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

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Summary

In **Chapter 1** a general introduction to food preservation and challenges is given. Food and its preservation are crucial for human existence. However, efficient preservation of food has many challenges. Microbes, such as filamentous fungi, survive various preservation techniques and subsequently contaminate food products. The abundantly present asexual spores (conidia) of filamentous fungi survive stressors applied in preservation techniques, due to their relatively high stress resistance. Therefore, it is crucial for future improvements in preservation techniques to understand the molecular mechanisms behind the high stress resistance of fungal spores. Additionally, conidia are highly heterogeneous in their ability to survive preservation techniques and in their capacity to contaminate foods, which could be explained by genetic differences between strains or species. Next to the genetic factors influencing conidial stress resistance, reports suggest that conidia from the same strain, i.e. being genetically identical, can be heterogeneous in their ability to survive preservation treatments. Therefore, the work described in this thesis focused on the identification of genetic and environmental factors determining the heterogeneity in preservation stress resistance of *A. niger* conidia.

In **Chapter 2**, the impact of strain diversity on the sorbic acid resistance of 100 *A. niger* strains was investigated. The most sorbic acid sensitive wild-type strain, isolated from grape, was sorbic acid sensitive due to a disruption in the gene coding for transcription factor SdrA. Additionally, 240 *A. niger* strains deleted in a single putative transcription factor were screened on their weak acid stress resistance. Multiple single knock-out strains showed sensitivity to weak acid stress, and specifically a strain lacking the *warB* gene was sensitive to a large plethora of weak acids used as food preservatives. The *warB* gene has an additive effect on the weak acid stress resistance of *A. niger* when compared to previously reported transcription factors involved in weak acid stress resistance; *sdrA* and *warA*. This study revealed the importance of transcription factors as overall stress response regulators during weak acid stress in *A. niger*.

In **Chapter 3**, the impact of strain diversity on the heat resistance of *P. variotii*,

P. roqueforti and *A. niger* conidia was investigated. Heat resistance was quantified as D-values for each strain. The maximum difference in D-value between strains of a single species was a factor 5 to 8 and therefore considered consistent in the three fungal species investigated here. Statistically, the impact of strain difference is in the same order of magnitude when fungal spores are compared to bacterial spores. This indicates that the impact of strain difference on the heat resistance of spores is consistent between food spoiling species, and therefore predictable, which could help food industries model food spoilage risks.

In **Chapter 4**, the sequencing of the neotype *A. niger* strain CBS 554.65 is discussed. The *A. niger* neotype strain CBS 554.65 has a MAT1-2 locus, in contrast to most of the previously sequenced industrial strains that contain a MAT1-1 locus. The genetic architecture of the mating type loci MAT1-1 and MAT1-2 was compared. Many genes in the MAT1-1 mating type locus have a flipped orientation when compared to their homologues in the MAT1-2 locus. This altered orientation of the MAT1-1 locus is consistent when analysing 24 newly sequenced *A. niger sensu stricto* isolates, of which 12 were MAT1-1 and 12 were MAT1-2. The heterothallic fungus *A. niger* has so far been viewed as a purely asexual fungus, but the discovery of the flipped mating type locus might lead to new insights into the potential of *A. niger* to reproduce sexually. The CBS 554.65 strain is the first high-quality genome of a mating type MAT1-2 *A. niger* strain, making it a suitable reference strain to further investigate fungal development in *A. niger*.

In **Chapter 5**, the sequences of 24 *A. niger sensu stricto* strains were further analysed. A phylogenetic tree was made containing the 24 newly sequenced *A. niger sensu stricto* strains and eight previously sequenced *A. niger sensu stricto* strains obtained from literature. The *A. niger sensu stricto* strains could be divided into three clades, in which interestingly industrial enzyme producers clustered in clade A and organic acid producers clustered in clade B. The phylogenetic distances between strains was used to determine the likeliness of heterokaryon formation, and subsequent parasexual crossings were attempted (initially with the aim of performing bulk-segregant analysis to pinpoint genetic elements causing the heat or sorbic acid resistant phenotype of strains

analysed in Chapter 2 and Chapter 3). However, a wide-spread heterokaryon incompatibility was observed, even between closely related *A. niger sensu stricto* strains. Only a single parasexual crossing was successful, thereby creating a diploid strain containing both mating type loci. Unfortunately, no ascospores were found when sclerotium formation was induced in this strain. This diploid strain still provides us the unique opportunity to study the putative sexual cycle in *A. niger*.

In **Chapter 6**, the CRISPR/Cas9 genome editing techniques developed for *A. niger* were adopted to *P. variotii* and *P. roqueforti* in order to create DHN-melanin deficient mutant strains. Additionally, *kusA*$^{-}$ strains were developed in order to facilitate future gene replacement and gene knock-out studies in these two food spoilage fungi. The conidia of melanin mutant strains of the three food spoilers were tested for their heat and UV-C resistance. The melanin deficient strains were not altered in heat resistance compared to the wild-type strains in all three food spoilage fungi. However, the UV-C resistance was reduced in melanin mutant strains of the three food spoilage fungi, indicating that DHN-melanin protects conidia against UV-C but not heat stress in all three food spoilage fungi.

In **Chapter 7**, young conidia were investigated for their relative heat resistance compared to older conidia. Young conidia were significantly more heat sensitive than older conidia, and this sensitivity increased gradually with age. This gradual increase with age was also observed in the internal compatible solute levels, correlating with the observed gradual heat resistance increase. These results show that young conidia are still accumulating compatible solutes during the maturation process, and are therefore initially still relatively heat sensitive. Knock-out strains were created using CRISPR/Cas9 genome editing in order to further investigate the impact of compatible solutes on the heat resistance of conidia. Mature conidia of the *ΔmpdA ΔtpsACB* strain containing limited compatible solutes were more heat sensitive compared to the parental strain, comparable to the results obtained from young conidia from the parental strain. When investigating the germination of these conidia, the spores that contained limited compatible solutes germinated in higher percentages in 10 mM arginine and 0.1 mM

proline when compared to wild-type conidia. The conidia containing limited compatible solutes also showed consistently lower germination percentages in 10 mM glucose. Taken together, the germination kinetics and heat stress resistance of conidia depends on internal compatible solute concentrations, and these concentrations depend on the age of conidia. Therefore, in a population of conidia with different ages, some (young) conidia have significantly different germination kinetics and heat stress resistance when compared to other (old) conidia. This could potentially be an ecological advantage, a form of bet-hedging applied by the fungus, where the abundant population of airborne conidia consists of genetically identical cells that are heterogeneous in stress resistance and germination capacity dependent on internal compatible solute composition.

In **Chapter 8**, the impact of cultivation temperature on the heat resistance of the resulting conidia in *A. niger* is investigated. When mycelium is cultivated at higher temperatures, the resulting conidia contain more trehalose and are more heat resistant. However, the conidia of a trehalose null mutant still showed the increased heat resistance when the mycelium was cultivated at higher temperatures, without any correlating increase in the amount of internal trehalose. Therefore, a transcriptome and proteome study were conducted where conidia cultivated at 28°C, 32°C and 37°C were compared in order to find genetic elements that could explain the heat resistance increase when conidia are cultivated at higher temperatures. The comparison between conidia cultivated at 28C versus 37C was the most informative, and showed that only two genes were both significantly upregulated in transcripts and their translated proteins significantly more present. These two genes both encode predicted *hsp26/42*-type heat shock proteins. These two genes make promising candidates for the observed heat resistance increase in conidia when they were cultivated at higher temperatures.

Chapter 9 gives a detailed summary that places the work in broader context and gives hypotheses as well as potential future research topics to further our knowledge on the heterogeneity in preservation stress resistance of *A. niger* conidia.

The work described in this thesis enhanced our knowledge on preservation

stress resistance mechanisms in *A. niger*, and revealed environmental factors that influence this stress resistance. Conidia are diverse in preservation stress resistance, based on genetic differences (i.e. species or strain), but also age and cultivation temperature, which are in turn due to changes in internal compatible solute levels and heat shock proteins.