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Trehalose degradation and glucose efflux precede cell ejection during germination of heat-resistant ascospores of *Talaromyces macrosporus*

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Abstract *Talaromyces macrosporus* forms ascospores that survive pasteurization treatments. Ascospores were dense (1.3 g ml^{-1}), relatively dry [0.6 g H_2O (g dry weight)$^{-1}$] and packed with trehalose (9–17% fresh weight). Trehalose was degraded to glucose monomers between 30 and 100 min after heat activation of the spores. The maximal activity of trehalase was calculated as 400–520 nmol glucose formed min^{-1} (mg protein)$^{-1}$ as judged by measurements of the trehalose content of spores during germination. During early germination, glucose was released from the cell (10% of the cell weight or more). The intracellular concentration of glucose only peaked briefly. After 160–200 min, the protoplast encompassed by the inner cell wall was ejected through the outer cell wall in a very quick process. Subsequently, respiration of spores increased strongly. The data suggested that trehalose is primarily present for the protection of cell components as glucose is released from the cell. Then, an impenetrable outer cell wall is shed before metabolic activity increases.

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Introduction

Ascospores of the fungi *Byssochlamys nivea, Talaromyces macrosporus* and *Neosartorya fisheri* are very heat-resistant (Beuchat 1988) and survive considerable periods of heat above 85 °C. These fungi are relevant for food science since they cause spoilage of canned and pasteurized fruit products (Tournas 1994). Furthermore, the spores are constitutively dormant (Sussman 1966) and need a distinct external physical or chemical trigger, in this case heat, for germination (Lingappa and Sussman 1959; Katan 1985; Beuchat 1986). Other fungi (yeasts, the *Zygomycete Pilobolus*), by contrast, need glucose as the germination trigger (Thevelein 1984b; Bourret 1989).

Germination of several fungal species is associated with the breakdown of trehalose by the enzyme trehalase. These species include ascospores of the yeast *Saccharomyces cerevisiae* (Thevelein 1982, erronously designated in the paper as *Pichia pastoris*) and *Neurospora crassa* (Lingappa and Sussman 1959), *Schizosaccharomyces pombe* (Inoue and Shimoda 1981), and sporangiospores of several species of *Zygomycetes* (van Assche et al. 1972; Bourret 1989). Trehalase activity peaks shortly after activation (van Assche 1972; Thevelein 1984a) and is mediated by cAMP in yeast ascospores and *Zygomycete* sporangiospores (Thevelein 1984b). Conidia of *Aspergillus nidulans* contain trehalose, which is degraded to glucose and immediately metabolized to glycerol. The intracellular trehalose pool is degraded during germination by a neutral trehalase (D'Enfert and Fontaine 1997; D'Enfert 1999).

Insights into the mechanisms of germination of heat-resistant ascospores are limited, and investigation of this process may generate methods for the prevention of spoilage by these organisms. In this study, we identified different stages during the germination of ascospores of

Talaromyces macrosporus, including a massive efflux of glucose and a remarkable ejection phenomenon.

Materials and methods

Organisms, growth conditions, isolation and activation of ascospores

Talaromyces macrosporus Stolk and Samson (Frisvad et al. 1990) CBS 130.89 was grown on oatmeal agar (30 °C). For isolation of ascospores, fungal cultures were flooded with 10 mM ACES buffer (pH 6.8, N-[2-acetamido]-2-aminoethane-sulfonic acid; Sigma) supplemented with 0.05% Tween 80. The mycelium was removed with a Drigalski spatula and fruit bodies were disconnected by suction through a 0.9-mm hypodermic needle with a syringe. The suspension was sonicated briefly (3 times for 15 s, Elma Transsonic 460H, Singen, Germany) and filtered over sterile glass wool. Ascospores were spun down at 1,100×*g* (5 min), washed three times in buffer and counted with a heamocytometer (Burker-Turk). The fungal cultures used for the experiments were 5–7 weeks old. Ascospores were heated for 5–10 min at 85 °C in a waterbath (Julabo SW 20, 160 strokes/min) for activation of germination. Samples were cooled with a jet of water and incubated at 30 °C (160 strokes/min).

Dimensions of the ascospores

Length and width of ascospores (n=100) were measured by means of an ocular grid and were within the range estimated by Stolk and Samson (1972). Importantly, the inner cell diameter was also measured. From these data we calculated the internal cell volume (protoplast) and total cell volume (assuming a cell wall thickness of 0.5 µm, Stolk and Samson 1972) by regarding the spores as ellipsoidal and using the equation: $V=4/3\ \pi abc$, where a, b, c are the radii of the three axes of the ellipsoid.

The specific weight of ascospores was estimated by weighing 500 µl of an ascospore suspension on a microbalance and then subtracting the weight of exactly the same volume of buffer alone. The weight difference reflects the spore density which can be calculated with the volume of the cells known. For estimation of the dry weight (minimally seven repetitions were done) spores were dried overnight under a flow of air (3% relative humidity or less).

Optical density of ascospore suspensions

The optical density of ascospores was measured at 660 nm in reaction tubes in a spectrophotometer before and after activation. Suspensions were measured within a range of optical densities of 0.6–1.1 (about 10^6 cells/ml).

Analysis of trehalose and glucose in cell-free extracts of ascospores

Ascospores were broken by agitation with glass beads combined with sonication. Equal volumes of a dense spore suspension (approx. 1–2×10^8 cells/ml in ACES buffer) and glass beads (diameter <0.5 mm, rinsed in 37% HCl) were vortexed twice for 30 s. Between vortex treatments the suspension was sonicated for 45 s (Elma Transsonic 460H). Between agitation and vortexing the samples were kept on ice. The suspensions were centrifuged for 5 min (13,000×*g*) in an Eppendorf centrifuge and the pellet was used to determine the ratio of broken cells (by optical microscopy). The cell-free extract was filtered through a 0.45-µm sterile syringe filter (Corning) and samples were stored at –20 °C prior to analysis of sugars and protein.

Sugars present in the medium outside the germinating spores were determined after brief centrifugation (10 s, 13,000×*g*) of suspensions. The remaining pellet was subsequently resuspended in buffer, and cells were broken for the determination of intracellular sugars.

HPLC was carried out as described by (Kuo et al. 1988) using a Waters Sugar Pak-I column (6.5×300 mm). Samples were kept at 4 °C in the autosampler and diluted five to ten times for optimal separation. Results were analyzed by means of the Waters Millenium[32] chromatography manager. As standards, trehalose (Merck) and D-(+)-glucose (Sigma) were used.

Protein was estimated according to the method of Bradford (1976) and a Bio-Rad protein assay was used.

Microscopical analysis

Activated ascospores were incubated in 2% malt extract and ACES buffer in a rotary incubator (30 °C, 160 rpm, Gallenkamp). The number of ejected cells was counted at different time intervals using phase-contrast microscopy (Zeiss, Axioskop). The time at which 50% of the cells had undergone ejection was calculated after regression analysis of the linear part of the plotted curves (r^2>0.98). These experiments were also done in the presence of 30% glucose (w/w) and 1 M sorbitol in buffer. Spores were activated by heat in buffer alone and subsequently were brought into the osmoticum.

In other experiments, germinating cells were washed three times with buffer 90 min following the beginning of activation. In controls, cells were suspended in the supernatant. Then the number of ejected cells was counted over time.

Low-temperature scanning electron microscopy of ascospores was done as described by Dijksterhuis et al. (1992). Dormant and activated ascospores were placed upon small (<0.5 cm^2) excised squares of 5% malt extract agar (5% agar, 2% malt extract) and incubated at 30 °C. The agar squares were mounted in the specimen holder with a mixture of Cryoblock (Klinipath, Duiven, The Netherlands) and colloidal graphite (Emscope Laboratories, Ashford, UK). Images were acquired as bitmaps using the Semafore 3.02 software packet.

Biological oxygen monitoring of germinating ascospores

Suspensions were diluted in buffer or malt extract broth and equilibrated at 30 °C. Oxygen was measured with a Clark-type electrode (Davies 1962) during several stages of germination. Data were collected with an analog/digital converter, and oxygen consumption rates were calculated using Mathcad software (Mathsoft, Cambridge, Mass., USA).

Results

Ascospores are dense, relatively dry and contain large amounts of trehalose

The inner dimensions (within the cell wall) of the ellipsoidal spores were 5.9±0.4 µm by 4.6±0.4 µm. From this, we calculated an average internal volume as 67 fl/cell, and with the cell wall included as 89 fl. The amount of trehalose ranged from 11 to 21 pg/cell (n=5), which is 9–17% of the wet cell weight. The mass ratio between the protein and trehalose was close to 1:3 (range: 2.7–3.3 in four experiments). The density of the spores was calculated (knowing the volume and the number of cells) as 1.34 g/ml. With the total cell volume calculated as 89 fl, the cells have an average fresh weight of 120 pg. After drying, this was 75±2 pg. This suggests that the water content of the spores on a fresh-weight basis is approximately 38% [0.6 g H_2O (g dry weight)$^{-1}$].

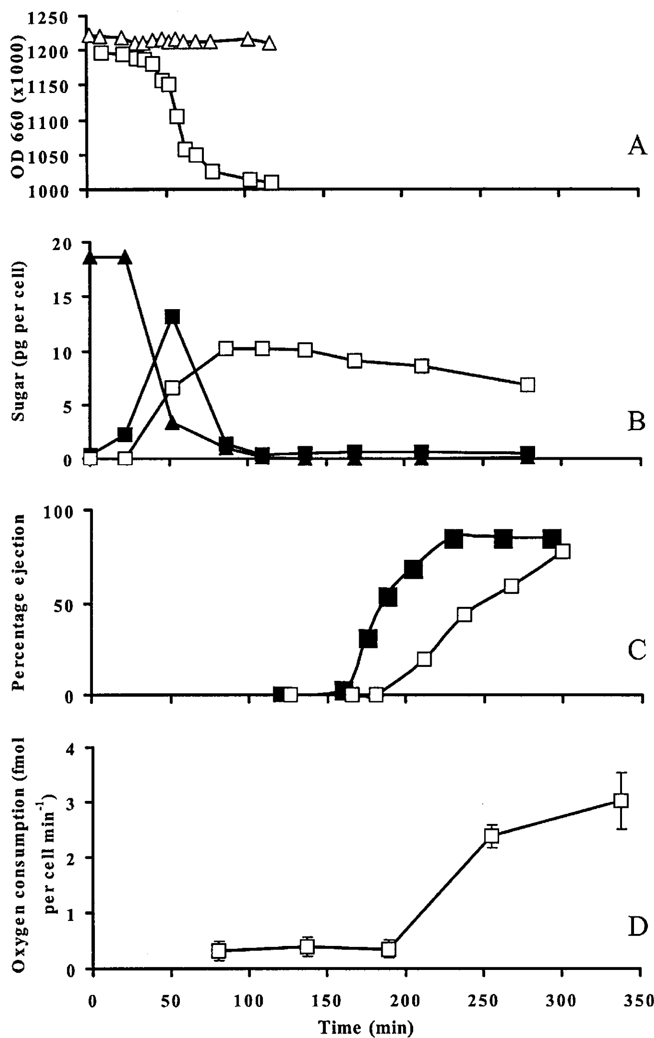

Fig. 1A–D Germination of ascospores of *Talaromyces macrosporus*. **A** Ascospores of *T. macrosporus* showed a distinct decrease in optical density after activation. The spores were incubated in tubes and heat-activated at 85 °C (*open squares*). In all experiments t=0 at the beginning of heat treatment. Controls invariably did not show any decrease (*open triangles*) in optical density. **B** Trehalose breakdown and subsequent glucose efflux during germination of ascospores in ACES buffer. Intracellular trehalose (*closed triangles*) was broken down into glucose, which only briefly accumulated inside the cell (*closed squares*), and then was measured in the supernatant outside the spores (*open squares*). **C** Ejection of the cell through the outer cell wall was delayed when 1 M sorbitol was added to ACES buffer (*open squares*) compared to the controls (*closed squares*). **D** Upon activation, ascospores showed a sudden increase of respiration after 150–200 min. These cells were incubated in ACES buffer

Ascospores show a distinct drop in optical density after heat activation

Heat activation was followed by a decrease in optical density at 660 nm of suspensions of ascospores. This process included a sudden and rapid drop between 40 and 60 min after the beginning of the experiment and a slower decrease thereafter (Fig. 1A). The decrease measured at t=92±8 min (in 17 experiments) was 16±1% of the initial optical density. Without exception, suspensions that were not activated showed no change in optical density. Identical kinetics were measured for spores in malt extract broth and buffer. The onset of the process was at 38±1 min (n=3) in malt extract broth and at 40±2 min (n=4) in buffer.

Trehalose is degraded to glucose after activation and released into the surrounding medium

Following heat activation, trehalose was invariably (six experiments) broken down within 2 h (Fig. 1B). Ascospores were disrupted and the amount of sugars was assessed by HPLC. In cell-free extracts, trehalose and glucose concentrations did not change in time, even when kept at 30 °C for 30 min. This indicates that the measured amounts of sugar indeed reflect the intracellular concentrations. Trehalose breakdown was complementary to the formation of glucose. The maximal rate of trehalose breakdown was calculated from samples taken during early germination and was used as an indication of combined trehalase activity. A rate of 210–260 nmol trehalose degraded min^{-1} (mg protein)$^{-1}$ (in 2 experiments) was calculated, which is equivalent to 420–520 nmol glucose formed min^{-1} (mg protein)$^{-1}$.

Surprisingly, glucose was also detected in supernatants of germinating intact ascospores, indicating an efflux of glucose from the cells (Fig. 1B). During initial germination, massive amounts (14 pg/cell, 12% of the cell weight) of glucose were released into the external medium. Trehalose was never observed outside germinating spores. Intracellular glucose levels only peaked after 51 min (average for 2 experiments) and then quickly dropped, corresponding with a rise in the external levels (Fig. 1B). During later stages of germination, glucose levels outside the cells decreased, which indicates uptake and metabolisation of the compound (Fig. 1B, and other experiments, not shown).

Rapid cell ejection through the outer cell wall and increased respiration of the spore occur during later stages of germination

After nearly 3 h, a sudden ejection of the cell, encompassed by the inner cell wall, through the outer cell wall (bearing the spines) was observed. In malt broth extract, 50% of the spores had undergone ejection at t=160± 15 min (n=7). In buffer alone this was markedly delayed, namely 201±20 min (n=6) after the start of the experiment (p<0.002, two-tailed t test). The proportion of cells that ejected per min, when the rate of the process was maximal, did not significantly differ (2%/min and 1.4%/min, respectively). In 1 M sorbitol in buffer the ejection process was delayed for more than 1 h (62 min, Fig. 1C), indicating that the phenomenon is responsive to a hyperosmotic environment. Ejection was also observed when 10–40% (w/w) glucose was supplied to the malt extract.

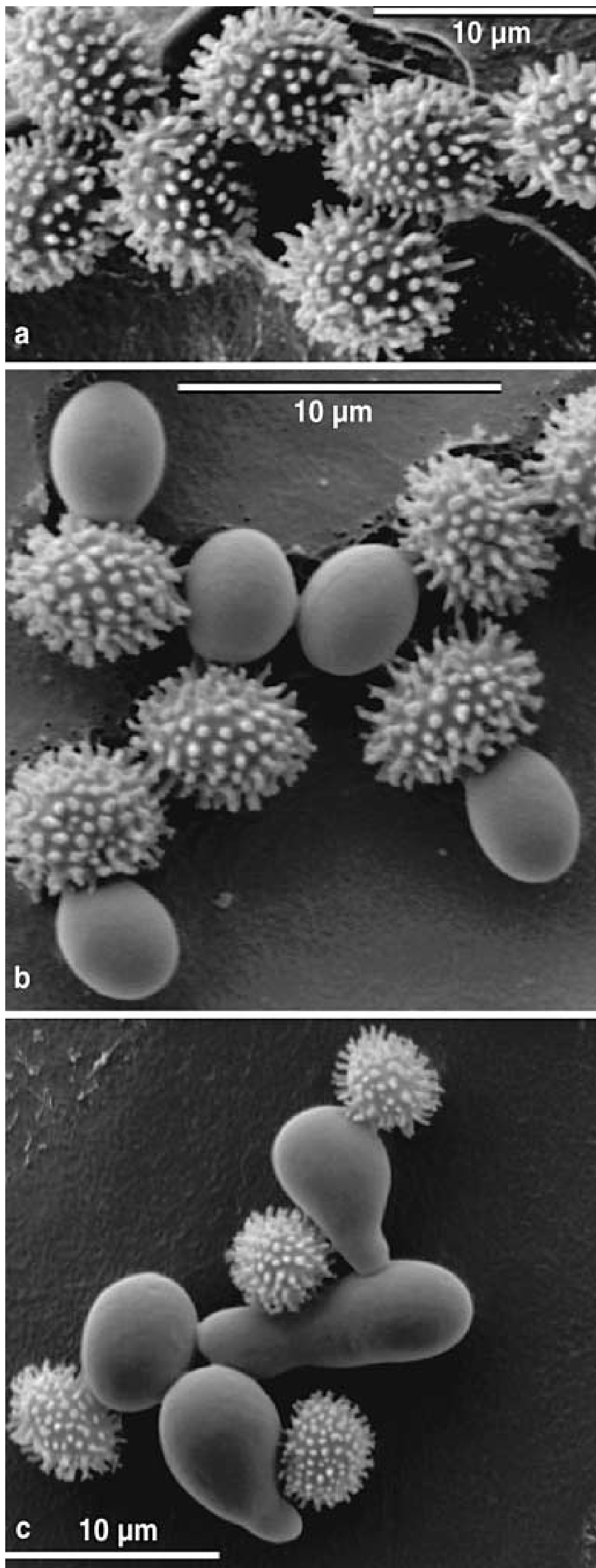

Fig. 2a–c Germination of ascospores of *T. macrosporus* on malt extract agar blocks as observed with low-temperature scanning electron microscopy. **a** Prior to activation, the spores showed their spinulose outer cell wall. **b** After 125 min, many cells showed ejection of the protoplast, which is encompassed by the inner cell wall (*bottom left*). **c** After 350 min, most cells formed a hyphal tip. *Bars* 10 µm

With 30% glucose the process was markedly delayed (32 min).

Figure 2a shows the spinulose ascospores before activation. After 125 min many spores had ejected their contents (Fig. 2b) and germ tubes were observed after 350 min (Fig. 2c). The ejection process included two stages, namely a slower one (minutes), during which the outer cell wall opened and the inner wall became partly visible (Fig. 3a), and a rapid one, in which cell ejection occurred in less than a second as was clear from optical microscopy (Fig. 3b). The ejected cell had very smooth features compared to the original outer cell wall of the ascospore. Figure 3c, d shows completely ejected cells. Notably, a sort of connection (ligament) with the emptied outer layer was present (Fig. 3c, d, arrow).

In another experiment we assessed whether the released glucose was important for cell ejection. Activated spores were washed three times in fresh buffer 90 min after activation, but cell ejection was not delayed. The cells also formed a germ tube during later stages of germination, albeit thinner and shorter than in the presence of glucose.

Germinating ascospores had low rates of respiration (0.2–0.3 fmol O_2 per cell min^{-1}) during early stages of germination. After 195–255 min (3 experiments, buffer) the level of respiration was markedly elevated and increased ten-fold to 3 fmol per cell min^{-1} after 6 h (see Fig. 1d). In malt extract broth, the elevation of respiration was earlier (175 min) and the increase was greater, namely to 6 fmol per cell min^{-1} after 6 h. This indicates that the high nutrient status of the environment results in a higher metabolic activity.

Discussion

Trehalose as a stress protectant in ascospores

The amount of trehalose observed in ascospores (9–17% wet cell weight) occurs also in anhydrobiotic organisms (desiccation-tolerant), e.g. nematodes (Madin and Crowe 1975) and cysts of the shrimp *Artemia* (Clegg 1986). Pollen accumulates another sugar, namely sucrose, in amounts up to 20% of the cell weight (Hoekstra 1992).

The abundance of trehalose in ascospores may be primarily dedicated to the protection of cell components (Wiemken 1990), making the cell competent to withstand complete dehydration (Beuchat 1992) and heat (Beuchat 1986). Trehalose protects membranes (Crowe et al. 1984) and proteins (Carpenter and Crowe 1988). Sugars prevent

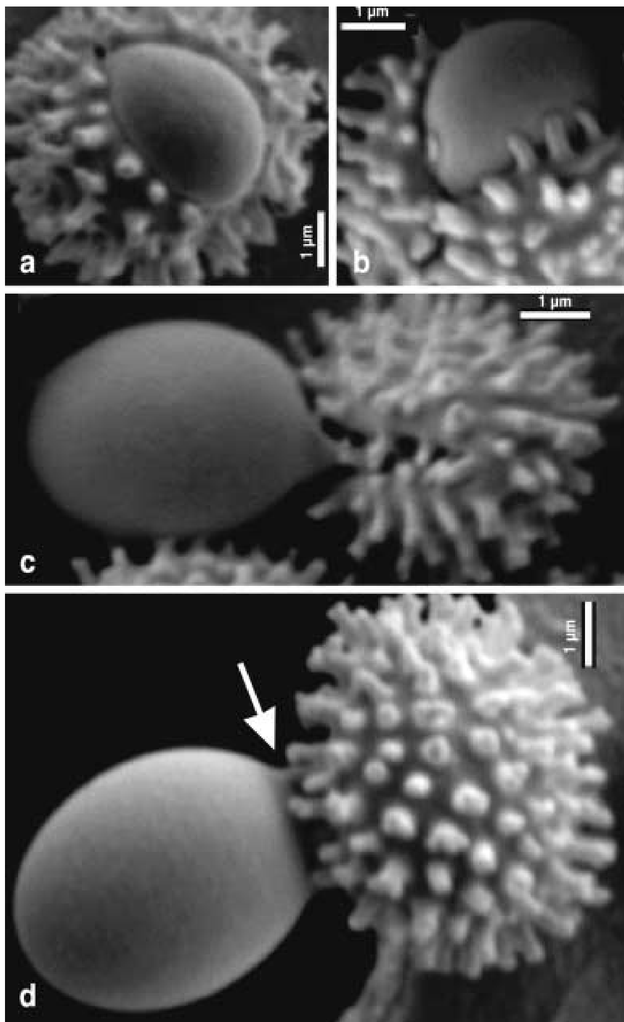

Fig. 3a–d Ejection through the outer ascospore cell wall occurred within a very short time. **a** Ascospore with split outer cell wall. **b** Actual ejection occurred within a second. **c** The outer cell wall was shed and remained cleaved because of the process. **d** Ejected cell connected with the outer cell wall by a ligament-like structure (*arrow*). *Bars* 1 µm

the formation of extended β-sheets of protein during drying as a mechanism to avoid denaturation (Prestrelski et al. 1993; Wolkers et al. 1998). In ascospores of *T. macrosporus* the ratio between protein and sugar was 1:3. This is a sufficient amount of sugar for protection. Wolkers et al. (1998) found that ratios of 2:1 to 1:1 were effective in the case of glucose, which was found to be more protective than sucrose. Hecker and Sussman (1973) found that trehalase from *Neurospora crassa* was maximally protected against heat (30 min, 65 °C) in 16% trehalose. This amount of trehalose fits very well within the range we calculated for *T. macrosporus* in this study.

Trehalose breakdown as the first step in the transition towards a vegetative cell

The first major step of the transition process from dormant and resistant ascospores to metabolising, growing fungal cells is the breakdown of trehalose to glucose monomers. In this study, maximal trehalase activity was calculated from the maximal difference in the trehalose content between two time points and related to the amount of protein. In *T. macrosporus* we estimated a trehalase activity of 210–260 nmol trehalose degraded min^{-1} (equivalent to 420–520 nmol glucose formed min^{-1}). These values were calculated from the amounts of these sugars present in the cell at the moment that the spores were disrupted. Our estimations resulted in much higher activities than observed in previous studies. Maximal activities of trehalase, as measured in an enzyme assay, ranged from 10 to 175 nmol glucose formed min^{-1} (mg protein)$^{-1}$ in cell-free extracts of sporangiospores of *Zygomycetes* (Thevelein et al. 1980, 1983; Thevelein 1984b; Bourret et al. 1989) and *Saccharomyces cerevisiea* (Gupta et al. 1987).

The onset of trehalose breakdown may have a major influence on the light-dispersing characteristics of the cells and hence the physical properties of the cytoplasmic matrix, as is suggested by the decrease in optical density during initial germination. Decreases in optical density were also measured in ascospores of yeast (Rousseau et al. 1973; Inoue and Shimoda 1981) and were similar to those measured in this study.

Normalization of intracellular sugar concentration after trehalose breakdown

Upon trehalose breakdown, the concentration of glucose inside the cell reached very high values (approx. 1 M) and then quickly dropped to low levels due to its release into the (micro)environment. Conidia of *A. nidulans* contain trehalose which is degraded in approximately 2 h during early germination. The formed glucose is directly metabolised into glycerol (D'Enfert and Fontaine 1997; D'Enfert et al. 1999). With *T. macrosporus*, glucose accumulates to high levels inside the cell. Unlike conidia, the ascospores of *T. macrosporus* do not metabolise glucose during early stages of germination.

High intracellular glucose levels may be detrimental for the regulation of metabolic routes (glycolysis). For instance, the ATP pool is exhausted when too much glucose is added to starving yeast cells (Teusink et al. 1998; Thevelein and Hohmann 1995). With *Talaromyces,* regulatory constraints of metabolic routes may require only low glucose levels inside the germinating spores, so that a very large glucose efflux, approx. 10% of the cell weight, is necessary. This is very remarkable when the natural habitat of *Talaromyces,* namely the soil, is taken into account. Here, other microorganisms may readily benefit from the glucose efflux. It suggests that trehalose is primarily present for protection of the cell, as was suggested

by Wiemken (1990). In addition, the released glucose can be taken up and metabolised by the cells during later stages of germination.

Cell ejection as a prerequisite for further germination

The next stage of the germination process is a dramatic ejection phenomenon. We suggest the name prosilition (from the Latin verb *prosilíre* which means "to jump out") to describe the quick ejection of the protoplast encompassed by the inner cell wall(s) through the outer layer(s). Explosive discharge after the rapid breakdown of trehalose has been observed with dormant microsporidia of the protist *Nosema algerae* (Undeen and van der Meer 1994; Undeen et al. 1987). After activation, this organism degraded 70% of its trehalose (present in amounts similar to those observed in *T. macrosporus*) and later the polar tube was expelled vigorously. With *T. macrosporus*, glucose was released from the spores within 100 min, which is approx. 1 h *before* the onset of prosilition. High concentrations of external glucose and sorbitol delayed prosilition, suggesting internal high osmotic pressures, which cannot be caused by an excess of internal glucose. Therefore, other compounds may be accumulated inside the cells.

What might be the reason for prosilition? The very distinct increase in the respiration rate after 2–3 h suggests that prosilition liberates the protoplast from the protective but impermeable outer cell wall layer. Ascospores of *Ascobolus crenulatus, Sordaria* spec, *Daldinia concentrica* and *Neosartorya fisheri* possess thick (approx. 0.5 µm), multi-layered cell walls(Read and Beckett 1996; Conner et al. 1987). In several fungi, ascospore germination includes a stage in which direct contact between the inner cell wall and the external environment increases strongly. *Daldinia concentrica* forms a "germinal slit" that opens during initial stages of germination (Beckett 1976a, b), and *Neosartorya fisheri* has a lateral groove that widens dramatically. (J. Dijksterhuis, unpublished observation). Very rapid events are observed during germination of the fungus *Hypoxylon fragiforme* in which the pigmented outer cell wall opens along a germinal slit in a process that takes about 10 s (Chapela et al. 1990). These spores, like those of *T. macrosporus*, are dormant and germinate after being discharged on host (beech) material. The inner cell wall is plastic, and this process may result in better contact of the spore with the host (Chapela et al. 1993). In general, prosilition may be a mechanism in ascospore germination that leads to an increased accessibility of the protoplast for nutrients or signaling compounds.

Becoming less protected

In this study, the gradual removal of protection during germination of highly resistant ascospores was followed. At this stage we do not know whether other protective elements, such as proteins, are involved in this process. The transition from a very dense, trehalose-packed, thick-walled ascospore to a metabolizing and growing fungal hyphae includes the breakdown of trehalose and the quick release of glucose monomers from the cell. In a later stage, the outer cell wall is shed in an ejection process termed prosilition. Trehalose breakdown seems to be relatively insensitive to environmental parameters, as it is independent of the medium (or the presence of azide, unpublished results). In contrast, prosilition is more responsive to external factors such as nutrients, pH, and osmolality. During and after prosilition the rate of respiration increases, and 3 h later the cells swell and form hyphal outgrowths.

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