

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

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CHAPTER 1

Introduction

Introduction

Food and the preservation of food are a crucial part of human existence and a central part of many human societies. Humans have been preserving food since pre-historic times. The oldest evidence of food preservation dates back to the Middle Pleistocene; archeological data suggests that prehistoric humans saved the bones of animals inside a cave (Israel) for delayed consumption of the bone marrow and grease safely stored inside [1]. Food preservation is necessary to protect food from microbial food spoilage, thereby securing food products for human consumption. Although food preservation has become more sophisticated since pre-historic times, still around 25% of the global food supply is lost, post-harvest, due to microbial spoilage [2]. A significant portion of this post-harvest food spoilage is due to filamentous fungi, commonly referred to as "moulds" [3.4]. Most isolated food spoilage moulds are part of the phylum Ascomycota and include several species belong to the genus *Penicillium*, *Paecilomyces* and *Aspergillus* [5]. In general preservation strategies are aimed at preventing the outgrowth of fungal spores. Both sexual (ascospores) and asexual (conidiospores) spores from these fungi are relevant for spoilage (see a recent review on food spoilage by heat-resistant molds [6]). In this thesis we focus on the asexual spores (conidia) of these food spoiling fungi.

Filamentous fungi produce asexually spores (conidia) that are everywhere

Filamentous fungi produce conidia as a means of reproduction. Conidia have general characteristics that are shared among all species, six of these characteristics are listed here. First, conidia are dormant structures, meaning they are thought to be metabolically inactive, although this has been topic of debate [7]. Second, the conidia are the offspring of the fungus, meant to germinate and thrive after arriving at a place where the proper conditions are met [8]. Third, conidia are per definition the asexual (clonal) offspring of the fungus, meaning that they are genetically identical to the parent. Fourth, to increase the chance of successful reproduction, large quantities of these asexual spores are spread into the environment. Conidia tend to be easily dispersed in these large quanti-

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ties through water or wind [8]. Fifth, conidia, meant for reproduction and long-distance travel through water and air, are heavily protected cells. They are meant to survive harsh conditions (drought, heat) and long periods of time before the proper conditions are met that support germination [9]. Sixth, the large airborne population of conidia is reported to be heterogenous with regards to their resistance against environmental stressors. Especially these last two characteristics make conidia well suited as reproductive structures for survival in nature, an therefore challenging structures to inactivate by commonly used preservation techniques.

Modern techniques of food preservation

In modern food preservation, various techniques are used to protect foods against spoilage by fungal spores. The most commonly used food preservation methods belong to one of three groups [10]. First, inactivation methods (for example inactivation by heating or applying radiation) are meant to kill the fungal spore and thereby protecting the food from spoilage. Second, growth inhibition methods (for example inhibition by adding preservatives or storing foods in cold environments) are meant to delay or prevent outgrowth of the fungus. Third, prevention methods (for example prevention by packaging) are meant to prevent recontamination after inactivation methods have been applied. In practice, these three methods are often combined where food is treated, packaged and subsequently stored in a way that inhibits fungal growth [11]. Studies have shown that 10 – 1000 conidia from genus Aspergillus are present in every cubic meter of air [12] and that these amounts vary with the season [13–15]. This makes preventing contamination nearly impossible. Even the air inside food factories contains many conidia that can potentially contaminate the food product throughout the production line. There are, however, ways to mitigate this challenge. Air filters are often installed inside clean rooms being part of these production lines [16]. Also, inactivation of contaminants present on the food product is commonly followed by instant packaging, thereby preventing contact with new potential contaminators from the air [17]. In this thesis, I will focus on the conidia of the food spoiler *Aspergillus niger* and I will specifically explore the mechanisms that make these conidia resilient against commonly used preservation techniques such as UV-C inactivation, heat inactivation and inhibition by weak acid preservatives.

The life cycle of Aspergillus niger

Most filamentous fungi belonging the Ascomycota are heterothallic, meaning that a haploid strain has a single mating type, commonly addressed as mating type MAT1-1 or MAT1-2 [18]. It requires fusion of two haploid strains of opposite mating type to form a diploid, which in turn, after going through meiosis, is able to form ascospores to complete the sexual cycle [19,20]. However, a sexual cycle of *A. niger* has not been observed yet and this organism is thought to be a truly asexual species, thereby only reproducing through conidia. Besides asexual and sexual reproduction, a third means of 'reproduction' has been explored under laboratory conditions in this fungus: the parasexual cycle [21]. In this life cycle, the offspring is not a perfect genetical clone, as in the asexual cycle, but is also not the result of meiosis as seen in the sexual cycle. In the parasexual cycle the diploid phase undergoes chromosomal reshuffling resulting in haploid offspring with chromosomes of either parent. An overview of the three life cycles is given in Figure 1.1.

Molecular genetics in Aspergillus niger

The filamentous fungus *A. niger* is most commonly known as a biotechnologically relevant producer of citric acid [24]. The production of almost all commercially available citric acid is produced by fermentation of *A. niger*, and its usefulness as a cell factory has since then been expanded to the production of other organic acids and proteins [25]. However, *A. niger* is also a common food contaminant, found for example in yoghurt, on grapes, coffee beans or the onion in your kitchen drawer [26–28]. The filamentous fungus *A. niger* has been used as a cell factory for decades, therefore our knowledge on the 1

organism is relatively large compared to other food spoiling moulds [25]. This includes our understanding of the genetics of *A. niger* and the genetic toolbox available for this organism, including CRISPR-Cas9 mediated genome editing [29–31], making it a prime candidate to use molecular genetic tools to study biological interesting aspects such as the resistance of food spoilage fungi towards food preservation techniques. In this thesis, we expand the genetic toolbox of *A. niger* to *Penicillium roqueforti* and *Paecilomyces variotii*, two other filamentous fungi that are common food spoilers. The research described in this thesis is part of a TIFN funded project entitled "Heterogeneity in spores of food spoilage fungi" in which the molecular mechanisms and heterogeneity of spores against food preservation strategies in *A. niger*, *P.roqueforti* and *P. variotii* are studied (https://topsectoragrifood.nl/project/heterogeneity-in-spores-of-food-spoilage-fungi/).

Compatible solutes are part of the fungal response to deal with harsh conditions

Finding the optimal preservation strategies against fungi can be challenging due to the large array of molecular stress resistance mechanisms present in these organisms. As mentioned above, conidia are relatively stress resistant cells and are able to survive common preservation techniques such as inactivation by heat. One of the major contributors to the high (preservation) stress resistance of fungal spores is considered to be the high concentrations of internal compatible solutes [32]. These molecules are defined as 'compatible' with the cytoplasm, as they accumulate to high concentrations without becoming toxic for the cell. These compatible solutes are thought to be quickly accumulated in vegetative cells upon encountering a stressful environment such as drought or heat. Different kind of molecules have been reported to act as compatible solutes, including trehalose, polyols, betaines and specific amino acids such as proline [33]. There are many synonyms to 'compatible solute' that are commonly used throughout the literature, such as osmoprotectant, osmolyte, chemical chaperone, co-solvent or kosmotropic solute. The most important characteristic of these compounds is that they protect the cell against stressors by stabilizing macromolecules (mainly proteins, but also structures

such as the plasma membrane). There are three main theories proposed on how these molecules protect macro-molecules.



Figure 1.1. Life cycle of *Aspergillus niger.* The sexual cycle depicted here has not yet been proven to exist in *A. niger,* therefore this depiction was based on the sexual cycle found in closely related species *Aspergillus tubingensis* [22,23]. Offspring of the fungus is either a genetic copy of the parent (asexual cycle), has undergone meiosis (sexual cycle), or has undergone haploidization and chromosome reshuffling (parasexual cycle).

One theory is the so-called 'water replacement theory' [34,35]. This theory states that the compatible solutes effectively replace water on a molecular level, creating water

bridges and thereby stabilizing proteins and membranes [36–38]. Another theory is the vitrification theory, also known as the 'glassy state theory' [39-41]. This theory proposes that compatible solutes in high concentrations establish a so-called glassy state inside the cell. This glassy state is a chemically steady state that looks like a glass, in which the non-covalent intramolecular bonds (such as water bridges) are preserved, thereby preventing denaturation of proteins. Intramolecular bonds in this state become extremely rigid, and therefore stable, even when the cell is treated with stressors such as extreme heat or cold. This is one of the main arguments raised as to why the compatible solute glycerol is so essential in keeping bacterial and fungal stocks alive in freezers kept at -80 °C [42]. A less often discussed theory is the preferential exclusion theory [43]. This theory stems from thermodynamics and has to do with the so-called 'folding equilibrium' of proteins in liquid. Depending on the amount of compatible solute present, the folding equilibrium will shift, making it thermodynamically more favorable for the protein to stay in the folded form. This has to do with the interaction between protein, water and compatible solute, where the water molecules are favored to interact with the compatible solute, thereby 'drying' or depriving the proteins from water molecules. This 'dried' and strongly folded protein would in turn be better protected against most forms molecular damage. Some adjusted versions of each theory exist, but these three form the basis for most theories currently proposed on the mode of action of compatible solutes [44]. It is also good to note that the three theories are not thought to be mutually exclusive [45].

Stress signaling pathways, transcription factors and their target genes

How does a fungus recognize it needs to protect itself against a stressor? Several kinase dependent signal transduction pathways are known in fungi that respond to stressors, often adapted to specific stressors the cell might face from the environment. These stress signaling pathways are best-described in *Saccharomyces cerevisiae* and *Candida albicans*, but overall are preserved among fungi, and some have been described in *Aspergillus* species as well [46]. Most stressors are first recognized by a receptor, many of which reside in the cell membrane [47]. Receptors directly activate a kinase-dependent signaling cascade machinery, belonging to either a two-component signaling (TCS) system or the mitogen-activated protein kinase (MAPK) dependent system. At the end of these, sometimes very complex, cascading pathways often resides a transcription factor (TF) which is activated. This protein activates or represses the expression of multiple genes, thereby creating a molecular response by the fungus to the observed environmental stressor. For example, upon sensing oxidative stress via the HOG pathway, the fungal cell will activate transcription factors that induce the production of catalases and superoxide dismutases [48,49], enzymes that can detoxify reactive oxygen species (ROS). In terms of conidial preservation stress resistance, transcription factor AtfA is important for the oxidative and heat stress resistance of conidia from Aspergillus fumigatus, Aspergillus nidulans and Aspergillus oryzae [50-53]. AtfA is known to regulate gene tpsA, important for trehalose accumulation in conidia. AtfA also regulates other genes encoding conidia specific proteins such as catalase catA, dehydrin dprA and "conidiation specific" gene conJ which are all thought to be involved in conidial stress resistance [54-56].

However, not all stress signaling pathways in fungi are fully understood, and some do not require a cell signaling cascade by kinases. The activation of the main heat shock response in *S. cerevisiae* is regulated by transcription factor "Heat shock factor 1" (Hsf1) and its homologues are conserved and active in filamentous fungi [57–59]. Upon heat shock, the Hsf1 proteins undergoes a confirmational change and forms a trimer, which is the activate form of the transcription factor, subsequently adjusting expression ~3% of all genes on the genome of *S. cerevisiae* [60], however it is not clear if additional molecules and steps are involved in this conformational change [61]. This transcription factor Hsf1 induces expression of heat shock proteins including Hsp26 and Hsp104, required for heat stress resistance [62]. Similarly, the main weak acid stress response in *S. cerevisiae* is regulated by "Weak acid resistance 1" (War1p) [63]. This transcription factor, important in the weak acid stress response, has direct interactions with the weak acid anions inside the fungal cell, leading to its activation [64,65]. Upon activation,

War1p induces the expression of Pdr12, an ABC-type efflux pump meant to extrude weak acid anions out of the cell [66].

The heterogeneity of Aspergillus niger conidia.

Food preservation becomes even more challenging when considering that conidia are heterogeneous in their stress resistance and germination capacity. Therefore, inactivation or inhibition of the germination of one conidium might not inactivate or inhibit another fungal conidium. The most well-described source of spore heterogeneity is caused by species and strain diversity. For example, the heat resistance of fungal conidia varies greatly depending on which species or strain is analysed [67,68]. However, a scarcely studied phenomenon is the heterogeneity in stress resistance and germination capacity of fungal spores within a single spore population, thus obtained from a single strain. Researchers have shown that conidia from a single A. niger strain are heterogeneous in their stress resistance towards sorbic acid [69] and heat [70]. It is remarkable that differences between individual conidia are observed when considering that these spores are clonal offspring and therefore genetically identical to each other (see Figure 1.1). Additionally, previous research has shown that cultivation temperature [71] and spore maturation [72] impact physical properties and potentially preservation stress resistance of Aspergillus conidia. Taken together with the fact that conidia are dispersed in large quantities, it illustrates the possibility of individual differences between conidia in terms of preservation or environmental stress resistance and germination capacity within a given spore population. These individual differences between conidia can be beneficial for survival of the fungus as a bet-hedging strategy, where individual conidia vary from one another, therein protected against specific stressors or able to germinate under specific conditions, ensuring propagation of the fungus. In this thesis, I will contribute to our understanding of the molecular mechanisms behind conidial stress resistance and heterogeneity between conidia of A. niger.

Scope and outline of this thesis

The conidia produced by filamentous fungi pose a constant threat to food preservation. Two major treatments to prevent food spoilage fungi from colonizing food are (1) the heat treatment of food, in order to heat inactivate the conidia, and (2) to include weak acid preservatives (especially sorbic acid) in the food, in order to inactivate the conidia or inhibit fungal growth. Fungi have evolved several mechanisms to survive heat stress and sorbic acid stress in order to survive and proliferate. In addition, earlier research has already indicated that different strains of the same species and spore populations from a single strain are highly heterogenic in their resistance to these stressors. The aim of the thesis is to study the heterogeneity in stress resistance of conidia between strains and between the spore populations of the same strain, and subsequently to identify important factors determining stress resistance.

In **Chapter 2** we investigate the strain diversity in stress resistance of *A. niger* against sorbic acid, a common chemical preservative used by food industries. The sorbic acid stress resistance of 100 *A. niger* strains isolated from various sources, including contaminated foods, was investigated. Additionally, a total of 240 transcription factor knock-out strains were screened for their weak acid stress resistance, revealing transcription factors that are involved in the weak acid stress response of *A. niger*. A novel transcription factor involved in weak acid stress resistance, named *warB*, was identified and further investigated.

In **Chapter 3** we investigate the role of strain diversity in the stress resistance of *P. variotii*, *P. roqueforti* and *A. niger* against heat. The heat stress resistance of ~20 strains of each species was investigated. The degree of variation in conidial heat resistance due to factor "strain" was quantified, and results were compared to previous reports in other organisms. The strain variability observed between conidia of the three food spoiling fungi were in the same order of magnitude as spores from bacterial species.

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Genomes of 24 *Aspergillus niger* strains displaying various sensitivities towards heat and weak acids were sequenced with the aim to perform genome wide association studies (GWAS) and bulk-segregant analyses in order to find genetic elements involved in conidial heat resistance and weak acid stress resistance. However, GWAS studies were inconclusive, providing too many target genes putatively involved in both heat resistance and weak acid stress resistance. Additionally, the parasexual crossing of *A. niger* strains, which is required for bulk segregant analysis, turned out to be highly challenging due to widespread heterokaryon incompatibility (described in **Chapter 5**). However, the 24 genomes of *A. niger* revealed an equal distribution of mating types in this presumably asexual species. As a first step to further explore the possible sexual state of *A. niger*, which would be interesting for many future genetic studies, including studies

on conidial stress resistance, a high-quality genome of the *A. niger* neotype strain CBS 554.65 containing the MAT1-2 mating type locus was analysed and presented in **Chap-ter 4**.

In **Chapter 5** the analysis and comparison of the 24 genomes of *A. niger sensu stricto* strains is described in more detail. Together with publicly available genomes, we show that the 32 *A. niger* genome sequenced strains cluster in three distinct phylogenetic groups. A successful parasexual cross between two *A. niger sensu stricto* strains with two different mating types is described, creating the first diploid *A. niger* strain containing both mating types.

In **Chapter 6** the CRISPR/Cas9 genome editing tool for *A. niger* was adopted for genetically less well characterised food spoiling fungi *P. roqueforti* and *P. variotii*. CRIS-PR/Cas9 was successfully implemented to create targeted mutations in a *pks* gene required for DHN-melanin synthesis. The role of melanin in mediating conidial heat stress and UV stress was studied in more detail using the generated melanin mutants in all three species.

In **Chapter 7** the impact of both the age of conidia and the internal compatible solute compositions of conidia on heat resistance and germination kinetics in *A. niger*

is described. Young conidia contained limited compatible solutes and were found heat stress sensitive. Knock-out strains deleted in trehalose and/or mannitol biosynthesis genes were made using CRISPR/Cas9 genome editing to investigate the role of compatible solutes in heat resistance and germination. The results indicate that trehalose and mannitol levels are important parameters in relation to mediating heat resistance and that the higher sensitivity to heat stress observed in relatively young conidia is due to low levels of compatible solutes.

In **Chapter 8** the impact of cultivation temperature on the resulting heat resistance of conidia was studied at transcriptome, proteome and physiology level. The analyses suggest that apart from increased trehalose levels also the induced expression of heat shock proteins could be responsible for the observation that conidia were more heat resistant when the mycelium was cultivated at 37 °C compared to 28 °C.

In **Chapter 9** the results are discussed and reflected upon. The genetic factors (species and strain) as well as the environmental factors (age, cultivation temperature) all contribute to the observed heterogeneity in preservation stress resistance of *A. niger* conidia. Internal compatible solutes, as well as protein levels from protective proteins such as heat shock proteins, potentially contribute to the individual differences observed between conidia.

The work described in this thesis may contribute in a further understanding of the genetic and environmental factors determining the heterogeneity observed in preservation stress resistance of *A. niger* conidia.

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