Trehalose Metabolism and Stress Resistance in *Aspergillus niger*

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Cover: Scanning electron microscopy image of a developing *A. niger* conidiophore. (photo: J. Dijksterhuis and Å. Svanström)

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Abstract

Food spoilage by filamentous fungi, moulds, is a global issue of great significance. Mould growth can cause food losses due to deterioration, and adverse health effects due to toxin production. Conidia, asexual spores, are the main factors of dispersal and infection in most food-relevant moulds. They therefore constitute important objects of study when trying to improve food safety. This thesis sought to increase understanding of conidial germination and stress resistance in the common food spoilage mould *Aspergillus niger*, by investigating the inhibitory properties of phenyllactic acid, a lactic acid bacteria metabolite, as well as the role and metabolism of trehalose. This sugar is a major component of spores and well known for its ability to protect proteins and membranes against various kinds of abiotic stress.

A thorough examination in *A. niger* revealed that it contains six genes involved in trehalose synthesis: *tpsA-C* and *tppA-C*. All genes were expressed during conidial germination, and conserved orthologues could be identified in related Aspergilli. However, when creating deletion mutants, only *ΔtpsA*, *ΔtppA* and *ΔtppB* had significantly lowered trehalose content. These three genes therefore seem to be most significant for trehalose accumulation. Degradation of internal trehalose is catalysed by trehalase; in *A. niger* this enzyme is encoded by *treB*. Studies of *ΔtppA*, *ΔtppB* and *ΔtreB* demonstrated that a functional synthesis as well as utilisation of trehalose is crucial for heat stress resistance of *A. niger* conidia. Inability to produce or degrade trehalose also negatively affects the ability of the fungus to generate conidia.

Lactic acid bacteria (LAB) have long been used to ferment foods, thereby extending the shelf life. Several LAB metabolites with antifungal properties have been identified, but the underlying mechanisms of inhibition are largely unknown. We have investigated the effect of phenyllactic acid (PLA) on growth and development of single *A. niger* conidia. Results showed that PLA inhibits moulds in a manner similar to that of weak acid preservatives, such as benzoic acid; but it also strongly restricts asexual development at sub-inhibitory concentrations. This effect is not seen by other acids, and therefore indicates a unique mechanism of action for PLA.

Keywords: Ascomycota, *Aspergillus niger*, bio-preservation, conidia germination, heat-stress, phenyllactic acid, trehalose.

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Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Åsa Svanström and Petter Melin (2013). Intracellular trehalase activity is required for development, germination and heat-stress resistance of *Aspergillus niger* conidia. *Research in Microbiology* 164(2), 91-99.

II Åsa Svanström, Martin Richard van Leeuwen, Jan Dijksterhuis and Petter Melin. Trehalose synthesis in *Aspergillus niger*: Identification and partial characterisation of six homologous genes, all with conserved orthologues in related species (manuscript).

III Åsa Svanström, Silvio Boveri, Emma Boström and Petter Melin. The lactic acid bacteria metabolite phenyllactic acid inhibits both radial growth and sporulation of filamentous fungi (manuscript in revision).

The contribution of Åsa Svanström to the papers included in this thesis was as follows:

I Took part in planning the study. Performed the majority of the laboratory work. Shared the responsibility for writing the manuscript.

II Took part in planning the study. Performed the majority of the laboratory work. Shared the responsibility for the initial draft and main writer of substantial parts of the manuscript.

III Took part in planning the study. Performed the majority of the laboratory work jointly with S. B. Shared the responsibility for writing the manuscript.

1 Introduction

Moulds, filamentous fungi, cause considerable loss of food and feed worldwide. The growth of mould is associated with spoilage and toxin production, which leads to discarding of vast amount of food, or consumption although it is unhealthy. In a world with growing population and where the natural resources are already strained this is a problem of increasing magnitude. (Pitt & Hocking, 2009)

Prevention of mould growth in food and feed include both pre- and post-harvest strategies. Examples of pre-harvest measures include choosing crop varieties that are resistant to moulds, crop rotation to break the infectious cycle, minimisation of plant susceptibility by reducing stress (for instance by irrigation or fertilisation) and spraying with fungicides. Post-harvest measures include use of chemical preservatives (for instance sorbic or benzoic acid); optimisation of storage conditions so that they are unfavourable for mould growth; and, use of antagonistic microorganisms such as yeast or lactic acid bacteria (LAB). LAB have been used by humans for a very long time to refine food and make it safer (Kabak *et al.*, 2006). Three aspects of LAB growth contribute to the reduction of spoilage organisms: competition for nutrients, decreased pH, and production of inhibitory metabolites (Dalie *et al.*, 2010). One such inhibitory metabolite is phenyllactic acid (PLA), which has been shown to have anti-fungal properties (Lavermicocca *et al.*, 2000). I have studied the potential of PLA as a food protectant and the factors that contribute to the protective capacity of this metabolite.

The asexual spore is the main mechanism of dispersal in many food spoilage fungi, and, therefore, plays a key role in food contamination and spoilage. Increased knowledge of fungal germination is, thus, an essential part in the efforts to make preservation and storage of food more efficient and to minimise mould damage. (Osherov & May, 2001)

To increase the understanding of fungal spores in different food contexts, I have studied the disaccharide trehalose. This sugar constitutes a substantial part of the dry weight of fungal spores, but is also found in several other fungal structures. During germination of spores, it is rapidly broken down, indicating its importance as an energy reserve. However, it has also been shown that trehalose has a more complex role, being important for spore survival in different situations. (Elbein *et al.*, 2003) The importance of this metabolite makes it worth studying, and furthermore, it might be useful as an antifungal target. For instance, in the human pathogen *Aspergillus fumigatus*, trehalose and trehalose metabolism are hypothesised as possible drug targets (Puttikamonkul *et al.*, 2010).

As a model organism for this thesis, *Aspergillus niger* was chosen. This commonly occurring food spoilage mould produces an abundance of spores that are spread by air. However, it is mainly a spoilage organism without serious side effects. The growth requirements are modest and the complete genome sequence has been published. (Pitt & Hocking, 2009; Pel *et al.*, 2007) All this combined makes *A. niger* a useful and convenient object of practical study in the laboratory.

1.1 Outline and aims

This thesis begins with a general background to the fungal kingdom followed by chapters on trehalose, fungal spores, food preservation and stress response in fungi. In these chapters, the general concepts as well as the specific results of this study are discussed. The aims of this work were to:

- Investigate trehalose metabolism in *A. niger* during conidial maturation and germination (**Papers 1** and **2**).
- Thoroughly elucidate the significance of specific genes and gene products in *A. niger* trehalose metabolism: catabolism (**Paper 1**) and synthesis (**Paper 2**).
- Evaluate the importance of a functional trehalose metabolism in different food relevant situations and as a stress protectant in *A. niger* (**Paper 1** and **2**).
- Study the ability of conidia to germinate in different stress conditions (**Papers 1**, **2** and **3**)
- Investigate the inhibitory properties of phenyllactic acid in fungi, to determine the MIC levels and to study the mode of function (**Paper 3**).
- Examine how stress from PLA affects the expression of a few relevant genes in *A. niger* (**Paper 3**).

2 The fungal kingdom

Fungi make up one of the major domains of eukaryotic organisms. Members of the fungal kingdom are highly diverse in terms of morphology, physiology, reproduction and ecology. Fungi are heterotrophic: they cannot fix carbon, and instead require organic carbon for their growth. In nature, they perform fundamental roles as carbon cycling organisms by decomposing organic matter. The downside for humans is that they are prevalent in food decay and degradation of building materials, for instance. Additional survival strategies of fungi include living in symbiosis with other organisms (mycorrhiza with vascular plants, and lichens with algae, for instance) and living as plant, animal or human pathogens. Humans have long utilised fungi for fermentation of food such as bread, soy sauce, wine and cheese, and more recently, for production of antibiotics and enzymes, and as model organisms for genetics and molecular biology. (Taylor *et al.*, 2004)

Although mycology (the study of fungi) is often considered a branch of botany, fungi are phylogenetically closer related to animals than to plants (Lutzoni *et al.*, 2004). Besides the difference that fungi are heterotrophic and plants are autotrophic, one important difference is the composition of the cell wall, which in fungi consists of chitin, and in plants, cellulose (Taylor *et al.*, 2004).

The fungal kingdom is estimated to comprise up to 1.5 million species, although the exact number is unknown. Traditionally, at least five phyla have been recognised within the Fungi; Chytridiomycota, Zygomycota, Glomeromycota, Ascomycota and Basidiomycota. However, recent phylogenetic studies have partially overthrown these distinctions, and the relationships are under debate (Medina *et al.*, 2011; Lutzoni *et al.*, 2004). In addition to the so-called true fungi, several other groups of organisms are sometimes referred to as “fungal”, e.g. Oomycota and Myxomycota, and have traditionally been studied by mycologists, although the organisms are not

closely related. Within the fungal kingdom, the Basidiomycota and the Ascomycota are monophyletic (they have one common ancestor) and they constitute an informal group, the dikaryomycetes. (Taylor *et al.*, 2004)

2.1 Ascomycota

The Ascomycota, comprising approximately 64000 described species, is the largest phylum in the fungal kingdom. Its members make up about 65 % of all known fungi. Of fungi that form lichens (with algae or cyano-bacteria), approximately 98 % are ascomycetes. The Ascomycota is a highly diverse phylum with species occurring in very varying niches and practically all ecosystems. (Schoch *et al.*, 2009; Taylor *et al.*, 2004)

Members in the Ascomycota employ two major growth forms: filamentous in the form of hyphae, or, as yeast. Filamentous species are multicellular and grow apically. The hyphae are compartmentalised by septa which are usually porated, allowing cytoplasmic transport between the different compartments. Yeasts are unicellular, mainly grow isotropically and multiply by budding or fission. There is no distinct phylogenetic relationship between the species growing in filamentous or yeast forms; on the contrary, many species in the Ascomycota are dimorphic with different growth forms in different life stages. (Crous *et al.*, 2009)

The Ascomycota includes many species with great impact for humans, both useful and economically important fungi, as well as species with negative effects, such as pathogens and/or toxin producers. Examples include: *Saccharomyces cerevisiae*, the yeast used for fermentation of wine and beer and leavening of bread; *Penicillium chrysogenum*, utilised for production of the antibiotic penicillin; *Candida albicans*, that causes diaper rash and vaginitis; and *Magnaporthe oryzae*, the rice blast fungus. (Taylor *et al.*, 2004)

Ascomycetes are defined as fungi that produce sexual spores, ascospores, in sac-like structures called asci (singular: ascus). They are therefore also called sac fungi. The asci of filamentous fungi are normally borne within a larger structure called an ascocarp or ascoma (plural: ascomata). The normally haploid hypha develops into a sexual reproduction structure by mating and becomes diploid by nuclear fusion. In some species, this can occur when only one specimen is involved, i.e. they are self-fertile (homothallic); other species are self-sterile (heterothallic), needing a partner with opposite mating type for sexual reproduction. In the ascus, the diploid nuclei undergo meiosis, producing haploid ascospores. The ascospores are commonly thick-walled and often resistant to heat, pressure and chemicals. (Crous *et al.*, 2009)

The typical member of the Ascomycota is able to reproduce both sexually, as described above, and asexually, producing asexual spores named conidia. However, a large proportion of the Ascomycota has no described sexual stage, reproducing entirely asexually. (Schoch *et al.*, 2009) For information on asexual reproduction see chapter 4 – Fungal spores.

2.2 Eurotiales

The Eurotiales are an order of Ascomycetes, within the class Eurotiomycetes, containing several well-known and commonly occurring fungi. Members of Eurotiales are characterised by thin-walled asci containing unicellular, often ornamented, ascospores. (Samson *et al.*, 2004)

Most Eurotiales are saprotrophic, excreting a large diversity of enzymes in order to break down proteins, lipids and carbohydrates. This makes them common causes of food spoilage, but it also allows us to utilise them industrially to produce enzymes and other metabolites. Other aspects of these fungi are their importance for research, being easily manipulated in the laboratory; they have been research tools for many years. (Geiser *et al.*, 2006)

Aspergillus and *Penicillium* are the most notable and well-studied genera within the Eurotiales. The two genera are closely related, both belonging to the Trichocomaceae family. They are both phialidic, i.e. the asexual spores are formed by specialised structures known as phialides. (Pitt & Hocking, 2009; Geiser *et al.*, 2006)

Both *Aspergillus* and *Penicillium* are among the dominant fungi worldwide, occupying a large variety of niches and being able to utilise diverse nutrient sources. *Aspergillus* as a genus is considered to be more tolerant of varied abiotic conditions, than *Penicillium*, being able to grow at low water activity and over a range of temperatures. (Krijgsheld *et al.*, 2013; Pitt & Hocking, 2009)

Aspergillus and *Penicillium* are the names traditionally given to the asexual (i.e. anamorphic) stage of the fungal life cycle. Most species in these genera only exist in this form, however, others also have a sexual (teleomorphic) stage. In those cases, two names have been in use, one for the sexual stage of the fungus and another for the asexual. For *Aspergillus*, the teleomorphic genera include *Eurotium*, *Neosartorya* and *Emericella*, and for *Penicillium* *Eupenicillium* and *Talaromyces*. (Samson *et al.*, 2004) However, the International Code of Botanical Nomenclature (that governs fungal nomenclature) recently agreed on the "one fungus: one name" principle. This initiated the work on a new classification system, in which only one name will

be used for all stages of a fungus, a process that is still ongoing. (Hawksworth, 2011; Houbraken & Samson, 2011)

2.2.1 Food spoilage and mycotoxins

Since the purpose of food and feed is mainly to be nutritious (in contrast to natural, nutrient-poor, systems, e.g. water and soil), they constitute a very rich habitat for microorganisms. The saprotrophic nature of many Eurotiales fungi, by which they can secrete vast amounts of enzymes that break down many types of macromolecules, makes them well equipped to live on all kinds of food. Combined with their ability to produce toxic metabolites, this contributes to their importance as food spoilage organisms. (Pitt & Hocking, 2009; Geiser *et al.*, 2006) Fungal spoilage is of great concern because of economical losses, shortage of food supply and adverse effects on human and animal health, especially in tropical and subtropical regions of the world. However, the magnitude of the problem is difficult to evaluate, as fluctuations, depending on food commodity, location, and climate, are large. It has been estimated that 25 % of all agricultural products are contaminated with mycotoxins, and economical losses due to fungal food spoilage in Australia are annually more than $10 million dollar. (Dagnas & Membre, 2013; Dantigny *et al*., 2005) *Aspergillus* and *Penicillium* species are among the most important agents of food spoilage worldwide. Generally, one can say that *Aspergillus* species dominate food spoilage in tropical regions, whereas *Penicillium* species are more widespread in temperate regions. (Pitt & Hocking, 2009)

Examples of foods with which fungi are commonly associated include fruits, vegetables, cereal grains, bread, dairy products and fermented products (Dagnas & Membre, 2013; Filtenborg *et al.*, 1996). Although some species of fungi are generalists and grow on many kinds of foods, it is common that only a small number of species can be found on a certain foodstuff. For example, spoilage of citrus fruits is strongly associated with *Penicillium digitatum* and *P. italicum*, whereas moulds growing on rice and peanuts are frequently identified as *Aspergillus flavus* or *A. niger*. (Frisvad *et al.*, 2007a)

Mould growth in food leads to quality loss such as off-smells and off-flavours, discoloration, visible mould growth, and loss of structure and texture. This is generally not dangerous, but will lead to rejection of the food by the consumer. A problem of greater magnitude is the potential production of mycotoxins during mould growth, which makes the food unhealthy. (Dagnas & Membre, 2013; Filtenborg *et al.*, 1996)

Mycotoxins are substances produced by fungi during secondary metabolism (i.e. they are chemicals that are not directly essential for growth). They are highly toxic to vertebrate animals when indigested, inhaled or absorbed

through the skin. (Frisvad *et al.*, 2007b) As for most other so-called secondary metabolites, production of mycotoxins is often linked to phases of fungal development, and more specifically, to sporulation. The reasons that fungi produce these metabolites are not fully elucidated and are complex; some substances directly stimulate spore-formation, others are believed to work as virulence factors or enhance the competitiveness of the fungal strain. (Calvo *et al.*, 2002)

Most mycotoxins are secreted by the mould, and in foodstuff with high water content, they rapidly diffuse and contaminate the whole product. Mycotoxins are generally very resistant to different chemical and physical treatments, meaning that once they are present in the food, they typically persist. (Filtenborg *et al.*, 1996) Today, more than 400 mycotoxins are known, of which only a small number are toxicologically well-described. The known toxic effects of mycotoxins are diverse, the most pronounced being immune suppression and carcinogenicity, but also, for example, liver, kidney and reproduction toxicity. (Creppy, 2002; Filtenborg *et al.*, 1996)

Several species of *Aspergillus* and *Penicillium* are well-known producers of mycotoxins. Some examples include: aflatoxin, the most potent naturally occurring carcinogen know, produced mainly by *A. flavus* but also by *A. parasiticus* and *A. nomius* among others; the nephrotoxin citrinin, produced by *P. verrucosum*, *P. expansum* and *P. citrinum*; and, ochratoxin A, which is a nephrotoxin and suspected genotoxin, produced by both *P. verrucosum* and *A. ochraceus* and closely related species, along with a few *A. niger* strains and related species such as *A. carbonarius*. (Frisvad *et al.*, 2007b; Creppy, 2002)

2.3 *Aspergillus niger*

In this thesis work *A. niger* has served as the model organism.

As indicated by its name, *A. niger* is distinguished by the dark coloured conidia, asexual spores, that give the colonies their black to brown-black colour seen in Figure 1.

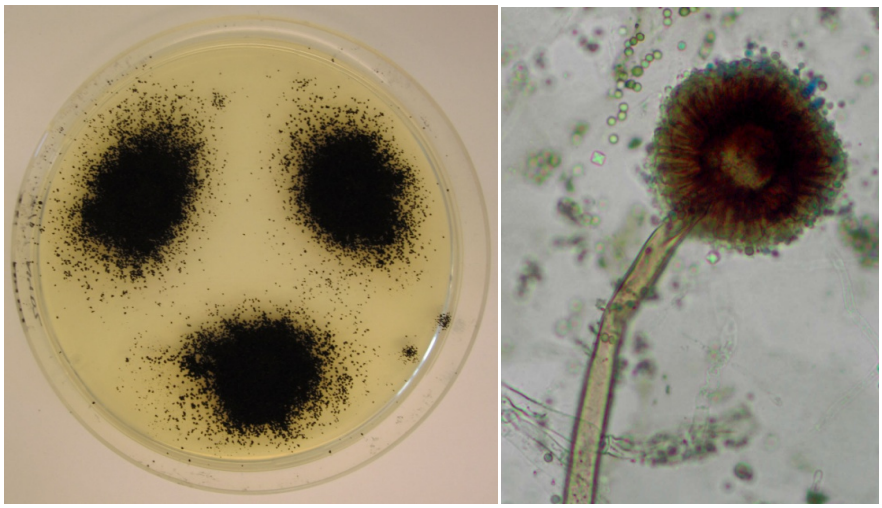

Figure 1. Morphology of *A. niger*: two weeks old colonies growing on minimal media (left) and microscopic picture of a conidiophore, the structure producing asexual spores (right).

A. niger is closely related to many other Aspergilli, some of which are impossible to distinguish from one another by morphology: these make up the so-called "*A. niger* species complex". Examples of species included in the complex are *A. brasiliensis*, *A. piperis*, *A. tubingensis* and *A. vadensis*. (Geiser *et al*., 2007; Pitt and Hocking 2009)

A. niger has a worldwide distribution and can grow under a wide range of abiotic conditions and on a large diversity of substrates. Growth temperatures range between 8-45°C, with optimum at 35°C. It is xerotolerant with reported growth at a_w[1] 0.77, and also tolerant of low pH, being able to grow at pH 2.0. *A. niger* is a common food contaminant and more prevalent in warmer climates. It is often isolated from fresh fruits and vegetables, sun-dried products, nuts, meats and cheese. It is mostly regarded as a spoilage organism, however, some strains can produce ochratoxin A under various conditions. (Pitt & Hocking, 2009)

1. Water activity: a measure of how efficiently the water present can take part in a chemical or physical reaction. Pure distilled water has the activity 1. (Adams & Moss, 2000a)

Although *A. niger* has been known to cause pulmonary aspergillosis in immune-suppressed patients, it is generally considered as a non-pathogenic fungus (Schuster *et al.*, 2002).

Besides being a spoilage organism, *A. niger* is widely used in industry. The most important fermentation product from *A. niger* is the organic acid, citric acid, with applications in foods, pharmaceuticals, cosmetics, etc. Other products include gluconic acid and enzymes such as pectinase, protease and amyloglucosidase. (Abarca *et al.*, 2004; Schuster *et al.*, 2002) *A. niger* is among the best studied fungal species; many molecular tools exist (Schuster *et al.*, 2002), and in 2007, the first complete genome (strain CBS 513.88) was sequenced and annotated (Pel *et al.*, 2007).

The *A. niger* wild-type used in the experiments of this thesis was a strain with short conidiophore stipes (*cspA1*) called N402 (Bos *et al.*, 1988).

3 Trehalose

Trehalose is a non-reducing disaccharide consisting of two glucose molecules joined by α,α-1,1-glycosidic linkages (see Figure 2). This sugar is widespread in nature and has been isolated from certain species of bacteria, plants, fungi and insects, but it does not occur in vertebrate animals. (Elbein *et al.*, 2003) Typically the levels of trehalose in these organisms are relatively low, but in various kinds of survival structures, such as insect eggs and fungal ascospores, the sugar is accumulated and can constitute as much as 10 % of cell dry weight. In so called anhydrobiotic organisms (that are able to survive long periods completely dehydrated), trehalose is commonly synthesised and accumulated. (Iturriaga *et al.*, 2009; Elbein *et al.*, 2003)

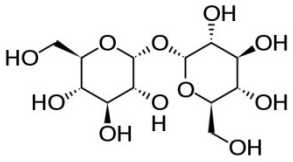

Figure 2. Structure of α,α-trehalose.

Trehalose was probably first discovered in 1832, when it was isolated from sclerotia of rye by Wiggers, who named the substance "mycose" (Iturriaga *et al.*, 2009; Wiemken, 1990). Since trehalose is rapidly degraded when resting structures goes into vegetative growth, early studies concluded that the main function of the sugar is as reserve carbohydrate for energy storage (Elbein, 1974). However, later studies have contributed to a drastically increased understanding of the diverse roles that trehalose plays in nature. These roles include regulation of carbon metabolism, and as a protectant against abiotic stress. (Elbein *et al.*, 2003)

Besides its wide distribution in nature, trehalose is also utilised in a range of biotechnical applications. Examples include as a stabiliser during dehydration and freezing, and as an additive in cosmetics. (Iturriaga *et al.*, 2009)

3.1 Synthesis

Trehalose is synthesised in several kinds of fungal structures, but particularly in spores, yeast cells going into stationary phase, and as a response to stress (Uyar *et al.*, 2010; Elbein *et al.*, 2003; Fillinger *et al.*, 2001; Crowe *et al.*, 1992)

There are five known synthesis pathways for trehalose. The one discovered first is also the most widely distributed, and it is the dominant pathway in fungi. It involves two enzymatic steps catalysed by trehalose-6-phosphate-syntahase (TPS) and trehalose-phosphate-phosphatase (TPP). The first intermediate in the reaction, trehalose-6-phosphate (T6P), is formed by the transfer of glucose from one UDP-glucose molecule to one glucose-6-phosphate, catalysed by TPS. In the next step, TPP catalyses the hydrolysis of the phosphate molecule, and trehalose is formed. An overview of the reaction can be seen in Figure 3. (Puttikamonkul *et al.*, 2010; Iturriaga *et al.*, 2009; Avonce *et al.*, 2006)

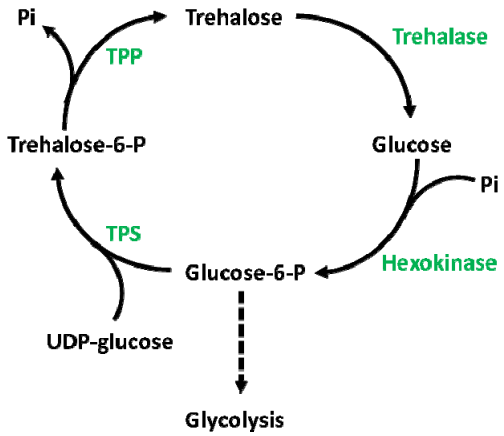

Figure 3. The most commonly occurring trehalose synthesis pathway in fungi, degradation and utilisation of trehalose is also indicated. Based on (Fillinger *et al.*, 2001)

A second synthesis pathway for trehalose in fungi has been hypothesised; trehalose phosphorylase (TreP or TP) catalyses the reaction where one glucose and one glucose-1-phosphate molecule form trehalose and inorganic phosphor. The gene encoding this protein has been found in several organisms, including

yeasts and the filamentous fungi *A. fumigatus* and *N. crassa*, but the biosynthetic reaction has only been shown *in vitro*. (Puttikamonkul *et al.*, 2010; Avonce *et al.*, 2006; Shinohara *et al.*, 2002)

Putative TPS/TPP pathway genes have been identified and more or less extensively characterised in several fungal species; an overview of orthologue and paralogue names can be seen in Table 1.

Table 1. Orthologue and paralogue names of TPS/TPP synthesising genes in a selection of fungi. Dash indicates non-described genes.

Species	TPS	TPP	Reference
A. niger	*tpsA-C*	*tppA-C*	**Paper II**; Wolschek & Kubicek, 1997
A. nidulans	*tpsA*	*orlA*	Fillinger *et al.*, 2001; Borgia *et al.*, 1996
A. fumigatus	*tpsA-D*	*orlA*	Al-Bader *et al.*, 2010; Puttikamonkul *et al.*, 2010)
Botrytis cinerea	*tps1*	-	Doehlemann *et al.*, 2006
Magnaporthe oryzae	*tps1*	-	Fernandez & Wilson, 2011
Candida albicans	*tps1*	*tps2*	Martinez-Esparza *et al.*, 2009; Alvarez-Peral *et al.*, 2002
S. cerevisiae	*tps1*	*tps2*	Gancedo & Flores, 2004

In the yeast *S. cerevisiae*, the gene products for trehalose synthesis are clustered in a "trehalose synthase complex". The TPS and TPP encoding genes (tps1 and tps2 respectively) are coupled with two regulatory subunits encoded by tsl1 and tps3. (Gancedo & Flores, 2004; Bell *et al.*, 1998; Reinders *et al.*, 1997) The TPS encoding gene, tps1, has a unique function as deletion of this gene totally abolishes the trehalose content, unlike deletion of any of the other genes. Deletion mutants of only one of the regulatory genes, tps3 or tsl1, have wild type levels of trehalose, whereas deletion of tps2 as well as double deletions of tps2, tps3 or tsl1 yield *ca* 30 % of the trehalose content. (Bell *et al.*, 1998)

3.1.1 In *Aspergillus* species

In *A. niger* there are six gene homologues to the enzymes of the trehalose complex in *S. cerevisiae*. Three of these are *tps1* orthologues: *tpsA*, *tpsB* and *tpsC*; and three are putative *tps2* orthologues: *tppA*, *tppB* and *tppC*. At least four of the six encoded proteins appear to interact and form a complex (TppA and TpsA-C) whereas TppB and TppC exist outside the complex. However, due to limitations of the experimental assay, these results are not entirely unambiguous, and it is possible that all gene-products are part of a complex. (**Paper II**)

Analysis of single gene deletion mutants of the six genes revealed that, under habitual conditions, *tpsA*, *tppA* and *tppB* had the greatest impact on

trehalose levels. These deletions lowered the trehalose content by 50 to 70 %, whereas deletion of the other genes did not affect trehalose significantly. (**Paper II**) According to Wolschek and Kubicek (1997) who investigated *A. niger tpsA* and *tpsB*, the first only functions in normal temperatures and the latter has a heat induced activity. This, and other types of regulated activates, might explain why the trehalose levels were not decreased in some of the deletion mutants in Paper II.

Unlike *ΔtpsA* and *ΔtppB*, which had wild type morphology, the phenotype of *ΔtppA* was severely affected. It displayed a drastic reduction in conidial formation, defective conidiophores, and trehalose-6-phosphate was accumulated in the mycelium. This clearly indicates that *tppA* encodes a T6P-phosphatase. (**Paper II**).

Among the Aspergilli, two other species have been studied with regard to trehalose synthesis.

In *A. nidulans*, one TPS encoding gene is denoted *tpsA*. A null mutant of this gene was depleted of both T6P and trehalose, and trehalose was not accumulated in response to stress. Therefore, *tpsA* seems to be a unique T6PS-encoding gene in *A. nidulans*, and this gene is indispensible for trehalose production. (Fillinger *et al.*, 2001) Disruptants of the TPP encoding gene of *A. nidulans*, *orlA*, contained wild type levels of trehalose and also accumulated the sugar in response to stress. On the other hand, levels of T6P were elevated in the disruptants. It therefore seems clear that *orlA* does encode a T6P-phosphatase, but that function can be partially replaced by other genes. (Borgia *et al.*, 1996)

In *A. fumigatus*, four TPS homologues have been identified and characterised, *tpsA – D*. Of these, *tpsC* and *tpsD* seems to be inactive copies with continuatively low gene expressions. On the other hand, either *tpsA* or *tpsB* was required for normal trehalose levels, as deletion of one of them did not alter the trehalose content, whereas it was abolished in a double mutant (*ΔtpsAΔtpsB*). (Al-Bader *et al.*, 2010) The TPP encoding gene of *A. fumigatus*, also denoted *orlA*, seems to have a similar function to the *orlA* gene of *A. nidulans*, as the trehalose levels of the deletion mutant were comparable with the wild type, but T6P was accumulated. A hypothesised explanation for the maintained trehalose value is that *A. fumigatus* can synthesise trehalose via the TP pathway (mentioned above), and two putative TP genes have been identified. (Puttikamonkul *et al.*, 2010)

Comparison of *A. niger* Tpp and Tps amino acid sequence with other published *Aspergillus* genomes showed that there are several, previously unidentified, homologues of the described genes among the Aspergilli, these are summarised in Table 2. (**Paper II**)

Table 2. Presence of different Tps and Tpp orthologues in Aspergilli

Subgenus	Section	Species	Tps	Tpp
NIDULANTES	Nidulantes	*A. nidulans*	A, D	A, B
		A. versicolor	A, D	A, B
		A. sydowi	A, D1, D2, D3, E	A, B
FUMIGATI	Fumigati	*A. fumigatus*	A, B, C, D	A, B, C
		N. fischeri	A, B, C, D	A, B, C
	Clavati	*A. clavatus*	A, B, C, D	A, B, C
CIRCUMDATI	Flavi	*A. flavus*	A, B, C	A, B, C
		A. oryzae	A, B, C	A, B, C
	Nigri	*A. acidus*	A, B, C	A, B, C
		A. aculeatus	A, B, C	A, B, C
		A. brasiliensis	C	A, B, C
		A. carbonarius	A, B, C	A, B, C
		A. kawachii	A, B, C	A, B, C
		A. niger	A, B, C	A, B, C
TERREI	Terrei	*A. terreus*	A, B	A, B

The fact that trehalose synthesis genes exist in multiple copies suggests that this pathway is of considerable importance for *Aspergillus* species. However, homologues of *tppA*, *tppB* and *tpsA* seem to have a unique position among these copies, as they are both conserved among all investigated Aspergilli (accept in *A. brasiliensis* which lack *tpsA*), and seem to have a greater impact on trehalose levels than other genes. Given that the trehalose levels in the *orlA* mutants of *A. nidulans* and *A. fumigatus* were maintained, it seems likely that TppB can partially replace the function of TppA.

3.2 Mobilisation

Two enzymes are known to catabolise trehalose: acid- and neutral trehalase, named because of their respective pH-optima. Acid trehalase is a secretory enzyme that breaks down external trehalose which serves as a carbon source; in fungi this enzyme is located in the cell wall. Internal trehalose is catabolised by the cytoplasmic enzyme neutral trehalase. In the reaction, one trehalose molecule is hydrolysed to two glucose molecules, see Figure 3. (d'Enfert *et al.*, 1999)

Turnover of internal trehalose is catalysed by neutral trehalase, and is triggered either when the sugar is no longer needed, i.e. when the stress condition under which trehalose was accumulated is lifted, or, upon induction,

such as germination of fungal spores or flight of insects, during which trehalose is used to provide energy. (Iturriaga *et al.*, 2009; Elbein *et al.*, 2003)

In *A. nidulans* and *A. niger*, neutral trehalase is synthesised by a gene denoted *treB* (Pel *et al.*, 2007; d'Enfert *et al.*, 1999). Deletion of the *A. niger treB* resulted in a strain that was unable to degrade internal trehalose during conidial germination and whose mycelium contained significantly more trehalose than wild type, supporting the importance of *treB* both after induction of germination and during vegetative growth (**Paper I**).

3.3 Trehalose functions in fungi

Trehalose and trehalose metabolism are vital to a range of cellular functions, besides serving as an energy source. Mutants in the various steps of trehalose metabolism are highly pleiotropic, displaying a range of phenotypic deviations. (Gancedo & Flores, 2004; Elbein *et al.*, 2003) In *A. niger*, trehalose and trehalose metabolism affect sporogenesis, conidial viability, and thermal stability (**Papers I** and **II**); this will be discussed further in later chapters. Additional traits of trehalose metabolism in Aspergilli include influence on virulence and cell wall biosynthesis (Al-Bader *et al.*, 2010; Puttikamonkul *et al.*, 2010; Fillinger *et al.*, 2001).

An aspect of trehalose synthesis that has received increasing attention in recent years is the role it plays in sugar sensing and growth regulation. Trehalose-6-phosphate has been shown to regulate glucose metabolism in yeasts as well as plants. One explanatory model for this is that T6P inhibits the enzyme hexokinase and subsequently glucose is blocked from glycolysis (see Figure 3). (Gancedo & Flores, 2004) In the rice blast fungus *Magnaporthe grisea* (*oryzae*), it has been shown that T6P-synthase is vital for both sugar metabolism and utilisation of nitrogen, as the enzyme regulates both gene expression and enzyme activities in these metabolic pathways (Wilson *et al.*, 2007).

The ability of trehalose to protect living cells against a variety of abiotic stresses has been demonstrated in several fungal species. For example, protective effects have been shown in conidia of *B. cinerea* and *A. nidulans* against heat (Doehlemann *et al.*, 2006; Fillinger *et al.*, 2001), blastoconidia of *C. albicans* against oxidative shock (Alvarez-Peral *et al.*, 2002), cells of *S. cerevisiae* against osmotic stress and heat (Hounsa *et al.*, 1998; Hottiger *et al.*, 1994), cells of *Cryptococcus laurentii* against low temperature (Li & Tian, 2006) and conidia of *A. fumigatus* against heat and oxidative shock (Al-Bader *et al.*, 2010).

3.3.1 Mechanisms behind protection

The bio-protective ability of trehalose mainly acts at two levels: the sugar protects lipid membranes at low water content so that they do not fuse or go into lipid phase transition; and, it stabilises proteins under conditions where the secondary structure would normally denature (Lins *et al*., 2004; Crowe *et al*., 1992).

The mechanisms behind the protective ability of trehalose are not fully elucidated, but three main hypotheses have been put forward:

1. The water replacement theory – during drying, water molecules are substituted by trehalose that form hydrogen bonds with the phospholipids of the membrane or the protein, thereby stabilising their native structure.
2. The mechanical entrapment theory – trehalose readily forms a glass, a bio-structure described as an amorphous solid with extremely high viscosity; when this matrix surrounds a bio-molecule, the conformation is retained.
3. The preferential exclusion theory – trehalose orders residual water molecules, thereby separating them from the bio-molecule, which then becomes more compact and therefore stabilised.

These theories are not necessarily mutually exclusive, but may all contribute to the observed effects of trehalose. (Jain & Roy, 2009; Lins *et al.*, 2004; Pereira *et al.*, 2004) It has been suggested that a prerequisite for the protective abilities of trehalose can be found in its extraordinary chemical and physical properties. However, the truth is that no such properties exist; it is more likely that all the properties of trehalose combined give rise to the superiority of this disaccharide as a stress protectant. (Jain & Roy, 2009; Crowe *et al.*, 2001)

4 Fungal spores

Fungi, like other organisms such as mosses, ferns and some bacteria, form spores to provide means of dispersal and to guarantee survival in times of unfavourable environmental conditions. The spore is a self-contained receptacle of genetic material that can develop in to a new colony when the circumstances are favourable. Within the fungal kingdom, Zygomycota, Basidiomycota and Ascomycota form both sexual spores – zygospores, basidiospores and ascospores, respectively – and asexual spores, called sporangispores in Zygomycota and conidia in Basidiomycota and Ascomycota. (Osherov & May, 2001)

Different kinds of spores often fulfil different purposes. In Ascomycota, for instance, the conidia are often produced in abundance and on aerial structures that facilitate distribution and spreading, whereas the ascospores are produced in lower numbers within the mycelium, and often have a higher survival capability as well as increased genetic diversity, which probably enhances survival in unstable environments. Many fungi are able to produce both sexual and asexual spores in the same colony (Chitarra & Dijksterhuis, 2007; Hoekstra, 2005).

The vast amounts of conidia produced by many species of Ascomycetes are commonly spread by air currents. When environmental conditions change, rapid dispersal is important for survival of the fungus. Therefore, the production of conidia (conidiation) is a swift process that involves relatively simple cellular transformations. (Ugalde & Corrochano, 2007) In *A. nidulans*, for instance, the time span between induction of conidiation to the first mature conidia is approximately 6-8 hours (Adams *et al.*, 1998). The circumstances that induce spore production differ between fungal species. Within the Ascomycota, exposure of hyphae to the air is often enough in *Penicillium* and *Aspergillus* species, whereas *Neurospora* species undergo conidiation as a

response to environmental induction such as carbon or nitrogen deficiency. (Ugalde & Corrochano, 2007; Roncal & Ugalde, 2003; Adams *et al.*, 1998)

4.1 Conidiation

Production of conidia generally occurs in the aerial hyphae that are no longer growing vegetatively, that is to say behind the growing edge of the colony. The older conidia will therefore be found in the centre of the fungal colony, and the younger, closest to the edge. (Krijgsheld *et al.*, 2013)

Conidiation in *Aspergillus* includes several well-defined morphological phases. After a certain period of vegetative growth, and if the colony is air-exposed, two kinds of aerial hyphal branches are formed in the centre of the colony. One type is similar to vegetative hyphae, whereas the other type can differentiate into conidiophore stalks by apical extension. The stalks have roughly twice the diameter of normal aerial hyphae, and, in contrast to these, they rarely branch, and emerge from a foot cell. This is a specialised, thick-walled structure, which lies at the same level as the substrate, thereby connecting the stalk to it. When apical growth of the stalk ceases, the top begins to swell, forming a spherical structure called the conidiophore vesicle. The foot cell, stalk and vesicle form a single unit (the conidiophore), since there are no septa in the structure. In so called biseriate species of *Aspergillus*, multiple nuclear divisions in the vesicle give rise to budding of metulae, elongated cells with one nucleus each. Division of this nucleus and further budding subsequently produce uninucleated phialides at the top of the metulae. The phialides are specialised reproductive cells producing daughter cells (conidia) by mitosis. The conidia are predominately uninucleate and formed in chains where each new one is produced closest to the phialide, pushing the chain upwards. In uniseriate species, the phialides are formed directly on the conidiophore vesicle, with no layer of metulae beneath. (Krijgsheld *et al.*, 2013; Adams *et al.*, 1998) An overview of asexual reproduction of *Aspergillus* can be seen in Figure 4.

The events that lead to conidiation in the closely related genus *Penicillium* are similar to those in *Aspergillus*, but with a few differences. The stalk is formed from aerial hyphae but does not emerge from a foot cell, and the conidiophore of *Penicillium* species is septated. No vesicle is formed; instead, the conidiophores may be branched in one or two steps (ter- or quaterverticillate) or bear the phialides directly on the stalk (monoverticillate). (Ugalde & Corrochano, 2007; Roncal & Ugalde, 2003)

The genetic regulation of conidiation in Aspergilli is complex and involves a vast number of genes and gene products; in colonies of *A. nidulans*, 1300

genes were found to be up-regulated during asexual development. A few examples of the many genes that have been studied and found to be of importance for conidiation include: *fluG*, which initiates asexual development; *brlA*, which regulates formation of conidiophores; and *wetA*, which is involved in conidial cell wall formation. (Krijgsheld *et al.*, 2013)

Genes in trehalose metabolism affect spore formation in *A. niger*. Deletion strains of *tppA* and *treB* produced 500 and 20-fold fewer conidia than the wild type, respectively (**Papers I** and **II**).

The morphology of *ΔtppA* was severely affected; the almost abolished conidial production was apparently due to malformation of the conidiophores. In contrast to *ΔtppA*, other deletion strains of *A. niger* (*ΔtpsA* and *ΔtppB*) displayed wild-type morphology in combination with lowered trehalose contents. This suggests that it is not the ability to produce trehalose, *per se*, which is important for conidiogenesis; rather, it is the accumulation of T6P that inhibits spore formation. (**Paper II**)

In the case of *ΔtreB*, the morphology of the conidiophores was unaffected, but decreased conidiation was accompanied by abnormal shape and size of the few produced, as well as reduced viability. This indicates that intracellular trehalase activity is crucial both for formation and function of conidia, but not for formation of conidiogenic structures. (**Paper I**)

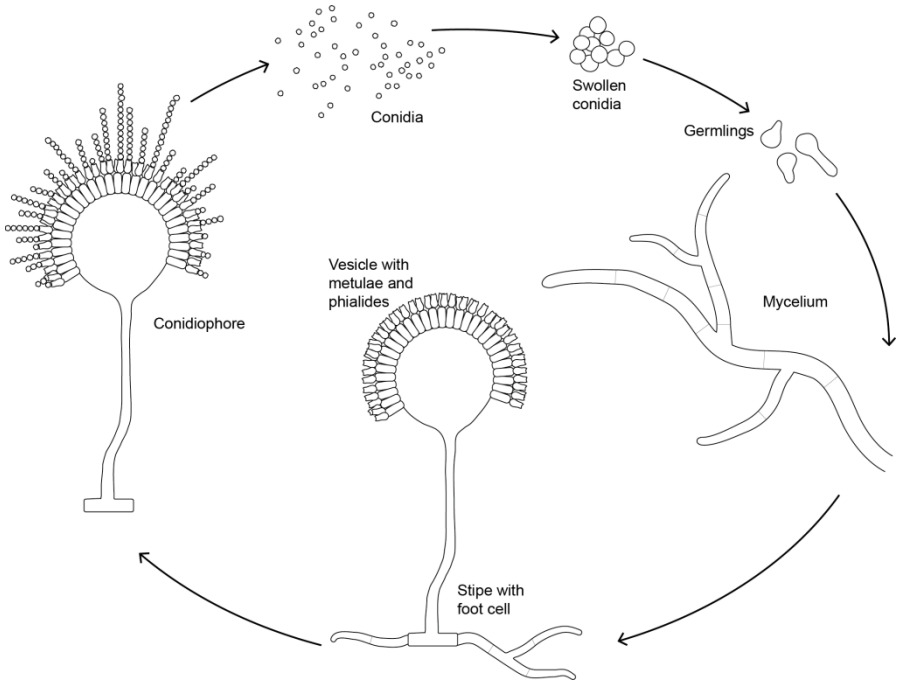

Figure 4. Asexual life-cycle of a typical *Aspergillus* species.

4.2 Maturation of conidia

Newly formed conidia display differences compared to older conidia, indicating that the maturation process is not completed during conidiation, but continues after release from the phialide. *A. niger* conidia harvested from 5 day old cultures exhibited higher sensitivity to heat stress compared to conidia from 14 days old cultures. However, the reproducibility of these results was very low, suggesting that the maturation process is irregular. After 14 days, the responses to heat stress had evened out and were reproducible. (Unpublished observation, Svanström and Melin) Moreover, conidia are not, as previously believed, completely dormant structures. (Aguilar-Osorio *et al.*, 2010) identified genes in the mannitol metabolism of *A. niger* that were exclusively expressed in dormant conidia, and we demonstrated that all identified trehalose synthesis genes of *A. niger* are expressed in dormant conidia as well as after induction of germination (**Paper II**). In *A. nidulans*, *vosA* has been shown to regulate the expression of trehalose synthesis genes and thereby affect conidial maturation. Conidia of a *ΔvosA* mutant were depleted of trehalose and they displayed rapidly decreasing viability. (Ni & Yu, 2007) Accordingly, conidia of an *A. niger vosA* deletion mutant contained approximately 10 % of the wild type level of trehalose (Unpublished observation, Svanström and Melin)

With regard to trehalose, *A. niger* conidia appear to become fully matured sometime in the time span 5 to 14 days. In 5 day old conidia, trehalose comprised approximately 2 % of the dry weight; after 14 days, this had increased to 5 %, a level which was then maintained during continued aging as can be seen in Figure 5. (**Paper II**)

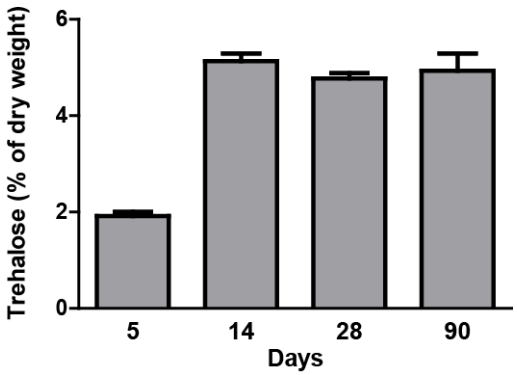

Figure 5. Trehalose content of wild-type *A. niger* conidia during aging.

4.3 Germination

To fulfil their purpose, it is important that fungal spores germinate only after they have been dispersed and only if the surrounding environment is favourable. Spores therefore have effective mechanisms to maintain dormancy. (Krijgsheld *et al.*, 2013)

Consequently, to break dormancy, the first stage of spore germination is activation. This is triggered by environmental factors that differ widely between species and genera. For some fungi, access to water, oxygen and carbon dioxide is sufficient. Others require nutrients such as an external carbon source and amino acids, and still others need a specific surface to germinate. (Osherov & May, 2001; d'Enfert, 1997)

When the requirements for activation are fulfilled, the spore starts isotropic growth, a stage called swelling. The surface properties of the spore change, increasing the capacity for adhesion to other spores and to the substrate. The spore takes up water, and metabolic activities, such as respiration and synthesis of RNA and proteins, are initiated. Trehalose is rapidly broken down to glucose, which enters glycolysis, subsequently giving rise to a glycerol pool. (Osherov & May, 2001; d'Enfert, 1997) In germinating *A. niger* conidia, this occurs during the first 3 hours of germination; after that period conidia are nearly depleted of trehalose. However, this quick decrease is followed by re-synthesis; after 6 hours, the level is approximately 50 % of that in dormant conidia (2.5 % *vs* 5 %), and after 12 hours of incubation, the levels are almost restored (see Figure 6). (**Paper I**)

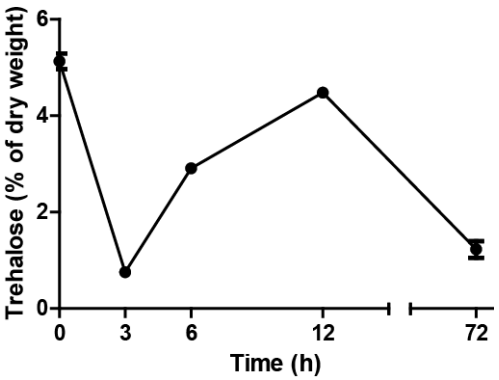

Figure 6. Trehalose content of wild-type *A. niger* conidia and during germination. Notice the scale break between 12 and 72 hours.

When swelling of conidia ceases, they proceed to polarised growth (see Figure 7). In the swollen spore, materials and machinery for production of the cell wall and plasma membrane are distributed throughout the cortex; these are now localised to a certain position on the cell wall, and a germ tube emerges. The germ tube continues to grow apically, septa are formed, and eventually the hypha will branch, creating additional directions of polarised growth – a fungal colony is formed (see Figure 4). (Momany, 2002)

In *A. niger* conidia, no morphological changes are observed within the first 3 hours of incubation, although the trehalose contents are nearly depleted during this initial period. Swelling is obvious in the majority of spores after 6 hours, and after 12 hours the germ tubes are typically ten times longer than the remaining conidia. (**Paper I**)

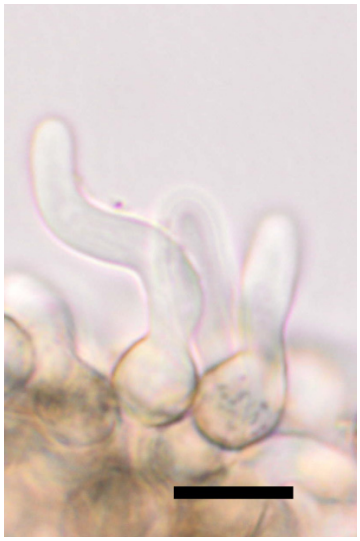

Figure 7. A. niger conidia after 9 hours of germination. The size-bar is 10μm.

5 Food preservation

Preventing the growth of unwanted microorganisms in food has always been a human concern and some of the methods still in use, drying for instance, are very old (Adams & Moss, 2000b).

When preserving food there are two major strategies: inactivation (killing) of the microorganisms, or inhibiting their growth. The latter is by far the most common, and involves changing physical or chemical factors of the food, thereby making it a poor habitat for microbial growth. Examples include lowering the water activity (by drying or adding solutes such as salt or sugar), low temperature storage, addition of preservatives, restriction of available oxygen, raising the level of carbon dioxide (by modified atmosphere packaging) and acidification. (Gould, 1996) In many kinds of foods, these methods are applied in so-called hurdles, where several factors combine to make the food safe. For instance, some fungi are well adapted to growing in cold and dry environments, so while these measures might reduce spoilage by prokaryotes, fungi can still thrive. Sole reliance on cold storage of a sausage might not prevent mould growth, but if it is also dried, salted and the pH is lowered, the shelf life is prolonged. (Adams & Moss, 2000b; Leistner, 2000)

The most widely used method to kill spoilage microorganisms in food is heating, pasteurisation and canning being two examples; certain newer techniques, like electromagnetic irradiation and high-pressure treatment, are also lethal to microbes (Gould, 1996).

To supplement food with a chemical substance that limits, or totally inhibits, microbial growth is an effective means of extending shelf life. Among the most commonly used types of chemical preservatives are naturally occurring weak organic acids such as acetate, propionate, sorbate and benzoate. The function of these acids is pH-dependent and the mechanisms behind this are sometimes referred to as “the weak acid theory”:

When the external pH is low (i.e. in acidic food), the acid exists in its undissociated form and is therefore lipophilic. This allows it to pass the microbial membrane. When inside the cell, the higher cytosolic pH leads to dissociation of the acid. The anionic part of the acid is charged and thus becomes trapped in the cell. The accumulation of protons acidifies the cell cytosol leading to stress for the organism. Furthermore, key steps in glycolysis are inhibited, leading to a drop in ATP levels. (Melin *et al.*, 2008; Plumridge *et al.*, 2004; Gould, 1996) In addition to this, weak organic acid preservatives have been shown to inhibit up-take of nutrients, such as amino acids and nucleotides, in microorganisms (Melin *et al.*, 2008; Bauer *et al.*, 2003; Hunter & Segel, 1973).

An ongoing trend is the demand for food that is perceived as more "natural", containing less additives and preservatives. The interest in alternative methods to extend shelf life is therefore great; one aspect of this is that the ancient method of fermentation receives increasing attention. (Schnürer & Magnusson, 2005; Gould, 1996)

Fermentation is a method to preserve as well as refine food by microorganisms. The vast majority of fermented foods are produced by lactic acid bacteria (dairy products, sourdough bread, pickled vegetables etc.) and fungi, both yeasts (beer, wine) and moulds (cheese, tempeh, soy sauce etc.) Early use of such processes was based on empirical knowledge rather than apprehension of the mechanisms behind it. The essential role played by microorganisms in fermentation was discovered when pasteurisation was developed in 1861. Besides improving the sensory qualities of the food, growth of beneficial microbes can prevent spoilage by other microorganisms. The mechanisms behind this include production of acids and antimicrobial compounds (such as bacteriocins and ethanol), as well as competition for nutrients and space. (Dalie *et al.*, 2010; Kabak *et al.*, 2006; Paul Ross *et al.*, 2002)

5.1 Lactic acid bacteria

Lactic acid bacteria (LAB) do not constitute a strict taxonomical group. However, they share many features, the most pronounced being the production of lactic acid. Genera include *Lactococcus*, *Lactobacillus*, *Enterococcus*, *Streptococcus*, *Leuconostoc* and *Pediococcus*. (Paul Ross *et al.*, 2002) Species of these genera naturally occur in nutrient rich environments and they are often isolated from various foods, such as dairy products and meat (Schnürer & Magnusson, 2005).

Lactic acid bacteria are strictly fermentative and can be divided into two groups depending on their metabolism; homofermentative and heterofermentative species. The main end product of homofermentative LAB is lactic acid, whereas heterofermentative LAB produce lactic acid as well as other organic acids, carbon dioxide and ethanol. Besides the main end products, many more substances are formed during fermentation, including antimicrobial compounds, flavour compounds, vitamins and anti-oxidants. (Schnürer & Magnusson, 2005; Paul Ross *et al.*, 2002)

In large scale food production today, defined starter cultures are almost always used for fermentation, unlike the more traditional method of "backslopping", whereby a portion of a previous, successful, batch of fermented food is used as inoculum (Leroy & De Vuyst, 2004).

5.1.1 Antifungal properties of lactic acid bacteria

Most research on food preservation by LAB is focused on the antibacterial effects, and the majority of LAB species are known to produce specific so-called bacteriocins. (Paul Ross *et al.*, 2002) However, the use of LAB for bio-preservation to control mould growth and mycotoxin production is of growing interest. (Dalie *et al.*, 2010; Schnürer & Magnusson, 2005; Ström *et al.*, 2005; Lavermicocca *et al.*, 2003)

Several LAB metabolites with antifungal properties have been identified, but the mechanisms of inhibition are largely unknown. Besides organic acids, the antifungal metabolites of LAB include: reuterin, a broad spectrum antimicrobial with effect against bacteria, yeasts and moulds; hydrogen peroxide, which is produced in the presence of oxygen and is a potent antimicrobial; hydroxylated fatty acids, that are known for their sensory qualities in food but also display a potent antifungal effect; and phenyllactic acid, which will be discussed in detail below. (Dalie *et al.*, 2010; Schnürer & Magnusson, 2005)

In addition to inhibiting the growth of moulds, LAB have been shown to reduce the levels of mycotoxins by either inhibiting their bio-synthesis, or by binding to and thereby neutralising them. (Dalie *et al.*, 2010; Kabak *et al.*, 2006)

When specifically introducing LAB as bio-protection in foods, choosing strains that produce effective antifungal metabolites could be a way of optimising the effect. (Valerio *et al.*, 2004) So far, most species with proven efficacy against mould belong to the *Lactobacillus* and *Lactococcus* genera, for instance *Lb. plantarum*, *Lb. reuteri* and *Lc. lactis*. (Dalie *et al.*, 2010)

5.1.2 Phenyllactic acid

Phenyllactic acid (PLA, see Figure 8) was first identified as an antifungal metabolite of LAB by Lavermicocca and co-workers (2000) when *L. plantarum* extracts were purified and analysed. PLA inhibits growth of several *Aspergillus*, *Penicillium*, *Eurotium* and *Fusarium* species. It has been shown that in sourdough bread made with a PLA-producing starter culture of *L. plantarum*, growth of *A. niger* and *P. roqueforti* were delayed for up to 7 days; shelf life was significantly longer than in bread made with cultures that did not produce PLA. (Lavermicocca *et al.*, 2003; Lavermicocca *et al.*, 2000) PLA has also been found to be active against moulds (*A. fumigatus* and *P. roqueforti* among others) in grass silage (Ström *et al.*, 2002).

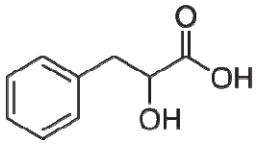

Figure 8. Structure of PLA

PLA has been isolated from several species of *Lactobacillus* (*L. coryniformis*, *L. sakei*, *L. reuteri*, *L. casei*, *L. fermentum*, *L. plantarum* and *L. acidophilus*, for instance), but also by *Pediococcus pentosaceus* and *Leuconostoc citreum*, among others (Gerez *et al*., 2013; Schnürer & Magnusson, 2005; Valerio *et al*., 2004; Ström *et al*., 2002). When added in pure form, relatively high concentrations (in the mg/ml range) of PLA are required to inhibit moulds. However, in systems where LAB acts as bio-preservation and lower concentrations of PLA are produced, this metabolite probably contributes to the antifungal effect in combination with other antifungal substances. (Schnürer & Magnusson, 2005; Ström *et al*., 2002). Furthermore, it is possible to increase the amount PLA produced by increasing the concentrations of phenylalanine and citrate in culture (Gerez *et al*., 2013; Valerio *et al*., 2004).

The functions by which PLA inhibits mould growth are largely unknown. However, PLA is an organic acid and the weak acid theory described above is applicable. This is supported by the fact that the effect of PLA is pH-dependent. In a “single-spore assay”, where the germination ability of conidia were tested on solid media containing various concentrations of PLA, 60 mM PLA was sufficient to completely inhibit the growth of *A. niger* conidia at pH 2.9 (that is, 60 mM = MIC, minimal inhibitory concentration). However, the same PLA concentration at pH 4.4 did not affect the germination at all compared to a control without PLA. This phenomenon is most probably explained by the fact that at pH 4.4, PLA exists in the dissociated form and is therefore unable to pass the cell membrane. The theory that PLA functions as a weak acid is further supported by the fact that a uridine auxotrophic strain (i.e. a strain that is unable to produce this nucleoside) of *A. niger* was oversensitive

compared to the wild type strain, a phenomenon previously observed for all weak acids with a carboxyl group (Melin *et al*., 2008). This suggests that the PLA inhibits uptake of nutrients in the same way as has been shown for other weak acids. (**Paper 3**)

In contrast to these findings, comparison with the weak acid preservative benzoic acid showed that PLA possesses an additional mode of function not displayed by other weak acids. At concentrations below the MIC, PLA inhibits radial growth and sporulation of *A. niger*, an effect not seen by benzoic acid. Moreover, unlike benzoic acid, PLA did not reduce the numbers of colonies formed at sub-inhibitory concentrations. These findings indicate that PLA have a unique mode of function, and rather than being fungicidal it specifically inhibits asexual development as well as growth. (**Paper 3**)

6 Stress

‘Abiotic stress’ imply environmental conditions that are harmful to organisms occupying the ecological niche in question. Examples that affect fungi include restriction of oxygen, adverse temperatures, changed osmolarity, and starvation, among many others. (Miskei *et al*., 2009; Magan & Aldred, 2007). Thus, many of the measures we take to preserve food could be thought upon as attempts to increase the abiotic stress on food-borne fungi.

Fungal spores are generally more resistant to stress than vegetative cells (Osherov & May, 2001). Unlike growing mycelium, the conidia of Aspergilli are, for instance, protected by a thick cell wall, and contain pigments like melanin (thought to protect against UV-radiation) and high levels of so-called compatible solutes, e.g. trehalose and mannitol, that are known to increase resistance to heat and drought (Krijgsheld *et al.*, 2013). As mentioned in chapter 4.2, dormant conidia are not completely resting structures but several genes are expressed. Furthermore, van Leeuwen and co-workers (2013) have shown that transcripts of many stress protective proteins are more highly expressed in dormant conidia, than after germination.

6.1 Stress response

When subjected to environmental stress, an organism will either die or survive, depending on its innate resistance. In the case where the organism survives the initial stress-state, changes in gene expression may lead to an altered phenotype, allowing continued, adapted, growth. (Smits & Brul, 2005) One example of adaptation is accumulation of trehalose, which has been detected as a stress response in many fungal species including *S. cerevisiae* (Hottiger *et al.*, 1994), *B. cinerea* (Doehlemann *et al.*, 2006), *Rhizopus oryzae* (Uyar *et al.*, 2010), *A. nidulans* (Fillinger *et al.*, 2001) and *A. fumigatus* (Al-Bader *et al.*, 2010). Another example is the existence of environmental stress-response

genes that encode proteins with specific protective functions, and whose expressions are linked to the corresponding situations. Many stress-response proteins have been annotated within the Aspergilli, osmotic-, oxidative- and thermal-stress proteins being the most common. (Miskei *et al.*, 2009)

One putative stress-response protein identified in several *Aspergillus* species is PhiA (Schachtschabel *et al.*, 2012; Glaser *et al.*, 2009; Melin *et al.*, 1999). In *A. nidulans*, the expression of *phiA* is induced by bafilomycin treatment. This is a macrolide antibiotic that inhibits V-ATPases and is produced by several species of the bacterial genus *Streptomyces*. Moreover, *phiA* is essential for asexual development: it encodes for a cell wall protein and is necessary for normal formation of phialides. (Melin *et al.*, 2003; Melin *et al.*, 1999) When conidia of *A. niger* are subjected to the LAB metabolite PLA, growth is reduced and conidiation is inhibited. These alterations of phenotype are followed by a strong up-regulation of *phiA*. PLA and bafilomycin are structurally dissimilar molecules, bafilomycin being considerably more complex, yet they result in up-regulation of the same gene. It therefore seems reasonable to assume that PhiA is a stress-response protein that is produced by fungi as a response to competitive stress from antagonistic bacteria. (**Paper 3**)

The expression of the trehalose metabolism genes, *tppA* and *treB*, are not affected when *A. niger* is subjected to PLA. Furthermore, the susceptibility to benzoic acid stress is not affected in deletion mutants of *tppB* and *treB* (with lowered and elevated trehalose levels respectively). Taken together, this suggests that trehalose does not play a role in the protection against weak organic acids. (**Papers 1, 2 and 3**)

6.2 Protective ability of trehalose in Aspergilli

As mentioned in chapter 3.3, trehalose functions as a stress protectant in a wide variety of fungi and is effective against stressors such as high and low temperature, oxidative shock and adverse osmolarity.

Among the Aspergilli, trehalose synthesis deletion mutants with considerably lowered or depleted trehalose levels have been created in *A. niger* (**Paper 2**, (Wolschek & Kubicek, 1997), *A. fumigatus* (Al-Bader *et al.*, 2010), and *A. nidulans* (Fillinger *et al.*, 2001), and their stress susceptibility has been evaluated. In addition, the trehalase gene, *treB*, has been shown to be of importance for stress resistance in *A. niger* and *A. nidulans* (d'Enfert *et al.*, 1999), **Paper1**).

Mutants with lowered or depleted trehalose levels were more sensitive to heat stress than wild types in all three species. In the *A. fumigatus ΔtpsAΔtpsB* double mutant, trehalose was undetectable and this mutant showed reduced

conidial viability, as well as reduced growth and conidiation at elevated temperatures (Al-Bader *et al.*, 2010). In *A. nidulans*, the single deletion of *tpsA* was sufficient to completely deplete trehalose, and at elevated temperatures, the deletion strain had an impaired capacity to germinate and form colonies. The survival of *ΔtpsA* and wild type germlings after heat shock was, on the other hand, equivalent. (Fillinger *et al.*, 2001) Results from *A. niger* show that the trehalose content does not have to be completely depleted to have a negative effect on thermo-tolerance. An *A. niger ΔtpsA* mutant contained about half as much trehalose as the wild-type, and mutant conidia were more sensitive to heat shock (note, in these experiments, trehalose was measured in the mycelium, whereas stress tests were performed on dormant conidia) (Wolschek & Kubicek, 1997). Deletion of *A. niger tppB* rendered a mutant strain in which conidia contained about one third of wild-type trehalose levels, and these were hypersensitive to heat, see Figure 9 (**Paper 2**).

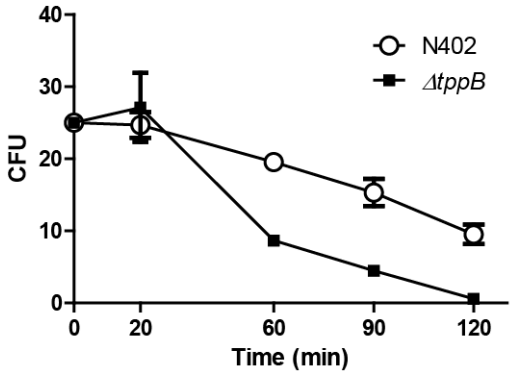

Figure 9. Survival of wild type and *ΔtppB* conidia after 20-120 min heat stress at 55 °C.

Disruption of the *A. nidulans treB* gene generated a mutant in which trehalose was not degraded during germination. When conidia of this strain (*treB*$^-$) were pre-incubated for 2 hours and then subjected to heat shock, the survival rates were significantly higher than in a *treB*$^+$ strain. Accordingly, deletion of the A. *niger treB* resulted in a mutant with severely reduced ability to degrade trehalose. In an experimental procedure similar to that in *A. nidulans treB*$^-$, where *A. niger* conidia were pre-incubated for 3 hours and then heat shocked, the survival of *ΔtreB* was significantly higher than the wild type. However, when heat shock was applied directly to dormant conidia, the *ΔtreB* strain showed significantly lower survival rates than wild type, see Figure 10. (**Paper 1**; d'Enfert *et al.*, 1999)

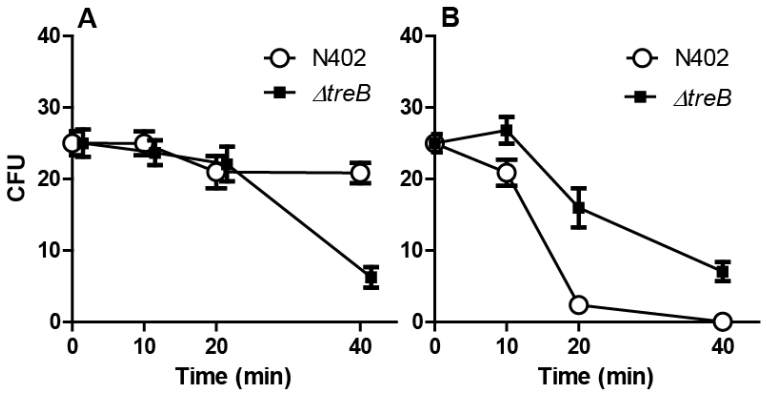

Figure 10. Survival of wild-type and *ΔtreB* after 40 min of heat stress. Dormant conidia stressed at 55°C (A, note that *ΔtreB* observations are nudged to avoid visual overlap), and conidia incubated for 3 hours prior to stress at 50°C (B).

The similarities of these results indicate that within the Aspergilli, trehalose plays an identical role in cellular protection against heat stress. Two experimental procedures have been used to test heat stress in these studies: growth in continuously elevated temperatures; or heat shock of conidia that were then grown in habitual temperatures. The only study that tested both assays was that with *A. nidulans ΔtpsA*. Those results suggested that in *A. nidulans*, trehalose has a role in resistance to continual exposure to sub-lethal stress, but not in protection against short exposure to lethal stress. In contrast, the results from *treB*$^-$ suggested that trehalose can protect *A. nidulans* cells against otherwise lethal stress. (Fillinger *et al.*, 2001; d'Enfert *et al.*, 1999) As only one of the two assays was employed in *A. fumigatus* and *A. niger*, it is difficult to draw any clear conclusions as to what is the true situation within the Aspergilli. It is, however, clear that trehalose can protect cells against heat stress. This is further supported by the results from *A. niger* and *A. nidulans*

treB mutants, suggesting that elevated levels of trehalose can protect against heat after the on-set of germination (3 *vs* 2 hours). On the other hand, the ability to degrade the accumulated trehalose proved to be crucial for stress recovery of dormant conidia of *A. niger*. (**Paper 2**, (d'Enfert *et al.*, 1999)

In contrast to the relatively similar outcomes previously described, oxidative shock did not give equivalent results in the three species: *A. fumigatus ΔtpsAB* (depleted of trehalose) was hyper-sensitive to oxidative shock, whereas neither *A. nidulans ΔtpsA* or *A. niger ΔtpsB* (depleted and with lowered trehalose content, respectively) showed any difference in survival compared to wild type (**Paper 2**) (Al-Bader *et al.*, 2010; Fillinger *et al.*, 2001). Rather than pointing to actual variations in the protective ability of trehalose among the three species, this likely depends on differences in experimental design. Parameters such as age of conidia, growth media, incubation temperature, and so on varied among the studies, and this naturally impairs comparison of the findings, but are difficult to avoid as different species have different demands.

7 Conclusions and future perspectives

The main findings of the present work were:

- In *A. niger*, *treB* is crucial for the ability to degrade trehalose, this ability in turn affects the survival of conidia after heat stress. However, the impact is twofold: dormant conidia of a *treB* deletion mutant are more resistant to heat stress than wild type conidia, whereas the inability to degrade trehalose is unfavourable for survival when incubated conidia (i.e. when dormancy is broken) are heat stressed. This duality indicates that while the raised level of trehalose protects proteins and membranes in dormant conidia, being able to utilise trehalose is important for stress recovery of conidia that have started to germinate. (**Paper 1**)
- There are six putative trehalose synthesis genes in *A. niger*, *tpsA*-*C* and *tppA*-*C*, and in closely related species there are orthologues to most or all of these genes (**Paper 2**).
- In *A. niger*, *tppA*, *tppB* and *tpsA* are most significant for functional trehalose synthesis. However, all *tps* and *tpp* genes are expressed both in conidia and during germination. (**Paper 2**)
- The main encoder of trehalose-6-phosphate-phasphatase in *A. niger* is *tppA*, and in addition to its essential role in trehalose bio-synthesis, this gene is also a pre-requisite for asexual development (**Paper 2**).
- As previously shown in other *Aspergillus* species, decreased trehalose content of *A. niger* conidia negatively affects their resistance to heat stress (**Paper 2**).
- PLA inhibits fungal growth, both through the same mechanism of action as other weak acids, but also by inhibiting asexual reproduction (conidiation). PLA stress triggers strong up-regulation of *phiA*, a gene known to affect phialide development and which is induced by macrolide antibiotics. This suggests that the inhibition of conidiation

likely occurs at the (mis)formation of spore-producing structures, rather than at formation of conidia *per se*. (**Paper 3**)

- Since food spoilage is often mediated by air-borne conidia, a "single-spore" assay is a relevant tool when characterising the inhibitory properties of potential anti-fungal compounds. Unlike other assays, this method facilitates evaluation of colony formation and conidiation which are both relevant in food contexts. (**Paper 3**)

Studying stress in fungi, or any organism, is not entirely easy as several factors and systems influence resistance – it is a complex matter.

In this thesis I have used two different approaches to study stress response and resistance. These can roughly be described as either deleting a gene that is believed to have an important function, applying stress to the deletion mutant and seeing if it was more sensitive than the wild type; or, applying stress to the wild type and screening for gene expressions which differ to that in the untreated control, thereby finding genes which function is relevant for the present stress.

A disadvantage of the first method is that deletion of one gene might trigger the fungus to activate other protective functions. For instance, a strain that is deficient in the trehalose metabolism might compensate this by increased production of other metabolites. In addition to trehalose, mannitol is an example of an important conidial constituent that has been shown to be essential for heat stress survival (Ruijter *et al.*, 2003). It is not unlikely that lack of trehalose can be partly compensated by overproduction of this or other metabolites. It would therefore be interesting to screen the trehalose deletion mutants for a wide variety of metabolites, to investigate how the lack of one affects the production of others.

Besides metabolites with similar function, there can be several different protective systems present with overlapping roles. It would then be easy to draw false conclusions – for instance, that trehalose is not important against oxidative shock, when indeed it might play a role, but not exclusively. One example of such overlapping functions is in the yeast *S. cerevisiae*, where the heat protective function of trehalose can be almost completely compensated by the heat shock protein 104, but in a double mutant of *tps1* and *hsp104*, the heat resistance is low (Elliott *et al.*, 1996).

In the second method, the key question is which genes to examine in detail. In the PLA experiments, results showed an effect on asexual reproduction and we therefore chose to analyse the expression of five genes known to affect sporulation. However, the reality is, of course, that a much greater number of genes are involved. To be able to further elucidate the function of PLA, it

would be worth screening a large set of genes involved in asexual reproduction, possibly by a method with larger output than the one used in Paper 3, for instance by using microarrays.

Besides the broad topic of stress, trehalose metabolism in *A. niger* still poses many questions. We have partially clarified the specific functions of the genes involved in trehalose synthesis, but some things remain to be investigated. For instance: why several genes are still expressed when their function is apparently redundant to trehalose production; how the genes are regulated; and under which environmental conditions they are expressed.

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