Mechanisms underlying extreme heat resistance of ascospores of *Neosartorya fischeri*

Timon Theo Wyatt
Januari 2014

Mechanisms underlying extreme heat resistance of ascospores of *Neosartorya fischeri*

Mechanismen ter bescherming van *Neosartorya fischeri* ascosporen tegen extreme hitte
(met een samenvatting in het Nederlands)

Proefschrift

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Promotor: Prof. dr. H. A. B. Wösten
Co-promotor: Dr. J. Dijksterhuis

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‘Things don’t have to change the world to be important’
- Steve Jobs -

‘If you would be a real seeker after truth, it is necessary that at least once in your life you doubt, as far as possible, all things’
- Rene Descartes -

‘A dirty joke is a sort of mental rebellion’
- George Orwell -

Voor elkaar

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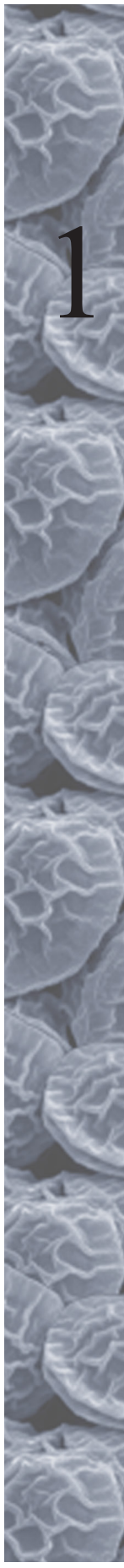
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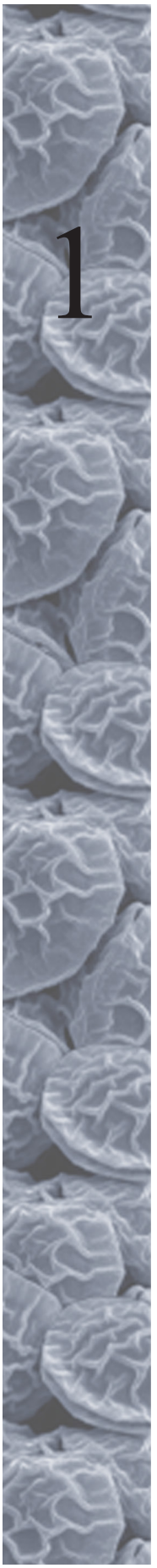

1 General Introduction

T.T. Wyatt, H.A.B. Wösten, J. Dijksterhuis

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The fungal kingdom

The fungi represent a morphological and physiological diverse kingdom with an estimated number of 1.5 million species (Hawksworth 1991; Hawksworth 2001). Traditionally, taxonomy of fungi was based on morphological and physiological properties, which usually didn't lead to a uniform classification. With the introduction of molecular techniques, DNA sequence analysis of functionally conserved genes became the standard for classification of fungi. However, for very closely related species polyphasic taxonomy is used, which consists of morphological, and physiological (i.e. the profile of primary and secondary metabolites) characterization. The introduction of multi-locus sequencing also helped to distinguish closely related species. Currently, the most accepted classification of the fungal kingdom consists of 1 subkingdom, 7 phyla, 10 subphyla, 35 classes, 12 subclasses, and 129 orders (Hibbett, Binder *et al.* 2007). Most fungi belong to the subkingdom Dikarya. This subkingdom is divided in the phyla Basidiomycota and Ascomycota, formerly known as Basidiomycetes and Ascomycetes. The other five phyla of the fungal kingdom are the Chytridiomycota, Neocallimastigomycota, Blastocladiomycota, Microsporidia, and Glomeromycota. Together with the 5 subphyla; Mucoromycotina, Entomophthoromycotina, Zoopagomycotina and Kickxellomycotina, they cover the fungal kingdom.

The vast majority of fungi grow filamentous, while a restricted number of species have adopted the unicellular growth mode. Filamentous growth occurs by means of apical (i.e. at the tip) extension of hyphae, which is accompanied by subapical branching. A network, called mycelium, is formed upon branching and fusion of hyphae. Fusion by means of anastomoses can occur via conidial anastomoses tubes (Gabriela Roca, Read *et al.* 2005), or between hyphae in the sub-peripheral zone of a colony (Glass, Rasmussen *et al.* 2004; Read, Lichius *et al.* 2009). Septation of hyphae is not common in "lower fungi" like Mucuromycotina and Chytridiomycota (Barr 2001; Letcher and Powell 2002). In contrast, hyphae of the Dikarya are divided into compartments by cross walls, the so-called septa. Cytoplasmic streaming between compartments within a hypha or between hyphae is possible through pores within the septa. Even whole cell organelles can stream through these septal pores (Bracker and Butler 1963). Transport of storage compounds such as glycogen and lipid droplets makes it possible to colonize nutrient poor environments from a food source. Both ascomycetes and basidiomycetes have developed strategies to (selectively) plug the septa of hyphae (van Peer, Müller *et al.* 2009; Bleichrodt, Vinck *et al.* 2012). Plugging of septa is for instance increased when the fungus is exposed to stress conditions such as a high temperature

The role of fungi in ecosystems

Fungi can grow saprobic and / or form mutual beneficial or parasitic interactions with other organisms. Mycorrhizal fungi live in a mutual beneficial collaboration with plants. The mycorrhizal fungus (for example the fly agaric) is associated

to the plant roots and has access to the sugars of the plant. In return, the fungus supplies the plant with nutrients (e.g. phosphates) and minerals. Endophytes that live inside plants also form a mutual beneficial interaction by helping the host to survive adverse conditions as drought or salt stress (Rodriguez, White Jr *et al.* 2009). They also form mycotoxins that may discourage herbivores to consume the host plant (e.g. in the case of the fungus *Pythomyces*). Pathogenic fungi also extract their nutrients from the host, but with detrimental effects. These fungi can establish a relationship keeping the host alive (biotrophic fungi), or killing it after which they feed on the host remnants (necrotrophic fungi). Many fungi become necrotrophic after an initial biotrophic stage. *Botrytis cinerea* is a true necrotrophic parasite of many plants species and causes necrosis in numerous fruits such as strawberries and grapes (Shlezinger, Doron *et al.* 2011). *Cladosporium fulvum* on the other hand is a biotrophic pathogen of tomato and grows in the extracellular space within leaves without causing necrosis in the host and without penetrating the host cells (Thomma, van Esse *et al.* 2005). Pathogenic fungi also affect animals and humans. One of the most well-known human (opportunistic) pathogenic fungi is *Aspergillus fumigatus*, which primarily causes infections in immuno-compromised individuals.

Stabilized life

Fungi and many other organisms are able to survive adverse conditions after stabilization of the cell and its constituents. Stabilization is a process in which biomolecules, cells or organisms become protected against stressors such as high osmolarity, temperature, drought and reactive oxygen species. As a result of stabilization, cells or organisms can survive extended periods of time in a virtually unchanged state. For example, bacterial spores arguably belong to the most stress resistant living structures, some of which claimed to be revived after tens of millions of years (Cano and Borucki 1995).

Single- or multi-celled eukaryotic organisms form survival structures or even survive as a whole. Many protists form cysts that survive dry periods in for instance soil. A number of well-known gut parasites are difficult to eradicate as the cysts are insensitive to antibiotic treatments. Nematodes, rotifers and tardigrates are small (in the realm of 100 µm to 1 mm) multicellular animals that can become dehydrated, a state dubbed as anhydrobiosis. This enables these organisms to survive prolonged periods of time, resuming biological activity upon rewetting. We all know the example of sea monkeys, the brine shrimps or *Artemia franciscana* that form diapauzed embryos in cysts that are able to survive drought and is used as "instant life" for educational purposes. Also larger organisms can be stabilized; entire plants survive drought as for example mosses or the "resurrection plants" (e.g. *Craterostigma plantagineum*; Bartels 2005).

In the kingdom of plants, stabilized structures have become an integral part of the life cycle. Plant seeds contain an embryo and storage components kept in a dried state and protected with a firm seed coat. Seeds isolated at archeological sites have

been reported to germinate after over one-thousand years of dormancy. Survival structures are also of great prominence in the fungal kingdom. The majority of fungal species form single- or multi-celled survival structures that play an important role in the worldwide distribution of fungi. The size of these structures is orders of magnitudes smaller than that of plant seeds. Fungi of the genera *Aspergillus*, *Cladosporium* and *Penicillium* are so well equipped in forming spores that they can be found in virtually every cubic meter of air. Other fungal species form the most stress-resistant eukaryotic cells described to date. The types of fungal spores, the way these cells protect themselves against stress, and how spores break their dormancy to become a vegetative cell are the topics of this Chapter.

Fungal survival structures for distribution in space and time

As non-mobile organisms, fungi have to deal with adverse conditions as depletion of nutrients and changes in abiotic conditions. Fungi accumulate protective compatible solutes in their mycelium to overcome stress and survive these unfavorable conditions. Further, small condensed micro-colonies that exhibit slow growth are able to survive stressful conditions (Staley, Palmer *et al.* 1982; Gorbushina 2003). A fungal mycelium can also differentiate by forming specialized survival structures known as a sclerotium. This is a firm, often rounded mass of hyphae with thickened cell walls (van den Boogert, Reinartz *et al.* 1989) and a strongly reduced metabolic activity. Some of the fungi that form sclerotia are notoriously widespread plant pathogens such as the basidiomycete *Rhizoctonia solani* and the ascomycetes *Sclerotinia sclerotiorum* and *Sclerotinia minor*. Under favorable conditions (moderate temperature and humidity), sclerotia can survive up to a few years in soil. Therefore, a 4-years crop rotation is used to diminish spoilage caused by *S. minor* (Adams and Ayers 1979). Sclerotia of *S. minor* tolerate some heat, especially in a dry environment. Germination is no longer observed after 1 week at 40 °C in humid soil, but still 28 % of the sclerotia germinate after 4 weeks at 40 °C in dry soil (Matheron and Porchas 2005). Despite the impact of sclerotia, the most common strategy for fungal survival is the formation of spores. Many different types of fungal spores can be distinguished, which are formed either asexually (conidia) or sexually (Fig. 1). The mechanisms underlying their formation and dispersion are also diverse (Fig. 2; van Leeuwen, van Doorn *et al.* 2010; McCartney and West 2007). For instance, the conidia forming conidiophores of fungi such as *Penicillium* and *Aspergillus* extend up to 100 µm into the air. This is suggested to be high enough to lift the chains of spores above the laminar airflow close to a surface into more turbulent air movements that may facilitate release of the conidia from the spore chains. Active ejection of spores is another strategy for spore release. This strategy is used to release sexual spores of many ascomycetes. The asci and ascospores of *Gibberella zeae* are forcibly ejected from the flask-like fruiting bodies (perithecia), in which high osmopressure leads to the highest accelerations of cells observed in a biological system (Trail *et al.* 2002; 2005). The launch speed is 34.5 m s^{-1} and results in an acceleration of 870.000 g.

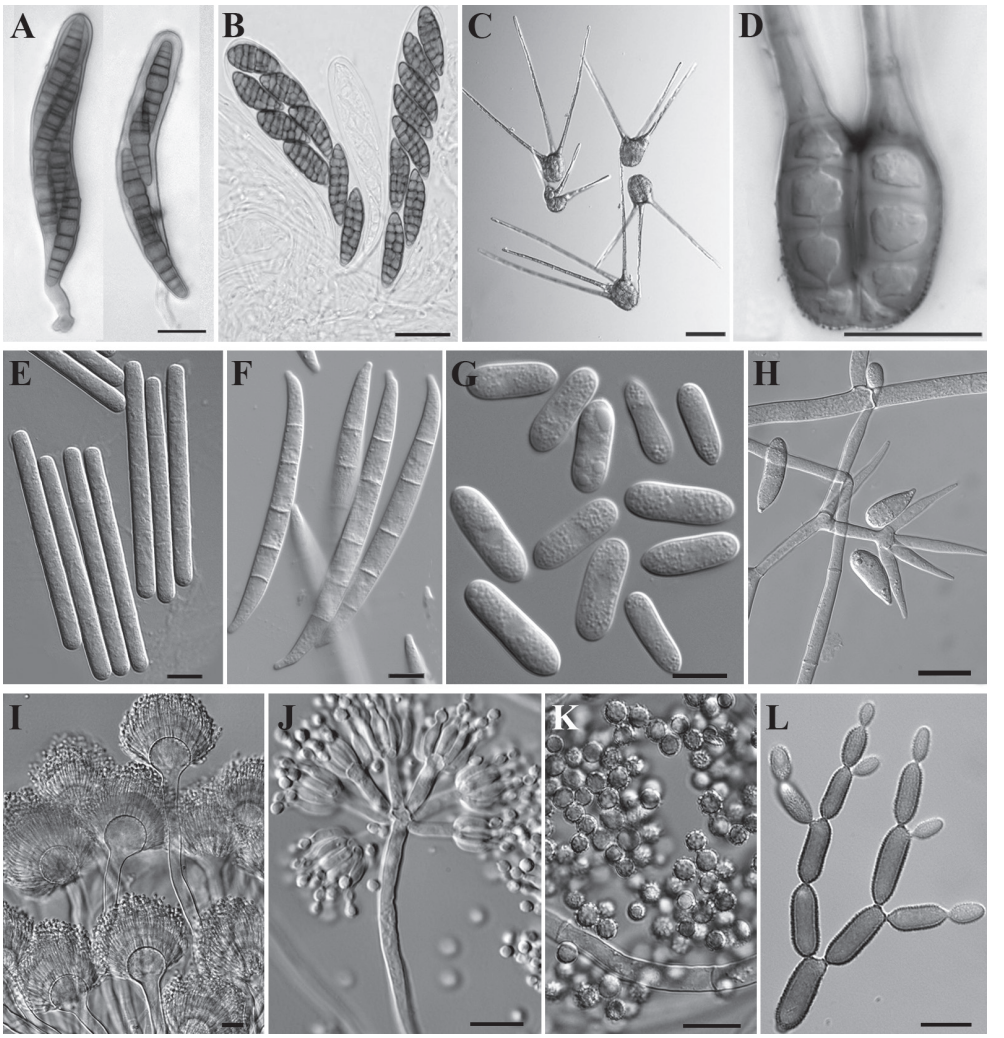

Figure 1. Variability of spore morphology and formation. Ascospores of *Oedohysterium sinense* and *Murispora rubicunda* (A, B), appendaged conidia of *Tetraplosphearia nagasakiensis* and *Tetraploa spp* (C, D), hyaline (macro)conidia of *Calonectria eucalypti*, *Cyanectria buxi*, *Colletotrichum rhombiforme* and *Cladobotryum paravirescens* (E-H), pigmented conidia and conidiophores of *Aspergillus pseudoterreus*, *Penicillium godlewski*, *Aspergillus pseudocaelatus* and *Cladosporium allii* (I-L). Bars represent 10 (E-G, I-L), 20 (A, B, D, H) and 50 (c) µm. Taken from Studies in Mycology, with courtesy. Fig. 1A is taken from Boehm *et al.*, 2009, Fig. 1B from Zhang *et al.*, 2009, Fig. 1C,D from Tanaka *et al.*, 2009, Fig. 1E from Lombard *et al.*, 2010, Fig. 1F from Schroers *et al.*, 2011, Fig. 1G from Damm *et al.*, 2012, Fig. 1H from Pöldmaa, 2011, Fig. 1I from Samson *et al.*, 2011, Fig. 1J from Houbraken *et al.*, 2011, Fig. 1K from Varga *et al.*, 2011, Fig. 1L from Bensch *et al.*, 2012.

Ascospores of *S. sclerotiorum* are released from asci on a cup-shaped fruiting body (apothecium). The synchronized release of many spores at the same time creates a flow of air that takes the spores much higher into the air than in case of the release of individual spores (Roper, Seminara *et al.* 2010). The mechanical stresses occurring during ascospore release cause co-ejection of the neighboring ascospores and can be regarded as a self-organizing process. This process might result in a 20-fold increase of range of cooperative spores compared to an individual release. It is clear that a number of ascospores sacrifice themselves in order to generate the draft that will disperse other spores over a larger distance. The authors even model and reflect on the effect of "cheating" where spores develop strategies of delaying release in order to belong to the lucky part of the spores that will be dispersed over a large distance.

Conidia of for instance *Verticillium fungicola* and *Fusarium oxysporum* are formed in dense clusters ("slimy heads") or between hyphae (Fig. 2). Such spores are less exposed to air flow. These spores are hyaline and are effectively distributed

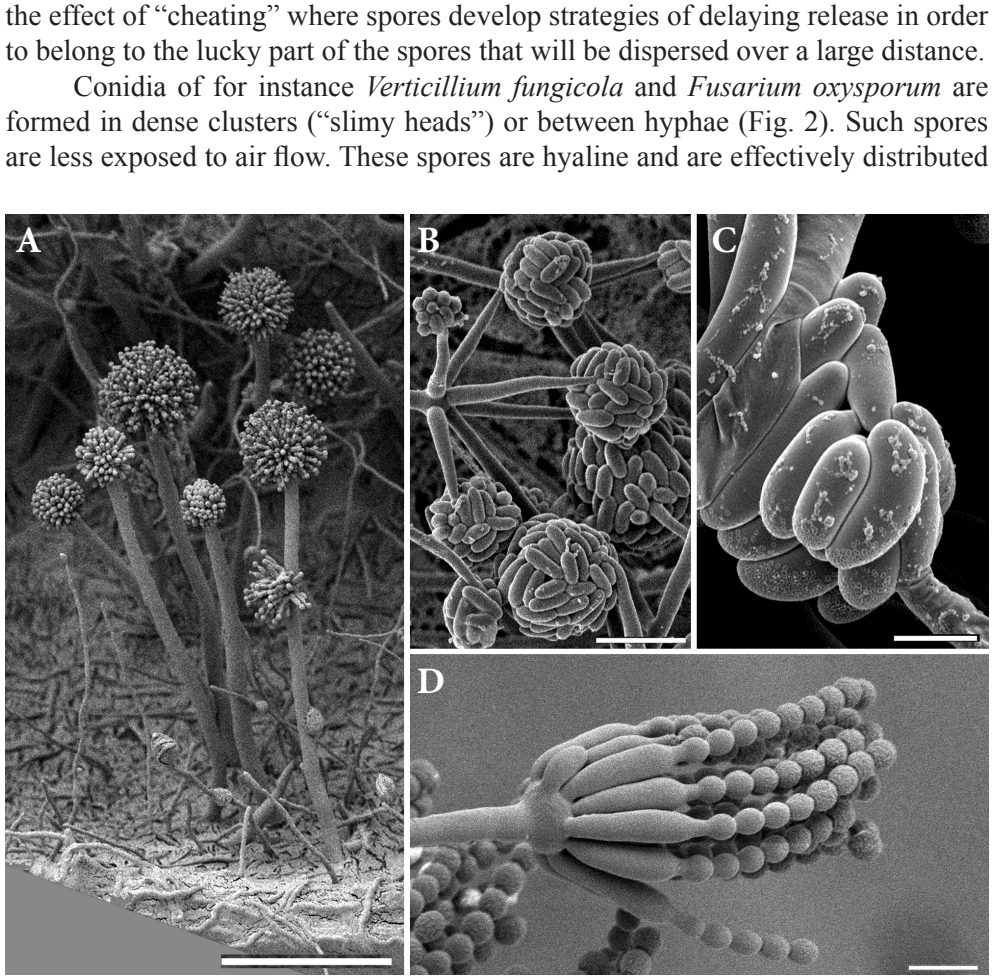

Figure 2. Formation of conidia by *Aspergillus niger* (A), *Verticillium fungicola* (B), *Fusarium oxysporum* (C) and *Penicillium vanoranjei* (D ; Visagie *et al.* 2013) as observed by cryoSEM. Numerous conidia are formed on erect conidiophores of *A. niger* and *P. vanoranjei*. Conidia of *F. oxysporum* and *V. fungicola* are formed in compact clusters that coalesce to form large aggregates of spores inside the mycelium. The bars represents 100 (A), 10 (B) and 5 (C, D) µm.

by water splashes and aerosols. Aero-aquatic fungi such as *Helicoon richonis* and *Helicodendron tubulosum* form conidia that are specialized to float on water (Michaelides and Kendrick 1982). These fungi grow on decaying leaves in sweet water ponds and are dispersed via the water surface. The conidia are very large (50-100 µm) and have shapes that resemble for instance electric filaments, barrels or beehives (Fig. 3). The capture of pockets of air during spore formation is thought to prevent sinking and settling of these "bubble trap propagules".

Fungal spores are not only distributed by wind or water, but also by other organisms. Earthworms, for example, disperse fungi by mixing soil layers. Fungal spores may also adhere to the surface of an insect, often mediated by hydrophobic interactions. Many fungi produce volatiles that can attract insects and thus increase the chance to adhere to their vector. For instance, 1-octen-3-ol is a known attractant of mosquito species (Cilek, Ikediobi *et al.* 2011). The sweet smell of *P. chrysogenum* and *Penicillium rubens* colonies may also attract insects. Certain plant diseases such as the Dutch Elm disease are distributed by bark beetles. The elm pathogen *Ophiostoma novo-ulmi* causes a change in the release of volatiles from the plant tissue during disease, which would attract the bark beetle *Hylurgopinus rufipes* (McLeod, Gries *et al.* 2005).

A very special way of distribution is observed in obligate endoparasitic fungi of nematodes. The menhir-shaped conidia of *Drechmeria coniospora* form a fibrillar layer on the tip that adheres strongly to sensory organs on the head of nematodes (Fig. 4). The conidia are adhering so strongly that numerous movements of the nematode do not shed the spores. These spores form an appressorium and subsequently penetrate

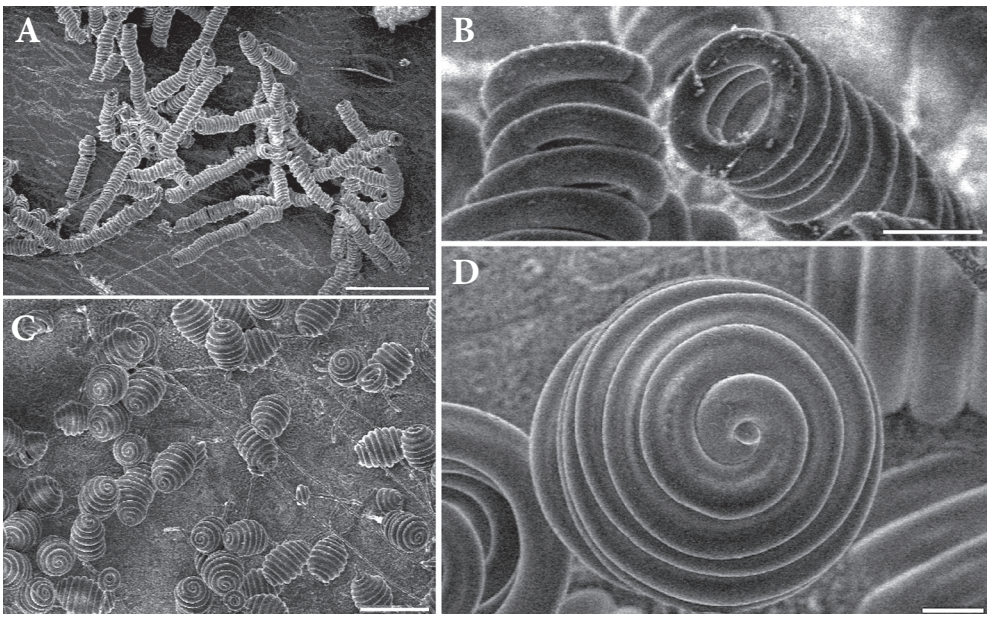

Figure 3. Morphology of conidia of the aero-aquatic fungi *Helicodendron tubulosum* (A,B) and *Helicoon richonis* (C,D). Bar represents 10 (A,C) and 50 (B,D) µm.

the nematode cuticle (Dijksterhuis *et al.* 1990; 1993; van de Boogert, Dijksterhuis *et al.*1992). During the process of infection the nematodes move freely for a period of approximately 30 h before they become moribund (Dijksterhuis,Veenhuis *et al.* 1991) and thus are a vector for distribution of the fungus. Subsequently, new conidiophores emerge through the cuticle of the nematode that form conidia that are dispersed by wind (Fig. 4B).

Not all fungal spores are dispersed to new substrates. Some spores are waiting for better growth conditions in a dormant state. *Talaromyces macrosporus* forms highly stress-resistant, dormant ascospores that are formed in closed fruiting bodies (cleistothecia) within the mycelium of the fungus. These spores are not readily released into the air. Ascospores of *T. macrosporus* survive at least 17 years when kept at room temperature in the dark (Nagtzaam and Bollen 1994).

The types of asexual and sexual spores and their stress resistance is the subject of the next two sections.

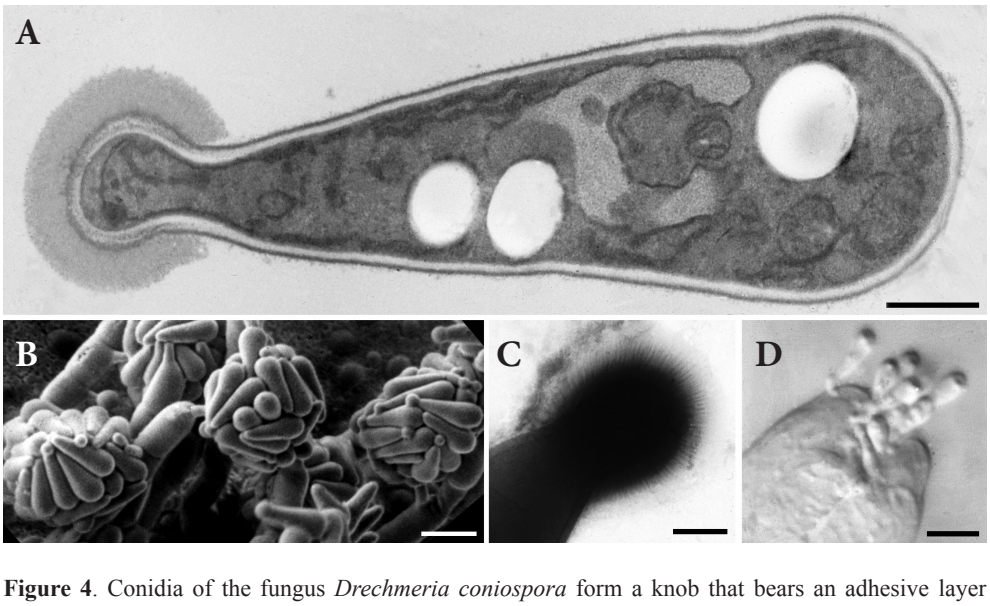

Figure 4. Conidia of the fungus *Drechmeria coniospora* form a knob that bears an adhesive layer that firmly attaches the spore to the cuticle of nematodes (A). The conidia are formed in clusters on conidiophores (B). The adhesive knob is fibrillar in nature (C) and attaches to sensory organs of the nematode *Panagrellus redivivus* (D). An outgrowth of a conidium has penetrated the cuticle (arrow). Bar represents 0.5 (A,C) or 5 (B,D) µm. Taken from Dijksterhuis *et al.* 1990 and Dijksterhuis *et al.* 1992.

Formation of spores

Asexual spores

Asexual spores are formed after mitosis (mitospores) without the involvement of meiosis. Fungi produce an enormous variety of asexual spores. Some species can produce several types of such spores. As an example, *Fusarium oxysporum* forms banana shaped macroconidia, smaller microconidia and thick-walled chlamydospores (Iida *et al.*,2008). The ratio between the formation of macro- and micro-conidia depends on environmental conditions and depends on the species (see Springer 1993). Other factors can also play a role. For instance, a reduced stability of the cell wall promotes formation of macroconidia in *F. verticilloides* (Li, Myung *et al.* 2006).

In the past, fungi were mainly classified by means of morphological and physiological parameters. One of the most important criteria was the formation of spores and their morphology. Sporangiospores are formed endogenously in a sporangium via cytoplasmic cleavage in the zygomycetes. Motile, flagellated spores of the chytridiomycetes are called zoospores and the term conidium is used for an asexual, non-motile (cf. zoospore) spore, usually caducous (easily dislodged) and not developed by cytoplasmic cleavage (cf. sporangiospore) or free-cell formation (cf. ascospore) (Kirk, Cannon *et al.* 2008). In the large majority of the cases the description conidia is used for fungi belonging to the phylum Ascomycota. The terminology associated with the morphology of conidia is elaborate and includes the spore shape, the presence of septa and ornamentation. If a species produces two types of conidia with a clearly different size, then the terms microconidia and macroconidia are used to distinguish these spores.

In general, two different modes of conidium formation (conidiogenesis) are distinguished, namely thallic- and blastic- conidiogenesis (Fig. 5, de Hoog, Guarro *et al.* 2000; Cole and Samson 1979). In thallic conidiogenesis a pre-existing cell differentiates into a conidium. If the whole cell is converted into a conidium this is called holothallic. Holothallic derived conidia usually have thick, melanised and often encapsulated cell walls. These conidia are in general referred to as chlamydospores. The chlamydospores of *G. zeae* are thick-walled cells formed between hyphal compartments that can persist up to 16 months in soil (Nyvall 1970; Son, Lee *et al.* 2012). Thallic conidiogenesis can also result in fragmentation of a cell into conidia, the so-called thallic-arthric mode that gives rise to arthrospores. In blastic conidiogenesis conidia differentiate via expansion from the conidiogenous cell. The cell wall of this cell is locally weakened and the developing conidium bulges out and is delineated by a septum. This is similar to the process of budding in yeasts. The general name for spores formed this way is blastospores. When expansion of a cell includes the complete cell wall of the conidiogenous cell, this is known as holoblastic conidium formation. In the case of enteroblastic conidium formation the cell wall of the conidiogenous cell is disrupted and the conidium appears through an opening in the cell wall. The formation of the conidial chain in the genus *Aspergillus* is an example of entero-blastic conidiogenesis.

Conidia can be formed at the base of a chain (as in *Penicillium*, Fig. 2D), which is called the basipetal mode of spore formation. Acropetal formation describes a chain that bears the youngest spores at the apex (as is the case with *Cladosporium*, Fig. 1L). Here, conidia are instrumental for the formation of spores, while in case of *Penicillium* maturation can start at the moment the spores are delimited from the conidiogenous cell.

Finally, asexual spores can be formed within structures that are called conidioma. These structures resemble fruiting bodies that form sexual spores. Conidiome formation is widespread among fungal genera, but was initially assigned to one group, the Coelomycetes containing species such as *Phoma* and *Colletotrichum*. There are different types of conidiomas including the pycnidium that encloses a layer of conidia-forming cells and that has an opening (ostiole) for release of the spores. The acervulus is a flat layer of fungal hyphae that forms conidia and often is immersed in the tissue of the plant host.

Sexual spores

Sexual development increases genetic diversity and promotes survival in a changing environment (Hoekstra 2005). Sexual development occurs widely through the fungal kingdom and the resulting spores have many different names. Zygospores are the sexual spores produced by Zygomycota in zygosporangia. The diploid motile zoospores are the result of the complex sexual reproduction of Chytridiomycota, while the basidiospores of Basidyomycota and the ascospores of Ascomycota are produced on basidia and within asci, respectively.

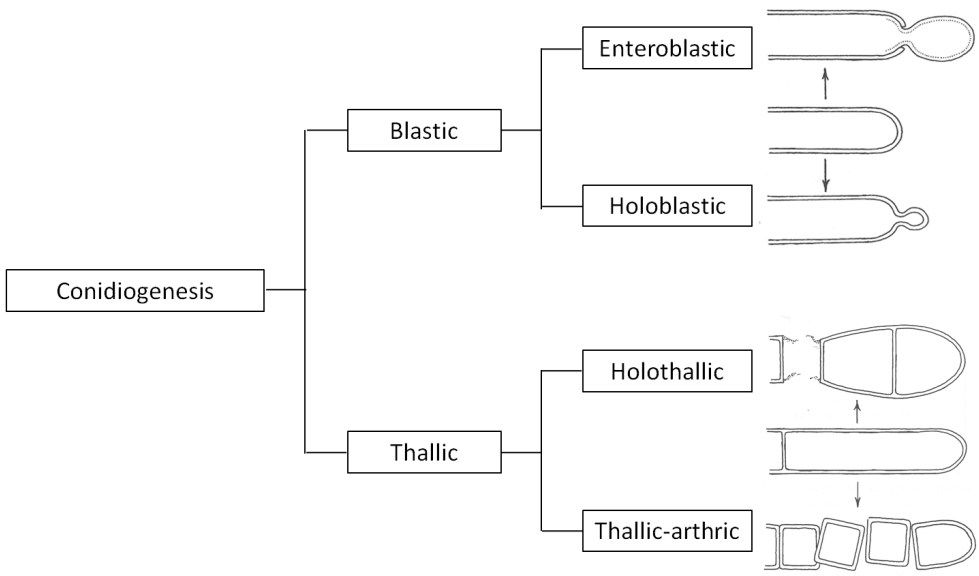

Figure 5. Different modes of conidia formation (Based on de Hoog *et al.* 2000, with courtesy).

The fruiting bodies of ascomycetes are called ascomata. The formation of ascospores, and the morphology of the ascospores, asci and the ascomata were traditionally very important for fungal classification. The ascomata are classified as cleistothecia, perithecia, apothecia and pseudothecia. Most of the extreme stress-resistant ascospores are formed within an enclosed fruiting body called cleistothecium. Cleistothecia of *Aspergillus nidulans* (*Emericella nidulans*) and *Neosartorya fischeri* are deliminated by means of a peridium, which surrounds the ascogenous system (Son and Yoon 2002). We found that the peridium of relatively young ascomata of *N. fischeri* were impermeable for chemical fixatives and consisted of hyphae that were connected to each other with an extracellular matrix (**Chapter 4**). The ascogenous cells within the peridium give rise to the ascus mother cells containing 8 ascospores after meiosis and one round of mitoses. In *A. nidulans*, the peridium is a prominent structure during very early stages of formation of the ascomata, but several layers lyse to make place for asci and ascospores (Son and Yoon 2002). There is a marked activity of ROS in the peridium at early stages of ascomata formation indicating that controlled cell death of part of the peridium is responsible for this process (Lara-Ortiz, Riveros-Rosas *et al.* 2003)

Some fungi have only a known asexual state. This has led to a dual nomenclature system in fungal systematics when at a later date the sexual state was identified (the dual life cycle is illustrated in Fig. 6). For instance, the teleomorphic genera

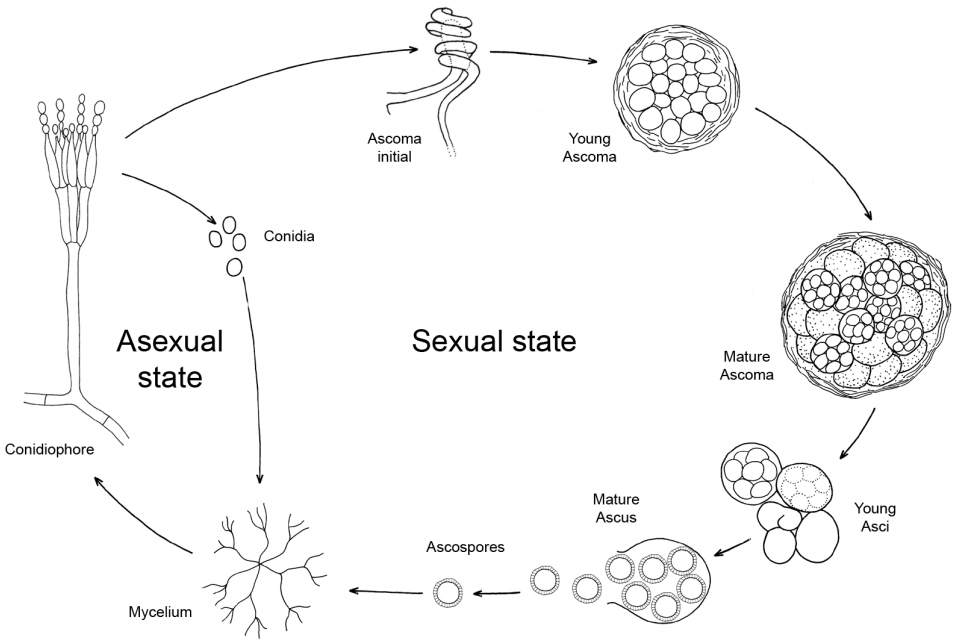

Figure 6. Life cycle of fungi belonging to the family *Trichocomaceae*, to which *Talaromyces*, *Byssochlamys* and *Neosartorya* belong. In these fungi, both the teleomorphic and anamorphic state are observed and, thus, both ascospores and conidia are produced. (Edited from Samson *et al.*, 2010 CBS laboratory manual series 2).

(with names based on the sexual structures) *Eupenicillium* and *Talaromyces* have a *Penicillium*-like anamorphic state (with names based on the asexual structures). The genera *Emericella*, *Neosartorya* and *Petromyces* have an *Aspergillus* anamorph and *Byssochlamys* a *Paecilomyces* anamorph. *Eupenicillium* and *Talaromyces* are genetically distinct and recently the relationship between the genera *Aspergillus*, *Penicillium*, *Byssochlamus* and *Talaromyces* was resolved by application of new nomenclature rules (Samson, Yilmaz *et al.* 2011; Houbraken and Samson 2011).

Stress resistance of spores

Conidia

The stress-resistance of conidia is in general higher than that of vegetative fungal cells. Waterborne conidia exhibit a number of cellular properties that are more similar to fungal vegetative cells than to dormant airborne conidia. For instance, airborne conidia show a low staining of ergosterol in the plasma membrane when compared to waterborne spores and have a higher cytoplasmic viscosity (van Leeuwen, van Doorn *et al.* 2010). Moreover, waterborne conidia are non-pigmented and show higher sensitivity for antibiotics than airborne conidia.

A number of examples of resistance of hydrated conidia to heat or oxidative stress are summarized in Table 1. Temperature inactivation of waterborne conidia of *Botrytis cinerea* (hyaline) and *Stagonospora nodorum* (from pycnidia, septated, hyaline) occurs within minutes at temperatures above 45 °C (Doehlemann, Berndt *et al.* 2006; Solomon, Tan *et al.* 2005). The median lethal temperature (LT_{50}) of *S. nodorum* conidia at 40 °C is 25 min (Solomon, Tan *et al.* 2005). *B. cinerea* conidia showed similar sensitivity with a LT_{50} of 8 min at 45 °C (Doehlemann, Berndt *et al.* 2006). The conidia of the insect pathogenic fungi *Beauveria bassiana* and *Paecilomyces fumosoroseus* are more resistant to heat (Wang, Lu *et al.* 2012; Ying and Feng 2004). More than 50 % of *B. bassiana* conidia survive 50 min at 45 °C and up to 2 h at 48 °C, while *P. fumosoroseus* conidia survive up to 15 min at 48 °C (Ying and Feng, 2004). More heat resistant are conidia of *Aspergillus* (*A. nidulans*, *A. niger* and *A. oryzae*). They survive 50 °C for minutes to even hours (Ruijter, Bax *et al.* 2003; Fillinger, Chaveroche *et al.* 2001; Sakamoto, Iwashita *et al.* 2009). The LT_{50} of conidia of *A. nidulans* and *A. oryzea* at 50 °C is 10 and 42 min, respectively (Fillinger, Chaveroche *et al.* 2001; Sakamoto *et al.* 2008; 2009), while 100 % of the conidia of *A. niger* still germinate after 2 h at this temperature (Ruijter, Bax *et al.* 2003). Baggerman and Samson (1988) reported a D_{59} of 3.3 minutes for conidia of *A. niger*. This means that 10 % of the conidia still survive a period of 3.3 minutes of heating at 59 °C. The resistance towards oxidative stress also shows variation among fungal species. 50 % of *A. nidulans* conidia survive 100 mM H_2O_2, while 60 % of *A. oryzae* conidia survive 30 min at 400 mM of this oxidative agent. *A. niger* conidia are not inactivated by a 1 h incubation in 1 mM NaOCl, while *S. nodorum* is highly sensitive to this condition. No survivors were measured after a 15 min exposure to 1 mM NaOCl (Solomon, Tan *et al.* 2005).

Table 1. Stress resistance of fungal conidia.

Species	Stress	Time	Survival	D-value, LT_{50}	Reference
Aspergillus nidulans	50 °C	>30 min	0 %	LT_{50} 10 min	Fillinger, 2001
	100 mM H_2O_2	>60 min	0 %	LT_{50} 30 min	Fillinger, 2001
Aspergillus niger	50 °C	2 h	100 %	LT_{50} >2 h	Ruijter, 2003
	Freeze thaw	1 x	76 %		Ruijter, 2003
	Lyophilization		64 %		Ruijter, 2003
	1 mM NaOCl	1 h	100 %		Ruijter, 2003
Aspergillus oryzae	50 °C	1 h	30 %	LT_{50} 42 min	Sakamoto, 2008
	400 mM H_2O_2	30 min	60 %		Sakamoto, 2008
	UV 254 nm	0.03 J cm^{-2}	50 %	LD_{50} 0.03 J cm^{-2}	Sakamoto, 2008
Beauveria bassiana	45 °C	50 min	>50 %	LT_{50} 51 min	Wang, 2012
	0-10 mM H_2O_2	24 h		LC_{50} 5.3 mM	Wang, 2012
	UV-B 312 nm	0.42 J cm^{-2}	50 %	LD_{50} 0.42 J cm^{-2}	Wang, 2012
	48 °C	>25-120 min	0 %	LT_{50} 10-60 min	Ying, 2004
Botrytis cinerea	45 °C	15 min	0 %		Doehlemann, 2006
	45 °C	10 min	20 %	LT_{50} 8 min	Doehlemann, 2006
Paecilomyces fumosoroseus	48 °C	>6-15 min	0 %	LT_{50} 3-6 min	Ying, 2004
Stagonospora nodorum	50 °C	15 min	0 %		Solomon, 2005
	40 °C	15 min	65 %	LT_{50} 25 min	Solomon, 2005
	40 °C	>60 min	0 %		Solomon, 2005
	1 mM NaOCl	15 min	0 %		Solomon, 2005

Airborne dormant conidia of *Aspergillus* and most probably also of *Penicillium* and *Paecylomyces* are moderate stress-resistant cells that survive drought, relatively high temperatures (1 h at 50 °C; several min at 60 °C (Ruijter, Bax *et al.*, 2003) and UV-radiation due to a melanized outer cell wall (Tiedt 1993; Jørgensen, Park *et al.* 2010). The dormancy of conidia of *A. nidulans* ensures that these cells survive 6 weeks in liquid (Fillinger, Chaveroche *et al.* 2001), but much longer in a dried state or when cooled.

Hydrated conidia of *Penicillium* chrysogenum, *P. italicum* or *P. digitatum* are inactivated (a log 2.5 to log 6 decrease) after a 4-days treatment at 30 °C in either 5 % ethanol vapor or 10 % ethanol solution (Dantigny, Tchobanov *et al.* 2005; Dao, Bensoussan *et al.* 2008). *P. chrysogenum* showed marked less inactivation compared to the other species. This makes sense as this species is more stress-resistant enabling them to grow at lower water activities. It is not clear whether conidia are still in their

dormant state after such a long period of treatment or have started some stage of germination. Dry-harvested conidia of all three species are more resistant for ethanol vapours than hydrated conidia (Dao and Dantigny 2009). Yet, the dry-harvested spores could be inactivated at higher vapour pressures (10 %; Dao, Dejardin *et al.* 2010). Mathematical models for the inactivation of fungal spores are reviewed (Dijksterhuis, Rodriquez de Massaguer *et al.* 2012) and can be used for different sporocidal conditions including heat, drying or vapour treatments (Dao and Dantigny 2009; Dao, Dejardin *et al.* 2010).

Ascospores

In general, ascospores survive heat, freezing, UV-radiation, high pressure, drought and oxidative stress better than conidia. Ascospores from the yeasts *Saccharomyces cerevisiae, Zygosaccharomyces bailii* and *Zygosaccharomyces chevalieri* that had been isolated from soft drinks and fruit products show a heat resistance at 60 °C that is 25-350 times higher than those of the corresponding vegetative cells (Put and de Jong 1980). Ascospores of species that are even more stress-resistant are mostly described within the family Trichocomaceae and show a large variation in morphology (Fig. 7). Heat resistance above 70 °C occurs in all main genera within this family, i.e. in *Eupenicillium*, *Neosartorya*, *Eurotium, Hamigera, Xeromyces, Byssochlamys, Thermoascus* and *Talaromyces* (Dijksterhuis 2007) and also in genera outside the Trichocomaceae namely *Neurospora* and *Leohumicola* (Lingappa and Sussman 1959;

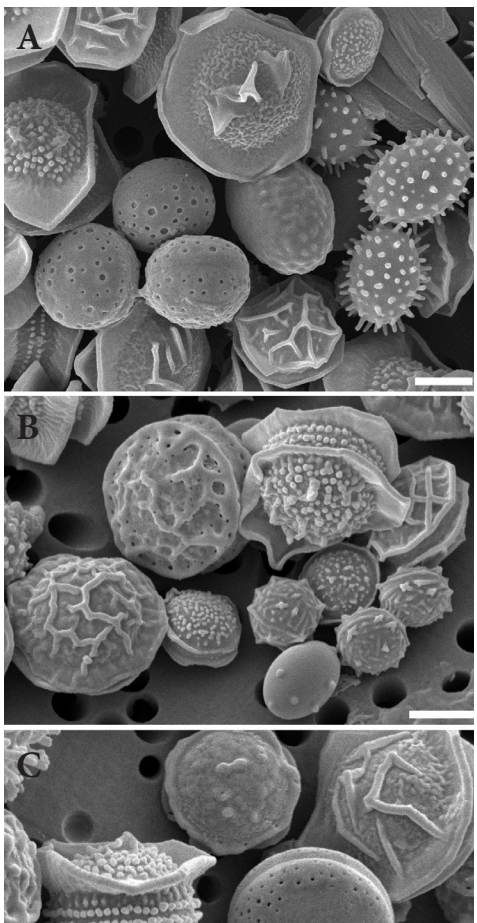

Figure 7. Cryo SEM micrographs illustrating the variability in morphology in a mixture of ascospores of 25 fungal species belonging to the genera *Neosartorya*, *Eurotium*, *Talaromyces* and *Thermoascus*. Bars represent 2 µm.

Hambleton, Nickerson *et al.* 2005). Such spores are characterized by a thick cell wall (≥ 0.5 µm) and high accumulation of polyols and / or sugars. For instance, ascospores of the fungus *T. macrosporus* contain 9-17 % wet weight of the disaccharide trehalose, have a water content of about 38 % (0.6 g water g^{-1} dry weight), and contain a protein : trehalose ratio of 1 : 3 (Dijksterhuis, van Driel *et al.* 2002).

T. macrosporus, Neosartorya spinosa and *Byssochlamys spectabilis* form ascospores that survive more than one hour at 85 °C and also ultra-high pressure treatments at 600 MPa (Butz, Funtenberger *et al.* 1996; Palou, Lopez-Malo *et al.* 1998; Reyns, Verbeke *et al.* 2003; Dijksterhuis and Teunissen 2004; Beuchat 1986; Splittstoesser and Splittstoesser 1977; Hecker and Sussman 1973; Dijksterhuis, Nijsse *et al.* 2007; Houbraken, Varga *et al.* 2008; Wyatt and Van Leeuwen unpublished results). These ascospores can be regarded as the most stress-resistant eukaryotic cells described to date. The D_{85} of ascospores of *N. fischeri* and *T. macrosporus* in aqueous solution varies from 10-100 min. This is in the same range as some bacterial spores such as those of *Bacillus subtilis* and explains why *Byssochlamys*, *Neosartorya* and *Talaromyces* still cause spoilage after pasteurization of food (Tournas 1994).

The heat resistance of ascospores increases with the sugar concentration of the surrounding medium (Splittstoesser and Splittstoesser 1977; Beuchat 1988a; King and Whitehand 1990). In addition, the pH and the presence of organic acids in the medium affect the survival after heat treatment. For instance, benzoic and sorbic acid have negative effects on heat resistance of *Talaromyces flavus* and *N. fischeri* (Beuchat 1988b; Rajashekhara, Suresh *et al.* 1998). A combination of different parameters may lead to an unpredictable heat resistance (Beuchat and Kuhn 1997). For instance, *Byssochlamys nivea, Byssochlamys fulva* and *N. fischeri* are approximately twice as heat resistant in tomato juice (pH of 4.2) when compared to phosphate buffer (pH 7.0; Kotzekidou 1997). The process of cultivation of fungi, for instance the age of the culture (Conner and Beuchat 1987ab; Beuchat 1988a), growth temperature (King and Whitehand 1990; Conner and Beuchat 1987b), and the composition of the growth medium (Beuchat 1988ab; Rajashekhara, Suresh *et al.* 1998; Splittstoesser and Splittstoesser 1977; King and Whitehand 1990; Conner and Beuchat 1987a) also affect the degree of heat resistance of ascospores. For instance, ascospores of *T. macrosporus* harvested from oatmeal-grown cultures are more heat resistant than ascospores from cultures grown on malt extract agar (Beuchat 1988a).

Heat resistance of *N. fischeri* increases with the age of the growing culture. Ascospores harvested from 11-day-old cultures are less heat resistant than those of 25-day-old cultures (D_{82} of ± 23 and > 60 min, respectively) (Conner, Beuchat *et al.* 1987). This difference correlates with the accumulation of mannitol and trehalose. Mannitol and trehalose cannot be detected in ascospores of 11-day-old cultures but they make up 2.8 % and 0.6 % of the dry weight of ascospores of 25-day-old cultures, respectively. Similarly, heat resistance of ascospores of *T. macrosporus* increases from 33 min for spores from 20-day-old cultures to 100 min for spores from cultures that were 67 days old (Dijksterhuis and Teunissen 2004). This increase also takes place during storage of harvested spores in a buffer solution at 30 °C but

is not observed at 10 °C. These data suggest that maturation of ascospores does not depend on the tissue of the ascomata surrounding the ascospores, but does require metabolic or physical processes.

Compounds protecting cell constituents

Compatible solutes

Fungi protect themselves against heat, drought and other stresses by accumulating compatible solutes. These solutes do not interfere with cell functioning even at high concentrations. Well-known compatible solutes are sugars, sugar alcohols (polyols), betaine and amino acids. Sugars and polyols accumulate to the highest levels in fungi. Glycerol is the main compatible solute that accumulates in the mycelium of fungi upon osmotic stress, illustrated by accumulation of this polyol when *A. nidulans* is exposed to salt stress (Hagiwara, Asano *et al.* 2007; Redkar, Locy *et al.* 1995; Kogej, Stein *et al.* 2007). *A. nidulans* strains impaired in glycerol synthesis show reduced growth under salt stress (de Vries, Flitter *et al.* 2003). Fungi accumulate also other polyols. Accumulation of glycerol, arabitol and erythritol was observed under salt stress in *A. oryzae* (Ruijter, Visser *et al.* 2004), while *A. niger* and *P. chrysogenum* accumulate the polyols glycerol and erythritol under this condition (Adler, Pedersen *et al.* 1982). Mannitol has also been shown to accumulate under salt stress but also upon exposure to heat and oxidative stress (Chaturvedi, Bartiss *et al.* 1997; Ruijter, Bax *et al.* 2003; Sakamoto, Iwashita *et al.* 2009; Voegele, Hahn *et al.* 2005; Managbanag and Torzilli 2002). Besides being an osmotic solute, mannitol is also a potent reactive oxygen species (ROS) scavenger (Shen, Jensen *et al.* 1997; Smirnoff and Cumbes 1989). Phytopathogenic fungi may use mannitol to suppress (ROS)-mediated plant defense strategies as it accumulates in the plant pathogens *Uromyces fabae* and *Alternaria alternata* during infection or contact with the host (Voegele, Hahn *et al.* 2005; Jennings, Ehrenshaft *et al.* 1998). Compatible solutes can also accumulate highly in spores. For example, mannitol and trehalose can make up 2.8 % and 0.6 % of the dry weight of ascospores of *N. fischeri* (Connor, Beuchat *et al.* 1987), while conidia of the insect pathogen *Metarrhizium anisopliae* contain 9-13 % dry weight of mannitol (Hallsworth and Magan 1996).

Trehalose is the most dominant solute sugar that accumulates in fungi upon stress. For instance, an increase of trehalose is seen after heat stress in the case of *Aspergillus pullulans* (Managbanag and Torzilli 2002) and *A. fumigatus* (Al-Bader, Vanier *et al.* 2010). Similarly, trehalose is involved in resistance against heat and oxidative stress in *N. crassa* (Bonini, Neves *et al.* 1995) and *A. nidulans* (Fillinger, Chaveroche *et al.* 2001). A relation between trehalose and drought- and cryo-tolerance is also observed in yeasts (Gadd, Chalmers *et al.* 1987; Gélinas, Fiset *et al.* 1989). Absence of either mannitol or trehalose reduces heat resistance and / or longevity of conidia of *A. niger* and *A. nidulans* (Fillinger, Chaveroche *et al.* 2001; Ruijter, Bax *et al.* 2003) as well as yeast cells (Wiemken 1990; DeVirgilio, Hottiger *et al.*1994). It thus seems that both mannitol and trehalose are needed for optimal

stress resistance of spores of several fungal species. Indeed, conidia of *A. oryzae*, *A. nidulans*, *A. niger* and *P. rubens* all contain mixtures of these compounds (Horikoshi and Ikeda 1966; D'Enfert and Fontaine 1997; van Leeuwen, Krijgsheld *et al.* 2013b; Bekker, Adan *et al.* 2012). *A. nidulans* and *A. oryzae* conidia contain 0.7-1.4 pg trehalose and 0.5-0.8 pg mannitol per spore (D'Enfert and Fontaine 1997; D'Enfert, Bonini *et al.* 1999; Sakamoto, Iwashita *et al.* 2009), which represent typically 4-6 % wet weight. Mannitol is the most abundant compatible solute in conidia of *A. niger*. This polyol makes up 10.9 % of the dry weight of the spore, while trehalose only represents 3.6 % (Ruijter, Bax *et al.* 2003). On the other hand, trehalose represents 24-32 % of the dry weight of ascospores of *T. macrosporus*. These spores contain much lower quantities of mannitol (Dijksterhuis, van Driel *et al.* 2002). The high trehalose content together with the low water content of the spores (38 %) introduces a very high viscosity inside the spores.

The composition of the compatible solutes inside spores depends on the environmental conditions during their formation. For instance, conidia of *A. nidulans* from cultures grown at potato dextrose agar with excess of glycerol or KCl have similar mannitol levels (4.4-4.6 % dry weight), but conidia from glycerol-containing medium also contain 6.3 and 2.7 % glycerol and erythritol (Hallsworth, Prior *et al.* 2003). Magan (2001; 2006) suggested that the composition of the spore impacts their ecological competence. Hallsworth and Magan (1994ab; 1996) studied this hypothesis with spores of insect pathogenic fungi that are used for biological control of insect pests. Conidia of *M. anisopliae* originating from dead insects are more virulent than those from rich artificial media. Quantities of sugars and polyols are different in such conidia (Magan 2001). Erythritol, mannitol and glucose accumulate in conidia from killed insects, while mannitol, glucose and trehalose are found in conidia formed on defined artificial media. Composition dependent ecological competence also exists in the case of conidia of *A. flavus* and *P. chrysogenum*. Spores of *P. chrysogenum* that are formed at a lower water activity (αw 0.95 compared to 0.99) have a shorter germination time (Nanguy, Perrier-Cornet *et al.* 2010; Judet, Bensoussan *et al.* 2008). In addition, conidia produced at a water activity of 0.90 are more resistant to ethanol vapor compared to those formed at a αw of 0.99 (Dao and Dantigny 2009). However, conidia of *P. chrysogenum* produced at 0.85 aw did not showed a further increase of resistance to ethanol vapor.

Mannitol and trehalose metabolism in fungi

In this section the metabolism of two important compatible solutes, mannitol and trehalose, is discussed. The biosynthesis of these molecules links to the glycolytic pathway and both synthesis and degradation can occur quickly.

Mannitol metabolism in fungi occurs via two different pathways. These pathways have been proposed to act in a cycle generating NADPH from NADH at the expense of ATP (Hult and Gatenbeck 1978). Actual proof for the cycle is lacking and its existence is still under debate (Singh, Scrutton *et al.* 1988; Ruijter, Bax *et al.*

2003; Solomon, Waters *et al.* 2007; Aguilar-Osorio, Vankuyk *et al.* 2010). The two metabolic pathways convert fructose-6-phosphate to mannitol via the intermediates fructose and mannitol-1-phosphate (Fig. 8). The primary pathway for mannitol synthesis occurs via the reduction of fructose-6p to mannitol-1p by the NAD(H) dependent enzyme mannitol 1-phosphate dehydrogenase (MPD) (Solomon, Waters *et al.* 2006; Velez, Glassbrook *et al.* 2007; Ruijter, Bax *et al.* 2003). Subsequently, mannitol-1p is dephosphorylated by the enzyme mannitol 1-phosphate phosphatase (MPP). The other pathway converts fructose-6p to fructose by fructose-6-phosphate phosphatase (FPP). Fructose is then reduced to mannitol by the NAD(P)H dependent mannitol dehydrogenase (MTD). The production of fructose-6p from fructose is catalyzed by an hexokinase

Two pathways are known for the synthesis of trehalose in fungi (Fig. 8; Iturriaga, Suarez *et al.* 2009; Paul, Primavesi *et al.* 2008). The main trehalose synthesis pathway involves two reactions. A glucose molecule is transferred from UDP-glucose to glucose-6-phosphate to form trehalose-6-phosphate, and subsequently trehalose-6-phosphate is dephosphorylated to form trehalose. These reactions are carried out by the trehalose synthesis complex. This complex of *S. cerevisiae* consists of the trehalose-6-phosphate synthase TPS1, the trehalose-6-phosphate phosphatase TPS2 and the regulatory subunit TSL1 (Tereshina 2005). Possibly, a fourth subunit (TPS3) stabilizes the complex. Trehalose synthesis in filamentous fungi is expected to resemble that in *S. cerevisiae*. However, the genomes of filamentous fungi (e.g. *Aspergillus* species) contain more TPS homologs. The second and less studied pathway to synthesize trehalose was discovered in the basidiomycete *Grifola frondosa* (Saito, Kase *et al.* 1998). This fungus forms trehalose from glucose-1-phosphate and glucose (Iturriaga, Suarez *et al.* 2009). This pathway may also exist in *N. crassa* (Shinohara, Correa *et al.* 2002) and *Aspergilli* (Pel, de Winde *et al.* 2007; Nierman, Pain *et al.* 2005) because the genomes of these fungi contain homologues of the trehalose synthase of *G. frondosa*.

Deletion of genes encoding enzymes involved in the synthesis of trehalose or mannitol lead to higher stress sensitivity of conidia. Total compatible solute quantity is not changed in the Δ*mpdA* strain of *A. niger* but trehalose levels are higher (11.5 % dry weight) and mannitol levels lower (4.0 % dry weight) (Ruijter, Bax *et al.* 2003). Notably, the Δ*mpdA* conidia do show a reduced viability after heating (50 °C). In addition, Δ*mpdA* conidia are more sensitive to freeze-thawing, drying and hypochlorite treatment.

Conidia of a Δ*tpsA* strain of *A. nidulans* show a clear reduction in long-term survival (50 days) in liquid at room temperature (Fillinger, Chaveroche *et al.* 2001) suggesting a role of trehalose in prolonged stabilization of biomolecules. Alternatively, trehalose might function as a storage compound enabling spores to survive long periods of time. Stress sensitivity of conidia can also be altered by deletion of genes that regulate compatible solute levels. This is illustrated with conidia of *A. oryzae*. Wild-type conidia contain 0.7-1 pg trehalose and 2 pg mannitol per spore (Sakamoto, Iwashita *et al.* 2009). Conidia of a strain in which the transcriptional regulator *aftA*

is deleted show reduced trehalose (0.4 pg) and mannitol (1 pg) levels. A similar trehalose level is found when the transcriptional activator *aftB* is inactivated but in this case the mannitol levels are increased (3 pg). The Δ*atfA* strain shows strongly reduced germination after 3 weeks of storage and this is not observed in the case of the Δ*atfB* strain. Δ*atfA* conidia are also much more sensitive for hydrogen peroxide.

Protective proteins

A variety of stresses induces upregulation of a class of proteins. Initially, these proteins were designated heat shock proteins (HSPs) as they were identified after

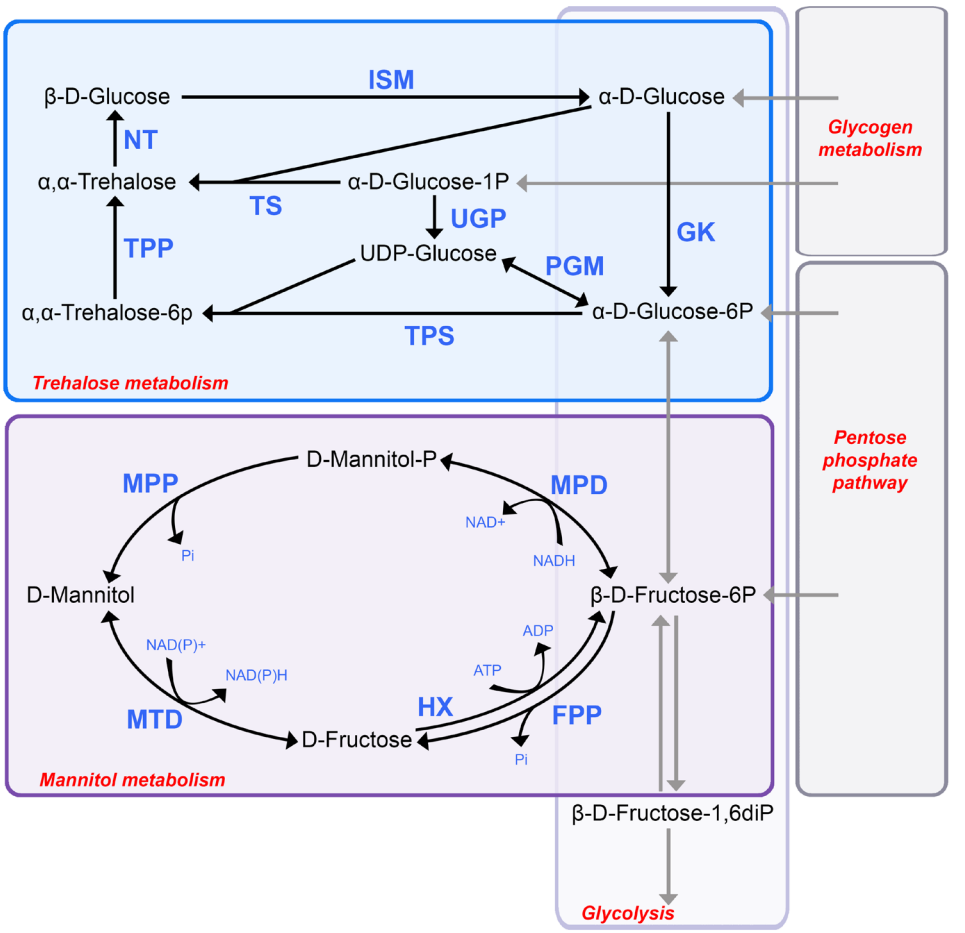

Figure 8. Trehalose and mannitol metabolism in fungi.
TREHALOSE METABOLISM. ISM: Isomerase, GK: glucokinase, PGM: phosphoglucomutase, UGP: UDP-glucose pyrophosphorylase, TS: Trehalose synthase, TPS: Trehalose-6-phosphate synthase, TPP: Trehalose-6-phosphate phosphatase, NT: Neutral trehalose
MANNITOL METABOLISM. MPD: Mannitol phosphate dehydrogenase, MPP; Mannitol phosphate phosphatase, FPP: Fructose phosphate phosphatase, MTD: Mannitol dehydrogenase, HX: Hexokinase.

heat stress. However, production of these proteins also occurs upon cold shock or desiccation treatments. The HSP family is a heterogeneous group of proteins with functions ranging from RNA methylation (Bugl, Fauman *et al.* 2000), ribosome protection (Korber, Stahl *et al.* 2000), proteolysis (Suzuki, Rep *et al.* 1997), to molecular chaperones (Gross 1996). Different groups of HSPs are distinguished including the ATP-dependent large chaperones Hsp100, Hsp90, Hsp70, Hsp60 and the ATP-independent small heat shock proteins (sHSP). Hsp70 and Hsp90 form a chaperone complex with a target protein and one or several co-chaperones (Picard 2002). This protects against aggregation or aberrant refolding as a result of stress (Gupta and Golding 1993; Helmbrecht, Zeise *et al.* 2000). The sHSPs also suppress protein aggregation (Haslbeck, Braun *et al.* 2004) and are characterized by a molecular mass of 12-43 kDa, a conserved α-crystallin domain, formation of large oligomers, and a dynamic quaternary structure (Haslbeck, Franzmann *et al.* 2005). The occurrence of sHSP has been reported in *S. cerevisiae* (Haslbeck, Franzmann *et al.* 2005) and filamentous fungi such as *N. crassa*, *Pisolithus sp* and *T. harzianum* (Montero-Barrientos, Cardoza *et al.* 2007; Ferreira, Totola *et al.* 2005; Plesofsky and Brambl 2002). Hsp12 is a small sized heat shock protein without an α-crystallin domain. It is expressed in yeast upon various stress conditions including heat and cold, osmotic stress, oxidative stress and the presence of ethanol (Praekelt and Meacock 1990; Pacheco, Pereira *et al.* 2009). Hsp12 is unfolded in solution, but forms α-helical structure when associated with the plasma-membrane (Welker, Rudolph *et al.* 2010; Singarapu, Tonelli *et al.* 2011). It shares characteristics with the group of hydrophilins and is also described as a LEA-like (Late Embryogenesis Abundant) protein (Garay-Arroyo, Colmenero-Flores *et al.*, 000). Hydrophilins are small, hydrophilic and unstructured proteins with a high glycine-content (> 6 %) (Garay-Arroyo, Colmenero-Flores *et al.* 2000). Hydrophilins include almost all Late Embryogenesis Abundant (LEA) proteins (92 %), dehydrins (Abba, Ghignone *et al.* 2006), as well as several conidiation related (Con) proteins (White and Yanofski 1993; Sachs and Yanofski 1991). LEA proteins widely occur in plants (Galau, Hughes *et al.* 1986; Dure, Greenway *et al.* 1981; Wolkers, McCready *et al.* 2001) and protect against protein aggregation during desiccation or freezing (Goyal, Walton *et al.* 2005). Like Hsp12 they are unstructured in solution and adopt α-helical structure upon dehydration (Shimizu, Kanamori *et al.* 2010; Popova, Hundertmark *et al.* 2011). Dormant conidia of *A. niger* contain relatively high levels of transcripts of genes involved in the synthesis of protective compounds including compatible solutes and protective hydrophilic proteins (Hsp9/12 and LEA-like proteins) (van Leeuwen, Krijgsheld *et al.* 2013a). These transcripts disappear at a very early stage of germination, i.e. after 2 h when spores start to swell. Similarly, transcripts of hsp12 and con-10, as well as a gene encoding a protein with a LEA-like domain are abundant in dormant *A. fumigatus* conidia and almost absent after germination (Suh, Fedorova *et al.* 2012). This indicates that these proteins are involved in protection or stabilization of conidia and that their mRNA is a left-over from the time the conidia were formed. Work of Hoi *et al.* indicates that dehydrins are involved in the tolerance

of *A. fumigatus* condidia to freezing (Hoi *et al.*, 2011; 2012). In addition conidia of the Δ*dprA* strain are sensitive to 2 mM H_2O_2 and killing by lung phagocytes of mice.

Null mutants of *tps1* (trehalose-6-phosphate phosphatase) and *hsp104* (heat shock protein 104) of *S. cerevisiae* affect heat shock sensitivity only moderately, while the double mutant shows little or no heat shock resistance (Elliott, Haltiwanger *et al.* 1996). This suggests that the functions of trehalose and Hsp104 are redundant and that the absence of one is compensated by the other. Cold adaptation of fungi is thought to be mediated by RNA-binding proteins with a cold shock domain (Fang and St. Leger 2010). In *M. anisopliae* two cold shock regulated proteins (i.e. Cpr1 and Cpr2) mediate protection against cold shock or oxidative stress. However, the mechanism of these RNA chaperones is unknown.

Other protective principles

Besides compatible solutes and intracellular proteins the cell wall provides protection. Conidia of *Aspergillus* and *Penicillium* have relatively thick cell walls compared to those of the vegetative hyphae. Different layers can be distinguished in the conidial cell wall by electron microscopy. This includes an ornamented outer electron dense (wharted) layer in the case of conidia of *A. niger*, *A. nidulans*, *A. oryzae* and *A. fumigatus* (Tiedt 1993; Tao and Yu 2011). The outer cell wall of many, if not all, hydrophobic conidia, including those of *Aspergillus* and *Penicillium* is covered with hydrophobins (Wösten 2001). Hydrophobins self-assemble at the cell wall - air interface into an amphipatic mosaic of amyloid-like fibrils (Wösten, de Vries *et al.* 1993; Wösten and Asgeirsdóttir 1994; Wösten and Schuren *et al.*1994; Wösten and de Vocht 2000). These fibrils are organized in 10 nm wide rods known as rodlets. They provide spores with a hydrophobic coating but the protein also affects the cell wall architecture (van Wetter, Wösten *et al.* 2000). The hydrophobic coating facilitates spore dispersal by wind and vectors (Wösten 2001) and also protects against the immune system (Aimanianda, Bayry *et al.* 2009). So far, evidence indicates that the rodlet layer does not provide an impermeable layer. The SC3 layer enables diffusion of proteins ≤ 200 Da (Wang, Shi *et al.* 2005). Transcripts of the hydrophobin genes *rodA* and the *dewA* gene accumulate in dormant conidia of *A. niger* (van Leeuwen, Krijgsheld *et al.* 2013a) but it has been suggested in *A. nidulans* that hydrophobins are produced by the phialides from which they diffuse to the outer surface of the conidia (Stringer, Dean *et al.* 1991). Successful deposition of the hydrophobin layers depends on the presence of an intact melanin-containing layer of the conidium (Pihet, Vandeputte *et al.* 2009; van Veluw, Teertstra *et al.* 2013) but has also been reported to depend on the presence of glucan in the cell wall (Scholtmeijer, de Vocht *et al.* 2009).

Melanin is a complex molecule that is specifically present in the spore cell wall and is thought to protect against UV-radiation (Singaravelan, Grishkan *et al.* 2008), and safeguards against the immune system (Heinekamp, Thywissen *et al.* 2013). Furthermore, melanin has an important function on the deposition of the rodlet layer (van Veluw, Teertstra *et al.* 2013) and influences spore adhesion and pellet formation (Priegnitz, Wargenau *et al.* 2012).

Biophysical aspects of cell protection

Several mechanisms have been described that explain the protective action of intracellular compatible solutes and protective proteins: (1) glass formation with high T_g, (2) high viscosity of the cytoplasm above the T_g, (3) replacement of water in dried membranes and proteins by hydroxyl groups (water-replacement hypothesis) and (4) kosmotropic effects (explained by the preferential exclusion theory) (Jain and Roy 2009). Most likely all these mechanisms contribute to heat resistance.

A glass is described as an amorphous solid. Thus, it does not have the regular structure that is characteristic for crystals. Glasses exhibit extreme high viscosity, which results in extremely reduced mobility of the molecules enclosed in it. As a consequence, diffusion and the rate of chemical reactions are low and protein unfolding and aggregation are inhibited. Therefore, a glass is very suitable to protect proteins and membranes and other biomolecules during prolonged periods of drought. Glasses are characterized by their transitional temperature (T_g). This is the temperature at which the glass transforms into a liquid phase, which is accompanied by a drop in viscosity (Fig. 9). In pure sugar glasses a second temperature dependent transition above T_g is observed, called the collapse temperature (T_c). This is accompanied with a further reduction in viscosity (Fig. 9) (Buitink, Hoekstra *et al.* 2000). Above T_c viscosity is low and protection of biomolecules and their structures is absent. In other words, there is an interval of relative high viscosity (protection) between T_g and T_c. The sugar composition of glasses strongly influences the T_g and the interval T_g - T_c. For example, glasses composed of high molecular weight (Mw) sugars have a higher T_g than low Mw sugars (Buitink, van den Dries *et al.* 2000; van den Dries, Besseling *et al.* 2000). Also the temperature interval between T_g and T_c is larger in the case of sugars with higher Mw. On the other hand, packing density is generally smaller in the case of high molecular weight sugars (Buitink and Leprince 2008; Buitink, van den Dries *et al.* 2000). In other words, the viscosity of glasses of low Mw sugars below T_g is higher than that of sugars with a higher Mw. As a consequence, movement of molecules in a glass of a sugar with low Mw is lower and protection is therefore expected to be better.

Glasses in biological systems are different from glasses formed by pure sugars (Bernallugo and Leopold 1995; Buitink and Leprince 2008; Buitink, van den Dries *et al.* 2000). Glasses in biological systems have a relatively high T_g, a large T_g – T_c temperature interval and a dense packing (Buitink, Hoekstra *et al.* 2000). These glasses can be mimicked by a glass composed of a mixture of sugars and proteins (Buitink, van den Dries *et al.* 2000). The inner content of plant seeds and fungal spores do indeed contain sugars and proteins but also other small molecules such as nucleotides, organic acids and salts. These small molecules can also impact glass properties. Citrate increases the glass transition temperature and the density of a glass of sucrose (Kets, Ijpelaar *et al.* 2004), while phosphate and citrate (Sundaramurthi and Suryanarayanan 2010; Al-Hussein and Gieseler 2012; Izutsu, Yomota *et al.* 2007) promote glass formation of mannitol and prevent it to crystallize (Izutsu, Yoshioka *et al.* 1993). Crystal formation of mannitol can occur at high concentration

and has a negative effect on lactate dehydrogenase (LDH) activity and stability (Al-Hussein and Gieseler 2012).

The water replacement theory assumes that compatible solutes like sugars replace water molecules upon desiccation (Figs 10 and 11). The hydroxyl groups of the sugars can form hydrogen bonds with proteins and lipids like water does. Removal of (almost) all water molecules therefore will not result in detrimental conformational changes of membranes and proteins. On the other hand, the preferential exclusion theory describes that a kosmotropic solute such as trehalose or sucrose is being repelled from the surface of a macromolecule such as a protein (Moelbert, Normand *et al.* 2004; Timasheff 2002). As a result, the concentration of the stabilizing compound is lower close to the protein than in solution (Fig. 12). This concentration difference is thermodynamically unfavorable because the entropy of the stabilizing compound is reduced (Arakawa, Prestrelski *et al.* 2001). Denaturation increases this unfavorable condition because the surface of the protein is enlarged during this process thereby reducing the entropy of the stabilizing molecules in solution even further. This explains why the native state of the protein is stabilized by kosmotropic molecules such as saccharides, amino acids and polyols. In contrast, chaotropic solutes show a higher concentration near macromolecules (preferential binding) and disrupt the water structure and destabilize proteins and membranes (Fig. 12). Conidia of the xerophilic fungus *Xeromyces bisporus* were harvested in chaotrope- or kosmotrope-supplemented solutions, and exposed to high temperature and pressure(Chin, Megaw *et al.* 2010). Conidia exposed to chaotropes lose viability at high temperature and high pressure, but there was virtually no loss of viability at low temperature. The opposie was observed for kosmotrope-treated conidia. It was concluded that the choice of solute (kosmotropic or chaotropic) influences the

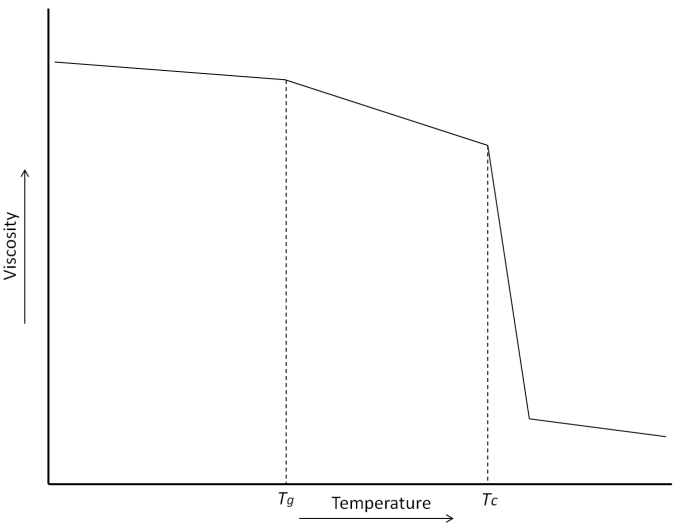

Figure 9. The change of viscosity in relation to the glass transition temperature (T_g) and collapse temperature (T_c).

temperature window of survival and growth.

It has been shown that protective proteins like hydrophilins interact with proteins and membranes upon drying. LEA proteins are unstructured in solution and some of these proteins adopt α helical structure upon drying (Hincha and Thalhammer 2012). This may increase the bonding capacity and makes them interact better with proteins and membranes, as described in the water replacement theory. As stated above, proteins can also increase the viscosity of glasses (Buitink and Leprince 2004; Buitink, van den Dries *et al.* 2000).

Airborne conidia encounter drying and rewetting situations that stress the cell in different ways. For example, the large majority of conidia of *P. chrysogenum* transferred through air (e.g. obtained by tapping a reversed colony) to dried gypsum

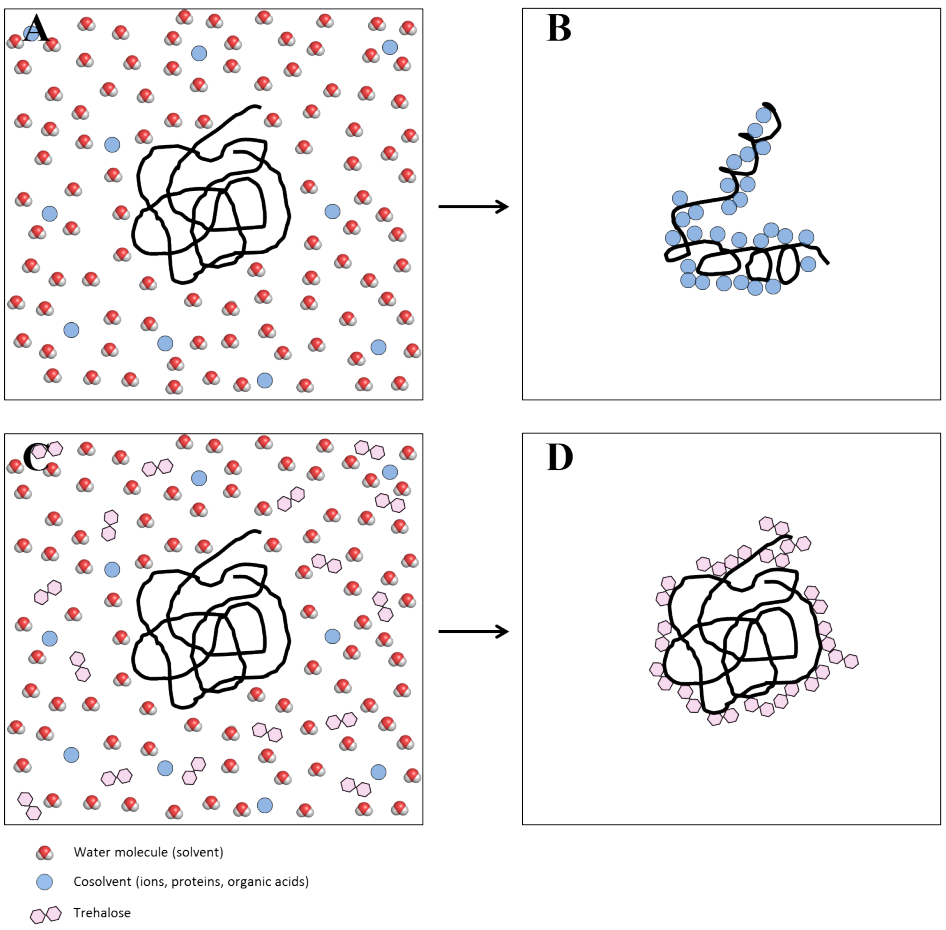

Figure 10. Water Replacement Theory. Hydrated proteins surrounded by water and cosolvents (A) or in solutions containing trehalose (B). Removal of water during dehydration results in unfolding (C) of protein. Sugars such as trehalose can replace water molecules by forming hydrogen bonds with the protein, thereby preserving its conformation (D).

are collapsed, but germinate after exposure of the gypsum to high humidity (Bekker, Adan *et al.* 2012). Drying introduces high molecule densities (known as crowding) as water disappears from the cell. This results in changes in the partitioning of molecules in cell components (e.g. membranes, Hoekstra, Golovina *et al.* 2001) and leads to altered properties of the cell constituents. Hydration of the dried spore causes imbibitional damage, another mechanism of cell stress. During transfer of the membrane from rigidity (gel-like) to semi-fluidity(liquid crystalline) these states occur simultaneously and extensive leakage occurs at the interface between these physical states (Hoekstra, Golovina *et al.* 2001). Conidia and ascospores must therefore possess protective mechanisms that enable the cells to survive these transitions.

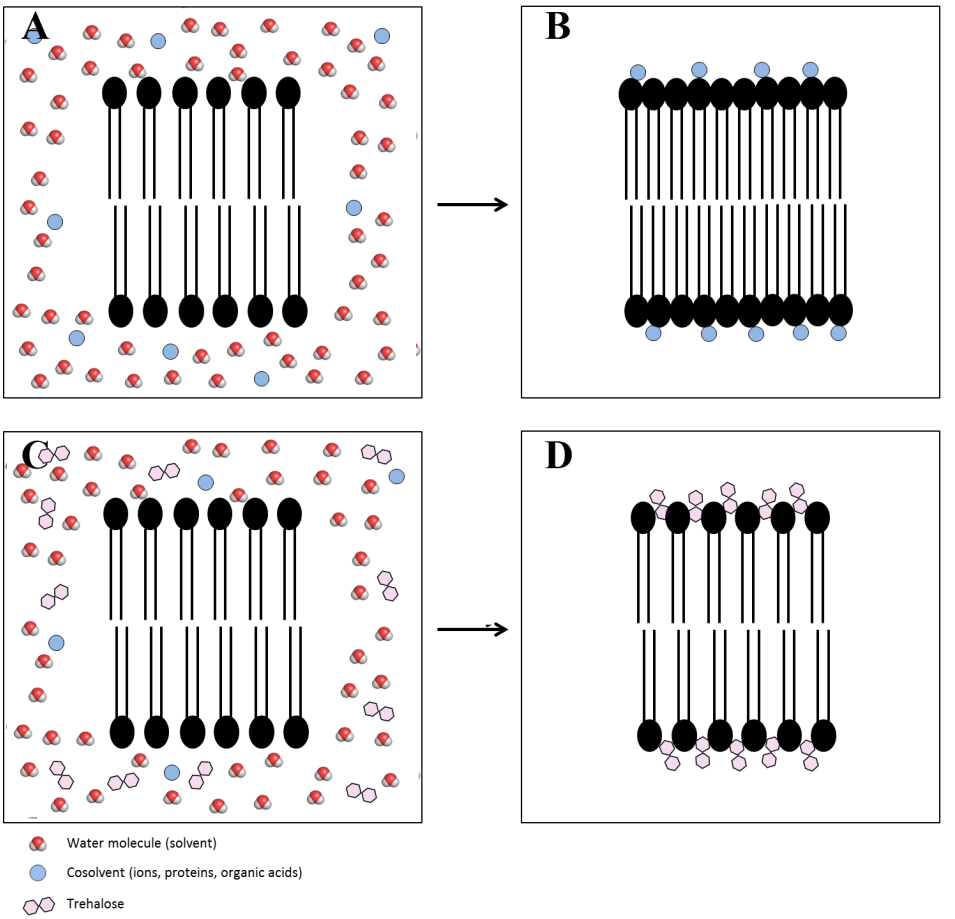

Figure 11. Water Replacement Theory. Hydrated membranes are surrounded by water and cosolvents in the absence (A) or presence (C) of trehalose. Removal of water during dehydration results in phase transition of the membrane from liquid to gel-like (B). Sugars such as trehalose can replace water molecules by forming hydrogen bonds with the lipid molecules, thereby preventing the phase transition (D).

When fungal spores are in an aqueous solution at room temperature no glassy state can be expected to exist as the concentrations of compatible solutes are too low and the water content too high. A sudden lowering of the temperature or a reduction of the water content might introduce a glass transition situation inside the cell, which virtually ends all processes in the cell. Such a glass is visible in cut dormant ascospores of *T. macrosporus* in cryoSEM (Fig. 13F). No intracellular features can be discerned during this condition. In contrast, cell organelles and membranes can be readily observed in frozen dormant conidia that are less stress resistant and that probably do not have an intracellular glass condition (Dijksterhuis, Nijsse *et al.* 2007). In nature, one can expect alternations between the glass and the hydrated states, which is a formidable challenge for life where all principles of protection discussed above, may act together.

Dormancy and activation

Dormancy

Dormancy is an important strategy to survive long periods of time. Stress-resistant ascospores can remain dormant for a long time, *T. flavus* ascospores still germinated after a 17-year-storage in the dark at room temperature (Nagtzaam and Bollen 1994). Two types of dormancy are distinguished (Sussman and Halvorson 1966). Exogenous dormancy depends on environmental conditions such as the absence of water and

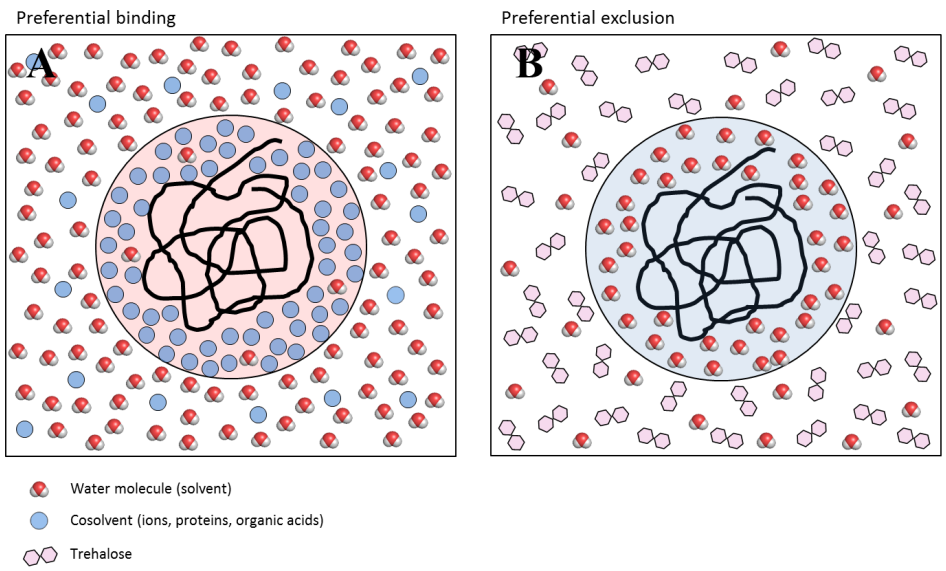

Figure 12. Schematic representation of kosmotropic and chaotropic effects. Preferential binding of a chaotropic co-solvent to a protein (A) will lead to protein unfolding. The preferential exclusion of a kosmotrophic co-solvent like trehalose near the protein surface (B) creates a thermodynamically unfavourable situation. Unfolding creates a larger protein surface, thereby further reducing the entropy of the co-solvent. This prevents the protein o unfold. Edited from Moelbert *et al.* (2004).

nutrients. On the other hand, constitutive dormancy involves internal conditions such as the presence of a self-inhibitor or conditions that create a metabolic block. Constitutive dormancy may also result from the build up of the cell wall and the cytosol that create a barrier for penetration of nutrients. Ascospores of *T. macrosporus* and other species even do not germinate when they are present in a rich medium for prolonged time. Their very thick cell wall and the dense cytoplasm present a formidable barrier for diffusion of nutrients. The viscosity of *T. macrosporus* and *N. fischeri* ascospores show values (far) above 10 cP (Dijksterhuis, Nijsse *et al.* 2007; **Chapter 2, 3**). In contrast, airborne and waterborne conidia that show exogenous dormancy are characterized by a viscosity of 3-4 and 2 cP, respectively (Dijksterhuis, Nijsse *et al.* 2007; van Leeuwen, van Doorn *et al.* 2010). A dense cytoplasm will also

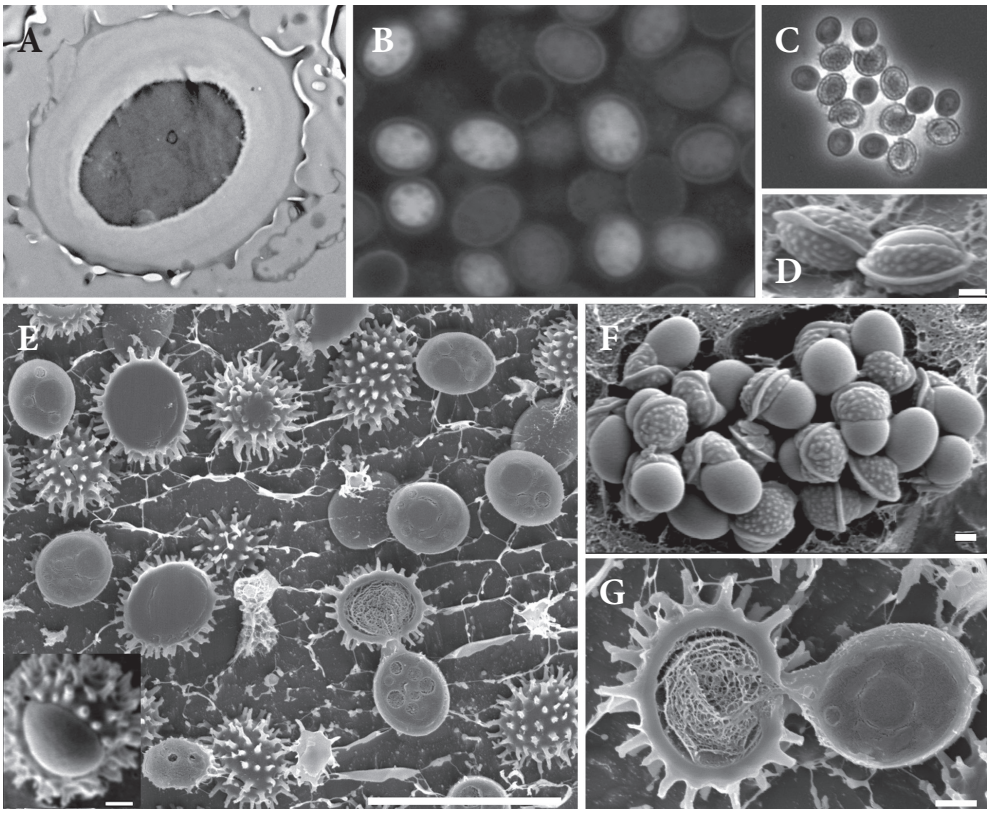

Figure 13. Ejection phenomena in the genus *Talaromyces*. Ascospores of *T. macrosporus* possess a very thick layered cell wall as is visible with TEM (A). Cytosolic GFP expression behind a gpd promotor results in fluorescent cytoplasm of ascospores as shown by confocal microscopy (B). The cell wall is also visible as a result of autofluorescence (arrow). Cell organelles are visible in these cells as non-fluorecent structures. Phase contrast microscopy shows prosilition of ascospores of *T. macrosporus* (C). Rupture of the cell wall in *T. stipitatus* (D) followed by ejection of the inner cell (E) as shown by cryoSEM. Cryo-planing of ascospores of *T. macrosporus* that have ejected (F). Dormant, not ejected ascospores do not show cell organelles. Inset, first stage of prosilition. Detail of an ejected cell that is still attached to the emptied outher cell wall (G). Cell organelles are visible.

restrict biochemcial processes. This would explain why the transcriptome of conidia of *A. fumigatus* has hardly changed after a year of dry storage (Lamarre, Sokol *et al.* 2008).

Activation

Ultimately, a spore will initiate germination. The release or breaking of dormancy is also called activation. Exogenous dormancy can be quickly broken upon availability of water either or not combined with the presence of the proper nutrients. Uredospores of rust fungi form germ tubes when exposed to pure water (Dijksterhuis 2003). This condition initiates polyribosome formation in airborne spores of *N. crassa* (Bonnen and Brambl 1983) and swelling of conidia of *A. niger* (Morozova, Kozlov *et al.* 2002). Ascospores of *S. cerevisiae* that are exposed to water show a metabolic rate of about 5 % of that of vegetative cells, but spores of other fungi may show much lower metabolic rates, if present at all. In the case of many fungi, nutrients are needed for further stages of germination. Conidia of *A. nidulans* require a C-source such as glucose, but not external phosphate and nitrogen (d'Enfert 1997; Osherov and May 2000). Dried sporangiospores of the tempeh fungus *Rhizopus oligosporus* remain dormant in the presence of water, but are activated to different extents in the presence of sole nutrients such as phosphate, amino acids, and glucose. Rich media that contain all these nutrients activate the largest proportion of the spores (Thanh and Nout 2004; Thanh, Rombouts *et al.* 2005).

Volatile compounds can also activate conidia to germinate as is observed in *P. digitatum* that causes post-harvest rot in citrus fruit. Wounded oranges produce several of such compounds, the mixture of it being most effective in conidial activation (Eckert and Ratnayake 2004). Activation can also be negatively affected by volatile compounds as is the case for 1-octen-3-ol that is produced by quite a number of fungi. The concentration of this compound can become relatively high at high densities of conidia of *P. paneum* and *A. nidulans* (Chitarra, Abee *et al.* 2004; Herrero-Garcia, Garzia *et al.* 2011). This suggests that 1-octen-3-ol acts as a fungal self-inhibitor that prevents premature germination of conidia on conidiophores or on substrates with a high conidial concentration. 1-octen-3-ol has a profound influence on protein expression patterns (Chitarra, Abee *et al.* 2005) and blocks isotropic growth, but had only mild physiological effects on germinating conidia in solution. The property of 1-octen-3-ol to repress activation of spores can be used to control infection of the button mushroom *Agaricus bisporus* with *Lecanicillium fungicola* (Berendsen, Kalkhove *et al.* 2013).

Ascospores of many stress resistant fungi do not show activation upon addition of water of and / or nutrients. Ascospores of *T. macrosporus* remain dormant after prolonged time in a complex (malt extract) medium and show virtually no respiration under this condition (Dijksterhuis *et al.* 2002; 2007). For these spores other methods are needed for activation. This can be a rigorous external trigger such as a short (5-10 min) heat treatment at 85 °C (Beuchat 1986; Dijksterhuis, van Driel *et al.* 2002)

or a high pressure treatment at 6000 Bar (Dijksterhuis and Teunissen 2004; Reyns, Verbeke *et al.* 2003). Germination of ascospores of different species is increased by several log cycles after a short heat treatment (e.g. Eurotium herbariorum at 60 °C, Splitstoesser, Lammers *et al.* 1989). The activation rate increases with higher temperatures in the case of *T. macrosporus* (Kikoku 2003). In nature, spores may be exposed to heat during or after a fire or after prolonged sunshine on a dark surface. It can not be excluded that the extreme triggers used in the laboratory may disrupt the factors that play a role to keep the cell dormant during extended periods in nature. Activation of spores is also realized by drying or by a combination of drying and heating. An activation treatment is not needed anymore when ascospores of *N. fischeri* are dried for 18 h at 40 °C (Beuchat 1992). This is not the case for *T. flavus*. Heating at 95 ºC at a relative humidity of 50 % (dry heat treatment) for 30 or 60 min activates *N. fischeri* ascospores. The temperature of the "recovery" buffer was crucial for the viable count obtained, which suggests an imbibitional damage effect (Gomez, Busta *et al.* 1989; Gomez, Pflug *et al.* 1993). Finally, ascospores of *Neurospora tetrasperma* are activated at 65 °C, but also upon exposure to chemical compounds like furfural (Lingappa and Sussman 1959; Eilers and Sussman 1970).

Possibly, a change in the properties of the membrane caused by chemical compounds, heat, or pressure break the dormancy of the ascospores (Sussman 1976; Hecker and Sussman 1973). In addition, high pressure treatment (6000 Bar) disrupts the thick outer cell wall of ascospores of *T. macrosporus* alleviating the barrier for uptake of molecules (Dijksterhuis and Teunissen 2004). Recent work in our laboratory indicates that a protein restricts the permeability of the cell wall of these ascospores (Wyatt *et al.*, unpublished results). Breaking of dormancy in heat treated ascospores of *T. macrosporus* results in irreversible changes. Activated spores cannot be brought back into dormancy not even after introduction of a glassy state realized by drying or cooling in liquid nitrogen (Dijksterhuis and Samson 2006). Instead, the ascospores immediately germinate in the presence of growth medium.

Changing from the dormant towards the vegetative state

In this section, the first stage of germination is discussed where the protective dormant state of the cell is changed into a vegetative cell capable of performing an active metabolism and cell division. Subsequent stages of germination including germ tube formation and elongation (polarized growth) are not covered in this section. For this we refer to Osherov and May 2001; Momany 2002; Harris and Momany 2004; Harris 2006; Magan, Aldred *et al.* 2013; and Krijgsheld, Bleichrodt *et al.* 2013. The first stage of germination includes: i) shedding of the conidial or ascospore cell wall (Tiedt 1993; Fontaine, Beauvais *et al.* 2010; Dijksterhuis, van Driel *et al.* 2002), ii) degradation of compatible sugars (trehalose and mannitol) (d'Enfert, Bonini *et al.* 1999; Fillinger, Chaveroche *et al.* 2001), which is accompanied by a decrease of the microviscosity of the cytoplasm (Dijksterhuis, Nijsse *et al.* 2007; van Leeuwen, van Doorn *et al.* 2010; van Leeuwen, Krijgsheld *et al.* 2013b), and iii) reorganization

of the transcriptome including major mRNA breakdown and selected upregulation of different gene categories (van Leeuwen, Krijgsheld *et al.* 2013a). Mannitol and trehalose are degraded upon germination of conidia and ascospores (Dijksterhuis, van Driel *et al.* 2002; van Leeuwen, Krijgsheld *et al.* 2013b; Thevelein 1984). At this stage, the demand for energy is high and mannitol and trehalose may act as carbon sources (Mandels, Vitols *et al.* 1965; Lewis and Smith 1967). Trehalose in fungi is degraded by trehalase into glucose. Neutral trehalase is involved in degradation of trehalose in conidia of *A. nidulans* (d'Enfert, Bonini *et al.* 1999), in ascospores of *T. macrosporus* (Dijksterhuis, van Driel *et al.* 2002), and in cells of *S. cerevisiae* (DeVirgilio, Hottiger *et al.* 1994), while acid trehalase of *A. nidulans* is used for the degradation of external trehalose (d'Enfert and Fontaine 1997). When the gene encoding neutral trehalase NTH1 is disrupted in yeast, trehalose is not degraded and a prolonged heat resistance is observed after the recovery of an initial heat shock (DeVirgilio, Hottiger *et al.* 1994). The degradation of mannitol and trehalose is associated with an (transient) increase of intracellular glycerol, which can be interpreted as a sign of metabolisation. Germinating conidia of the Δ*treB* strain of *A. nidulans*, in which the neutral trehalase gene is inactivated also show reduced mannitol degradation and only partial (30 %) glycerol accumulation. It should be noted that germination is not affected in this strain. The high levels of trehalose in ascospores of *T. macrosporus* are quickly degraded to glucose upon activation of the spores. A large proportion of the glucose is released into the surrounding medium (Dijksterhuis, van Driel *et al.* 2002; Dijksterhuis, Nijsse *et al.* 2007). This may prevent depletion of ATP levels due to excessive glucose conversion during the first steps of glycolysis.

Upon activation, nutrient uptake is readily observed. Amino acids such as arginine, methionine and proline and the purine xanthine are taken up in conidia of *A. nidulans* and *A. niger* during swelling (Tazebay, Sophianopoulou *et al.* 1995; 1997; Amilis, Cecchetto *et al.* 2004; te Welscher, van Leeuwen *et al.* 2012). Xanthine was taken up after 1 h of germination, coinciding with the upregulation of a gene encoding a high affinity purine transporter (Amilis, Cecchetto *et al.* 2004). A cytosine-purine permease similar to Fcy2 from yeast was abundantly present in dormant conidia and during germination. *FcyB* is also expressed highly in conidia of *A. nidulans* (Vlanti and Diallinas 2008). Proline transport can be detected after 2 h of germination and increases rapidly peaking after 5 h, just before germ tube emergence. A micro-array analysis of conidia of *A. niger* shows a relatively strong up-regulation of genes of several neutral amino acid transporters and sugar transporters at very early germination (2 h). *A. niger* (Van Leeuwen, Krijgsheld *et al.* 2013a) and *A. fumigatus* (Lamarre, Sokol *et al.* 2008) conidia also show upregulation of genes involved in protein synthesis within 2 h after activation. Initiation of protein synthesis can be considered a hallmark of early stages of germination. This is also illustrated by the finding that the protein synthesis inhibitor cycloheximide prevents initial stages of germination, while inhibitors of the cytoskeleton, and DNA- and RNA synthesis do not prevent this process (Osherov and May 2000).

The first stage of germination also involves changes in the cell wall and the

plasma membrane (Van Leeuwen, Smant *et al.* 2008). The rodlet layer on conidia progressively disappears during the first few hours of germination (Dague, Alsteens *et al.* 2008). Changes can be even more dramatic. The cell wall of several species of *Talaromyces* ruptures and the inner cell is ejected within a second. This phenomenon is dubbed "prosilition" (Fig. 13E). A sudden exposure of an inner cell wall to the surrounding medium is also observed in several other ascospore forming fungi (see Dijksterhuis, van Driel *et al.* 2002) such as in *Neosartorya*. In this case, shedding occurs in a more gradual way by splitting of the outer cell wall into two halves (Wyatt *et al.* unpublished results).

Applied mycology and fungal spores

Fungal spores bring biochemical processes to a very slow pace and thus protect the cell against many stressors. Studying the mechanisms of cell protection can be used to prevent fungal infections and food spoilage. Spores are often the infective propagules of fungi on substrates like food and organisms. Food spoilage by fungi causes enormous losses of food products worldwide (Dijksterhuis, Houbraken *et al.* 2013). Prevention of these losses is an important area of research with respect to the need for food in the decades to come. Fungal conidia are more resistant to many stressors than vegetative cells and an increase of sensitivity for e.g antifungal compounds is expected to occur during subsequent germination. Induction of germination in combination with stress could be a novel strategy to prevent fungal infections or spoilage of food. Dormant conidia of *A. fumigatus* are insensitive to 50 μg ml^{-1} of the polyene antibiotic amphotericin B methyl ester. This insensitivity decreases to 20 and 1-2 μg after 2- and 4 h, respectively (Russel, Kerridge *et al.* 1975; 1977). Conidia of *A. niger* and P. discolor are able to survive 20 h at moderate concentration of natamycin. In fact, they germinate in high numbers after removal of the antifungal compound (van Leeuwen, Krijgsheld *et al.* 2010).

Thesis outline

Eradication of ascospores of heat-resistant fungi is only possible by means of prolonged heating. Such procedures affect the taste and quality of the product and lead to increased energy costs. Moreover, consumers prefer minimal processed food. Short pasteurisation times (blanching) and absence of preservatives are therefore preferred. However, heat-resistant ascospores survive these treatments. In fact, dormancy is actually broken by short pasteurisation treatments and will induce spoilage. An alternative preservation method uses high pressure (≥ 400 MPa), but this also induces germination of *T. macrosporus* (Reyns, Verbeke *et al.* 2003; Dijksterhuis and Teunissen 2004). Unraveling the mechanisms underlying heat resistance of fungal spores will help to develop novel strategies to prevent fungal food spoilage. Moreover, these studies may identify bioactive molecules that can be used to improve stability of therapeutic and industrial proteins, vaccines and micro-organisms that are applied in the industry and agriculture. Improved stability results

in a more active product, in a longer shelf-life, and the potential use of higher storage temperatures.

Research on heat resistant ascospores has focused on external factors influencing the heat resistance. In this Thesis, I studied the role of internal sugars, polyols and small proteins in the survival of ascospores during exposure to heat and drought. *N. fischeri* was used as a model system. Its genome is sequenced, the spores are stress resistant and it is regularly found as a cause of food spoilage.

Chapter 2 describes the identification of novel trehalose-based oligosaccharides (TOS). These oligosaccharides consist of a trehalose core with one, two or three glucoses linked via an α-1,6 bond. TOS are found in ascospores of various species of the class Eurotiomycetes, but seem to be most abundant in the genera *Neosartorya* and *Thermoascus*. *N. fischeri* ascospores contain TOS and trehalose and are more viscous and more resistant to the combined stress of heat and desiccation than the ascospores of *T. macrosporus* that contain predominantly trehalose. Synthesized TOS were less effective in protecting proteins and blastospores against heat and drought than trehalose. TOS inside the context of the cell may be important for their functioning.

Chapter 3 related redox stability, accumulation of compatible solutes and heat resistance of ascospores. Stress resistance of ascospores is gradually acquired during their development. Accumulation of compatible solutes (3.9 to 12.1 pg spore^{-1}), increase of viscosity (5.4 to 21.2 cP) and reduction of bulk water is characteristic for the first phase of acquirement of stress resistance. The second phase is accompanied with an increase of trehalose and TOS, while the total concentration of compatible solutes and viscosity doesn't change. It is also described that dry heat storage does not affect cytoplasmic mobility, but it does affect the redox stability in hydrophobic cytoplasmic environments.

In **Chapter 4** the role of MpdA in *N. fischeri* was studied. Its gene is mainly expressed in ascospores and cleistothecia. Deletion of *mpdA* reduced mannitol levels to as low as 15 % of the wild type, whereas trehalose levels increased more than 400 %. Absence of MpdA had no effect on vegetative growth and formation of cleistothecia, irrespective of exposure to heat and oxidative stress. However, in the absence of the protein ascospore formation was impaired.

The role of the hydrophilins LeamA, Hsp12A, and Hsp12B of *N. fischeri* was assessed in **Chapter 5**. Heterologous expression of these proteins in *Escherichia coli* resulted in increased tolerance against salt and osmotic stress. Purified LeamA, Hsp12A, and Hsp12B protected LDH against dry heat and freeze-thaw cycles. Deletion of *leamA* decreased the sensitivity of *N. fischeri* ascospores to dry heat. These results indicate that the three hydrophilins LeamA, Hsp12A, and Hsp12B are involved in the mechanism of heat resistant ascospores of *N. fischeri*.

Results are summarized and discussed in **Chapter 6**.

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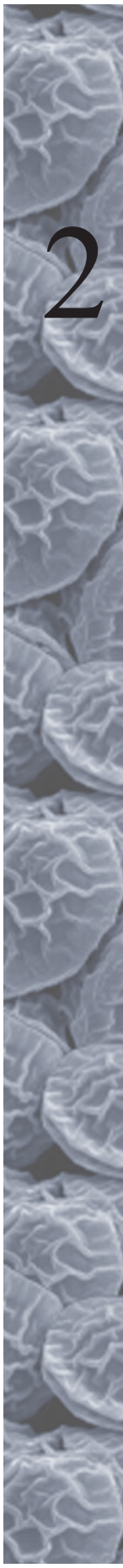
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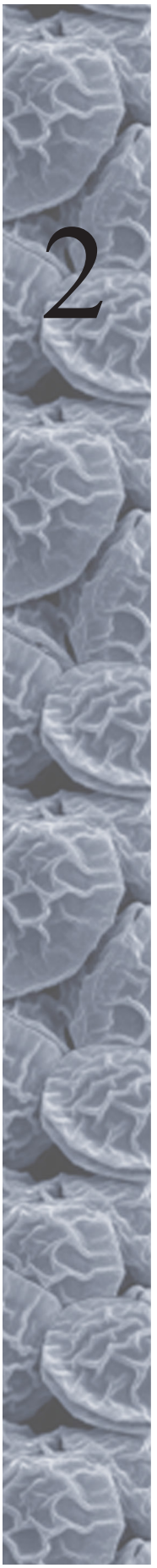

2 Novel trehalose-based oligosaccharides from extreme stress-tolerant ascospores of *Neosartorya fischeri* (*Aspergillus fischeri*) protect against heat

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Abstract

Ascospores of *Neosartorya*, *Byssochlamys* and *Talaromyces* can be regarded as the most stress-resistant eukaryotic cells. They can survive exposure to 85 °C for 100 min or more. Here we describe the identification and characterization of novel trehalose-based oligosaccharides (TOS) in members of the order Eurotiales, to which *Neosartorya*, *Byssochlamys*, and *Talaromyces* belong. These oligosaccharides consist of a trehalose backbone with 1, 2 or 3 glucose molecules attached via an α-1,6 linkage. The tetra- and pentasaccharide, dubbed neosartose and fischerose, respectively, have not been reported in nature before. *Neosartorya fischeri* ascospores that contain TOS as well as trehalose are more viscous and more resistant to the combined stress of heat and desiccation than the ascospores of *Talaromyces macrosporus* that contain predominantly trehalose. TOS glasses have a higher glass transition temperature (T_g) than trehalose and form a more stable glass with crystallizing molecules such as mannitol. Our data indicate that TOS are important for prolonged stabilization of cells against stress. These molecules may also be applied to extent the shelf life of micro-organisms, vaccines or other pharmaceutical or industrial products.

Introduction

Extremely stress-resistant ascospores are found among the fungal genera *Neosartorya*, *Byssochlamys* and *Talaromyces*. These spores resist high temperature, pressure, and desiccation (Beuchat 1986; Dijksterhuis and Samson 2002; Reyns, Veraverbeke *et al.* 2003; Dijksterhuis and Teunissen 2004; Dijksterhuis and Samson 2006; Dijksterhuis, Nijsse *et al.* 2007; Houbraken, Dijksterhuis *et al.* 2012) and can be regarded as the most stress-resistant eukaryotic cells described to date. Their temperature resistance is similar to that of bacterial spores of *Bacillus subtilis* by surviving a treatment of 85 °C for 100 min or more (Dijksterhuis and Teunissen 2004). These ascospores therefore survive pasteurization and cause spoilage of food products (Tournas 1994). The pasteurization treatment can even break the dormancy of these ascospores leading to germination of these survival structures (Devirgilio, Muller *et al.* 1991; Reyns, Veraverbeke *et al.* 2003; Dijksterhuis and Teunissen 2004; Dijksterhuis and Samson 2006). Humidity is an important determinant for stress resistance. Ascospores of *Neosartorya fischeri* exposed to extreme heat (95 °C) at a relative humidity of 30 % had a D-value (decimal reduction time, the time that is required to kill 90 % of the spores) that was almost 200 times higher than that of spores exposed at a relative humidity of 75 % (Gomez, Pflug *et al.* 1994).

Compatible solutes protect cells against stresses such as drought and high temperature. These molecules do not interfere with cellular functioning even when present at high concentration. Compatible solutes include polyols, sugars, betaines and amino acids. The sugar trehalose and the polyols mannitol, glycerol, arabitol and erythritol are the major solutes in fungal cells. Glycerol is the main compatible solute that accumulates upon osmotic stress in fungi (Redkar, Locy *et al.* 1995; Hagiwara, Asano *et al.* 2007; Kogej, Stein *et al.* 2007), but also erythritol, mannitol or arabitol can be found (Managbanag and Torzilli 2002; Ruijter, Visser *et al.* 2004). Stress-resistant ascospores contain large amounts of trehalose and mannitol (Conner, Beuchat *et al.* 1987; Dijksterhuis, van Driel *et al.* 2002). For instance, *Talaromyces macrosporus* ascospores accumulate trehalose up to 17 % of the fresh weight and also mannitol is abundant (Dijksterhuis, van Driel *et al.* 2002). Trehalose and mannitol are also the most abundant solutes in conidia (asexual spores) when grown on rich media (oatmeal agar, malt extract agar or Sabouraud dextrose agar) (Tereshina, Memorskaya *et al.* 2000; Fillinger, Chaveroche *et al.* 2001; Ruijter, Bax *et al.* 2003; Tereshina, Kovtunenko *et al.* 2004; Doehlemann, Berndt *et al.* 2006; Solomon, Waters *et al.* 2006; Wang, Lu *et al.* 2012). Decrease of either trehalose or mannitol leads to increased stress sensitivity of conidia (Fillinger, Chaveroche *et al.* 2001; Ruijter, Bax *et al.* 2003; Sakamoto, Arima *et al.* 2008; Wang, Lu *et al.* 2012). This indicates that both compounds are important for stabilization of the biomolecules within these spores.

The stabilizing effect of compatible solutes is best studied for trehalose. This compound is thought to have the most superior protective properties of sugars (Sola-Penna and Meyer-Fernandes 1998; Kaushik and Bhat 2003). The non-reducing nature of trehalose makes it less reactive, which is one of the prerequisites for successful

stabilization. Trehalose provides protection of complete micro-organisms, but also enzymes, membranes and DNA in vitro (Crowe, Crowe *et al.* 1984; Yoshinaga, Yoshioka *et al.* 1997; Kandror, Deleon *et al.* 2002; Jain and Roy 2010) against a wide variety of stresses, like heat, freezing, desiccation, radiation, and oxidative stress (Hottiger, Boller *et al.* 1989; Wiemken 1990; Devirgilio, Hottiger *et al.* 1994; Yoshinaga, Yoshioka *et al.* 1997; An, Iwakiri *et al.* 2000; Benaroudj, Lee *et al.* 2001; Fillinger, Chaveroche *et al.* 2001). Functioning of trehalose is thought to be based on several principles including a high glass transition temperature (T_g) (Sun and Davidson 1998; Buitink and Leprince 2004), the ability to replace water using its hydroxyl groups (water-replacement hypothesis) (Crowe, Crowe *et al.* 1984; Crowe and Crowe 1992) and its stabilizing effect on the water structure and intermolecular interactions in biomolecules as a result of preferential exclusion (kosmotrophic effect) (Timasheff 2002; Moelbert, Normand *et al.* 2004; Jain and Roy 2009).

A biological glass provides stability to the cell due to extreme reduction of movement of molecules (Crowe, Carpenter *et al.* 1998). Glass formation (vitrification) occurs during drying, or when cooled rapidly. Glass formation depends on the concentration of the solutes and the amount of water present, because water acts as plasticizer (Roos and Karel 1990; Wolkers, Oldenhof *et al.* 1998). The temperature influences the melting of the glass (T_g). Trehalose has a high glass transition temperature (T_g = 108 °C) compared to other disaccharides (e.g. 67 °C in case of sucrose) and it forms already a glass at room temperature at a water content of 10 % (Chen, Fowler *et al.* 2000). Trehalose also has a larger binding capacity of water molecules than other disaccharides (Lerbret, Bordat *et al.* 2005), although it has the same number of hydroxyl groups. This indicates that trehalose is better able to interact with water molecules, which may also explain why it is such a good replacing agent for water during desiccation. Being a kosmotropic solute, trehalose is excluded from the surface of proteins and membranes when water concentration decreases. This preferential exclusion stabilizes hydrophobic interactions and the protein surface area is minimized during the process of drying preventing denaturation and functional loss (Elbein, Pan *et al.* 2003; Jain and Roy 2010). Polyols and other oligosaccharides (consisting of 2 or more moieties) also protect against various stresses via similar mechanisms as trehalose. For example, the polyol mannitol provides excellent protection against heat inactivation in solution (Ortbauer and Popp 2008), but due to it's tendency to crystallize mannitol gives poor protection against (freeze) drying (Izutsu, Yoshioka *et al.* 1993; Al-Hussein and Gieseler 2012).

Here, we describe novel trehalose-based oligosaccharides (TOS) that are abundantly present in ascospores of part of the fungal species belonging to the order Eurotiales. These TOS are characterized by their non-reducing nature and high glass transition temperature. They are proposed to protect the cells against drought and heat.

Material and methods

Strain, growth conditions and culture media

Fungal strains from the order Eurotiales (Supplementary Table 1) were grown at 25-40 °C on oatmeal agar (OA) or malt extract agar (MEA40S) (Samson and Houbraken 2010). Inoculation was done from a glycerol stock solution of conidia (106 spores ml-1). *N. fischeri* (CBS 317.89) and *T. macrosporus* (CBS 580.72) cultures were routinely grown at 30 °C on OA. Ascospores of these species were heat activated for 2 min at 85 °C (Dijksterhuis, van Driel *et al.* 2002). Agar medium was inoculated by spreading 100 µl of a heat-activated suspension containing 10^7 ascospores ml^{-1}. After 40 days of growth ascospores were harvested by collecting fungal material from cultures with a glass spatula. The mixture of hyphae and ascomata was transferred to 9 ml ice-cold 10 mM ACES (N-(2-acetamido)-2-aminoethanesulfonic acid) buffer, pH 6.8, supplemented with 0.02 % Tween-80 (Sigma-Aldrich, Zwijndrecht, the Netherlands), after which 1 cm^3 of sterile glass beads (1:1 ratio of beads with a diameter of 0.10-0.11 mm and 1.0 mm) was added. Ascospores were released from cleistothecia by vortexing for 1-2 min and sonification for 5 min using a Ultrasonic cleaner 2510E-MT (Branson Ultrasonics Corporation, Danbury, USA). Filtration through sterile glass wool removed the mycelial debris and remnants of the ascomata. The spores were washed three times with ice-cold ACES buffer with a centrifugation step (5 min, 1100 g) after each washing step. If not immediately used for experiments, pellets of ascospores were stored in ACES buffer at -80 °C.

Compatible solutes

The polyols mannitol and glycerol and the sugars glucose, trehalose, sucrose, verbascose and stachyose were ordered by Sigma-Aldrich. Isobemisiose, neosartose and fischerose were synthesised by the group of Dr. Nicole Snyder and Prof Peter Seeberger (Berlin, Germany). The first batch of isobemisiose used for NMR was acquired from Dr. T. Nishimoto and Dr. H. Watanabe of the Glycoscience Institute of Hayashibara Biochemical Laboratories.

Monitoring heat resistance of ascospores

Ascospores were heated in solution (wet heat) or after vacuum drying (dry heat). After the heat treatment the germination percentage was measured with one of the following methods. Heat-treated spores were diluted to 10^4 and 10^3 spores ml^{-1}. 100 µl was spread on MEA plates and incubated for 2-3 days at 30 °C. Germination percentage was based on the number of colony forming units. Alternatively, heat-treated ascospores were inoculated on 1-2 mm thin slices of MEA (10^6 spores ml^{-1}) placed on an objective glass. The MEA slides were incubated for 14-16 h at 30 °C in a water-saturated container, after which the germinated spores were counted by light microscopy (Zeiss Axioskop 2 plus microscope). A spore was considered to be germinated when a germ tube (initial) was visible. At least 100 spores were

evaluated in triplicate.

Wet-heat treatment: Ascospores were suspended in hot ACES buffer (85 °C). The ascospore suspension (10^6 spores ml^{-1}) was immediately transferred to a 85 °C water bath and shaken at 150 rpm. the spore suspension was cooled after 0, 2, 10, or 30 min by adding ice-cold ACES buffer to a final concentration of 10^3 spores ml^{-1}, after which MEA plates were inoculated. Alternatively, the spore suspension was cooled on ice and 10 µl was used to inoculate a MEA slice positioned on an object glass. Germination was determined microscopically as described above.

Dry-heat treatment: Ascospores (10^7 spores in 10 µl) were vacuum dried for 1 h (Savant SpeedVac DNA 110 Concentrator, Thermo Scientific, Erembodegem-Aalst, Belgium) in a 1.5 ml Eppendorf tube. The dry spores were kept for 7 days at ambient temperature (25 °C) and ambient humidity (relative humidity (R.H.) of 45-85 %) called the ambient-dry treatment. Alternatively, spores were kept for 7 days at 25 °C in a desiccator filled with silica with a R.H. of 0.5-2 %, the so-called extreme dry condition. After incubation the dried spores were heated at 25, 60, 70 or 80 °C for 1 h in a heat block. Subsequently, the ascospores were resuspended in ACES buffer (10^6 spores ml^{-1}) and heated at 85 °C for 0-30 min at 150 rpm in a water bath to evaluate heat activation and subsequent thermal inactivation as described above.

Phylogenetic analysis of ascospore producing species within the family Trichocomaceae

Genomic DNA was extracted from mycelium of fungal strains (Supplementary Table 1) that had been grown for 3-5 days on MEA agar plates using the UltraClean Microbial DNA Isolation kit (MO BIO Laboratories, Inc., USA). The ITS and LSU fragments were amplified and sequenced as described (Houbraken, Due *et al.* 2007). The sequences were aligned with Muscle within the Mega 5.1 software package (Tamura, Peterson *et al.* 2011). Genes were concatenated in SEAview (Galtier, Gouy *et al.* 1996; Gouy, Guindon *et al.* 2010). PAUP 4.10b (Swofford 2002) was used to test compatibility between the two databases. The appropriate substitution model for the Maximum Likelihood phylogenetic analysis was calculated with Mega 5.1 (Tamura 1992) using the T92 model (Tamura 1992) with gamma distributed with invariant sites. Statistical support for branch nodes was calculated using a bootstrap analysis of a 1000 replicates. The *Talaromyces* clade was used as outgroup.

Cell free extracts of ascospores

Ascospores were frozen in liquid nitrogen and transferred to a stainless steel grinding jar (Qiagen, Venlo, The Netherlands) cooled with liquid nitrogen and homogenized with the Qiagen Tissuelyser (2 min at 30 strokes sec^{-1}). 0.5-1 ml milliQ water was added and grinding was continued for an additional 2 min at 30 strokes sec^{-1}. Samples were thawed, transferred to a 2 ml Eppendorf tube, and centrifuged at 4 °C for 30 min at 10.000 g. The supernatant was heated for 30 min at 95 °C to inactivate oligosaccharide degrading enzymes and centrifuged again for 30 min at 10.000 g.

The supernatant was filtered (0.2 µm acrodisc Cr 13 mm Syringe filter, Pall Life Science, Mijdrecht, the Netherlands) and stored at -80 °C until used for further analysis.

High-performance liquid chromatography (HPLC)

The amount of glucose, trehalose, isobemisiose, neosartose, fischerose, glycerol, and mannitol in cell free extracts were determined using a HPLC system (Waters, Etten-Leur, the Netherlands) equipped with a 2414 refractive index (RI) detector, a 515 HPLC pump, a pump control module II, a 717 plus autosampler and a cation-exchange column Sugar-Pak I. The miliQ mobile phase had a flow of 0.5 ml min^{-1}. Sample volumes of 10 µl were run for 20 min using a column and RI detector temperature of 50 °C. Peak integrations and calculations were performed by the Empower software (Waters). Retention time of the peaks was compared with those of 0.01-0.50 % w/v trehalose, isobemisiose, neosartose, fischerose, mannitol, glucose and glycerol.

Gas-liquid chromatography

Mono- and disaccharides and alditols in lyophilized cell free extracts were trimethylsilylated (pyridine:hexamethyldisilazane:trimethylchlorosilane 5:1:1, 30 min, rt), and analyzed by gas-liquid chromatography - electron ionization mass spectrometry (GLC-EIMS) (temperature program: 200-280 °C, 4 °C min^{-1}, 15 min 280 °C) using an AT-1 column (30 m x 0.25 mm, Alltech, Grace, Deerfield, United States) and a Fisons Instruments GC 8060/MD 800 system (Interscience, Breda, The Netherlands). For analysis of monosaccharides (i.e. also those contained in di- and oligosaccharides), samples were first subjected to methanolysis (1 M methanolic HCl, 24 h, 85 °C) before they were trimethylsilylated and analyzed by GLC-EIMS (temperature program: 140-240 °C, 4 °C min^{-1}) (Kamerling and Vliegenthart 1989).

Thin-layer chromatography (TLC)

Ascospore cell free extracts and partially purified oligosaccharide preparations were spotted (2 µl) on TLC sheets (Merck Kieselgel 60 F254, 20 x 20 cm) and run using 2:1:1 n-butanol : acetic acid : water as the mobile phase. Sugar containing compounds were visualized by orcinol / sulfuric acid staining (100 mg orcinol monohydrate, 95 ml methanol, 5 ml sulfuric acid) using glucose, trehalose, raffinose, verbascose and stachyose as a standard.

Gel-filtration chromatography

Oligosaccharides were purified by fractionation of extracts on a Bio-Gel P2 column (50 x 2 cm and 100 x 1.5 cm, Bio-Rad Laboratories, Veenendaal, the Netherlands). The gel-filtration system was composed of a peristaltic pump p-1 (Pharmacia Fine Chemicals, New Jersey, United States), a 2238 UVICORD SII UV detector (LKB

Bromma, Bromma, Sweden) and a BD40 channel chart recorder (Kipp & Zonen, Delft, the Netherlands). The mobile phase (10 mM NH4HCO3) had a flow of 15 ml h^{-1} and peaks were detected at 206 nm. Fractions were collected and evaluated by TLC (see above). Fractions containing the same oligosaccharide were pooled and freeze dried.

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS)

MALDI-TOF-MS experiments were performed using an Axima mass spectrometer (Shimadzu Kratos, Kyoto, Japan) equipped with a nitrogen laser (337 nm, 3 ns pulse width). Positive-ion mode spectra were recorded using the reflector mode at a resolution of 5000 FWHM and delayed extraction (450 ns). The accelerating voltage was 19 kV with a grid voltage of 75.2 %. The mirror voltage ratio was 1.12, and the acquisition mass range was 200-1100 Da. Samples were prepared by mixing 0.5 µl sample with 0.5 µl aqueous 10 % 2,5-dihydroxybenzoic acid on the target.

NMR spectroscopy

Resolution-enhanced 1D/2D 500-MHz ^{1}H NMR spectra were recorded in D_2O on a Bruker DRX-500 spectrometer (Wormer, the Netherlands) at a probe temperature of 300 K (27 °C). Prior to analysis, samples were exchanged twice in D_2O (99.9 atom % D, Cambridge Isotope Laboratories, Andover, MA) with a lyophilisation step in between, and then dissolved in 0.6 ml D_2O. Suppression of the HOD signal was achieved by applying a WEFT pulse sequence for 1D experiments and by a pre-saturation of 1 s during the relaxation delay in 2D experiments. The 2D TOCSY spectra were recorded using an MLEV-17 mixing sequence with spin-lock times of 20-200 ms. The 2D ROESY spectra were recorded using standard Bruker XWINNMR software (Wormer, the Netherlands) with a mixing time of 200 ms. The carrier frequency was set at the downfield edge of the spectrum in order to minimize TOCSY transfer during spin-locking. Natural abundance 2D ^{13}C-^{1}H HSQC experiments were recorded without decoupling during acquisition of the ^{1}H FID. Resolution enhancement of the spectra was performed by a Lorentzian-to-Gaussian transformation for 1D spectra or by multiplication with a squared-bell function phase shifted by π / (2.3) for 2D spectra, and when necessary, a fifth order polynomial baseline correction was performed. Chemical shifts (δ) are expressed in ppm by reference to internal acetone (δ 2.225 for ^{1}H and δ 31.07 for ^{13}C). NMR data were processed using in-house developed software (J.A. van Kuik, Bijvoet Center, Department of Bio-Organic Chemistry, Utrecht University).

Micro-viscosity determination with electron spin resonance (ESR) spectroscopy

Cytoplasmic micro-viscosity of spores was determined by ESR spectroscopy as previously described (Dijksterhuis, Nijsse *et al.* 2007; van Leeuwen, van Doorn

et al. 2010). Perdeuterated TEMPONE (4-oxo-2,2,6,6-tetramethylpiperidine-N-oxy) (Sigma, St. Louis, MO) was used as a spin label. Potassium ferricyanide (FC) [$K_3Fe(CN)_6$] was used to quench the extracellular spin label signal. The final concentration of TEMPONE and FC in samples was 1 mM and 120 mM, respectively. At these concentrations the narrow line spectrum of TEMPONE originates exclusively from spin probe molecules in the cytoplasm, and therefore can be used to characterize cytoplasmic viscosity. The ESR spectra were recorded with a X-band 300E ESR spectrometer (Bruker Analytik, Rheinstetten, Germany).

The rotational correlation time (τC) of TEMPONE in the cytoplasm of ascospores was calculated from the ESR spectra making use of the equation $\tau C = K\Delta W_{+1}(\sqrt{h_{+1}}/h_{-1}-1)$, where K is a constant (Kuznetsov, Wasserman *et al.* 1971) with a value of $6.5\ 10^{-10}$ s, ΔW_{+1} is the peak-to-peak width of the low-field (left-hand) line of the spectra (in gauss) and h_{+1},and h_{-1} are the heights of the low-field (left hand) and high-field (right hand) lines, respectively (Kivelson 1960). The cytoplasmic micro-viscosity was calculated from the rotational correlation time using the Stokes-Einstein relationship $\tau R = 4\pi(a)^3\eta/3kT$, where a is the molecular radius of TEMPONE, η is the effective viscosity, k is the Bolzmann constant and T is the absolute temperature in Kelvin. The molecular radius of TEMPONE is usually defined as 3 Å (Keith and Snipes 1974).

Fourier transform infrared (FTIR) spectroscopy

Glasses were formed by drying 2.5 µl of a solution containing a total of 50 mg sugar and / or mannitol ml^{-1} on circular CaF_2 windows (2 x 13 mm) for at least 1 week in a cabinet that was continuous purged with dry air at a RH of 3 % at 24 °C. Infrared absorption of the samples was measured using a Perkin–Elmer (Massachusetts, USA) series 1725 Fourier transform infrared spectroscope equipped with an external beam facility to which a Perkin–Elmer IR-microscope was attached. The microscope was equipped with a narrowband mercury–cadmium–telluride LN_2 (liquid nitrogen)-cooled IR-detector. The temperature was regulated by a computer-controlled device activating the LN_2 pump, in conjugation with a power supply for heating the cell. The temperature of the sample was recorded separately using a PT-100 element that was located very close to the sample windows. The acquisition parameters were 4 cm^{-1} resolution, with 32 co-added interferograms, at a 3600-100 cm^{-1} wavenumber range.

Spectral analysis was described by Wolkers *et al.* (Wolkers, Oldenhof *et al.* 1998; Wolkers, McCready *et al.* 2001) making use of the infrared Data Manager Analytical Software. The FTIR spectra were measured from -10 to 140 °C with a temperature increase of 1.5 °C min^{-1}, back to -10 °C at a speed of 2 °C min^{-1}, and then to 140 °C again with a temperature increase of 1.5 °C min^{-1}. The melting of glasses was monitored by the position of the band region between 3600 and 3000 cm^{-1} (OH stretching vibration, νOH) and the region between 1300 and 1000 cm^{-1} (OH bending vibration, δOH). The band position was calculated as the average of

the spectral positions (n = 50) at 75 % of the total peak height. The relation between the wavenumber of the OH stretching band as function of the temperature of the samples was visualized in a plot. The point of crossing between the regression lines in both solid-like and liquid regions of the plot was used to estimate T_g (Wolkers, Oldenhof *et al.* 1998). The rate of change of the vibrational energy with temperature (cm^{-1} $°C^{-1}$), is defined as the wavenumber-temperature coefficient (WTC) and gives information on the average strength of hydrogen bonding between the compatible solutes (Wolkers, Oldenhof *et al.* 1998; Kets, Ijpelaar *et al.* 2004).

Stabilization of the protein LDH and blastospores

Rabbit L-lactic dehydrogenase (LDH) type XI (Sigma-Aldrich) was dissolved in 100 mM Na_2HPO_4. 6.6 units LDH was vacuum dried in an 1.5 ml Eppendorf tube for 1 h in a Savant SpeedVac DNA 110 Concentrator and exposed to 60 °C for 18 h in an oven (Model 0035, Horo, Stuttgart-Hedelf, Germany). This "dry-heat" exposure was done in the presence of 50-200 µg compatible solute (sugar or mannitol) per sample. After exposure, the samples were resuspended in 50 µl demi water. LDL activity was measured by determining the reduction of NADH at 340 nm after 1 min incubation in assay buffer [100 mM Na_2HPO_4 buffer, 0.1 mM β-NADH, 2 mM sodium pyruvate] (All products, Sigma-Aldrich).

Dried Blastospores (10^9 cfu g^{-1}) of *Trichoderma harzianum* T-22 (Trianum-P; Koppert, Berkel en Rodenrijs, the Netherlands) were washed three times with ice-cold 10 mM ACES buffer (N-(2-acetamido)-2-aminoethanesulfonic acid; pH 6.8) containing 0.02 % Tween-80 (Sigma-Aldrich) and pelleted by centrifugation for 5 min at 1100 g. A total of 10^4 spores was vacuum dried for 1 h in a Savant SpeedVac DNA 110 Concentrator in a volume of 20 µl in the presence of 50-200 µg compatible solute and then exposed to 45 °C for 24 h in a closed 2.0 ml Eppendorf tube in an oven (Model 0035, Horo). After heating, the spores were resuspended in 40 µl MEB and inoculated on 1-2 mm thin slides of MEA, which were positioned on objective glass slides. The percentage of germination was determined microscopically after 16 h incubation at 30 °C.

Results

Heat resistance of ascospores

A low rate of germination of *N. fischeri* ascospores is observed (0.33 ± 0.19 %) in the absence of a heat treatment at 85 °C. Several seconds of exposure to this temperature was sufficient to partially activate germination. Over 50 % of the ascospores germinated after a heat treatment as short as 20 s at 85 °C (data not shown). Maximal activation (95.1 ± 2.9 %) of *N. fischeri* ascospores was observed with a 2 min treatment at 85 °C (Fig. 1A). No *N. fischeri* spores survived after a 30 min treatment at 85 °C. Similar results were obtained with *T. macrosporus* ascospores. No germination of *T. macrosporus* ascospores was observed without heating (Fig. 1A). Partial activation occurred by a 20 s heat treatment (not shown), while maximal

activation (94.1 ± 4.1 %) was obtained after a heat treatment of 10 min. In contrast to *N. fischeri*, spores of T *macrosporus* even survived a heat treatment of 30 min (germination 93.3 ± 3.8 %).

In the next set of experiments, the effect of drying and heating on *N. fischeri* and *T. macrosporus* ascospores was tested. The ambient-dry spores were 1 h vacuum dried and kept for 7 days at 22-25 °C at a RH of 45-85 %, while the silica-dry spores were treated similarly but kept at a RH of 0.5-2 %. After drying, the spores were incubated for 1 h at 25, 60, 70 or 80 °C (dry heat) and subsequently their viability was measured microscopically after heat activation in ACES buffer (2 min at 85 °C). The silica-dry spores of *N. fischeri* and *T. macrosporus* were in general less sensitive to heat than the ambient-dry spores (Fig. 1B). Germination of ascospores of *N. fischeri* was 97 ± 0.4 % and 98 ± 0.9 % in the case of ambient and silica dried spores, respectively, after a 1 h treatment at 25 °C. These values were 85 ± 2.8 % and 77 ± 4.0 % in the case of the ascospores of *T. macrosporus*, respectively. Heat treatment for 1 h at higher temperature resulted in decreased germination. *N. fischeri* ascospores stored at ambient RH showed 68 ± 4.9 %, 42 ± 3.7 %, and 20 ± 2.5 %

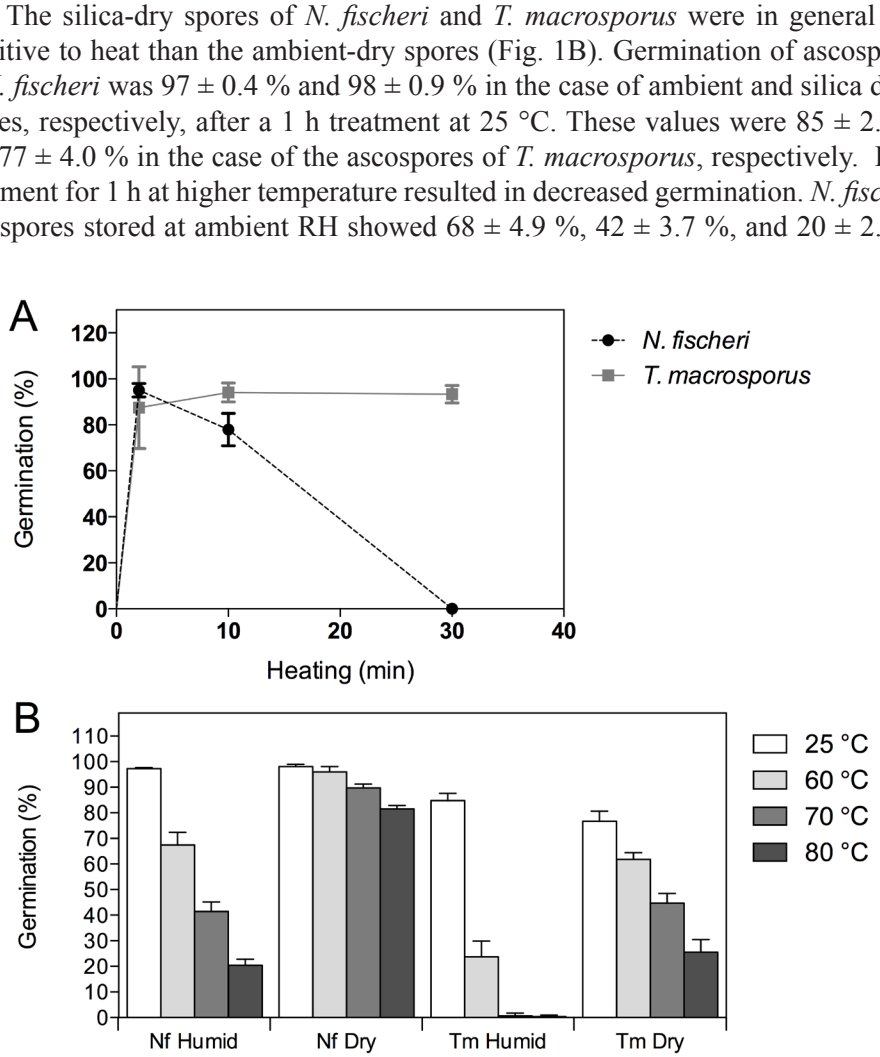

Figure 1. Germination (%) of *N. fischeri* and *T. macrosporus* ascospores after heating for 0-30 min at 85 °C in ACES buffer (A) or after drying, storage for 1 week at 22-25 °C at a RH of 45-85 % (humid) or 0.5-2 % (dry), a 1 h exposure at 25, 60, 70 or 80 °C in the absence of water, and a heat activation for 2 min at 85 °C in ACES buffer (B).

germination when incubated at 60, 70 and 80 °C, respectively. The silica-dry *N. fischeri* ascospores showed higher survival rates (96 ± 2.1 %, 90 ± 1.5 % and 82 ± 1.3 %, respectively). Germination of *T. macrosporus* ascospores was much more affected by the heat treatments. Exposure at 60, 70 and 80 °C resulted in germination of 24 ± 6.2 %, 0.6 ± 1.1 % and 0.3 ± 0.6 % for the ambient-dry spores and 62 ± 2.6 %, 45 ± 3.8 and 26 ± 4.9 % for the silica-dry ascospores, respectively.

Micro-viscosity of ascospores

ESR spectra of ascospores containing the spin probe TEMPONE were used for calculation of the (micro)viscosity of the cytoplasm. These spectra are a superposition of broad and narrow-line spectra. The narrow-line spectrum originates from TEMPONE that is present inside the cell. The broad component is a signal from TEMPONE / FC that is located extracellularly (residing in the cell wall and the medium). This component has to be subtracted from the recorded spectrum to obtain the narrow line spectrum, from which the micro-viscosity can be calculated (Fig. 2; Table 1). The calculated micro-viscosities before heating and cooling were 15.8 and 10.5 cP for *N. fischeri* and *T. macrosporus* ascospores, respectively (Table 1). These values were 14.2 and 9.8 cP after heating / cooling, respectively. After heating and cooling the ESR spectra remained intact and still contained narrow lines. However, the signal was less intense, which indicates a reduction of the amount of paramagnetic spin probe molecules.

Identification of oligosaccharides in ascospores of N. fischeri

Cell free extracts of ascospores from 40-day-old cultures of three independent isolates of *N. fischeri* (Fig. 3A) and *T. macrosporus* (Fig. 3B) were analyzed by HPLC to identify compatible solutes. The elution-patterns of the isolates of *T. macrosporus* were dominated by a peak with the same retention time as trehalose (RT 7.9 min). In addition, a small peak at the position of mannitol (RT 13.8 min) was observed. Some unknown components with a RT of 4.5-6 min and 14.8 min were also present. The cell free extract of *N. fischeri* also showed trehalose and mannitol peaks that were significantly lower and higher, respectively, than that of *T. macrosporus*. The HPLC pattern of *N. fischeri* was characterized by three additional peaks with a RT of 6.0, 6.4 and 6.9 min (Fig. 3A). As with *T. macrosporus* some other peaks were present at

Table 1. Effective cytoplasmic viscosity calculated from the rotational correlation time of intracellular TEMPONE of *N. fischeri* (Nf) and *T. macrosporus* (Tm) ascospores.

	Rotation correlation time (s)		Viscosity (cP)	
	Before heating	After heating	Before heating	After heating
Nf	$4.3\ 10^{-10}$	$3.9\ 10^{-10}$	15.8	14.2
Tm	$2.9\ 10^{-10}$	$2.7\ 10^{-10}$	10.5	9.8
water	$0.24\ 10^{-10}$	n.a.	0.89	n.a.

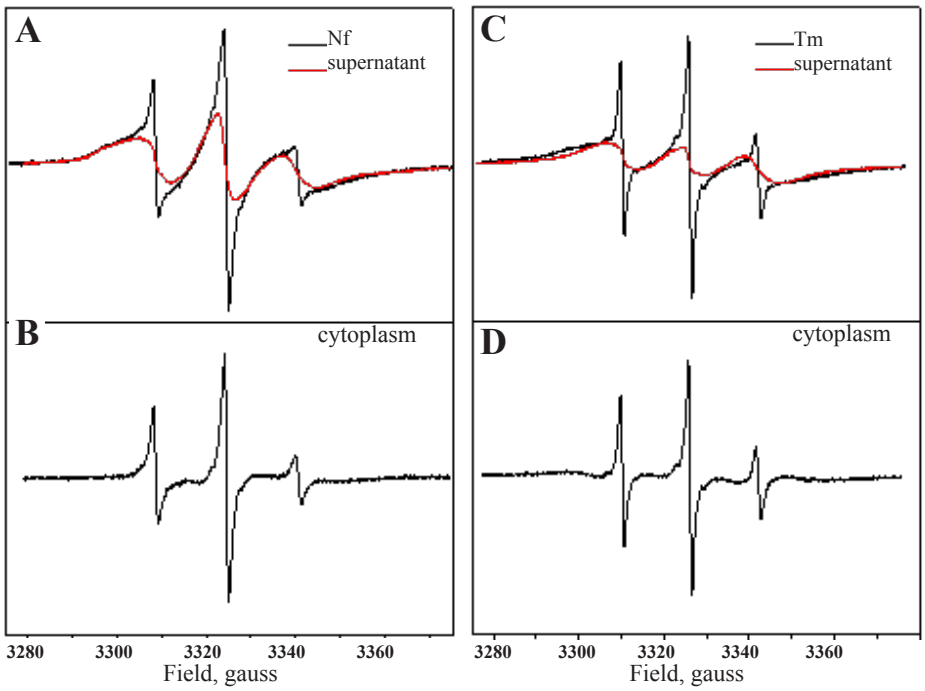

Figure 2. ESR of ascospores of *N. fischeri* (A, B) and *T. macrosporus* (C, D). The spectra of TEMPONE are composed of a signal originating from the cell wall and the medium (A, C) and an intracellular signal. The latter is calculated by subtracting the signal of the cell wall and the medium from the total signal (B, D). Supernatant is the extracellular solution in which the spores are suspended and composed of demi water, TEMPONE, and Fc.

4.5-6 min and 14.8 min.

Trehalose (α,α-trehalose) is characterized by an α-1,1-α glucoside bond. Trehalose has two anomers: Neotrehalose (α,β-trehalose) and isotrehalose (β,β-trehalose), which only differ in the orientation of the hydroxyl group of the anomeric carbon. The presence of mannitol and a disaccharide of 2 glucose molecules in *N. fischeri* (mol ratio of 1:0.5) and *T. macrosporus* (mol ratio 1.0:2.7) ascospores was confirmed by analysis of dried aliquots of cell free extracts with GLC-EIMS. The anomeric configuration of trehalose (Fig. 3C) was confirmed by ^{1}H-NMR spectroscopy (data not shown). No free glucose or other mono / disaccharides and alditols (e.g. arabitol) were identified by GLC. Note that GLC cannot detect saccharides with a degree of polymerization (DP) larger than 2 (see below).

Methanolysis degrades oligosaccharides into their monomers. Methanolysis followed by GLC on cell free extracts of *T. macrosporus* showed only glucose stemming from trehalose. Cell free extracts of *N. fischeri* showed a very high ratio of glucose compared to mannitol (25:1). Far more glucose was present than expected if solely trehalose had been degraded to glucose by methanolysis. This suggested the presence of glucose-containing oligosaccharides larger than trehalose. In order to demonstrate the presence of these oligosaccharides, TLC and MALDI-TOF-MS

were performed. TLC indeed showed the presence of larger oligosaccharides in the cell free extract (Fig. 3D). The oligosaccharides ran at the same height as the plant disaccharide sucrose, the trisaccharide raffinose, the tetrasaccharide verbascose and the pentasaccharide stachyose. MALDI-TOF-MS confirmed the presence of tri-, tetra- and pentasaccharides in the cell free extract of *N. fischeri* ascospores by showing the corresponding pseudomolecular ions $[M+Na]^+$ and $[M+K]^+$ (Table 2). These data also suggest the presence of a hexasaccharide in lower amounts.

Quantification of soluble sugars and polyols in N. fischeri and T. macrosporus ascospore extracts

The amount of compatible solutes in ascospores was calculated from HPLC analysis using calibration curves (Fig. 4A). For the quantification of the newly discovered oligosaccharides we determined their structure and used synthesized oligosaccharides for calibration (see below). *N. fischeri* accumulated 2.8 ± 0.2 pg spore^{-1} mannitol,

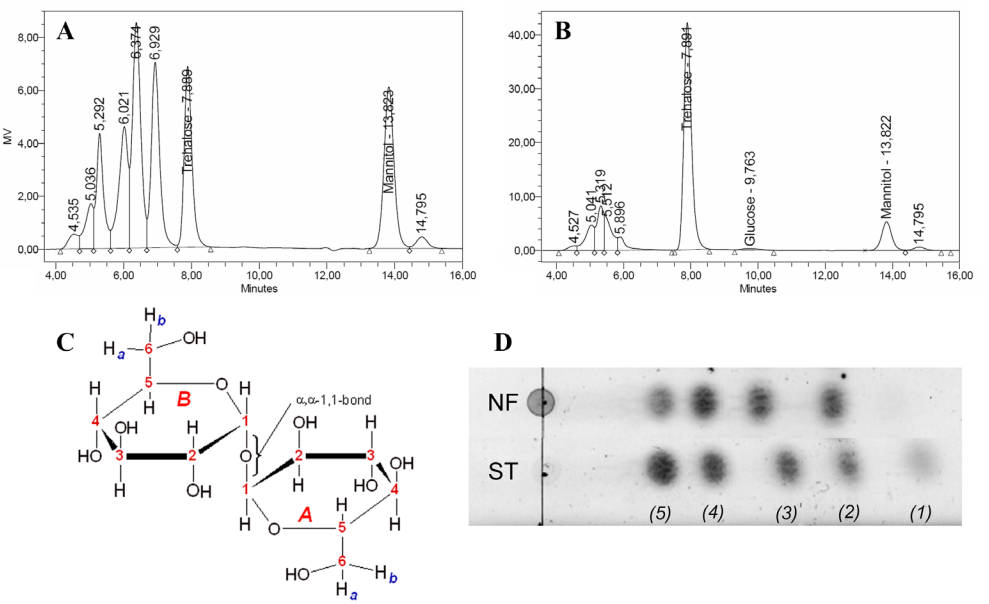

Figure 3. HPLC patterns of cell free extracts from *N. fischeri* (A) and *T. macrosporus* (B) ascospores that had been isolated from 40-day-cultures. Besides trehalose and mannitol, the *N. fischeri* extract has peaks with shorter retention times than trehalose. The solutes with RT; 6.0, 6.4 and 6.9 were identified as trehalose-based oligosaccharides (TOS). (C) Structure of trehalose, a disaccharide formed by two α-glucose units (A and B in red) linked by a 1,1 glucosidic bond. The protons are numbered as the number of the carbon to which it is linked. The two protons connected to C-6s are numbered 6a & 6b. The α or β definition involves the position of the hydroxyl group of the anomeric carbon (C-1). The anomeric protons (H-1) attached to the anomeric carbon are the least shielded protons, which gives clearly distinct NMR values when compared to the other protons (see results). (D) TLC of *N. fischeri* ascospore extract (NF) and a sugar standard (ST) consisting of glucose (1), trehalose (2), raffinose (3), stachyose (4) and verbascose (5). *N. fischeri* spores contain oligosaccharides of different size; a disaccharide, trisaccharide, tetrasaccharide and pentasaccharide.

Table 2. MALDI TOF MS of cell free extracts of *N. fischeri* (Nf) and *T. macrosporus* (Tm) ascospores.

-saccharide	M(Da)	$[M+Na]^+$	$[M+K]^+$	Nf	Tm
Mannitol	182	205	221	+	+
Di- (trehalose)	342	365	381	+++	+++
Tri- (3)	504	527	543	++	-
Tetra- (4)	666	689	705	+	-
Penta- (5)	828	851	867	+	-
Hexa- (6)	990	1013	1029	+/-*	-

*A very small amount of hexasaccharide seems to be present, but this couldn't be confirmed by TLC or HPLC.

2.8 ± 0.2 pg spore^{-1} trehalose, 2.7 ± 0.2 pg spore^{-1} of the trisaccharide, 4.3 ± 0.4 pg spore^{-1} of the tetrasaccharide and 2.0 ± 0.3 pg spore^{-1} of the pentasaccharide. *T. macrosporus* accumulated mainly trehalose (16.1 ± 3.4 pg spore^{-1}) with 2.7 ± 0.7 pg spore^{-1} mannitol and 1.0 ± 1.2 pg spore^{-1} glucose. Glucose was most likely the result of trehalose degradation in the spore extract after grinding. The dimensions of the ascospores were determined to calculate the concentration of the compatible solutes in the ascospores. To this end, dimensions of 101 and 45 ascospores of *N. fischeri* and *T. macrosporus* were measured by light microscopy, respectively. Cell wall thickness of ascospores of *N. fischeri* was measured using staining with carboxy fluoresceine, while autofluorescence of the cell wall was used in the case of *T. macrosporus* (Supplemental Figure 1). *N. fischeri* ascospores have the shape of a sphere ($V = 4/3\ \pi r^3$). The cell has a diameter of 4.0 ± 0.2 μm (r = 2 μm) excluding a cell wall that is approximately 0.3 μm thick. The shape of the *T. macrosporus* ascospores is oblate spheroid ($V = 1/6\ \pi ab^2$, with a = long and b = short dimension) (Dijksterhuis and Samson 2002). The inner cell has a short diameter of 4.5 ± 0.2 μm (a = 2.2 μm) and a long diameter of 5.2 ± 0.3 μm (b = 2.6 μm). The cell wall was approximately 0.4 μm thick (ornamentation not included). These data result in a volume of the cytoplasm of 34 ± 4 fl and 55 ± 8 fl for ascospores of *N. fischeri* and *T. macrosporus*, respectively. Taking these values into account, *N. fischeri* ascospores contain 449 ± 28 mM mannitol (Fig. 4B). The concentration of the sugars was less being 220 ± 17 mM, 156 ± 11 mM, 187 ± 18 mM and 70 ± 11 mM for trehalose, and the tri-, tetra-, and pentasaccharide, respectively. The ascospores of *T. macrosporus* contained 269 ± 27 mM mannitol, 774 ± 163 mM trehalose and 96 ± 115 mM glucose. Evidenced by the standard deviation, the *T. macrosporus* samples showed considerable variation in the accumulation of compatible solutes. Especially the amount of glucose varied probably due to variation in trehalose degradation after grinding of the ascospores.

NMR spectroscopy of the ascospore oligosaccharides

Oligosaccharides of cell free extracts of *N. fischeri* ascospores were separated on a Bio-Gel P2 column. Fractions were collected and monitored by TLC. Fractions containing one particular oligosaccharide were pooled, lyophilized and used for

structural analysis by NMR. The 1D ^{1}H-NMR spectra of the di-, tri-, tetra- and pentasaccharide are depicted in Fig. 5. The spectrum of the disaccharide was confirmed to represent α,α-trehalose. The molecule is depicted in Fig. 3C where the positioning of the protons is shown. The signals of the anomeric protons (H-1s) of the glucose residues (labelled as A and B in Fig. 3 and 5) of α,α-trehalose combine to one split peak at position (δ 5.182, 3J1,2 4.0 Hz) as a result of the symmetrical structure of the molecule (Fig. 3C). This holds also for the other individual ring protons as assigned and listed in Table 3 (Ohta, Pan *et al.* 2002; Lin, van Halbeek *et al.* 2007). The H-2s and H-4s (linked to the C-2s and C-4s) have characteristic chemical shifts at δ 3.634 and δ 3.436, respectively.

The spectrum of the trisaccharide was identical to that of the reference compound Glc-(α-1,6)-α,α-trehalose. The anomeric protons of the two glucose residues (labelled A and B in Fig. 3C and 5) of α,α-trehalose still overlap at δ 5.199 (3J1,2 3.9 Hz) and there is an extra anomeric signal at δ 4.960 (3J1,2 3.9 Hz), stemming from the α-1,6 linked glucose residue (labelled C in Fig. 5). The ratio of the signals of the anomeric protons is 2:1 as expected. The complete 1H assignment (Table 3) is in agreement with the assignment of reference compounds, which contain an α-1,6 linked glucose residue (Table 4).

The 1D ^{1}H-NMR spectra of the tetra- and pentasaccharides show the presence of an α,α-trehalose (H-1s, δ 5.199/5.20) linked to additional glucose residues with anomeric proton signals (δ 4.9753 and 4.975, 3J1,2 4.0 Hz). This corresponds to

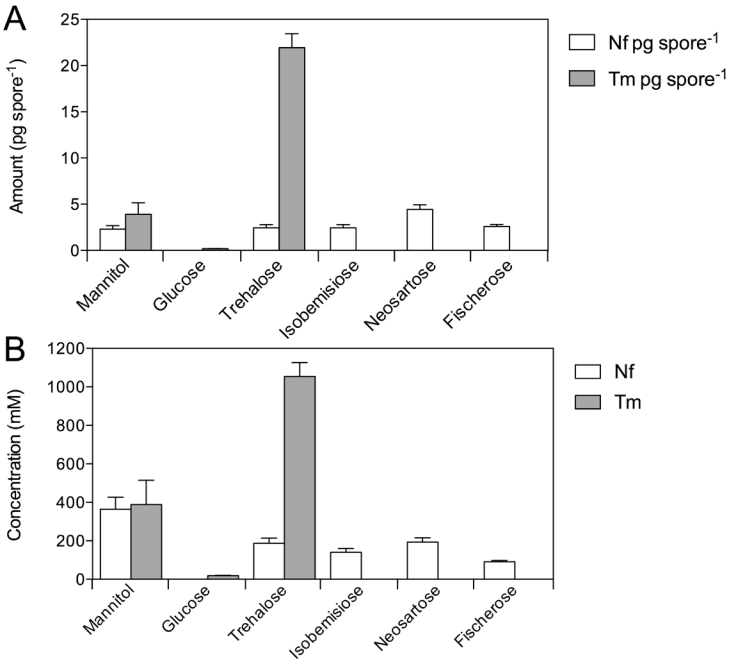

Figure 4. Compatible solutes in *N. fischeri* (non-shaded bars) and *T. macrosporus* (grey-shaded bars) ascospores expressed as pg spore^{-1} (A) and mM (B).

the anomeric proton signals of α-1,6 linked glucose residues (δ 4.96/4.97, 3J1,2 4.0 Hz)(van Leeuwen, Leeflang *et al.* 2008). 2D-NMR spectroscopy was used to assess whether these α-1,6 linked glucose residues are on one side or both sides of trehalose (see below). The 1D ^{1}H-NMR spectrum (Fig. 5) of the tetrasaccharide showed the signal of anomeric protons of the two α-1,1-α linked glucose residues (labelled as A and B in Fig. 5 and 6) of α,α-trehalose at δ 5.199/5.202 (3J1,2 3.8 Hz). The splitting of this signal and the difference in chemical shift of the anomeric proton signals of the two α-1,6 linked glucose residues (labelled as C and D in Fig. 5 and 6) at δ 4.973 and δ 4.961 (3J1,2 3.9 Hz), respectively, already suggest an asymmetrical structure as depicted in Fig. 4. The anomeric ratio of A : B : C : D of 1 : 1 : 1 : 1 agrees with a tetrasaccharide.

The TOCSY (200 ms), HSQC and ROESY (200 ms) 2D-NMR spectra of the tetrasaccharide are presented in Fig. 7. The ^{13}C chemical shifts are shown in Table 5 and used for the HSQC spectra. Starting points for the interpretation of the TOCSY spectra were the anomeric signals of the various residues. Comparison of TOCSY spectra with increasing mixing times (20, 40, 80, 200 ms) allowed the assignments of the sequential order of the chemical shifts belonging to the same spin system. Connectivities from H-1 to H-2,3,4,5,6a,6b were traced for residues A, B, C and D (Fig. 7) but due to overlap, some uncertainties could not be resolved on the basis of the TOCSY data alone. Additional assignments and confirmation of the assignments were obtained from ROESY cross-peaks and by correlating the ^{1}H resonances to the corresponding ^{13}C resonances in the HSQC spectrum. All ^{1}H and ^{13}C chemical shifts of the A, B, C and D glucose residues (Fig. 7) have been assigned by 2D-NMR experiments (Table 3, 5) and compared to reference data (Table 4).

The ^{1}H chemical shifts of the set of residues A (H-1, δ 5.199) and B (H-1, δ 5.202) reflect an α,α-trehalose residue, of which one of the glucose residues (in this case B) is substituted at C-6, suggested by the δ values of B H-4, B H-5 and B H-6a. The TOCSY C H-1 track (H-1, δ 4.973) showed the complete scalar coupling network H-1,2,3,4,5,6a,6b (Fig. 7), corresponding with the values for a [1→6)-α-D-Glcp-(1→6)-] residue (Tables 3 and 4). The carbon positions of the B C-6a,b (δ 65.9) and C C-6a,b (δ 65.9) in the HSQC spectrum suggest that these atoms are involved in a glycosidic bond (downfield shift Δδ ~5 ppm). The ^{1}H and ^{13}C chemical shifts of the set of residue D (H-1, δ 4.961) are characteristic for a terminal [α-D-Glcp-(1→6)-] residue (Tables 3 and 4). In the ROESY spectrum, the anomeric H-1 of residues A, B, C and D revealed intra-residual cross-peaks with their H-2, due to the α-configuration of these glucose residues. The determination of the sequence of the glucose residues could be established through the assignment of the inter-ROE cross-peaks. On the ROESY C H-1 track, the inter-residual cross-peak at δ 3.97 and δ 3.75 indicated the glycosidic linkage between C(1→6)B. Furthermore, the ROE cross-peaks between D H-1 and C H-6a/6b (δ 3.97/3.76) can be observed (encircled in Fig. 7), indicating the glycosidic linkage between D(1→6)C. Remarkable are the inter-residual ROE cross peaks between A H-1 and B H-5 (δ 4.02), and between B H-1 and A H-5 (δ 3.82), suggesting a three dimensional structure of the α,α-trehalose

residue, having those protons in close proximity of each other. Based on all NMR data, the structure as depicted in Fig. 6 can be accepted as the primary structure for the trehalose-based tetrasaccharide.

The 1D ^{1}H-NMR spectrum (Fig. 5) of the pentasaccharide also showed the signal of the anomeric protons (H-1s) of the two α-1,1-α linked glucose residues (A and B) of α,α-trehalose at δ 5.199/5.201 (3J1,2 3.8 Hz). Furthermore, three other α-anomeric signals could be observed at δ 4.975 (3J1,2 3.7 Hz) and δ 4.965 (3J1,2 3.8 Hz), in the ratio 2:1 (labeled as C, C' and D in Fig. 6). The increase of the signal at δ 4.975, stemming from the anomeric proton of a [1→6)-α-D-Glcp-(1→6)-] residue, according to the NMR data of the tetrasacharide, suggests an elongation of the 1,6 linked glucose chain connected to trehalose by one glucose residue. Confirming evidence was obtained by 2D NMR spectroscopy (Fig. 8) as follows. All ^{1}H and ^{13}C chemical shifts of the A, B, C, C' and D glucose residues (Fig. 8) have been assigned (Table 3) and compared to reference data (Table 4).

TOCSY spectra with increasing mixing times (20, 40, 80, 200 ms), and additional information from ROESY and HSQC experiments, allowed the assignments of the sequential order of the chemical shifts belonging to the same spin system for residues A, B, C, C' and D (Fig. 8). The 1H chemical shifts of the set of residues A (H-1, δ 5.199) and B (H-1, δ 5.201) reflect the α,α-trehalose residue. The mostly overlapping TOCSY C/C' H-1 track (H-1s, δ 4.975), revealed the complete scalar coupling network H-1,2,3,4,5,6a,6b (Fig. 8), corresponding with the values for [1→6)-α-D-Glcp-(1→6)-] residues (Tables 3 and 5). On the TOCSY A/B H-1 track (H-1, δ 5.20), the complete scalar coupling network H-1,2,3,4,5,6a,6b (Fig. 8), was found, in agreement with the values as found for the α,α-trehalose residue in the tetrasaccharide (Tables 3 and 4). The carbon positions of the B C-6a,b (δ 65.9) and C/C' C-6a,b (δ 65.9) in the HSQC spectrum suggest again that these atoms are involved in a glycosidic bond (downfield shift Δδ ~5 ppm). The ^{1}H and ^{13}C chemical shifts of the set of residue D (H-1, δ 4.961) are characteristic for a terminal [α-D-Glcp-(1→6)-] residue (Tables 3 and 4). In the ROESY spectrum, the anomeric H-1 of residues A, B, C, C' and D (Fig. 8) revealed intra-residual cross-peaks with their H-2, due to the α-configuration of these glucose residues. The determination of the sequence of the glucose residues could be established through the assignment of the inter-ROE cross-peaks. On the ROESY C/C' H-1 track, the inter-residual cross-peak at δ 3.97 and δ 3.75 indicated the glycosidic linkage between C(1→6)B and the glycosidic linkage between C'(1→6)C. Furthermore, the ROE cross-peaks between D H-1 and C' H-6a/6b (δ 3.97/3.76) can be observed, indicating the glycosidic linkage between D(1→6)C'. As seen in the ROESY spectrum of (4), also in (5), inter-residual ROE cross peaks between A H-1 and B H-5 (δ 4.02), and between B H-1 and A H-5 (δ 3.82) were observed (Fig. 8), suggesting a three dimensional structure of the α,α-trehalose residue, having those protons in close proximity of each other.

Based on all NMR data, the primary structure for the trehalose-based pentasaccharide is similar to the tetrasaccharide, having an extra α-1,6 linked

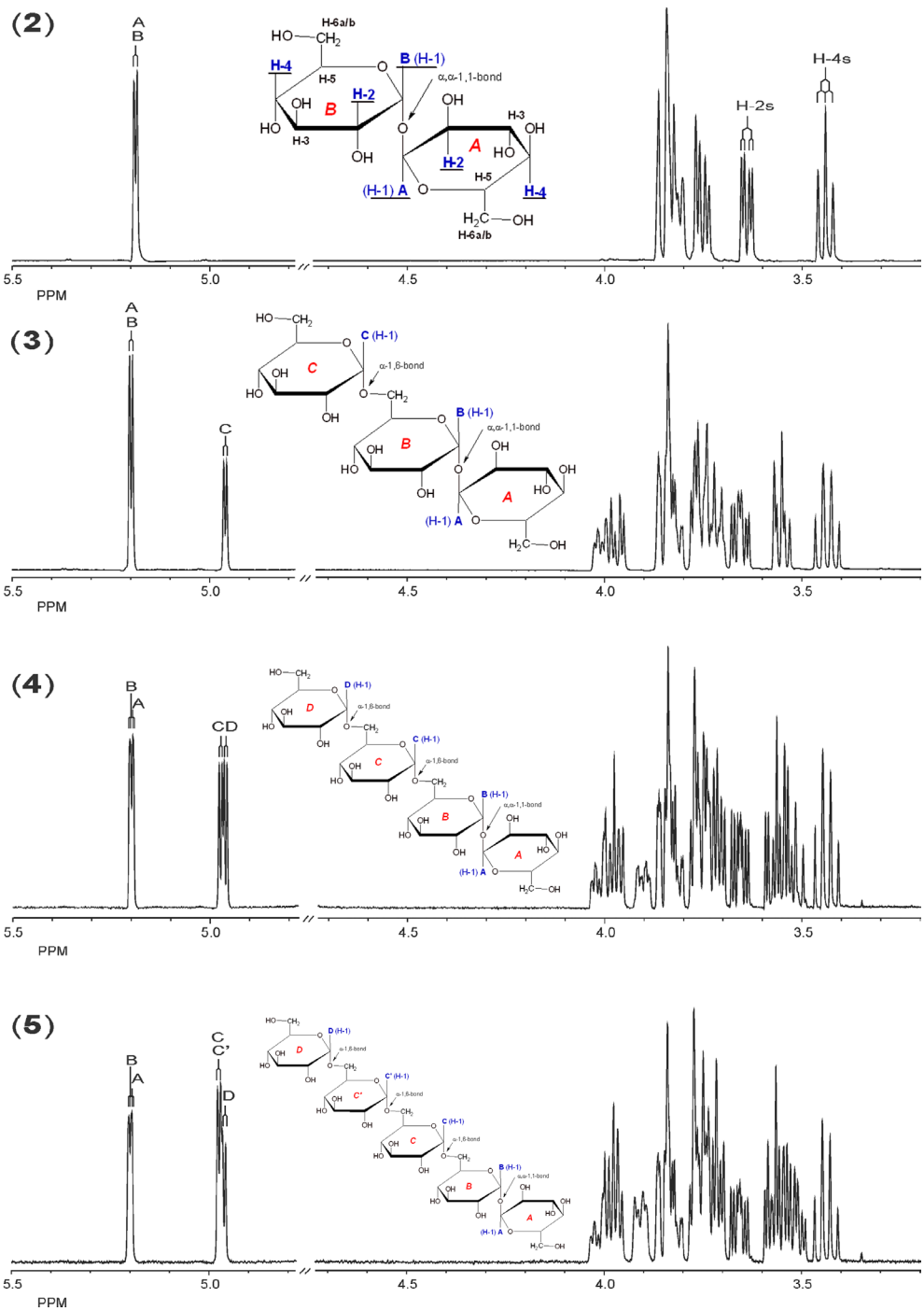

Figure 5. 1D ^{1}H-NMR spectra of the di- (2), tri- (3), tetra- (4) and pentasaccharide (5) isolated from a cell free extract of *N. fischeri* ascospores.

Table 3. ^{1}H chemical shifts of D-glucopyranose residues of the di-, tri-, tetra-, and pentasaccharides isolated from ascospores of *N. fischeri*, referenced to internal acetone (δ 2.225).

Residue		**(2)**	**(3)**	**(4)**	**(5)**
A	H-1	5.182	5.199	5.199	5.199
	H-2	3.62	3.65	3.65	3.65
(1→1)-α-Glcp	H-3	3.84	3.84	3.84	3.84
	H-4	3.436	3.447	3.448	3.448
	H-5	3.82	3.82	3.82	3.82
	H-6a	3.85	3.84	3.84	3.84
	H-6b	3.74	3.75	3.76	3.76
B	H-1	5.182	5.199	5.202	5.201
	H-2	3.62	3.67	3.67	3.67
α-Glc*p*-(1→1)	H-3	3.84	3.85	3.85	3.85
	H-4	3.436	3.54	3.54	3.55
	H-5	3.82	4.02	4.02	4.02
	H-6a	3.85	3.97	3.97	3.98
	H-6b	3.74	3.75	3.75	3.76
C	H-1	-	-	4.973	4.975
	H-2	-	-	3.58	3.58
→6)-α-Glc*p*-(1→6)	H-3	-	-	3.71	3.72
	H-4	-	-	3.53	3.53
	H-5	-	-	3.90	3.92
	H-6a	-	-	3.97	3.98
	H-6b	-	-	3.75	3.76
C'	H-1	-	-	-	4.975
	H-2	-	-	-	3.58
→6)-α-Glc*p*-(1→6)	H-3	-	-	-	3.72
	H-4	-	-	-	3.53
	H-5	-	-	-	3.92
	H-6a	-	-	-	3.98
	H-6b	-	-	-	3.76
D	H-1	-	4.960	4.961	4.964
	H-2	-	3.55	3.55	3.55
α-Glc*p*(1→6)	H-3	-	3.73	3.73	3.73
	H-4	-	3.425	3.428	3.428
	H-5	-	3.72	3.72	3.72
	H-6a	-	3.84	3.84	3.84
	H-6b	-	3.76	3.76	3.76

Table 4. ^{1}H chemical shifts of D-glucopyranose residues of CASPER database and reference compounds.

Residue		CASPER (www.casper.organ.su.se)		α,α-trehalose (Lin *et al.*, 2007)	α,α-trehalose (reference compound)	α-Glc-(1→6)-α,α-trehalose (ref. comp.)
(1→1)-α-Glcp	H-1	-		5.18	5.182	5.199
	H-2	-		3.64	3.62	3.65
	H-3	-		3.84	3.84	3.84
	H-4	-		3.43	3.436	3.447
	H-5	-		3.81	3.82	3.82
	H-6a	-		3.85	3.85	3.84
	H-6b	-		3.75	3.74	3.75
α-Glc*p*-(1→1)	H-1	-		5.182	5.199	5.199
	H-2	-		3.62	3.67	3.67
	H-3	-		3.84	3.85	3.85
	H-4	-		3.436	3.54	3.54
	H-5	-		3.82	4.02	4.02
	H-6a	-		3.85	3.97	3.97
	H-6b	-		3.74	3.75	3.75
			*			
→6)-α-Glc*p*-(1→6)	H-1	4.97	***4.970***	-	-	-
	H-2	3.59	***3.57***	-	-	-
	H-3	3.74	***3.72***	-	-	-
	H-4	3.54	***3.49***	-	-	-
	H-5	3.90	***3.93***	-	-	-
	H-6a	3.98	***3.98***	-	-	-
	H-6b	3.77	***3.78***	-	-	-
α-Glc*p*-(1→6)	H-1	4.96	***4.961***	-	-	4.960
	H-2	3.57	***3.55***	-	-	3.55
	H-3	3.74	***3.72***	-	-	3.73
	H-4	3.44	***3.42***	-	-	3.425
	H-5	3.73	***3.72***	-	-	3.72
	H-6a	3.86	***3.85***	-	-	3.84
	H-6b	3.77	***3.78***	-	-	3.76

*Italic and bolt numbers are from the reference compound isomaltotriose

glucose residue in the glucose chain linked to α,α-trehalose (Fig. 6). We propose the names neosartose and fischerose for the newly found tetra- and pentasaccharide, respectively. The trisaccharide isolated from *N. fischeri* was already dubbed isobemisiose (Hendrix and Salvucci 2001).

Glass transition temperature and density of sugar / polyol solutions

Samples composed of trehalose, isobemisiose, neosartose, fischerose, sucrose, stachyose, verbascose, and mannitol and mixtures thereof were analysed by FTIR spectrometry. Spectra were recorded from -10 to 140 °C, back from 140 to -10 °C, and again from -10 to 140 °C. The glass transition temperature (T_g) and the wave number-temperature coefficient (WTC) were deduced from the FTIR spectra. WTC

Table 5. ^{13}C chemical shifts of D-glucopyranose residues of the isolated di-, tri-, tetra-, and pentasaccharides isolated from ascospores of *N. fischeri*, referenced to the methylcarbon of internal acetone (δc 31.07).

Residue		(2)	(3)	(4)	(5)
A	C-1	93.8	93.8	93.8	93.8
	C-2	71.7	71.4	71.3	71.5
(1→1)-α-Glcp	C-3	73.2	73.2	73.2	73.1
	C-4	70.3	70.1	70.1	70.0
	C-5	72.8	72.8	72.8	72.8
	C-6	61.2	60.8	60.7	60.7
B	C-1	93.8	93.8	93.8	93.8
	C-2	71.7	71.4	71.3	71.5
α-Glc*p*-(1→1)	C-3	73.2	73.2	73.2	73.1
	C-4	70.3	70.1	70.1	70.0
	C-5	72.8	71.0	71.0	71.0
	C-6	61.2	65.6	65.9	65.9
C	C-1	-	-	98.2	98.2
	C-2	-	-	72.0	72.1
→6)-α-Glc*p*-(1→6)	C-3	-	-	72.5	72.5
	C-4	-	-	70.1	70.0
	C-5	-	-	70.8	70.8
	C-6	-	-	65.9	65.9
C'	C-1	-	-	-	98.2
	C-2	-	-	-	72.1
→6)-α-Glc*p*-(1→6)	C-3	-	-	-	72.5
	C-4	-	-	-	70.0
	C-5	-	-	-	70.8
	C-6	-	-	-	65.9
D	C-1	-	98.2	98.2	98.2
	C-2	-	72.0	72.0	72.1
α-Glc*p*(1→6)	C-3	-	73.7	73.8	73.8
	C-4	-	70.1	70.1	70.0
	C-5	-	73.8	73.8	73.8
	C-6	-	60.7	60.7	60.7

is defined as the rate of change of the vibrational energy with temperature (cm^{-1} $°C^{-1}$). Notably, the T_g of the first series recorded from -10 to 140 °C, T_{g1}, differed significantly of the second recording from -10 to 140 °C (note that this is the third series). The latter values (T_{g2}) are in line with published values (e.g trehalose, raffinose). The T_{g2} and WTC_2 values will be discussed first. T_{g2} increased with the DP in the case of the fungal and plant sugars: trehalose < isobemiose < neosartose < fischerose and sucrose < raffinose < stachyose < verbascose (Table 6). The T_{g2} of sucrose had the lowest value (56.6 °C), while fischerose had the highest T_{g2} value (124.4 °C). Notably, the T_{g2} of the plant sugars were lower compared to their fungal counterparts. For instance, T_{g2} of sucrose is 42 °C lower than that of trehalose, and T_{g2}

of raffinose is 13 °C lower than that of isobemisiose (Table 6). Moreover, mixtures of trehalose and mannitol with the fungal tri-, tetra- and pentasaccharides had a much higher T_{g2} compared to these mixtures with the plant counterparts.

Table 6. T_g and WTC of mannitol (Man), trehalose (Tre), isobemisiose (Iso), neosartose (Neo), fischerose (Fis), sucrose (Suc), raffinose (Raf), stachyose (Sta), verbascose (Ver) and mixtures thereof as determined by FTIR spectrometry.

	T_{g1}	T_{g2}	WTC_1	WTC_2
Mannitol (Man)*	n.a.	n.a.	n.a.	n.a.
Trehalose (Tre)	48.1	108	0.239	0.272
Isobemisiose (Iso)	44.3	120.4	0.185	0.200
Neosartose (Neo)	39.1	123	0.184	0.228
Fischerose (Fis)	38.8	125.4	0.195	0.256
Tre + Man (1:1)	26.6	115	0.200	0.246
Iso + Neo + Fis (1:1:1)	43.1	123.4	0.172	0.234
Tre + Iso + Neo + Fis (1:1:1:1)	40.6	122.8	0.175	0.223
Tre + Iso + Neo + Fis + Man (1:1:1:1:1)	44.2	115.1	0.187	0.207
Iso + Neo + Fis + Man (1:1:1:1)	43.4	52.7	0.185	0.208
Sucrose (Suc)	43.8	65.6	0.215	0.200
Raffinose (Raf)	40.1	107.8	0.213	0.264
Stachyose (Sta)	39.5	119.2	0.207	0.225
Verbascose (Ver)	33.8	123.8	0.213	0.273
Suc + Man (1:1)	21.6	63	0.200	0.179
Raf + Sta + Ver (1:1:1)	45.8	123.2	0.217	0.262
Suc + Raf + Sta + Ver (1:1:1:1)	41.2	98.1	0.213	0.252
Suc + Raf + Sta + Ver + Man (1:1:1:1:1)	44.6	52.2	0.218	0.216

* Due to crystallization of mannitol no values could be obtained

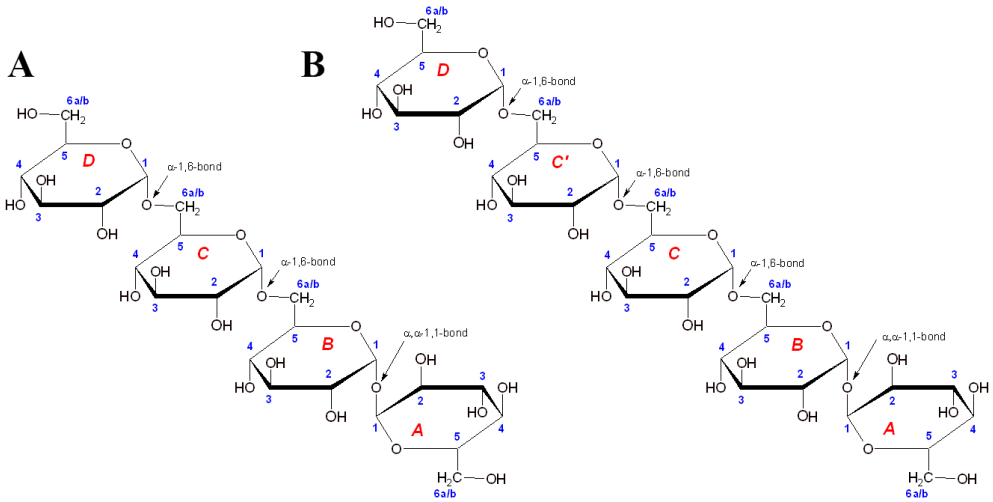

Figure 6. Schematic representation of the trehalose-based tetrasaccharide (left) and pentasaccharide (right) dubbed neosartose and fischerose, respectively. The protons are numbered in blue.

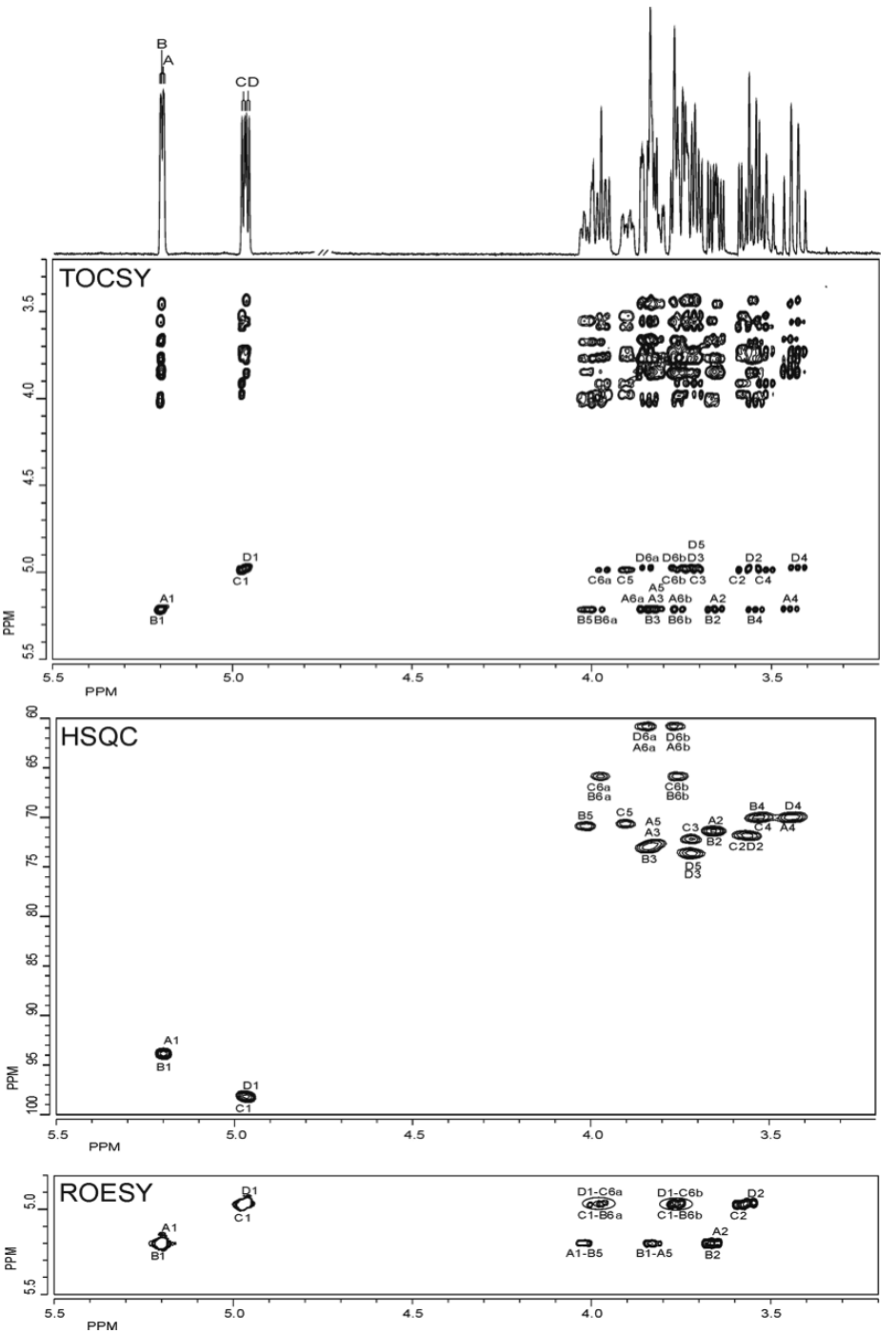

Figure 7. TOCSY, HSQC and relevant part of ROESY of the tetrasaccharide from a cell free extract of *N. fischeri* ascospores.

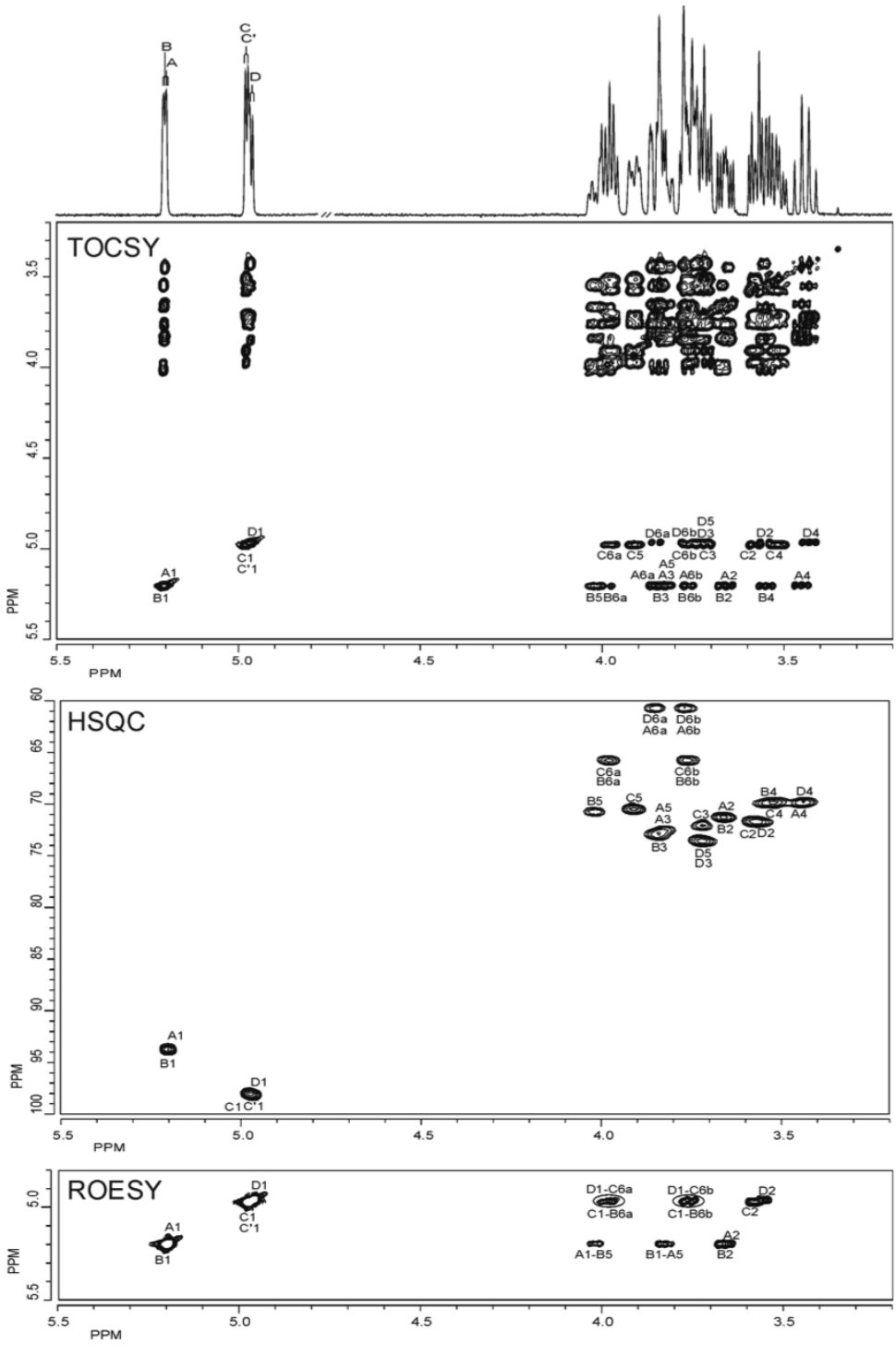

Figure 8. TOCSY, HSQC and part of ROESY of the pentasaccharide from a cell free extract of *N. fischeri* ascospores.

The WTC value represents the strength of hydrogen bonding in a glass. High WTC values indicate weaker hydrogen bonding and a glass that has a higher degree of freedom for rearrangement (Wolkers, Oliver *et al.* 2004). Thus higher WTC values indicate a less dense glass. Trehalose, raffinose, verbascose and fischerose showed the highest WTC_2 values. Glasses of other pure solutes or mixtures had lower values with by far the lowest value for the sucrose / mannitol mixture (Table 6). This indicates that the glass of the sucrose / mannitol mixture has the highest density.

T_{g1} and T_{g2} values were highly different (Table 6). The T_{g1} values ranged between 21.6 and 48.1 °C for trehalose and the sucrose / mannitol glass, respectively. T_{g1} decreased with increasing DP of both fungal and plant sugars: Trehalose > isobemiose > neosartose > fischerose and sucrose > raffinose > stachyose > verbascose (Table 6). The mixtures had a relatively high T_{g1} value. These data suggest that the way a glass has been formed has a large influence on Tg and henceforth the protective properties of a glass. The same holds for the WTC parameters. The WTC_1 values are all lower than the WTC_2, with highest values for trehalose, sucrose-based oligosaccharides

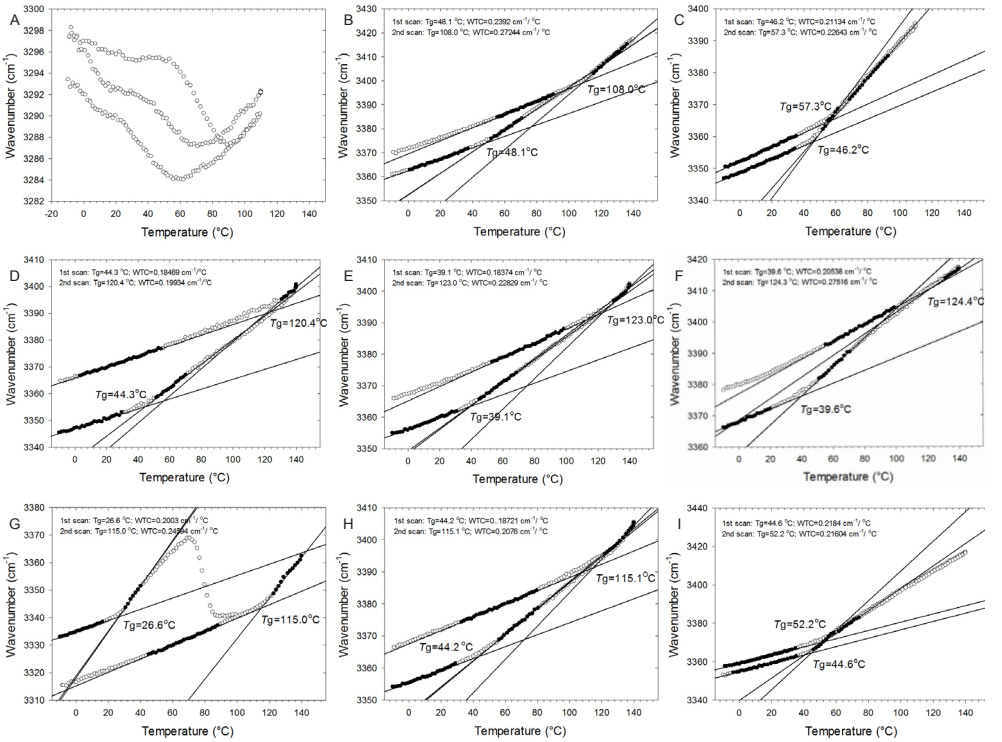

Figure 9. Linear regression of the wavenumber of the OH stretching band as function of the temperature of the samples consisting of mannitol (A), trehalose (B), sucrose (C), isobemisiose (D), neosartose (E), fischerose (F), trehalose + mannitol (G), man + tre + iso + neo + fis (H) and man + suc + raf + sta + ver (I). No regression line could be determined in the mannitol sample due to crystallization (A). Mannitol crystallization was also observed in the trehalose + mannitol mixture (G). The intersection of the regression lines represents the glass transition temperature with T_{g1} and T_{g2} determined from the 1st and 2nd scan, respectively. The steepness of the regression line corresponds with the WTC value (cm^{-1} $°C^{-1}$).

(0.207-0.239 cm^{-1} $°C^{-1}$) and low values for the trehalose-based oligosaccharides and its mixtures (0.172-0.195 cm^{-1} $°C^{-1}$). The lower WTC values of the trehalose-based oligosaccharides indicate tighter packed hydrogen bonds and a more densely packed glass structure.

Regression lines could not be obtained for T_g or WTC in the case of mannitol, trehalose + mannitol and sucrose + mannitol samples (Fig. 9). The sharp peak (around 3250 cm^{-1}) of the FTIR absorbance spectra of these samples indicates that mannitol crystallization has occurred (Fig. 10A,D). The peaks of fischerose (Fig. 10B) or the 1st scan of the mannitol + trehalose (Fig. 10C) sample are less sharp and indicate an amorphous state. The presence of mannitol results in a strong decrease of the T_{g2} of several sugar mixtures, but this effect was low in case of the sugar mixtures that were observed in ascospores of *N. fischeri*. This suggests that the tendency of mannitol to crystallize is suppressed in mixtures of TOS, but not (or less) in mixtures of sucrose-based oligosaccharides (SOS) such as the raffinose family oligosaccharides (RFO's).

Stabilization of Trichoderma harzianum conidia and LDH against heat

Bio-protective properties of isobemisiose, neosartose and fischerose were compared to that of trehalose, sucrose, RFO's, and mannitol. To this end, LDH and blastospores of T. harzianum were dried and subsequently heated as described above. After 18 h at 60 °C LDH had only 4.7 % of its initial activity (Fig. 11). Heat-exposed LDH was more active when oligosaccharides had been present. Activity was 13.2 – 99.6 % of that of the non-heat treated control. The activity of isobemisiose after exposure

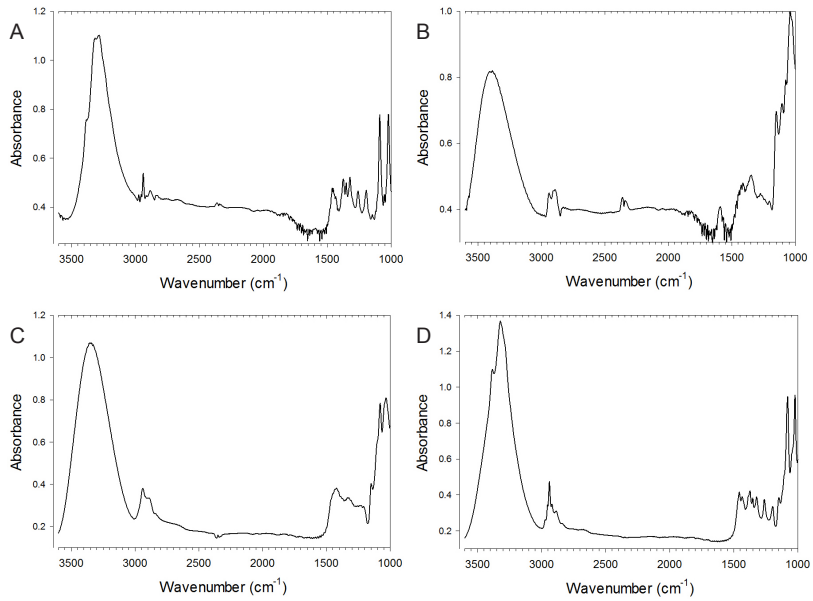

Figure 10. FITR absorbance spectrum of mannitol (A), fischerose (B) and trehalose + mannitol (C, D). The spectra of A, B and D are from the 2nd scan, while C is of the 1st scan. The change of peak shape of the trehalose + mannitol sample (C, D) is due to crystallization. The mannitol sample (A), but not fischerose (B) shows also crystallization.

(13.2 %) was not significant different from the control (4.7 %). Similarly, only 14.2 % LDH activity remained after exposure with neosartose. Fischerose protected LDH better (60.8 %) than mannitol (37.6 %). The mixtures of trehalose with TOS (22.8 %) or trehalose with TOS and mannitol (18.6 %) showed lower protection than the trehalose with mannitol mixture (97.9 %). Also very high protection was observed when trehalose (98.2 %), sucrose (99.2 %), raffinose (99.6 %) or stachyose (99.6 %) were added to LDH before drying (Fig. 11A).

Dried blastospores of T. harzianum were stored for 24 h at 45 °C, resuspended in ACES buffer and inoculated on an agar slice. None of the spores survived this treatment, whereas more than 90 % of the blastospores germinated after drying without storage at 45 °C (Fig. 11B). The presence of trehalose during incubation at 45 °C resulted in survival of 35.4 % of the spores, while presence of sucrose resulted in only 6.3 % survival. Two of the RFO's namely raffinose and stachyose

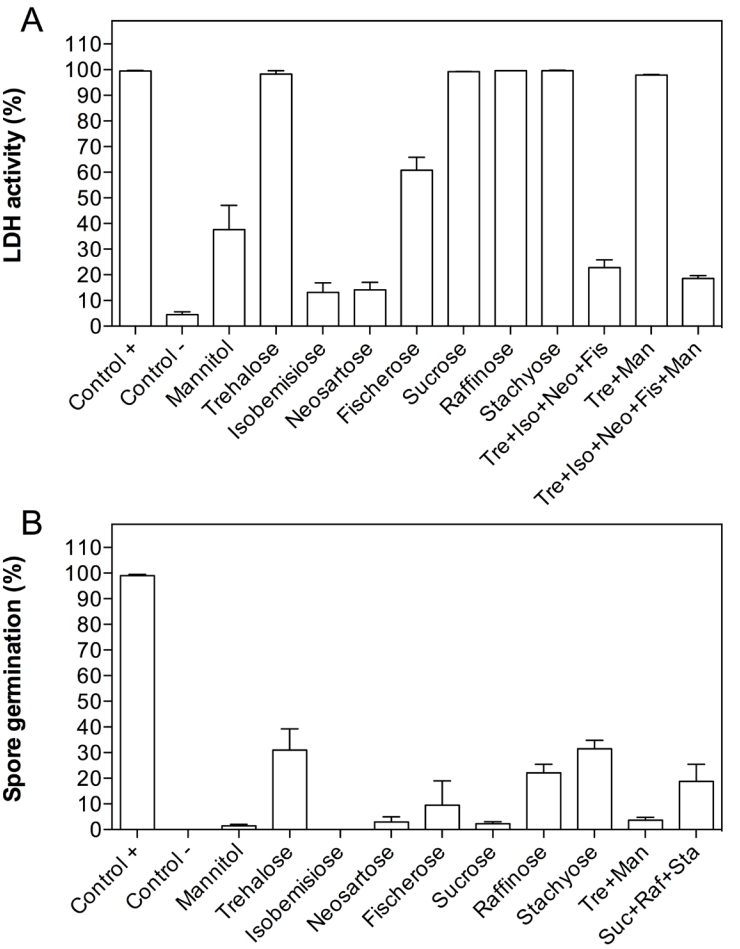

Figure 11. LDH activity (A) and viability of *T. harzianum* blastospores (B) after drying and exposure to 60 °C for 18 h (A) or 45 °C for 24 h (B).

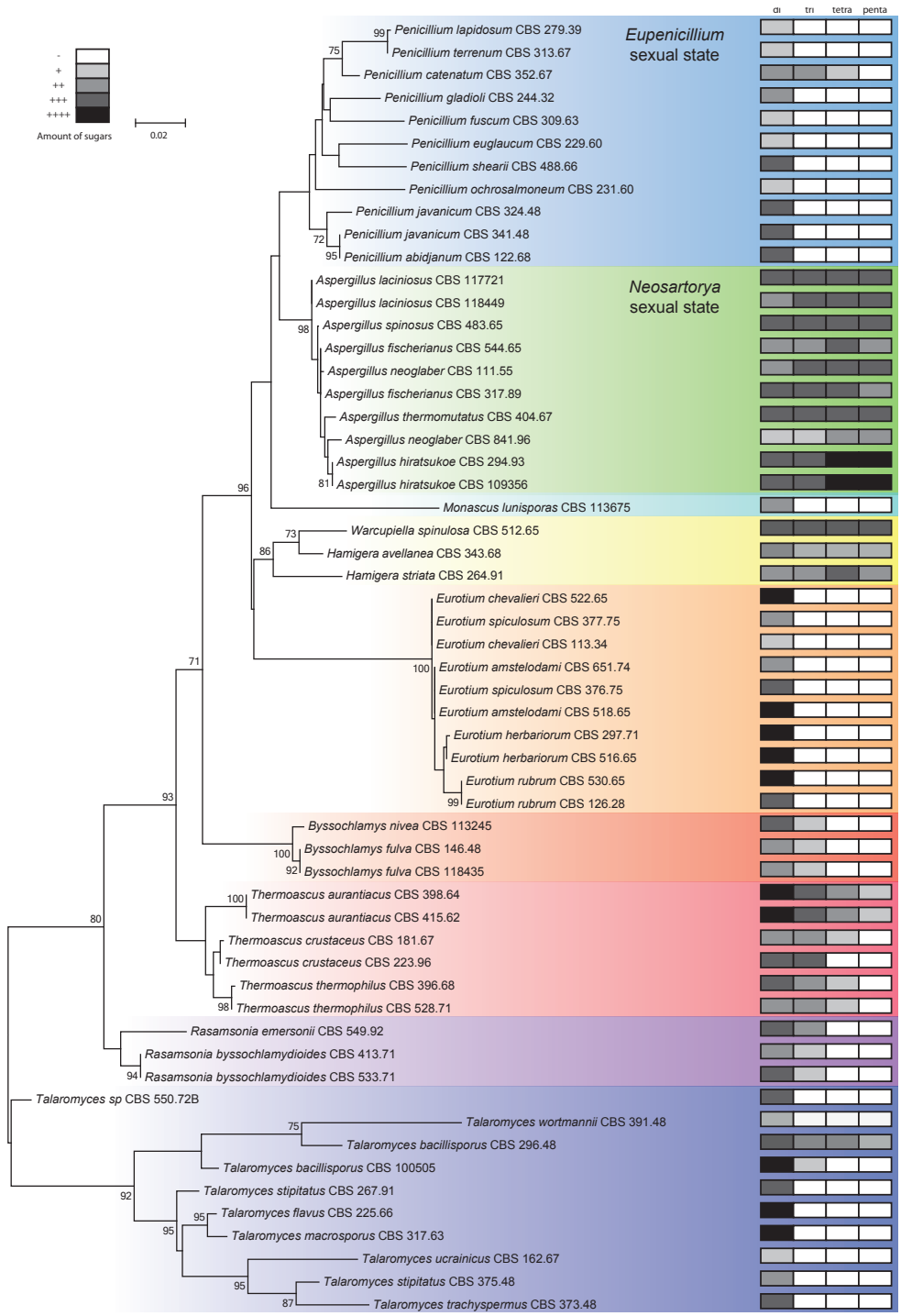

Figure 12. Phylogenetic tree of the Trichocomaceae family indicating the presence or absence of trehalose and TOS in the ascospores.

protected 39 % of the cells. The other sugars hardly protected the spores with 6.6, 2.3, 2.9 and 1.5 % survival for verbascose, isobemisiose, neosartose, and fischerose, respectively. Also mannitol showed a low protection with 3.25 % survival. Taken together, these data show that TOS are able to protect LDH against dry heat to some extent, but not blastospores.

Occurrence of trehalose-based oligosaccharides (TOS) in species of the order Eurotiales

A multi-locus phylogenetic tree was constructed with DNA sequences from the ribosomal internal translated spacer (ITS) and ribosomal large subunit (LSU) obtained from various Eurotiales belonging to the genera *Byssochlamys*, *Eupenicillium* (*Penicillium*), *Eurotium*, *Hamigera* / *Warcupiella*, *Monascus*, *Neosartorya* (*Aspergillus*), *Rasamsonia*, *Talaromyces*, and *Thermoascus* (Fig. 12). This selection includes well-known producers of extreme heat-resistant ascospores such as *Byssochlamys nivea*, *Byssochlamys fulva*, *T. macrosporus*, *Talaromyces flavus* and *N. fischeri*. The presence of oligosaccharides in the ascospore extracts was analysed by TLC. Note that polyols such as mannitol can't be detected with this method. All six tested species (10 strains) of the genus *Neosartorya* showed a TLC pattern of oligosaccharides identical to that of *N. fischeri*. HPLC analysis of the ascospore extracts confirmed the presence of isobemisiose, neosartose and fischerose (data not shown). According to TLC, three species of the *Hamigera* / *Warcupiella* clade contained four oligosaccharides, which had the same retention time as trehalose, isobemisiose, neosartose and fischerose (Supplementary Table 1). Two *Byssochlamys* and two *Rasamsonia* species contained a disaccharide and trisaccharide and ascospores of three studied *Thermoascus* species accumulated oligosaccharides larger than a disaccharide. *Thermoascus aurantiacus* accumulated a di-, tri-, tetra-, and pentasaccharide, while *Thermoascus thermophilis* strains and *Thermoascus crustaceus* accumulated a di- and trisaccharide or a di-, tri-, and tetrasaccharide dependent on the strain used. In contrast, the ascospores of the majority of the species from *Eurotium*, *Eupenicillium*, *Monascus*, and *Talaromyces* only showed a band with the retention time of trehalose. *Eupenicillium catenatum* and *Talaromyces bacillisporus* accumulated a di- and trisaccharide. Another isolate from *T. bacillisporus* (CBS 296.48) accumulated four oligosaccharides, which suggest the presence of a similar quartet of compounds as within the genus *Neosartorya*. Sequencing revealed that the two *T. bacillisporus* strains were genetically different, and must be different *Talaromyces* species. These data show that oligosaccharides are a hallmark of the genus *Neosartorya*, but also occur in other groups within the order Eurotiales and the family Trichocomaceae.

Discussion

Here we report the identification of novel oligosaccharides that are abundant in the ascospores of the fungus *N. fischeri*. Our data suggests that these oligosaccharides are wide spread in the order Eurotiales. The molecules consist of glucose moieties, have a DP (degree of polymerization) of 3-5 and are characterized by a trehalose (Glcα1-1αClc) core. One, two or three glucose moieties are linked to one side of the trehalose by means of α-1,6 bonds. These molecules, collectively dubbed as trehalose-based oligosaccharides (TOS) are called isobemisiose (DP 3), neosartose (DP 4) and fischerose (DP 5). Occurrence of isobemisiose has previously been reported in the whitefly *Bemisia argentifolii* (Hendrix and Salvucci 2001). On the other hand, neosartose and fischerose have never been reported to occur in nature before. There are indications that small amounts of even a TOS with DP 6 is present in the ascospores of *N. fischeri*.

TOS distinct of isobemisiose, neosartose, and fischerose have been described in the literature. *B. argentifoli* not only forms isobemisiose but also produces bemisiose. This trisaccharide consists of trehalose and a glucose molecule linked by a α-1,4 bond. Bemisiose is also formed by the bacterium *Mycobacterium smegmatis* (Besra, McNeil *et al.* 1993; Ohta, Pan *et al.* 2002; Tropis, Meniche *et al.* 2005) together with a number of other TOS including trehalose to which a glucose is linked via a β-1,6 bond. This TOS was also present in a cell free extract of the yeast *Saccharomyces cerevisiae* (Iwahara, Takegawa *et al.* 1993). The bacterium *Sinorhizobium meliloti* has been reported to produce a number of TOS with 1-4 glucose moieties α-1,2 linked to trehalose (Hisamatsu, Yamada *et al.* 1985; Breedveld and Miller 1994; Brique, Devassine *et al.* 2010). *S. meliloti* accumulates a number of TOS when grown in hyperosmolarity (Brique, Devassine *et al.* 2010). It is suggested that these TOS are the precursor of trehalose, which is released after hydrolysis of TOS. More is known about the function of oligosaccharides that are produced by plants. Most research is done on the sucrose-based oligosaccharides (SOS), including fructans and the raffinose family oligosaccharides (RFO's) (Valluru and van den Ende 2008). These molecules consist of a core of sucrose (i.e. glucose linked to fructose via an α-1,2-β bond). Fructans are polymers of fructose molecules with a sucrose molecule to the reducing end. RFO's consist of sucrose with one, two or three galactose moieties α-1,6 linked to the glucose moiety of sucrose and each other. The oligosaccharides are called raffinose, stachyose and verbascose, respectively. Striking is the similarity with their fungal homologs, our TOS consist of trehalose to which one, two or three glucose moieties are connected with an α-1,6 linkage. Fructans and RFO's are believed to act as membrane protectors under stress (Hincha, Zuther *et al.* 2002; Hincha, Zuther *et al.* 2003) via direct hydrogen binding (Milhaud 2004; Beck, Fettig *et al.* 2007). A flexible bond, such as the α-1,6 bond, between the saccharide moieties could be essential for insertion of the sugars between the lipids (Valluru and van den Ende 2008). Other authors claim that fructans and RFO's protect against oxidative-stress and lipid oxidation (Cacela and Hincha 2006; Agati, Matteini *et al.* 2007; Nishizawa, Yabuta *et al.* 2008; van den Ende and Valluru 2009). The concentration

of raffinose, stachyose and verbascose increase during maturation of plant seeds (Kuo, Vanmiddlesworth *et al.* 1988; Blackman, Obendorf *et al.* 1992; Bernallugo and Leopold 1995). Seed maturation in turn is correlated with increased stress-tolerance, longevity and glass formation (Brenac, Horbowicz *et al.* 1997).

Like SOS, TOS may also protect against abiotic-stress. The presence of TOS correlated with a thermophilic or thermotolerant nature of the fungus (note that the production of heat-resistant spores is not linked to a thermophilic or thermotolerant nature). A fungus is regarded as thermophilic when it can grow at or above 50 °C and not below 20 °C. Thermotolerant species have a maximum growth temperature at 45-50 °C and a minimum growth temperature below 20 °C (Cooney and Emerson 1964). *Neosartorya*, *Thermoascus* and *Rasamsonia* are well known thermotolerant or thermophilic fungi (Houbraken unpublished work; Mouchacca 1997; Mouchacca 2007; Houbraken, Spierenburg *et al.* 2012) and also *Byssochlamys* species are known to be moderate thermotolerant and able to grow at temperatures above 40 °C. *Hamigera* species can be found in hot climates and several species are known to be able to grow above 40 °C. *T. bacillisporus* that also seems to accumulate TOS is also (moderate) thermotolerant (Stolk and Samson 1972).

The question is what the advantage would be of accumulation of (mixtures) of TOS when compared to trehalose alone. Ascospores of *T. macrosporus* (trehalose accumulation) survived 1 h at 85 °C, while *N. fischeri* (TOS accumulation) could not. Strikingly, ascospores of *N. fischeri* survived desiccation and dry heat clearly better than *T. macrosporus*. This suggests that TOS may function in the protection of spores against drought and subsequent heat. Upon drying, the interior of the ascospore forms a biological glass and the properties of this glass is expected to function in spore survival during prolonged periods of high (between 30 and 50 °C) temperatures. The cytoplasm of ascospores is a matrix of proteins, membranes, DNA, RNA, organic acids, inorganic molecules and sugars with different polymerization. The stabilizing glass in ascospores formed during drying has other properties than a glass composed of a mixture of oligosaccharides. The properties of glasses in plants corresponds more to a glass composed of a sugar protein mix than solely a mixture of oligosaccharides (Buitink and Leprince 2008). Also other molecules (e.g. inorganic molecules, organic acids and amino acids) influence the properties of the glass, as was shown for citrate (Kets, Ijpelaar *et al.* 2004). The molecules that contribute to glass formation and its properties collectively determine the protective capacity. Thus, the context of the TOS inside the ascospores impacts their properties and thereby their function in the cell.

FTIR experiments showed that glasses prepared from pure oligosaccharides or mixtures of TOS, trehalose and / or mannitol behave different after heating during the first scan. T_{g1} values are invariably lower than the literature (T_{g2}) values. Heating in the first scan could remove any residual water molecules and / or rearrange the molecules in the glass leading to the so-called 'matured' glass, which has a higher melting temperature. As expected T_{g2} correlates with the DP (Table 6). To our surprise Tg1 seems to be negatively correlated with the DP. An explanation for this could be

that glass 'maturation' occurs faster in glasses composed of lower DP molecules. The T_{g1} of the TOS was higher than that of the corresponding plant homologs. However, the most remarkable finding was that mannitol did not crystallize in a TOS / trehalose mixture, but did so in a 1:1 mannitol / trehalose and mannitol / sucrose mixture. The T_g of mannitol / TOS mixtures was also much higher than the mannitol / RFO mixtures. It is tempting to speculate that one of the functions of TOS is preventing mannitol to crystallize in the cytosol. Mannitol has an important function in protection in water-containing environments and with its small size it costs less carbon to synthesize. Trehalose seems to be most effective in a dry state or in the phase between hydrated and dehydrated (low humidity). The combination of these compatible solutes would provide protection at strongly fluctuating water availabilities. So far, we could not show improved protection of LDH or blastospores in the presence of TOS and we have to conclude that functioning of these compounds is occurring in the context of other cell components in ascospores of *N. fischeri*.

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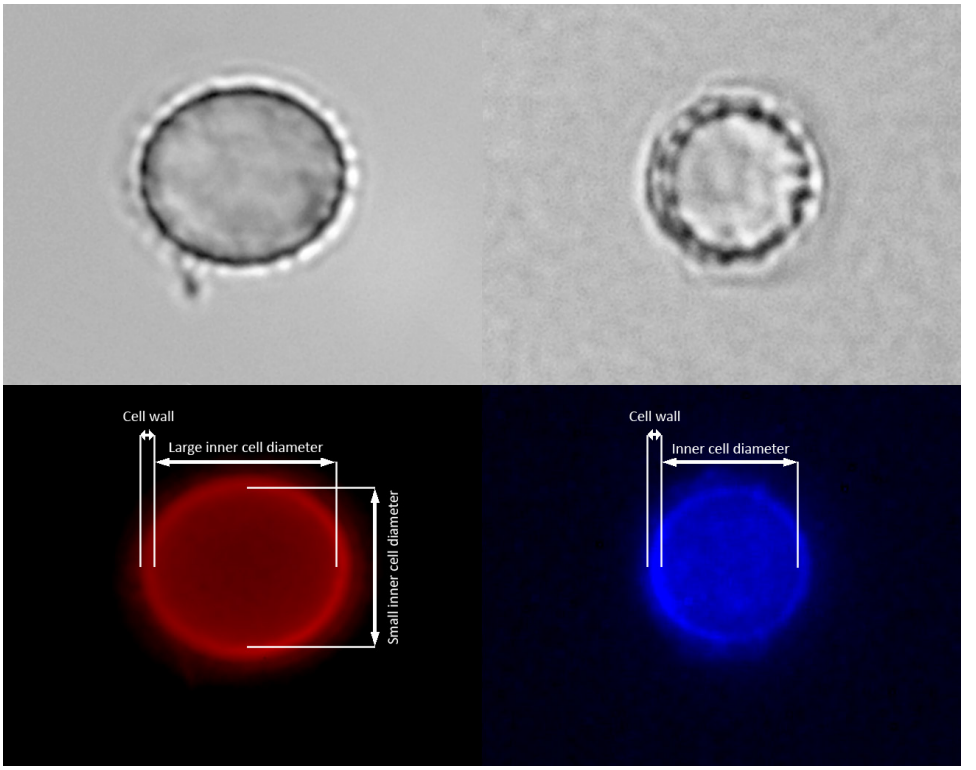
Supplemental Table 1. Strains used to study occurrence of trehalose (Tre), isobemisiose (Iso), neosartose (Neo), and fischerose (Fis) in ascospore extracts.

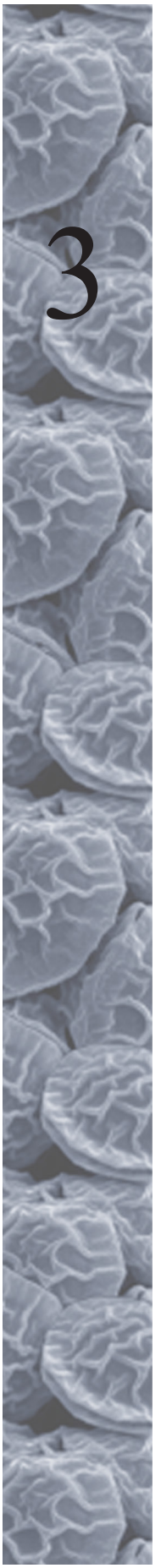
Species	Strain CBS #	Growth Media	°C	T_{max} °C [ref]	Ratio of sugars (%) Tre	Iso	Neo	Fis
Byssochlamys fulva	132.33	OA	30	45 [1]	>0	>0	0	0
Byssochlamys fulva	11845	OA	30	45 [1]	>0	>0	0	0
Byssochlamys nivea	100.11	OA	30	40 [1]	>0	>0	0	0
Eupenicillium abidjanum	122.68	OA	25	>37 [2]	100	0	0	0
Eupenicillium catenatum	352.67	OA	25	>37 [2]	56	23	13	7
Eupenicillium crustaceum	244.32	OA	25	<37 [2]	100	0	0	0
Eupenicillium euglaucum	229.6	OA	25		100	0	0	0
Eupenicillium javanicum	341.48	OA	25	>37 [2]	100	0	0	0
Eupenicillium javanicum	324.48	OA	25	>37 [2]	100	0	0	0
Eupenicillium lapidosum	279.39	OA	25		100	0	0	0
Eupenicillium ochrosalmoneum	231.60	OA	25	>37 [2]	100	0	0	0
Eupenicillium pinetorum	309.63	OA	25	<37 [2]	100	0	0	0
Eupenicillium shearii	488.66	OA	25	37 [2]	100	0	0	0
Eupenicillium terrenum	313.67	OA	25	37 [2]	100	0	0	0
Eurotium amstelodami	518.65	MEA40S	25	43-46 [3]	100	0	0	0
Eurotium amstelodami	651.74	MEA40S	25	43-46 [3]	100	0	0	0
Eurotium chevalieri	113.34	MEA40S	25	37-49 [3]	100	0	0	0
Eurotium chevalieri	522.65	MEA40S	25	37-49 [3]	100	0	0	0
Eurotium herbariorum	516.65	MEA40S	25	37-40 [3]	100	0	0	0
Eurotium herbariorum	297.71	MEA40S	25	37-40 [3]	100	0	0	0
Eurotium rubrum	530.65	MEA40S	25		100	0	0	0
Eurotium rubrum	126.28	MEA40S	25		100	0	0	0
Eurotium spiculosum	377.75	MEA40S	25		100	0	0	0
Eurotium spiculosum	376.75	MEA40S	25		100	0	0	0
Hamigera avellanea	343.68	OA	30		0	33	33	33
Hamigera striata	264.91	OA	30		n.a.	n.a.	n.a.	n.a.
Warcupiella spinulosa	512.65	OA	25		n.a.	n.a.	n.a.	n.a.
Neosartorya fischeri	544.65	OA	30	51-52 [3]	24	20	34	22
Neosartorya fischeri	317.89	OA	30	51-52 [3]	28	26	32	14
Neosartorya glabra	111.55	OA	30		18	27	34	21
Neosartorya glabra	841.96	OA	30		17	17	33	33
Neosartorya hiratsukae	294.93	OA	30	43 [4]	15	16	34	36
Neosartorya hiratsukae	109356	OA	30	43 [4]	20	15	34	31
Neosartorya laciniosa	117721	OA	30	>45 <50 [5]	24	27	32	18
Neosartorya laciniosa	118449	OA	30	>45 <50 [5]	17	27	33	24
Neosartorya pseudofischeri	404.67	OA	30		34	24	27	15
Neosartorya spinosa	483.65	OA	30		25	25	30	20
Rasamsonia byssochlamydioides	413.71	OA	40		n.a.	n.a.	n.a.	n.a.
Rasamsonia byssochlamydioides	533.71	OA	40		n.a.	n.a.	n.a.	n.a.
Rasamsonia emersonii	549.92	OA	40	55 [6]	79	21	0	0

Talaromyces bacillisporus	296.48	OA	36	45 [6]	47	30	15	8
Talaromyces bacillisporus	100505	OA	36	45 [6]	100	0	0	0
Talaromyces flavus	225.66	OA	30	40 [6]	100	0	0	0
Talaromyces macrosporus	317.63	OA	30	40 [6]	100	0	0	0
Talaromyces sp.	550.72B	OA	30	<40 [6]	100	0	0	0
Talaromyces stipitatus	375.48	OA	30	<40 [6]	100	0	0	0
Talaromyces stipitatus	267.91	OA	30	<40 [6]	100	0	0	0
Talaromyces trachyspermus	373.48	OA	30	40 [6]	100	0	0	0
Talaromyces ucrainicus	162.67	OA	30	<40 [6]	100	0	0	0
Talaromyces wortmannii	391.48	OA	30	<40 [6]	100	0	0	0
Thermoascus aurantiacus	398.64	OA	40	55-62 [3]	75	18	6	1
Thermoascus aurantiacus	415.62	OA	40	55-62 [3]	69	17	7	8
Thermoascus crustaceus	181.67	OA	40	55 [7]	n.a.	n.a.	n.a.	0
Thermoascus crustaceus	223.96	OA	40	55 [7]	63	37	n.a.	0
Thermoascus thermophilus	528.71	OA	40		70	30	0	0
Thermoascus thermophilus	396.68	OA	40		72	28	n.a.	0

References T_{max}: [1]Houbraken (unpublished), [2]Pitt (1979), [3]Domsch (1980), [4]Koutroutsos (2010), [5]Malejczyk (2013), [6]Stolk (1972), [7]Morgenstern (2012)



Supplemental Figure 1. Diameter and thickness of the cell wall of *T. macrosporus* (left) and *N. fischeri* (right) ascospores. The ascospore cell wall of *N. fischeri* was made visible by staining with 5-(and-6)-carboxyfluorescein. The ascospore cell wall of *T. macrosporus* is highly auto-fluorescent and no fluorescent dye was needed.



3

Two-stage maturation of extreme heat-resistant ascospores of *Neosartorya fischeri* (*Aspergillus fischeri*) involves reduction of bulk water and accumulation of trehalose and trehalose-based oligosaccharides

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Abstract

Neosartorya fischeri ascospores survive stresses such as high temperature (85 °C) and drought (<0.5 % RH). In this study, acquisition of stress resistance during maturation of *N. fischeri* ascospores was related to accumulation of compatible solutes, the presence of bulk water, and redox stability. Ascospores of 11-day-old cultures were killed by a 2 min treatment at 85 °C, while spores of 15-50 day-old cultures survived this treatment. Spores of 50-day-old cultures even resisted a 50 min treatment at 85 °C. Individual ascospores isolated from 11- and 15-day-old cultures contained 3.9 pg (454 mM) and 12.1 pg (1027 mM) compatible solutes, respectively. This amount increased to 15.4 pg (1051 mM) in ascospores of 50-day-old cultures. The composition of the compatible solutes in the ascospores changed during growth of the culture. Glycerol levels had disappeared in ascospores of 15-day-old-cultures, while mannitol levels decreased after day 20. In contrast, the relative amount of trehalose and trehalose-based oligosaccharides increased until 50 days of culturing. Bulk water, as measured by electron spin resonance (ESR) spectroscopy, was much higher in spores of 11-day-old cultures when compared to spores of 15- to 50-day-old cultures. Ascospore maturation also coincided with increased redox stability. This stability gradually increased during maturation. Dry heat storage of 3 days at 60 °C didn't affect the spin probe immobility or the redox stability of the polar cytoplasmic environment of dried ascospores. However, the redox stability of the more hydrophobic cytoplasmic environment (possibly in the proximity of lipid membranes) did decrease due to dry heat storage. Taken together, this study distinguishes two maturation stages of ascospores. The first stage is accompanied by a reduction of bulk water in the spores, the second stage is characterized by an increase of trehalose and TOS. Redox stability build up was observed during both stages.

Introduction

Many fungi belonging to the order Eurotiales form sexual ascospores within ascogenous cells that reside in the fruiting bodies (cleistothecia). These ascospores often survive high temperature (Dijksterhuis 2007) and drought (**Chapter 2**). For instance, ascospores of *Neosartorya fischeri* survive 85 °C for more than 10 min in an aqueous environment (Beuchat 1986; Houbraken, Dijksterhuis *et al.* 2012). Moreover, they survive a relative humidity (RH) lower than 0.5 % at a temperature of 60 °C for more than 7 days in a dried state (**Chapter 2**). These properties make that fungi such as *N. fischeri* survive mild food preservation treatments such as pasteurization, resulting in food spoilage. Knowledge about the mechanisms of heat resistance is required to minimize food spoilage by heat-resistant fungi. In addition, this knowledge may provide novel strategies to stabilize cells, vaccines, and biomolecules such as proteins.

Factors contributing to stress resistance of ascospores include a thick cell wall, low water content, high viscosity, and accumulation of protective compatible solutes (Dijksterhuis 2007; **Chapter 2**). Ascospores of *N. fischeri* and *Talaromyces macrosporus* exhibit a high cytoplasmic viscosity compared to conidia (asexual spores) (Dijksterhuis, Nijsse *et al.* 2007; **Chapter 2**). High cytoplasmic viscosity slows down the rate of metabolic reactions and therefore low amounts of reactive metabolites will be produced. Reactive metabolites such as oxygen radicals damage proteins, membranes and other cell components. The main compatible solutes that accumulate in heat-resistant ascospores of *T. macrosporus* are trehalose and mannitol (Dijksterhuis, Nijsse *et al.* 2007). Ascospores of *Neosartorya* species such as *N. fischeri* not only contain these compatible solutes but also trehalose-based oligosaccharides (TOS) (**Chapter 2**). These sugars consist of a trehalose core with 1 (isobemisiose), 2 (neosartose), or 3 (fischerose) glucose groups α-1,6 linked to one side of the molecule. TOS with a degree of polymerization (DP) of 3-5 also occur in ascospores of other genera within the Eurotiales (**Chapter 2**). Their presence correlated with the ability to grow at high temperature (thermophilic / thermotolerant growth style). TOS or sucrose-based oligosaccharides (SOS) (DP > 2) also accumulate in yeast (Iwahara, Takegawa *et al.* 1993), bacteria (Brique, Devassine *et al.* 2010), insects (Hendrix and Wei 1994; Wei, Hendrix *et al.* 1996), and plants (Kuo, Vanmiddlesworth *et al.* 1988; Blackman, Obendorf *et al.* 1992). It has been proposed that these molecules function as antioxidant (Chen and Yan 2005; van den Ende and Valluru 2009) and / or as protectors of membranes during the processes of dehydration and rehydration (Hincha, Zuther *et al.* 2002; Cacela and Hincha 2006). We have found indications that TOS are involved in survival of ascospores in dry heat (i.e. heat after drying of the spores). *T. macrosporus* ascospores, which do not contain TOS, were more sensitive to dry heat than *N. fischeri* ascospores (**Chapter 2**). Conversely, *T. macrosporus* ascospores were more resistant to high temperatures when present in solution (wet heat).

Conner *et al.* (1987) studied ascospore maturation of *Neosartorya glabra*. It was observed that ascospores from 25-day-old cultures were more stress resistant

than ascospores of 11-day-old cultures. This was accompanied by an increase of compatible solutes and changes in protein composition and cell wall structure (Conner, Beuchat *et al.* 1987). Here, we studied the composition of mixtures of compatible solutes, the cytoplasmic viscosity, and redox stability of ascospores during maturation of ascospores of *N. fischeri*. The data indicate that acquirement of stress resistance is a two-stage process. In the first stage bulk water in the spore is reduced, while in the second stage trehalose and TOS levels increase and mannitol levels decrease.

Material and methods

Strain, growth conditions and culture media

Cultures of *N. fischeri* (CBS 317.89) were routinely grown at 30 °C on oatmeal agar (OA) (Samson and Houbraken 2010). 10^6 ascospores were taken up in 100 µl 10 mM ACES [N-(2-acetamido)-2-aminoethanesulfonic acid] buffer, pH 6.8, with 0.02 % Tween-80 (Sigma-Aldrich, Zwijndrecht, the Netherlands). They were activated with a 2 min treatment at 85 °C and inoculated on 20 ml OA in a Petri dish. After 11 to 50 days of growth, fungal material of 3 Petri dishes was collected with a glass spatula and taken up in 9 ml ice-cold ACES buffer. Approximately 1 cm^3 of sterile glass beads (diameters 0.10-0.11 mm and 1.0 mm in a 1:1 ratio) was added and the fungal material was homogenized by vortexing (2 x 1 min) and sonication (5 min) using a Ultrasonic cleaner 2510E-MT (Branson Ultrasonics Corporation, Danbury, USA). Debris was removed by filtration through sterile glass wool and the ascospores were spun down at 5 °C at 1100 g for 5 min using a swing out rotor. The ascospores were taken up in ice-cold ACES buffer and the washing step was repeated 2 times. If not immediately used for experiments, the pellets of ascospores were stored in ACES buffer at -80 °C.

Germination of ascospores

Heat activated ascospores were inoculated in 200 ml malt extract broth (MEB) (Oxoid, Badhoevedorp, the Netherlands) using a final concentration of 2 10^7 spores ml^{-1}. The cultures were incubated at 30 °C in 250 ml Erlenmeyer flasks at 150 rpm. Samples (10 ml) were collected after 0-6 h culturing and immediately cooled on ice. The ascospores were pelleted at 5 °C for 5 min at 1100 g using a swing out rotor. They were washed twice with ice-cold ACES buffer as described above. The samples were frozen in liquid nitrogen and stored at -80 °C until further processing.

Heat inactivation of ascospores

Ascospores (50 µl ACES buffer containing 10^6 spores ml^{-1}) were heated for 0-70 min at 85 °C in 0.5 ml Eppendorf tubes in a water bath. The ascospores were cooled on ice and germination was assessed on malt extract agar (MEA) (Samson and Houbraken 2010) plates or on 1-2 mm thin MEA slices placed on top of object glasses using

10^2 spores and 10^4 spores, respectively. Colony forming units on the plates were counted after incubation for 2 d, while germination on the glass slides was quantified microscopically after incubation for 16 h.

Sugar and polyol analysis

Dormant and germinating (0-6 h) ascospores (± 2 10^8 spores) from 11- to 50-day-old cultures were broken using the Qiagen Tissuelyser (2 min at 30 strokes sec^{-1}) in a stainless steel grinding jar (Qiagen, Venlo, The Netherlands) cooled with liquid nitrogen. Grinding was resumed for an additional 2 min after adding 1 ml milli-Q water. Samples were thawed, transferred to a 2 ml Eppendorf tube and centrifuged at 10.000 g for 30 min at 4 °C. The supernatant was heated for 30 min at 95 °C and centrifuged again at 10.000 g for 30 min. The supernatant was filtered (0.2 µm Acrodisc Cr13mm Syringe filter, Pall Life Science, Mijdrecht, the Netherlands) and stored at -80 °C until further analysis.

Quantitative analysis of saccharides and polyols was carried out by high-performance liquid chromatography (HPLC) using a Sugar-Pak I cation-exchange column (Waters, Etten-Leur, the Netherlands) (**Chapter 2**). The column was heated (50 °C) during separation (Column heater, Waters) and the oligosaccharides and polyols were detected with an IR detector (2414 refractive index detector, Waters). The flow of the mobile phase (0.1 mM Ca EDTA) was 0.5 ml min^{-1} using a 515 HPLC pump and the pump control module II (Waters). Empower software (Waters) was used for peak integration and calculations. Trehalose, isobemisiose, neosartose, fischerose, mannitol, glucose and glycerol (0.01-0.50 % w/v) were used as reference.

Electron spin resonance (ESR) spectroscopy

PD-TEMPONE (perdeuterated 2,2,6,6-tetramethylpiperidone-N-oxyl), TEMPO (2,2,6,6- tetramethylpiperidine-1-oxyl) and TEMPOL (4-hydroxy-2,2,6,6-tetramethyl-piperidine-1-oxyl) (Fig. 1C) were purchased from Sigma (St. Louis, MO, USA) and used for ESR spectroscopy. Spectra of the spin probes were recorded with an X-band 300E ESR spectrometer (Bruker Analytik, Rheinstetten, Germany) using a modulation amplitude of 1 gauss and 2-5 mW power. Under these conditions spectra were not over-modulated and the signal was not saturated. The ESR spectra are the first derivatives of the absorbance spectra.

ESR can be used to determine the micro-viscosity, because the shape of ESR spectra is influenced by the environment of the spin label (Keith and Snipes 1974; Knowles, Marsh *et al.* 1976; Keith, Snipes *et al.* 1977). TEMPONE was used for ESR of ascospores in an aqueous solution (Dijksterhuis, Nijsse *et al.* 2007). To this end, ascospores were incubated for 1 min at RT in milli-Q water containing 1 mM TEMPONE and 120 mM $K_3Fe(CN)_6$ (FC) (Fig. 1C). After a 1 min centrifugation at 2000 g the supernatant was removed and the pellet with the remaining fluid was taken up with a Hamilton syringe. The spore pellet was transferred to a glass capillary with a diameter of 2 mm and stopped with cotton wool.

TEMPOL and TEMPO were used for ESR of dry spores (10^6 spores 50 µl in milli-Q water) in the absence of FC. FC is not necessary in this case because after drying a low amount of extracellular spin probe is present. The used spin probe concentration provides good signal / noise ratio and at the same time avoids concentration broadening of the spectra. Ascospores were labelled by resuspending ascospore pellets in 5 mM spin probe solutions followed by an incubation of 1 min at RT. The ascospores were pelleted (1 min, 2000 g) and excesses supernatant was removed. The spin-labelled spores were vacuum dried in 1.5 ml Eppendorf tubes for 1 h (Savant SpeedVac DNA 110 Concentrator, Thermo Scientific, Erembodegem-Aalst, Belgium) and stored for 3 d at room temperature (RT) or 60 °C at ambient humidity. The dry spores were transferred to a glass capillary with a diameter of 2 mm for ESR measurements.

The broadened TEMPONE spectrum was subtracted from the recorded spectrum (Fig. 1) to obtain the narrow lines from TEMPONE, which resides in the bulk aqueous phase of the spore cytoplasm (Fig. 1). The broad lines originate from paramagnetically broadened TEMPONE molecules. In our samples paramagnetically broadened TEMPONE is recorded from a sample that contained ascospore cell wall labelled with TEMPONE in the presence of FC. FC was used to broaden ESR spectra of extracellular TEMPONE (Chesnut 1977).

The rotational correlation time (τC) of intracellular spin probe in a liquid phase

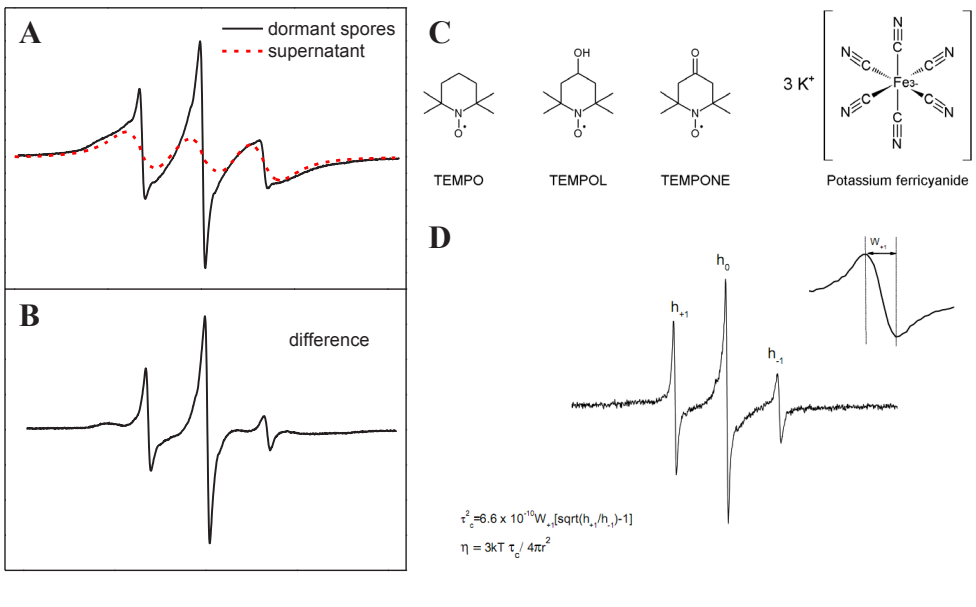

Figure 1. Decomposition of the spectrum of TEMPONE of *N. fischeri* ascospores in ACES buffer (A) and the resulting spectrum of TEMPONE in the cytoplasm at room temperature (B). The structure of the spin probes TEMPO, TEMPOL and TEMPONE, and the broadening agent potassium ferricyanide (C). Supernatant was composed of demiwater and spinprobe and Fc. ESR spectrum of TEMPONE in *N. fischeri* ascospores (D). Calculations of the rotational correlation time and related viscosity is based on the shape of ESR spectra of the spin label in the cytoplasm of the spores.

was calculated using the equation: $\tau C = K\Delta W_{+1}(\sqrt{(h_{+1}/h_{-1})} -1)$, where K is a constant with the value 6.7 10^{-10} s (Kuznetsov, Wasserman *et al.* 1971), ΔW_{+1} is the peak-to-peak width of the low-field (left-hand) line in gauss and h_{+1} and h_{-1} are the heights of the low-field (left-hand) and high-field (right-hand) lines, respectively (Fig. 1D) (Kivelson 1960). This formula contains only low-field (h_{+1}) and high-field (h_{-1}) lines because these lines are not influenced by the presence of other free radical signals such as melanin. If present, the low- and high-field lines are also the best resolved from the anisotropic spectra of immobilized spin label. With the Stokes-Einstein relationship; $\tau R = 4\pi(a)^3\eta / 3kT$, the cytoplasmic viscosity can then be derived from the rotational correlation time (τR), where a is the particle radius of the spin probe molecule, η is de effective viscosity, k is de Bolzmann constant and T is the absolute temperature in Kelvin (Fig. 1D). The radius of TEMPONE, TEMPOL and TEMPO is approximately 3 Å (Keith and Snipes 1974).

The cytoplasm of dried spores is in a glassy state. The degree of spin probe immobilization (TEMPOL and TEMPO) was determined from the solid-type spectra using ΔW_0 (central line width) and distance between two outermost extremes $2A_{max.}$ The change of the number of paramagnetic centers (number of paramagnetic spin probe molecules) in a sample can be used to estimate redox conversion of the spin label. As the shape of the ESR spectrum changes with temperature due to viscosity, the height of the spectral lines cannot be used to quantify the number of paramagnetic centres. To overcome this, the relative number of TEMPONE molecules in a sample was calculated by double integration of the ESR spectrum. Because the ESR spectrum is the first derivative of the absorption spectrum, the first integration gives the absorption spectrum, and the second derivative gives the area under the absorption spectrum, which is proportional to the number of spin probe molecules.

Metabolic processes inside the cell can reduce the spin probe TEMPONE to paramagnetically silent hydroxylamine. This redox conversion is indicative for metabolic activity. The permeability of the membrane for neutral spin probes as TEMPONE is very high and diffusion in and out the cell is much faster than the formation of paramagnetically silent spin probe molecules (Miller 1978). Therefore, the number of paramagnetic molecules (the intensity of the ESR signal) is the result of an equilibrium between spin probe reduction to hydroxylamine in the cell, hydroxylamine diffusion out of cells, its re-oxidation (by FC) and diffusion back into cells. To prevent re-oxidation of spin probe and estimate the metabolic activity of ascospores we measured in the absence of FC. The height of the central peak is a measurement of the signal strength.

Results

Compatible solutes in ascospores during maturation and germination

Colonies of *N. fischeri* formed ascospores after 3-4 days of cultivation on OA. The amount of glycerol, mannitol, trehalose, isobemisiose, neosartose and fischerose was determined in cell free extracts of ascospores harvested from 11- to 50-day-old

Table 1: Amount (pg) and concentration (mM) of solutes per ascospore of 11- to 50-day-old cultures.

Culture age (d)	11		15		20		25		32		40		50	
	pg	mM	pg	mM	pg	mM	pg	mM	pg	mM	pg	mM	pg	mM
Fischerose	0.7	24	1.7	61	2.1	73	2.2	76	2.1	74	2.3	80	2.5	87
Neosartose	0.5	23	2.9	128	3.7	163	3.3	144	3.2	142	3.4	147	4.3	188
Isobemisiose	0.4	24	2.0	117	2.3	135	2.4	138	2.4	139	2.7	157	3.0	173
Trehalose	1.2	92	2.0	153	2.2	172	2.2	173	2.6	199	3.0	236	3.8	293
Glycerol	0.7	214	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0
Mannitol	0.5	77	3.5	568	3.8	614	3.4	549	2.9	468	2.7	431	1.9	310
Total amount	**4.0**	**454**	**12.1**	**1027**	**14.1**	**1157**	**13.5**	**1080**	**13.2**	**1022**	**14.1**	**1051**	**15.5**	**1051**

cultures (Table 1). The total amount of these compatible solutes was 3.9 pg (454 mM) per ascospore of 11-day-old cultures. This amount had increased to 12.1 pg (1027 mM) in 15-day-old cultures. During further maturation the amount increased to a maximum of 15.5 pg (1051 mM) per ascospore of 50-day-old cultures. The composition of the polyols and oligosaccharides changed markedly during maturation (Table 1). The ascospores of 11-day-old cultures contained 0.7 pg glycerol. This solute was absent during later stages of culturing. Trehalose was the main solute in ascospores of 11-day-old cultures with 1.2 pg per spore. Levels of isobemisiose, neosartose, fischerose (all $\leq$ 0.7 pg spore^{-1}) and mannitol (0.5 pg spore^{-1}) were lower. All compounds showed increased abundance in ascospores of 15-day-old cultures with 3.5 pg spore^{-1} (568 mM) for mannitol and $\geq$ 1.7 pg for isobemisiose, neosartose, fischerose, and trehalose. The amount of TOS and trehalose increased further when cultures became older; i.e. 2.5-4.3 pg for TOS and 3.8 pg for trehalose per ascospore of 50-day-old cultures. In contrast, mannitol levels had decreased to 1.9 pg per spore (Table 1).

In order to study the dynamics of solute concentration during germination, ascospores of 40-day-old cultures were heat-activated and incubated in MEB medium. Morphological changes were not observed with light microscopy during the first three hours after heat activation. Swelling, accompanied with a slow disruption of the thick outer cell wall along the equatorial ridge, took place between 3 and 6 h of incubation and germ tubes had formed after 6 h (data not shown). Levels of mannitol, TOS and trehalose had already decreased from 13.6 to 4.8 pg spore^{-1} 30 min after heat activation (Table 2; concentrations of these solutes were not calculated because of the changes in cell volume due to swelling and germ tube formation between 3 and 6 h of incubation). The amount of TOS dropped approximately two-fold, while levels of trehalose and mannitol had even decreased four-fold. This was accompanied by the appearance of glucose (1.3 pg spore^{-1}), most probably as a result of degradation of the oligosaccharides. After one hour, levels of mannitol, TOS and trehalose had further decreased to a total amount of 1.3 pg spore^{-1}. Levels of isobemisiose, neosartose and fischerose dropped to 0.2, 0.3, and 0.1 pg spore^{-1} respectively, while levels of mannitol and trehalose had decreased to 0.4 and 0.3 pg

Table 2: Amount (pg) of solutes per activated ascospore of 40-day-old cultures 0-360 min after heat activation.

Time after activation (min)	0	30	60	90	120	180	240	360
Fischerose	1.7	0.8	0.1	0.0	0.0	0.0	0.0	0.0
Neosartose	4.1	1.8	0.3	0.1	0.1	0.1	0.0	0.0
Isobemisiose	2.5	1.0	0.2	0.1	0.0	0.0	0.0	0.0
Trehalose	2.6	0.6	0.3	0.1	0.1	0.0	0.0	0.0
Glucose	0.0	1.3	0.7	0.3	0.1	0.1	0.0	0.0
Glycerol	0.0	0.0	0.0	0.3	0.3	0.3	0.2	0.1
Mannitol	2.7	0.6	0.4	0.4	0.3	0.0	0.0	0.0
Total amount	**13.6**	**6.1**	**2.0**	**1.3**	**0.9**	**0.5**	**0.2**	**0.1**

spore^{-1}. These solutes had disappeared 360 min after heat activation. Glycerol started to appear after 90 min with an amount of 0.3 pg spore^{-1} and gradually decreased to 0.1 pg spore^{-1} between 180 and 360 min after heat activation.

Heat resistance of ascospores during maturation and germination.

The heat resistance of ascospores of 11- to 50-day-old cultures in ACES buffer was determined. To this end, ascospores were subjected to 85 °C for 0-60 min, after which they were plated (Fig. 2). Ascospores from 11-day-old cultures did not survive 2 min at 85 °C, while ascospores of 15-day-old cultures did survive this treatment (Fig. 2). More mature (older) ascospores showed a gradual increase in heat resistance. Some ascospores from 50-day-old cultures even survived 50 min at 85 °C.

Loss of heat resistance during germination was determined by subjecting heat-activated ascospores of 40-day-old cultures to a second heat treatment (85 °C) at different time points after activation (0-4 h) (Fig. 3). As expected, dormant (t = 0 h) and activated ascospores (30 min) were not affected by this heat treatment (240 s at 85 °C), but after 1 h the majority of the cells was killed (81 ± 5 %, Fig. 3). Resistance to 85 °C was completely lost 1.5 h after heat activation, even a 30 sec treatment at 85 °C killed all activated ascospores.

Viscosity of the cytoplasm of ascospores changes maturation and germination

The viscosity of the cytoplasm of ascospores during maturation and germination was studied with ESR spectroscopy. Fig. 1B shows the narrow line spectrum from intracellular TEMPONE. The narrow lines indicate fast rotation of the spin probe molecules. The distance of 16.1 gauss between the narrow lines of the ESR spectrum is characteristic for a polar (aqueous) environment (Golovina and Hoekstra 2002). Therefore, this spectrum can be attributed exclusively to the spore cytoplasm. The heights of the low-field and high-field lines and the width of the low-field line can be used to calculate a rotational correlation time and related effective cytoplasmic viscosity (see Material and Methods). The cytoplasmic viscosity of ascospores from

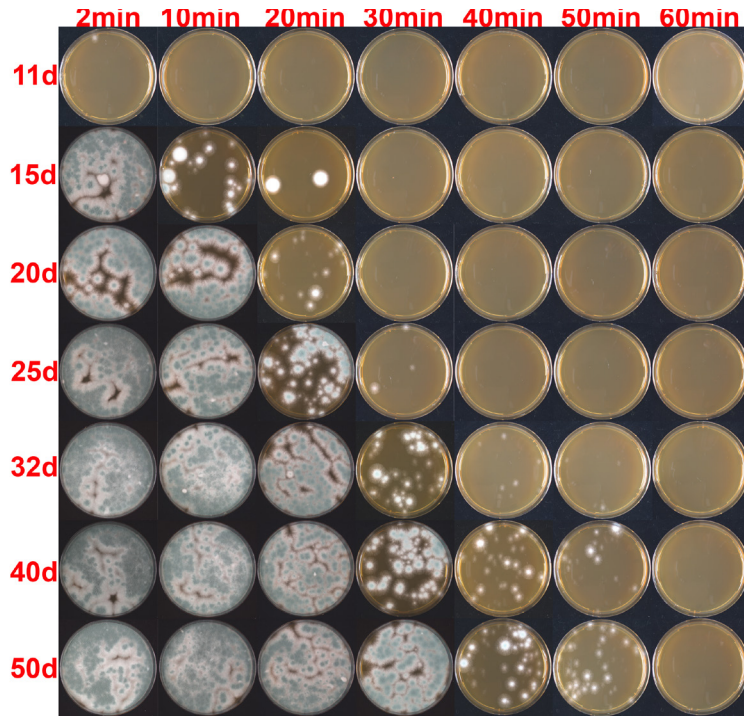

Figure 2. Survival of ascospores of *N. fischeri* from 11-50-day-old cultures after a 2-60 min treatment at 85 °C.

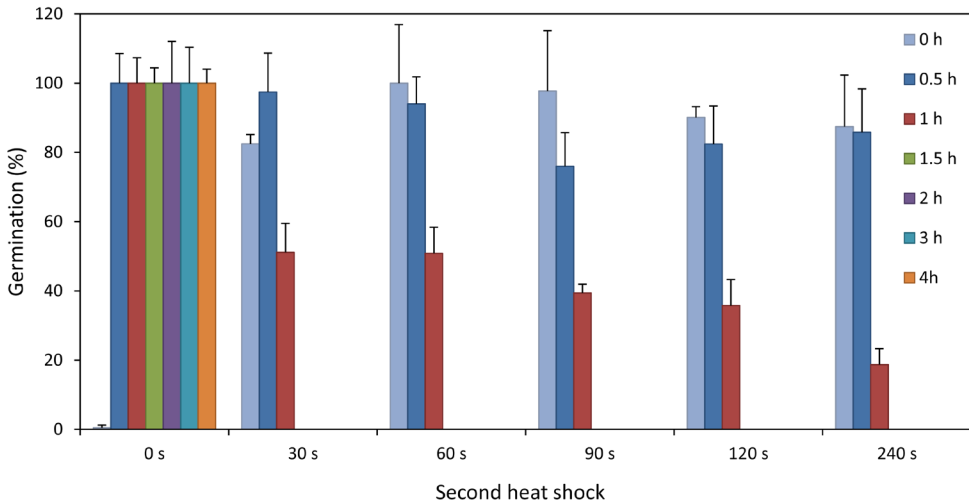

Figure 3. Heat sensitivity of ascospores from 40-day-old cultures 0-4 h after heat activation (2 min at 85 °C). The viability of the germinating ascospores was measured after a second exposure at 85 °C for 0-240 s.

Table 3. Rotation correlation time (τc), effective cytoplasmic viscosity (η), and the degree of anisotropy at 295 K calculated from the spectra of TEMPONE of ascospores of *N. fischeri* of 11-50-day-old cultures.

Culture age	Rotation correlation time τc (s) x10^{-10}	Viscosity η (cP)	Anisotropy h_0/h_{+1}
11d	1.45	5.4	1.40
15d	5.8	21.2	2.38
20d	5.7	20.8	2.08
25d	5.9	21.73	2.15
32d	5.0	18.4	2.10
40d	4.9	18.0	2.21
50d	5.6	20.3	2.17

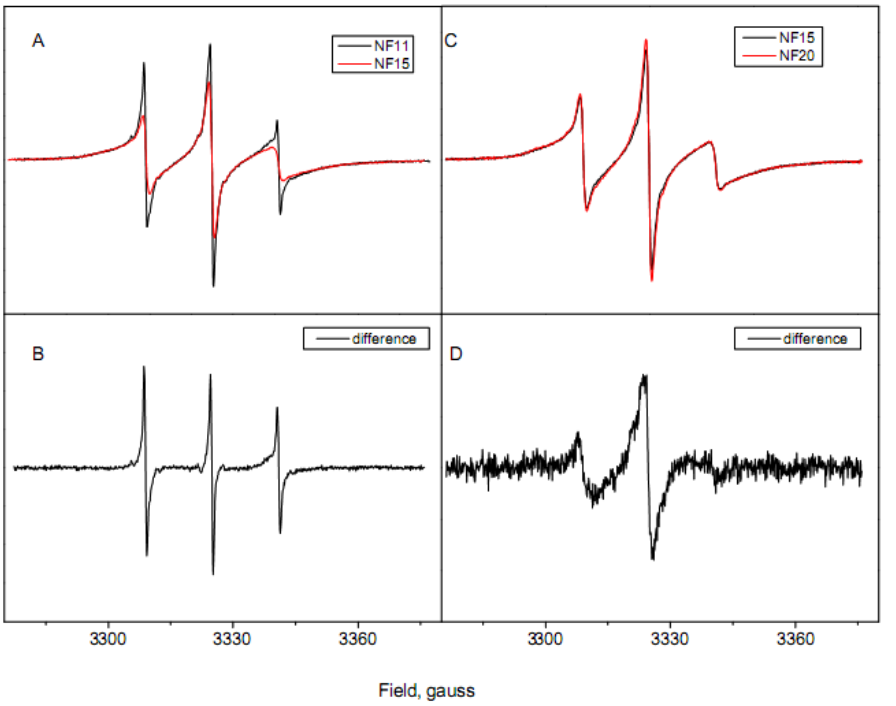

Figure 4. TEMPONE spectra of *N. fischeri* spores from 11- and 15-day-old cultures (A), from 15- and 20-day-old cultures (C), and their difference spectra, (B) and (D), respectively. Spectrum D is enlarged.

15-day-old cultures was almost 4 times higher than that of 11-day-old cultures (5.4 and 21.2 cP, respectively) (Table 3). Viscosity of spores of older cultures was similar to that of 15-day-old cultures (Table 3). The ESR-spectra of the ascospores of 15-day-old cultures was subtracted from that of ascospores of 11-day-old cultures (Fig. 4A). This difference spectrum (Fig. 4B) is typical for the fast isotropic rotation of TEMPONE molecules in bulk water (Golovina and Hoekstra 2002). The differences in ESR spectra of TEMPONE from ascospores of 15- and 20-day-old-cultures

(Fig. 4C,D) and older cultures (data not shown) are negligible. Taken together, fast isotropic motion of the spin label as in ascospores of 11-day-old cultures disappeared in spores of cultures of 15 days and older.

The cytosol of dormant ascospores of 40-day-old-cultures had a viscosity of about 20 cP (Fig. 5A). This value dropped two-fold within 60 min after activation. The steep decrease in viscosity slowed down between 1 and 2 h (from 10 to 9 cP) followed by a second phase of reduction to 4 cP after 3 hours. The viscosity decreased

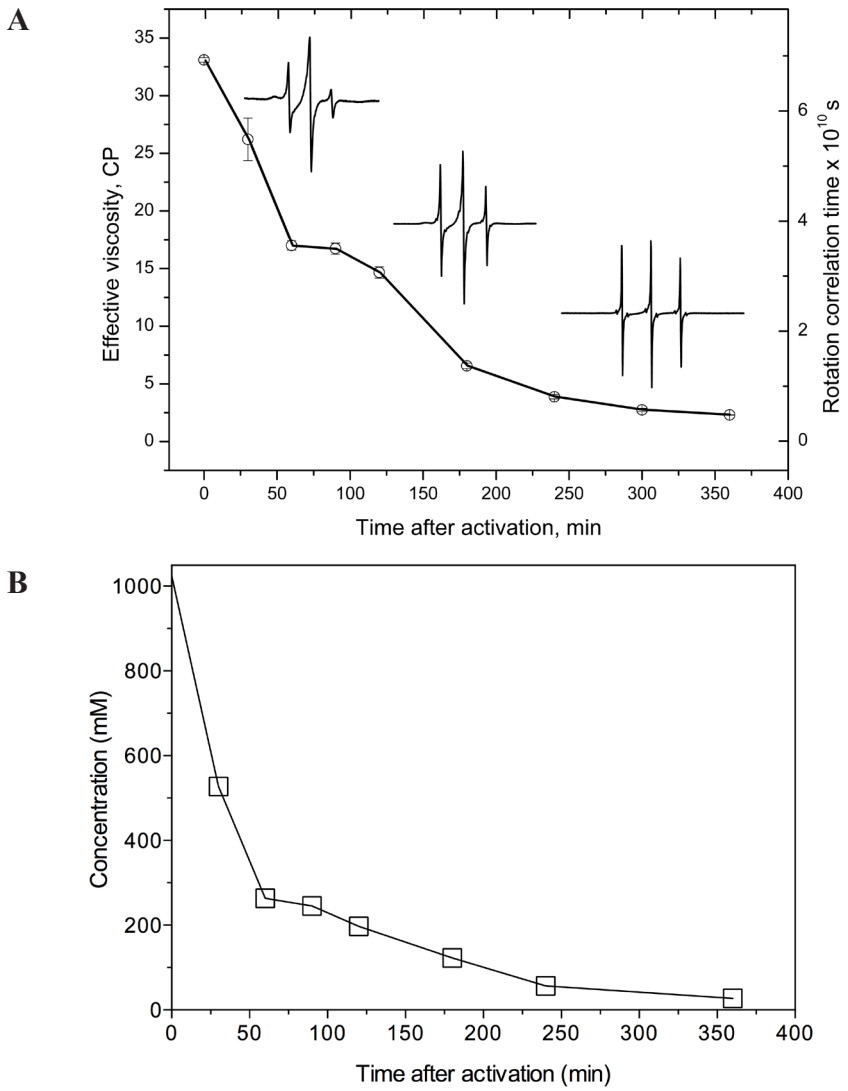

Figure 5. Effective viscosity of the cytoplasm of ascospores of 40-day-old cultures during germination (A) calculated from the rotation correlation time of TEMPONE. The cytoplasmic viscosity of ascospores during germination decreased in two steps, which correlates with the concentration of compatible solutes in the spores (B).

gradually to 1.1 cP 6 h after activation (Fig. 5A). This value is close to the viscosity of the cytosol of the vegetative mycelium.

Redox stability of developing spores using ESR spectroscopy

The redox stability of an environment is assumed to be correlated with the rate of redox conversion. The protection of the spin probe against redox conversion by the interior of the ascospores was evaluated at different stages of maturation. In our experiments the double integral of the ESR spectrum (the integrated intensity) is used as a measure of spin probe concentration in the cell. ESR spectra were acquired from ascospores of cultures of different age at temperatures from 250 (-23 °C) to 375 K (102 °C). The integrated intensities of the spectra, normalized to the values at 295 K (22 °C), were plotted against the temperature for dormant hydrated ascospores from 11-50-day-old cultures (Fig. 6). Three phases of temperature dependence can be discerned. At temperatures below 260 K (-13 °C) (and 265 K / - 8 °C for spores of 11-day-old cultures) the integrated intensity of the ESR spectra against temperature decreased fast with increasing temperature. The fast change of intensity is correlated with a vitrified state of the cytoplasm as the shape of the spectrum is specific for

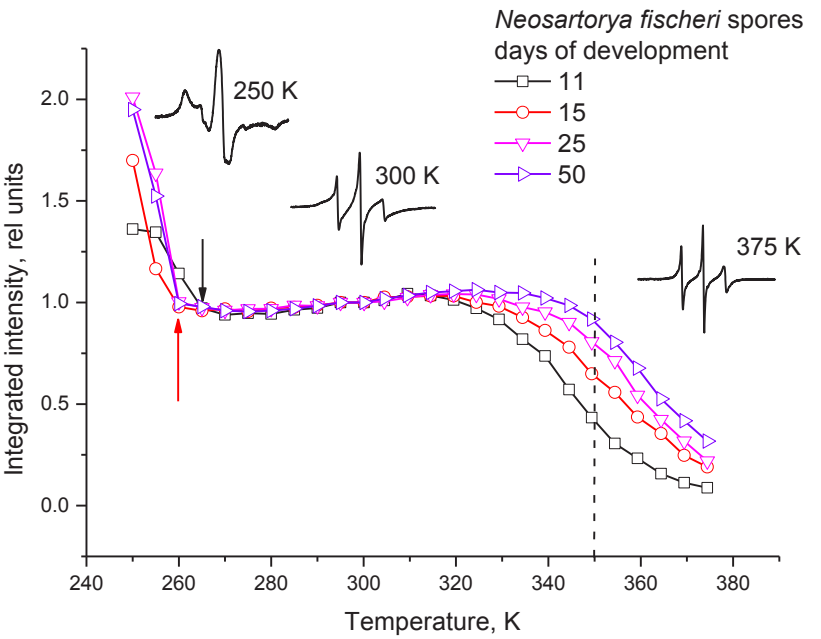

Figure 6. The temperature dependence of the integrated intensity of ESR spectra of TEMPONE in ascospores of *N. fischeri* from 11-50-day-old cultures. The shape of the ESR spectra of TEMPONE in spores of 50-day-old cultures at 250 K, 300 K and 375 K are shown. The arrows at low temperature indicate the temperature of water vitrification. Integrated intensities of the ESR spectra were normalized to the integrated intensity at 295 K. The signal strength at 350 K is stronger in older spores (dashed line).

spin probe in a solid environment. The intensities at 250 K (-23 °C) are highest in the case of 25- and 50-day-old cultures. The second phase (between 260 / 265 and 315 K) shows a nearly fixed signal strength, irrespective of spore age. Above this temperature (third phase) the integrated intensity decreases, and the temperature at which the decrease starts, depends on spore age. This phase begins at lower temperature in young ascospores, which clearly suggests that young ascospores have a higher redox conversion (activity) and hence a lower redox stability.

The degree of spin probe redox conversion can be expressed as the ratio between integrated intensity of the spectrum at 350 K (77 °C) and that at room temperature (300 K, 27 °C; Fig. 7). The Boltzmann fit of these data has an adjusted R-Square of 0.93184. The decrease of the spectral intensity after heating was irreversible, meaning that these silent molecules can't be re-oxidized by FC into paramagnetic TEMPONE. Thus at high temperatures (> 60 °C) paramagnetic TEMPONE is conversed to paramagnetic silent molecules other than hydroxylamine. Most reduction (93.5 % from the initial intensity) was observed in ascospores of 11-day-old cultures, while in ascospores of 50-day-old cultures 73 % of the signal was lost (Fig. 7, insert). This clearly shows that the redox stability at 350 K had increased with age.

The signal stability in ascospores was determined in the absence of FC (see Material and Methods). Dormant ascospores of 40-day-old cultures in water did

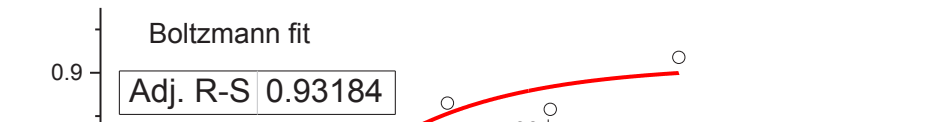

Figure 7. Normalized integrated intensity of TEMPONE spectra at t=350 K ($IntInt_{350}/IntInt_{300}$, dashed line in Fig. 7) of ascospores of *N. fischeri* from 11-50-day-old cultures fitted to a Boltzmann equation with a adjusted R-square (Adj. R-S) of 0.93184 . Insert: Intensity of TEMPONE spectra after heating (during recording) followed by cooling of the ascospores in % from the initial intensity.

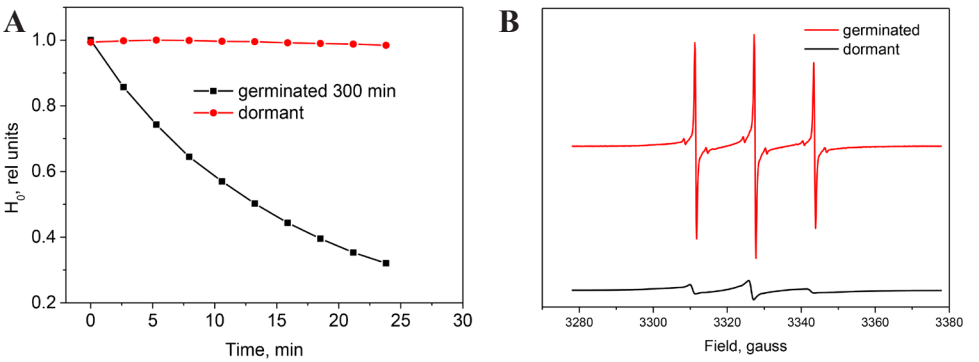

Figure 8. Kinetics of TEMPONE reduction in dormant and 5-h-germinated spores in the absence of FC in the extracellular space (A) and TEMPONE spectra in dormant and germinating spores normalized to the same number of spin probe molecules in the presence of FC (B).

not show any reduction of spin probe signal within 25 min of incubation at room temperature, while germinating ascospores showed a distinct reduction of the signal 5 h after activation (Fig. 8). These results indicate a stable signal strength and absence of metabolic activity in dormant ascospores.

Structural and chemical stability of dried ascospores

The spin labels TEMPOL and TEMPO were used to study viscosity and redox stability within dried *N. fischeri* ascospores. Both spin probes are water-soluble, but TEMPOL is more hydrophilic than TEMPO. These probes are expected to reside in a more hydrophilic or hydrophobic environment of the cytoplasm, respectively (Kocherginsky, Swartz *et al.* 1995). Fig. 9 shows the spectra of TEMPOL and TEMPO in dry *N. fischeri* ascospores of 40-day-old-cultures at room temperature. Previous ESR spectra of TEMPONE were characteristic for a spin probe in an aqueous environment with a relatively high mobility (Fig. 1 and 4). The ESR spectra of TEMPOL and TEMPO in Fig. 9 are typical for a spin probe in a solid amorphous environment or glassy state (see also Fig. 6, 250 K) (Golovina, Hoekstra *et al.* 2001). The degree of immobilization of TEMPOL and TEMPO was estimated by the distance between the outermost peaks of the ESR spectrum ($2A_{max}$, Fig. 9) and from the peak-to-peak width of the central line (W_0 as in Fig. 1D). The spectrum of TEMPOL in dry spores at room temperature has a larger $2A_{max}$ (67.7 gauss) than that of TEMPO (65.9 gauss). Thus, TEMPOL molecules are more immobilized than TEMPO. This is also shown by the larger value of W_0 (Table 4). Similar values of both components were observed in ascospores stored at room temperature or 60 °C (3 days, Table 4) (67.7 gauss for TEMPOL and 65.8-65.9 gauss for TEMPO) and W_0 (8.8-9.0 gauss for TEMPOL and 6.2-6.3 gauss for TEMPO). Both spin probes showed an increased mobility at higher temperatures (Fig. 10). Plotting of $\Delta 2A_{max} = 2A_{max(T)} - 2A_{max(220\ K)}$ against temperature shows a lower mobility of TEMPOL compared to TEMPO at increasing temperatures of measurement.

The effect of heat-storage on the redox stability of the dry spores was also studied. The integrated intensity of spectra of both spin probes (see above) was plotted (Fig. 10B). All curves had a biphasic appearance with an increase of spin probe oxidation with a temperature above 335 K (58 °C). The integrated intensity of TEMPOL spectra only slowly decreases at T > 335 K in ascospores that were kept at RT or were heat stored. However, the integrated intensity of TEMPO spectra decreased markedly from 295 K (22 °C) on and more so at 335 K. Invariably, dry heat storage resulted in lower values indicating a higher sensitivity for oxidation of

Table 4. Spin probe immobilization as indicated by the $2A_{max}$ and W_0 of the ESR spectra of the spin probes TEMPOL (TL) and TEMPO (TO) in dry *N. fischeri* ascospores that had been stored for 3 d at room temperature (RT) or 60 °C. The immobilization of the spin probe is related to the structure of the environment where it resides.

	$2A_{max}$, gauss	W_0, gauss
TL: RT	67.68	8.79
TL: 60 °C	67.68	8.98
TO: RT	65.92	6.15
TO: 60 °C	65.82	6.25

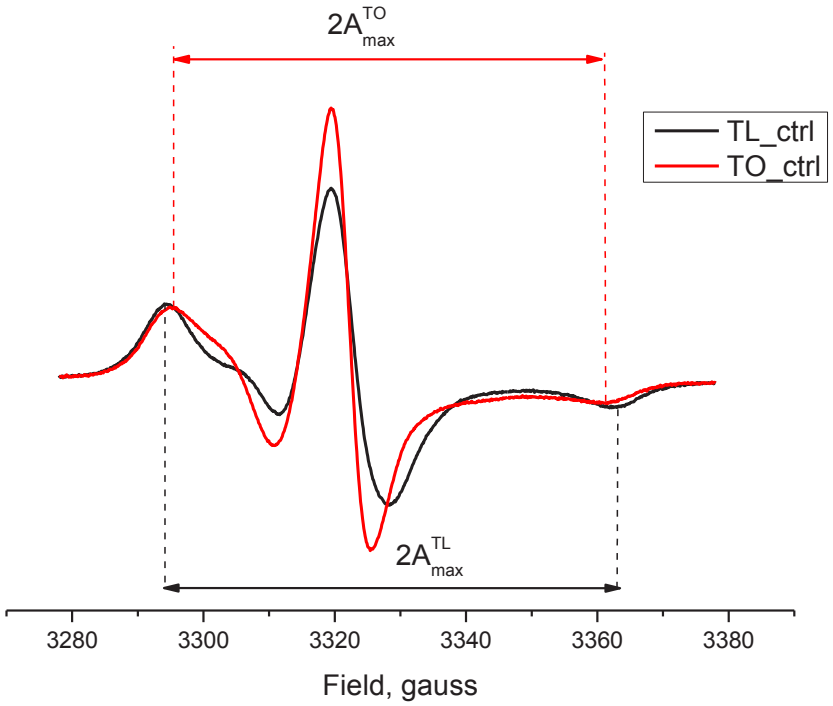

Figure 9. Spectra of TEMPOL (TL) and TEMPO (TO) in the cytoplasm of dried *N. fischeri* ascospores (from 40-day-old cultures) at room temperature. The distance between two outermost extremes $2A_{max}$ is an indicator of the degree of spin probe immobilization and local polarity.

TEMPO molecules in its more hydrophobic micro-environment. From these data it is concluded that TEMPO in dry spore cytoplasm is less redox stable than TEMPOL and more sensitive after dry heat storage (Fig. 10B).

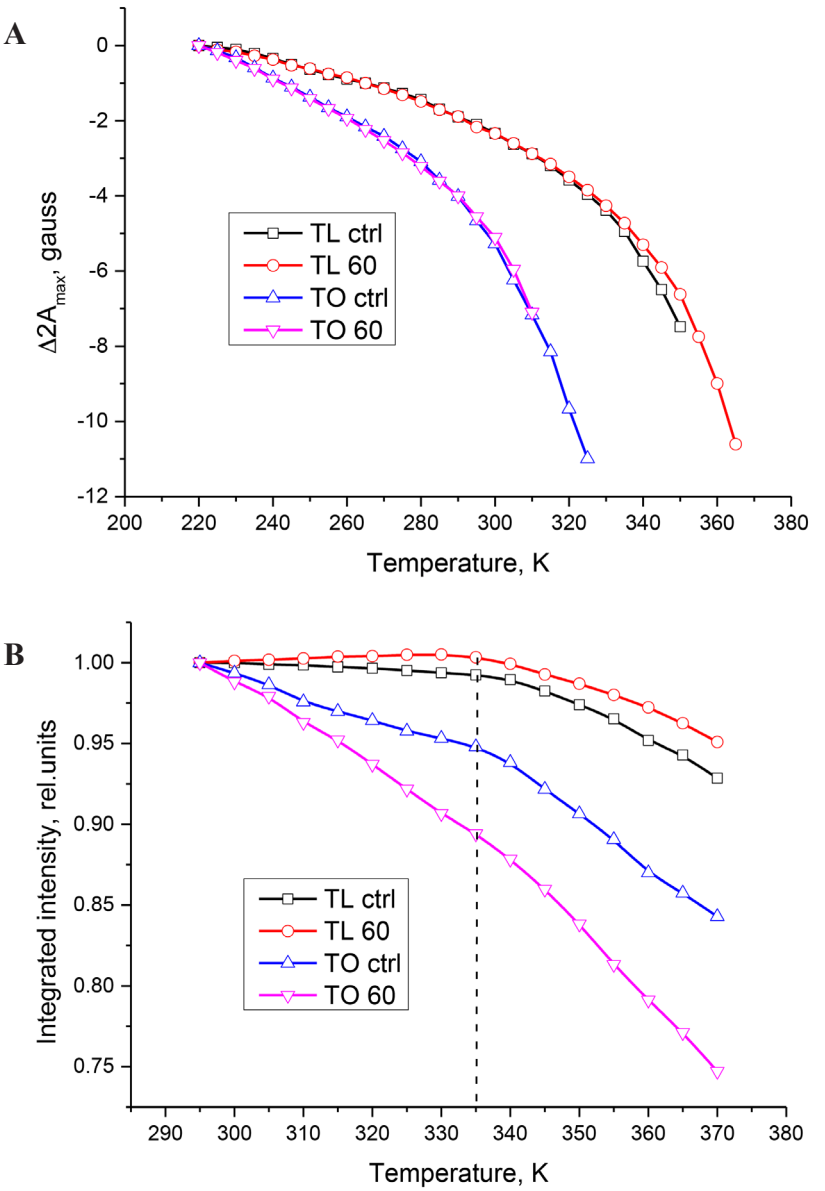

Figure 10. The temperature dependence of TEMPOL (TL) and TEMPO (TO) immobilization (A) and the temperature dependence of spectral integrated intensity of TEMPOL (TL) and TEMPO (TO) in dry spores stored at room temperature (ctrl) and at 60 °C. Immobilization is expressed as $\Delta 2A_{max} = 2A_{max(T)} - 2A_{max(220)}$ and the intensity is normalized to the intensity at 295 K. At T > 335 K (dashed line in B) the rate of redox conversion increases in all samples.

Discussion

Ascospore maturation of *N. fischeri* is characterized by the acquisition of extreme stress resistance. Little is known about the mechanisms that play a role in this process. In this study maturation of ascospores was related to the accumulation of compatible solutes, the viscosity of the cytoplasm, the presence of bulk water (water that only interacts with other water molecules), and redox stability (low rate of redox reactions). Our results distinguish two phases in ascospore development. A relatively short early phase that takes place in cultures of 11-15 days followed by a much longer phase that takes place between 15-50 days. During the first phase, accumulation of compatible solutes (from a total of 0.45 to 1.0 M), a large increase of viscosity (5.1 to 21.2 cP), disappearance of bulk water, acquisition of stress (heat) resistance, and an increase of redox stability occur. The second phase of maturation is characterized by a decrease of mannitol and an increase of trehalose and TOS, accompanied by a further increase of redox stability and stress (heat) resistance. The total amount of compatible solutes as well as viscosity did not change much during this stage (approximately 14 pg spore^{-1} and 20 cP respectively). Mannitol concentration was highest in ascospores from 20-day-old cultures, while trehalose and the TOS isobemisiose, neosartose and fischerose were most abundant in ascospores of 50-day-old cultures. Therefore, the increase of stress resistance in the second phase seems to depend on the increase of trehalose and TOS.

Several observations suggest that the (micro)viscosity of the cytoplasm of fungal survival structures correlates with stress resistance (Dijksterhuis, Nijsse *et al.* 2007; van Leeuwen, van Doorn *et al.* 2010). Water-dispersed conidia of *Fusarium oxysporum* and *Verticillium fungicola* have a low viscosity (2 cP) and have lower stress resistance when compared to air-dispersed conidia of *Penicillium discolor* and *Aspergillus niger* (3-4 cP) (van Leeuwen, van Doorn *et al.* 2010). Constitutively dormant and extreme stress-resistant fungal and bacterial spores exhibit an even higher level of cytoplasmic viscosity (10-33 cP) (de Vries 2006; Dijksterhuis, Nijsse *et al.* 2007; **Chapter 2**). Winther *et al.* (2012) observed that the effective viscosity derived from tumbling time (rotation correlation time) of trehalose molecules at different concentrations increases linearly up to approx. 300 mM, but at higher concentrations increases markedly faster. This was more prominently observed when the effective viscosity was plotted against the trehalose concentration (Winther, Qvist *et al.* 2012). This indicates that accumulation of solutes above a certain concentration might be an important factor in the increase of viscosity and hence stress resistance of the spores. Our data, however, show a strong correlation between the intracellular viscosity and the total amount of compatible solutes during maturation and germination of ascospores. Even relatively small changes in the total amount of compatible solutes as in the case of ascospores of 32-day-old cultures (13.2 pg and 18.4 cP) compared to 20-day-old cultures (13.2 and 14.1 pg per spore, respectively) are accompanied with similar changes in viscosity (18.4 and 20.8 cP, respectively). Viscosity and amount of compatible solutes also correlated strongly during germination of ascospores, as illustrated by a precise congruence between

total solute concentration and intracellular viscosity in time (see Fig. 5). The marked increase of viscosity during early maturation coincides with the disappearance of bulk water. Trehalose that increased nearly 2 times in concentration during early maturation is known to be highly effective in reducing the amount of bulk water (Lerbret, Bordat *et al.* 2005). Mannitol (7.4-fold) and TOS levels (4.2-fold) increased even stronger. They may also have a role in bulk water removal.

High intracellular viscosity results in a relatively low speed of adverse chemical reactions (Roos 1995) such as the production of reactive radicals. The analysis of the redox conversion of spin probes in dormant ascospores provided an opportunity to study the protection of molecules within the matrix of the cell at different conditions as e.g. temperature or after drying. The signal intensity of TEMPONE at temperatures above 320 K (47 °C) remains highest in the oldest ascospores (of 50 day-old cultures). This suggests that radicals may be formed at a lower rate or that reactions of radicals with TEMPONE occur at a lower rate in older spores. Thus, the redox stability of the spore cytoplasm increased with age. Mannitol, trehalose and oligosaccharides are suggested to play a role in protection against oxidative stress (Shen, Jensen *et al.* 1997; Benaroudj, Lee *et al.* 2001; Valluru and van den Ende 2008; van den Ende and Valluru 2009). Additional or superior protective properties of trehalose and TOS in this respect could explain the accumulation of these solutes.

Dried ascospores of *N. fischeri* (containing trehalose and TOS) survive a heat treatment better than dried ascospores of *T. macrosporus* (containing only trehalose; **Chapter 2**). Less than 10 % of *T. macrosporus* ascospores survive 1 day at 60 °C, while approximately 15 % of the *N. fischeri* spores survived even after 15 days at 60 °C (T.T. Wyatt, unpublished results). The process of drying includes crowding of solutes in the remaining water, resulting in disturbances in the structure of proteins and membranes (Hoekstra, Golovina *et al.* 2001). Compatible solutes protect these molecules by direct interaction via their hydroxyl groups (water replacement theory) or by preferred exclusion of solutes around proteins and membranes (kosmotrophic effect) (Jain and Roy 2009). Membranes of ascospores of *N. fischeri* may be the Achilles heel during drought and heat stress. Dry heat storage resulted in lower redox stability of the spin probe TEMPO that is expected to reside in the proximity of the membranes. The signal intensity of TEMPOL on the other hand remained higher, suggesting that the cytosol protects spin probes better against oxidation than membrane environments. The membranes and the environment in the proximity of the membranes might be more sensitive to harmful (redox) reactions, because the membrane can be still in a liquid phase as is shown for the membrane in dry wheat seeds (Golovina and Tikhonov 1994). In plants, sucrose-based oligosaccharides (SOS) are believed to act as membrane protectors under stress via direct hydrogen binding, in which the chain length is important for the insertion into the membrane (Hincha, Zuther *et al.* 2002; Hincha, Zuther *et al.* 2003; Milhaud 2004; Beck, Fettig *et al.* 2007). It is also claimed that these plant oligosaccharides protect against oxidative-stress and lipid oxidation (Cacela and Hincha 2006; Agati, Matteini *et al.* 2007; Nishizawa, Yabuta *et al.* 2008; van den Ende and Valluru 2009). TOS show

similarities with SOS belonging to the raffinose family oligosaccharides (RFOs), which accumulate during seed maturation (Kuo, Vanmiddlesworth *et al.* 1988). These RFOs have like our TOS a DP of 3-5, α-1,6 linkages and their accumulation is related to increased stress resistance (Brenac, Horbowicz *et al.* 1997). The specific function of TOS in ascospores might be similar to the suggested function of SOS, which is providing protecting of membranes against desiccation (Hincha, Zuther *et al.* 2003).

Acknowledgement

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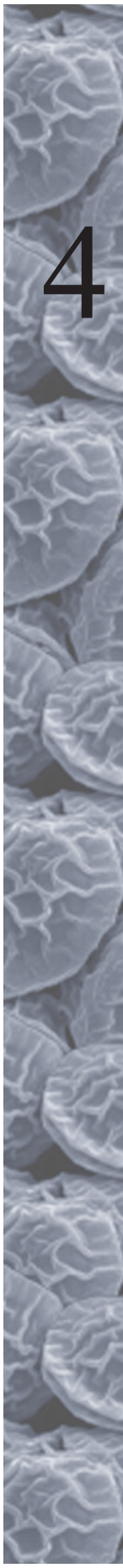
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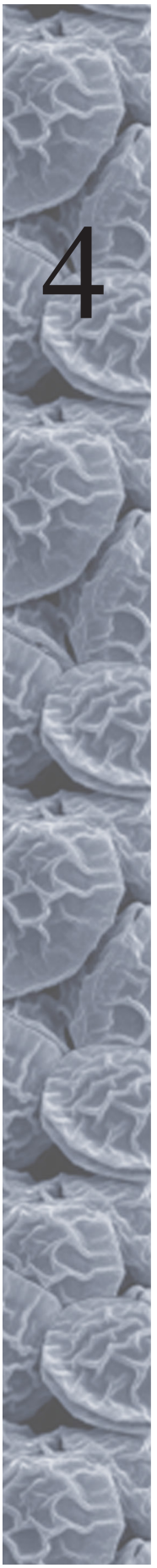


4 Mannitol is essential for the development of stress-resistant ascospores in *Neosartorya fischeri* (*Aspergillus fischeri*)

Based on T.T. Wyatt, M.R. van Leeuwen, H.A.B. Wösten, J. Dijksterhuis (2013) "Mannitol is essential for the development of stress-resistant ascospores in *Neosartorya fischeri* (Aspergillus fischeri)." In revision.

Abstract

The polyol (sugar alcohol) mannitol is one of the main compatible solutes in *Neosartorya fischeri* and accumulates in conidia and ascospores. Here, it is shown that biosynthesis of mannitol in *N. fischeri* mainly depends on mannitol 1-phosphate dehydrogenase (MpdA). Reporter studies demonstrated that *mpdA* is moderately expressed in aerial hyphae and conidiophores, while expression in cleistothecia and ascospores was high. Deletion of *mpdA* reduced mannitol as much as 85 % of the wild type. Trehalose levels had increased in the mutant to over 400 %, but trehalose based oligosaccharides were totally absent. Decreased mannitol accumulation had no effect on mycelial growth irrespective of heat- or oxidative stress. On the other hand, the conidia of the Δ*mpdA* strain were more sensitive to these stresses when compared to the wild-type. The most distinct phenotype of *mpdA* deletion was the complete absence of fully formed ascospores. However, formation of cleistothecia, asci and ascospore initials was not affected. The cleistothecia of the Δ*mpdA* strain did collapse after drying. After 6 days of development many ascospores showed a distorted cell contents and malformed ascospore cell wall. Moreover, the ascus cell wall remained intact in mutant strains. In contrast, the wild type ascus cell wall disappeared which was accompanied by accumulation of numerous ascospores in the ascomata. Addition of the Mpd inhibitor nitrophenide to the wild type strain also resulted in disturbed ascospore formation. Taken together, these results show that mannitol has a role in sexual development of *N. fischeri*.

Introduction

The acyclic sugar alcohol mannitol is one of the most abundant compatible solutes in nature and can be found in bacteria (Chalfan, Levy *et al.* 1975), algae (Kremer 1976; Benamotz and Avron 1983; Armstrong and Smith 1998), lichens (Armstrong and Smith 1998), plants (Stoop, Williamson *et al.* 1996) and fungi (Jennings 1984). The term compatible solute implies that mannitol can accumulate intracellularly without interfering with cell metabolism. A wide variety of functions have been described for mannitol in fungi including carbon storage (Corina and Munday 1971), maintaining reduction potential (Hult, Veide *et al.* 1980), water absorption (Velez, Glassbrook *et al.* 2007; Nehls, Gohringer *et al.* 2010), heat-stress protection (Allaway and Jennings 1970; Chaturvedi, Flynn *et al.* 1996; Solomon, Waters *et al.* 2007; Albrecht, Guthke *et al.* 2010), protection against oxidative stress (Shen, Hohmann *et al.* 1999; Voegele, Hahn *et al.* 2005; Solomon, Waters *et al.* 2007; Velez, Glassbrook *et al.* 2007), and tolerance against osmotic stress (Chaturvedi, Wong *et al.* 1996; Chaturvedi, Bartiss *et al.* 1997). Moreover, mannitol plays a role in the pathogenesis of various plant and animal pathogens by acting as a Reactive Oxygen Species (ROS) scavenger thereby neutralizing plant and animal defense (Chaturvedi, Flynn *et al.* 1996; Chaturvedi, Wong *et al.* 1996; Jennings, Ehrenshaft *et al.* 1998). Mannitol also plays a role in asexual sporulation (Ruijter, Bax 2003; Solomon, Waters *et al.* 2006), spore germination (Voegele, Hahn *et al.* 2005; Solomon, Waters *et al.* 2006), and ascospore discharge (Trail and Xu 2002; Trail, Xu *et al.* 2002). The precise function of mannitol in fungi, however, is still not fully established (Solomon, Waters *et al.* 2007; Aguilar-Osorio, Vankuyk *et al.* 2010).

It has been proposed that fungal mannitol metabolism is organized in the so-called mannitol cycle (Hult and Gatenbeck 1978). This cycle would consist of two metabolic pathways that convert fructose-6-phosphate to mannitol via the intermediates fructose or mannitol-1-phosphate (Fig. 1). Various studies have shown that mannitol synthesis mainly occurs via the reduction of fructose-6p to mannitol-1p by the NAD(H) dependent mannitol 1-phosphate dehydrogenase (MPD; EC 1.1.1.138) (Ruijter, Bax *et al.* 2003; Solomon, Waters *et al.* 2006; Velez, Glassbrook *et al.* 2007). This reaction (Fructose-6p + NADH $<\underline{\text{MPD}}>$ Mannitol-1p + NAD^+ H^+) can act in two directions. Subsequently, mannitol-1p is dephosphorylated to mannitol by the enzyme mannitol 1-phosphate phosphatase (MPP; EC 3.1.3.22). The other pathway converts fructose-6p to fructose by fructose-6-phosphate phosphatase (FPP; EC 2.7.1.1/4; Fructose-6p + $\underline{\text{FPP}}>$ Fructose + phosphate). Fructose is then reduced to mannitol by the NAD(P)H dependent MTD (EC 1.1.1.138 or EC 1.1.1.68, Fructose + NAD(P)H + H^+ $<\underline{\text{MTD}}>$ Mannitol + $NAD(P)^+$). The reversed reaction of FPP; the production of fructose from fructose-6p (fructose + ATP $\underline{\text{HX}}>$ Fructose-6p + ADP) is catalyzed by hexokinase (EC 2.7.1.1). It should be noted that the existence of the mannitol cycle is still debated (Singh, Scrutton *et al.* 1988; Ruijter, Bax *et al.* 2003; Solomon, Waters *et al.* 2007). For example, the genes encoding MpdA and MtdA in *Aspergillus niger* are not localized in the same part of the colony. Gene *mpdA* is only expressed in vegetative mycelia, while *mtdA* is expressed in conidiospores (Aguilar-

Osorio, Vankuyk *et al.* 2010).

Deletion of *mpdA* in *A. niger* results in a decrease of mannitol and an increase of trehalose in conidia (Ruijter, Bax *et al.* 2003). The conidia of the Δ*mpdA* strain exhibit increased stress sensitivity. Similarly, deletion of *mpd* in *Beauveria bassiana* results in decreased UV-, H_2O_2- and heat resistance of conidia. Moreover, *mpd* deletion affected asexual sporulation and germination of conidia in this fungus (Wang, Lu *et al.* 2012). In the wheat pathogen *Stagonospora nodorum mpd1* is required for the asexual sporulation but has no role in stress resistance of spores (Solomon, Tan *et al.* 2005; Solomon, Waters *et al.* 2006). Supplementing mannitol to the medium restored sporulation almost to wild type levels (Solomon, Waters *et al.* 2006). In this study we show that mannitol has a role in stress resistance of conidia and in the maturation of ascospores in *Neosartorya fischeri*. These data present for the first time genetic evidence that mannitol plays a role in sexual development in fungi.

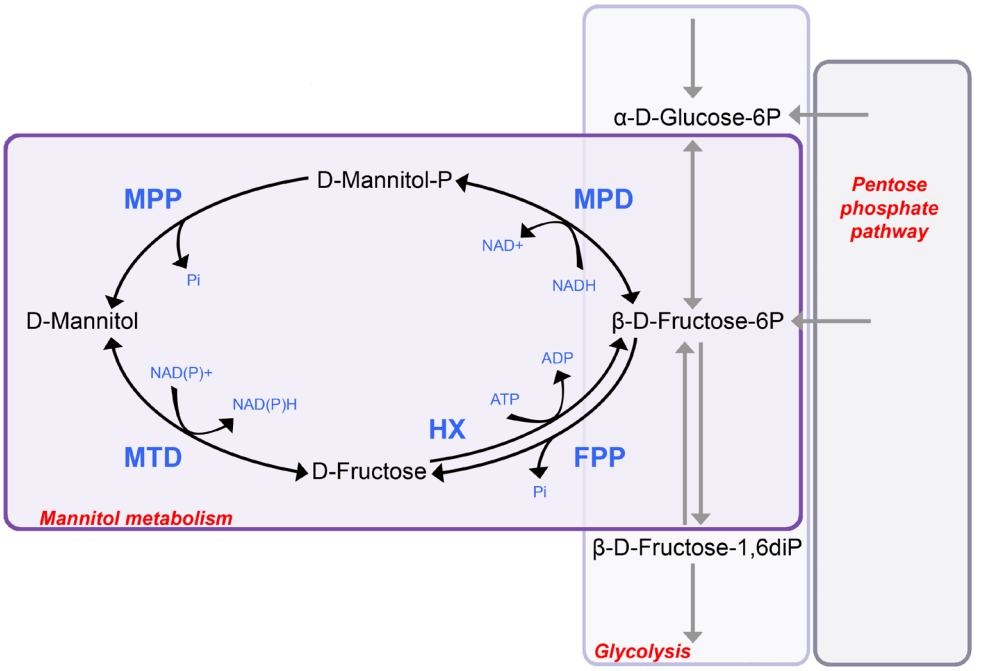


Figure 1. Mannitol metabolism in ascomycota as proposed by Hult and Gatenbeck (1978). MPD: NAD(H) dependent mannitol 1-phosphate dehydrogenase (EC 1.1.1.17); MPP: mannitol 1-phosphate phosphatase (EC 3.1.3.22); MTD: NAD(H) or NADP(H) mannitol dehydrogenase (EC 1.1.1.138 and EC 1.1.1.68); HX: hexokinase (EC 2.7.1.1); FPP: fructose 6-phosphate dehydrogenase (EC 2.7.1.1/4). The predicted genes encoding MTD in *N. fischeri* are *mtdA* (NFIA_090670) and *mtdB* (NFIA_101920) that are NADP(H) and NAD(H) dependent, respectively.

Material and methods

Strain, growth condition and culture media

Cultures of *N. fischeri* (CBS 317.89 CBS-KNAW, The Netherlands) and *N. fischeri* Δ*mpdA* were grown at 30 °C on Oatmeal agar (OA) (BD Difco, Breda, The Netherlands) or Malt extract agar (MEA) (BD Difco). Ascospores or conidia were harvested by gently scraping cultures with a glass spatula and were subsequently taken up in 9 ml ice-cold 10 mM ACES (N-(2-acetamido)-2-aminoethanesulfonic acid) buffer, pH 6.8, supplemented with 0.02 % Tween-80 (Sigma-Aldrich, Zwijndrecht, The Netherlands). The ascospore solution was supplemented with approximately 1 cm^3 of sterile glass beads (1 : 1 ratio of beads with diameters of 0.1 mm and 1.0 mm) and homogenized by vortexing for 2 min and sonification for 5 min using an Ultrasonic cleaner 2510E-MT (Branson Ultrasonics Corporation, Danbury, USA). The mycelial debris was removed by filtration through sterile glass wool. The spores were pelleted by centrifugation for 5 min at 1100 g (Mistral 400 centrifuge, Fisons plc, Ipswich, United Kingdom) and washed three times with ice cold ACES buffer. Pellets of ascospores were stored at -80 °C and conidia were stored on ice in ACES buffer before further processing. Agar medium was inoculated with ascospores (10^6) and mycelial plugs in the case of the wild type and the Δ*mpdA* strain, respectively. Ascospores were activated to germinate by a heat treatment (2 min, 85 °C) prior to inoculation.

Stress resistance of conidia and mycelium was measured on MEA. The germination percentage was determined after exposure of conidia to 50 °C for 0-4 h or exposure to 0-10 mM H_2O_2 for 1 h. The treated conidia were put on MEA slices and incubated for 14-16 hours at 25 °C. The germination percentage was determined by light microscopy. Stress resistance of mycelium of the *N. fischeri* wild type and Δ*mpdA* strains was measured by growing at 30-45 °C for 2 days or by adding 0-6 mM H_2O_2 to the medium. The activity of MpdA was inhibited by adding 0-1000 µM nitrophenide (Bis(3-nitrophenyl) disulfide (Sigma-Aldrich) (Allocco, Nare *et al.* 2001) dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich) to MEA. The final concentration DMSO in the media was 2 %.

The size and the number of cleistothecia were determined after 30 days incubation at 30 °C on OA. At least 100 cleistothecia were measured, and 5 frames were counted. The calculations (standard deviation) were done in Microsoft excel.

Polymerase Chain Reaction (PCR)

PCR was performed with Phusion Hot Start (Bioke, Leiden, The Netherlands) for amplification of fragments for cloning, while BioTaq (Gentaur, Eersel, The Netherlands) was used to check integration of constructs in the genomic DNA of transformants. PCR was performed according to the manufactures instructions. In the case of Phusion Hot Start an initial denaturation of 3 min at 98 °C was used followed by 35 cycles consisting of denaturation for 10 s at 98 °C, annealing for 30 s at 55-70 °C, and extension at 72 °C for 30 s per kb of fragment. In the case of BioTaq

an initial denaturation of 3 min at 95 °C was used followed by 35 cycles consisting of denaturation for 1 min at 95 °C, annealing for 30 s at 55-70 °C, and extension at 72 °C for 30 s per kb. In both cases PCR was completed with a final extension of 7 min at 72 °C.

Plasmid construction

DNA fragments were amplified using genomic DNA of *N. fischeri* CBS 317.89 as target DNA and primers (Biolegio, Nijmegen, The Netherlands) listed in Table 1. Amplified DNA was purified from 1 % agarose gels using the QIAquick Gel Extraction Kit (QIAGEN, Venlo, The Netherlands) and tailed at 70 °C for 30 min in a 20 µl reaction mixture containing 1-2 µg purified DNA, 1 unit Biotaq (GC biotech B.V., Alphen aan den Rijn, The Netherlands), 1 x NH_4 reaction buffer (delivered together with Biotaq), 0.2 mM dATP, and 2.5 mM $MgCl_2$. The tailed DNA fragments were ligated with linear pGEM-T vector (pGEM-T easy vector system II, Promega, Leiden, The Netherlands) and introduced in JM109 competent cells. Plasmid DNA was isolated with PureLink Quick Plasmid MiniPrep Kit (Invitrogen, Breda, The Netherlands).

Construction of the mpdA deletion strain

The 1.5 kb upstream and downstream flanking regions of the *mpdA* open reading frame of *N. fischeri* were amplified with the primer combinations mpdUP-fw#37 / mpdUP-rv#38 and mpdDWN-fw#19 / mpdDWN-rv#20 (Table 1), respectively. The PCR products were introduced in pGEM-T resulting in plasmids pGEM-mpdUP and pGEM-mpdDWN. A 1.1 kb upstream flank was isolated from pGEM-mpdUP by digestion with HindIII-XbaI. This fragment was cloned in front of the hygromycin resistance cassette of PAN7-1 (Punt, Oliver *et al.* 1987) by cutting with the same enzymes. This resulted in PAN7-1-mpdUP. PAN7-1-mpdUP was digested with NheI-BglII, after which the 1.2 kb NheI-BglII downstream sequence of *mpdA* from pGEM-mpdDWN was introduced. This resulted in the Δ*mpdA*-pan7-1 plasmid, which was cut with HindIII and BglII. The hygromycin cassette flanked by the up and downstream regions of the *N. fischeri mpdA* gene was purified from gel and introduced in the CBS 317.89 strain.

Construction of pMPD-dTomato expression in the CBS 317.89 strain

The 1.5 kb upstream region of *mpdA* was amplified by PCR using the primer combination pMPD-fw#156 / pMPD-rv#157. The product was purified from gel, tailed and ligated in pGEM-T to obtain plasmid pGEM-pMPD. Plasmid phNmrrdTomato containing the *dTomato* gene (Shaner, Campbell *et al.* 2004) between the promoter region of *hNmrr* of *A. niger* and the *trpC* terminator of *A. nidulans* was digested with NcoI and NotI to replace the *hNmrr* promoter for that of *mpdA* contained on a 1.5 kb fragment NcoI / NotI fragment. The resulting expression vector pMPDdTomato contains the *dTomato* gene under control of the *mpdA* promoter of *N. fischeri*.

Transformation of N. fischeri

N. fischeri was transformed with linearized Δ*mpdA*-pan7-1 plasmid, or circular DNA of pMPDdTomato. The latter construct was co-transformed with PAN7-1. Protoplast formation was based on the protocol described by de Bekker *et al.* (2009). Activated ascospores (10^6-10^7) were used to inoculate 250 ml malt extract broth (MEB) (BD Difco, Breda, The Netherlands) containing 100 µg ml^{-1} ampicillin using a 0.5 l Erlenmeyer flask. After 16-20 h at 30 °C and 200 rpm the mycelium was homogenized for 1 min in a Waring blendor at full speed and 125 ml homogenate was added to 125 ml fresh MEB with ampicillin. After incubation for 16 h at 30 °C and 200 rpm, the mycelium was filtered over sterilized nylon gauze and washed with 0.9 % NaCl. The mycelium (2-3 g wet weight) was resuspended in 0.2 M phosphate / 0.8 M sorbitol buffer with 5 mg ml^{-1} lysing enzymes (L1412, Sigma-Aldrich), 0.15 units ml^{-1} chitinase (C6137, Sigma-Aldrich), and 460 units ml^{-1} β-glucuronidase (G0751, Sigma-Aldrich). Protoplasts were harvested after shaking at 130 rpm for 1.5-3 h and incubation at 37 °C. Mycelial debris was removed by filtration over glass wool. The protoplasts were centrifuged at 4 °C for 10 min at 2000 g after adding ice-cold 0.2 M phosphate / 1.33 M sorbitol buffer. Protoplasts were washed with 50 mM $CaCl_2$, 10 mM Tris / HCl, pH 7.5, 1.33 M sorbitol (STC) and resuspended in STC at a concentration of 10^8 protoplasts ml^{-1}. 2 10^7 protoplasts (200 µl) were transformed with 1-5 µg transformation DNA (either or not in the presence of 10-20 µg co-transforming DNA). To this end, 50 ul of PEG (25 % PEG-6000 in 50 mM $CaCl^2$, 10 mM Tris / HCl, pH 7.5) was added to protoplasts and incubated at RT for 20 min. This was followed by adding 2 ml PEG buffer and incubation at RT for 5 min. After addition of 4 ml of STC, 10 ml of pre-warmed (50 °C) minimal medium (de Vries, Burgers *et al.* 2004) containing 0.95 M sucrose and 0.6 % agar (MMST) was added to half of the mixture and poured onto minimal medium containing 0.95 M sucrose, 1.2 % agar and 200 µg ml^{-1} hygromycin (Hygrogold, InvivoGen, Toulouse, France). The transformation plates were incubated for 3-5 days at 30 °C. For the second selection the transformants were transferred to minimal medium plates containing 1 % glucose and 200 µg ml^{-1} hygromycin.

PCR screening of transformants

Genomic DNA of transformants was isolated with the UltraClean Microbial DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, USA) to check for deletion of *mpdA*. PCR with the primer combination mpdPresence-fw#76 / mpdPresence-rv#77 (Table 1) amplifies a 526 bp fragment of *mpdA*, indicative for the presence of a wild type copy of the gene. Primer combinations mpdUPintegr-fw#46 / mpdUPintegr-rv#61 and mpdDWNintegr-fw#60 / mpdDWNintegr-rv#47 (Table 1) resulted in fragments of 1246 bp and 1850 bp in the case the hygromycin resistance cassette had integrated within *mpdA*.

Sugar analyses

Sugar analysis was performed on cultures from various ages. Fungal material,

Table 1. Primers used in this study

		sequence
mpdUP-fw#37	AAGCTTATTGGATCTCGATCGGTTACG	*HindIII* restriction site
mpdUP-rv#38	TCTAGAGGGTCGATGGCTAGTTACTTC	*XbaI* restriction site
mpdDWN-fw#19	GCTAGCAGGTTTGAACCGCTCGATTACGAACC	*NheI* digestion site
mpdDWN-rv#20	AGATCTAAAAGGTACGGACGTGCCTTCCCCCT	*BglII* digestion site
mpdPresence-fw#76	CGAGCAGCACACCGATAAGG	
mpdPresence-rv#77	AGGCCCGATGAAGCGTTC	
mpdUPintegr-fw#46	CCTGATCCGAGACCCGAAC	
mpdUPintegr-rv#61	GCTCCGTAACACCCAATACGC	
mpdDWNintegr-fw#60	CTTCTCTCTGCGTCCGTCC	
mpdDWNintegr-rv#47	CTGGCAGTGGCTAGATGACG	
pMPD-fw#156	GATCGCGGCCGCGGTGAGAGAACGGAAGAGAAATG	NotI digestion site
pMPD-rv#157	GATCCCATGGGATGTTGTGTTGAACGAGTTGGA	NcoI digestion site

consisting of aerial hyphae, cleistothecia, and conidiophores, were removed from the agar medium with a glass spatula. The samples were frozen in liquid nitrogen, freeze dried, and homogenized with the Qiagen Tissuelyser (2 min at 30 strokes sec^{-1}) in a stainless steel grinding jar (Qiagen, Venlo, The Netherlands) cooled with liquid nitrogen. 0.5-1 ml milli Q water was added and grinding was repeated for an additional 2 min at 30 strokes sec^{-1}. Samples were thawed, transferred to a 2 ml Eppendorf tube and centrifuged at 4 °C for 30 min at 10.000 g. The supernatant was heated for 30 min at 95 °C and centrifuged again 30 min at 10.000 g. The supernatant was filtered (0.2 µm acrodisc Cr13mm Syringe filter, Pall Life Science, Mijdrecht, The Netherlands) and stored at -80 °C until used for further analysis.

Quantitative analysis of saccharides and polyols was carried out by high-performance liquid chromatography (HPLC) equipped with a Sugar pak I cation-exchange column (Waters, Etten-Leur, The Netherlands). The column and detector were kept at 50 °C with a column heater (Waters) and internal heater respectively. The carbohydrates were detected with an IR detector (2414 refractive index detector, Waters), and the flow of the mobile phase (MQ with 0.1 mM Ca EDTA) was maintained at 0.5 ml min^{-1} with the 515 HPLC pump and the pump control module II (Waters). Samples were injected using the 717 plus autosampler (Waters). Peak integrations and calculation were performed with Empower software delivered by Waters. The retention time of the peaks were compared with those of 0.01-0.50 % w/v trehalose, mannitol, glucose and glycerol.

Light Microscopy

Cultures of *N. fischeri* were imaged at low magnification using a Nikon binocular (SMZ1500) equipped with a Nikon HR Plan Apo 1x lens and a Nikon DS-Fi1 digital

camera run by Nikon NIS-Elements D 3.0 software. Micrographs of individual cleistothecia were taken with a Zeiss Axioskop 2 plus microscope equipped with a Nikon DS-Fi1 digital camera run by Nikon NIS-Elements D 3.0 software.

Fluorescence microscopy

Nuclei were stained with 4'-6-diamidino-2-phenylindole dihydrochloride (DAPI). To this end, cleistothecia were squashed between two object glasses, incubated for 90 min with DAPI (1.5 µg ml^{-1}) in vectrashield mounting medium (Vector laboratories, Burlingame, USA) and washed 3 times with phosphate buffered saline (PBS). Fluorescence was captured with a Zeiss Axioplan II microscope using a Zeiss Plan-ApoChromat 100x / 1.4 oil objective, a Zeiss Plan-NeoFluar 40x / 0.75 objective or a Zeiss AchroPlan 10x / 0.25 objective. The G365 (FT396, LP420, BP450-490 (FT510, LP520) and BP510-560 (FT580, LP590) filters were used for DAPI, GFP and dTomato. Images were captured with a Zeiss AxioCam MRc digital camera run by Zeiss AxioVision 4.

Electron microscopy (LTSEM and TEM)

Cleistothecia were covered with 2 % agarose that was pre-warmed at 40 °C. Small cubes (1x3 mm) were cut by means of a stainless razor blade (GEM products, Orange Park, Florida, USA) and glued with KP-Cryoblock (Klinipath, Duiven, The Netherlands) in a 1 cm ø copper cup. After snap-freezing in a nitrogen slush, samples were transferred to a JEOL 5600LV scanning electron microscope (JEOL, Tokyo, Japan) via an Oxford CT1500 Cryostation. Ice was removed by sublimation by heating the sample stage to -85 °C. Samples were sputter coated with gold for 3 min. Micrographs were acquired at an acceleration voltage of 5 kV using 30 averaged fast scans (SCAN 2 mode) or a single scan at the SCAN 4 mode.

For transmission electron microscopy colonies were covered with 2 % agarose and cleistothecia were cut. This was necessary for entrance of the fixatives into the fruiting bodies. The agarose blocks were fixed in 4 % paraformaldehyde in Sorensen phosphate buffer (pH 7.2, 100 mM) for 2 h with gentle agitation. During the first 30 min the samples were kept in a desiccator under vacuum for removal of air. Subsequently, the sample was transferred to 2.5 % glutaraldehyde and kept overnight while gently shaken (10 times min^{-1}, VXR basic Vibrax, IKA Werke GmbH, Staufen, Germany). Samples were post-fixed in 1 % OsO4 for 1 h, dehydrated in an alcohol series and subsequently embedded in QUETOL resin (EMS, Hatfield, PA, USA). 70-80- nm sections were obtained with a Reichert Ultracut S ultramicrotome (Leica, Wien, Austria) using a Diatome diamond knife (Diatome, Hatfield, PA, USA). Sections were transferred to a 75 mesh copper grid coated with 1 % formvar and imaged in a JEOL 1011 transmission electron microscope (Jeol, Tokyo, Japan). Micrographs were acquired with a Veleta high resolution TEM camera (ResAlta Research Technologies, Golden, USA) and processed with Adobe Photoshop CS 5.5.

Results

Characterization of mpdA of N. fischeri

The gene encoding MpdA of *N. fischeri* (*mpdA*: NFIA_086010;) (Wortman, Fedorova *et al.* 2006) contains two preserved pfam domains, a substrate specific catalytic domain, the mannitol dehydrogenase C-terminal domain (pfam08125), and the mannitol dehydrogenase Rossmann domain (pfam01232). The Rossmann domain is specific for binding of the co-enzyme nicotinamide adenine dinucleotide (NAD^+) and contains the conserved consensus motif G-x-G-x-x-G (Fig. 2) (Lesk 1995). The C-terminal domain contains conserved amino acids important for binding of the substrate mannitol-1p and for catalysis of the reaction (Fig. 2). Nitrophenide is thought to modify the thiol group of cysteines (Allocco, Nare *et al.* 2001), thereby impairing the function of Mpd. We found one conserved cysteine, most likely the target of nitrophenide (Fig 2).

Localization of mpdA expression

The 1.5 kb upstream region of the predicted *mpdA* coding sequence was placed in front of the *dTomato* reporter gene. The resulting expression construct pMPDdTomato was introduced in *N. fischeri* and strain pMPDdT2b was selected as a representative transformant. Dormant ascospores of this strain were highly fluorescent (Fig. 3A-C), but the fluorescence was lost during germination (Fig. 3D-F). Leading hyphae showed no detectable fluorescence (Fig. 3G-I), in contrast to aerial mycelium in the middle of the colonies (Fig. 3J-L). The fluorescence was especially high in cleistothecia (Fig. 3F-G) and to a lesser extent in conidiophores (Fig. 3M-O). On the other hand, conidia were not fluorescent (Fig. 3M-O). Taken together, these data show that the *mpdA* promoter of *N. fischeri* is especially active in sexual structures.

Functional analysis of mpdA

The deletion construct Δ*mpdA*-pan7-1 was introduced in *N. fischeri*. In 3 out of 50 transformants (dubbed Δ*mpdA*70, Δ*mpdA*73 and Δ*mpdA*76) the *mpdA* coding sequence could not be amplified by PCR (Supplemental Fig. 1). In contrast, fragments were obtained in the case of these transformants using primers that annealed within the hygromycin resistance cassette and outside the flanking sequences of *mpdA* (see Material and Methods for details). This showed that the *mpdA* coding sequence was replaced by the hygromycin resistance cassette. The three deletion strains all showed defects in ascospore development (see below). Strain Δ*mpdA*70 was selected for further study.

Initial stages of development were similar for wild type and Δ*mpdA*. Early stages of cleistothecia formation are characterized by the formation of a spherical body, approximately 250-350 μm in diameter, which is enveloped by several layers of cells (the peridium). The mature cleistothecia consist of hyphae embedded in an extracellular matrix in which numerous asci are formed (Fig. 4). Both the mutant

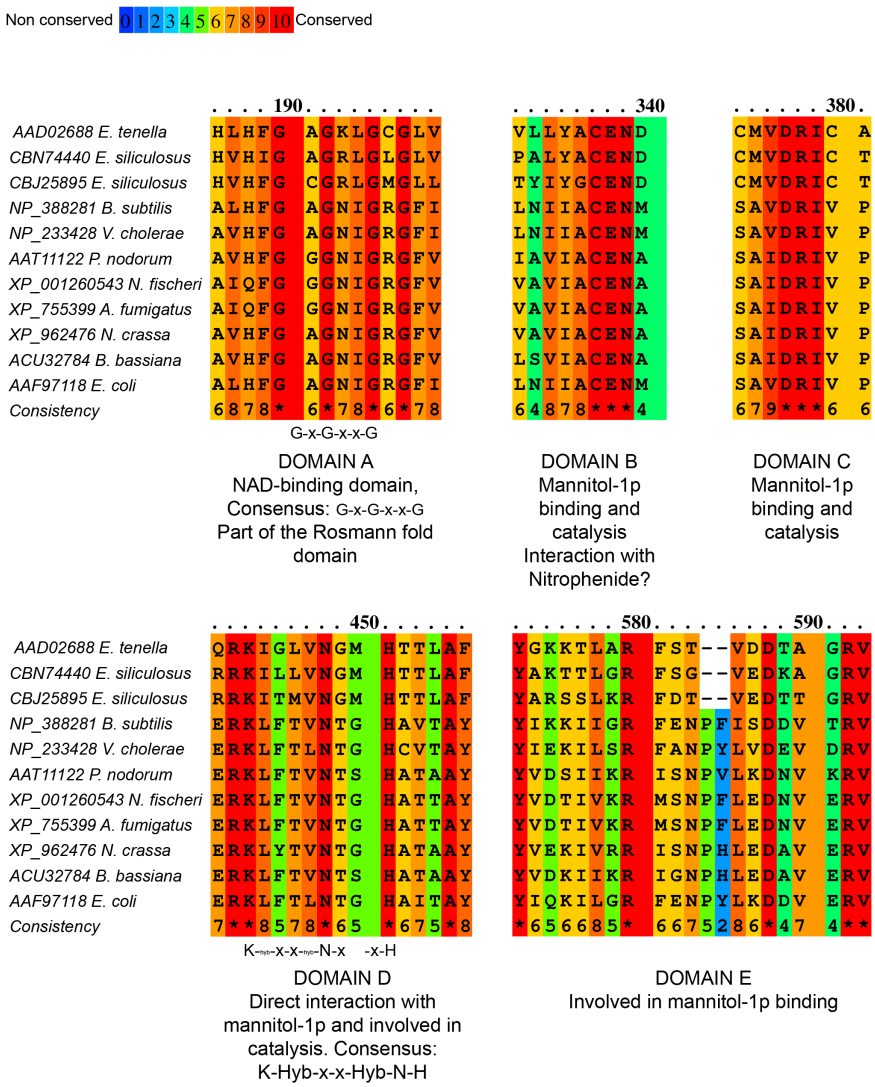
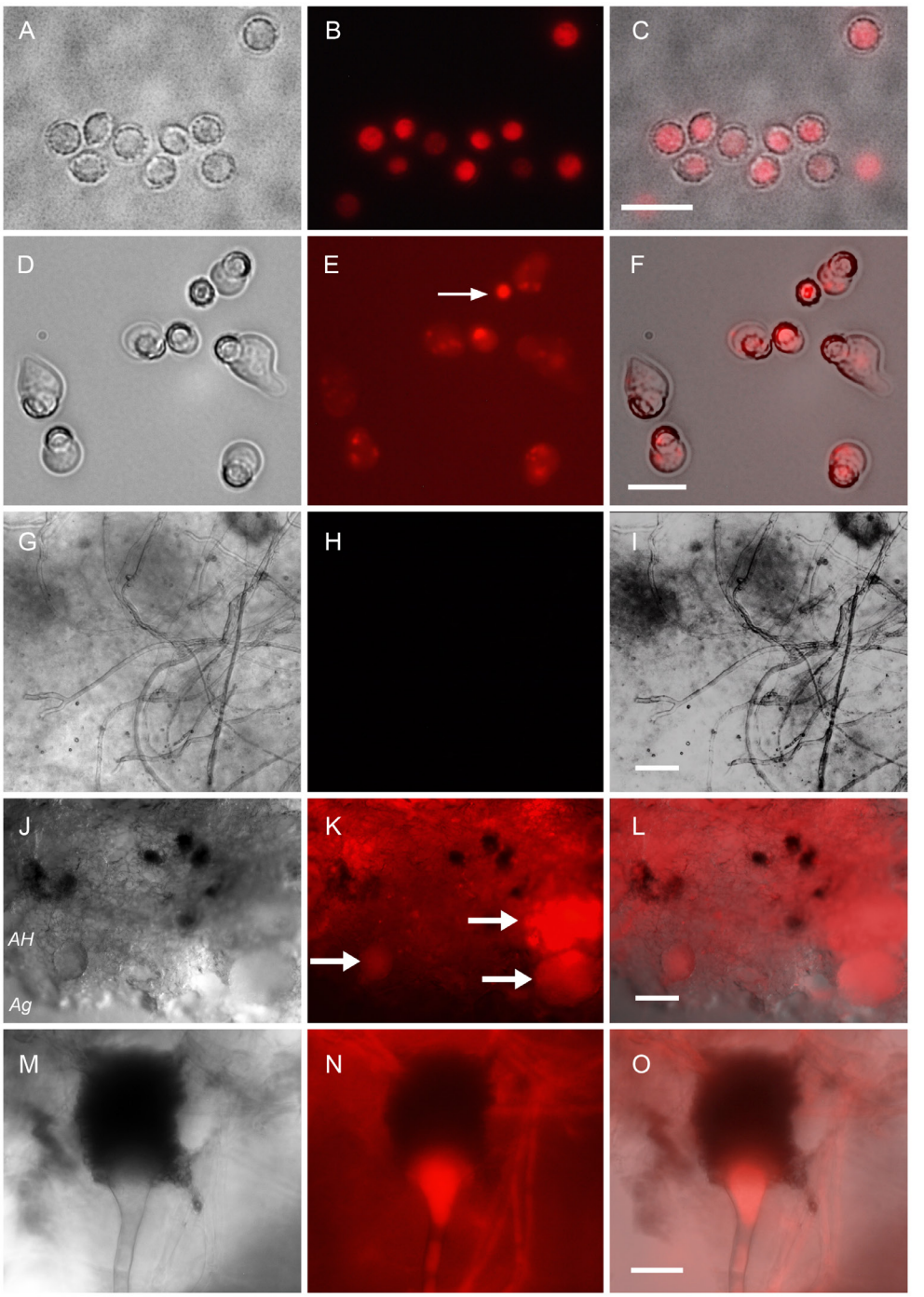


Figure 2. Conserved domains in Mpd involved in NAD^+ binding (domain A), mannitol-1p binding (domain B, C, D and E) and catalysis (domain B, C and D). The inhibition of Mpd activity by nitrophenide might be due the modification of the thiol group of Cys-337 (domain B).

On the next page: **Figure 3**. Expression of *mpdA* of *N. fischeri* by using dTomato as a reporter. Dormant (A-C) and heat activated ascospores (D-F) of 13-day-old cultures are highly fluorescent, but fluorescence decreases during germination. Leading hyphae of a 5-day-old mycelium (G-I) do not express *mpdA*. In contrast, aerial hyphae (AH: surrounding and being part of the cleistothecia) and especially the cleistothecia (J-L) are fluorescent. Fluorescence of vegetative hyphae in the agar (Ag) is low. Fluorescence is also observed in conidiophores, but not in conidia (M-O). The thin arrow points to an ascospore, the thick arrows to cleistothecia. Bar represents 10 µm (A-F), 50 µm (G-L), and 100 µm (M-O).


A
B
C
D
E
F
G
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I
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AH
Ag
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and the wild type formed equal amounts of cleistothecia after 30 days at 30 °C, with 95.5 ± 6.8 and 95.3 ± 3.6 cleistothecia per frame for the wild type and mutant

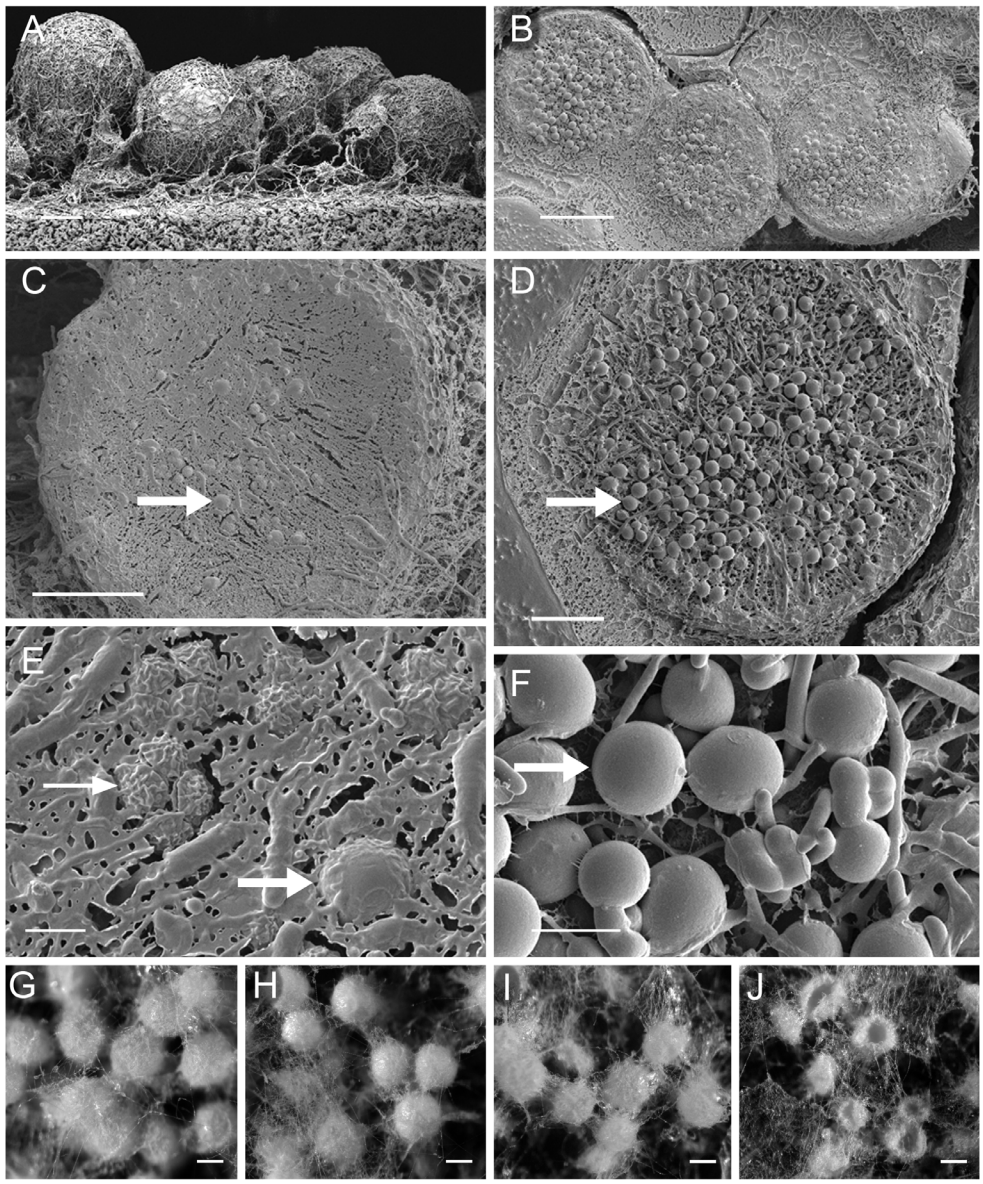

Figure 4. Dissection of cleistothecia of 10-day-old cultures of wild type (A, C, E) and strain Δ*mpdA* (B, D, F). The Δ*mpdA* cleistothecia are significant smaller but no clear morphological differences are observed (A, B). More asci are found in Δ*mpdA* cleistothecium (D) compared to the wild type (C). Close ups show numerous ascospores in the wild type (E) and only asci in the mutant (F). The thin arrow points to ascospores, thick arrows to asci. Cleistothecia of *N. fischeri* wild type (G, H) and Δ*mpdA* (I, J) of 21-day-old cultures. Drying of the cultures results in collapse of the mutant cleistothecia (J) but not of those of the wild type (H). Bars represent 150 µm (G, H, I, J), 100 µm (A, B), 50 µm (C, D), 10 µm (F) and 5 µm (E).

respectively. The diameter of the cleistothecia of 30-day-old wild type colonies was 326 ± 46 µm, while that of the Δ*mpdA* strain was 288 ± 39 µm. All cleistothecia of the deletion strain from cultures older than 20 days, but none of the wild type strain collapsed after a drying treatment (30-60 min) in a laminar flow cabinet (Fig. 4G-J). Moreover, ascospores could not be isolated from Δ*mpdA* cultures, while $>10^8$ ascospores were obtained from one culture of the wild-type contained in a Petri Dish. Addition of 1-4 % mannitol to the media did not restore ascospore formation.

Cryo electron microscopy showed that cleistothecia of wild type cultures older than 5 days contained many ascospores. Asci were no longer observed in such cultures. In contrast, the Δ*mpdA* strain contained many asci but no ascospores (Fig. 4F). In some asci small premature spores seemed to be present. DAPI staining revealed multiple nuclei in asci of 10-day-old wild type and Δ*mpdA* cultures (Fig. 5E-H).

Transmission electron micrographs showed that both wild type and mutant

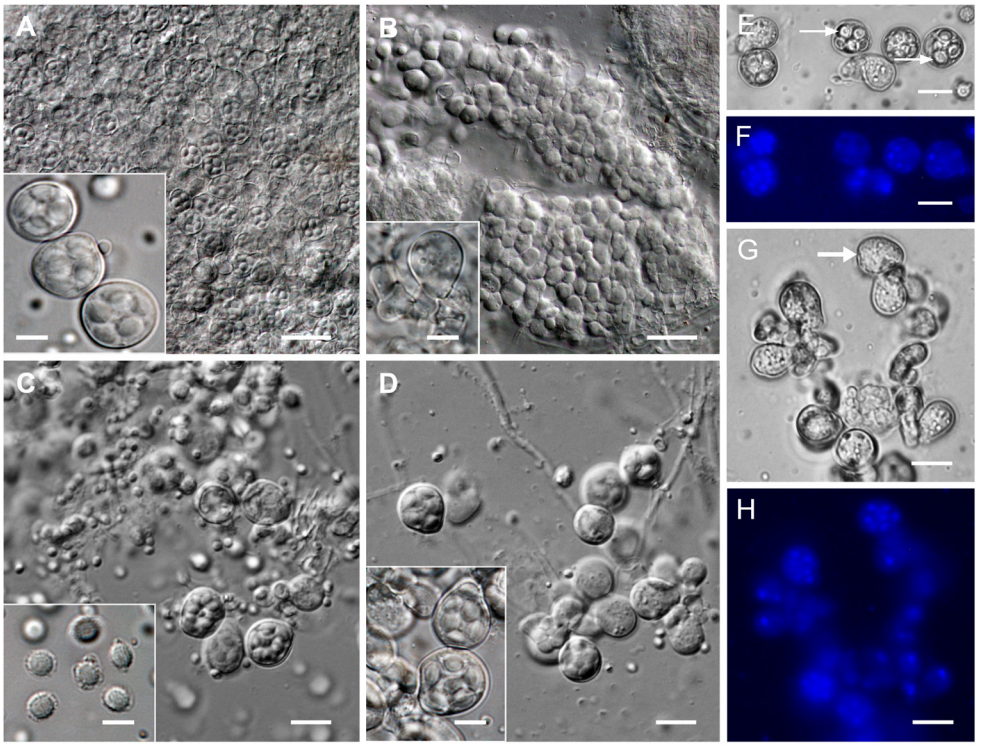

Figure 5. Ascospores in asci released from 7-day-old (A) and 10-day-old (C) cultures grown in the absence of nitrophenide. Asci from 7-day-old (B) and 10-day-old (D) cleistothecia grown in the presence of 1000 µM nitrophenide. Inserts are close ups and show an empty ascus (B), premature ascospores in asci (A, D) and released ascospores (C). Nuclear staining of asci of 10-day-old cultures of wild type (E, F) and Δ*mpdA* (G, H). DAPI staining shows 8 nuclei in wild type asci (F) and Δ*mpdA* asci (H). (E) The wild type ascospores inside the asci are almost fully developed (arrows). (G) No mature ascospores are observed in mutant asci (arrow). However, a similar number of nuclei are present in asci of the wild type and mutant strain (F, H). Bar represents 20 µm (A, B) and 10 µm (C-H). Bars in inserts represent 5 µm (A-D)

cleistothecia were bound by a peridium that consisted out of several layers of cells that were very intimately connected. This layer was impermeable for fixatives and only cleistothecia that were cut (see Material and Methods) showed successful structural preservation. Wild type asci contained ascospores with a regular shape and clear ornamentation on a thick cell wall (Fig. 6A,E). Numerous asci were observed within a cleistothecium of the mutant strain (Fig. 6D) and two types could be discerned. Firstly asci, with a number of relatively thin walled, incompletely formed ascospores that contained nuclei, ER, small lipid droplets and mitochondria (Fig. 6B). In addition, vacuoles were observed in these cells. Secondly, many asci contained

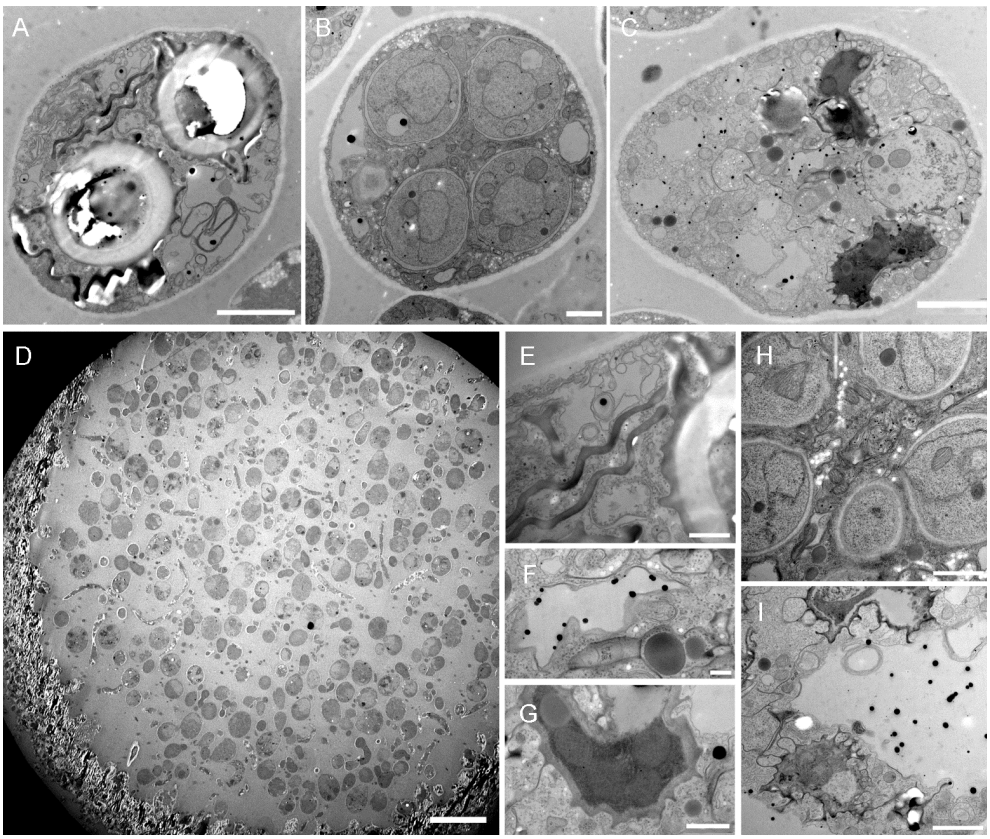

Figure 6. Transmission electron microscopy of wild type and mutant cleistothecia. (A) Wild type ascus with fully formed thick walled ascospores. The cytoplasm of these spores was not effectively preserved due to the impermeable cell wall. (B) Ascus of the Δ*mpdA* strain with four profiles of incomplete ascospores in this section and some electron dense inclusions. (C) Ascus of the Δ*mpdA* strain containing distorted ascospores and numerous electron dense inclusions. (D) Overview of a Δ*mpdA* cleistothecium delineated with a peridium and containing many asci and a number of hyphae. (E) Detail of (A) showing a thick cell wall (arrow) of a wild type ascospore and a tangential cut through the outer rim of another spore. (F) Detail of (C) showing a vacuole with electron dense particles. (G) Distorted ascospore in mutant ascus with irregular thinned cell wall (thin arrow). (H) Ascus of the Δ*mpdA* strain showing electron dense inclusions in mitochondria (arrow heads). (I) Vacuole in an ascus of the mutant strain with inclusions and distorted ascospores. Bars represent 20 µm (A), 2 µm (B, D), 1 µm (C, H, I), 0.5µm (E, G) and 0.2 µm (F).

irregularly shaped compartments that showed distorted ornamentation (Fig. 6C,G). These compartments were electron dense. All asci, either containing incompletely formed or distorted ascospores were characterized by the presence of small, very electron dense particles that were mostly present in vacuoles and mitochondria (Fig. 6B,C,F-H). These observations show that formation of ascospores is delayed and disrupted in the mutant strain. Abortion of the ascospores is correlated with the presence of electron dense inclusions.

The Δ*mpdA*70 strain was still able to produce asexual spores. It produced even 50-70 % more conidia after 10 days. The mutant conidia showed similar germination percentages (99.1 ± 0.1 %) as the wild type conidia (96.5 ± 0.6 %) when grown on OA at 30 °C). However, after 4 h exposure at 50 °C the germination percentage of wild type conidia was 75 %, while it was only 45 % in the case of the mutant (Fig. 7A). The difference was less obvious when exposed to H_2O_2. The germination percentage of Δ*mpdA*70 ascospores at 5 mM H_2O_2 was 72.3 ± 1.0 % compared to 84.0 ± 4.1 % in the case of the wild type. No significant differences were seen at 7.5 mM, with germination percentages of 8.6 ± 3.8 and 7.2 ± 1.1 % for the wild type and mutant conidia, respectively. Minimal germination was seen for both wild type and mutant conidia at 10 mM (1.3 ± 0.5 % and 0.6 ± 1.0 % respectively). Taken together, deletion of *mpdA* seems to result in slightly more heat and oxidative sensitive conidia. The dry weight of the mycelium of *N. fischeri* grown at 30 °C was

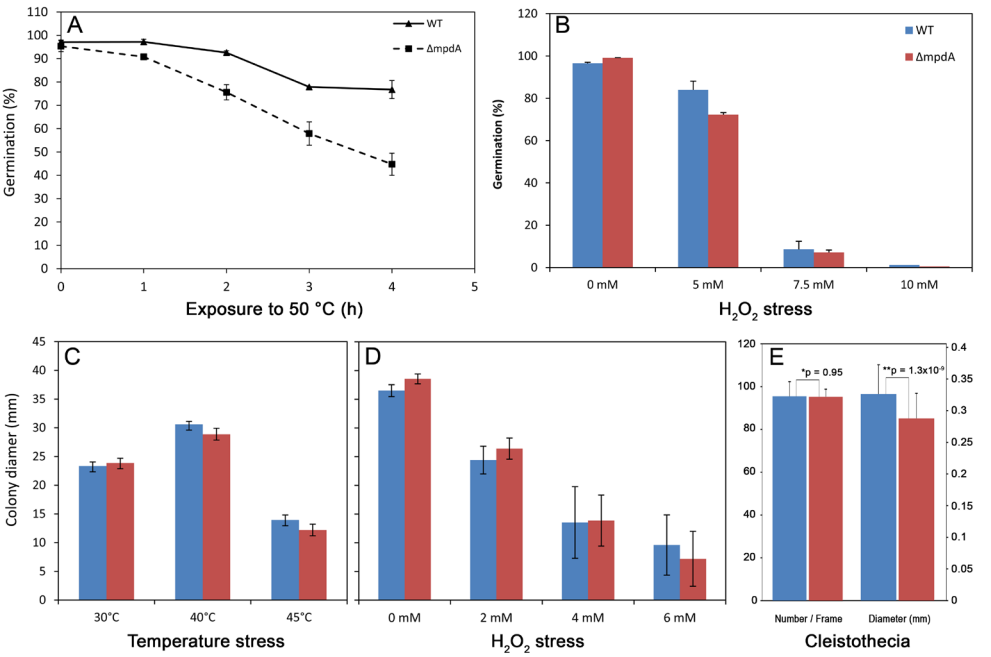

Figure 7. Germination percentage of wild type and Δ*mpdA* conidia when exposed for 0-4 h to 50 °C (A) or for 1 h to 0-10 mM H_2O_2 (B). Radial growth of 2-day-old wild type and Δ*mpdA* colonies at various temperatures (C). Radial growth of 3-day-old colonies upon exposure to 0-6 mM H_2O_2 (D) (E) Number and diameter of cleistothecia from 30-day-old wild type and Δ*mpdA* cultures grown on OA.

equal when wild type and the Δ*mpdA* strain were compared (data not shown). This was also the case for radial growth at 30, 40 and 45 °C (Fig. 7C), irrespective of the presence of 2-6 mM H_2O_2 (Fig. 7D). Apparently, mannitol has no major influence on vegetative growth when exposed to heat or oxidative stress.

The presence of mannitol and other sugar compounds in cultures was evaluated by means of HPLC analysis. The carbohydrate content of the complete mycelium was measured in 5- to 30-day-old colonies. Cultures of the Δ*mpdA* strain contained less mannitol (0.8-1.2 % of the dry weight) compared to the wild type (2.2-7.8 %) (Fig. 8). The wild type exhibited a marked decrease of mannitol from 7.2 % to 2.2 % between 4 and 10 days after inoculation. The mannitol levels in the wild type

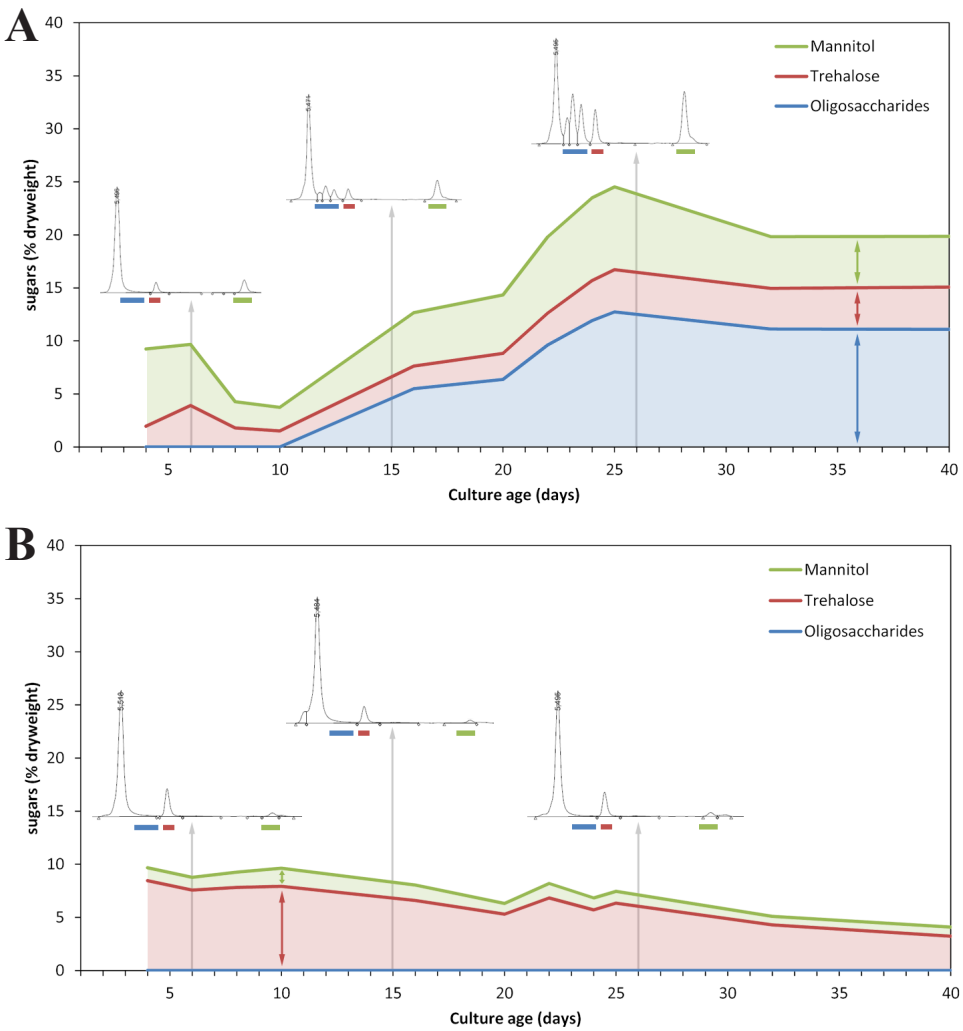

Figure 8. Trehalose, mannitol, TOS and total soluble sugars/polyols in the *N. fischeri* wild type (A) and Δ*mpdA*70 (B) strains grown on OA for 4-40 days. The amounts of sugars are stacked, so the highest line equals the total amount of sugar in the complete aerial part of the colonies. Oligosaccharides are completely absent, and only traces of mannitol are found in the mutant (Thin green line in graph B).

increased gradually again to 8 % between 10 and 25 days after inoculation, which correlated with ascospore maturation. During later stages of ascospore maturation mannitol levels slightly decreased to 5 %. Throughout culturing, mannitol levels remained ≤ 1.2 % in the Δ*mpdA* strain. In contrast, trehalose levels were clearly enhanced compared to the wild type at all stages except ascospore maturation. The highest amount of trehalose in cells of the mutant was observed after 4 days and was over 8 % dry weight. During development it gradually declined to 4 %. The wild type showed a gradual increase from 2 to 5 % between 8 and 40 days with the exception of an initial peak at 4 % after 6 days.

The total amount of sugars in the colony (consisting of aerial mycelium and if present cleistothecia and conidiophores), including mannitol, trehalose, and trehalose based oligosaccharides (TOS) (**Chapter 2**) showed an initial decline from 10 to 5 % of the dry weight (between 0-10 days), followed by an increase to maximal 25 % in 25-day-old wild type colonies. In case of the Δ*mpdA* strain a gradual decline throughout the development was observed from 11.5 to less than 5 % of the dry weight (5-40 days) (Fig. 8). Taken together, *mpdA* deletion has a strong influence on the accumulation of sugars and polyols during all stages of development of *N. fischeri*.

The MPD inhibitor nitrophenide (3-Nitrophenyl disulfide) inhibits sexual sporulation of oocysts of the apicomplexan parasite Eimeria tenella (Allocco, Nare *et al.* 2001). Nitrophenide (100-1000 µM) also caused a dose-dependent inhibition of ascospore formation on MEA in *N. fischeri*. The strongest inhibition was seen on 1000 µM. In this case, no ascospores had been formed in 6-day-old cultures. Nitrophenide did not affect the asexual reproduction as well as the formation of cleistothecia (Fig. 5A-D). These data confirm the role of mannitol as was shown by deletion of *mpdA*.

Discussion

This study shows that deletion of the *mpdA* gene results in strongly reduced levels of mannitol during all stages of development of *N. fischeri*. The reduced level of mannitol correlated with increased formation of conidia, that showed a higher sensitivity to heat and oxidative stress, and in a developmental block during maturation of ascospores. The reduced levels of mannitol did not result in reduced vegetative growth, not even after exposure to heat or oxidative stress. This agrees with previous findings in other fungi (Ruijter, Bax *et al.* 2003; Solomon, Tan *et al.* 2005; Solomon, Waters *et al.* 2006; Dulermo, Rascle *et al.* 2010; Wang, Lu *et al.* 2012). Only in the case of *Alternaria alternata* a minimal effect on colony growth was observed (Velez, Glassbrook *et al.* 2007).

The impact of inactivation of *mpd* on asexual development varies among different species of fungi, but the increased formation of conidia in *N. fischeri* seems to be unique. Possibly, more nutrients are available for the production of conidia when the production of ascospores is disrupted. Deletion of *mpd* had a negative effect on conidia formation in case of the fungi *B. bassiana* and *S. nodorum*, the latter not sporulating at all in planta (Solomon, Tan *et al.* 2005; Solomon, Waters *et al.* 2006; Wang, Lu *et al.* 2012). No effect was observed on sporulation and germination when *mpd* was deleted in *B. cinerea* and *A. alternata* (Velez, Glassbrook *et al.* 2007; Dulermo, Rascle *et al.* 2010). Conidia of *A. niger* and *B. bassiana* were more sensitive to stress when *mpd* was deleted (Ruijter, Bax *et al.* 2003; Wang, Lu *et al.* 2012). So far, effects of inactivation of *mpdA* on sexual development had not been reported.

We found that MpdA was most highly expressed within ascospores and cleistothecia, which suggests a pivotal role in sexual development. Indeed, the Δ*mpdA* strain did not form any ascospores within the asci. DAPI staining and electron microscopy showed that the defect of ascospore development occurred after meiosis. The failure to differentiate the cell wall of individual ascospores was correlated with a retainment of the ascus cell wall. The latter had disappeared in case of the wild type, which resulted in a relatively high number of asci in the cleistothecia of the mutant strain. Moreover, drying resulted in collapsed cleistothecia of the Δ*mpdA* strain but not of the wild type. This structural problem might be the result of the absence of ascospores in the cleistothecium, which also explains the smaller size of Δ*mpdA* strain cleistothecia. The apicomplexan parasite *Eimeria tenella*, showed disruption of development of sporocysts in the oocyst, when Mpd was inhibited. Sporocysts are also survival structures that develop from a fertilized zygote, the ookinete, and therefore are sexual structures like ascospores. That Mpd plays such an important role in the formation of sexual survival structures from organisms so distantly related to fungi indicates that mannitol might be a general factor in development.

How can we explain the impact of mannitol on ascospore maturation? First of all, mannitol could serve as an energy source. Such a role has already been suggested in 1965 by Horikoshi *et al.* (Horikoshi, Iida *et al.* 1965). Mannitol is abundant (2.5 % of the wet weight) in *A. oryzae* conidia and decreases rapidly upon germination when

endogenous respiration levels are high (Horikoshi, Iida *et al.* 1965). Mannitol also accumulates in ascospores of *Talaromyces macrosporus* and *N. fischeri*, (up to 9 %), and is degraded quickly after breaking of dormancy (T.T Wyatt and J. Dijksterhuis, unpublished work). We also observed a decrease of mannitol levels during early (day 6-10) ascospore formation in contrast to the mannitol levels in the mutant that remained low throughout these stages. This hints that mannitol usage is important for effective ascospore production. Accumulated solutes as mannitol could even play a role in prolonged maturation of ascospores that occur in buffer solution outside the cleistothecia (Dijksterhuis and Teunissen 2004).

Secondly mannitol can also serve as a reservoir of reducing power (Hult, Veide *et al.* 1980). The mannitol dehydrogenases Mpd and Mtd depend on the coenzymes NAD(H) or NADP(H) (Fig. 1). The ratios between NAD^+ / NADH and $NADP^+$ / NADPH are important for the redox balance of the cell. NADPH for example is also a substrate for the NOX family of NADPH oxidases that produce the reactive oxygen species (ROS) superoxide. ROS is known to play a role in germination (Lledias, Rangel *et al.* 1998) and asexual sporulation (Hansberg, de Groot *et al.* 1993). Notably, NoxA of *A. nidulans* has been shown to be essential for sexual sporulation. Deletion of NoxA blocks the differentiation of fruiting bodies. NoxA generates ROS in peridial layers during development (Lara-Ortiz, Riveros-Rosas *et al.* 2003). Many peridial layers are formed around the initials of the ascogenous cells and part of these layers die and dissolve, which result in space for the developing asci and supporting hyphae (Sohn and Yoon 2002). This suggests a role for programmed cell death (PCD) during cleistothecia development (PCD in fungi is reviewed by Ramsdale, 2008). There are more examples of PCD during ascospore formation. For instance, half of the originally eight ascospores of *Coniochaeta tetraspora* are degenerated (Raju and Perkins 2000). Asci are present in high numbers in the mutant strains after 5-6 days, while numerous fully formed ascospores with a thick ornamented impenetrable cell wall without visible asci are present in the wild type. This suggests that the ascus cell wall and contents disappear during proper ascospore formation. This also could be the result of PCD following completion of ascospore formation. PCD seems to be initiated in the ascospores of the Δ*mpdA* strain as they appear malformed, with uncompleted cell wall, distorted ornamentation and electron dense contents. Earlier developmental stages show ascospore initials with cell constituents with intact ultrastructure. It might be possible that a PCD signal is also entering the ascospores due to a slower development caused by low mannitol levels. This is ultimately leading to abortion of these cells. These assumptions suggest a link between the reducing agent mannitol and the ROS induced process PCD.

Mannitol might act as a signal molecule in *Arabidopsis thaliana*. This organism normally does not produce this polyol (Chan, Grumet *et al.* 2011). Exposure to salt stress increases the expression of a variety of stress-inducible genes in the wild type strain. This induction is enhanced in a mannitol producing strain of *A. thaliana*. Mannitol may also act as a signal molecule in fungi (Solomon *et al.* 2006), but more research is clearly needed to evaluate such a role for this molecule.

Deletion of *mpdA* in *N. fischeri* also resulted in an increase of trehalose up to 400 % between 4 and 20 days of development. This phenomenon was previously observed in other fungi (Ruijter, Bax *et al.* 2003; Solomon, Tan *et al.* 2005; Solomon, Waters *et al.* 2006; Velez, Glassbrook *et al.* 2007; Dulermo, Rascle *et al.* 2010; Wang, Lu *et al.* 2012). Trehalose may take over the function of mannitol during vegetative growth and may also compensate for the absence of this compatible solute in conidia, thereby reducing but not fully compensating the loss of stress resistance. Trehalose is a well-known stress protector (Hottiger, Boller *et al.* 1989; Crowe, Reid *et al.* 1996; Fillinger, Chaveroche *et al.* 2001) and is important in longevity of *A. nidulans* conidia (Fillinger, Chaveroche *et al.* 2001) and stress sensitivity of *A. nidulans* and *B. cinerea* conidia (Fillinger, Chaveroche *et al.* 2001; Doehlemann, Berndt *et al.* 2006). Clearly, an increase of trehalose does not recover the effects of mannitol depletion in ascospore formation in *N. fischeri*. Future studies should reveal the specific and overlapping roles of mannitol, trehalose and other compatible solutes in growth and development of ascomycetes in general and *N. fischeri* in particular.

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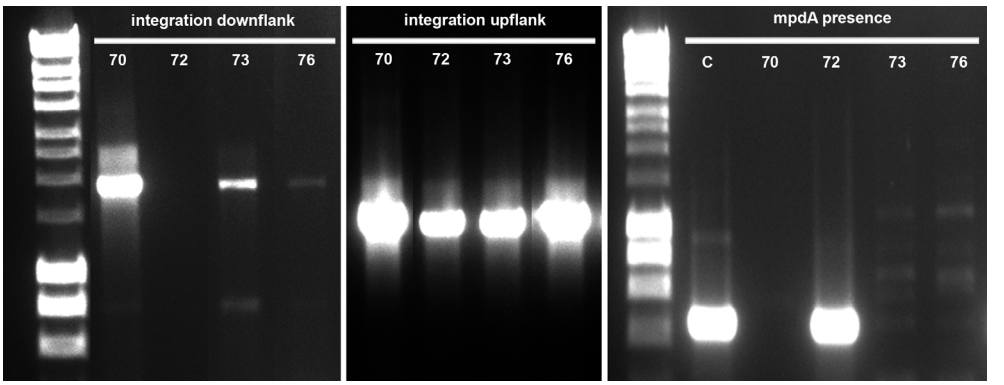
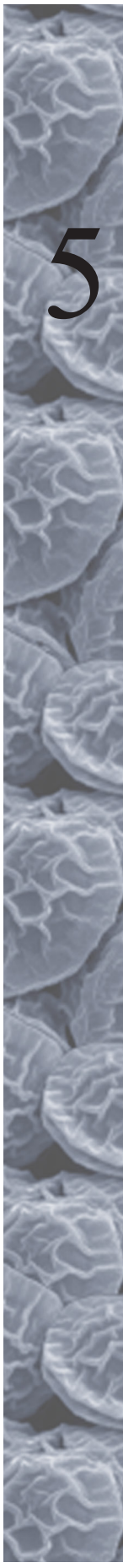
Supplemental table and figure

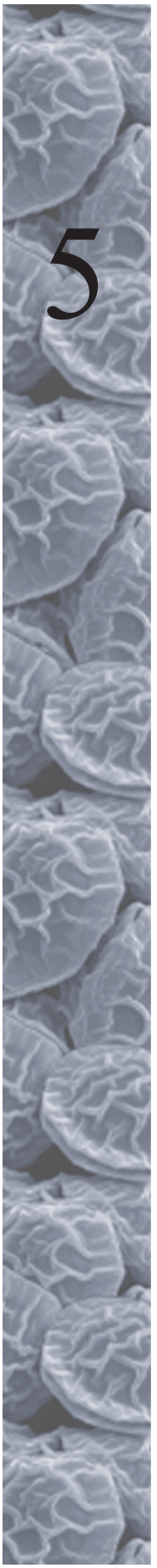
Supplemental Table 1. Blasf of the *N. fischeri* MpdA to the Mtld of bacteria and Mpd of protista, algae and fungi

Protein Number	Organism	Size	Query coverage	E value	Max identity
NP_233428	*Vibrio cholerae*	384aa	94%	4.00E-79	37%
NP_839297	*Shigella flexneri*	382aa	93%	2.00E-86	39%
AAF97118	*Escherichia_coli*	366aa	93%	4.00E-88	40%
NP_388281	*Bacillus subtilis*	373aa	95%	1.00E-99	42%
AAD02688	*Eimeria_tenella*	617aa	56%	1.00E-04	56%
CBJ25895	*Ectocarpus siliculosus*	525aa	61%	4.00E-05	67%
CBN74440	*Ectocarpus siliculosus*	553aa	56%	8.00E-05	23%
AAG09209	*Cryptococcus neoformans*	361aa	17%	1.10E-01	40%
AAT11122	*Phaeosphaeria nodorum*	390aa	99%	0.00E+00	63%
ACU32784	*Beauveria bassiana*	391aa	100%	1.00E-168	60%
XP_962476	*Neurospora crassa*	398aa	98%	6.00E-173	62%
XP_755399	*Aspergillus fumigatus*	388aa	100%	0.00E+00	97%



Supplemental Figure 1. PCR confirming deletion of *mpdA* in *N. fischeri*. Flanks of the deletion fragment are integrated homologously in the case of Δ*mpdA*70, Δ*mpdA*73 and Δ*mpdA*76. In the case of Δ*mpdA*72 the upstream flank has integrated but the coding sequence of *mpdA* gene is still present. Numbers correspond to the *mpdA* mutant and C represents wild type genomic DNA.


5



5 The role of the hydrophilins LeamA, Hsp12A, and Hsp12B in stress tolerance of *Neosartorya fischeri* (*Aspergillus fischeri*)

M. R. van Leeuwen, T. T. Wyatt, T. M. van Doorn, L. G. Lugones, H. A. B. Wösten, and J. Dijksterhuis

Abstract

Hydrophilins are proteins that occur in all domains of life. They protect cells and organisms against stress. Hydrophilins have a glycine content > 6 % and a hydrophilicity index > 1. They include most of the Late Embryogenesis Abundant (LEA) proteins and the heat shock protein Hsp12. Late Embryogenesis Abundant (LEA) proteins are highly expressed during seed maturation in plants and are associated with tolerance against desiccation, high and low temperature, and oxidative or osmotic stress. Hsp12 of *Saccharomyces cerevisiae* is induced by stress conditions and was shown to function in freeze and heat resistance. In this study, the role of genes encoding a predicted LEA-like protein (LeamA) and two Hsp12 proteins (Hsp12A, Hsp12B) was investigated in the filamentous fungus *Neosartorya fischeri*. Heterologous expression of these hydrophilins resulted in increased tolerance against salt and osmotic stress in *Escherichia coli*. These proteins were also shown to protect lactate dehydrogenase against dry heat and freeze-thaw cycles in vitro. We were unable to delete the genes encoding Hsp12A and Hsp12B in *N. fischeri*. Deletion of *leamA* caused diminished viability of sexual ascospores after drought and heat. To conclude, our results indicate for the first time that Leam and Hsp12 proteins protect a filamentous fungus against stresses such as desiccation, extreme temperature, and osmotic stress.

Introduction

Adverse environmental conditions including low and high temperatures and variations in osmotic potential confront many organisms with a limitation of cellular water. Removal of water from living cells is highly detrimental because of its impact on the conformation of proteins and membranes (Hoekstra, Golovina *et al.* 2001). Moreover, lack of water induces formation of potentially damaging reactive oxygen species that harm the cell during or after drying (Crowe and Crowe 1992). To resist water stress, desiccation-tolerant organisms have evolved protective mechanisms including intracellular accumulation of compatible solutes and protective proteins. Compatible solutes include sugars, polyols, amino acids and derivatives thereof. They stabilize cellular structures and restore the osmotic balance without interfering with cellular metabolism. Intracellular accumulation of the disaccharides sucrose and trehalose and the polyols mannitol and sorbitol have been implicated in protection against dehydration in a variety of organisms (DeVirgilio, Hottiger *et al.* 1994; Leslie, Israeli *et al.* 1995; Shen, Hohmann *et al.* 1999; Billi, Wright *et al.* 2000; Ruijter, Bax *et al.* 2003).

Protective proteins bind to and protect other proteins, membranes, and RNA (Warner, Brunet *et al.* 2004) during stress. Many of these protective proteins are classified as hydrophilins. Hydrophilins have a glycine content > 6 % and a hydropilicity index > 1 (Garay-Arroyo, Colmenero-Flores *et al.* 2000). They include 92 % of the Late Embryogenesis Abundant (LEA) proteins (Battaglia, Olvera-Carrillo *et al.* 2008) and a heat shock protein (HSP) called Hsp12. LEA proteins have been extensively studied. They were first described in the cotton plant *Gossypium hirsutum* as proteins accumulating at high levels during late stages of embryo development in seeds (Dure, Greenway *et al.* 1981; Galau, Hughes *et al.* 1986). LEA proteins generally occur in plants. LEA-like proteins have also been identified in bacteria (Volker, Engelmann *et al.* 1994; Stacy and Aalen 1998), protozoa (Katinka, Duprat *et al.* 2001), algae (Honjoh, Yoshimoto *et al.* 1995; Tanaka, Ikeda *et al.* 2004), nematodes (Browne, Tunnacliffe *et al.* 2002), yeast (Garay-Arroyo, Colmenero-Flores *et al.* 2000), insects (Kikawada, Nakahara *et al.* 2006), crustaceans (Wang, Meng *et al.* 2007), filamentous fungi (Sachs and Yanofsky 1991; White and Yanofsky 1993; Garay-Arroyo, Colmenero-Flores *et al.* 2000; Abba, Ghignone *et al.* 2006; Hoi, Lamarre *et al.* 2011; Wartenberg, Voedisch *et al.* 2012) and archea (Campos, Cuevas-Velazquez *et al.* 2013). LEA(-like) proteins show absence or low amount of Cys and Trp and relatively high levels of Ala, Glu, Lys, Arg, and Thr residues (Dure 1993ab; Garay-Arroyo, Colmenero-Flores *et al.* 2000). Five to nine subclasses of LEA proteins have been distinguished based on amino acid sequence and conserved motifs (Dure, Crouch *et al.* 1989).

For instance, group 3 LEA proteins are between 7.2 and 67.2 kDa and are characterized by a repeating 11-mer sequence motif containing Ala, Asp and / or Glu with Lys appearing every 11 aa (Dure 1993ab; Tunnacliffe and Wise 2007). Structure prediction programs suggest a high degree of folding for LEA proteins and other hydrophilins, mainly into amphipatic α-helices (Dure, Crouch *et al.* 1989). However,

these proteins show a high degree of unordered structure in solution (Goyal, Tisi *et al.* 2003; Dyson and Wright 2005; Tompa 2005; Mouillon, Gustafsson *et al.* 2006; Kovacs, Kalmar *et al.* 2008; Welker, Rudolph *et al.* 2010) but fold during dehydration or in the presence of a partner molecule such as cations or macromolecules (Dyson and Wright 2002; Goyal, Tisi *et al.* 2003; Tolleter, Jaquinod *et al.* 2007; Shimizu, Kanamori *et al.* 2010; Welker, Rudolph *et al.* 2010; Popova, Hundertmark *et al.* 2011). The folded hydrophilins stabilize phospholipids and membranes (Sales, Brandt *et al.* 2000; Tolleter, Jaquinod *et al.* 2007; Shih, Huang *et al.* 2010; Welker, Rudolph *et al.* 2010; Shih, Hsieh *et al.* 2012), have molecular chaperone activity (Goyal, Walton *et al.* 2005; Liu, Chakrabortee *et al.* 2011; Chakrabortee, Tripathi *et al.* 2012; Furuki, Shimizu *et al.* 2012) and form stable glasses by interaction with non-reducing oligosaccharides (Wolkers, McCready *et al.* 2001; Kalemba and Pukacka 2007).

The HSP group is the most heterogeneous group of stress protective proteins. It consists of large ATP-dependent chaperones and the small HSPs (sHSPs). Hsp12 of *Saccharomyces cerevisae* is a hydrophilin and as such structurally and functionally different from all other known HSPs. Hsp12 is soluble at high temperatures (80 °C) and it is induced by osmotic shock and when entering into the stationary phase (Mtwisha, Brandt *et al.* 1998; Praekelt and Meacock 1990; Sales, Brandt *et al.* 2000; Motshwene, Welker, Rudolph *et al.* 2010). Hsp12 is upregulated during exposure to stress such as high ethanol concentrations, glucose starvation and cell wall stress (Stone, Matarese *et al.* 1990; Jamieson, Rivers *et al.* 1994; Piper, Talreja *et al.* 1994). Deletion of the gene encoding Hsp12 had no effect on growth and viability of *S. cerevisiae* at various temperatures (Praekelt and Meacock 1990). Pacheco *et al.* (2009) showed that the accumulation of intracellular trehalose in *hsp12* null mutants compensated for the deletion of Hsp12. Overexpression of *hsp12* in a trehalose-6-phosphate synthase (TPS1) null mutant increased the freeze and heat resistance of the cells (Pacheco, Pereira *et al.* 2009). Hsp12 has also been proposed to have a role in barotolerance, desiccation protection and membrane stability (Sales, Brandt *et al.* 2000; Motshwene, Karreman *et al.* 2004; Welker, Rudolph *et al.* 2010; Dang and Hincha 2011).

LEA-like and Hsp12 proteins have not been functionally studied in filamentous fungi. We here present evidence that these hydrophilins function in stress resistance in *Neosartorya fischeri*. This fungus is a common food spoilage agent and produces extreme stress resistant sexual spores.

Materials and Methods

Organisms and growth conditions

N. fischeri strain CBS 317.89 was grown at 30 °C on oatmeal agar (OA) or malt extract agar (MEA) (Samson and Houbraken 2010). Cultures were inoculated with 10^5 heat-activated ascospores by spreading spores on the agar surface. To isolate ascospores, ascomata were harvested by scraping fungal colonies (± 30 days old or otherwise stated) from OA plates and collecting them in 9 ml ice-cold 10 mM ACES (N-(2-acetamido)-2-aminoethanesulfonic acid; Sigma-Aldrich, Zwijndrecht, the Netherlands) buffer, pH 6.8, containing 0.02 % Tween-80. After adding 1 cm^3 sterile glass beads (1:1 ratio of beads with diameters of 0.10 mm and 1.0 mm diameter). Ascomata were broken by vortexing (2 x 1 min) and sonification (5 min) using an Ultrasonic 2510E-MT cleaner (Branson Ultrasonics Corporation, Danbury, USA). Mycelium debris was removed by filtration through sterile glass wool. Ascospores were washed three times with ice-cold ACES buffer with a centrifugation step (1100 g, 5 min, 5 °C, swing out rotor) after each washing step. Ascospore pellets (10^8) were stored at -80 °C. Before inoculation, ascospores were taken up in 10 mM ACES buffer and activated by heat treatment for 2 min at 85 °C.

Construction of Escherichia coli cells expressing LeamA, Hsp12A and Hsp12B

The DNA coding sequences of LeamA (gene NFIA_065760), Hsp12A (gene NFIA_007970), and Hsp12B (gene NFIA_058420) of *N. fischeri* (Nierman, Pain *et al.* 2005) were synthesized introducing *BamHI* and *XhoI* restriction sites before the start and after the stop codon, respectively (Baseclear, Leiden, The Netherlands). The DNA fragments were inserted in pUC57 (Fermentas, St. Leon-Rot, Germany) and verified by sequencing. The coding sequences were then ligated with *BamHI/ XhoI* digested pET28a(+) (Merck, Beeston, UK). This resulted in expression vectors pET28a-065760, pET28a-007970, and pET28a-058420 that encompass a hydrophilin gene fused with a N-terminal His-tag. The expression vectors were introduced into *E. coli* BL21(DE3) (Merck, Beeston, UK) yielding BL21-LeamA, BL21-Hsp12A, BL21-Hsp12B. Introduction of the empty pET28a(+) vector served as a control.

Purification of hydrophilins from E. coli BL21-LeamA, BL21-Hsp12A, BL21-Hsp12B

BL21-LeamA, BL21-Hsp12A, and BL21-Hsp12B were grown overnight at 37 °C and 200 rpm in a 250 ml Erlenmeyer flask containing 50 ml lysogeny broth (LB) (Bertani 1951), supplemented with 50 μg ml^{-1} kanamycin (Sigma-Aldrich). The cultures were adjusted to OD_{600} 0.05 with fresh medium and incubated for 2 h at 37 °C and 200 rpm to reach exponential growth phase. Isopropyl-β-D-1-thiogalactopyranoside (IPTG; Sigma-Aldrich) was added to a final concentration of 1 mM and growth was prolonged for an additional 4 h. Cells were harvested at 4000 g for 15 min at 4 °C and kept at -20 °C until further processing. The hydrophilins were purified using the Ni-NTA spin kit (Qiagen, Venlo, The Netherlands) with some modifications. In short,

cells from 5 ml culture medium were resuspended in 700 µl NPI-10 lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 2 % Triton X-100, pH 8.0) and incubated on ice for 30 min. Cells were broken by sonication for 15 min and heating at 100 °C for 15 min. The lysate was centrifuged at 12.000 g for 30 min at 4 °C. The supernatant was loaded onto a Ni-NTA spin column that had been equilibrated with 600 µl NPI-10 and centrifuged for 2 min at 890 g. After centrifugation for 5 min at 270 g, the Ni-NTA spin column was washed twice with NPI-20 (NPI-10 with 20 mM imidazole) and once with NPI-20 without Triton X-100. The protein was eluted in two steps with 300 µl NPI-500 (50 mM NaH_2PO_4, 300 mM NaCl, 500 mM imidazole, pH 8.0) by centrifugation for 2 min at 890 g. The protein solution was dialyzed three times against deionized water (ddH_2O) for a total of 20 h in Spectra / Por dialysis tubing with a cut off of 3500 Da (Spectrum, Breda, The Netherlands) and freeze dried. Purification of the proteins was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to standard methods using 15 % gels (Sambrook and Russell 2001). Gels were stained with Gelcode blue stain reagent (Fisher Scientific, Landsmeer, The Netherlands). The identity of the purified proteins was confirmed by mass spectrometry (Alphalyse, Odense, Denmark). Proteins were quantified with the Novagen BCA protein assay kit (Merck Millipore, Amsterdam, the Netherlands).

Stress resistance of E. coli BL21-LeamA, BL21-Hsp12A, BL21-Hsp12B

Overnight cultures of *E. coli* BL21-LeamA, BL21-Hsp12A, and BL21-Hsp12B were diluted to OD_{600} 0.05 in 50 ml LB contained in a 250 ml Erlenmeyer flask and supplemented with 50 µg ml^{-1} kanamycin. After 2 h incubation at 37 °C and 200 rpm, IPTG was added to a final concentration of 1 mM and growth was prolonged for 4 h. Cells were pelleted (1 min, 1000 g) and taken up in 200 µl (OD_{600} = 0.003) LB containing 50 µg ml^{-1}kanamycin and 1 mM IPTG, and either or not containing 500 mM NaCl, 500 mM KCl, 130 mM $MgCl_2$ or 1300 mM sorbitol (Sigma-Aldrich). Cells were incubated at 37 °C and 200 rpm in a 96-wells flat bottom microtiter plate placed in a box with wet paper towels to prevent evaporation. OD_{600} was measured with a FLUOstar Optima (BMG LabTech, Ortenberg, Germany) microtiter plate reader after 20 h.

LDH activity assay

L-lactate dehydrogenase (LDH) from rabbit muscle (Sigma-Aldrich) was dissolved (0.4 mg ml^{-1}) in 100 mM dibasic sodium phosphate buffer pH 7.5. 10 µl of the protein solution was added to 50 µl demineralized water either or not containing 4-20 mg ml^{-1} hydrophilin. The protein mixture was dried for 1 h under vacuum using a Savant SpeedVac DNA 110 Concentrator (Thermo Scientific, Breda, The Netherlands) and incubated for 16 h at 60 °C. To determine enzyme activity, 1 ml of assay buffer (100 mM dibasic sodium phosphate buffer, pH 7.5, 100 mM NADH, 2 mM sodium pyruvate) was added to the dried proteins. LDH activity was monitored by NADH

absorbance at 340 nm using a Biochrom Libra S22 Visible Spectrophotometer (Cambridge, UK).

Construction of ΔleamA

Upstream and downstream flanking regions of *leamA* (NFIA_065760) were amplified by PCR with primer combinations LeamA up F / LeamA up R and LeamA down F / LeamA down R (Table 1) resulting in 1100 and 1250 bp fragments, respectively. The amplicons were extracted from gel using the Qiaquick Gel Extraction Kit (Qiagen) and A-tailed for 30 min at 70 °C in 20 µl containing 1-2 µg purified DNA, 1 unit BioTaq (Gentaur, Eersel, the Netherlands), 1 x NH_4 reaction buffer (delivered together with BioTaq), 0.2 mM dATP and 2.5 mM $MgCl_2$. The A-tailed amplicons were ligated with pGEM-T vector (Promega, Leiden, The Netherlands) and introduced into *E. coli* JM109. *BglII* / *NheI* and *XbaI* / *HindIII* fragments representing the up- and downstream flanking regions of *leamA*, respectively, were introduced in pAN7-1 that contains the hygromycin B resistance cassette (Punt, Oliver *et al.* 1987). The resulting deletion construct was named pAN7-1-Δ*leamA*.

Transformation of N. fischeri

Protoplasts of *N. fischeri* were generated as described (de Bekker, Wiebenga *et al.* 2009) with some minor modifications. In short, heat-activated ascospores (10^7) were introduced in a 500 ml Erlenmeyer flask containing 250 ml malt extract broth (MEB) (BD Difco, Breda, The Netherlands) supplemented with 100 µg ml^{-1} ampicillin. After incubation for 24 h at 30 °C and 200 rpm, the mycelium was homogenized in a Sorvall Omni Mixer (Thermo scientific) for 1 min at full speed. Half the homogenate was mixed with 125 ml MEB. After incubation for 16 h at 30 °C and 200 rpm, the mycelium was filtered over sterile nylon gauze and washed with 0.9 % NaCl. 2.5 g of mycelium (wet weight) was resuspended in 20 ml 0.2 M sodium phosphate buffer, pH 6, containing 0.8 M sorbitol, 0.15 units ml^{-1} chitinase (C6137, Sigma-Aldrich), 460 units ml^{-1} β-glucuronidase (G0751, Sigma-Aldrich) and 5 mg ml^{-1} lysing enzymes (L1412, Sigma-Aldrich). The mycelial suspension was incubated for up to 3 h at 37 °C and 150 rpm. Protoplasts were harvested by filtration over sterile glass wool. Cold 0.2 M sodium phosphate buffer, pH 6, containing 1.33 M sorbitol was added to the protoplasts to a total volume of 45 ml. After centrifugation for 10 min at 2000 g and 4 °C, protoplasts were washed with STC (50 mM $CaCl_2$, 10 mM Tris HCl, pH 7.5, 1.33 M sorbitol) and resuspended in STC at a concentration of 10^8 protoplasts ml^{-1}. 2 10^7 protoplasts (200 µl) were mixed with 50 µl PEG buffer (50 mM $CaCl_2$, 10 mM Tris HCl, pH 7.5, 25 % PEG-6000) and 1-5 µg pAN7-1-Δ*LeamA* that had been linearized with *BglII* and *HindIII*. After 20 min at RT, 2 ml PEG buffer was added followed by incubation for 5 min at RT. Subsequently, 4 ml STC was added and 50 % of it was mixed with 10 ml pre-warmed (60 °C) minimal medium (de Vries, Burgers *et al.* 2004) containing 0.95 M sucrose, 0.6 % agar and 250 µg ml^{-1} hygromycin B (HygroGold, InvivoGen, Toulouse, France). The mixture was poured onto minimal

medium containing 0.95 M sucrose, 1.2 % agar and 250 µg ml^{-1} hygromycin B and incubated for 3-5 days at 30 °C. Transformants were grown on a second selection plate containing minimal medium with 1 % glucose and 250 µg ml^{-1} hygromycin B. Absence of *leamA* in transformants was confirmed by PCR using primer sets LeamA pres F / LeamA pres R, LeamA up int F / LeamA up int R and LeamA down int F / LeamA down int R (Table 1).

Table 1. Primers used in this study

Name	Sequence 5'→ 3'	
LeamA up F	GCG**AGATCT**CTTATACCCCTGCATGTCTCGA	Introducing a *BglII* restriction site
LeamA up R	GCG**GCTAGC**TTCCGGGCCATCTTGGAGGT	Introducing a *NheI* restriction site
LeamA down F	**TCTAGA**CGCAGTTGAGATTGCCTCGGCAGAATGAAG	Introducing a *XbaI* restriction site
LeamA down R	**AAGCTT**CATGGCTGAATCACGAGATGACTACCGTGAG	Introducing a *HindIII* restriction site
LeamA pres F	ATGTCTTCTCTCGCACGCTTCGCTCC	Checking presence of *leamA*
LeamA pres R	TCACATGTTACCCATCTCCTTTGCCTTGCC	Checking presence of *leamA*
LeamA up int F	AGGACGGCGAACTGGCGGCTGGAA	Checking integration of deletion construct
LeamA up int R	TTACTCAGCCCTTCTCTCTGCGTCCGT	Checking integration of deletion construct
LeamA down int F	TTCGGGAGACGAGATCAAGCAGATCAACGG	Checking integration of deletion construct
LeamA down int R	CAAAGGGTCACGTGCAGGGACCCAT	Checking integration of deletion construct

Drought and heat stress of ΔleamA

Plugs taken from the peripheral zone of 10-day-old colonies of wild type and the deletion strain Δ*leamA* were placed on OA plates and incubated at 24, 27, 30, 33, 36, 40, 45, and 50 °C. After 4 days colony diameter was measured.

A number of 107 ascospores of 40-day-old colonies were pelleted by centrifugation (5 min, 1100 g). After removing the supernatant, spores were dried in a Savant SpeedVac (DNA 110 Concentrator, Thermo Scientific) for 30 min without heating. After 3 and 10 days at 25 °C, the ascospores were subjected to a 1-h-heat shock at 60, 70, or 80 °C followed by rehydration with 500 µl ACES buffer. The surviving ascospores were activated by a heat treatment for 2 min at 85 °C. Germination was analysed by inoculating a thin layer (± 1 mm) of MEA, placed on top of an object glass, with 10 µl of ascospores, subsequently covered with a cover glass. The ascospores were incubated overnight at 25 °C. The number of germinated ascospores was monitored with a Zeiss 206 Axioskop 2 plus microscope equipped with a Nikon DS-Fi1 digital camera run by Nikon NIS207 Elements D 3.0 software.

In silico analysis

Aliphatic index and the grand average of hydropathicity (GRAVY) were computed with ExPASy ProtParam (Artimo, Jonnalagedda *et al.* 2012). The hydropathy plot

was constructed according to the Kyte and Doolittle algorithm (Kyte and Doolittle 1982) using the ExPASy ProtScale with a 9-residue window length (moving average) (Artimo, Jonnalagedda *et al.* 2012). The degree of protein disorder was calculated with the web-based FoldIndex application (Prilusky, Felder *et al.* 2005) implementing the algorithm of Uversky *et al.* (2000). The subcellular localization of LeamA was predicted using PREDOTAR (Small, Peeters *et al.* 2004), MitoProt II (Claros and Vincens 1996), PSORT II (Nakai and Horton 1999) and TargetP (Emanuelsson, Nielsen *et al.* 2000). The secondary structure was computed using SOPMA (Geourjon and Deleage 1995), GOR4 (Garnier, Gibrat *et al.* 1996), and SIMPA96 (Levin 1997). The physicochemical properties of protein helices were calculated with HeliQuest (Gautier, Douguet *et al.* 2008). Repeated motif detection in the LeamA protein sequence was performed with the TRUST server from IBIVU (Szklarczyk and Heringa 2004).

Results

In silico analysis of LeamA, Hsp12A and Hsp12B

The genome of *N. fischeri* contains one gene encoding a predicted LEA-like protein and two genes encoding Hsp12 proteins. The open reading frame of the predicted LEA gene, *leamA* (NFIA_065760; NCBI-geneID: 4589955), is 423 bp in size and is interrupted with one intron. The ORF encodes a protein of 140 amino acid residues with a predicted molecular mass of 14.8 kDa and a theoretical pI of 9.5. PREDOTAR, PSORT II, MitoProt II and TargetP predicted the protein to reside in mitochondria with 79, 74, 78, and 91 % probability, respectively. The latter two programs identified a N-terminal pre-sequence of 39 amino acids (Fig. 1C, in red). The open reading frames of the predicted Hsp12 genes *hsp12A* (NFIA_007970; NCBI-geneID: 4591922) and *hsp12B* (NFIA_058420; NCBI-geneID: 4584904) are both 270 bp in length, each interrupted with two introns. They encode proteins with 90 amino acid residues with a molecular mass of 9.7 and 9.3 kDa and estimated pI's of 5.6 and 8.1, respectively. Localization signals could not be identified in Hsp12A and Hsp12B.

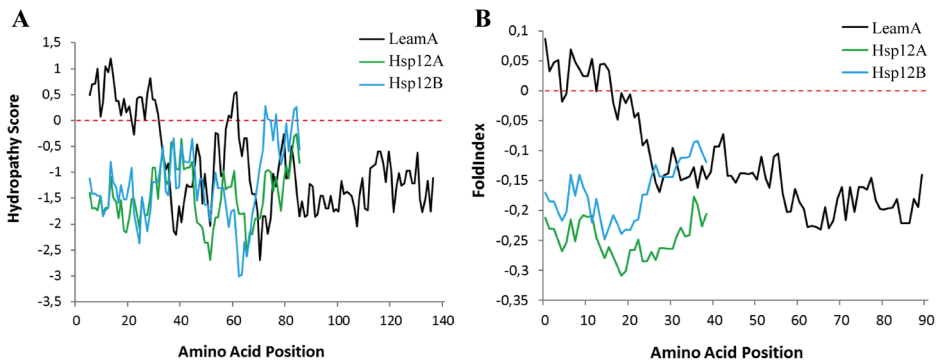
The LeamA, Hsp12A and Hsp12B proteins are devoid of Cys and Trp but show relatively high numbers of the positively charged basic residues Arg, Lys, and His (20.0, 15.7, and 20.2 %, respectively, for LeamA, Hsp12A and Hsp12B) and the negatively charged acid / amide residues Asn, Asp, Gln, and Glu (18.5, 28.1, and 23.6 %, respectively). Moreover, polar amino acids (Arg, Asn, Asp, Gln, Glu, Lys, Ser and Thr) were found to be highly represented with a total of 54.2, 68.5, and 62.9 % of the amino acid residues for LeamA, Hsp12A, and Hsp12B respectively. The TRUST server from IBIVU found three 11-mer motif repeats and a truncated 11-mer motif at the C-terminal part of LeamA (Figure 1C; Table 2), which is a characteristic of a LEA group 3 protein (Dure 1993ab). Comparison of the 11-mer motif of LeamA of *N. fischeri* with homologues of the related ascomycetes *Aspergillus fumigatus*, *Aspergillus clavatus*, *Aspergillus niger*, *Aspergillus nidulans*,

Talaromyces stipitatus, and *Penicillium chrysogenum* showed a consensus amino acid sequence of KGKAKEXAGEA, which was (mostly) repeated 4 times (with a truncated motif at the end, Table 2).

The solubility of proteins can be expressed by the grand average of hydropathicity (GRAVY) index (Kyte and Doolittle 1982). It is calculated as the sum of hydropathy values of all the amino acids, divided by the number of residues in the sequence. Positive GRAVY is indicative of a hydrophobic protein, while negative GRAVY indicates a hydrophilic protein. LeamA is strongly hydrophilic with a GRAVY value of -0.8, while Hsp12A and Hsp12B are even more hydrophilic with values of -1.4 and -1.2, respectively. Like the GRAVY index, the Kyte and Doolittle hydropathy plot revealed that the LeamA protein is strongly hydrophilic, except for the N-terminal pre-sequence that showed hydrophobic properties (Figure 1A).

Table 2. Alignment of the 11-mer repeat sequence of the LEA-like proteins of ascomycetes related to *N. fischeri* and their consensus sequence.

Species	Accession number	11-mer repeat sequence										
Neosartorya fischeri	A1D6R8_NEOFI	K	G	E	A	S	E	K	A	G	E	T
		K	G	K	A	S	E	T	L	G	E	A
		K	G	K	A	K	E	V	Y	G	E	A
		K	G	K	A	K	E	M				
Aspergillus fumigatus	Q4WY27_ASPFU	K	G	E	A	S	E	K	A	G	E	T
		K	G	K	A	K	E	T	L	G	E	V
		K	G	K	A	K	E	V	F	G	E	A
		K	G	K	A	K	E	M				
Aspergillus clavatus	A1CKQ2_ASPCL	K	G	D	A	A	E	V	A	G	K	S
		K	G	K	A	E	E	A	L	G	S	A
		K	G	T	A	K	E	V	Y	G	E	A
		K	G	K	A	K	E	M				
Aspergillus niger	A2QDJ8_ASPNC	K	G	K	A	E	E	A	L	G	T	A
		K	G	K	A	K	E	A	A	G	E	V
		K	G	K	A	K	E	T	A	G		
Aspergillus flavus	B8N1P4_ASPFN	K	S	K	A	E	E	T	L	G	S	A
		K	G	K	T	E	E	T	L	G	S	A
		K	G	K	A	K	E	T	L	G	E	A
		K	G	K	A	K	E	T	V			
Emericella nidulans	Q5B986_EMENI	Q	K	E	A	K	E	K	A	G	E	L
		K	G	D	A	A	E	L	A	G	K	A
		K	G	K	G	E	E	V	A	G	E	A
		K	G	K	A	K	E	V	A	G	E	F
Talaromyces stipitatus	B8M6I2_TALSN	E	G	K	A	K	E	V	A	G	E	A
		K	G	K	A	H	E	L	E	G	K	A
		K	G	K	A	E	E	L				
Penicillium chrysogenum	B6H4P1_PENCW	K	G	E	A	S	E	Y	A	G	K	A
		K	G	E	A	S	E	Y	A	G	K	V
		K	G	E	T	A	E	Y	A	G	K	G
		K	G	K	A	E	E	A				
- +	**Consensus**	**K**	**G**	**K**	**A**	**K**	**E**	**x**	**A**	**G**	**E**	**A**



C

MSSLARFAPLTARVAAVRTTTPAFTAAGSRFISSTPKNEKGPVEATKDTLKKADRVISDNLVK
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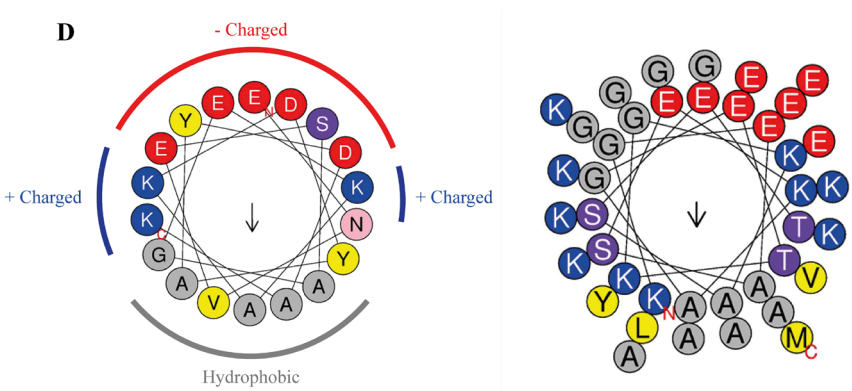
AKEVYGEAKGKAKEMGNM



E

Hsp12A
MSDTGRKDFSTKAQEKMTPDSQKSTMDKMKETVTDATDRLTGSAQGDNQKSYSQQAYDS
VRGETDNQTHGSSTETMGQKAKNAMGLGDH

Hsp12B
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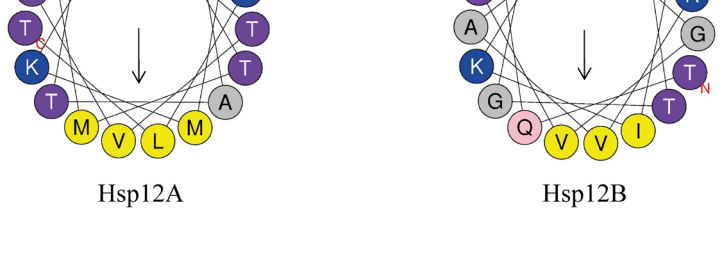

Previous page: **Figure 1**. In silico analysis of LeamA, Hsp12A, and Hsp12B. (A) Hydropathy plot with the amino acids plotted on the x-axis beginning at the N-terminus and using a 9-residue window length. Regions with a hydropathy score below zero are regarded as hydrophilic. (B) Degree of protein disorder according to the web based FoldIndex application showing unfolded (FoldIndex < 0) and folded (FoldIndex > 0) regions. (C) Amino acid sequence of LeamA showing the putative mitochondrial localization signal (red) and the C-terminal 11-mer repeats (underlined). (D) Helical wheel projection of the 11-mer repeat of LeamA and the LEAM 101-119 region of *Pisum sativum* (Tolleter *et al.*, 2007) showing the structural analogy between their amphipathic helices. (E) Amino acid sequence of Hsp12A and Hsp12B showing the regions predicted to form α-helices (underlined) of which the largest (red) are represented as a helical wheel projection.

Numerous proteins are shown to be unfolded under physiological conditions and the graphic web server FoldIndex (Prilusky, Felder *et al.* 2005) predicts whether a protein assumes a defined fold or whether it is intrinsically unfolded. FoldIndex predicts that the LeamA protein is intrinsically unfolded except for its N-terminal pre-sequence (Fig. 1B). Similarly, Hsp12A and Hsp12B are predicted to be intrinsically unfolded. The secondary structure of LeamA, computed with SOPMA, GOR4, and SIMPA96 revealed a proportion of α-helices of 75, 61, 59 %, respectively. This proportion was lower in the case of Hsp12A and Hsp12B with 47.2, 38.2, 48.9 % and 49.4, 23.6, 35.6 %, respectively. HeliQuest predicts that the repeated 11-mer motif forms an amphipathic α-helix similar to that of the helix motif of the mitochondrial LEAM protein from pea (*Pisum sativum*) (Tolleter, Jaquinod *et al.* 2007) (Fig. 1D). The α-helices of Hsp12A and Hsp12B also are predicted to have amphipathic properties (Fig. 1E).

Heterologous expression of LeamA, Hsp12A and Hsp12B

LeamA, Hsp12A and Hsp12B were expressed as His-tagged proteins in *E. coli* BL21. Protein bands of approximately 18, 14, and 14 kDa were observed in the Ni-NTA purified protein fractions of BL21-LeamA, BL21-Hsp12A, and BL21-Hsp12B, respectively (Fig. 2A). These bands were excised from gel and their identity was confirmed by mass spectrometry.

Viability measurements after drying *E. coli* BL21-LeamA, BL21-Hsp12A, and BL21-Hsp12B were not successful, because of difficulties to grow the bacteria after drying. Therefore, salinity and osmotic stress tolerance of BL21-LeamA, BL21-Hsp12A, BL21-Hsp12B and the control strain BL21-pET28a was determined. No difference in growth was observed when the strains were incubated in LB. Density of all cultures was reduced upon exposure to salinity or osmotic stress. However, the OD_{600} was significantly higher in cells expressing LeamA, Hsp12A or Hsp12B (Figure 2B). The OD_{600} values of Bl21-Leam were 22.9-, 14.0-, 2.7-, and 1.5-fold higher than that of the control strain BL21-pET28a after 20 h of growth in the presence of 500 mM NaCl, 500 mM KCl, 130 mM $MgCl_2$ and 1300 mM sorbitol, respectively. These values were 22.0-,14.3-, 2.7-, and 1.6-fold, and 21.4-, 16.7-, 2.8-, and 1.6-fold higher in the case of BL21-Hsp12A and BL21-Hsp12B, respectively (Fig. 2B). These results indicate that heterologous expression of LeamA, Hsp12A and Hsp12B improve salt and osmotic tolerance of *E. coli* cells with a similar efficiency.

Stress protection of LDH enzyme activity

LDH is sensitive to stress conditions such as heat and freeze-thaw cycles (Carpenter and Crowe 1988; Dong, Prestrelski *et al.* 1995). Activity of LDH was monitored in the presence or absence of partially purified LeamA, Hsp12A, and Hsp12B (see Fig. 2A) to assess whether these hydrophilins could protect the enzyme. At low oxygen levels, LDH catalyzes the interconversion of pyruvate and lactate with concomitant interconversion of NADH and NAD^+. The conversion of pyruvate was measured by a decrease in absorbance at 340 nm due to the oxidation of NADH. The heat stabilization of LDH by LeamA, Hsp12A and Hsp12B was assessed after drying the protein mixtures and subsequent heating. Drying (45 min under vacuum) of LDH alone decreased its activity by 3 % but drying in combination with heating for 18 h at 60 °C resulted in a 91 % decrease in activity. LDH activity after drying and heating was 36, 35, and 50 % of the original activity at a mass ratio of 10 : 1 (hydrophilin : LDH) and 62, 73, 84 % at a mass ratio of 40 : 1 for LeamA, Hsp12A, Hsp12B, respectively (Fig. 3A). Addition of lysozyme, that has a size similar as the hydrophilins of *N. fischeri*, resulted in 22 and 50 % LDH activity after dry heat at a mass ratio of 10 : 1 and 40 : 1, respectively. Bovine serum albumin (BSA) did not protect LDH under these conditions (Fig. 3A).

LDH maintained 42 and 11 % of its enzymatic activity after 2 and 6 freeze-thaw cycles, respectively (Fig. 3B). These values were 91 and 45 % when LeamA was

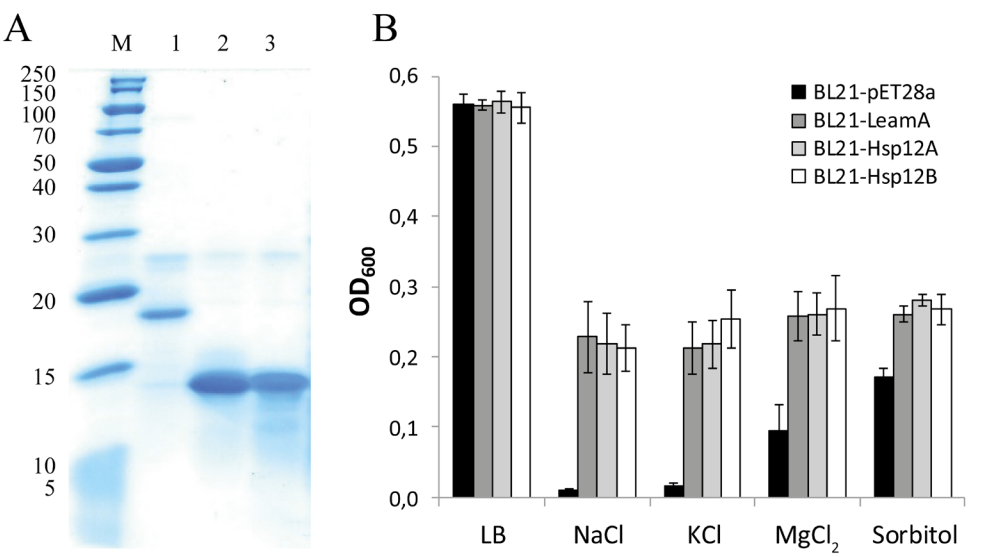

Figure 2. Heterologous expression of LeamA, Hsp12A and Hsp12B in *Escherichia coli* BL21. (A) The hydrophilins were extracted from BL21–LeamA, BL21–Hsp12A and BL21–Hsp12B cells, partially purified with a Ni-NTA spin column, and separated by SDS-PAGE. LeamA (lane 1), Hsp12A (lane 2), and Hsp12B (lane 3). (B) Viability of BL21–LeamA, BL21–Hsp12A and BL21–Hsp12B in LB or under high salinity (500 mM NaCl, 500 mM KCl or 130 mM $MgCl_2$) or osmolarity (1.3 M sorbitol). OD_{600} was measured after 20 h of growth. Average and standard deviations are based on four independent experiments.

present at a 20 : 1 ratio. Hsp12A, Hsp12B and the cryoprotectant BSA (Merino *et al.*, 2011) were able to prevent LDH inactivation nearly completely (3, 1 and 0 % loss of activity, respectively, after 6 freeze-thaw cycles). In contrast, presence of lysozyme resulted in 63 and 17 % activity after 2 or 6 freeze-thaw cycles, respectively (Fig. 3B). These results show that LeamA, Hsp12A and Hsp12B protect LDH against different stresses, whereas lysozyme and BSA only protect against dry heat and freeze-thawing, respectively.

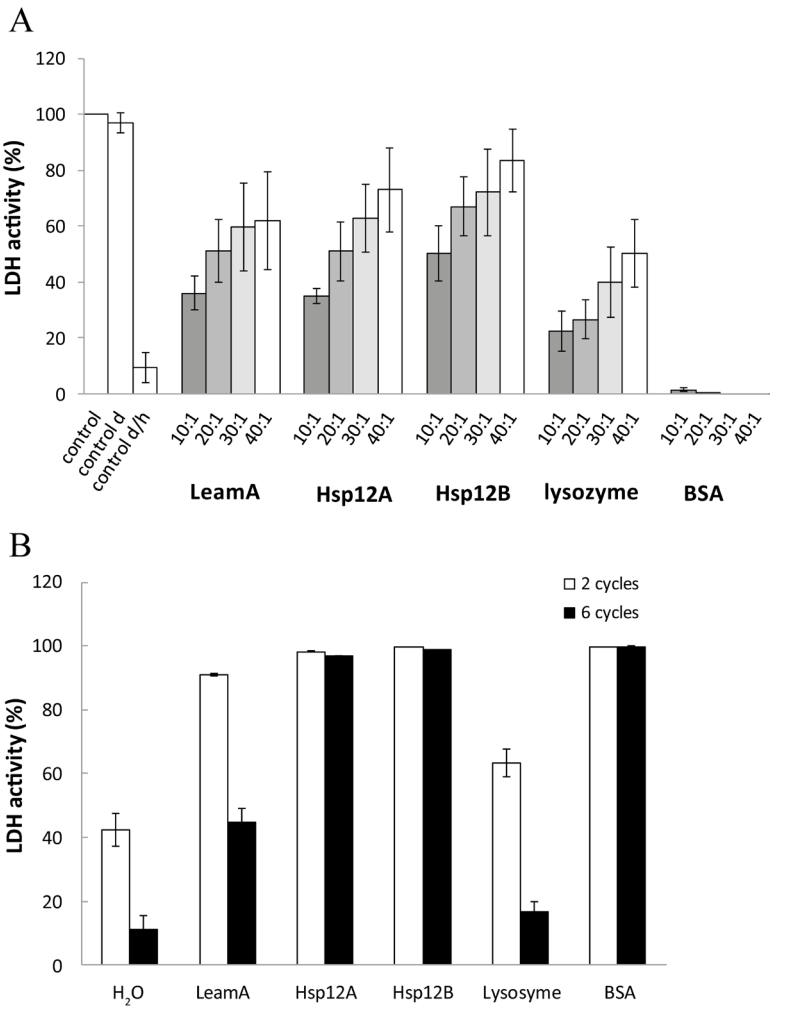

Figure 3. LDH protection by LeamA, Hsp12A and Hsp12B against heat and cycles of freezing in liquid nitrogen and thawing at ambient temperature. (A) Relative residual LDH activity was measured at OD_{340} after drying and exposure at 60 °C for 18 h in the absence or presence of LeamA, Hsp12A and Hsp12B in various ratios (protein protectant : LDH). The control was maintained at 7 °C, dried (d), or dried and heated (d/h). (B) Relative residual LDH activity after 2 and 6 freeze-thaw cycles in the absence or presence of LeamA, Hsp12A and Hsp12B (protein protectant : LDH ratio 20:1). Lysozyme and BSA served as controls.

Deletion of leamA

We were unable to delete the *hsp12A* and *hsp12B* genes of *N. fischeri*. However, the coding sequence of LeamA of *N. fischeri* was successfully replaced for the Hygromycin B resistance cassette using the pAN7-1-Δ*leamA* deletion vector. The absence of *leamA* and the correct integration of pAN7-1-Δ*leamA* were confirmed by PCR (Data not shown). Radial extension was not affected in the Δ*leamA* strain when compared to the wild type at a growth temperature of 24-50 °C (Fig. 4A). The

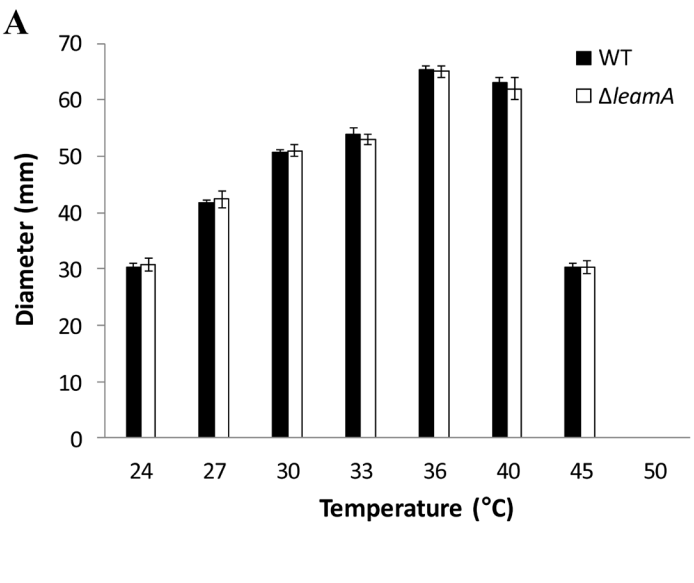

Figure 4. Heat and drought tolerance of Δ*leamA*. (A) Colonies of WT and Δ*leamA* were grown from mycelial plugs on oatmeal agar at a temperature varying from 24 to 50 °C. (B) Ascospores of WT and Δ*leamA* were dried and either or not subjected to a 1-h-dry-heat shock at 60, 70 or 80 °C followed by heat activation for 2 min at 85 °C in an aqueous solution. Average and standard deviations are based on biological triplicates. 'Control' are non-dried ascospores not subjected to a dry heat shock. 'No heat shock' are dried ascospores not subjected to a dry heat shock.

optimal and maximal growth temperature was 36 °C and 45 °C, respectively, for both strains.

The viability of wild type and Δ*leamA* ascospores was determined under drought and heat stress. Ascospores of 40-day-old cultures of the wild type strain showed similar germination levels after 1 h drying under vacuum when compared to non-dried ascospores (92 and 93 %, respectively). The survival rate was 78 % when the dried ascospores had been heated for 1 h at 60 °C. This decreased to 74 % and 64 % when the spores had been incubated at 70 °C and 80 °C, respectively (Fig. 4B). Drying of Δ*leamA* ascospores of 40-day-old cultures resulted in a viability of 58 % while viability of the untreated control was 82 %. Drying followed by heat shock (60, 70, and, 80 °C) resulted in survival of 36, 28 and 16 %, respectively (Fig. 4B). Taken together, these data show that LeamA promotes survival during exposure to drought and heat.

Discussion

Ascospores of fungi belonging to the order Eurotiales can be highly stress resistant (Dijksterhuis 2007). For instance, ascospores of *N. fischeri* survive 85 °C for more than 10 min in an aqueous environment (Beuchat 1986; Houbraken, Dijksterhuis *et al.* 2012). Moreover, these spores survive a relative humidity (RH) < 0.5 % at 60 °C for more than 7 days in a dried condition (**Chapter 2**). It has been shown that stress resistance of *N. fischeri* increases during ascospore maturation. The first stage of maturation is accompanied by a reduction of bulk water in the spores, while the second stage is characterized by an increase of trehalose and trehalose based oligosaccharides (**Chapter 3**). Here, the role of the hydrophilins LeamA, Hsp12A and Hsp12B of *N. fischeri* was studied in relation to stress resistance. This is the first functional characterization of such proteins in filamentous fungi.

The genome of *N. fischeri* encodes one LEA-like protein and two Hsp12 proteins, called LeamA, Hsp12A and Hsp12B. Other species of the order Eurotiales including *A. fumigatus*, *A. nidulans*, *T. stipitatus* and *P. chrysogenum* also contain a single LEA-like protein and one or two Hsp12 proteins. In silico analysis revealed that LeamA, Hsp12A, and Hsp12B of *N. fischeri* can be classified as hydrophilins (Dure 1993ab; Garay-Arroyo, Colmenero-Flores *et al.* 2000; Battaglia, Olvera-Carrillo *et al.* 2008). These proteins are intrinsically unstructured, highly hydrophilic, and contain a high content of Gly. Moreover, they lack Cys and Trp residues, while Ala, Glu, Lys, Arg, and Thr residues are relatively abundant. Absence of Cys and Trp is common in thermophilic proteins. Comparing mesophilic proteins and thermophilic proteins reveals that Cys is often replaced by Ala/Val/Ile, while Trp is converted in Tyr (Gromiha, Oobatake *et al.* 1999). LeamA, Hsp12A and Hsp12B indeed did not precipitate, at least partly, upon a 15 min treatment at 100 °C. LeamA contains three C-terminal 11-mer motif repeats (and a truncated one). Repeats of a 11-mer motif is characteristic for LEA class 3 proteins (Dure 1993ab; Tunnacliffe and Wise 2007). The consensus sequence of the repeated motif found in this study (KGKAKEXAGEA)

shows the characteristic presence of Lys (K), Glu (E), Ala (A) and Gly (G) and is predicted to form an amphipatic α-helix. Similarly, Hsp12A and Hsp12B are predicted to form amphipathic α-helical structures. This is expected to occur upon drying, and exposure to lipid layers (Tolleter, Jaquinod *et al.* 2007; Welker, Rudolph *et al.* 2010). The hydrophobic and hydrophilic sides of the amphiphilic α-helices are assumed to interact with the membrane core and the negatively charged heads, respectively (Tolleter, Jaquinod *et al.* 2007; Tolleter, Hincha *et al.* 2010; Welker, Rudolph *et al.* 2010). By doing so, they would protect membranes. PsLEAm of pea enhances stability of liposomes against desiccation, rehydration and freezing. Its protective capacity depends on phospholipid composition. The mitochondrial lipid cardiolipin in particular enhances protection (Tolleter, Hincha *et al.* 2010).

A stretch of 39 amino acids at the N-terminus of LeamA is predicted to represent a mitochondrial signal sequence. Mitochondrial localized LEA-like proteins have been identified in the brine shrimp *Artemia franciscana* (Menze, Boswell *et al.* 2009) and in pea (*P. sativum*) (Grelet, Benamar *et al.* 2005; Tolleter, Jaquinod *et al.* 2007). Future studies should confirm the localization of LeamA in mitochondria of *N. fischeri*. Hsp12A and Hsp12B are predicted to be localized in the cytoplasm because they lack localization signals. Hsp12 of *S. cerevisiae* has been localized in the cytosol (Huh, Falvo *et al.* 2003;Welker, Rudolph *et al.* 2010), but also in the cell wall, where it is suggested to act as a plasticizer (Sales, Brandt *et al.* 2000; Motshwene, Karreman *et al.* 2004; Welker, Rudolph *et al.* 2010). Cytosolic and cell wall Hsp12 have been localized at either side of the plasma membrane (Welker, Rudolph *et al.* 2010). Taken together, LeamA and the Hsp12 proteins may protect the membranes of mitochondria and the plasma membrane, respectively. LeamA, Hsp12A and Hsp12B of *N. fischeri* also protected LDH in vitro during desiccation, and exposure to heat and freeze-thaw cycles. Similarly, PsLEAm of pea protects the mitochondrial matrix enzymes fumarase and rhodanese in vitro against desiccation (Grelet, Benamar *et al.* 2005).

Deletion of *leamA* increased the sensitivity of *N. fischeri* ascospores to a combination of drought and heat. Presence of this protein in the ascospores should be confirmed in future studies. Transcripts of the homologue of *leamA* of *A. niger* (A2QDJ8_ASPNC: Table 2) are highly present in dormant conidia and conidia kept for 8 h in 10 µM of the antifungal compound natamycin. Its mRNA levels rapidly decrease at initiation of germination (van Leeuwen, Krijgsheld *et al.* 2013). This suggests that the homologue of LeamA also has a role in stress tolerance (van Leeuwen, Krijgsheld *et al.* 2013). Transcripts of the hydrophilin gene *con-6* also accumulate in conidia, ascospores and microconidia of *Neurospora crassa* (Springer and Yanofsky 1992; White and Yanofsky 1993). Levels of the encoded protein rapidly decrease within 2 h of germination and is undetectable after 16 h (White and Yanofsky 1993).

We have not been able to inactivate *hsp12A* and *hsp12B* in *N. fischeri*. Possibly, these genes are essential. Hsp12A and Hsp12B are predicted to function at distinct positions in the cell when compared to LeamA. Compatible solutes like trehalose

and trehalose based oligosaccharides are expected to function at the same location as Hsp12A and Hsp12B. It would be interesting to assess whether compatible solutes act synergistically with these hydrophilins.

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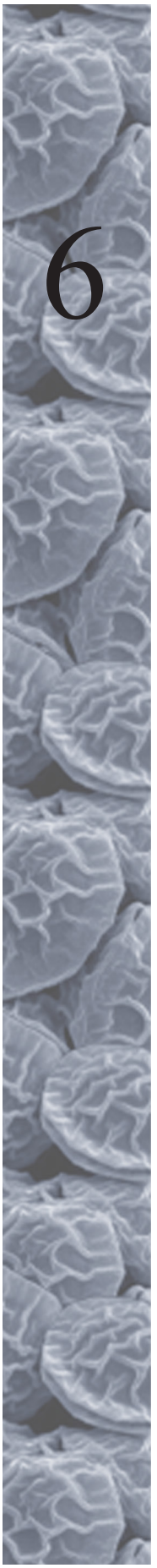

6 Summary and General Discussion

General Discussion

Fungi are an important food source. Mushrooms like champignons and Quorn represent reproductive and vegetative forms of fungi, respectively. Fungi are also involved in the production of food products. For instance, fungal fermentation of soy results in soy sauce and tempeh, fermentation of wheat results in bread and beer, and fermentation of grape extract results in wine. Fungi are also negatively associated with food. Fungal food spoilage is distasteful and can negatively affect human health due the production of toxic compounds, so-called mycotoxins. Reliable estimations of the worldwide financial costs due to fungal spoilage are hard to find. Yet, it is generally accepted that this amounts billions of euro each year. Mild heat treatments are used to minimize fungal spoilage. Pasteurized food products, especially canned fruit and fruit juices, are still prone to spoilage by fungi that produce extreme heat-resistant sexual spores, known as ascospores (Olliver and Rendle 1934; Hull 1939). For instance, heat-resistant ascospores of *Neosartorya fischeri* survive more than 10 minutes at 85 °C (**Chapter 2, 3**), while ascospores of *Talaromyces macrosporus* can survive more than 2 hours at this temperature (Dijksterhuis, van Driel *et al.* 2002). In fact, pasteurization can activate dormant heat-resistant ascospores to germinate, thereby initiating spoilage of the food product. Heat activation may be functional in nature after a fire. Competitors have been killed by the high temperatures, while there is plenty of nutrients available after the fire. Knowledge of the mechanisms underlying heat-resistance of ascospores is thus of fundamental and applied interest. *N. fischeri* was selected as the model system in this Thesis. It is a common and well-studied food spoilage agent, it is closely related to the human pathogen *Aspergillus fumigatus*, it can be genetically modified, and its genome sequence is available.

Heat-resistant ascospores of *T. macrosporus* are also resistant to high pressure (Reyns, Veraverbeke *et al.* 2003; Dijksterhuis and Teunissen 2004), drought (**Chapter 2**), and chemical stresses (pH stress, salt stress). Stress-tolerant organisms often use intracellular accumulation of compatible solutes as a mechanism to protect themselves against adverse conditions. Compatible solutes including sugars (sucrose, trehalose, raffinose), polyols (glycerol, mannitol, sorbitol), betaines, and amino acids (glycine, proline) stabilize cellular structures and proteins without interfering with cellular metabolism. Proteins can also protect organisms against stress by interacting with other proteins, membranes and RNA (Warner, Brunet *et al.* 2004). Many of these protective proteins are classified as hydrophilins. Hydrophilins have a glycine content > 6 % and a hydropilicity index > 1 (Garay-Arroyo, Colmenero-Flores *et al.* 2000). They include most of the Late Embryogenesis Abundant (LEA) proteins (Battaglia, Olvera-Carrillo *et al.* 2008), as well as conidiation related proteins (like Con-6), dehydrins, and the heat shock protein Hsp12. Hsp12 of *Saccharomyces cerevisae* was identified as a protein that is induced by stress conditions (Nisamedtinov, Lindsey *et al.* 2008). LEA(-like) proteins occur in all domains of life (Hand, Menze *et al.* 2011) and have been extensively studied. They were first described in the cotton plant *Gossypium hirsutum* as proteins accumulating at high levels during late stages of embryo development in seeds (Dure, Greenway *et al.* 1981; Galau, Hughes *et al.* 1986).

The aim of this Thesis was to study mechanisms underlying heat resistance of ascospores of *N. fischeri*. It is shown that stress resistance in *N. fischeri* increases during ascospore maturation. The first stage of maturation is accompanied by a reduction of bulk water in the spores, while the second stage is characterized by an increase of trehalose and trehalose based oligosaccharides (TOS) (**Chapter 3**). These TOS may protect ascospores especially to dry heat (**Chapter 2**). It is also shown that a LEA-like protein is involved in stress resistance of ascospores of *N. fischeri*, while two Hsp12 proteins seem to fulfill a similar role (**Chapter 5**). The polyol mannitol was shown to be essential for the formation of ascospores (**Chapter 4**). This made it impossible to study its role in stress resistance of ascospores. Understanding the formation and functioning of trehalose, TOS, hydrophilins and mannitol may provide new leads to prevent food spoilage by heat-resistant fungi. These molecules themselves could also be used to stabilize industrial important micro-organisms, enzymes, and / or vaccines. External addition of these compounds could increase their shelf life and protect against abiotic stresses.

Identification of compatible solutes in ascospores of N. fischeri

Intracellular glycerol accumulation has been associated with osmotic stress in *S. cerevisiae* (Nevoigt and Stahl 1997), while intracellular accumulation of the disaccharides sucrose and trehalose and the polyols mannitol, sorbitol, and glycerol has been related to protection against desiccation, heat and oxidative stress in a variety of organisms such as tardigrades (Hengherr, Heyer *et al.* 2008), bacteria (Billi, Wright *et al.* 2000), insects (Hendrix and Salvucci 1998), plants (Blackman, Obendorf *et al.* 1992), yeast (Devirgilio, Hottiger *et al.* 1994; Shen, Hohmann *et al.* 1999) and filamentous fungi (Singer and Lindquist 1998; Benaroudj, Lee *et al.* 2001; Fillinger, Chaveroche *et al.* 2001; Ruijter, Bax *et al.* 2003; Cao, Wang *et al.* 2008). Mannitol and trehalose are present in high amount in extreme heat-resistant ascospores. The combined concentration of these compounds in *T. macrosporus* ascospores is more than 1 M (Dijksterhuis, van Driel *et al.* 2002) (**Chapter 2**), while it can be as high as 800 mM in the ascospores of *N. fischeri* (**Chapter 3**). Analysis of *N. fischeri* ascospore extracts by high-performance liquid chromatography (HPLC) also showed the presence of glycerol and several unknown solutes (**Chapter 2**). Glycerol was present in ascospores of 11-day-old cultures but had disappeared in ascospores of 15 day-old cultures or older (**Chapter 3**). The molecular structure of the unknown solutes was resolved with thin layer chromatography (TLC), mass spectrometry and nuclear magnetic resonance spectroscopy. These molecules are characterized by a trehalose (Glcα1-1αClc) core to which one (isobemisiose), two (neosartose) or three (fischerose) glucose moieties are α-1,6 linked (**Chapter 2**). These trehalose-based oligosaccharides were collectively called trehalose-based oligosaccharides (TOS). Isobemisiose had previously been identified in the white fly *Bemisia argentifolii* (Hendrix and Salvucci 2001) but neosartose and fischerose had never been reported to occur in a living system.

TOS were absent in *N. fischeri* cultures upon disruption of the mannitol 1-phosphate dehydrogenase gene *mpdA*. Inactivation of this gene did not affect vegetative growth and formation of conidia, fruiting bodies (ascomata), and asci (**Chapter 4**, see below). However, development of ascospores was affected after meiosis had occurred. This indicates that TOS are specifically present in ascospores. Species belonging to the clades *Byssochlamys*, *Hamigera* / *Warcupiella*, *Neosartorya*, *Rasamsonia*, and *Thermoascus* were shown to accumulate similar, if not identical, oligosaccharides, according to their HPLC and TLC patterns (**Chapter 2**). *Neosartorya*, *Rasamsonia* and *Thermoascus* are well-known thermotolerant or thermophilic fungi (Houbraken unpublished work; Mouchacca 1997; Mouchacca 2007; Houbraken, Spierenburg *et al.* 2012). These fungi are thus expected to occur in environments with high temperature. Their ascospores should therefore also be able to endure hot conditions.

Role of compatible solutes in ascospores

Disruption of *mpdA* reduced mannitol levels in *N. fischeri* cultures as much as 85 %, while trehalose levels had increased > 400 %. Inactivation of *mpdA* had no effect on mycelial growth, even when exposed to heat or oxidative stress. Possibly, trehalose compensates for the absence of mannitol. Conidia however, did show increased sensitivity to heat and oxidative stress. Deletion of mpd in other filamentous fungi also often leads to a decrease of mannitol levels and increased levels of trehalose without affecting mycelial growth. Yet, in contrast to *N. fischeri* this is often accompanied with a defect in asexual sporulation (Ruijter, Bax *et al.* 2003; Solomon, Tan *et al.* 2005; Solomon, Waters *et al.* 2006; Dulermo, Rascle *et al.* 2010; Wang, Lu *et al.* 2012). The most pronounced effect of *mpdA* inactivation in *N. fischeri* was the absence of mature ascospores. Such a role in filamentous fungi had not been reported before. Asci of the Δ*mpdA* strain of *N. fischeri* did produce premature ascospores. These spores, however, were malformed with an incompleted cell wall, distorted ornamentation, and electron dense contents. The exact role of mannitol in ascospore maturation in *N. fischeri* is not yet clear. Mannitol has been proposed to act as a carbon storage molecule in conidia (Horikoshi, Iida *et al.* 1965). Mannitol may have a similar role in ascospores of *N. fischeri*. It may be used as energy source during ascospore maturation (Dijksterhuis and Teunissen 2004). Mannitol may also act as a signalling molecule based on the finding that it induces expression of stress-related genes in *Arabidopsis thaliana*. (Chan, Grumet *et al.* 2011). Finally, mannitol may function in ascospore development by acting as a reservoir of reducing power (Hult, Veide *et al.* 1980). NAD^+/NADH and $NADP^+$/NADPH ratios can be adapted by mannitol synthesis and breakdown (see Figure 1, **Chapter 4**). NADPH is a substrate of Nox that produces the reactive oxygen species (ROS) superoxide. NoxA of *A. nidulans* has been shown to be essential for differentiation of fruiting bodies by generating ROS in peridial layers that surround the initials of the ascogenous cells (Lara-Ortiz, Riveros-Rosas *et al.* 2003). Part of these layers die and dissolve

to generate space for the developing asci and supporting hyphae (Sohn and Yoon 2002). ROS may induce programmed cell death (PCD) in these layers. PCD may also induce degradation of the ascus cell wall and its contents during proper ascospore formation. Wild-type ascospores may be protected against PCD but the ascospores of the Δ*mpdA* strain may be sensitive. Possibly, a PCD signal enters the ascospores of the Δ*mpdA* strain due to a slower development caused by low mannitol levels. This would lead to abortion of these cells.

Two phases were distinguished in heat resistance acquirement in ascospores of *N. fischeri* (**Chapter 3**). The first phase takes place in 11-15-day-old cultures. Acquirement of heat resistance is accompanied with an increase in the total amount of compatible solutes (trehalose, TOS, and mannitol) from about 400 mM to 1 M, an increase in viscosity (from 5.1 to 21.2 cP), and a reduced amount of bulk water. The second phase takes place in 15-50 day-old cultures. Viscosity and concentration of compatible solutes do no change during this phase. However, redox stability and heat resistance of the spores still increased. This is accompanied with a change in the composition of the compatible solutes. The mannitol level reaches its maximum at a culture age of 20 days, while trehalose and TOS levels increase to a maximum in spores of 50-day-old cultures. This indicates that trehalose and TOS play an important role in redox stability and heat resistance in the second phase of development.

Constitutively dormant and extreme stress resistant fungal and bacterial spores exhibit high cytoplasmic viscosity (approx. 10-20 cP) (de Vries 2006; Dijksterhuis, Nijsse *et al.* 2007; **Chapter 2, 3**). Drying of the ascospores of *N. fischeri* leads to a glassy state of the cytoplasm with an even higher viscosity (**Chapter 3**), with high protection during anhydrobiosis and dry heat (**Chapter 2**). Formation of a glass could thus further increase the stability and longevity of an organism. Important properties of a glass are the glass (-liquid) transition temperature; T_g, and the glass density. A high T_g is essential for prolonged stabilization in a dry condition (Buitink and Leprince 2004). The density of the glass depends on the interactions between the molecules. Denser glasses might give more structural support, while a less dense glass might fold better around its target molecule(s), thereby giving a better protection. The physical properties of glasses that were composed of mannitol, trehalose, isobemisiose, neosartose and fischerose were studied with Fourier Transform Infrared (FTIR) spectroscopy (**Chapter 2**). In general, the T_g of the first scan (T_{g1}) differed from the second and third scan (T_{g2}). For instance, T_{g1} of trehalose and isobemisiose were 48.1 and 44.3 °C, respectively, while T_{g2} was 108 and 120.4 °C. Heating in the first scan could remove residual water molecules that act as an plasticizer and / or rearrange the molecules in the glass. This rearrangement leads to so-called 'matured' glass, which has a higher melting temperature. T_{g2} corresponds to the T_g found in the literature. The question is if T_{g1} values are more relevant than T_{g2} values for glasses in nature. In nature glasses will never be heated until 140 °C as is done during FTIR scanning, thus most likely never reach T_{g2} values. Unless the glass is formed at a very slow speed, because the speed of drying is an important factor for the formation of a stable glass (Hoekstra, internal conversation).T_{g2} is correlated with the degree of

polymerization (DP), while T_{g1} negatively correlated with DP. Thus, TOS showed a higher T_{g2} than trehalose, while they showed a lower T_{g1} (48.1, 44.3, 39.1, and 38.8 °C for trehalose, isobemisiose, neosartose, and fischerose respectively). Packing density of TOS glasses (WTC_2-values) also decreased with DP. However, a glass of pure trehalose had a lower density than the glasses composed of TOS (**Chapter 2**). Thus, other characteristics than solely the size of the molecule are important for the density of a glass, like the flexibility of the molecule or ability to form hydrogen bonds.

Trehalose is an effective stabilizing solute because of its relatively high T_g, its ability to replace water molecules that interact with biomolecules, its non-reducing behavior (not reactive), and its ability to form hydrates during water sorption (Rossi, Buera *et al.* 1997). TOS may have similar properties as trehalose but even may have additive value in protection against stress. The plant homologs of TOS are sucrose-based oligosaccharides (SOS). SOS consist of sucrose to which 1 (raffinose), 2 (stachyose), or 3 (verbascose) galactose moieties are connected via α-1,6 linkages, respectively. SOS are believed to act as membrane protectors via direct hydrogen binding (Hincha, Zuther *et al.* 2002; Hincha, Zuther *et al.* 2003; Milhaud 2004; Beck, Fettig *et al.* 2007). A longer chain length than that of sucrose would be important for insertion into the membrane, where a flexible random coil structure of the oligosaccharide reduces steric hindrance. TOS could have a similar role as SOS. Notably, ascospores of *N. fischeri* that contain trehalose and TOS survived desiccation and dry-heat better than *T. macrosporus* that contains mainly trehalose. In contrast, *T. macrosporus* ascospores survived wet heat better when compared to *N. fischeri* (**Chapter 2**). These results suggest that TOS function in the protection against the combined stress of drought and heat. TOS didn't provide better in vitro protection of lactate dehydrogenase (LDH) or fungal blastospores to both dry heat (drought combined with heat) and wet heat (heat in an aqueous environment) than trehalose, but seemingly operate in complex and specific mixtures of biomolecules.

Storage of dried spores at room temperature (RT) or 60 °C for 3 days didn't have any effect on the mobility of the spin probes TEMPO and TEMPOL in the spores according to the ESR spectra. This indicates that the storage didn't affect the cytoplasmic structure of the ascospores. However, an increase of the redox conversion of TEMPO, but not of TEMPOL, was observed after storage at 60 °C (**Chapter 3**). The hydrophobic nature of TEMPO suggests that this spin probe resides in the proximity of the membrane, while the hydrophilic TEMPOL resides in the cytosol. The more redox unstable environment of TEMPO suggests that the membranes are more vulnerable to storage at 60 °C. This proposed Achillus heel of the ascospore may be protected by TOS; in other words it may even be less stable in their absence.

Protection of small soluble proteins

Various proteins have evolved to protect against stress conditions. Many of these protective proteins are classified as hydrophilins (Garay-Arroyo, Colmenero-Flores

et al. 2000). The variety and number of hydrophilins in plants is very high. Most of their LEA proteins are hydrophilins. They are classified into 5-9 different groups, each consisting of many representatives (Battaglia, Olvera-Carrillo *et al.* 2008). For instance, the genome of *Arabidopsis* contains 51 genes encoding LEA proteins (Hundertmark and Hincha 2008). The number of hydrophilins in filamentous fungi seem to be much lower. Four different types of hydrophilins have been identified in filamentous fungi, namely Hsp12 (**Chapter 5**), the conidiation related genes (like Con-6) (White and Yanofsky 1993), the dehydrin(-like) genes (Abba, Ghignone *et al.* 2006; Hoi, Beau *et al.* 2012) and the LEA-like proteins. The genome of *N. fischeri* contains 3 genes with a Con-6 domain, 2 genes encoding a predicted Con-10 protein, 2 genes encoding a predicted dehydrin, one gene predicted to encode a LEA-like protein (LeamA), and two genes encoding a predicted Hsp12 (Hsp12A and Hsp12B). LeamA, Hsp12A and Hsp12B were studied in **Chapter 5**. They are predicted to be intrinsically unstructured, to be highly hydrophilic, and to contain a high glycine content. Hsp12A and Hsp12B do not contain localization signals and are therefore predicted to reside in the cytosol. Hsp12 of *S. cerevisiae* has also been found in the cell wall. As a result it can localize close to each side of the plasmamembrane (Sales, Brandt *et al.* 2000; Huh, Falvo *et al.* 2003; Motshwene, Karreman *et al.* 2004; Welker, Rudolph *et al.* 2010). LeamA is predicted to have a N-terminal mitochondrial targeting motif. The presence of an 11-mer repeat classifies LeamA of *N. fischeri* as a LEA group 3 protein (Dure 1993; Dure 2001; Shimizu, Kanamori *et al.* 2010; Yamaguchi, Tanaka *et al.* 2012).

As mentioned above, hydrophilins are intrinsically disordered proteins (Kovacs, Kalmar *et al.* 2008; Boucher, Buitink *et al.* 2010; Thalhammer, Hundertmark *et al.* 2010; Tompa and Kovacs 2010; Popova, Hundertmark *et al.* 2011; Singarapu, Tonelli *et al.* 2011; Chakrabortee, Tripathi *et al.* 2012; Kovacs and Tompa 2012). Their folding is induced by desiccation, the presence of particular cations or macromolecules, or by an increase in the hydrophobicity of the environment (Dyson and Wright 2002). The repeat of LeamA is predicted to form amphipathic α-helices. Such α-helices are also predicted to be formed by Hsp12A and Hsp12B of *N. fischeri*. This would agree with previous findings showing that Hsp12 of *S. cerevisiae* (Welker, Rudolph *et al.* 2010; Singarapu, Tonelli *et al.* 2011), Leam of *Pisum sativum* (Tolleter, Jaquinod *et al.* 2007), and heat-soluble proteins of anhydrobiotic tardigrades (Yamaguchi, Tanaka *et al.* 2012) form amphipathic α-helices. The hydrophobic part of these α-helices interacts with the membrane core and the clustered positive charged amino acids interact with the negatively charged heads (Tolleter, Jaquinod *et al.* 2007; Tolleter, Hincha *et al.* 2010; Welker, Rudolph *et al.* 2010). As a result, LEA proteins have been reported to stabilize phospholipids and membranes upon desiccation (Tolleter, Jaquinod *et al.* 2007; Shih, Huang *et al.* 2010; Shih, Hsieh *et al.* 2012). For instance, Leam proteins protect the mitochondrial membrane during drying (Grelet, Benamar *et al.* 2005; Tolleter, Jaquinod *et al.* 2007; Tolleter, Hincha *et al.* 2010). Moreover, LEA proteins have molecular chaperone activity (Goyal, Walton *et al.* 2005; Chakrabortee, Tripathi *et al.* 2012; Furuki, Shimizu *et al.* 2012),

and form a stabilizing glass together with non-reducing oligosaccharides (Wolkers, McCready *et al.* 2001; Kalemba and Pukacka 2007). One of the α-helices of Hsp12 of *S. cerevisiae* is essential for its interaction with the cell wall (Welker, Rudolph *et al.* 2010).

Increased sensitivity against heat and osmotic and oxidative stress was observed when *hsp12* was deleted in yeast (Welker, Rudolph *et al.* 2010). Hsp12A and Hsp12B are also expected to function in stress resistance of *N. fischeri*. *Escherichia coli* expressing one of these proteins were more tolerant to osmotic stress. Moreover, these hydrophilins protected LDH in vitro against dry heat (45 min vacuum drying and 16 h at 60 °C) and freeze thaw cycles (**Chapter 5**). Unfortunately, we were unable to inactivate the genes encoding Hsp12A and Hsp12B of *N. fischeri*. We cannot exclude that they are essential. Gene *leamA* could be deleted in *N. fischeri*. Its inactivation resulted in decreased viability of ascospores after exposure to dry heat (**Chapter 5**). Moreover, it protected *E. coli* to osmotic stress. A mitochondrial localization may explain why LeamA function cannot be complemented by compatible solutes or Hsp12A and Hsp12B that reside in the cytosol. Possibly, hydrophilins such as Hsp12A and Hsp12B may function in a network together with compatible solutes or may even physically interact. This would explain why exhaustive dialysis couldn't remove sugars that interacted with a LEA protein from wheat (Walters, Ried *et al.* 1997). Further, proteins which primary function is not protection may also provide "less specific" protection, as we observed protection of LDH against stress by lysozyme and BSA. Proteins and compatible solutes operate in the context of the whole cell and interaction between membranes, ions, metabolites, and larger biomolecules may act together in protection of the cell. The properties of protective molecules may therefore serve best or act only in this context.

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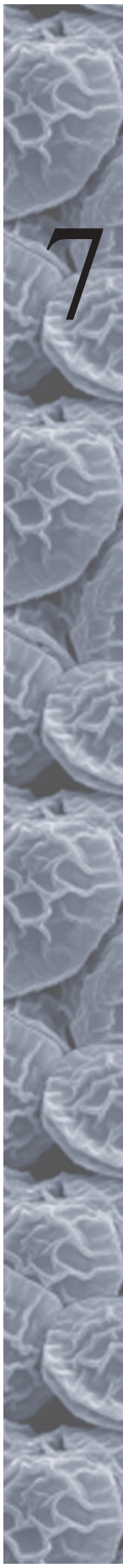
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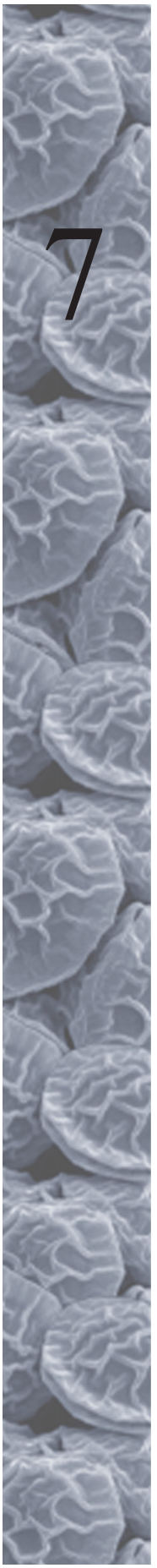

7 Appendix

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Nederlandse samenvatting

Introductie

Schimmels zijn een belangrijke voedselbron. Zowel het reproductieve stadium (paddenstoelen, truffels etc.) als het vegetatieve mycelium (netwerk van schimmeldraden, zoals gebruikt voor de producten tempeh, Quorn) vormen een rijke bron van voedsel. Schimmels zijn ook betrokken bij de productie van voedingsmiddelen. Bijvoorbeeld, verschillende soorten schimmels spelen een belangrijke rol in de bereiding van sojasaus en tempeh. De fermentatie van tarwe resulteert in brood en bier, en vergisting van druiven in wijn. Schimmels worden ook negatief geassocieerd met voedsel. Voedselbederf door schimmels is niet alleen onsmakelijk, maar is ook gevaarlijk voor de menselijke gezondheid als gevolg van de productie van giftige stoffen, de zogenaamde mycotoxinen. Schattingen van de wereldwijde financiële kosten als gevolg van schimmelbederf liggen in de orde van miljarden euro's per jaar. (Milde) hitte behandelingen (sterilisatie en pasteurisatie) worden gebruikt om schimmelbederf te minimaliseren. Gepasteuriseerde voedingsmiddelen, vooral ingeblikt fruit en vruchtensappen, zijn nog steeds gevoelig voor schimmelbederf door extreem hittebestendige sporen die door sommige schimmelsoorten worden gevormd. Schimmels van de familie Eurotiales vormen ascosporen, die voortkomen uit de seksuele cyclus en worden gevormd in kleine, bolvormige vruchtlichamen. De hittebestendige sporen overleven temperaturen tot bijna het kookpunt. Hittebestendige sporen bevinden zich voornamelijk in een rusttoestand en ontkiemen niet zonder een korte hittebehandeling. Daardoor kan pasteurisatie, wat zelf ook een korte hittebehandeling is, de ontkieming van hittebestendige sporen starten en het bederf van voedsel induceren. Hitteactivering zou in de natuur kunnen voorkomen door middel van een (bos) brand. Voordelig is dat mogelijke concurrenten dan zijn gedood door de hoge temperaturen en er genoeg voedingsstoffen beschikbaar zijn na de brand. Kennis van de mechanismen die ten grondslag liggen aan de hittebestendigheid van sporen is van fundamenteel en toegepast belang. In dit proefschrift werd de schimmel *Neosartorya fischeri* geselecteerd als modelsysteem. Het is een veel voorkomende en goed bestudeerde voedselbederf gerelateerde schimmel en nauw verwant met het menselijke pathogeen *Aspergillus fumigatus*. De genoomsequentie van de schimmel is bekend en beschikbaar en *N. fischeri* kan genetisch worden gemodificeerd.

Een van de manieren voor cellen om zo stressresistent te zijn wordt bereikt door het ophopen van zogenaamde “compatibele oplosstoffen” om zich te beschermen tegen stress. Dit zijn goed oplosbare verbindingen en kunnen bestaan uit suikers (zoals sucrose, trehalose, en raffinose), polyolen (zoals glycerol, mannitol, en sorbitol), betaïnen en aminozuren (zoals glycine en proline). Deze stoffen stabiliseren de osmotische balans en de cellulaire structuren die proteïnen en membranen bevatten zonder de celstofwisseling te schaden. Daarnaast kunnen kleine oplosbare eiwitten ook organismen beschermen tegen stress zoals een groep van hydrofiele (waterminnende) eiwitten, de “hydrophilines”. Drie eiwitten van *N.*

fischeri genaamd LeamA, Hsp12A en Hsp12B behoren tot de hydrophilines.

Het doel van dit proefschrift was om de mechanismen die ten grondslag liggen aan de (hitte)stressbestendigheid van ascosporen van *N. fischeri* te bestuderen. Er wordt aangetoond dat stressbestendigheid in *N. fischeri* toeneemt tijdens het rijpen van ascosporen. Deze rijping (of maturatie) kan in twee fases worden ingedeeld. De eerste fase gaat gepaard met een vermindering van de hoeveelheid vrij (bulk) water in de sporen, terwijl de tweede fase wordt gekenmerkt door een toename van trehalose en trehalose-bevattende oligosachariden (TOS) (**Hoofdstuk 3**). TOS kunnen ascosporen vooral beschermen tegen droge hitte (**Hoofdstuk 2**). Het is ook aangetoond dat het eiwit LeamA betrokken is bij stressbestendigheid van ascosporen van *N. fischeri*, terwijl twee Hsp12 eiwitten een soortgelijke rol lijken te vervullen (**Hoofdstuk 5**). Er werd eveneens aangetoond dat de polyol mannitol essentieel is voor de vorming van ascosporen (**Hoofdstuk 4**). Dit maakte het onmogelijk om de rol van mannitol in de bestendigheid tegen stress van ascosporen te bestuderen. Het begrijpen van de vorming en het functioneren van trehalose, TOS, hydrophilines, en mannitol kunnen nieuwe aanknopingspunten bieden om voedselbederf door hittebestendige schimmels te voorkomen. Deze moleculen kunnen ook worden gebruikt om industrieel belangrijke micro-organismen, enzymen en / of vaccins te stabiliseren. Externe toevoeging van deze verbindingen konden hun houdbaarheid verhogen en bescherming bieden tegen abiotische stress.

Identificatie van compatibele oplosstoffen in ascosporen van N. fischeri

Mannitol en trehalose zijn in grote hoeveelheden aanwezig in extreem hittebestendige ascosporen. De gezamenlijke concentratie van deze stoffen in *Talaromyces macrosporus* ascosporen is meer dan 1 M (**Hoofdstuk 2**), terwijl dit in de ascosporen van *N. fischeri* wel 800 mM kan zijn (**Hoofdstuk 3**). Analyse van *N. fischeri* ascospore extracten door middel van vloeistofchromatografie (HPLC) toonde de aanwezigheid van glycerol en verscheidene onbekende compatibele oplosstoffen (**Hoofdstuk 2**). Glycerol was aanwezig in ascosporen van 11 dagen oude culturen, maar afwezig in ascosporen van 15 dagen of ouder culturen (**Hoofdstuk 3**). De moleculaire structuur van de onbekende compatibele oplosstoffen werd opgelost met dunne laag chromatografie (TLC), massaspectrometrie en nucleaire magnetische resonantie (NMR) spectrometrie. De moleculen worden gekenmerkt door een trehalose (Glcα1-1αClc) kern waaraan een (isomebiosiose), twee (neosartose) of drie (fischerose) glucosemoleculen zijn verbonden via een α-1, 6 binding (**Hoofdstuk 2**). Deze trehalose bevattende oligosachariden worden gezamenlijk TOS genoemd. Het bestaan van de suikers neosartose en fischerose was nog nooit eerder vermeld in een levend systeem. TOS waren afwezig in een *N. fischeri* stam die het gen coderend voor mannitol 1-fosfaat dehydrogenase (MpdA) dat belangrijk is voor de ophoping van mannitol in de cel niet tot expressie brengt. Deletie van dit gen had geen invloed op de vegetatieve groei en de vorming van aseksuele sporen (conidia), vruchtlichamen (ascomata) en de seksueel bevattende cellen (asci) (**Hoofdstuk 4**, zie hier onder).

Echter, de ontwikkeling van ascosporen werd volledig gestopt nadat meiose had plaatsgevonden. Dit geeft aan dat TOS specifiek zijn voor ascosporen. Soorten die tot de geslachten *Byssochlamys*, *Hamigera* / *Warcupiella*, *Neosartorya*, *Rasamsonia* en *Thermoascus* behoren, bleken soortgelijke, zo niet identieke, oligosachariden op te hopen volgens hun HPLC en TLC patronen (*Hoofdstuk 2*). *Neosartorya*, *Rasamsonia* en *Thermoascus* zijn bekend thermotolerante of thermofiele schimmels en worden dus verwacht in omgevingen met hoge temperaturen. Hun ascosporen moet dus ook in staat zijn om warme en / of droge omstandigheden te doorstaan.

Rol van compatibele oplosstoffen in ascosporen

Deletie van *mpdA* verminderde de hoeveelheid mannitol in *N. fischeri* culturen met maar liefst 85%, terwijl de hoeveelheid trehalose steeg met tot meer dan 400% ten opzichte van wild type *N. fischeri* culturen. Deletie van het *mpdA* gen had geen effect op de groei van mycelium (schimmeldraden), zelfs niet bij blootstelling aan hitte of oxidatieve stress. Mogelijk compenseert trehalose voor de afwezigheid van mannitol. Conidia toonden wel verhoogde gevoeligheid voor warmte en oxidatieve stress. Het meest uitgesproken effect van *mpdA* deletie was de volledige afwezigheid van rijpe ascosporen. Een dergelijke rol van mannitol in filamenteuze schimmels was nog niet eerder gerapporteerd. Asci (sporenzakjes) van de Δ*mpdA* stam van *N. fischeri* bevatten onvolledig ontwikkelde ascosporen. Deze sporen waren misvormd met een incomplete celwand en bevatten elektrondichte structuren. De precieze rol van mannitol in *N. fischeri* ascospore rijping is nog onduidelijk.

Er werden twee fasen onderscheiden in de ontwikkeling van de stressbestendigheid in ascosporen van *N. fischeri* (**Hoofdstuk 3**). De eerste fase vindt plaats tussen 11 en 15 dagen na inoculatie. De ascosporen worden hittebestendig wat gepaard gaat met een verhoging van de totale concentratie compatibele oplosstoffen (trehalose, TOS, en mannitol) van ongeveer 400 mM tot 1 M, een toename van de viscositeit (5.1 tot 21.2 cP) en een verminderde hoeveelheid bulk water. De tweede fase vindt plaats tussen 15 en 50 dagen. De viscositeit en de concentratie compatibele oplosstoffen veranderen niet veel in deze fase. Echter, de redox stabiliteit en hittebestendigheid van de sporen worden nog hoger. Dit gaat gepaard met een verandering in de samenstelling van de compatibele oplosstoffen. Terwijl mannitol een maximum bereikt in cultures van 20 dagen oud, wordt het maximum van trehalose en TOS bereikt in 50 dagen oude cultures. Dit geeft aan dat trehalose en TOS een belangrijke rol spelen in de ontwikkeling van de redox stabiliteit en hittebestendigheid tijdens de tweede fase.

Extreem stressbestendige schimmelsporen in een vloeistofomgeving vertonen hoge cytoplasmatische viscositeit in rusttoestand (ca. 15-20 cP; **Hoofdstuk 2, 3**). Droging van de ascosporen van *N. fischeri* leidt tot een glasachtige toestand van het cytoplasma met een nog hogere viscositeit (**Hoofdstuk 3**). Dit gaat gepaard met een verhoogde hittebestendigheid (**Hoofdstuk 2**). Het vormen van een glas kan een goede bescherming bieden tegen de verschillende effecten van het verdwijnen

van water uit een levende cel. Belangrijke eigenschappen van een glas zijn de glas (-vloeistof) overgangstemperatuur, T_g, en de glas-dichtheid. Het smeltgedrag en de pakking van glazen die waren samengesteld uit mannitol, trehalose, isobemisiose, neosartose en fischerose werden bestudeerd met Fourier Transform Infrared (FTIR) spectroscopie gedurende verschillende temperaturen (**Hoofdstuk 2**). Opmerkelijk was het grote verschil in smelttemperatuur, T_g wanneer het glas voor de eerste keer werd verwarmd. Deze eerste scan (T_{g1}) was sterk verschillend van de tweede en derde scan (T_{g2}). Bijvoorbeeld, de T_{g1} van trehalose en isobemisiose waren respectievelijk 48.1 en 44.3 °C, terwijl de T_{g2} 108 en 120.4 °C was. Verklaringen hiervoor kunnen de verwijdering van resterende watermoleculen uit het glas zijn en / of een verandering van de structuur van een glas na smelten. Deze herschikking leidt tot zogenaamd "gerijpt" of "potentieel" glas, die een hogere transitietemperatuur heeft. De T_g waarden in de literatuur komen overeen met de T_{g2} waarden. De vraag is of de T_{g1} waarden relevanter zijn dan de T_{g2} waarden voor de glazen die gevormd worden in de natuur. De temperaturen waaraan glazen in de natuur worden blootgesteld zullen normaliter zelden of nooit oplopen tot 140 °C zoals wordt gedaan tijdens de FTIR scanning. Hoogstwaarschijnlijk zullen natuurlijk gevormden glazen nooit de T_{g2} waarden bereiken. Verder is de T_{g2} positief gecorreleerd met de polymerisatiegraad (DP, hoeveelheid suikergroepen) van de oligosachariden, terwijl T_{g1} negatief correleert met de DP. Zo toonden de TOS een hogere T_{g2} dan trehalose, terwijl de T_{g1} van de TOS lager was (48.1, 44.3, 39.1, en 38.8 °C voor respectievelijk trehalose, isobemisiose, neosartose en fischerose). De pakkingsdichtheid van TOS glazen (WTC_2-waarden) daalde eveneens met de DP. Echter, een puur trehalose glas heeft een lagere dichtheid dan de glazen welke bestaan uit TOS (**Hoofdstuk 2**). Andere kenmerken dan alleen de grootte van het molecuul zijn dus van belang voor de dichtheid van een glas, zoals de flexibiliteit van de moleculen of het vermogen om waterstofbindingen te vormen.

De rol van TOS in ascosporen kan mogelijk worden herleid uit de observatie dat ascosporen van *N. fischeri* (die trehalose en TOS bevatten) droging en droge hitte beter overleven dan *T. macrosporus* ascosporen die voornamelijk trehalose bevatten. Echter, de ascosporen van *T. macrosporus* overleven vochtige hitte beter in vergelijking met *N. fischeri* ascosporen (**Hoofdstuk 2**). Deze resultaten suggereren dat TOS belangrijk zijn voor de bescherming tegen een gecombineerde stress van droogte en hitte. Echter, in vitro bood TOS geen betere bescherming van lactaat dehydrogenase (LDH) of schimmel blastosporen tegen zowel droge hitte (drogen in combinatie met hitte) en vochtige hitte (verhitten in een waterige omgeving) dan trehalose.

Het bij 60 °C bewaren van bij kamertemperatuur (RT) gedroogde sporen gedurende 3 dagen had geen effect op de mobiliteit van de moleculen van de spin probes TEMPO en TEMPOL in *N. fischeri* ascosporen gemeten met Electron Spin Resonance spectroscopie. Dit betekent dat deze behandeling geen invloed had op de cytoplasmatische structuur van de ascosporen. Wel werd een verhoging van de redox omzetting (oxidatie en reductie) van TEMPO, maar niet die van TEMPOL,

waargenomen na opslag bij 60 °C (**Hoofdstuk 3**). Door de meer hydrofobe aard van de spin probe TEMPO zou deze zich in de nabijheid van membranen kunnen bevinden, terwijl de hydrofiele spin probe TEMPOL zich in het cytosol bevindt. De meer redox instabiele omgeving van TEMPO suggereert een hogere gevoeligheid van de membranen voor opslag bij 60 °C. Het lijkt erop dat de membranen de Achilleshiel van ascosporen zijn, die kunnen worden beschermd door TOS. Met andere woorden het kan nog minder stabiel zijn in hun afwezigheid.

Bescherming door kleine oplosbare eiwitten

Verschillende eiwitten zijn geëvolueerd om te beschermen tegen stress. Veel van deze eiwitten zijn hydrofiel en maken deel uit van de zogenaamde "hydrophilines". LEA (late embryonic abundant) eiwitten bijvoorbeeld, komen veel voor in zaden van planten. Het genoom van *N. fischeri* bevat enkele genen die coderen voor eiwitten die geclassificeerd worden als hydrophilines. Dit zijn 3 genen met een Con-6 domein, 2 genen coderend voor een voorspeld Con-10 eiwit, 2 genen coderend voor een dehydrine, een gen coderend voor een LEA-achtig eiwit (LeamA), en twee genen coderend voor een Hsp12 eiwit (Hsp12A en Hsp12B). LeamA, Hsp12A en Hsp12B werden bestudeerd in **Hoofdstuk 5**. Zij zijn naar verwachting ongevouwen in oplossing, zeer hydrofiel, en hebben een hoog glycine gehalte. Hsp12A en Hsp12B hebben geen lokalisatiesignaal en hun voorspelde locatie is daarom in het cytosol. LeamA heeft een N-terminale signaalsequentie kenmerkend voor een eiwit in het mitochondrium. De aanwezigheid van een herhaling van een 11-mer motief in LeamA van *N. fischeri* classificeert het als een LEA groep 3 eiwit. Het motief is voorspeld om amfifatische α-helices te vormen en ook Hsp12A en Hsp12B van *N. fischeri* zijn voorspeld om α-helices te vormen. Deze helices kunnen een interactie aangaan met membranen en zo mogelijk bescherming bieden.

Hsp12A en Hsp12B lijken ook een functie in stressbestendigheid van *N. fischeri* te spelen. Bacteriecellen van *Escherichia coli* die deze eiwitten tot expressie brengen zijn beter bestand tegen osmotische stress. In vitro beschermen deze hydrophilines bovendien het LDH enzym tegen droge hitte (45 min vacuüm drogen en 16 uur bij 60 °C) en vries-dooi cycli (**Hoofdstuk 5**). Helaas waren we niet in staat om de genen die coderen voor Hsp12A en Hsp12B in *N. fischeri* uit het genoom te verwijderen. We kunnen niet uitsluiten dat ze essentieel zijn. Het gen coderend voor LeamA kan worden gedeleteerd in *N. fischeri*. De deletie resulteerde in een verminderde levensvatbaarheid van ascosporen na blootstelling aan droge hitte (**Hoofdstuk 5**). Bovendien beschermde intracellulair LeamA *E. coli* tegen osmotische stress. De mitochondriale lokalisatie kan verklaren waarom de afwezigheid van LeamA niet kan worden opgevangen door compatibele oplosstoffen of door Hsp12A en Hsp12B, die zich allemaal in het cytosol bevinden. Mogelijk kunnen de hydrophilines Hsp12A en Hsp12B wel samenwerken of een fysieke interactie aangaan met de oplosstoffen. Andere eiwitten zoals lysozym en BSA, die niet als beschermende eiwitten bekend staan, bieden ook protectie van LDH tegen stress. Eiwitten en compatibele

oplosstoffen werken mogelijk in de context van de gehele cel; een complex mengsel van membranen, eiwitten en andere grotere biomoleculen, ionen en metabolieten. Deze zouden samen kunnen werken in de bescherming van de cel. De eigenschappen van beschermende moleculen kunnen misschien alleen optreden in deze context.

Curriculum Vitae

Timon Wyatt was born on July 15th 1981 in Winschoten, The Netherlands. He followed his secondary education at the Christiaan Huygens College in Eindhoven, The Netherlands and graduated in 2000 with a VWO-level diploma. In September of the same year he started with the Architecture study at the Technical University of Eindhoven. In September 2002, he began with his coursework in biology at the Utrecht University. Timon obtained his bachelor degree in 2005, and immediately started the Biomolecular Sciences Master's at the Utrecht University. As part of his studies he did an internship at the CBS-KNAW Fungal Biodiversity Centre at Utrecht University under the supervision of Dr. J. Dijksterhuis and Dr. L.G. Lugones, followed by a second internship in the Mycorrhiza Research Group at the Estación Experimental del Zaidín CSIC in Spain. This internship was performed under the supervision of Dr. M. Jose Pozo and Utrecht University advisor Prof. Dr. C.M.J. Pieterse. Timon obtained his master's degree in March 2008. In November of the same year he started his PhD within the Applied and Industrial Mycology group at the CBS-KNAW and the Molecular Microbiology group of the Department of Biology at Utrecht University under the supervision of Dr. J. Dijksterhuis and Prof. Dr. H.A.B. Wösten. The research on the mechanism of extreme heat resistant ascospores in *Neosartorya fischeri* is described in this thesis and Technologiestichting STW provided financial support during this period. In July 2013, Timon started working for another 3 months at the CBS-KNAW Fungal Biodiversity Centre in Utrecht. During this time he worked on the same project as his PhD, to provide extra data for the submission of a patent on novel protective compounds.

List of publications

Wyatt, T.T., M.R. van Leeuwen, H.A.B. Wösten, J. Dijksterhuis (2013). "Mannitol is essential for the development of extreme stress resistant ascospores of *Neosartorya fischeri*." Under revision

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Bekker, M., H.P. Huinink, O.C. Adan, R.A. Samson, T.T. Wyatt, and J. Dijksterhuis (2012). "Production of an extracellular matrix as an isotropic growth phase of *Penicillium rubens* on gypsum." Applied and Environmental Microbiology **79**(19): 6930-6937

de Román, M., I. Fernández, T.T. Wyatt, M. Sahrawy, M. Heil, and M.J. Pozo (2011). "Elicitation of foliar resistance mechanisms transiently impairs root association with arbuscular mycorrhizal fungi." Journal of Ecology **99**: 36-45.

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