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

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CHAPTER 9

Discussion

Discussion

The importance of transcription factors in the sorbic acid resistance of *A. niger*

In Chapter 2, the strain variability in sorbic acid resistance of 100 *A. niger* strains was investigated. We show that the minimal inhibitory concentration of undissociated sorbic acid towards *A. niger* can vary between 2 mM and 7 mM and depends on which strain was tested and which medium was used. A recent study conducted on three *A. niger* strains found MIC values between 2.88 mM – 4.80 mM undissociated sorbic acid [1]. These values are indeed consistent with our data, and fall nicely into the average MIC values found for 100 *A. niger* strains, where we show that the average *A. niger* strain has a MIC value between 2.9 ± 0.41 mM and 4.8 ± 0.83 mM depending on the medium. However, it is important to mention the outliers, such as CBS 113.50 that can even grow in the presence of 7 mM undissociated sorbic acid, as this strain might be the specific *A. niger* strain consistently found contaminating your food product. It is impossible to predict which *A. niger* strain will be encountered in which food production pipelines, and whether the average MIC among the 100 *A. niger* isolates presented here also represents the average *A. niger* conidium found in conidial populations within any given food production plant. In this thesis we show that strain diversity needs to be taken into account when designing effective preservation strategies, and in recent years other researchers have addressed this as well [2].

Transcription factors are important in the weak acid stress response of *A. niger*. The most sorbic acid sensitive wild-type strain CBS 147320 had a premature stop codon in the *sdrA* gene, and complementation of this strain with the *sdrA* gene from lab strain N402 increased the sorbic acid resistance. So, the natural strain variation found in *A. niger* isolates may in part be due to (point) mutations occurring in the transcription factors involved in the sorbic acid response. Additionally, in Chapter 2 we show that complementation of the *sdrA* gene in CBS 147320 leads to transformants with increased sorbic acid resistance, and that two transformants showed an even higher increase in sorbic acid resistance compared to the other transformants. This high sorbic acid resistance

could be due to the donor DNA, containing the complete *sdrA* gene, being inserted more than once into the genome of CBS 147320. Higher expression of *sdrA* may lead to higher sorbic acid resistance in those strains, which could also play a role in the high sorbic acid resistance observed in some *A. niger* isolates. Taken together, the results indicates that transcription factors play an important role in the sorbic acid stress response and contribute to the strain variability in sorbic acid stress resistance of *A. niger* isolates.

The screening of 240 transcription factor knock-out strains revealed the importance of multiple transcription factors, including WarB, in the weak acid stress response of *A. niger*. The *ΔwarB* strain was sensitive to sorbic, benzoic, cinnamic, propionic and acetic acid stress. Additionally, the triple knock-out strain lacking *sdrA, warA* and *warB* was more sorbic acid sensitive than any of the double knock-out strains, indicating that all three transcription factors contribute synergistically to the sorbic acid stress response.

Strain variability in heat resistance

Strain variability was also seen in heat resistance of conidia. In Chapter 3 we investigated the heat resistance of conidia isolated from *P. variotii*, *P. roqueforti* and *A. niger*. The conidia of *P. variotii* have the highest decimal reduction values, meaning that conidia from this species are generally the most heat resistant. When analysing heat resistance of three strains within *P. variotii* the D_{60}-values were 3.7 ± 0.08 minutes, 5.5 ± 0.35 minutes and 22.9 ± 2.00 minutes, which are the most heat resistant conidia known to date [3]. In order to ensure proper heat inactivation of conidia within a food production pipeline, the most heat resistant strain needs to be taken into account, since it is impossible to predict which specific strain will contaminate any given food product. The most heat resistant strain has a D-value that is roughly 6 times higher than the most heat sensitive strain in *P. variotii*. To put this heat resistance difference in perspective; after 23 minutes of 60°C heat stress 10% of the conidia from the heat resistant strain survive and only 0.0001% of the conidia of the heat sensitive strain survive. This difference in heat resistance between strains is also observed in *P. roqueforti* and *A. niger*. In fact, the impact of

strain on the variability of conidial heat resistance is consistent between the three fungi, suggesting that fluctuations in heat resistance due to strain is of a consistent factor, and therefore predictable, within filamentous fungi. Food industry can use these results to model and subsequently predict how the factor 'strain' could potentially impact heat stress resistance of conidia from any given filamentous fungus species.

The possibility of a sexual cycle in *Aspergillus niger*

Parasexual crossings between *A. niger sensu stricto* wild-type strains were attempted in order to use a bulk-segregant approach to pinpoint genes responsible for sorbic or heat resistant conidia. Unfortunately, parasexual crossings were often unsuccessful and heterokaryons could not be obtained, most likely due to heterokaryon incompatibility between most of the wild-type *A. niger sensu stricto* strains. Only 1 in 24 parasexual crossings attempted between wild-type strains were successful. This finding is in agreement with previous reports on the heterokaryon incompatibility between strains from *Aspergillus* section *Nigri* [4,5]. In our work, we limited the parasexual crossings between whole-genome sequenced *A. niger sensu stricto* strains that were genetically similar according to the phylogenetic tree (Chapter 5), but still encountered widespread heterokaryon incompatibility. Therefore, even between genetically similar *A. niger sensu stricto* strains, vegetative (heterokaryon) incompatibility is often observed.

However, vegetative incompatibility does not influence sexual compatibility in filamentous fungi. In fact, many heterothallic ascomycetes, especially heterothallic Aspergilli, show vegetative incompatibility between strains of different mating-type, such is the case for *Aspergillus flavus* and *Aspergillus heterothallicus* [6,7], which are still able to form sexual crosses. Therefore, vegetative incompatibility does not per definition affect the sexual reproduction, hence two heterokaryon incompatible strains could still potentially undergo sexual reproduction with each other (for more information, see Pál et al. [4]). Some of the molecular mechanisms behind this interplay between vegetative and sexual (in)compatibility between strains has been demonstrated in *Neuros-*

pora crassa, in which the *tol* gene mediates the mating-type associated heterokaryon incompatibility [8]. To conclude, even though a widespread vegetative incompatibility is observed between *A. niger sensu stricto* strains (Chapter 5), there is still a form of genetical exchange to be explored between *A. niger* strains; the sexual cycle. The discovery of a sexual state in *A. niger* would therefore open up the possibility to pinpoint genetic elements causing sorbic and heat resistance of conidia, by performing a bulk-segregant analysis using the sexual cycle.

No sexual state of *A. niger* has been observed to date. However, the equal distribution of mating types, and the equal distribution of these mating types throughout the phylogenetic tree (Chapter 4 and Chapter 5), indicate exchange of genetic material in nature. If *A. niger* would be a purely asexual fungus, and genetic exchange through the parasexual cycle is blocked, the presence of both MAT1-1 and MAT1-2 containing strains spread throughout the phylogenetic tree would be unexplainable. The genetical diversity observed between *A. niger sensu stricto* strains in SNPs is on average 6 ± 2 SNPs/kb (Chapter 5), which is not very different from ascomycetes with known sexual cycles. The diversity is higher than the average of 1.3 - 2.6 SNPs/kb found between 95 environmental and clinical strains from *Aspergillus fumigatus* [9]. This suggests ongoing genetical exchange between *A. niger* strains in nature, which due to heterokaryon incompatibility cannot be generated by chromosome shuffling through vegetative crossings. A closely related black Aspergillus species, *Aspergillus tubingensis*, has recently been described as having a sexual cycle [10,11]. Taken together, I hypothesize that a sexual cycle of *A. niger* is probably present and active in nature, however we currently do not know the right conditions to trigger it. By revealing the flipped orientation of the MAT1-1 locus in *A. niger* in Chapter 4, and creating a stable diploid strain between isolates of different mating types in Chapter 5, we gained more insight into the differences between *A. niger* and well-established sexual cycles of other closely related filamentous fungi such as *A. tubingensis*. Perhaps the unusual orientation of the MAT1-1 locus makes the fungus currently unable to perform a sexual cycle *in vitro*, and requires a unique activator or triggering molecule to start the sexual cycle. The ability to form a sta-

ble diploid containing two mating-types in the heterothallic species *A. niger* is, as far as I know, unique and suggests that this species, or at least the two parental strains, have no heterokaryon incompatibility mediated by the mating-type genes themselves, in contrast to this well-described phenomenon in *N. crassa* [12]. This finding indicates that genes involved in mating type gene heterokaryon incompatibility known from *N. crassa*, such as mediator *tol* [8,13], are probably not active in *A. niger*, although additional research is needed to confirm this. The unique diploid will enable to studies on environmental conditions and genetical modifications that could potentially trigger meiosis in *A. niger*.

Compatible solutes accumulate during conidial maturation on the spore chain and affect both stress resistance and germination

The conidium of *A. niger* is well-protected against heat stress, which is due to internal compatible solute composition (as shown in Chapter 7) and possible due to the presence of specific heat shock proteins (as shown in Chapter 8). The melanin present on the cell wall does not protect the cell against heat stress (as shown in Chapter 6), but it does protect the cell against UV-C radiation. All factors that contribute to a high heat stress resistance of the *A. niger* conidium, at least those studied in this thesis, are accumulating inside the cell, and are molecules or proteins that are suggested to stabilize macro-molecules [14–19]. Interestingly, in Chapter 7 we show that trehalose and polyols, the main compatible solutes found in *A. niger* conidia, are only present in small quantities in young conidia and increase in time with conidial age, thereby suggesting compatible solute accumulation is part of the conidial maturation. Taken together with the recent discovery that *A. nidulans* conidia are still transcriptionally active while attached to the spore chain [20], confirms the possibility that these compatible solutes are still actively accumulating inside the conidia after the conidia are formed, while being attached to the spore chain. Additionally, we show that germination kinetics are directly linked to internal compatible solute composition, where both a knock-out strain with conidia containing limited compatible solutes and young conidia with limited compatible solutes germinate better than

eight days old wild-type conidia in MM containing 10 mM arginine or 0.1 mM proline as the sole carbon and nitrogen source. We suggest in Chapter 7 that this could be a form of bet-hedging, where the conidia from a fungal colony are heterogenous in age and are therefore heterogenous in internal compatible solute composition. Some conidia are immature and contain limited internal compatible solutes, therefore these conidia are heat stress sensitive, but germinate better in low concentrations alanine, proline or arginine. Whereas some conidia are matured and contain high amounts of internal compatible solutes, therefore these conidia are heat resistant and germinate better in high concentrations of glucose. It suggests that a certain percentage of spores are primed for quick reproduction with minimal nutrient requirements, whereas other spores are primed for long term survival and stay dormant until ideal conditions are met.

The role of heat shock proteins in the heat resistance of *Aspergillus niger* conidia

In Chapter 8, we show that cultivation temperature increases conidial heat resistance and this correlates with both trehalose, Hsp20 and Hsp30 accumulation. The functionality of the Hsp20 and Hsp30 homologue proteins in *A. niger* still needs to be confirmed, however it is interesting to note that these two proteins are the best homologues of the two heat shock proteins found most upregulated in *A. nidulans* when conidia attached to the spore chain are heat treated. This suggests that the impact of cultivation temperature on conidial heat resistance might be established during this time-frame observed in *A. nidulans*, namely after the conidia are formed. Additionally, we show in Chapter 8 that a knock-out strain lacking the *hsp104* homologue produces heat sensitive conidia. It is interesting to note that small heat shock proteins of the *hsp26/42*-type (which the two upregulated proteins in Chapter 8 belong too) have a different mode of action than Hsp104 in *S. cerevisiae*, but both play a pivotal role in the heat stress response of yeast. In *S. cerevisiae*, Hsp26 and Hsp42 are referred to as “anti-aggregases” and bind proteins that were unfolded (due to heat shock or other stressors), thereby preventing aggregation of the unfolded proteins [21]. The yeast protein Hsp104 is unique and referred to as a dis-

aggregase or “unfoldase” and is the only yeast protein known to pull protein aggregates apart [22]. These three proteins are central in the main heat shock response of yeast, both Hsp26 and Hsp104 are regulated by Hsf1 and Msn2/4 [23], as reviewed in [24].

The effect of environmental factors during conidiation on spore heterogeneity

In Chapter 8, we describe accumulation of trehalose and heat shock proteins inside conidia upon cultivation at higher temperatures, which may seem solely beneficial for the cell. However, there might be trade-offs present; in *A. fumigatus* conidia cultivated at higher temperatures had significantly less DHN-melanin and were therefore more sensitive to UV-C resistance [25]. Reduced coloration was also observed in *A. niger* during growth at higher cultivation temperatures in our studies (data not shown). Perhaps these findings indicate that conidia are prepared for their current environment, during the maturation on the spore chain. This hypothesis is illustrated in Figure 9.1. Perhaps both compatible solute compositions and heat shock proteins concentrations are dependent on the environmental cues received, specifically during conidiation. Wang *et al.* have shown that conidia, while attached to the spore chain, are transcriptionally active in response to a heat shock. The study showed that conidia accumulate small heat shock proteins upon heat shock in *A. nidulans*, homologues of the Hsp20 and Hsp30 proteins found in Chapter 8 to accumulate inside conidia when the mycelium is cultivated at increased temperatures. Taken together, this could suggest that the cultivation temperature mainly impacts the heat resistance of conidia after their formation (by enhancing trehalose and heat shock protein accumulation), while on the spore chain, before they enter dormancy. However, more research is needed to confirm these hypotheses.

The impact of cultivation conditions during sporulation on the stress resistance has not been investigated in great detail, but could potentially be impactful and relevant for challenge tests undertaken in food industries. For example, conidia formed during a high cultivation temperature have increased heat resistance [25,26] (and Chapter 8), which means that food industries located in warmer climates face more food spoilage

due to heat resistant conidia than those located in colder climates, even when the conidia are from the same species or even strain. It would be interesting to know the other environmental cues that the conidia of the fungus can respond to during conidiation that would influence conidial resistance. A recent study shows that the nutrients the mycelium feeds on have an impact on the germination kinetics of the resulting conidia in *A. fumigatus* [27], similar results were obtained in Penicillium species [28]. Another recent study in *Penicillium rubens* showed that the water activity during conidiation affects the germination kinetics of the resulting conidia, when presented with conditions with low relative humidity [29].

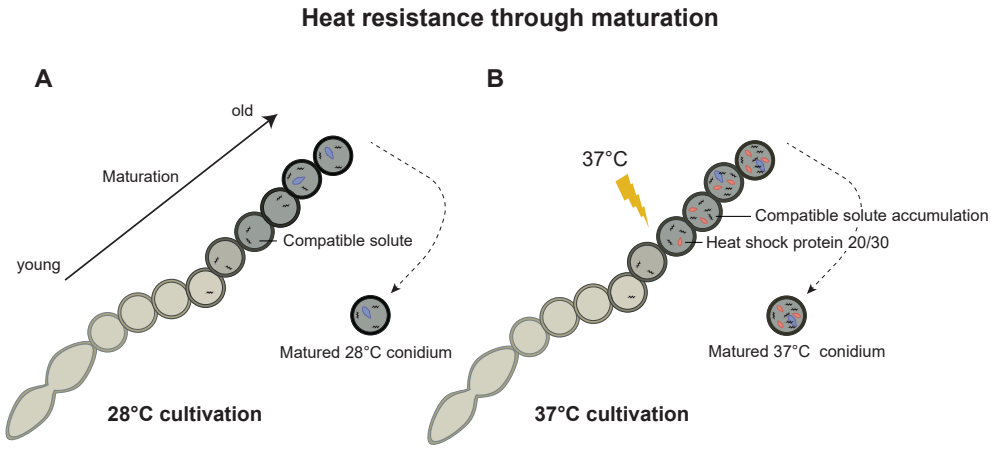

Figure 9.1. Heat resistance through maturation hypothesis. During maturation conidia are still transcriptionally active and respond to environmental cues as observed by Wang *et al.* [20] in *A. nidulans*. **A.** Conidia show maturation with age, compatible solutes start accumulating after conidia are formed (Chapter 7). **B.** At increased cultivation temperature (37°C), the conidia transcriptionally respond to the 'heat shock' environment, like described by Wang *et al.* [20], by accumulating extra trehalose and extra heat shock proteins (Hsp20 and Hsp30) in order to increase the heat resistance of the conidia (Chapter 8). As a result, a fungal colony from a single strain consists of conidia with varying heat resistance, dependent on both age and cultivation temperature experienced during conidiation.

Relevant to our study, also other research showed the impact of cultivation conditions on the stress resistance and germination of resulting conidia. The entomopathogenic filamentous fungus *Metarhizium robertsii* is used as a biocontrol agent in crop protection [30,31]. Conidia of this species are used as a product, sold to local farmers

to protect crops. Therefore, researchers and industries have been interested in generating preparations of long-lasting conidia from *Metarhizium* species, that are stress resistant in order to survive long-term storage conditions [32–34]. Previous research on *M. robertsii* has shown that growth conditions of the mycelium, i.e. the production process of these conidia (since they are a commercial product in this case), impact the stress resistance of resulting conidia [35,36]. The authors describe that the complexity of the medium, the water activity of the medium, osmotic stressors, illumination, hypoxic conditions and a heat shock during conidiation were all able to influence either the heat resistance or UV-B tolerance of the resulting conidia of *M. robertsii*. Additionally, the environmental conditions experienced during conidiation also impacted germination speeds and virulence of these conidia, which is understandably relevant for the use of *M. robertii* conidia as a natural biocontrol agent [37]. From this, we could conclude that the environmental conditions experienced during conidiation of consistently lead to changes in physical properties of conidia among filamentous fungi, including Aspergillus, Penicillium and Metharizium species.

Conclusion

Conidia are variable in their stress resistance and germination, either due to the species they belong to (Chapter 3 & 6), or the strain of a particular species they belong to (Chapter 2 & 3), or the age or environmental cues conidia of a particular strain received during conidiation (Chapter 7 and Chapter 8).

In this thesis, I show the effect of transcription factors on the natural variability in sorbic acid stress resistance of *A. niger* wild-type strains (Chapter 2), and identified novel weak acid response regulator WarB. Parasexual crosses were performed that revealed widespread heterokaryon incompatibility between *A. niger sensu stricto* strains and in the process, I created a unique diploid strain containing two mating types that can be used for future studies on sexual reproduction and/or heterokaryon incompatibility in *A. niger* (Chapter 5). The correlation between conidial age and internal compatible solute composition is shown (Chapter 7), and how compatible solutes are important for the wild-type heat stress resistance and germination kinetics. The relation between internal compatible solute composition and the germination kinetics of conidia should be further explored and could lead to new insights into molecular mechanisms behind spore germination and spore heterogeneity. Additionally, both transcriptomic and proteomic analysis on dormant conidia were performed, revealing the importance of heat shock proteins in the heat resistance of *A. niger* conidia.

The importance of transcription factors, compatible solutes and heat shock proteins described in this thesis provide new insight into molecular mechanisms behind food spoiling conidia, and could possibly be used as targets by food industry in future food preservation techniques.

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