Genetic and environmental factors determining heterogeneity in preservation stress resistance of Aspergillus niger conidia

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CHAPTER 7

Dissecting the pivotal role of mannitol and trehalose as compatible solutes in heat resistance of conidia, germination, and population heterogeneity in the filamentous fungus *Aspergillus niger*

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Manuscript in preparation

Abstract

Populations of conidia are naturally heterogeneous, which is a challenge for food preservation strategies. Variation in maturation and thereby spore age of conidia causes heterogeneity within a conidial population. In this research, we investigate the impact of spore age and compatible solutes on the heat stress resistance of *A. niger* conidia. Young conidia from *A. niger* were found to contain reduced amounts of compatible solutes, were more heat sensitive and had altered germination kinetics. To further investigate the impact of compatible solutes on the heat resistance and germination of *A. niger* conidia, knock-out strains were made of trehalose biosynthesis genes (*tpsABC*) as well as the genes putatively involved in the mannitol cycle (*mpdA, mtdAB*) using CRISPR/Cas9 genome editing. A total of twenty-five knock-out strains and five complemented strains containing most of the potential combinations of these six genes were made. Conidia of these knock-out strains were tested for their relative heat resistance, compatible solute profiles and germination capacity. The strain in which four genes (*tpsABC* and *mpdA*) were deleted, contained no measurable amount of trehalose and limited left-over mannitol in its conidia and were found to be sensitive to heat treatments. Conidia lacking compatible solutes, either because they are young or from this four-fold knock-out strain, have higher germination percentages in 10 mM arginine and 0.1 mM proline when compared to wild-type conidia, but lower germination percentages in 10 mM glucose. Taken together, it is concluded that differences in intracellular concentration of compatible solutes within a clonal spore population can affect heterogeneity in heat resistance and germination of *A. niger* conidia.

Introduction

A population of asexual fungal spores (conidia) can be highly heterogeneous, which poses a challenge for food preservation strategies [1]. The conidia are a constant concern in relation to food spoilage, impacting food production [2,3]. Many fungal species produce numerous conidia that are dispersed by air. Every cubic meter of air contains conidia of genera *Cladosporium*, *Penicillium* and *Aspergillus* [4], and this aerial population of conidia varies with the season [5,6]. Apart from species variation, conidia within a population can differ from each other even though they belong to the same species or even strain [1]. This is due to the history of the conidium, and specifically the growth conditions of the mycelium from which the conidia originate. Conidiation during increased cultivation temperature is known to decrease melanin concentrations as well as increase internal compatible solute concentrations, thereby impacting heat stress resistance of the conidia of *Paecilomyces variotii*, *Penicillium roqueforti* and *Aspergillus fumigatus* [7–10]. Additionally, the composition of the medium on which the mycelium feeds during conidiation impacts internal compatible solute concentrations, germination kinetics and conidial stress resistance [11–13]. Yet another source of heterogeneity is introduced by the difference in age of conidia. Culture age is reported to affect the transcription profile and physical properties of conidia, such melanin and compatible solute composition [9,14,15]. Therefore, the change in physical properties and compatible solute compositions due to spore age differences could impact stress resistance to various (preservation) stressors.

An important parameter in stress resistance of fungal cells is the amount and composition of internal compatible solutes [16,17]. These compounds can be quickly accumulated and degraded in the cytoplasm of vegetative cells in response to stress conditions. Compatible solutes like glycerol, erythritol and arabitol are suggested to protect fungal cells against osmotic stress, whereas trehalose and mannitol are shown to be important for protection against heat, cold and drought [17,18]. The biosynthesis routes of trehalose and mannitol in Aspergilli are tightly linked to glycolysis and have been

largely described [19,20], see Figure 7.1.

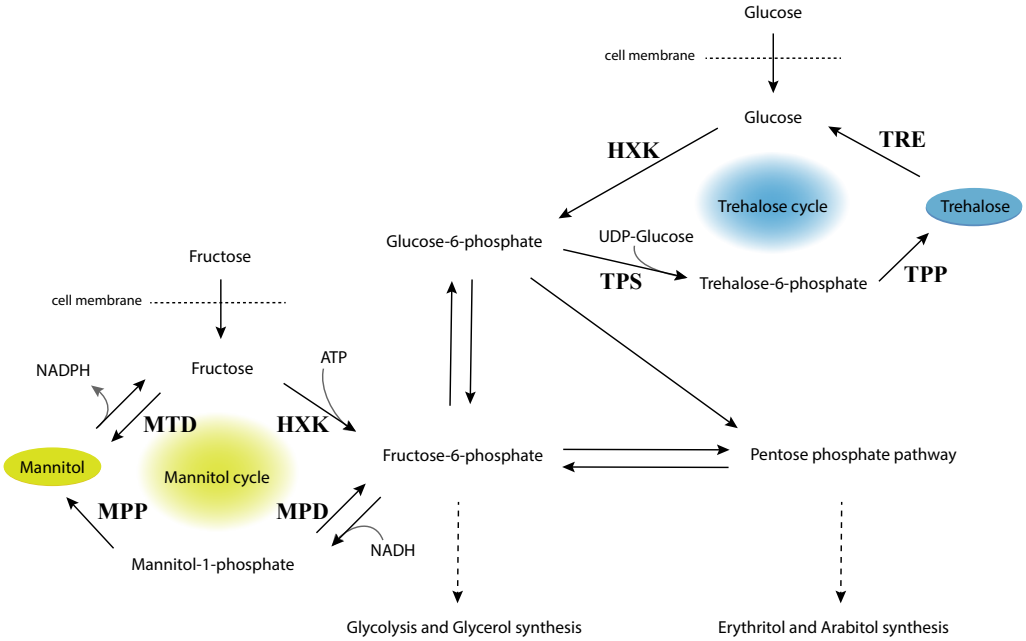

Figure 7.1. Mannitol and trehalose cycles in Aspergilli. The biosynthesis of trehalose in Aspergilli, as depicted in blue, starts with the phosphorylation of glucose to glucose-6-phosphate by hexokinase activity (HXK). Glucose-6-phosphate is converted to trehalose-6-phosphate by trehalose-6-phosphate synthase (TPS). Trehalose-6-phosphate phosphatase (TPP) is able to dephosphorylate trehalose-6-phosphate, yielding trehalose. The catabolism of trehalose is catalyzed by trehalase (TRE) yielding glucose. As represented by the yellow mannitol cycle, mannitol in Aspergilli is mainly synthesized from fructose, which is converted to fructose-6-phosphate by HXK activity. Subsequently, fructose-6-phosphate may be reduced to mannitol-1-phosphate by mannitol-1-phosphate dehydrogenase (MPD). Finally, the dephosphorylation of mannitol-1-phosphate is catalyzed by mannitol-1-phosphate phosphatase (MPP), thereby yielding mannitol. The catabolic conversion of mannitol to fructose by mannitol dehydrogenase (MTD) completes the cycle. Both cycles are connected to glycolysis. Additionally, there is a connection with the pentose phosphate pathway and subsequently erythritol and arabitol synthesis. This figure was adapted from figures shown by Svanström *et al.* (2003) and Ruijter *et al.* (2003).

The role of both mannitol and trehalose in stress resistance of conidia of *A. niger* has been studied previously. Trehalose is responsible for about 4-5% of the conidial dry weight of *A. niger* conidia [21]. Three trehalose-6-phosphate synthase genes (*tpsABC*) and three trehalose-6-phosphate phosphatase genes (*tppABC*) have been

identified in *A. niger* [22]. For the catabolism of trehalose during germination, the *treB* gene was identified, encoding a neutral trehalase [21]. Additionally, *treA* encodes an acid trehalase, presumably involved in the extracellular degradation of trehalose during vegetative growth [23,24]. Deletion of the *tpsA* gene in *A. niger* leads to a 56% reduction of the mycelial trehalose content, compared to wild-type [25]. In addition, *A. niger* ΔtpsA conidia show reduced heat tolerance and a 50% decrease in conidial trehalose content, whereas *tpsB* and *tpsC* deletions showed less effect on both the trehalose content and heat resistance.

Besides trehalose, mannitol is involved in determining stress resistance of *A. niger* conidia. Mannitol is the predominant compatible solute present in *A. niger* conidia and responsible for about 10-15% of the conidial dry weight [26]. The mannitol dehydrogenase gene A (*mtdA)* is suggested to be responsible for the mannitol dehydrogenase activity in *A. niger* [27], whereas the mannitol-6-phosphate dehydrogenase (MPD) enzyme is encoded by *mpdA* [20]. In the *A. niger ΔmpdA* strain, only 30% of the original mannitol concentration is present inside conidia. Furthermore, ΔmpdA conidia show increased sensitivity to stressors like heat and oxidative stress.

The role of trehalose and mannitol in relation to germination has not been well studied so far. In *A. niger*, the concentrations of both trehalose and mannitol drops drastically during the first hours of germination [26,28] suggesting that these two compatible solutes could play a role in the energy household of the mould during early germination. However, whether there is an actual requirement for the presence of the compatible solutes trehalose and mannitol as storage sugars for efficient germination of conidia has not yet been investigated.

Here we describe the impact of conidial age on heat resistance of *A. niger* conidia. We found that young conidia are heat sensitive and contain low concentrations of compatible solutes. A gradual increase in heat resistance and compatible solute concentrations is observed that correlates with an increase in spore age. In order to further investigate the role of compatible solutes, knock-out strains deleted in genes involved in

the trehalose and mannitol cycle were constructed in order to investigate the role of both trehalose and mannitol in the heat resistance. We found that young conidia and conidia from knock-out strain SJS128 (*ΔmpdA, ΔtpsABC*) both lacked most compatible solutes. Conidia lacking most compatible solutes were heat sensitive and were found to significantly differ in germination kinetics when compared to wild-type conidia. Conidia with limited internal compatible solutes show higher germination percentages and speeds in 10 mM arginine, 0.1 mM proline and 1 mM alanine, but lower germination percentages and speeds in 10 mM glucose. Taken together, conidia of *A. niger* show heterogeneity in heat stress resistance and germination kinetics based on internal compatible solute composition.

Results

The impact of culture age on heat resistance and germination of *A. niger* conidia

Previous reports have focussed on the comparison between conidia with age differences of several days [9,14,15]. However, most conidia are formed during the first three days following inoculation after which many spore chains reach their maximum capacity of approximately 20 conidia per chain [15]. In this study, we compared conidia harvested from plates that were inoculated for different amounts of time in hours rather than days. Conidia harvested from plate cultures incubated for 39 hours, will be referred to as 39h conidia.

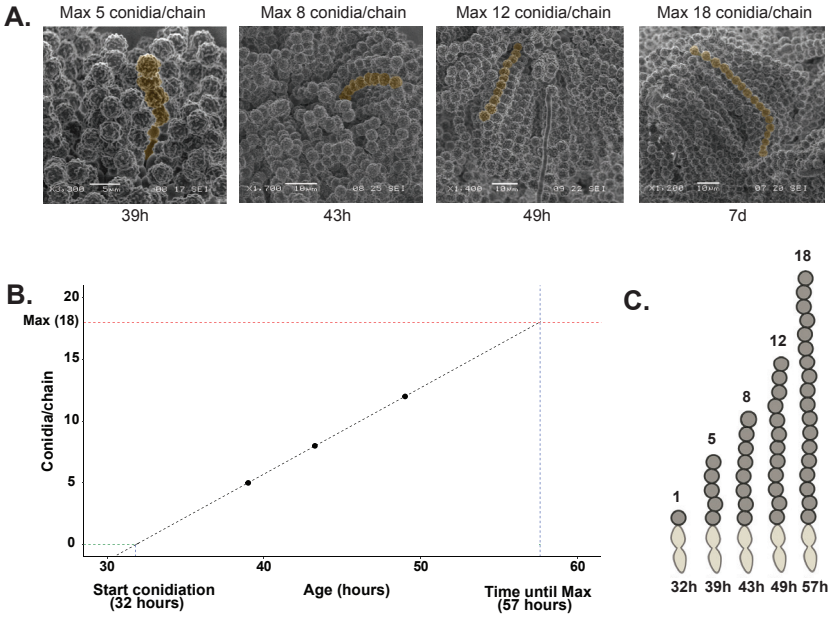

Figure 7.2. Maximum spore chain length of *Aspergillus niger* followed through time. Scanning electron microscopy pictures were analyzed taken from conidiophores of *A. niger* taken from MEA plates (in hours after inoculation). Additionally, a 7 days old plate was taken as a control. **A.** Spore chain lengths were manually counted for at least 15 conidiophores per sample and scanned for the longest chain. The longest possible chain per plate was noted for each time point and highlighted in the picture. **B.** The maximum chain lengths found suggests a constant conidiation speed, calculated to be around ~80 minutes/conidium. Assuming conidiation speed is constant, the first conidium was formed ~32 hours after inoculation and the first spore chain reached its maximum ~57 hours after inoculation. **C.** A representation of the maximum chain lengths found for each time point. A single spore chain takes ~25 hours to reach maximum capacity.

Scanning electron microscopy (SEM) pictures were taken of conidiophores from confluently plated MEA plates incubated for 39h, 43h, 49h and 7 days to see how many conidia are formed during these incubation times (Figure 7.2). The largest spore chain length found from plates incubated for 7 days contained 18 conidia, which is similar to the 20 conidia per chain reported for *A. niger* before [15]. Based on the maximum number of conidia on the chain at the different time points, conidial formation on the spore chain seemed of consistent speed under these conditions (MEA plates, incubated at 28°C) and was found to be around 1 conidium per 80 minutes (Figure 7.2B). From these results it was extrapolated that the first conidium on the first phialide was formed ~32 hours after inoculation and the spore chain hits maximum capacity at ~57 hours after inoculation, which suggests that the formation of a full spore chain takes ~25 hours under these conditions.

In an additional experiment using the exact same conditions as a above but with a slightly different timing, internal compatible solute concentrations and the heat resistance of conidia harvested at 38h, 43h, 46h, 53h and 64h were determined (Figure 7.3). A correlation is seen between the absence of compatible solutes and the heat sensitivity of young conidia. The 38h conidia were significantly more heat sensitive and contained very limited amounts of compatible solutes when compared to older conidia. The heat resistance and compatible solute concentrations of the conidial population increased gradually when conidia were harvested after longer times of growth. The maximum number of conidia found on the largest spore chain at each time point (Figure 7.2) was compared to the heat resistance (Figure 7.3). On plates where the largest spore chain contained eight conidia (as found for 43h conidia), conidia were still considered heat sensitive, since no colony forming units (CFUs) were found when plating 10^6 43h conidia after a heat treatment of 57°C for 10 minutes (Figure 7.3B). Therefore, the heat resistance of conidia is still increasing on plates where a maximum spore chain length of eight conidia was found. These results indicate that compatible solute concentrations, and therefore the heat resistance of conidia, are still increasing during conidial maturation. The maturation, in terms of heat resistance, only occurs while conidia are attached

to the spore chain, since dry harvested 43h conidia no longer show increased heat resistance when incubation times are extended (Figure 7.S1).

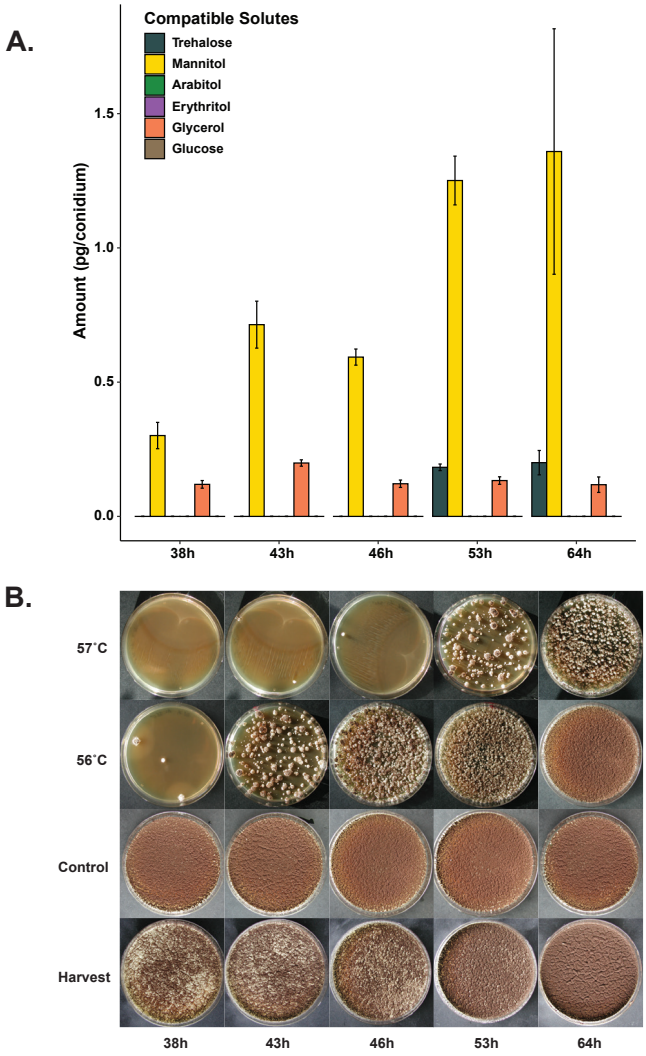

Figure 7.3. Young conidia are sensitive to heat and contain very little compatible solutes. **A.** HPLC analysis of $1*10^8$ cracked conidia revealed only limited amounts of compatible solutes are present inside young conidia. The level of compatible solutes increases when the age of the conidia increases. **B.** Heat treatment assay (Figure 7.S5) results, showing that young spores are sensitive to heat stress. 10^6 conidia were heat treated for 10 minutes at either 56°C, 57°C or room temperature (control) and subsequently plated confluently. After 5 days pictures are taken and visualized here, the amount of colony forming units observed gives an indication of the amount of conidia that survived the heat treatment. A picture of the harvesting plates has been included to show the level of conidiation present on the initial plates from which the conidia were harvested to perform the heat treatment assay.

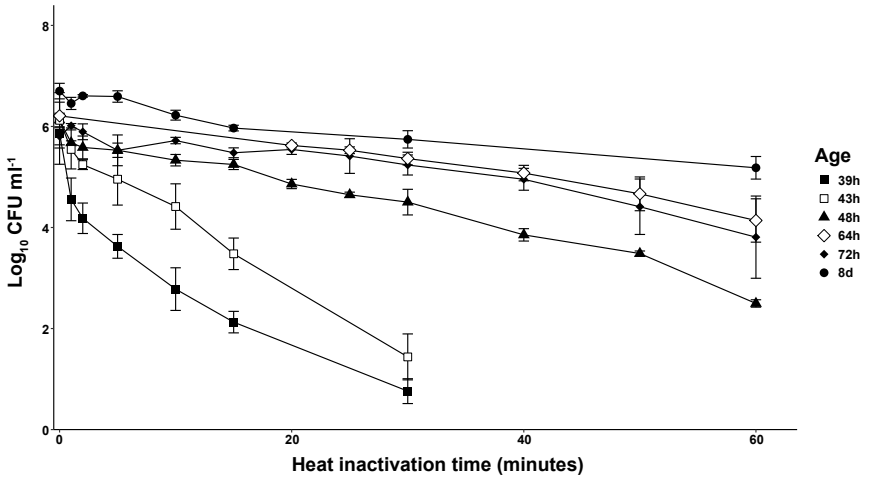

Figure 7.4. Heat inactivation curves of *Aspergillus niger* N402 conidia of different ages. Here the inactivation curves of N402 conidia from different ages 39h (■), 43h (□), 48h (▲), 64h (◊), 72h (♦) and 8d (●) are visualized. Heat treatments were done in a water bath at 54°C. Samples were taken for each time point, put directly on ice and subsequently serially diluted and plated. CFUs were counted after 5 days of growth. Measurements were done in biological triplicates. Linear regression lines were drawn from these curves in order to calculate decimal reduction values (Table 7.1). Heat resistance of conidial populations gradually increases when the age increases, which is in agreement with the heat treatment assay as shown in Figure 7.3.

Table 7.1. Decimal reduction values of *Aspergillus niger* conidia heat treated at 54°C.

Strain	Age	D_{54} value ± SD (minutes)
N402	8d	41.4 ± 7.3
N402	72h	36.8 ± 13.9
N402	64h	30.8 ± 7.2
N402	48h	16.4 ± 2.2
N402	43h	7.1 ± 1.0
N402	39h	5.3 ± 0.6
MA234.1	8d	52.3 ± 25.8
SJS126	8d	22.1 ± 7.7
SJS128	8d	3.3 ± 1.0
SJS132	8d	22.9 ± 5.4
SJS134	8d	22.4 ± 3.5

In this table the quantified results of the heat inactivation curve experiments are summarized as decimal reduction values (D-values). D-values are listed in minutes: average ± the standard deviation. The D-values were based on a log-linear model, calculated from heat inactivation curves shown in Figure 7.4 (N402 conidia of various ages) and shown in Figure 7.7 (conidia of various knock-out strains).

In addition to determining the sensitivity of conidia to a 10 min heat shock at 56° C and 57° C (Figure 7.3B), heat inactivation curves at 54° C of conidia were made to further quantify the heat resistance of young vs older conidia (Figure 7.4). From these graphs, D-values were calculated based on the linear regression model (Table 7.1). The 39h conidia were significantly more sensitive to heat stress ($D_{54°C}$ = 5.3 ± 0.6 minutes) when compared to 8d conidia ($D_{54°C}$ = 41.4 ± 7.3 minutes) and, in agreement with Figure 7.3, heat resistance of conidial populations gradually increased with conidial age.

Compatible solute profiles and heat stress resistance of conidia of knock-out strains

To address the role of mannitol and trehalose as compatible solutes in relation to heat sensitivity of conidia, *A. niger* strains were constructed in which the genes involved in mannitol and trehalose cycles were deleted. Strains lacking compatible solutes, in which multiple genes were deleted simultaneously, were constructed using a recently described CRISPR/Cas9 genome editing system [29]. Single knock-out strains were made in which putative mannitol dehydrogenases were deleted *(*Δ*mtdA*, Δ*mtdB*), the mannitol-phosphate dehydrogenase was deleted *(*Δ*mpdA*) and in which the trehalose-6-phosphate synthase encoding genes were deleted *(*Δ*tpsA*, Δ*tpsB* and Δ*tpsC*). Additional knock-out strains lacking any of these six genes were made in several combinations (Table 7.2). Additionally, complemented strains of a subset of knock-out strains with interesting phenotypes were constructed. Correct deletion and complementation of all transformants was confirmed by diagnostic PCR (Figure 7.5).

Ruijter *et al.* have shown that a *ΔmpdA* strain in *A. niger*, lacking the main mannitol biosynthesis enzyme MpdA, produces conidia containing ~70% less mannitol which are heat sensitive when compared to wild-type conidia [20]. Ruijter *et al.* postulate that the residual mannitol in this strain could be produced by a dual function of the mannitol dehydrogenases, which are thought to mainly catalyse the conversion from mannitol back to fructose, but could also catalyse the conversion from fructose to mannitol [30]. We confirmed that conidia from a Δ*mpdA* strain, as described by Ruijter *et al.*, contain

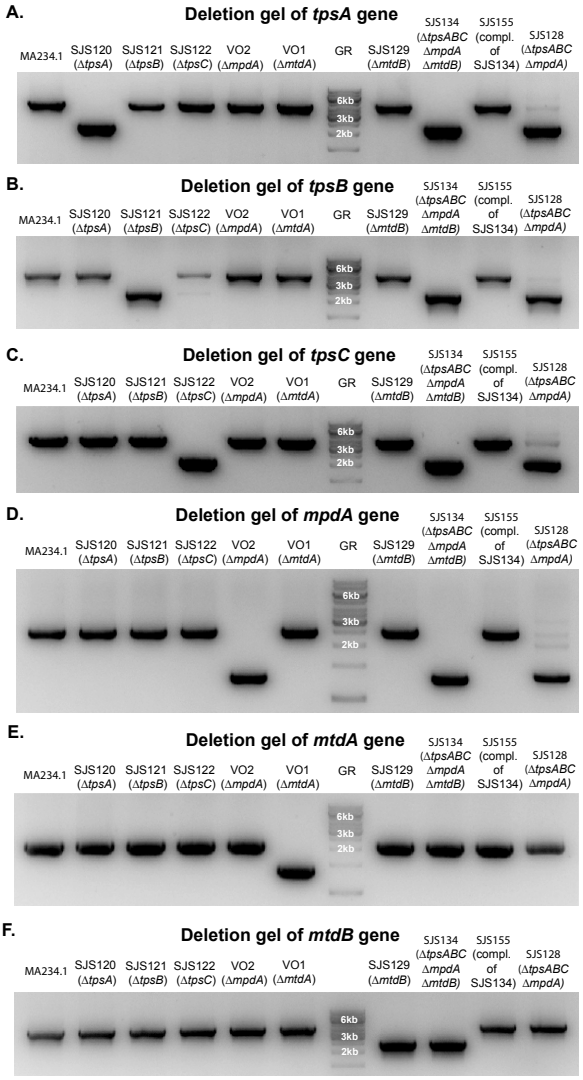

Figure 7.5. Diagnostic PCR confirming deletions in knock-out strains. Each column on the gel represents a knock-out strain. Each individual gel amplified one gene. The GeneRuler lane (GR) contains the ladder. **A.** Deletion of the *tpsA* gene. If the gene is present, a band size of 4118 bps is expected. If the gene is absent a band size of 2189 bps is expected. **B.** Deletion of the *tpsB* gene. If the gene is present, a band size of 3994 bps is expected. If the gene is absent a band size of 2281 bps is expected. **C.** Deletion of the *tpsC* gene. If the gene is present, a band size of 3894 bps is expected. If the gene is absent a band size of 2196 bps is expected. **D.** Deletion of the *mpdA* gene. If the gene is present, a band size of 2499 bps is expected. If the gene is absent a band size of 1345 bps is expected. **E.** Deletion of the *mtdA* gene. If the gene is present, a band size of 2176 bps is expected. If the gene is absent a band size of 1387 bps is expected. **F.** Deletion of the mtd*B* gene. If the gene is present, a band size of 4233 bps is expected. If the gene is absent a band size of 2347 bps is expected.

less mannitol (~65% compared to wild type), more trehalose (~300% compared to wild type) and are heat sensitive compared to conidia of the parental strain (Figure 7.S2). Conidia of the triple knock-out strain lacking *mtdAB* (two putative mannitol dehydrogenases) and *mpdA* were similar to the Δ*mpdA* strain in heat resistance and internal compatible solute profile. These results suggest that only the *mpdA* gene has a direct effect on the internal compatible solute composition of conidia. Surprisingly, all obtained knock-out strains lacking genes involved in the mannitol cycle were still able to produce some amount of mannitol indicating that additional (currently unknown) genes are involved in the mannitol biosynthesis in *A. niger*.

Additionally, the HPLC analysis on conidia of strains lacking *tps* genes revealed that the Δ*tpsAB* and Δ*tpsABC* strains were devoid of (measurable) internal trehalose and were heat sensitive (Figure 7.S3). Therefore, both *tpsA* and *tpsB* are essential for trehalose biosynthesis under standard laboratory conditions. Taken together, we show that the reduction of internal mannitol or trehalose concentrations both lead to heat sensitive conidia compared to wild type, indicating that both mannitol and trehalose are important for the heat stress resistance of *A. niger* conidia.

Conidia from strains deleted in both trehalose and mannitol cycle genes had the largest changes in compatible solute profiles (Figure 7.6A). Specifically, the ΔmpdA and Δ*tpsABC* four-fold knock-out strain (named strain SJS128) resulted in conidia with a drastically changed compatible solute profile. No trehalose and only very little mannitol was detected, which are normally the two most pre-dominant compatible solutes inside *A. niger* conidia. Deletion of the *mtdB* gene in the four-fold knock-out strain SJS128 (*ΔtpsABC*, *ΔmpdA*), creating the five-fold knock-out strain SJS134 (*ΔtpsABC*, *ΔmpdA*, *ΔmtdB*), restored mannitol concentrations to wild-type level. This indicates that the *mtdB* gene encodes for a mannitol dehydrogenase being able to catalyse the conversion of mannitol to fructose, as removing this dehydrogenase results in accumulation of mannitol in these conidia. Additionally, the five-fold knock-out strain SJS134 (*ΔtpsABC*, *Δmpda*, *ΔmtdB*) accumulates a large amount of arabitol, indicating that if genes in both the trehalose and mannitol cycle are being deleted, the conidia accumulate arabitol. All com-

binations in which the deletion of the *mtdA* gene was added did not result in a change in compatible solute composition inside the conidia, and therefore strains in which *mtdA* had been additionally deleted were left out of any further experiments (Figure 7.S4).

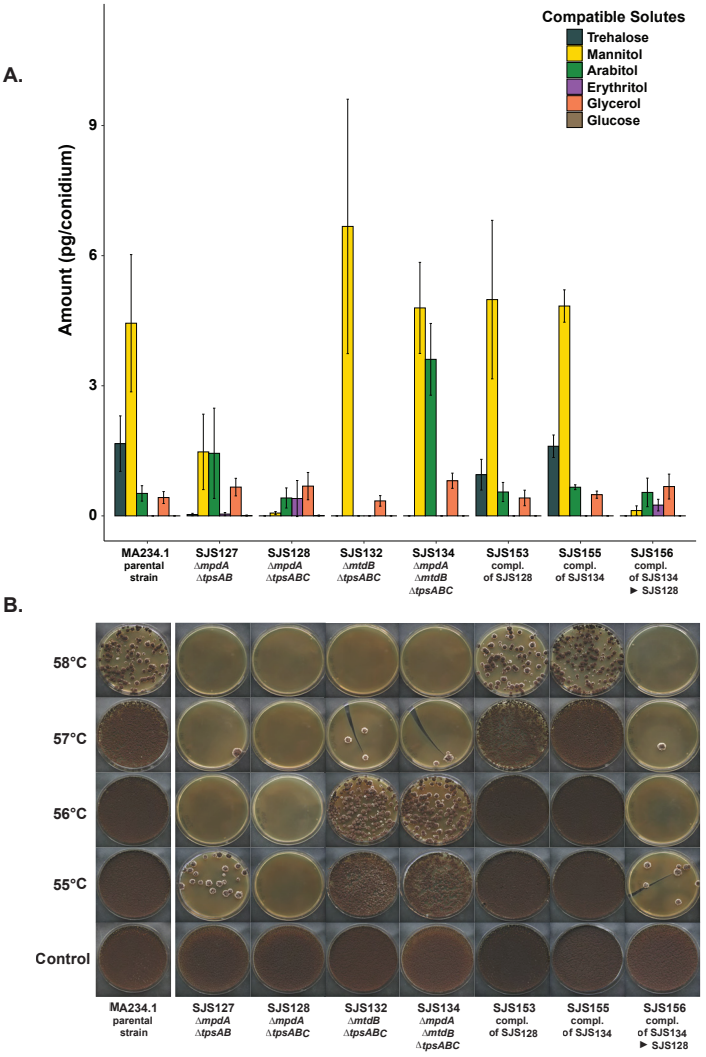

Figure 7.6. Internal compatible solute composition and heat resistance of conidia from *A. niger* trehalose and mannitol knock-out strains. Conidia were freshly harvested from MEA plates grown for 8 days at 28°C. Measurements were performed in biological triplicates. **A.** Internal compatible solute composition of conidia from knock-out strains as determined by HPLC analysis. The conidia from strain SJS128 were most impacted in their trehalose and mannitol concentrations. The SJS127 strain still produces a sliver of trehalose when compared to SJS128, which shows that the *tpsC* gene has impact on trehalose production and overall compatible solute profile of the conidia. Addition of an extra *mtdB* mutation in SJS128 restores mannitol back to wild-type

level and the knock-out strain SJS134 and this strain additionally accumulates arabitol. Complementation strains SJS153 and SJS155 show restoration of the compatible solute profile back to wild type. Strain SJS156 in which only the *mtdB* gene is restored in the SJS134 background re-introduces the SJS128-like compatible solute composition in the conidia. **B.** Heat treatments were applied to 10^6 conidia for 10 minutes in a thermocycler (Figure 7.S5). After heat treatments conidia were plated on plates containing MEA+0.05% triton x-100. Plates were grown for 5 days after which the pictures were made as shown above. The Δ*mpdA*, Δ*tpsABC* mutations (strain SJS128) has the largest effect on the heat resistance of *A. niger* conidia as zero colonies were found after a relatively mild heat stress of 55°C was applied. This correlates with a change in internal compatible solute composition in this strain, showing no trehalose and almost completely abolished mannitol concentrations. When introducing an extra *mtdB* deletion (creating SJS134), the heat resistance of the conidia is partially restored. Conidia of the complementation strains have restored heat resistance as the conidia survive 58°C similarly to parental strain MA234.1. Additionally, when complementing the five-fold knock-out strain SJS134 back to the four-fold knock-out strain SJS128 the heat sensitive phenotype returns.

Heat resistance of the knock-out strains lacking multiple genes in the trehalose biosynthesis route and the mannitol cycle was assessed using a heat treatment assay (Figure 7.6B). The largest impact on heat resistance was found in the four-fold knock-out strain SJS128 (Δ*tpsABC*, Δ*mpdA*). The HPLC analysis showed that this strain lacked detectable levels of trehalose and contained limited levels of mannitol indicating that the low level of compatible solutes is correlated with heat sensitivity. None of the 10^6 conidia from strain SJS128 survived 10 minutes at 55°C, indicating enhanced heat sensitivity when compared to the other knock-out strains and the parental strain, again emphasizing that both mannitol and trehalose contribute to the heat resistance of *A. niger* conidia. Conidia of strain SJS127 (Δ*tpsAB*, Δ*mpdA*), which still contains the *tpsC* gene in comparison to strain SJS128, were more heat sensitive as wild-type conidia but not as heat sensitive as conidia from strain SJS128. The presence of the gene *tpsC* in SJS127 has a major impact on the compatible solute profile, in contrast to the previous comparison between the Δ*tpsAB* and Δ*tpsABC* strains (Figure 7.S3), since the conidia from strain SJS127 (Δ*tpsAB*, Δ*mpdA*) still contain mannitol, arabitol and a small but measurable amount of trehalose when compared to SJS128 (Δ*tpsABC*, Δ*mpdA*). The heat sensitivity of conidia from strains SJS132 (Δ*tpsABC*, Δ*mtdB*) and SJS134 (Δ*tpsABC*, Δ*mpdA,*

Δ*mtdB*), containing mostly mannitol and arabitol, is comparable to that of the trehalose null strain SJS126 (Δ*tpsABC*) (Figure 7.S3).

All complementation strains (SJS149.2, SJS152.3 – SJS155) restored both the compatible solute profile and the heat resistance of conidia back to wild type concentrations. As an extra control, the five-fold knock-out strain SJS134 (*ΔtpsABC*, *Δmpda*, *ΔmtdB*) was complemented with only the *mtdB* gene re-creating the genotype of four-fold knock-out strain SJS128 (this complemented strain is named SJS156). The conidia of this strain SJS156 were again lacking in compatible solutes and showed the heat sensitive phenotype similar to that of the original four-fold knock-out strain SJS128 (Figure 7.6).

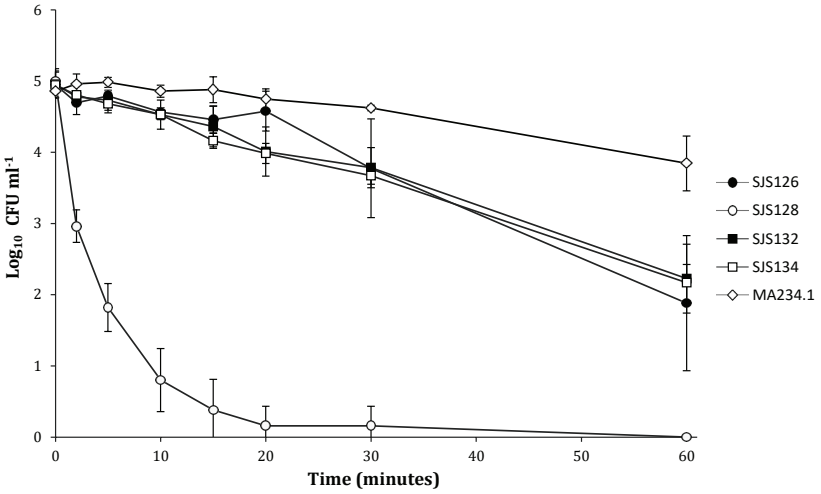

Figure 7.7. Inactivation curves of conidia from *A. niger* knock-out strains altered in their compatible solute concentrations. Here the inactivation curves of conidia from strains MA234.1 (ΔkusA, ◊) parental strain, SJS126 (ΔtpsABC, ●), SJS128 (ΔmpdA ΔtpsABC, ○), SJS132 (ΔmtdB ΔtpsABC, ■) and SJS134 (ΔmpdA ΔmtdB ΔtpsABC, □) are visualized. Conidia of these strains were subjected to heat stress in a heat bath at 54 °C and sampled for up to 60 minutes. Mean values of three biological replicates are shown; standard deviations are indicated by error bars. The conidia of the three knock-out strains SJS126, SJS132 and SJS134 were all similarly affected in their heat resistance, while the conidia of knock-out strain SJS128 were very heat sensitive. D-values were calculated based on linear regression (Table 7.1). During 1 hour of 54°C heat stress wild-type conidia showed ~1 log reduction (1 in 10^1 conidia survive), knock-out strains SJS126, SJS132 and SJS134 showed ~3 log reduction (1 in 10^3 conidia survive) while knock-out strain SJS128 showed, based on linear regression, ~15 log reduction (1 in 10^{15} conidia survive).

After assessing the heat resistance of the conidia from the knock-out strains, additional heat inactivation experiments, that followed heat inactivation in time, were performed to further quantify conidial heat resistance of a subset of heat sensitive strains. Strains SJS126 (Δ*tpsABC*), SJS128 (Δ*tpsABC*, Δ*mpdA*), SJS132 (Δ*tpsABC*, Δ*mtdB*) and SJS134 (Δ*tpsABC*, Δ*mpdA*, Δ*mtdB*) as well as their parental strain MA234.1 were analysed. Survival of conidia during a 54°C heat exposure was followed in time (Figure 7.7) and D-values were calculated from linear regression lines based on this data (Table 7.1). All four knock-out strains have a decreased conidial heat resistance when compared to their parental strain (MA234.1), confirming the earlier findings shown in Figure 7.6 and Figure 7.S3. The conidia of strains SJS126, SJS132 and SJS134 were all similarly sensitive to heat, with D_{54}-values of 22.1 ± 7.7, 22.9 ± 5.4 and 22.4 ± 3.5 minutes respectively when compared to the parental strain MA234.1, which had a D_{54}-value of 52.3 ± 25.8 minutes. To put this in perspective in terms of spore survival; these three knock-out strains display a 3-log reduction after 60 minutes (1 in 10^3 conidia survives the heat treatment) when compared to 1-log reduction (1 in 10^1 conidia survive the heat treatment) observed in the parental strain. The conidia of strain SJS128 (Δ*tpsABC*, Δ*mpdA*) were the most heat sensitive with a decimal reduction value of D_{54} = 3.9 ± 1.0 min when compared to their parental strain D_{54} = 52.3 ± 25.8 min. Therefore, a 60 minutes heat stress of 54°C would result in a ~1 log reduction in the amount of viable conidia in the parental strain (1 in 10^1 conidia survives the heat stress), and a ~13 log reduction in the four-fold knock-out strain SJS128 (1 in 10^{13} conidia survives the heat stress). These results in combination with the obtained compatible solute profiles (Figure 7.6A) suggest that both mannitol and trehalose play a pivotal role in the heat resistance of *A. niger* conidia.

Conidia lacking compatible solutes germinate different from wild-type conidia

Compatible solutes are often referred to as storage sugars, required for the early stages of germination [26,31–33]. Germination efficiency of knock-out strains lacking compatible solutes was tested by plating ~100 conidia (counted using a Bio-Rad TC20™ automated cell counter) on MEA plates in triplicates to get an initial indication of germination and CFUs (Table 7.S1). These results suggest a near 100% germination on MEA plates

for wild-type conidia, as well as for all compatible solute lacking knock-out strains created in this study. From this result it was concluded that all conidia lacking compatible solutes are viable and are capable of germinating under nutrient-rich conditions on MEA plates.

To further investigate if there is any effect on the swelling and germ tube formation of conidia with altered compatible solutes concentrations, germination experiments were performed following germination on defined NaPS medium (25 mM Na_2HPO_4/NaH_2PO_4 (pH 6.0), 2 mM $MgSO_4$) testing four germination triggering molecules; glucose, alanine, proline and arginine. The swelling and germination of conidia was tracked using oCelloscope imaging, based on previous work [34]. The asymmetric model [35] was used to describe germination of conidia. The maximum germination percentage after 24 hours (P_{max}) and the time in hours until 50% of the observed germination was reached (τ) were calculated from this model, and all germination characteristics are summarized in Table 7.S2. Strains tested included lab strain N402, parental strain MA234.1 (*ΔkusA*), compatible solutes lacking four-fold knock-out strain SJS128 (*ΔtpsABC*, *ΔmpdA*), complemented knock-out strain SJS153 and 38h conidia from lab strain N402. The P_{max} values were used to compare the germination percentages after 24 hours of conidia that have wild-type levels of compatible solutes (N402, MA234.1, SJS153) versus conidia with altered compatible solute composition (SJS128 and 38h N402 conidia) (Figure 7.8).

All conditions with higher germination percentages (P_{max} = the maximum germination percentage, determined at t = 24 hours) also showed significantly higher germination speeds (theta = time until 0.5, or half, of P_{max} is reached), as is seen in Table 7.S2. Additionally, the swelling and germ tube formation percentages follow the same trend. Therefore, conidia that were triggered for germination and showed swelling, also formed germ tubes given time in the conditions tested. Conidia of MA234.1 and SJS153 germinated the same in all tested conditions. A slight difference in germination percentage was found between wild-type strain N402 and the *ΔkusA* strains MA234.1 and SJS153 in the 10 mM glucose condition, where N402 germinated slightly better (~35%) than the two *ΔkusA* strains (~26%). However, the largest differences in germination percentages

were seen when comparing 38h conidia and SJS128 conidia to the other three strains. In conditions with relatively high glucose and high amino acid concentrations, 8d N402 wild-type conidia showed relatively high germination percentages (93% in 10 mM proline, 60% in 10 mM alanine and 35% in 10 mM glucose). In these conditions, the 38h N402 conidia have significantly lower germination percentages (49% in 10 mM proline, 48% in 10 mM alanine and 7% in 10 mM glucose) than the 8 days N402 conidia. Interestingly, the 38h conidia show significantly higher germination percentages (26% instead of 11% in 10 mM arginine and 18% instead of 8% in 0.1 mM proline) than 8d conidia when germinating in 10 mM arginine and 0.1 mM proline. Overall, the results show that 38h conidia have higher germination percentages in 10 mM arginine and 0.1 mM proline, but lower germination percentages in 10 mM glucose and 10 mM alanine compared to 8d conidia.

Conidia from strain SJS128 (*ΔmpdA*, *ΔtpsABC*), containing no trehalose and very limited mannitol (Figure 7.6A), showed significantly higher germination percentages than parental strain in 10 mM arginine, 0.1 mM proline and 1 mM alanine (22% versus 14%, 29% versus 7% and 27% versus 13%, respectively), but a significantly lower germination percentage than the parental strain in 10 mM glucose (12% versus 27%, respectively). Therefore, similar to the observations for young conidia, the conidia from strain SJS128 showed significantly different germination on all four germination triggers. In both 38h conidia and SJS128 conidia germination percentages were higher than wild-type conidia in 10 mM arginine and 0.1 mM proline, and lower than wild-type conidia in 10 mM glucose. In conclusion these data indicate that although young conidia do not show identical germination kinetics to conidia from strain SJS128, they do show similar trends.

Additional experiments are needed to fully elucidate the effect of compatible solutes on the germination kinetics of *A. niger* conidia. However, it is safe to conclude that young conidia germinated differently from old(er) conidia in all four conditions tested. Additionally, conidia from four-fold knock-out strain SJS128 (containing no trehalose and limited mannitol) also germinated significantly differently from wild-type conidia in all four

conditions tested. Therefore, the results indicate that internal compatible solutes have an impact on the germination kinetics of *A. niger* conidia.

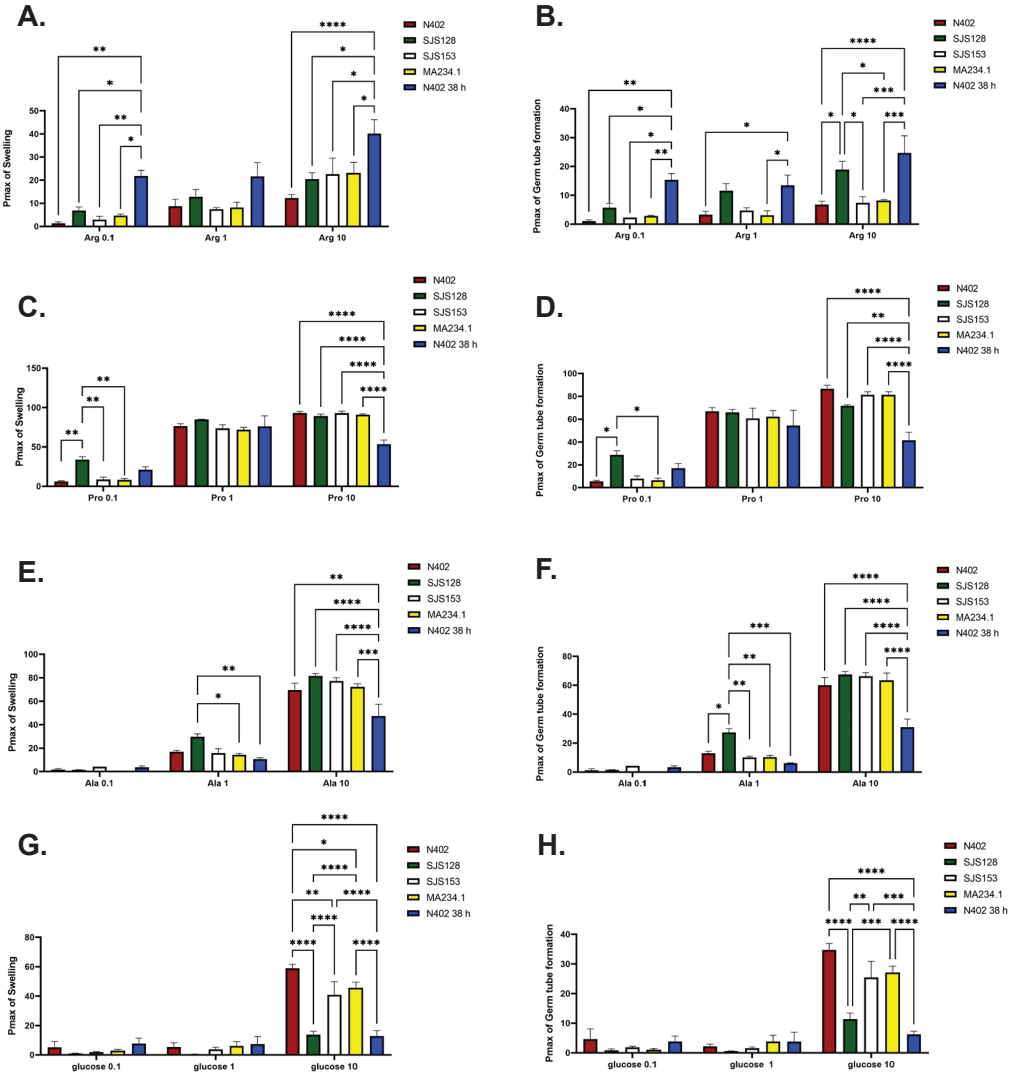

Figure 7.8. Swelling and germ tube formation percentages (P_{max}) after 24 hours on NaPS with glucose, alanine, proline and arginine. All four molecules (arginine, proline, alanine and glucose) were added as 0.1 mM, 1 mM and 10 mM. Conidia were harvested from MEA plates incubated for 8 days or 38 hours at 28°C. Germination experiments were done in triplicates and grouped, a Two-way ANOVA and subsequently Tukey's test for multiple comparisons were performed to calculate significant differences between the strains ($p<0.05$). All significantly higher germination percentages are accompanied by significantly higher germination speeds (theta), see Table 7.S2. The germination percentages follow the pattern seen in the swelling germination per-

centages, indicating that no strain or conditions causes swelling without subsequently germination. Germination percentages of conidia of compatible solute mutant SJS128 (*ΔmpdA*, *ΔtpsABC*) were higher than parental strain MA234.1 or wild-type strain N402 in NaPS medium with 10 mM arginine, 0.1 mM proline and 1 mM alanine. Similarly, young conidia had higher germination percentages than 8d old conidia (N402) in 10 mM arginine and 0.1 mM proline (but not alanine). Both young conidia and SJS128 conidia had lower germination percentages in glucose when compared to the controls. Swelling percentages on arginine (**A**), proline (**C**), alanine (**E**) and glucose (**G**) were visualized. Similarly, germ tube formation percentages on arginine (**B**), proline (**D**), alanine (**F**) and glucose (**H**) were visualized.

Discussion

Filamentous fungi grow as colonies, where young conidia are located on conidiophores at the edge of the colony, and matured conidia reside in the middle of the colony [15]. Therefore, the conidial population from a single fungal colony is highly heterogeneous with respect to their age. In this study, we show that young *A. niger* conidia harvested from MEA plates 38 hours after confluent inoculation have significantly less internal compatible solutes and are sensitive to heat stress compared to their older counterparts (Figure 7.3). The heat resistance and compatible solute concentrations of the conidia increased gradually when strains were grown for longer times. This indicates that during the early hours of conidiation, *A. niger* conidia do not contain the expected high concentrations of compatible solutes (yet), even though conidia harvested from these plates are viable and the spore chains already contain multiple conidia (Figure 7.2 & Figure 7.3). Therefore, we conclude that compatible solute accumulation and the observed heat resistance increase are part of the conidial maturation process in *A. niger*.

A recent study showed that conidia are still subject to change and mature after their formation, while being attached to the spore chain [36]. In agreement with Wang and colleagues, our findings suggest the accumulation of internal compatible solutes, and subsequently heat resistance increase, occurs after conidia are formed. When 43h conidia were dry harvested and kept for further incubation, a heat resistance increase was no longer observed, indicating that attachment to the spore chain is crucial for proper heat resistance maturation of *A. niger* conidia. Several pre-culturing conditions are known to impact conidial compatible solute concentrations and even germination kinetics. A recent study in *A. fumigatus* showed that conidia from a single strain can differ in germination kinetics, depending on the growth medium the mycelium was feeding on during conidiation [37]. Moreover, conidia harvested from plates cultivated at a high(er) temperature have high(er) heat resistance in both *A. fumigatus* and *P. roqueforti*, and this heat resistance increase correlates with an increase in the amount of internal trehalose in both species [7,9]. Here we show for the first time that compatible solute accumulation occurs after conidial formation, suggesting that the increased accumulation

of trehalose inside conidia due to high cultivation temperature also occurs after conidial formation when the conidia are still attached to the chain.

We propose that the compatible solute composition of any given conidium is dependent on the amount of time that has passed since its formation, and the environmental cues received during this time. Moreover, this difference in compatible solute composition has a direct effect on the germination kinetics of the conidium (Figure 7.8). Conidia from knock-out strain SJS128 (*ΔmpdA*, *ΔtpsABC*), containing limited compatible solutes, have a different germination profile from wild type in all tested conditions, showing higher germination percentages than wild type in 10 mM arginine, 0.1 mM proline and 1 mM alanine, and lower germination percentages than wild type in 10 mM glucose. This is in agreement with a study done on *A. nidulans*, where conidia of a *ΔtpsA* strain, lacking all internal trehalose, were shown to have a reduced germ tube formation speed during carbon (glucose) limiting conditions [38]. These authors conclude that internal trehalose concentration of conidia impacts germination, but that trehalose is not essential for the overall germination process. Indeed, in our study we show that strain SJS128 (*ΔmpdA*, *ΔtpsABC*), lacking all trehalose and most mannitol, is still able to germinate with 10 mM glucose, 1 mM alanine and even in nutrient limited NaPS medium containing 0.1 mM proline and arginine, suggesting that both internal trehalose and mannitol pools are not essential for germ tube formation of *A. niger* conidia.

Conidia of *A. niger* show differences in internal compatible solute composition based on age, but also based on environmental cues received during conidiation, such as cultivation temperature (see Chapter 7). This suggests that the fungus employs a form of bet-hedging, where conidial populations from a single fungal colony are diverse in their compatible solute composition, based on age and environmental cues received during conidiation, which has a direct impact on germination kinetics of conidia (Figure 7.8). This heterogeneity among *A. niger* conidia form a single colony could be considered a survival strategy and an ecological advantage, since there is a natural distribution of conidia from the same colony with different germination kinetics. It is interesting to hypothesize about the possibility that the fungus can shift between these types of conidia,

depending on the environmental cues it has experienced during conidiation.

The compatible solutes trehalose and mannitol both significantly contribute to the heat resistance of *A. niger* conidia. The impact of trehalose on heat resistance is best illustrated by the conidia of strain SJS126 (Δ*tpsABC*), which are devoid of trehalose and are more sensitive to heat stress than wild-type conidia (Figure 7.S3), while no significant increase in any other compatible solute is observed. The impact of mannitol on heat resistance is best illustrated by the four-fold knock-out strain SJS128 (Δ*tpsABC*, Δ*mpdA*), which lacks most compatible solutes including most mannitol and is very heat sensitive, compared to the four-fold knock-out SJS132 (Δ*tpsABC*, Δ*mtdB*) that contains mainly a large amount of mannitol (Figure 7.6). Conidia of knock-out strains SJS132 (Δ*tpsABC*, Δ*mtdB*) and SJS134 (Δ*tpsABC*, Δ*mpdA,* Δ*mtdB*) each have comparable heat resistance (Table 7.1), but have different compatible solute profiles (Figure 7.6). This suggests that arabitol and glycerol, which are the only significant compatible solute differences between SJS132 and SJS134 ($p = 0.02$ and $p = 0.02$, respectively based on a Student's t-test), do not significantly impact heat resistance of *A. niger* conidia.

The D-value (54°C) of 39h conidia, lacking most compatible solutes (Figure 7.3A), and the D-value of strain SJS128 (*ΔmpdA*, *ΔtpsABC*), lacking all trehalose and most mannitol (Figure 7.6A), were 5.3 ± 0.6 minutes and 3.3 ± 1.0 minutes, respectively. The 39h conidia are not identical to 8d conidia of knock-out strain SJS128 in terms of compatible solute composition and heat resistance, but do show similar trends. Both contain low concentrations of compatible solutes, are relatively heat sensitive with comparable D-values, and both germinate significantly different compared to wild-type conidia (Figure 7.8).

It would be informative to create conidia containing wild-type levels of trehalose but no mannitol, however deletion of the genes *mpdA*, *mtdA* and *mtdB* did not result in conidia unable to produce any mannitol (conidia of triple knock-out strain SJS139 contained ~80% of wild-type mannitol concentration) (Figure 7.S2). Previously, it was suggested that the mannitol dehydrogenases can act to either synthesize and/or metabolize mannitol, which would explain the remaining ~30% mannitol observed in the

ΔmpdA strain when compared to wild type [20]. However, the conidia of a strain where the two putative mannitol dehydrogenases (*mtdA*, *mtdB*) have been additionally deleted (SJS139) still contain mannitol, suggesting that either additional mannitol dehydrogenases are present, or an alternative biosynthesis route is producing the leftover mannitol in these strains.

The *mtdA* deletion did not alter mannitol concentrations whereas the *mtdB* deletion did (Figure 7.S4), this suggests that *mtdB* rather than *mtdA* codes for the main mannitol dehydrogenase in *A. niger*. This also suggests that a mannitol cycle could still be active in *A. niger,* contrasting a previous report stating that such cycle does not exist in *A. niger* based on the spatial differentiation of the MpdA and MtdA enzymes [27]. Additionally, we noted that HPLC data and heat resistance data of strains only deleted in genes that are part of the trehalose cycle (*tpsABC*), suggested that *tpsC* is not important for trehalose biosynthesis (Figure 7.S3). However, the presence of the *tpsC* gene did have a significant impact on the internal compatible solute composition and heat resistance when a *ΔtpsAB, ΔmpdA* strain was compared with a *ΔtpsABC, ΔmpdA* strain (Figure 7.6). Therefore, only when genes were deleted in both the trehalose and mannitol cycles was the importance of *tpsC* for the biosynthesis of trehalose observed. Similarly, the importance of *mtdB* for the metabolism of mannitol was only observed in a strain deleted in genes from both the trehalose and mannitol cycles (Figure 7.S4B).

Additional experiments are needed to fully understand the impact of internal compatible solutes on the germination of *A. niger* conidia. Future research could focus on the molecular mechanisms behind the observed changes in germination of conidia with limited internal compatible solutes. Dijksterhuis and colleagues have shown that the microviscosity inside conidia decreases during the germination process and that compatible solutes play an important role in the internal microviscosity of conidia [39,40]. Perhaps the conidia of the *ΔtpsAB, ΔmpdA* strain and the young conidia have decreased internal microviscosity, thereby impacting the germination process or trigger, which would be an interesting topic for future studies.

Materials and Methods

Standard growth media and conditions used

The strains used in this study are listed in Table 7.2. Media were prepared as described previously [41]. Minimal medium (MM) contained 1% (w/v) glucose and 1.5% agar, supplemented with hygromycin (100 µg ml^{-1}) when needed. Transformation plates contained minimal medium + sucrose (MMS) 32.5% (w/v) sucrose and 1.5% agar, supplemented with hygromycin (200 µg ml^{-1}) and caffeine (500 µg ml^{-1}). Malt extract agar (MEA, Oxoid) contained 3% (w/v) malt extract, 0.5% (w/v) mycological peptone and 1.5% agar. In order to harvest conidia, strains were first inoculated on MEA plates and grown for 8 days at 28 °C. Conidia were harvested by adding 13 mL of physiological salt buffer (PS, 0.9% (w/v) NaCl and 0.02% (v/v) Tween-80 in demi water), after which the conidia were carefully scraped from the plate using a cotton swab. The resulting spore solution was filtrated through a sterilized filter (Amplitude™ Ecocloth™ Wipes, Contec Inc., Spartanburg, SC, USA) in order to remove mycelial debris.

Table 7.2. List of strains used in this study

Name	Genotype	Parental strain	Reference
N402	*cspA1, amdS-*		[45]
MA234.1	*cspA1, ΔkusA::DR-amdS-DR*	N402	[42]
VO1	*cspA1, ΔkusA::DR-amdS-DR, ΔmtdA*	MA234.1	This study
VO2	*cspA1, ΔkusA::DR-amdS-DR, ΔmpdA*	MA234.1	This study
VO3	*cspA1, ΔkusA::DR-amdS-DR, ΔmtdA, ΔmpdA*	MA234.1	This study
SJS120	*cspA1, ΔkusA::DR-amdS-DR, ΔtpsA*	MA234.1	This study
SJS121	*cspA1, ΔkusA::DR-amdS-DR, ΔtpsB*	MA234.1	This study
SJS122	*cspA1, ΔkusA::DR-amdS-DR, ΔtpsC*	MA234.1	This study
SJS123	*cspA1, ΔkusA::DR-amdS-DR, ΔtpsAB*	SJS121	This study
SJS124	*cspA1, ΔkusA::DR-amdS-DR, ΔtpsAC*	MA234.1	This study
SJS125	*cspA1, ΔkusA::DR-amdS-DR, ΔtpsBC*	SJS121	This study
SJS126	*cspA1, ΔkusA::DR-amdS-DR, ΔtpsABC*	SJS121	This study
SJS127	*cspA1, ΔkusA::DR-amdS-DR, ΔmpdA, ΔtpsAB*	SJS123	This study
SJS128	*cspA1, ΔkusA::DR-amdS-DR, ΔmpdA, ΔtpsABC*	SJS126	This study
SJS129	*cspA1, ΔkusA::DR-amdS-DR, ΔmtdB*	MA234.1	This study
SJS130	*cspA1, ΔkusA::DR-amdS-DR, ΔmtdB, ΔmpdA*	VO2	This study
SJS131	*cspA1, ΔkusA::DR-amdS-DR, ΔmtdB, ΔtpsAB*	SJS123	This study

Name	Genotype	Parental strain	Reference
SJS132	*cspA1, ΔkusA::DR-amdS-DR, ΔmtdB, ΔtpsABC*	SJS126	This study
SJS133	*cspA1, ΔkusA::DR-amdS-DR, ΔmtdB, ΔmpdA, ΔtpsAB*	SJS127	This study
SJS134	*cspA1, ΔkusA::DR-amdS-DR, ΔmtdB, ΔmpdA, ΔtpsABC*	SJS128	This study
SJS135	*cspA1, ΔkusA::DR-amdS-DR, ΔtpsAB, ΔmtdA*	SJS123	This study
SJS136	*cspA1, ΔkusA::DR-amdS-DR, ΔtpsABC, ΔmtdA*	SJS126	This study
SJS137	*cspA1, ΔkusA::DR-amdS-DR ΔtpsABC, ΔmpdA, ΔmtdA*	SJS128	This study
SJS138	*cspA1, ΔkusA::DR-amdS-DR, ΔmtdAB*	SJS129	This study
SJS139	*cspA1, ΔkusA::DR-amdS-DR, ΔmtdAB, ΔmpdA*	SJS130	This study
SJS141	*cspA1, ΔkusA::DR-amdS-DR, ΔmtdAB, ΔtpsABC*	SJS132	This study
SJS142	*cspA1, ΔkusA::DR-amdS-DR, ΔmtdAB, ΔmpdA, ΔtpsABC*	SJS134	This study
SJS149.2	*cspA1, ΔkusA::DR-amdS-DR, mpdA* T669A, T672G	VO2	This study
SJS152.3	*cspA1, ΔkusA::DR-amdS-DR, tpsC* T93A, A96T	SJS126	This study
SJS153	*cspA1, ΔkusA::DR-amdS-DR, tpsA* T258C, G273A	SJS128	This study
SJS154	*cspA1, ΔkusA::DR-amdS-DR, tpsB* G267A, G270A	SJS132	This study
SJS155	*cspA1, ΔkusA::DR-amdS-DR, tpsA* T258C, G273A, *tpsB* G267A, G270A	SJS134	This study
SJS156	*cspA1, ΔkusA::DR-amdS-DR, ΔmpdA, ΔtpsABC*	SJS134	This study

Conidial age experiments

The age of conidia was defined as the amount of time passed since inoculation. Hence, conidia harvested from a plate that had been incubated for 38 hours are referred to as 38h conidia. The precise amount of time passed may vary a maximum of 15 minutes from their designated age. To prevent heterogeneity in the spore population, conidia were plated out confluently. All plates were inoculated confluently using sterilized glass beads. All plates were incubated at 28°C. All conidial age experiments were done using MEA plates.

CRISPR/Cas9 mediated genome editing approach

Using marker-free CRISPR/Cas9 mediated gene editing [29], ORFs were deleted from the non-homologous end-joining (NHEJ)-deficient *A. niger* strain MA234.1 [42]. All primers used in this study are listed in Table 7.3. The sgRNA targeting the gene of interest (GOI) was created essentially as described previously [29]. All plasmids used in this study are listed in Table 7.4. The DNA repair fragments were obtained by amplifying both the 5' and the 3' flanks of the GOI from the parental strain MA234.1, followed by fusion

PCR. Primers contained an overhang creating a novel 23 base pairs region replacing the original open reading frame (ORF). This 23 base pairs overhang was identical for all deletions and resulted in the replacement of the original gene by a new unique artificially created CRISPR/Cas9 target sequence named KORE1 (sequence: CCGGCTTATATTG-GTACCACTCC). Complementation of the knock-out strains was done by re-inserting the original gene on the original locus, using a CRISPR/Cas9 containing vector targeting the KORE1 sequence to cut open the original loci. In this case, repair DNA was a PCR product amplified from the original gene including both 5' and 3' flanks with the following alteration; In order to show that the correct complementation was obtained, two silent mutations were introduced into the original gene (created during amplification by using primers with overhangs containing these point mutations) before re-insertion the original gene into the genome. In this way, sequencing of the gene confirms that the obtained strain is indeed a complemented, and not a contamination of the genetically identical original parental strain MA234.1.

Table 7.3. List of primers used in this study.

Primer	Sequence	Purpose
pTE1_for	CCTTAATTAAACTCCGCCGAACGTACTG	Creation sgRNA on plasmids
pTE1_rev	CCTTAATTAAAAAAGCAAAAAAGGAAGGTACAAAAAAGC	Creation sgRNA on plasmids
tpsA_2_fw	CAGCTGTCGCTTCTCCCATCGTTTTAGAGCTAGAAATAGCAAG	3' *tpsA* gRNA (target)
tpsA_2_rv	GATGGGAGAAGCGACAGCTGGACGAGCTTACTCGTTTCG	5' *tpsA* gRNA (target)
TS1_tpsA_fw	GTTGTTGCTCGTTAAGTCGGGG	5' tpsA flank (repair DNA fragment)
TS1_tpsA_rv	ATAAGCCAGTCTCGCCCTTTGTGATTGTTCAACGGCCGAGGATC	5' tpsA flank (repair DNA fragment)
TS2_tpsA_fw	GATCCTCGGCCGTTGAACAATCACAAAGGGCGAGACTGGCTTAT	3' tpsA flank (repair DNA fragment)
TS2_tpsA_rv	GCCGGAACTACTCTTGTCCCTT	3' tpsA flank (repair DNA fragment)
DIAG_tpsA_5'_fw	TTGGTCTTGTAGGGGTAGCTGC	Diagnostic PCR tpsA deletion
DIAG_tpsA_3'_rv	GGTGGTTTTTACTGCTGGGGTG	Diagnostic PCR tpsA deletion
tpsB_fw	TTTGTCGTCCATGAACACCGGTTTTAGAGCTAGAAATAGCAAG	3' tpsB gRNA (target)
tpsB_rv	CGGTGTTCATGGACGACAAAGACGAGCTTACTCGTTTCG	5' tpsB gRNA (target)
TS1_tpsB_fw	CCATCTGTCTGCCTGTCCTTCA	5' tpsB flank (repair DNA fragment)
TS1_tpsB_rv	CTCCTTTCGCTCTGCTCTCCATTCTCTTTGGCGAACACAAGCAC	5' tpsB flank (repair DNA fragment)
TS2_tpsB_fw	GTGCTTGTGTTCGCCAAAGAGAATGGAGAGCAGAGCGAAAGGAG	3' tpsB flank (repair DNA fragment)

Primer	Sequence	Purpose
TS2_tpsB_rv	TAGACACCCGAACCAGCAGATG	3' tpsB flank (repair DNA fragment)
DIAG_tpsB_5'_fw	CAACCGCAACCGCTACTACTTC	Diagnostic PCR tpsB deletion
DIAG_tpsB_3'_rv	CCGGACCAAGGGATGCTAAAGA	Diagnostic PCR tpsB deletion
tpsC_2_fw	TCGCTGAAAAAGGTCGACGGGTTTTAGAGCTAGAAATAGCAAG	3' *tpsC* gRNA (target)
tpsC_2_rv	CCGTCGACCTTTTTCAGCGAGACGAGCTTACTCGTTTCG	5' *tpsC* gRNA (target)
TS1_tpsC_fw	TGCTCGAGTCTGAGTCTGAAGC	5' tpsC flank (repair DNA fragment)
TS1_tpsC_rv	GCAGCTCGAAGCATTGCAATTGAGAGACCGTTGGAAGGCTGAAC	5' tpsC flank (repair DNA fragment)
TS2_tpsC_fw	GTTCAGCCTTCCAACGGTCTCTCAATTGCAATGCTTCGAGCTGC	3' tpsC flank (repair DNA fragment)
TS2_tpsC_rv	AGCTGGAAGGCGATTGTAGGTT	3' tpsC flank (repair DNA fragment)
DIAG_tpsC_5'_fw	AATGAATGTGTGTGGGTGCTGC	Diagnostic PCR tpsC deletion
DIAG_tpsC_3'_rv	AAACTGGGAGCGATGCATGAAC	Diagnostic PCR tpsC deletion
mtdA_fw	GCTGGCAAGACAGCCAGCAGGTTTTAGAGCTAGAAATAGCAAG	3' mtdA gRNA (target)
mtdA_rv	CTGCTGGCTGTCTTGCCAGCGACGAGCTTACTCGTTTCG	5' mtdA gRNA (target)
GOI5_mtdA_fw	CGGTTTGTTTCGGTCTTACGGG	5' mtdA flank (repair DNA fragment)
XXGOI5_mtdA_rv	GGAGTGGTACCAATATAAGCCGGCGCGGTCGGATAGAAAATAATGT	5' mtdA flank (repair DNA fragment)
XXGOI3_mtdA_fw	CCGGCTTATATTGGTACCACTCCTGATTGAGGTAGAGATGAGTTTGGT	3' mtdA flank (repair DNA fragment)
GOI3_mtdA_rv	CTGCAACGTCACTTAGTGGCTG	3' mtdA flank (repair DNA fragment)
DIAG_mtdA_3'_rv	GCATGCTTGACGTACGGATTGT	Diagnostic PCR mtdA deletion
DIAG_mtdA_5'_fw	CCCCTTGATTTCTCTCCAGCCA	Diagnostic PCR mtdA deletion
mtdB1_fw	GAATTTGTCGCAAATCGTGGGTTTTAGAGCTAGAAATAGCAAG	3' mtdB gRNA (target)
mtdB1_rv	CCACGATTTGCGACAAATTCGACGAGCTTACTCGTTTCG	5' mtdB gRNA (target)
5_mtdB_fw	ATCAAGGGATGGAAGGGGTTGG	5' mtdB flank (repair DNA fragment)
5_ mtdB _rv	GGAGTGGTACCAATATAAGCCGGGCGGTGTAATTTACCTCTTTGTCGG	5' mtdB flank (repair DNA fragment)
3_ mtdB _fw	CCGGCTTATATTGGTACCACTCCTGGGAGGATGAAGGAGGAAGGA	3' mtdB flank (repair DNA fragment)
3_ mtdB _rv	AGGTGGCACATGTTCGGTATCA	3' mtdB flank (repair DNA fragment)
DIAG_MtdB_3'_rv	CGACCAGATCCTCGAAGGGCCA	Diagnostic PCR mtdB deletion
DIAG_MtdB_5'_fw	CTTGCGGAATTTGCGTGGCCAC	Diagnostic PCR mtdB deletion
mpdA_fw	CGATGAACTTGAGAATGTGGGTTTTAGAGCTAGAAATAGC	3' mpdA gRNA (target)
mpdA_rv	CCACATTCTCAAGTTCATCGGACGAGCTTACTCGTTTCGT	5' mpdA gRNA (target)
GOI5_mpdA_fw	TAGTCGCGAGGGAGTCAAGTTG	5' mpdA flank (repair DNA fragment)
NEW_GOI5_mpdA_rv	GGAGTGGTACCAATATAAGCCGGATTCCGAGTCGATCACCTGCAT	5' mpdA flank (repair DNA fragment)
XXGOI3_mpdA_fw	CCGGCTTATATTGGTACCACTCCAGTGGAAGTCTGATAGTAGAAGGGA	3' mpdA flank (repair DNA fragment)
GOI3_mpdA_rv	TTTGGATTGGCTTGGATTGGGC	3' mpdA flank (repair DNA fragment)

Primer	Sequence	Purpose
DIAG_mpdA_3'_rv	AATCAACCGGGACCATGACTGT	Diagnostic PCR mpdA deletion
DIAG_mpdA_5'_fw	CCGACATGGTGATTGCGTCTTC	Diagnostic PCR mpdA deletion
tpsA_KORE2_fw	CAAGAATTACATACCTATGAAGGACAAAGGGCGAGACTGGCTT	Complementation with different KORE (no repair) for SJS156
tpsA_KORE2_rv	CCTTCATAGGTATGTAATTCTTGGATTGTTCAACGGCCGAGGA	Complementation with different KORE (no repair) for SJS156
tpsB_KORE3_fw	TTTGTTCACAGTCCTCATTAAGGATGGAGAGCAGAGCGAAAGG	Complementation with different KORE (no repair) for SJS156
tpsB_KORE3_rv	CCTTAATGAGGACTGTGAACAAATGACTGCAGCTTTTTCCTTG	Complementation with different KORE (no repair) for SJS156
tpsC_KORE4_fw	AACACCGTTTACCCCCTTAAGGGCAATTGCAATGCTTCGAGCT	Complementation with different KORE (no repair) for SJS156
tpsC_KORE4_rv	CCCTTAAGGGGGTAAACGGTGTTAGAGACCGTTGGAAGGCTGA	Complementation with different KORE (no repair) for SJS156
mpdA_KORE5_fw	TCAGTCTATCCGTTTCTTGACGGAGTGGAAGTCTGATAGTAGA	Complementation with different KORE (no repair) for SJS156
mpdA_KORE5_rv	CCGTCAAGAAACGGATAGACTGATTGCTACTGTCGCAAACTGT	Complementation with different KORE (no repair) for SJS156
SJS_compl_tpsC_seq_rv	ACGTATTGCCATTCCGTCGGAG	sequence silent mutations tpsC gene
compl_tpsA_fw	CTCATCATCAATGAACACCGGCACG	Create complementation with 2 silent mutations in tpsA gene
compl_tpsA_rv	CGTGCCGGTGTTCATTGATGATGAG	Create complementation with 2 silent mutations in tpsA gene
seq_tpsA_fw	GCCGATCTCCTCACGCAGCATC	sequence silent mutations tpsA gene
seq_tpsA_rv	CGGTGGCCTGGTCAGTGGACTA	sequence silent mutations tpsA gene
compl_tpsB_fw	CAGTTTATCATCCATGAACACCGGG	Create complementation with 2 silent mutations in tpsB gene
compl_tpsB_rv	CCCGGTGTTCATGGATGATAAACTG	Create complementation with 2 silent mutations in tpsB gene
seq_tpsB_fw	AGACAAGGTCTCCTTCCCGGGC	sequence silent mutations tpsB gene
seq_tpsB_rv	TCCATGTCCTCTGGTGGCCTGG	sequence silent mutations tpsB gene
seq_mtdB_fw	ACCCGCATCGTGTCTCTCACCA	sequence silent mutations mtdB gene
seq_mtdB_rv	GTGACGACAGGCCAGGAGTCCT	sequence silent mutations mtdB gene
seq_mpdA_fw	CTCGTCGTCACCAGGCACGTTC	Create complementation with 2 silent mutations in mpdA gene
seq_mpdA_rv	CTCAGCAAGCCCCCAACAGTGG	Create complementation with 2 silent mutations in mpdA gene
mpdA_comsil_fw	CCAAAGTAGGCCGTTGTAGCATGGCTGGTGTTGACAGTGA	sequence silent mutations mpdA gene
mpdA_comsil_rv	CTACAACGGCCTACTTTGGACACTTCCGGGGCAAGAAGAT	sequence silent mutations mpdA gene
SJS_tpsC_complementationSilent_fw	GTGACTAGTCCACCACTGGAGAGGGAAGATTCGTATCCCC	Create complementation with 2 silent mutations in tpsC gene
SJS_tpsC_complementationSilent_rv	CTCCAGTGGTGGACTAGTCACATCTTTATCGGGATTGACT	Create complementation with 2 silent mutations in tpsC gene
compl3_mtdB_rv	GCGAGGGCAGCGTAGAGGAAACCGAAAGTGGTACGGGGGG	Create complementation with 2 silent mutations in mpdA gene
compl2_mtdB_fw	CCCCCCGTACCACTTTCGGTTTCCTCTACGCTGCCCTCGC	Create complementation with 2 silent mutations in mpdA gene

Table 7.4. Plasmids used in this study

Plasmid	Technical name	Gene	An# (gene)	Gene name	Target sequence	Reference
pFC332	pFC332	-	-	-	-	[46]
pTLL108.1	pTLL108.1	-	-	-	-	[29]
pTLL109.2	pTLL109.2	-	-	-	-	[29]
pFC332_*mtdA*-sgRNA	pVO1	NRRL3_04005	An15g05450	*mtdA*	GCTGGCAAGACAGCCAGCAG	This study
pFC332_*mtdB*-sgRNA	pSJS4	NRRL3_08606	An03g02430	*mtdB*	GAATTTGTCGCAAATCGTGG	This study
pFC332_*mpdA*-sgRNA	pVO2	NRRL3_05796	An02g05830	*mpdA*	CGATGAACTTGAGAATGTGG	This study
pFC332_*tpsA*-sgRNA	pSJS2	NRRL3_11571	An08g10510	*tpsA*	TCGCGGGTTGACGAAACAAT	This study
pFC332_*tpsB*-sgRNA	pSJS3	NRRL3_04893	An07g08710	*tpsB*	TTTGTCGTCCATGAACACCG	This study
pFC332_*tpsC*-sgRNA	pSJS4	NRRL3_00777	An14g02180	*tpsC*	TCGCTGAAAAAGGTCGACGG	This study
pFC332_KORE1-sgRNA	pTL71.1	-	-	KORE1	CCGGCTTATATTGGTACCACTCC	This study

Transformation techniques for the creation of compatible solute deficient knock-out strains

Protoplastation and subsequent transformation of fungal cells was performed according to a previously described PEG-mediated transformation protocol [41]. CRISPR/Cas9 mediated transformation was done essentially as described previously [29]. In short, each transformation used 2 µg of Cas9-sgRNA plasmid together with 0.5 µg of repair DNA. Transformed cells were incubated at 30 °C on selective MMS containing 200 µg ml^{-1} hygromycin for approximately 3 days (until colonies are visible). Transformants were inoculated on MM selection plates containing 100 µg ml^{-1} hygromycin. To allow loss of the Cas9-sgRNA plasmid, transformants were subsequently grown on MM without selection pressure. Finally, each transformant was grown on both MM and MM containing 100 µg ml^{-1} hygromycin, in order to verify whether the Cas9-sgRNA plasmid was lost when selection pressure was removed. Genomic DNA of transformants was subsequently isolated with a phenol-chloroform based protocol as described previously [41]. Diagnostic PCR was used to confirm deletion of the targeted genes. The presence of the silent point mutations in the complemented strains was confirmed by sequencing the PCR fragments.

The internal compatible solutes investigated with HPLC analyses

Conidia were harvested using PS buffer. All knock-out strains were analysed in biological triplicates. Spore suspension concentrations were determined with a Bio-Rad TC20 Cell Counter. A total of $2*10^8$ cracked conidia per sample were used for HPLC analysis. Conidial suspensions were centrifuged at 13000 rpm at 4°C for 15 minutes after which supernatant was removed. The pellet was immediately frozen in liquid nitrogen. Two metal beads (QIAGEN, 3 mm diameter) were added to the pellets and the tubes were subsequently loaded into a Tissuelyser II (QIAGEN). The cracking of the conidia was performed for 1 minute at 30 Hz. A total of 1 mL of miliQ was added to the crushed samples and subsequently heated in a water bath at ~95°C for 30 minutes. Samples were then centrifuged for 15 minutes at 13.000 rpm. Supernatant was filtered through a 0.2 µm Acrodisc filter and stored in -20°C for a maximum of one week. Samples were thawed and transferred to HPLC tubes and subsequently analysed by HPLC. Two sugar pack columns (Waters), installed in series, were used to analyse the samples. Pure compounds of trehalose, mannitol, glycerol, glucose, erythritol and arabitol were serially diluted and measured as a reference for each HPLC analysis. Compatible solute amounts were calculated by taking the area under the corresponding retention peak and comparing this area to the references.

Germination analyses of *A. niger* conidia using the oCelloScope

Conidia were used directly after harvesting. Spore solutions were counted and diluted to obtain $2 \cdot 10^4$ per well of a 96 wells suspension culture plate (Greiner bio-one, Cellstar 655185, www.gbo.com) in 150 µl NaPS (25 mM Na_2HPO_4/NaH_2PO_4 (pH 6.0), 2 mM $MgSO_4$) with 0.1 mM, 1 mM or 10 mM glucose, alanine, proline or argenine. All experiments were done using three biological replicates. Germination of the conidia was monitored at 28 °C using an oCelloScope imager (Biosense Solutions, www.biosense-solutions.dk) with UniExplorer software version 8.1.0.7682-RI2 as described in [34]. In short, objects were scanned every hour for 24 hours, starting 1 hour after inoculation to allow settling of the conidia. Conidial aggregates, and non-conidial objects at t = 1 h were manually removed from the data set and conidia were followed in time based on

their X and Y coordinates using the fast k-nearest neighbour (KNN) searching algorithm from the R package 'FNN'[43]. This was done from t = x to t = x+1 and vice versa. In addition, neighbour distance of an object was not allowed to exceed 27.5 µm (i.e. 50 pixels) between 2 adjacent time points. The lineage was discontinued if these conditions were no longer met. The objects were classified as resting or germinating conidia at t = 1 h, resting conidia had a surface area of < 150 pixels and circularity > 0.93. Germinating conidia had circularity ≤ 0.93 and had a surface are of > 150. Each sample followed 229-478 conidia, an average was calculated for each condition and listed as part of Table 7.S2. Two-way ANOVA and Tukey's multiple comparison tests were used for statistical analysis and were deemed significant if $p \leq 0.05$.

Modelling of germination kinetics

The asymmetric model [35] was used to describe germination (P) and germination time τ (h) as a function of time, (Eq. 1).

$$P = P_{max}\left(1 - \frac{1}{1+\left(\frac{t}{\tau}\right)^{d}}\right) \qquad \text{(Eq. 1).}$$

P_{max} is the maximal percentage of conidia that germinate (the asymptotic value of P at $t \rightarrow +\alpha$). Germination time τ (h) is the time at which $P = 0.5 \cdot P_{max}$, while d is a shape parameter that can be correlated to the heterogeneity of the population. A low d reflects a population where conidia have more variable individual germination times. To estimate the model parameters of the asymmetric model, three biological replicates (≥ 200 conidia per condition) were fitted together with the R package GrowthRates [44] using the Levenberg-Marquardt algorithm. Parameters were limited to P ≥ 0 and ≤ 120 %, τ ≥ 1 and ≤ 16, d ≥1 and ≤30 when fitting the model. Objects that had an object area > 300 pixels and that had decreased in size were excluded from the data set. Missing objects represent resting spores (R) that are lost during the analysis (i.e. that were no longer detected at t ≥ 2 h because the object had moved or was obscured for instance by germlings of other spores) before they germinated. Size and circularity data of all objects were used for the parameter estimation until the time point when hyphal growth started to obscure resting spores.

Heat treatment assays using a thermo cycler

Heat treatment assays were applied to test for changes in the heat resistance of conidia, see Figure 7.S5 for a detailed overview. The strains were confluently plated on MEA plates and subsequently grown for 8 days at 28°C unless noted otherwise. Measurements were done in biological triplicates. Freshly harvested conidia were diluted in PS buffer and spore suspension concentrations were measured using a Bio-Rad TC20™ automated cell counter. A total of $1*10^6$ conidia inside a volume of 100 µl PS buffer was used for each heat treatment assay. The heat treatment was applied in a thermocycler for 10 minutes at 55 °C, 56 °C, 57 °C or 58 °C. Controls were taken which were subjected to room temperature for 10 minutes. After heat treatment, the conidia were confluently plated on MEA containing 0.05% (v/v) Triton® X-100. Plates were incubated for five days at 28°C, after which pictures were taken. The number of colony forming units (CFUs) per plate is considered a readout of the amount of conidia that survived the heat treatment.

Heat inactivation experiments using a water bath

In order to further quantify the heat resistance, heat inactivation curves of selected knock-out strains were made. These measurements were done in biological triplicates. Freshly harvested conidia were diluted in PS and spore concentrations were measured using a Bio-Rad TC20™ automated cell counter. A volume of 19.8 mL of PS buffer inside an Erlenmeyer was pre-heated in a water bath (Julabo Corio c-bt27) to 54°C. A total volume of 200 µl spore suspension was added to obtain a final concentration of $1x10^7$ spores ml^{-1} inside each Erlenmeyer for the heat inactivation experiment. Samples were taken after 0, 2, 5, 10, 15, 20, 30 and 60 minutes of exposure to heat stress. The samples were serially diluted into 10^6, 10^5, 10^4 and 10^3 spores ml^{-1}, after which 100 µl of each dilution was inoculated on MEA containing 0.05% (v/v) Triton® X-100. Plates were incubated for seven days at 30 °C, after which the number of colony forming units (CFUs) per plate was counted. The results were used to calculate and subsequently plot inactivation curves based on a log-linear fit. The linear regression line in turn was used to calculate a decimal reduction value (D-value).

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Additional files

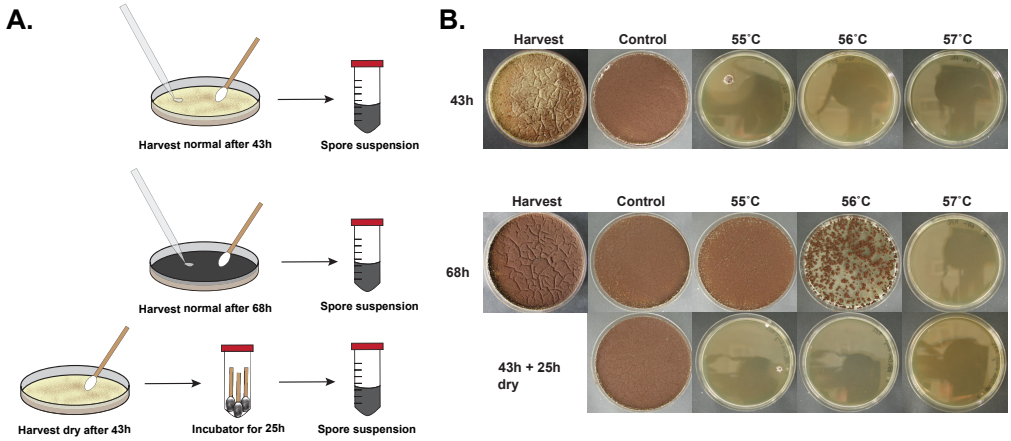

Figure 7.S1. Heat resistance increase no longer observed after 43h conidia detached from the mycelium. Three types of conidia were tested for their heat resistance: 43h conidia, 68h conidia and 43h conidia harvested dry and subsequently incubated for an additional 25 hours. **A.** The methodology of the experiment. The 43h and 68h conidia were harvested in PS buffer as normal. However, some 43h conidia were harvested dry using sterilized cotton buds and subsequently stored inside falcon tubes, and returned to the 28°C incubator for another 25 hours. After 25 hours PS buffer was added and the heat resistance assay was performed as normal. **B.** The heat resistance results, tested with the heat treatment assay (Figure 7.S5). The 43h conidia do not survive 10 minutes at 55 °C or 56 °C, whereas 68h conidia do. When 43h conidia are dry harvested and kept inside a falcon tube, returned to the incubator for another 25h and subsequently tested for heat resistance. No increase in heat resistance is observed for dry harvested conidia if compared to the 43h conidia. These results show that maturation, at least in heat resistance, does no longer occur after conidia are detached, by dry harvesting, from the mycelium.

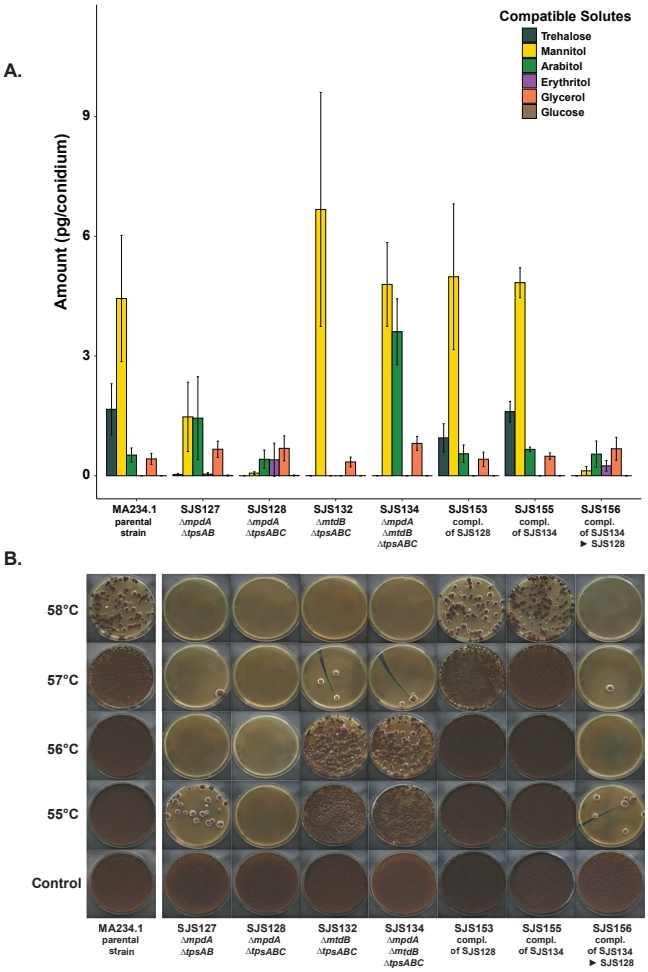

Figure 7.S2. Internal compatible solute composition and heat resistance of conidia from *A. niger* mannitol knock-out strains. Conidia were freshly harvested from MEA plates grown for 8 days at 28°C. Measurements were performed in biological triplicates. **A.** Internal compatible solute composition of conidia from mannitol knock-out strains lacking genes in the mannitol cycle. Only the deletion of the *mpdA* gene resulted in a change of internal sugar composition inside conidia of *A. niger*. Strain VO2 (***Δ****mpdA*) contains less mannitol and more trehalose as has been observed before [20]. The internal compatible solute composition of the strain SJS139 (*mpdA, mtdAB*) was the same as VO2 (*mpdA*). As such, no effect of both *mtdA* or *mtdB* deletion on compatible solute composition of conidia was seen in these knock-out strains. **B.** Heat treatments were applied for 10 minutes to 10^6 conidia (Figure 7.S5). After heat treatment, conidia were plated on plates containing MEA+0.05% triton x-100. Plates were grown for 5 days after which the pictures were made as shown above. The Δ*mpdA* mutation has the largest effect on the heat resistance of *A. niger* conidia as the CFUs is less than 100 when heat stress of 57°C is applied. The strain SJS139, in which *mpdA* and both mannitol dehydrogenases *mtdA* and *mtdB* have been knocked out, is comparable in heat resistance to the Δ*mpdA* single knock-out strain, suggesting that *mtdA* and *mtdB* do not significantly contribute to the heat resistance of *A. niger* conidia.

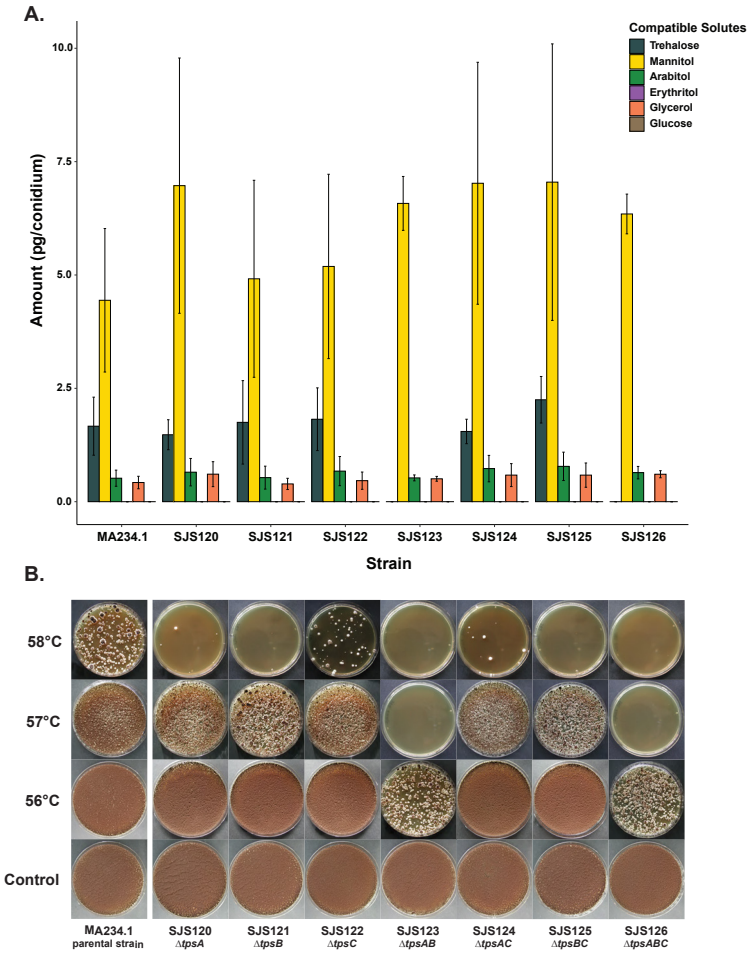

Figure 7.S3. Internal compatible solute composition and heat resistance of conidia from *A. niger* trehalose knock-out strains. Conidia were freshly harvested from MEA plates grown for 8 days at 28°C. Measurements were performed in biological triplicates. **A.** Internal compatible solute composition of conidia from trehalose knock-out strains as determined by HPLC analysis. Conidia from strains SJS123 (***Δ**tpsAB*) and SJS126 (***Δ**tpsABC*) were significantly impacted in their internal compatible solute composition, as they produced no measurable amount of trehalose. No significant increase in another type of sugar was measured in these two strains. **B.** Heat treatments were applied for 10 minutes to 10^6 conidia (Figure 7.S5). After heat treatments conidia were plated on plates containing MEA+0.05% triton x-100. Plates were grown for 5 days after which the pictures were made as shown above. All conidia from knock-out strains lacking *tps* genes showed at least a slight decrease in heat resistance. However, the largest drop in heat resistance was observed in strains SJS123 (***Δ**tpsAB*) and SJS126 (***Δ**tpsABC*). This drop in conidial heat resistance corresponds with the decrease and overall absence of trehalose inside these conidia. Overall, a clear link is seen between internal trehalose concentration and conidial heat resistance.

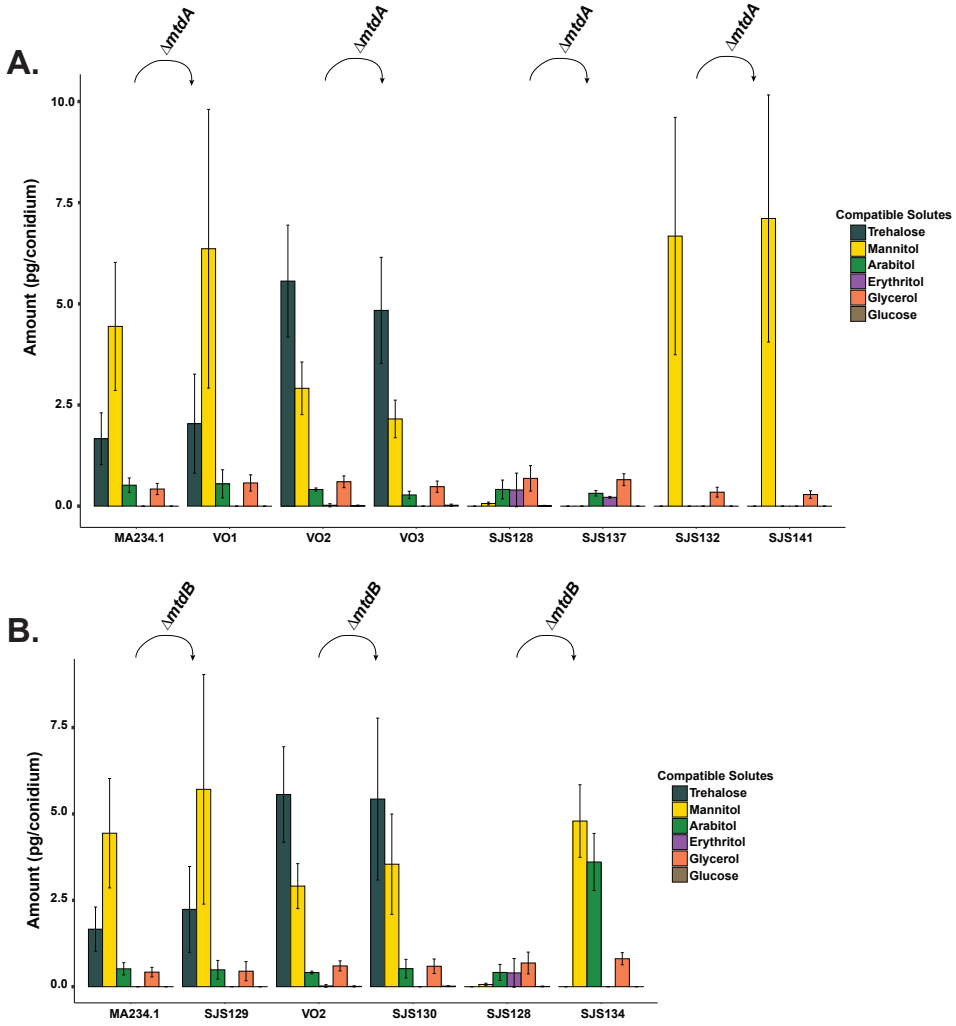

Figure 7.S4. The *mtdA* gene does not have an impact on internal compatible solute composition of conidia. The genes *mtdA* and *mtdB* have been deleted in many different backgrounds. Here we give an overview of the effect of the individual mutations on the internal compatible solute profiles of conidia. In all cases, the parental strain without the *mtdA* or *mtdB* mutation is given first, followed by the strain in which the *mtdA* or *mtdB* gene is additionally knocked-out. **A.** Here the effect of the *mtdA* deletion is visualized. When comparing *ΔmtdA* strains with their respective parental strains, no significant changes in compatible solute profiles were found in any of the knock-out strains where an extra *ΔmtdA* deletion was made. **B.** Here the effect of the *mtdB* deletion is visualized. Initially, in single double or triple knock-out strains the absence of the *mtdB* gene does not show any significant impact on the compatible solute profiles. However, when adding the *ΔmtdB* deletion to the strain SJS128 (*ΔmpdA*, *ΔtpsABC*) therefore becoming strain SJS134 (*ΔmpdA, ΔmtdB, ΔtpsABC*) a clear change in compatible solute profile is seen.

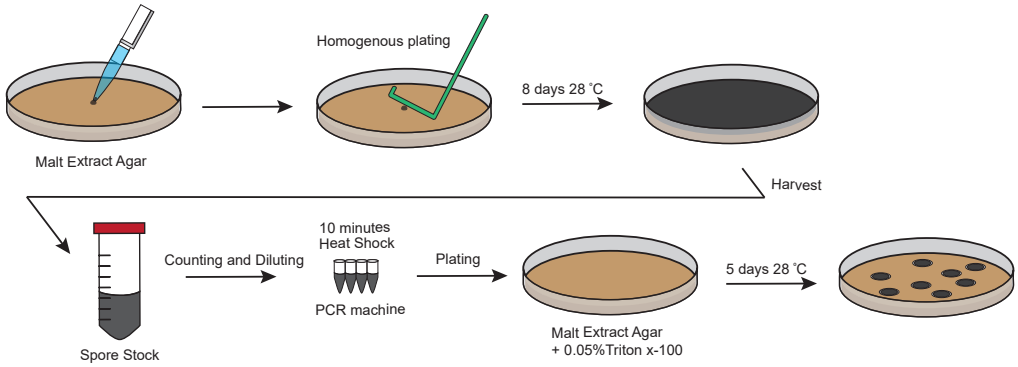

Figure 7.S5. Heat treatment assay protocol. All strains were plated homogenously and subsequently grown on MEA for 8 days (or other amounts of time in days/hours depending on the experiment) at 28°C. Conidia were harvested in PS buffer and subsequently counted using a Bio-Rad Automated Cell Counter. A total volume of 100 µl with $1*10^6$ conidia were heat treated per PCR tube in a thermocycler for 10 minutes. After heat treatment the 100 µl is plated homogenously on MEA + 0.05% Triton X-100 and grown for 5 days at 28°C after which CFUs are counted and pictures were taken.

Table 7.S1. Colony forming units of *A. niger* conidia from knock-out strains plated on MEA.

Strain	Average (CFUs)	Stdev (CFUs)
N402	107	±19
SJS126 (*ΔtpsABC*)	107	±19
SJS128 (*ΔmpdA, ΔtpsABC*)	88	±8
SJS132 (*ΔmtdB, ΔtpsABC*)	92	±11
SJS134 (*ΔmpdA, ΔmtdB, ΔtpsABC*)	73	±17

Conidia were counted with a Bio-Rad TC20™ automated cell counter and diluted until 100 conidia/100µl and subsequently confluently plated. This experiment was done in biological triplicates. No significantly lower amount ($p<0.05$ student t-test) of colonies were formed when conidia from knock-out strains were plated and compared to wild-type conidia of strain N402.

Table 7.S2. Germination kinetics of conidia of *Aspergillus niger* strains with altered internal compatible solute compositions. Maximum germination or swelling percentage after 24 hours (P_{max}), the time in hours until 50% germination/swelling (theta) and the heterogeneity parameter between conidia (d) are given with their corresponding confidence interval in between brackets.

Swelling							
Strain name	**Genotype**	**Treatment**	**Pmax (%)**	**Theta (hours)**	**d**	**Missing**	**Total**
MA234.1	*ΔkusA*	0.1 mM Ala	NA	NA	NA	8	309
N402	Wild type	0.1 mM Ala	2.22 [-1.65;6.08]	15.92 [-14.99;46.8]	2 [-0.79;4.79]	5	326
N402 38h	Young conidia of wild type	0.1 mM Ala	6.08 [-1.45;13.61]	20 [7.81; 32.19]	4.55 [-0.17; 9.28]	12	370
SJS128	*ΔmpdA*, *ΔtpsABC*	0.1 mM Ala	1.88 [-2.78;6.54]	20 [-12.56; 52.56]	3.24 [-2.34; 8.82]	7	497
SJS153	Complementation of SJS128 to MA234.1 (*tpsA* T258C, G273A)	0.1 mM Ala	1.37 [-0.08;2.81]	5.06 [-3.54; 13.67]	2 [-4.53; 8.53]	8	329
MA234.1	*ΔkusA*	0.1 mM Arg	4.23 [3.06;5.39]	5.83 [3.37; 8.29]	2 [0.5; 3.5]	7	359
N402	Wild type	0.1 mM Arg	2.5 [-2.58;7.58]	20 [-0.95; 40.95]	4.31 [-2.79; 11.4]	9	396
N402 38h	Young conidia of wild type	0.1 mM Arg	32.62 [3.31;61.93]	20 [2.07; 37.92]	2.03 [0.99; 3.08]	29	362
SJS128	*ΔmpdA*, *ΔtpsABC*	0.1 mM Arg	6.36 [5.18;7.54]	13.16 [11.56; 14.76]	5.6 [2.5; 8.71]	28	500
SJS153	Complementation of SJS128 to MA234.1 (*tpsA* T258C, G273A)	0.1 mM Arg	3.48 [0.18;6.77]	14.81 [4.19; 25.43]	3.44 [-1.03; 7.91]	12	353
MA234.1	*ΔkusA*	0.1 mM Glucose	3.65 [0.88;6.41]	13.84 [1.52; 26.15]	2 [0.53; 3.47]	16	356
N402	Wild type	0.1 mM Glucose	5.28 [0.38;10.17]	5.81 [-2.42; 14.04]	2 [-3.06; 7.06]	24	348
N402 38h	Young conidia of wild type	0.1 mM Glucose	8.37 [2.96;13.78]	11.77 [3.58; 19.96]	2.48 [0.17; 4.8]	10	353
SJS128	*ΔmpdA*, *ΔtpsABC*	0.1 mM Glucose	0.99 [-0.1;2.09]	17.65 [8.44; 26.86]	5.54 [-3.33; 14.41]	10	464
SJS153	Complementation of SJS128 to MA234.1 (*tpsA* T258C, G273A)	0.1 mM Glucose	2.96 [0.13;5.78]	13.39 [-1.84; 28.63]	2 [0.06; 3.94]	16	396
MA234.1	*ΔkusA*	0.1 mM Pro	7.98 [6.06;9.9]	12.86 [10.68; 15.04]	4.86 [1.79; 7.93]	21	361
N402	Wild type	0.1 mM Pro	6.59 [3.58;9.61]	13.22 [5.99; 20.44]	2 [1.05; 2.95]	9	374
N402 38h	Young conidia of wild type	0.1 mM Pro	20.75 [15.15;26.36]	13.23 [10.56; 15.9]	4.04 [1.85; 6.23]	43	344
SJS128	*ΔmpdA*, *ΔtpsABC*	0.1 mM Pro	34.48 [30.54;38.43]	9.22 [8.12; 10.31]	4.4 [2.57; 6.22]	66	445
SJS153	Complementation of SJS128 to MA234.1 (*tpsA* T258C, G273A)	0.1 mM Pro	10.49 [1.84;19.14]	14.28 [2.99; 25.56]	2.56 [0.22; 4.91]	20	340

Strain name	Genotype	Treatment	Pmax (%)	Theta (hours)	d	Missing	Total
MA234.1	*ΔkusA*	1 mM Ala	13.8 [12.14;15.46]	10.86 [9.62; 12.1]	3.51 [2.54; 4.48]	19	324
N402	Wild type	1 mM Ala	17.52 [13.59;21.45]	10.91 [8.23; 13.6]	2.55 [1.61; 3.49]	27	388
N402 38h	Young conidia of wild type	1 mM Ala	16.35 [3.88;28.82]	20 [9; 30.99]	2.91 [1.45; 4.37]	23	349
SJS128	*ΔmpdA*, *ΔtpsABC*	1 mM Ala	29.9 [27.88;31.93]	10.12 [9.48; 10.77]	4.93 [3.72; 6.15]	56	477
SJS153	Complementation of SJS128 to MA234.1 (*tpsA* T258C, G273A)	1 mM Ala	16.06 [11.64;20.47]	13 [9.57; 16.44]	2.73 [1.71; 3.75]	28	353
MA234.1	*ΔkusA*	1 mM Arg	11.03 [3.85;18.21]	16.09 [8.33; 23.84]	3.29 [0.89; 5.7]	9	272
N402	Wild type	1 mM Arg	12.88 [-5.44;31.19]	20 [-2.06; 42.06]	2.68 [0.27; 5.1]	11	386
N402 38h	Young conidia of wild type	1 mM Arg	21.3 [13.48;29.12]	12.09 [8.3; 15.88]	3.64 [0.9; 6.37]	37	292
SJS128	*ΔmpdA*, *ΔtpsABC*	1 mM Arg	13.18 [9.95;16.41]	12.56 [10.29; 14.83]	4.66 [1.7; 7.63]	51	510
SJS153	Complementation of SJS128 to MA234.1 (*tpsA* T258C, G273A)	1 mM Arg	11.25 [4.82;17.67]	20 [11.16; 28.84]	2.69 [1.72; 3.66]	8	356
MA234.1	*ΔkusA*	1 mM Glucose	5.97 [2.93;9.01]	7.07 [2.13; 12]	2.25 [-0.65; 5.16]	8	322
N402	Wild type	1 mM Glucose	5.49 [1.65;9.32]	7.83 [0.49; 15.17]	2.2 [-1.16; 5.56]	7	311
N402 38h	Young conidia of wild type	1 mM Glucose	4.89 [0.47;9.31]	6.39 [-2.17; 14.94]	2 [-2.49; 6.49]	4	344
SJS128	*ΔmpdA*, *ΔtpsABC*	1 mM Glucose	0.54 [0.34;0.74]	3.49 [0.93; 6.05]	2 [-1.03; 5.03]	12	487
SJS153	Complementation of SJS128 to MA234.1 (*tpsA* T258C, G273A)	1 mM Glucose	3.77 [2.84;4.7]	5.31 [3.37; 7.25]	3.23 [-0.51; 6.96]	7	370
MA234.1	*ΔkusA*	1 mM Pro	71.89 [69.84;73.95]	5.69 [5.47; 5.91]	4.27 [3.59; 4.94]	20	321
N402	Wild type	1 mM Pro	76.37 [73.18;79.55]	6.24 [5.91; 6.56]	4.67 [3.58; 5.77]	22	280
N402 38h	Young conidia of wild type	1 mM Pro	80.63 [59.95;101.31]	13.72 [10.67; 16.77]	3.04 [1.96; 4.12]	37	267
SJS128	*ΔmpdA*, *ΔtpsABC*	1 mM Pro	85.13 [80.44;89.82]	6.97 [6.52; 7.43]	4.57 [3.28; 5.86]	14	427
SJS153	Complementation of SJS128 to MA234.1 (*tpsA* T258C, G273A)	1 mM Pro	73.76 [70.71;76.8]	6.23 [5.91; 6.55]	4.68 [3.59; 5.77]	21	315
MA234.1	*ΔkusA*	10 mM Ala	72.81 [69.25;76.37]	4.83 [4.46; 5.21]	3.37 [2.52; 4.22]	22	250
N402	Wild type	10 mM Ala	69.55 [66.12;72.98]	6.25 [5.85; 6.65]	4.07 [3.05; 5.09]	23	266
N402 38h	Young conidia of wild type	10 mM Ala	56.62 [16.74;96.5]	10.37 [0.89; 19.85]	2 [0; 4]	21	183
SJS128	*ΔmpdA*, *ΔtpsABC*	10 mM Ala	82.29 [77.12;87.46]	8.54 [7.94; 9.13]	3.83 [2.99; 4.66]	25	353
SJS153	Complementation of SJS128 to MA234.1 (*tpsA* T258C, G273A)	10 mM Ala	77.32 [75.56;79.07]	5.82 [5.64; 6]	3.82 [3.38; 4.26]	23	271

Strain name	Genotype	Treatment	Pmax (%)	Theta (hours)	d	Missing	Total
MA234.1	*ΔkusA*	10 mM Arg	32.95 [4.39;61.5]	20 [5.79; 34.21]	2.52 [1.17; 3.87]	11	277
N402	Wild type	10 mM Arg	20.02 [9.17;30.88]	20 [13.9; 26.1]	3.87 [2.27; 5.47]	12	276
N402 38h	Young conidia of wild type	10 mM Arg	40.33 [33.06;47.61]	14.12 [12.29; 15.95]	3.91 [2.7; 5.13]	33	245
SJS128	*ΔmpdA*, *ΔtpsABC*	10 mM Arg	23.68 [17.43;29.94]	16.41 [14.13; 18.7]	5.19 [2.9; 7.49]	6	219
SJS153	Complementation of SJS128 to MA234.1 (*tpsA* T258C, G273A)	10 mM Arg	32.47 [-17.83;82.77]	20 [-8.36; 48.36]	2.23 [0.19; 4.27]	10	289
MA234.1	*ΔkusA*	10 mM Glucose	46.11 [42.82;49.4]	4.35 [3.81; 4.89]	3.18 [1.98; 4.38]	25	313
N402	Wild type	10 mM Glucose	59.06 [56.76;61.36]	4.75 [4.47; 5.04]	4.21 [3.22; 5.19]	21	286
N402 38h	Young conidia of wild type	10 mM Glucose	12.58 [7.37;17.79]	5.49 [1.95; 9.03]	2 [-0.38; 4.38]	32	281
SJS128	*ΔmpdA*, *ΔtpsABC*	10 mM Glucose	14.91 [9.43;20.38]	9.75 [5.11; 14.39]	2.06 [0.87; 3.26]	38	458
SJS153	Complementation of SJS128 to MA234.1 (*tpsA* T258C, G273A)	10 mM Glucose	40.98 [36.26;45.7]	4.67 [3.82; 5.52]	4.06 [1.27; 6.84]	26	229
MA234.1	*ΔkusA*	10 mM Pro	91.97 [85.26;98.69]	5.31 [4.74; 5.89]	3.33 [2.16; 4.5]	16	288
N402	Wild type	10 mM Pro	98.02 [82.39;113.64]	4.14 [2.96; 5.32]	2 [0.84; 3.16]	10	237
N402 38h	Young conidia of wild type	10 mM Pro	54.93 [46.94;62.92]	9.6 [8.17; 11.03]	3.82 [2.16; 5.49]	51	270
SJS128	*ΔmpdA*, *ΔtpsABC*	10 mM Pro	89.86 [83.88;95.84]	6.67 [6.12; 7.23]	3.91 [2.69; 5.12]	18	472
SJS153	Complementation of SJS128 to MA234.1 (*tpsA* T258C, G273A)	10 mM Pro	93.45 [88.98;97.93]	5.28 [4.91; 5.64]	3.85 [2.86; 4.84]	17	298
MA234.1	*ΔkusA*	NaPS	0.17 [-0.08;0.41]	19.96 [13.6; 26.31]	15.73 [-44.8; 76.26]	6	325
N402	Wild type	NaPS	3.99 [-2.07;10.04]	10.41 [-10.08; 30.91]	2 [-2.29; 6.29]	3	252
N402 38h	Young conidia of wild type	NaPS	8.51 [0.58;16.44]	20 [6.1; 33.9]	2.8 [1.12; 4.49]	5	349
SJS128	*ΔmpdA*, *ΔtpsABC*	NaPS	0.18 [0.1;0.27]	10.97 [5.91; 16.03]	29.97 [-120.49; 180.44]	10	492
SJS153	Complementation of SJS128 to MA234.1 (*tpsA* T258C, G273A)	NaPS	1.69 [-0.72;4.1]	11.64 [-6.81; 30.09]	2.38 [-2.4; 7.17]	5	331
Germ tube formation							
Strain name	**Genotype**	**treatment**	**Pmax (%)**	**Theta (hours)**	**d**	**Missing**	**Total**
MA234.1	*ΔkusA*	0.1 mM Ala	NA	NA	NA	8	309
N402	Wild type	0.1 mM Ala	1.75 [-3.29; 6.79]	20 [-10.99; 50.99]	4.1 [-5.2; 13.39]	5	326
N402 38h	Young conidia of wild type	0.1 mM Ala	5.52 [-0.79; 11.82]	20 [9.44; 30.56]	4.91 [-0.02;9.85]	12	370

Strain name	Genotype	treatment	Pmax (%)	Theta (hours)	d	Missing	Total
SJS128	*ΔmpdA*, *ΔtpsABC*	0.1 mM Ala	1.88 [-2.78; 6.54]	20 [-12.56; 52.56]	3.24 [-2.34; 8.82]	7	497
SJS153	Complementation of SJS128 to MA234.1 (*tpsA* T258C, G273A)	0.1 mM Ala	1.45 [-0.41; 3.31]	6.39 [-5.75; 18.53]	2 [-4.36;8.36]	8	329
MA234.1	*ΔkusA*	0.1 mM Arg	2.31 [1.79; 2.84]	3.27 [1.72; 4.83]	2 [0.04; 3.96]	7	359
N402	Wild type	0.1 mM Arg	1.89 [-4.19; 7.98]	20 [-43.91; 83.91]	2.04 [-1.71; 5.79]	9	396
N402 38h	Young conidia of wild type	0.1 mM Arg	16.4 [10.64; 22.16]	14.23 [10.55; 17.92]	3.73 [1.62; 5.84]	29	362
SJS128	*ΔmpdA*, *ΔtpsABC*	0.1 mM Arg	5.84 [4.51; 7.16]	14.33 [12.54; 16.13]	6.68 [2.04;11.32]	28	500
SJS153	Complementation of SJS128 to MA234.1 (*tpsA* T258C, G273A)	0.1 mM Arg	3.08 [-4.42; 10.58]	20 [-11.87; 51.87]	3.25 [-2.25; 8.76]	12	353
MA234.1	*ΔkusA*	0.1 mM Glucose	1.14 [0.75; 1.53]	11.09 [7.94; 14.23]	5.77 [-1.99; 13.53]	16	356
N402	Wild type	0.1 mM Glucose	4.94 [-1.28; 11.16]	8.24 [-6.14; 22.62]	2 [-2.73; 6.73]	24	348
N402 38h	Young conidia of wild type	0.1 mM Glucose	4.07 [2.29; 5.85]	12.31 [8.3; 16.32]	4.85 [-1.09; 10.79]	10	353
SJS128	*ΔmpdA*, *ΔtpsABC*	0.1 mM Glucose	0.91 [-1.57; 3.38]	20 [-8.09; 48.09]	4.31 [-5.23; 13.85]	10	464
SJS153	Complementation of SJS128 to MA234.1 (*tpsA* T258C, G273A)	0.1 mM Glucose	2.83 [-0.79; 6.46]	17.61 [-6.45; 41.67]	2 [0.24; 3.76]	16	396
MA234.1	*ΔkusA*	0.1 mM Pro	7.47 [4.44; 10.49]	16.22 [12.73; 19.72]	5.23 [1.55; 8.91]	21	361
N402	Wild type	0.1 mM Pro	7.91 [-0.03; 15.86]	20 [2.81; 37.19]	2.4 [0.94; 3.86]	9	374
N402 38h	Young conidia of wild type	0.1 mM Pro	17.84 [11.47; 24.2]	14.42 [11.14; 17.7]	4.63 [1.33; 7.93]	43	344
SJS128	*ΔmpdA*, *ΔtpsABC*	0.1 mM Pro	28.56 [25.96; 31.16]	12.22 [11.42; 13.02]	5.86 [3.97; 7.75]	66	445
SJS153	Complementation of SJS128 to MA234.1 (*tpsA* T258C, G273A)	0.1 mM Pro	10.91 [-1.53; 23.34]	18.48 [3.56; 33.41]	3.14 [0.24; 6.04]	20	340
MA234.1	*ΔkusA*	1 mM Ala	12.62 [10.3; 14.95]	16.61 [15.19; 18.02]	6.35 [4.02; 8.69]	19	324

Strain name	Genotype	treatment	Pmax (%)	Theta (hours)	d	Missing	Total
N402	Wild type	1 mM Ala	13.78 [12.02; 15.53]	14.55 [13.54; 15.55]	6.63 [4.15; 9.12]	27	388
N402 38h	Young conidia of wild type	1 mM Ala	7.35 [5.33; 9.37]	16.7 [14.04; 19.37]	4.41 [2.77; 6.04]	23	349
SJS128	*ΔmpdA*, *ΔtpsABC*	1 mM Ala	27.11 [25.48; 28.74]	13.88 [13.38; 14.38]	5.96 [4.93; 7]	56	477
SJS153	Complementation of SJS128 to MA234.1 (*tpsA* T258C, G273A)	1 mM Ala	9.98 [8.85; 11.1]	14.74 [13.85; 15.62]	6.57 [4.47; 8.67]	28	353
MA234.1	*ΔkusA*	1 mM Arg	4.38 [2.34; 6.42]	18.56 [15.99; 21.13]	11.28 [-2.05; 24.61]	9	272
N402	Wild type	1 mM Arg	4.4 [1.6; 7.21]	17.97 [12.71; 23.23]	5.63 [0.64; 10.63]	11	386
N402 38h	Young conidia of wild type	1 mM Arg	13.6 [10.11; 17.09]	12.2 [9.77; 14.62]	4.48 [1.51; 7.46]	37	292
SJS128	*ΔmpdA*, *ΔtpsABC*	1 mM Arg	11.59 [9.71; 13.47]	13.71 [12.42; 15.01]	7.16 [2.98; 11.33]	51	510
SJS153	Complementation of SJS128 to MA234.1 (*tpsA* T258C, G273A)	1 mM Arg	6.36 [-2.54; 15.26]	20 [-6.27; 46.27]	2.17 [0.4; 3.95]	8	356
MA234.1	*ΔkusA*	1 mM Glucose	4.09 [1.25; 6.93]	8.36 [0.34; 16.39]	2 [-0.56; 4.56]	8	322
N402	Wild type	1 mM Glucose	2.19 [1.73; 2.65]	8.68 [6.76; 10.61]	5.69 [-0.14; 11.51]	7	311
N402 38h	Young conidia of wild type	1 mM Glucose	3.69 [-0.25; 7.64]	7.12 [-3.83; 18.07]	2 [-2.73; 6.73]	4	344
SJS128	*ΔmpdA*, *ΔtpsABC*	1 mM Glucose	0.49 [0.4; 0.57]	4.65 [1.83; 7.48]	30 [-216.74; 276.73]	12	487
SJS153	Complementation of SJS128 to MA234.1 (*tpsA* T258C, G273A)	1 mM Glucose	1.02 [0.56; 1.48]	4.56 [1.1; 8.03]	2.21 [-1.51; 5.93]	7	370
MA234.1	*ΔkusA*	1 mM Pro	75.58 [56.6; 94.56]	16.12 [13.1; 19.14]	3.26 [2.36; 4.17]	20	321
N402	Wild type	1 mM Pro	68.09 [63.27; 72.9]	12.05 [11.39; 12.7]	4.79 [3.82; 5.75]	22	280
N402 38h	Young conidia of wild type	1 mM Pro	73.4 [35.86; 110.95]	17.82 [13.35; 22.29]	5.19 [1.67; 8.71]	37	267
SJS128	*ΔmpdA*, *ΔtpsABC*	1 mM Pro	105.01 [70.83;139.19]	19.79 [16.02; 23.57]	3.73 [2.8; 4.66]	14	427
SJS153	Complementation of SJS128 to MA234.1 (*tpsA* T258C, G273A)	1 mM Pro	60.37 [50.32; 70.42]	11.88 [10.29; 13.46]	4.43 [2.47; 6.39]	21	315

Strain name	Genotype	treatment	Pmax (%)	Theta (hours)	d	Missing	Total
MA234.1	*ΔkusA*	10 mM Ala	61.98 [58.54; 65.42]	13.46 [13.01; 13.92]	6.6 [5.35; 7.86]	22	250
N402	Wild type	10 mM Ala	60.05 [55.18; 64.92]	12.65 [11.96; 13.33]	6.56 [4.54; 8.58]	23	266
N402 38h	Young conidia of wild type	10 mM Ala	47.95 [-6.93; 102.83]	20 [0.75; 39.25]	2.45 [0.74; 4.16]	21	183
SJS128	*ΔmpdA*, *ΔtpsABC*	10 mM Ala	67.65 [65.14; 70.16]	12.87 [12.56; 13.18]	6.54 [5.65; 7.43]	25	353
SJS153	Complementation of SJS128 to MA234.1 (*tpsA* T258C, G273A)	10 mM Ala	65.62 [61.85; 69.39]	12.91 [12.43; 13.38]	6.88 [5.35; 8.4]	23	271
MA234.1	*ΔkusA*	10 mM Arg	13.63 [7.17; 20.09]	20 [16; 24]	5.51 [3.03; 7.98]	11	277
N402	Wild type	10 mM Arg	11.12 [4.49; 17.75]	20 [15.21; 24.79]	5.87 [2.39; 9.34]	12	276
N402 38h	Young conidia of wild type	10 mM Arg	25.72 [18.54; 32.9]	14.95 [12.47; 17.43]	4.93 [2.2; 7.66]	33	245
SJS128	*ΔmpdA*, *ΔtpsABC*	10 mM Arg	21.92 [16.45; 27.39]	16.42 [14.38; 18.46]	5.71 [3.08; 8.34]	6	219
SJS153	Complementation of SJS128 to MA234.1 (*tpsA* T258C, G273A)	10 mM Arg	12.75 [4.55; 20.95]	20 [15.67; 24.33]	7.4 [1.7; 13.1]	10	289
MA234.1	*ΔkusA*	10 mM Glucose	27.25 [25.88; 28.62]	7.2 [6.78; 7.61]	5.08 [3.69; 6.48]	25	313
N402	Wild type	10 mM Glucose	34.58 [33.38; 35.78]	7.01 [6.74; 7.28]	5.57 [4.42; 6.71]	21	286
N402 38h	Young conidia of wild type	10 mM Glucose	6.73 [3.78; 9.69]	14.23 [7.96; 20.49]	2.42 [1.29; 3.55]	32	281
SJS128	*ΔmpdA*, *ΔtpsABC*	10 mM Glucose	11.77 [7.92; 15.62]	12.13 [8.59; 15.67]	3.32 [1.32; 5.32]	38	458
SJS153	Complementation of SJS128 to MA234.1 (*tpsA* T258C,G273A)	10 mM Glucose	25.49 [22.85; 28.14]	6.67 [5.95; 7.39]	6.91 [1.99; 11.83]	26	229
MA234.1	*ΔkusA*	10 mM Pro	80.97 [78.63; 83.3]	11.75 [11.49; 12.01]	5.95 [5.3; 6.6]	16	288
N402	Wild type	10 mM Pro	93.16 [75.27; 111.06]	9.94 [7.89; 11.99]	2.95 [1.72; 4.17]	10	237
N402 38h	Young conidia of wild type	10 mM Pro	48.99 [35.06; 62.92]	16.59 [13.98; 19.2]	4.77 [2.75; 6.8]	51	270
SJS128	*ΔmpdA*, *ΔtpsABC*	10 mM Pro	74.37 [71.07; 77.68]	14.91 [14.57; 15.26]	6.76 [5.91; 7.62]	18	472
SJS153	Complementation of SJS128 to MA234.1 (*tpsA* T258C, G273A)	10 mM Pro	81.13 [78.57; 83.69]	11.54 [11.26; 11.81]	6.87 [5.89; 7.85]	17	298

Strain name	Genotype	treatment	Pmax (%)	Theta (hours)	d	Missing	Total
MA234.1	*ΔkusA*	NaPS	NA	NA	NA	6	325
N402	Wild type	NaPS	1.72 [0.57; 2.87]	4.81 [-0.48; 10.09]	2 [-2.32; 6.32]	3	252
N402 38h	Young conidia of wild type	NaPS	7.26 [1.81; 12.72]	20 [10.01; 29.99]	3.19 [1.54; 4.84]	5	349
SJS128	*ΔmpdA*, *ΔtpsABC*	NaPS	0.18 [0.1; 0.27]	10.97 [5.91; 16.03]	29.97 [-120.49; 180.44]	10	492
SJS153	Complementation of SJS128 to MA234.1 (*tpsA* T258C, G273A)	NaPS	0.82 [0.61; 1.04]	12.98 [10.71; 15.25]	30 [-37.23; 97.23]	5	331