after 18-24h there was a significant increase in fungal trehalose levels (Fig. 1A). These time points correspond to the onset of developmental competence and subsequent conidiation, respectively (34). Conidia also had high trehalose content (data now shown). To determine if the increase in fungal trehalose might correspond to changes in trehalose synthase gene transcription, we examined the mRNA levels of the putative trehalose-6-phosphate synthase genes *tpsA*, *tpsB*, *tpsC* and *tpsD* using real-time RT-PCR. Levels of *tpsA* and *tpsB* mRNA were expressed throughout the course of development, and increased in parallel with the accumulation of trehalose in maturing hyphae (Fig. 1B). In contrast, expression of *tpsC* and *tpsD* remained low at all time points. Collectively, these results suggest that transcriptional upregulation of *tpsA* and *tpsB* may contribute to trehalose
accumulation during hyphal development and that these two genes may play a dominant role in trehalose biosynthesis during development.

To determine the effects of environmental stressors on trehalose content in *A. fumigatus*, actively growing exponential phase hyphae were subjected for 1h to oxidative shock with 100mM H$_2$O$_2$, osmotic shock with 0.5M NaCl or heat shock at 50°C. Only heat shock at 50°C was associated with an increase in hyphal trehalose content (Fig. 2A). Real-time RT-PCR assays of *tps* gene expression under these conditions showed that this increase correlated with increased expression of *tpsB*, but not the other *tps* genes (Fig. 2B). Interestingly, oxidative stress caused a significant decrease in *tpsB* mRNA levels and was not associated with an increase in trehalose content. However, unlike the other shock conditions, exposure to peroxide resulted in a growth arrest in hyphae as determined by a reduction in galactomannan release and stable dry weight for 12 hours after exposure (data not shown), and this may explain the lack of induction of trehalose biosynthesis under these conditions. Collectively, these results suggest that trehalose may play an important role in protecting the fungus from heat stress. Furthermore, *tpsB* is the trehalose synthase gene with the highest expression levels under these conditions, suggesting it may play a dominant role in trehalose biosynthesis in *A. fumigatus* under conditions of thermal stress.

Since *tpsA* and *tpsB* were considerably homologous to *tps* genes of other fungi and were significantly expressed during stress and development, we constructed mutant strains in which *tpsA* and *tpsB* were deleted both singly and in combination. To verify the specificity of our findings, each mutant was complemented with a wild-type allele of the disrupted gene, and the ΔtpsAB double mutant was complemented with a wild-type allele of *tpsA*. These strain sets were then used to determine the role of TpsA and TpsB in fungal development, stress response and virulence.
Either tpsA or tpsB are sufficient for trehalose accumulation in hyphae and conidia. We first tested the effects of deletion of tpsA, tpsB or both genes on trehalose levels during development. No significant difference in trehalose content between wild-type A. fumigatus and single mutant strains ∆tpsA and ∆tpsB was observed at any time point (Fig. 3A). In contrast, the double mutant strain ∆tpsAB produced no detectable trehalose, even after prolonged incubation. Complementation of the ∆tpsAB mutant with tpsA restored production of trehalose to wild-type levels (Fig. 3B). Similarly, the trehalose content of ∆tpsA, ∆tpsB and ∆tpsAB::tpsA conidia was similar to that of the wild-type strain, while that of the ∆tpsAB mutant was very low (Fig. 3C).

Since trehalose normally accounts for up to 15% of the biomass of conidia (46), we investigated whether the absence of trehalose in the ∆tpsAB conidia might result in a reduction of their size. By flow cytometric analysis, we observed a 30% reduction in the forward scatter (FSC) of conidia from the ∆tpsAB mutant as compared with those isolated from the wild-type, single mutant and complemented strains (Fig. 4). This reduction in size is likely in part due to trehalose deficiency, as complementing the ∆tpsAB mutant with tpsA restored both the trehalose content and size of the conidia.

Since an increase in trehalose levels was also observed in wild-type A. fumigatus in response to heat stress, we next compared the trehalose response of all strains during heat shock. When actively growing hyphae were exposed to heat shock at 50°C for 1h, similar increases in trehalose content were found in wild-type, ∆tpsA and ∆tpsB hyphae. In contrast, no trehalose was detectable in ∆tpsAB hyphae (Fig. 3D). Complementation of the ∆tpsAB mutant with tpsA restored production of trehalose under these and all other conditions (Fig. 3E).
These results suggest that \textit{tpsA} and \textit{tpsB} have redundant roles in trehalose biosynthesis in \textit{A. fumigatus}. We therefore tested the \textit{ΔtpsB} single mutant for a compensatory increase in expression of \textit{tpsA} or the other putative trehalose-6-phosphate synthase genes \textit{tpsC} and \textit{tpsD}.

Expression of \textit{tpsA}, \textit{tpsC} and \textit{tpsD} was measured in the \textit{ΔtpsB} mutant under the condition of heat shock, a condition which resulted in a significant increase in \textit{tpsB} expression and trehalose production in the wild-type and the \textit{ΔtpsB} mutant of \textit{A. fumigatus}. Under these conditions, deletion of \textit{tpsB} was not associated with the upregulation of \textit{tpsA}, \textit{tpsC} or \textit{tpsD} mRNA expression as compared with untreated controls and the wild-type strain (data not shown). The absence of compensatory upregulation of \textit{tpsA}, \textit{tpsC} or \textit{tpsD} suggests that although transcriptional regulation of \textit{tps} gene function may contribute to trehalose synthase regulation in development and in shock, there are likely post-translational mechanisms of regulation that allow for the TpsA protein to functionally compensate for the lack of TpsB and increased trehalose production.

**Normal germination requires either \textit{tpsA} or \textit{tpsB}.** \textit{A. nidulans} mutants deficient in \textit{tpsA}, a single-copy trehalose-6-phosphate synthase, have a germination delay at lower temperatures, (16). We therefore examined the \textit{A. fumigatus} wild-type strain and \textit{ΔtpsA}, \textit{ΔtpsB} and \textit{ΔtpsAB} mutant strains for their ability to germinate at 37°C. In YEPD medium, we observed at least a 2 to 3h germination delay for the \textit{ΔtpsAB} mutant as compared to the wild-type and single mutant strains, which was reversed by complementation of \textit{ΔtpsAB} with an intact copy of \textit{tpsA} (Fig. 5). Supplementation of media with exogenous trehalose had no effect on the delayed germination of the \textit{ΔtpsAB} mutant (data not shown).
**tpsA or tpsB are required for A. fumigatus thermotolerance.** We found that the ∆tpsAB mutant had undetectable trehalose content, even when exposed to high temperature. To determine the functional consequences of this deficiency, we compared the growth of wild-type, ∆tpsA, ∆tpsB, ∆tpsAB and ∆tpsAB::tpsA strains at 37°C and 50°C. Growth of all strains was indistinguishable at 37°C (Fig. 6A). However, at 50°C the ∆tpsAB mutant was severely delayed in colony development, and had reduced radial growth and conidiation relative to the other strains (Figs. 6A and 6B). Moreover, radial growth and conidiation of the ∆tpsAB mutant did not reach wild-type levels despite prolonged incubation (data not shown). Microscopic examination confirmed that ∆tpsAB conidia were able to germinate, grow and produce phenotypically normal reproductive structures at 50°C; however, this process was markedly delayed as compared to the wild-type or single mutant strains (data not shown). In addition, at 50°C, only 20% of the conidia of the ∆tpsAB mutant were viable as compared with over 80% of the wild-type or single mutant conidia (Fig. 6C). Furthermore, ∆tpsAB conidia that were viable were delayed in growth and conidiation by about 1 day as compared to other strains. Supplementation of the medium with trehalose, sorbitol or glycerol was unable to reverse these thermosensitive phenotypes (data not shown). Globally, these results suggest that tpsA and tpsB are redundant genes which have a role in thermotolerance.

**A. fumigatus deficient in tpsA and tpsB is susceptible to oxidative shock.** Trehalose is an important antioxidant. Mutant strains of *A. nidulans* that are deficient in trehalose have increased susceptibility to prolonged sub-lethal exposure to oxidative stress (16), and similar mutant strains of *C. albicans* and *C. neoformans* also have increased susceptibility oxidative shock (1, 22). Even though trehalose levels in *A. fumigatus* did not change in response to oxidant stress (Fig. 3).
we tested the viability of wild-type, ΔtpsA, ΔtpsB, ΔtpsAB and ΔtpsAB::tpsA swollen conidia to sub-lethal and lethal exposure to oxidative stress. When conidia of the various strains were exposed to a sub-lethal dose of 1.5mM H₂O₂, there was no difference in their viability (data not shown). However, exposure of conidia to 100mM H₂O₂ for 10 minutes resulted in a marked reduction of viability of the ΔtpsAB mutant as compared with the single mutants and the wild-type strain (Fig. 7). Again, this phenotype was reversed by complementation of the ΔtpsAB mutant with tpsA. Thus, basal production of trehalose by the products of tpsA and tpsB is likely necessary for protection of *A. fumigatus* from severe oxidative stress.

*A. fumigatus* mutants deficient in *tpsA* and *tpsB* are hypervirulent. Since the ΔtpsAB mutant had delayed germination and was more susceptible to oxidative stress compared to the wild-type or single mutant strains, we hypothesized that this strain might also be impaired in virulence. To test this hypothesis, we compared the virulence of wild-type, ΔtpsA, ΔtpsB, ΔtpsAB and ΔtpsAB::tpsA strains in a corticosteroid treated murine model of invasive aspergillosis. The survival of mice infected with either the ΔtpsA or ΔtpsB mutants was indistinguishable from mice infected with the wild-type strain (Fig. 8A). Surprisingly, the survival of mice infected with the ΔtpsAB double mutant strain was significantly shorter than those infected with the wild-type strain. The hypervirulence of the ΔtpsAB strain was abrogated when the ΔtpsAB double mutant was complemented with a single copy of tpsA.

To confirm these results, the fungal burden and pulmonary inflammatory response to infection with the wild-type, ΔtpsAB and ΔtpsAB::tpsA strains was examined in an independent set of experiments. Consistent with the survival studies, mice infected with the ΔtpsAB mutant displayed an increased fungal burden as measured by galactomannan content (Fig. 8B), as well
as increased pulmonary inflammation as measured by myeloperoxidase levels (Fig. 8C). As in the survival studies, complementation of the ΔtpsAB mutant with an intact allele of tpsA reduced pulmonary fungal burden and pulmonary inflammation.

Finally, to test if the hypervirulence of the ΔtpsAB mutant strain was a consequence of increased pulmonary delivery of the smaller conidia of this mutant, we examined the pulmonary fungal burden of the mice sacrificed immediately post-inhalation in the previous experiments. Mice infected with the ΔtpsAB mutant strain did not have a higher pulmonary fungal burden immediately post inhalation as compared with those infected with the wild-type strain (5.4 X 10³ cfu/mouse for the ΔtpsAB mutant strain versus 7.5 X 10³ cfu/mouse for strain Af293; n=8; p>0.3). Thus, the hypervirulence of the ΔtpsAB strain is unlikely due to higher levels of pulmonary infection during inhalation.

Collectively these data strongly suggest that tpsA and tpsB are not required for virulence, and may indeed moderate the virulence of A. fumigatus in vivo.

The hypervirulence of the ΔtpsAB mutant strain may result from changes in the cell wall composition leading to altered host cell interactions. The majority of hypervirulent A. fumigatus mutants that have been reported to date have displayed alterations in cell wall architecture and composition (13, 23, 33). To determine if the hypervirulent phenotype of the ΔtpsAB double mutant might be related to changes in cell wall architecture, transmission electron microscopy was performed to examine the cell wall of the ΔtpsAB mutant strain. Consistent with the observations from other hypervirulent strains, the cell wall of both hyphae and conidia of the ΔtpsAB mutant strain exhibited a loss of the electron dense outer layer, and conidia had an increased electrolucent zone compared with wild-type A. fumigatus (Figure 9A). In addition, the
ΔtpsAB mutant had increased susceptibility to calcofluor white and caspofungin as compared with the wild-type or ΔtpsAB::tpsA complemented strain (Figure 9B,C). This effect was specific to cell wall active compounds as the ΔtpsAB strain displayed normal susceptibility to voriconazole and amphotericin B (data not shown).

To determine if these changes in the cell wall were due to altered expression of known virulence modulating factors, we performed a real-time RT-PCR analysis of the expression of *A. fumigatus* ags3, ace2, and ecm33 genes. Deletion of each of these genes has been associated with alterations in cell wall architecture and increased virulence in murine models of invasive pulmonary aspergillosis(13, 23, 33). Expression of *fks1* was also examined in light of the hypersusceptibility to caspofungin seen with the ΔtpsAB strain. Real-time RT-PCR analysis of these genes revealed a statistically significant reduction in the expression of ags3, which encodes an α-glucan synthase, but not any of the other three genes (Figure 9D). These results suggest that a decrease in ags3 activity may contribute to alterations in the cell wall composition of the ΔtpsAB mutant.

Finally, to determine if these alterations in cell wall composition may influence interactions with immune cells, we examined the effect of tpsAB deletion on the phagocytosis of conidia by the RAW264.7 macrophage cell line. Upon microscopic examination, macrophages infected with conidia of the ΔtpsAB mutant were found to bind and phagocytose conidia much less efficiently as compared with those of the wild-type and ΔtpsAB::tpsA complemented strains (Figure 10A-C). To confirm these results we performed flow cytometric analysis of macrophages after 4 hours of infection with conidia of each of the strains. Macrophages infected with the ΔtpsAB mutant strain exhibited significantly lower complexity than those infected with the wild-type or ΔtpsAB::tpsA strains, confirming a reduction in the number of ΔtpsAB conidia that were
cell-associated (adherent or phagocytosed) with the macrophages (Figure 10D). Collectively these results suggest that alterations in the cell wall of the \( \Delta tpsAB \) mutant result in adherence and phagocytosis of the fungus, leading to hypervirulence.

**DISCUSSION**

Trehalose is an important reserve carbohydrate in fungal development and metabolism, contributing to energy requirements in cell processes such as glycolysis, sporulation and germination. As a result, trehalose deficient mutants of multiple fungal species have notable developmental defects. *S. cerevisiae* deficient in *TPS1* sporulate poorly, while *C. albicans* deficient in *TPS1* are impaired in yeast to hyphal transformation, an important virulence factor in this pathogenic yeast (14, 48). Similarly, *A. nidulans* \( \Delta tpsA \) mutants are delayed in germination when glucose or fructose is the sole carbon source (16). We found that *A. fumigatus* mutants deficient in both *tpsA* and *tpsB* were also delayed in germination. However, the germination delay of the *A. fumigatus* \( \Delta tpsAB \) mutant was unaffected by trehalose supplementation or growth in rich media. These results suggest the possibility that, in addition to mediating the synthesis of trehalose, *A. fumigatus* TpsA and TpsB may play a more complex role in governing fungal metabolism and development. It has been hypothesized that the *S. cerevisiae* Tps1 protein could have a direct regulatory role in glycolysis by interacting with glucose transport and sugar kinase mechanisms in the cell (12). Therefore, it is possible that *A. fumigatus* TpsA and TpsB may also be involved in regulation of glycolysis or other crucial cell processes.

In addition to serving as a reserve carbohydrate in development, trehalose protects actively growing cells from environmental injury. Thermosensitivity is a common defect of
trehalose deficient fungi including *S. cerevisiae* (18), *C. albicans* (1, 42), and *A. nidulans* (8, 16).

In wild-type *A. fumigatus*, trehalose levels increased during exposure to 50°C, and the ∆tpsAB double mutant had a severe defect in growth and viability at this temperature. Also, conidia of the ∆tpsAB mutant were markedly less viable at 50°C, with only 20% of ∆tpsAB conidia able to undergo germination. Those ∆tpsAB conidia that were able to germinate developed phenotypically normal hyphae and conidiophores; however, the developmental process was delayed by about 1 day. This germination block was insensitive to supplementation with glycerol, contrasting with findings in *A. nidulans*. Thus, TpsA and TpsB have an important role in the thermostability of *A. fumigatus*.

In *A. nidulans*, trehalose levels increase with oxidant stress and trehalose deficient ∆tpsA mutants have increased susceptibility to this stress condition (16). We found that trehalose levels in *A. fumigatus* were unchanged by oxidant stress. Despite this finding, the ∆tpsAB double mutant had increased susceptibility to oxidant stress. Thus, while inducible trehalose synthesis plays an important role in mediating thermotolerance in *A. fumigatus*, basal trehalose levels proved resistance to oxidative stress.

Our results suggest that regulation of *tps* activity in *A. fumigatus* occurs by both transcriptional and post-translational mechanisms. Increases in trehalose levels in the wild-type strain correlated directly with *tpsA* and *tpsB* mRNA levels during development and with *tpsB* transcript levels during heat shock. These results suggest that transcriptional regulation is important for governing trehalose metabolism in *A. fumigatus*, in contrast with findings in other fungi, where post-translational modifications of the trehalose synthase complex are thought to govern trehalose homeostasis (45, 49). On the other hand, our studies with the ∆tpsB single mutant suggest that post-translational regulation of trehalose-6-phosphate synthase activity can
also occur in *A. fumigatus*. In this mutant strain, trehalose levels increased normally during heat shock and development, despite an absence of TpsB, and without any detectable increase in the mRNA levels of *tpsA* (or *tpsC/D*). Since deletion of *tpsA* in addition to *tpsB* abrogated the increase in trehalose content under these conditions, these findings suggest that a post-translational modification of TpsA activity mediates compensation for a lack of TpsB.

In most fungi, including *A. nidulans*, a single copy trehalose-6-phosphate synthase is required for trehalose biosynthesis (16). In *A. niger*, two trehalose-6-phosphate synthase genes have been reported: *tpsA* which is more highly expressed during vegetative growth and *tpsB* whose expression increased significantly during heat shock and was barely detectible during vegetative growth (47). The contribution of each of these genes to trehalose biosynthesis has not been studied. Our results show that, like in *A. niger*, two genes encode trehalose-6-phosphate synthase in *A. fumigatus* with significant protein homology to *tpsA* and *tpsB* counterparts in *A. niger* and *A. nidulans*. We found that expression patterns of *tpsA* and *tpsB* in *A. fumigatus* were similar to those in *A. niger* except that *A. fumigatus* *tpsB* was highly expressed during both development and heat shock. Disruption of both *tpsA* and *tpsB* in *A. fumigatus* was required to block trehalose biosynthesis under all conditions, indicating redundancy between these two genes. This finding is consistent with our observed high level of sequence conservation between *tpsA* and *tpsB*. Differences in sequence between these two genes were distributed uniformly along their length, suggesting that these genes might represent a remote gene duplication event. This redundancy of Tps function may reflect the critical importance that thermotolerance plays in the life cycle of *A. fumigatus*, which commonly grows in high temperature compost.

During infection, *A. fumigatus* is exposed to conditions of both oxidative stress and nutrient depletion. We found that *A. fumigatus* conidia deficient in *tpsA* and *tpsB* had increased
susceptibility to severe oxidative shock with hydrogen peroxide as well as delayed germination, suggesting that this strain might be impaired in virulence. Surprisingly, we found that the trehalose deficient ΔtpsAB mutant strain did not display a reduction in virulence in a murine model of invasive aspergillosis, and was in fact hypervirulent. Mice infected with the ΔtpsAB mutant strain had a shorter survival and a higher fungal burden than mice infected with the wild-type parent strain. Furthermore, the higher fungal burden was associated with higher levels of pulmonary inflammation as measured by total pulmonary myeloperoxidase. Complementation of the ΔtpsAB mutant with a single copy of tpsA resulted in lower fungal burdens and a prolongation of survival to wild-type levels. These results contrast sharply with the role of trehalose-6-phosphate synthases in other fungi such as C. albicans and C. neoformans, in which Tps activity is required for normal virulence (30, 48).

The majority of hypervirulent strains of A. fumigatus that have been reported display abnormalities in cell wall content and architecture which have been hypothesized to lead to altered interactions with host cells (13, 23, 33) Consistent with these reports, we also observed that the hypervirulent phenotype of the tpsAB strain was associated with alterations in the cell wall, and that these changes were associated with a significant reduction in macrophage adherence and phagocytosis. Similar results were observed with Candida albicans mutants deficient in tps1, in which alterations in cell wall composition were visible by transmission electron microscopy (22). In C. albicans, these changes were accompanied by a reduction in adherence to and phagocytosis by macrophages, although the magnitude of these effects was less marked than we found with A. fumigatus, and did not result in an overall increase in virulence.

Although the mechanism by which A. fumigatus TpsA and TpsB govern cell wall synthesis is not yet elucidated, we observed a reduction in mRNA expression of the putative glucan synthase
ags3 in the \( \Delta tpsAB \) mutant strain. Deletion of ags3 has been associated with hypervirulence in a non-neutropenic model of invasive aspergillosis very similar to the one used in this study. However, a decrease in Ags3 expression is likely not the only mechanism by which trehalose influences the cell wall, since the conidia of the \( \Delta tpsAB \) mutant display different cell wall architecture than those deficient in Ags3. Ags3 deficient conidia were found to have an increase in the outer electron dense cell layer of the cell wall, in contrast to the decrease seen with the \( \Delta tpsAB \) mutant strain. Further, Ags3 deficient conidia were more resistant to oxidative shock, unlike conidia of the \( \Delta tpsAB \) mutant. Thus, it is likely that additional genes that govern cell wall synthesis, or their protein products, are dysregulated in the tpsAB mutant.

Collectively, our results indicate that TpsA and TpsB are redundant trehalose-6-phosphate synthase proteins which both have a role in development, thermosensitive growth and oxidative stress, and cell wall architecture of \( A. fumigatus \). Furthermore, our results suggest that trehalose is linked to a variety of processes in fungi that both promote and reduce virulence. While in other fungi such as \( C. albicans \) and \( C. neoformans \) the overall effect of trehalose is to promote virulence, the opposite seems to be true in \( A. fumigatus \) where an absence of Tps activity promotes virulence, likely through altered cell wall synthesis. Although the mechanism by which TpsA and TpsB modulate virulence in \( A. fumigatus \) is not yet completely defined, our results suggest that these alterations in cell wall composition lead to enhanced evasion of host immune responses. The mechanism by which alterations in trehalose biosynthesis affect cell wall synthesis are under active study in our group.

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REFERENCES


FIGURE LEGENDS

FIG. 1. Trehalose levels increase in mature hyphae and correlate directly with expression of tpsA and tpsB. Developmental time courses were performed with wild-type A. fumigatus grown in YEPD media. Samples of hyphae were isolated at the indicated time points. (A) Trehalose content of hyphae at the indicated time points. (B) RNA expression of trehalose synthase genes during development as assessed by real-time RT-PCR. Data were normalized to TEF1 expression. All results are expressed as mean plus or minus standard error, and represent at least three different experiments performed on different days. * indicates a statistically significant difference relative to 8 hour hyphae (p<0.05).

FIG. 2. Trehalose content of hyphae increases in response to heat shock and directly correlates with an increase in expression of tpsB. Wild-type hyphae were grown for 12 hours, and then exposed to shock with either 100mM H2O2, 0.5M NaCl or at 50°C for one hour. (A) Trehalose content of hyphae at the indicated time points. (B) RNA expression of trehalose synthase genes during development as assessed by real-time RT-PCR. Data were normalized to TEF1 expression. All results are expressed as mean plus or minus the standard error, and represent at least three different experiments performed on different days. * indicates a statistically significant difference relative to control hyphae (p<0.05).

FIG. 3. Trehalose production is abrogated in ∆tpsAB hyphae and conidia. (A) Trehalose content of hyphae isolated from the indicated strains during a developmental time course. Note, that data from the ∆tpsAB mutant are omitted at 8 hours since this organism had not produced hyphae at this time point. (B) Complementation of the ∆tpsAB mutant restores trehalose content
during development. (C) Trehalose content of conidia of the indicated strains. (D) Trehalose content was measured in hyphae grown for 12 hours and then incubated at 50°C for 1 hour. Control hyphae were incubated at 37°C for 1 hour. To compensate for the germination delay of the ΔtpsAB mutant, hyphae were pre-grown for 15 hours before heat shock. (E) Complementation of the ΔtpsAB mutant restores trehalose content during heat shock. All experiments were repeated in triplicate on three separate days and are presented as mean plus or minus the standard error. * indicates a statistically significant difference, (p<0.05), when compared to the wild-type strain at any time point.

FIG. 4. Conidia from the ΔtpsAB mutant are smaller than those from other strains.
Conidial size of indicated strains was assessed by flow cytometry. About 10^6 conidia of each strain was chemically fixed for analysis and the estimated volume of conidia was measured by forward scatter (FSC) using the wild-type strain as a reference.

FIG. 5. The ΔtpsAB mutant is delayed in germination at 37°C. Germination of wild-type, ΔtpsA, ΔtpsB, ΔtpsAB and ΔtpsAB::tpsA conidia was monitored hourly in YEPD medium. For each time point, 100 cells were scored for each strain and % germination was assessed. Data represent the mean of two independent experiments plus or minus the standard error.

FIG. 6. The ΔtpsAB mutant is delayed in growth and development, and significantly less viable at 50°C. Serial dilutions of conidia of the indicated strains were plated on Aspergillus minimal glucose medium and grown at 37°C or 50°C. (A) Photograph of colonial morphology of strains grown for 2 days at 37°C. (B) Photograph of colonial morphology of strains grown for 3
days at 50°C. (C) Conidia of the indicated strains were plated on *Aspergillus* minimal glucose medium at 37°C and at 50°C, and fungal colonies were counted daily for 5 days. The % viability was measured by expressing counts at 50°C as a percent of counts at 37°C for the same strain. Experiments were repeated in triplicate on three independent occasions. Data are presented as the mean plus or minus the standard error.

**FIG. 7.** The △tpsAB mutant is highly susceptible to oxidative shock. Swollen conidia of the indicated strains were exposed to 100mM H₂O₂ for 10 minutes, and then recovered on *Aspergillus* minimal glucose medium. Fungal colonies were then enumerated after two days of incubation. Viability was expressed as the mean number of fungal colonies for each strain on plates inoculated with conidia exposed to oxidative shock as a percent of those on plates inoculated with untreated conidia. Data are presented as the mean plus or minus the standard error. All experiments were repeated in triplicate on three separate occasions. * indicates a statistically significant, (p<0.05), difference in survival relative to the wild-type strain.

**FIG. 8.** The △tpsAB mutant is hyper-virulent in a murine model of invasive aspergillosis.

(A) Survival of mice infected with the indicated strains. Mice were immunosuppressed with cortisone acetate and infected intranasally with *A. fumigatus* wild-type, △tpsA, △tpsB, △tpsAB and △tpsAB::tpsA strains. 8 infected mice per strain were monitored for survival relative to a group of 8 uninfected mice. Data represent combined results from two independent experiments. * indicates a statistically significant difference (p<0.05) relative to wild-type and △tpsAB::tpsA strains using the log-rank test. (B) The concentration of galactomannan (GM) and (C) myeloperoxidase (MPO) was measured from the lungs of mice infected with *A. fumigatus* wild-
type, \( \Delta \text{tps}\text{AB} \) and \( \Delta \text{tps}\text{AB::tps}\text{A} \) strains after 4 days of infection. Results represent a median plus or minus an interquartile range of 5 to 8 mice per strain. § indicates a statistically significant difference, \( p<0.05 \), relative to the wild-type strain using the Wilcoxon Rank Sum Test.

**FIG 9. Either tpsA or tpsB is required for normal cell wall architecture in *A. fumigatus*.**

(A) Transmission electron microscopy of conidia and hyphae of the *A. fumigatus* strains demonstrates a loss of the electron dense outer layer (arrows) in both conidia and hyphae of the \( \Delta \text{tps}\text{AB} \) mutant. Scale bars indicate 200nm. (B) 10-fold serial dilutions of Af293, \( \Delta \text{tps}\text{AB} \), and \( \Delta \text{tps}\text{AB::tps}\text{A} \) conidia were spot inoculated on plates of *Aspergillus* minimal glucose medium containing 75 \( \mu \text{g/ml} \) of Calcofluor white. Photographs of each plate were taken after strains were grown for 2 days at 37°C. (C) Relative growth of individual strains in the presence of varying concentrations of caspofungin, (D) RNA expression of cell wall active genes as assessed by real-time RT-PCR. Data were normalized to TEF1 expression. All results are expressed as mean plus or minus standard error, and represent at least three different experiments performed on different days. * indicates a statistically significant difference relative to wild-type Af293 \( p<0.05 \).

**Figure 10: Either tpsA or tpsB are required for normal phagocytosis by macrophages.**

Cultures of RAW264.7 cells were infected with conidia of the indicated *A. fumigatus* strains and incubated for 4h. (A-C). Photomicrographs of RAW264.7 cultures infected with conidia of wild-type Af293 (A), \( \Delta \text{tps}\text{AB} \) (B) or \( \Delta \text{tps}\text{AB::tps}\text{A} \) strains (C). Arrows indicated non-adherent, unphagocytosed, extracellular conidia. (D) Flow cytometric analysis of macrophage cells infected as in A-C. Side-scatter kinetics were determined as a measure of the number of cell-associated conidia. Bars indicate 50\( \mu \text{m} \) in size.
TABLE 1. Primers used in this study

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A. WT
\( \Delta \text{tpsA} \)
\( \Delta \text{tpsB} \)
\( \Delta \text{tpsAB} \)
\( \Delta \text{tpsAB::tpsA} \)

Minimal Medium 37°C

B. WT
\( \Delta \text{tpsA} \)
\( \Delta \text{tpsB} \)
\( \Delta \text{tpsAB} \)
\( \Delta \text{tpsAB::tpsA} \)

Minimal Medium 50°C

C. % viability vs. Time (days)
**A.**

- Af293 conidia
- ΔtpsAB conidia
- Af293 hyphae
- ΔtpsAB hyphae

**B.**

- 10^4
- 10^3
- 10^2

- Af293
- ΔtpsAB
- ΔtpsAB::tpsA

**C.**

- Relative Growth (%)
- Caspofungin concentration (ug/ml)

- 0
- 16
- 32

- Af293
- ΔtpsAB
- ΔtpsAB::tpsA

**D.**

- Relative gene expression
- ags3
- ace2
- acm33
- fks1

- Af293
- ΔtpsAB
- ΔtpsAB::tpsA