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Metabolic activity in dormant conidia of *Aspergillus niger* and developmental changes during conidial outgrowth

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ABSTRACT

The early stages of development of *Aspergillus niger* conidia during outgrowth were explored by combining genome-wide gene expression analysis (RNAseq), proteomics, Warburg manometry and uptake studies. Resting conidia suspended in water were demonstrated for the first time to be metabolically active as low levels of oxygen uptake and the generation of carbon dioxide were detected, suggesting that low-level respiratory metabolism occurs in conidia for maintenance. Upon triggering of spore germination, generation of CO_2 increased dramatically. For a short period, which coincided with mobilisation of the intracellular polyol, trehalose, there was no increase in uptake of O_2 indicating that trehalose was metabolised by fermentation. Data from genome-wide mRNA profiling showed the presence of transcripts associated with fermentative and respiratory metabolism in resting conidia. Following triggering of conidial outgrowth, there was a clear switch to respiration after 25 min, confirmed by cyanide inhibition. No effect of SHAM, salicylhydroxamic acid, on respiration suggests electron flow via cytochrome *c* oxidase. Glucose entry into spores was not detectable before 1 h after triggering germination. The impact of sorbic acid on germination was examined and we showed that it inhibits glucose uptake. O_2 uptake was also inhibited, delaying the onset of respiration and extending the period of fermentation. In conclusion, we show that conidia suspended in water are not completely dormant and that conidial outgrowth involves fermentative metabolism that precedes respiration.

1. Introduction

Conidia are asexual spores formed by moulds in enormous numbers, up to 10^9 per cm^2 of a mature colony. They are primarily formed for widespread distribution with the intention that at least a few conidia will arrive in suitable environments and will develop into mycelia. Conidia are designed to survive the physical traumas present during their distribution, particularly in their resistance to UV-light, extreme heat, and dehydration (drought) (Krijgsheld et al., 2013a). It has been shown that dry conidia of *Aspergillus fumigatus* and *Aspergillus nidulans* remain fully viable for at least a year (Osherov and May, 2001; Lamarre et al., 2008).

Aspergillus niger is a widely-distributed filamentous fungus, forming colonies with large clusters of black conidia. Conidia remain dormant in air or in water and tests on limited numbers of species have shown that the germination of conidia requires a triggering mechanism. Germination triggering for most fungi is related to sugars, amino acids and inorganic salts (Osherov and May, 2001). A combination of D-glucose and water is sufficient to initiate germination of *A. nidulans* conidia (Osherov and May, 2000) while specific pyranose sugars and L-amino acids have been shown to trigger germination in *A. niger* (Hayer et al., 2013, 2014) irrespective of their potential metabolism. There was no evidence of conidial influx of the triggering compounds, and it is probable that the triggering structures outside the spores activate signalling pathways, such as cAMP/protein kinase A (PKA), RasA, or Ca^{2+}/calmodulin (Osherov and May, 2000, 2001; Krijgsheld et al., 2013b). The triggering/signalling results in isotropic conidial swelling and self-adhesion, followed by establishment of polarity, and the formation of a germ tube, and then mycelial growth

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(Momany and Taylor, 2000; Osherov and May, 2000). Transcriptome analysis of dormant conidia of *A. nidulans*, *A. fumigatus* and *A. niger* revealed high levels of pre-packaged mRNA in the conidia (Lamarre et al., 2008; Novodvorska et al., 2013; van Leeuwen et al., 2013a), including transcripts from genes involved in fermentation, gluconeogenesis, and the glyoxylate cycle.

The fermentation process is widely associated with yeasts, particularly the brewing yeast *Saccharomyces cerevisiae*, while most moulds appear to require abundant oxygen for growth (Miller and Golding, 1949). In reality, large numbers of yeast species do not ferment (Barnett et al., 2000) but generate energy using respiration mechanisms, and very few mould species grow anaerobically. Traditionally, fermentation was measured by collection of gas in inverted Durham tubes in broth cultures. Yeasts employing respiration also generate CO_2 from the tricarboxylic acid cycle but this is exactly balanced by the uptake of oxygen by the mitochondria, for the cytochrome oxidase, resulting in no nett detection of gas. Fermenting yeasts absorb low levels of oxygen for use in biosynthetic reactions, particularly related to sterols and lipids (Fraenkel, 2011). The suggestion of fermentation in dormant mould conidia (Lamarre et al., 2008; Novodvorska et al., 2013; van Leeuwen et al., 2013a) is supported by the observations that no oxygen consumption was detected in *A. fumigatus* resting conidia, and oxygen uptake was detected after 3 h of germination (Taubitz et al., 2007). Respiration has been shown to be essential during the germination process (Brambl, 1975; Stade and Brambl, 1981). Anaerobic environments inhibit conidial germination although germination of conidia is inhibited at much lower oxygen levels that the inhibition of mould growth (Miller and Golding, 1949). The action of respiratory chain inhibitors, Rotenone and Antimycin A, allowed the swelling of conidia but not full germination to hyphae (Taubitz et al., 2007) and active mitochondria were detected in swollen spores and germlings. It was demonstrated that 1 mM cyanide inhibited respiration via the cytochrome *c* oxidase while the AOX (alternate oxidase) system, transferring electrons from ubiquinol to oxygen and bypassing the cytochrome *c* complex (Kirimura et al., 1999), was inhibited specifically by 1 mM SHAM. Fungal conidia provide a means of dissemination of the fungus in nature but also a source of infection of plants, animals and insects, and a means of contamination of food. The early stages of conidial development provide crucial events in pathogenesis and contamination and also provide handles for combatting disease and spoilage. For example, natamycin, an effective food preservative, specifically binds to ergosterol and inhibits germ tube formation (van Leeuwen et al., 2013b). In this study, we sought to improve the understanding of the early developmental events of conidial germination and took *A. niger* as our model system. Experiments were carried out to determine the metabolic activities in dormant conidia of *A. niger*, and during the early stages of the germination process, and to relate those metabolic events to a genome-wide analysis of gene expression and protein complement in the conidia. In addition, the action of the weak-acid food preservative, sorbic acid, was applied to study the mode of its action as full expression analysis of conidia exposed to sorbic acid has not previously been reported in moulds. Sorbic acid acted as a membrane active compound and inhibited the conidial germination process.

2. Material and methods

2.1. Media and growth conditions

A. niger strain N402 (Bos et al., 1988) was grown at 28 °C on *Aspergillus* complete medium at pH 4 (ACM) with composition as described previously (Novodvorska et al., 2013). Conidia were collected after 6–9 days by washing the agar slopes using 0.01% (w/v) Tween 80 at laboratory temperature, filtered through sterile synthetic wool and counted using a haemocytometer.

2.2. Warburg manometry

Respiration and fermentation in conidia were detected using constant-volume Warburg manometry. All experiments were carried out using single side-arm manometer flasks. 2 ml of conidia (10^9/ml) re-suspended in water were pipetted into the flask and 0.4 ml water or 20% KOH in the centre well. Growth inhibitors and/growth media were put in single arm and then added to conidia at the appropriate time. Two control flasks contained water only. The spore suspension flask was warmed up for 10 min before measurements took place. Each experiment was performed in both technical and biological duplicates at 28 °C.

2.3. Trehalose assay

The trehalose assay was done in duplicates using cytosolic extracts prepared from dormant and germinating conidia over a 4 h period (0 h, 0.5 h, 1 h, 2 h and 4 h) ±1 mM sorbic acid. 10^9 conidia were collected by centrifugation, washed with sterile water and re-suspended in 1 ml 0.25 M Na_2CO_3 and 0.5 ml glass beads and disintegrated in a Sartorius dismembrator for 4 min at 2000 rpm. Supernatants were used to assay for trehalose content using a commercially available kit according to the supplier's instructions (Megazyme International, Ireland Ltd). The amount of trehalose was expressed in pg/spore.

2.4. Protein extraction, LC-MS/MS and data analysis

Conidia germinated in liquid ACM media ±1 mM sorbic acid for 1 and 5 h at 28 °C, were collected, washed in 50 mM of Tris-HCl pH 7.5 and snap frozen in liquid nitrogen. Pellets from all 4 time points (T1, T1SA, T5, T5SA) with 0.5 ml glass beads were separately disintegrated in a Sartorius dismembrator for 4 min at 2000 rpm. Soluble protein fractions were extracted by adding 1 ml of TRIS buffer pH 7.5, vortexed for 1 min, boiled for 5 min at 95 °C and supernatant was transferred into 2 ml collection tubes. Insoluble protein fractions were extracted by adding 1 ml of protein extraction buffer (4% w/v SDS, 100 mM Tris buffer pH 7.5, 100 mM DTT) to the pellet and treated the same way. Soluble and insoluble protein fractions from the same time point were combined and subjected to 10 min trichloroacetic acid precipitation on ice, collected by centrifugation and washed twice with ice-cold acetone. The protein pellet was then air-dried and dissolved in 50 mM TRIS buffer pH 7.5. Each sample was prepared in duplicate. Proteins in 35–70 μg of total protein extract were separated on 1D SDS-PAGE gels. After destaining, gels were cut to 1 mm pieces and the proteins were reduced, alkylated and digested by trypsin. Peptides were then collected by rounds of incubation with 100% acetonitrile, then 0.5% (w/v) formic acid at 37 °C for 15 min before being vacuum-dried and subsequently being solubilized in solution (0.1% formic acid, 3% acetonitrile). 50% of the material was injected, using a Dionex Ultimate 3000 HPLC, onto a PepMap100 C18 2 cm × 75 μm I.D. trap column (ThermoFisher Scientific) at 5 μl min^{-1} in 0.1% (w/v) formic acid, 2% (w/v) acetonitrile at 35 °C in the column oven, 6 °C in the autosampler. Components in the sample were separated, over a 2 h HPLC run (containing a 70 min separation gradient) using mass spectrometry (van Munster et al., 2014). The resulting spectra were searched using SequestHT (ThermoFisher Scientific) against a custom *A. niger* 588.13 database (http://www.aspergillusgenome.org/download/sequence/A_niger_CBS_513_88/current/), and a decoy database, within the Proteome Discoverer 1.4 software package