The effect of natamycin on the transcriptome of conidia of *Aspergillus niger*

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Abstract: The impact of natamycin on *Aspergillus niger* was analysed during the first 8 h of germination of conidia. Polarisation, germ tube formation, and mitosis were inhibited in the presence of 3 and 10 µM of the anti-fungal compound, while at 10 µM also isotropic growth was affected. Natamycin did not have an effect on the decrease of microviscosity during germination and the concomitant reduction in mannitol and trehalose levels. However, it did abolish the increase of intracellular levels of glycerol and glucose during the 8 h period of germination.

Natamycin hardly affected the changes that occur in the RNA profile during the first 2 h of germination. During this time period, genes related to transcription, protein synthesis, energy and cell cycle and DNA processing were particularly up-regulated. Differential expression of 280 and 2586 genes was observed when 8 h old germlings were compared with conidia that had been exposed to 3 µM and 10 µM natamycin, respectively. For instance, genes involved in ergosterol biosynthesis were down-regulated. On the other hand, genes involved in endocytosis and the metabolism of compatible solutes, and genes encoding protective proteins were up-regulated in natamycin treated conidia.

Key words: antibiotics, *Aspergillus niger*, conidia, germination, natamycin, transcriptome.

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INTRODUCTION

Conidia are stress-resistant dispersal vehicles that are produced by many fungal species. Fungi belonging to the genera *Aspergillus* and *Penicillium* produce large numbers of airborne conidia. These conidia easily contaminate and colonise food, which explains why *Aspergillus* and *Penicillium* are among the most important food-spoiling organisms. Preservatives as sorbic acid and natamycin (Plumridge *et al.* 2004, Stark 2007) prevent fungal growth in or on a food source. There are clear indications that dormant conidia are more resistant to antifungal compounds than growing hyphae. Dormant conidia of *Aspergillus fumigatus* survive concentrations of 50 µg/mL of the polyene antibiotic amphotericin B, but become sensitive to 20 and 1–2 µg/mL of the antifungal after 2 and 4 h of germination, respectively (Russel *et al.* 1975, 1977). Similarly, conidia of *A. niger* and *Penicillium discolor* survive a treatment with 45 µM of the polyene antibiotic natamycin, which equals ten times the minimal inhibitory concentration for germinating conidia. Notably, conidia start to germinate upon removal of the antibiotic (van Leeuwen *et al.* 2010).

It is the aim of this study to evaluate the cellular mechanisms that explain these variations in antifungal sensitivity. Novel insights may lead to new prevention strategies of fungal contamination in agriculture and the food industry. As a model system the antifungal compound natamycin that is used in the food industry (Stark 2007) is used. In contrast to other polyene antifungals, natamycin does not induce membrane permeability (Te Welscher *et al.* 2008, van Leeuwen *et al.* 2009). It does inhibit endocytosis in germinating conidia of *P. discolor* in a time and dose dependent manner (van Leeuwen *et al.* 2009). Moreover, natamycin interferes with vacuole fusion in yeast cells as well as filamentous fungi (Te Welscher *et al.* 2010). Very recent work has shown that natamycin also reversibly inhibits transport of different nutrient molecules into the cell (Te Welscher *et al.* 2012).

In order to study the changes that occur in conidia that are challenged with antifungal compounds, the transcriptome of conidia of *Aspergillus niger* was studied in the presence of natamycin and compared with data of untreated germinating conidia. Recently, RNA profiles of dormant and germinating conidia of *A. niger* were reported (van Leeuwen *et al.* 2013). It was shown that the RNA composition of dormant conidia was most distinct when compared to conidia that had been germinating for 2, 4, 6, and 8 h. Dormant conidia contain high numbers of transcripts of genes involved in formation of protecting components such as trehalose, mannitol, heat shock proteins and catalase. Transcripts of the functional gene classes protein synthesis, cell cycle and DNA processing and respiration were over-represented in the up-regulated genes after 2 h of germination, whereas metabolism and cell cycle and DNA processing were over-represented in the up-regulated genes after 4 h of germination. No functional gene classes were over- or under-represented in the differentially expressed genes after 6 and 8 h of germination. From these data it was concluded that the RNA profile of conidia changes especially during the first 2 h of germination and that this coincides with protein synthesis and respiration.

Studies in Mycology

We here show that 3 and 10 µM natamycin hardly affect the transcriptome during the first 2 h of germination, but it inhibits further stages of germination as judged by several cellular parameters. The transcriptome after 8 h was less affected when spores were kept in 3 µM natamycin compared to those treated in a concentration of 10 µM. For instance, genes involved in endocytosis, and genes involved in protection of conidia were up-regulated. On the other hand, genes involved in ergosterol biosynthesis were down-regulated.

MATERIALS AND METHODS

Organism and growth conditions

The *A. niger* strain N402 (Bos *et al.* 1988) and its derivative RB#9.5 were used in this study. The latter strain expresses a gene encoding a fusion of sGFP and the histone protein H2B under regulation of the *mpdA* promoter (R. Bleichrodt, unpubl. results). For spore isolation, strains were grown for 12 d at 25 °C on complete medium (CM) containing per liter: 1.5 % agar, 6.0 g $NaNO_3$, 1.5 g KH_2PO_4, 0.5 g KCl, 0.5 g $MgSO_4$, 4.5 g D-glucose, 0.5 % casamino acids, 1 % yeast extract and 200 µl trace elements (containing per liter: 10 g EDTA, 4.4 g $ZnSO_4 \cdot 7H_2O$, 1.0 g $MnCl_2 \cdot 4H_2O$, 0.32 g $CoCl_2 \cdot 6H_2O$, 0.32 g $CuSO_4 \cdot 5H_2O$, 0.22 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 1.5 g $CaCl_2 \cdot 2H_2O$, and 1.0 g $FeSO_4 \cdot 7H_2O$). Conidia were harvested in ice-cold ACES-buffer (10 mM ACES, 0.02 % Tween-80, pH 6.8), filtered through sterile glass wool, washed in ice-cold ACES-buffer and resuspended in CM (van Leeuwen *et al.* 2013). The conidia were kept on melting ice until further processing on the same day. An aliquot of 3.10^9 conidia were added to 300 ml CM in 500 ml Erlenmeyers. Cultures were shaken at 125 rpm in the absence or presence of 3 or 10 µM natamycin. Stock solutions of natamycin (10 mM) were freshly made in 85 % DMSO (Brik 1981).

Transcriptome analysis

Data analysis was performed on biological triplicates, each based on three cultures. At each time point, 15 ml of each of the three cultures was pooled. The (germinating) conidia were pelleted at 1100 g at 5 °C for 5 min and immediately frozen in liquid nitrogen. RNA extraction, cDNA labeling, microarray hybridisation and data analysis were done as described (van Leeuwen *et al.* 2013). The array data has been deposited in NCBI's Gene Expression Omnibus (Edgar *et al*. 2002) and is accessible through GEO Series accession number GSE36440 (www.ncbi.nlm.nih.gov/geo/).

HPLC analysis

Dormant, germinating or treated conidia ($5 \cdot 10^7$ - $1 \cdot 10^8$) were frozen at -80 ºC and homogenised with a Qiagen Tissuelyser® (2 min at 30 strokes /sec: Qiagen, Venlo, The Netherlands) using a stainless steel grinding jar cooled with liquid nitrogen. After an additional round of grinding with 1 ml milliQ, the samples were thawed and quickly transferred to a 2 ml Eppendorf tube. Samples were centrifuged at 4 °C for 30 min at 20.817 g. The supernatant was stored at -80 °C until analysis. Prior to HPLC analysis samples were filtered through an Acrodisc® 0.2 µm PTFE syringe filter (Sigma-Aldrich, Zwijndrecht, The Netherlands). A volume of 10 µl was subjected to HPLC analysis, using a Waters 717 plus autosampler equipped with a 515 HPLC pump with control module II (Waters Corporation, Etten-Leur, The Netherlands). The mobile phase consisting of 0.1 mM Ca EDTA in water was maintained at a flow rate of 0.5 ml/min. The Sugar Pak I Ca^+ cation-exchange column was kept at 65 °C with a Waters WAT380040 column heater module (Laborgerätebörse GmbH, Burladingen, Germany). Sugars and polyols were detected with an IR 2414 refractive index detector (Waters Corporation, Etten-Leur, The Netherlands). As standards, trehalose, mannitol, D-(+)-glucose, glycerol, erythritol and arabitol were used (Sigma-Aldrich, Zwijndrecht, The Netherlands). Peak integrations and quantitative calculations were performed with the Waters Empower software (Waters Corporation, Etten-Leur, The Netherlands).

ESR spectroscopy

Germinating conidia were centrifuged at 8000 rpm for 2 min. The supernatant was discarded and the conidia were resuspended in 25 µl perdeuterated TEMPONE-potassium ferricyanide solution (1 mM and 120 mM, respectively). Micro-viscosity was determined and calculated as described in (van Leeuwen *et al.* 2010).

Fluorescence microscopy

Samples of liquid cultures were placed on poly-l-lysine (Sigma) coated cover slips (van Leeuwen *et al.* 2008). The medium was removed and the cover slips with the immobilised conidia were placed upside-down onto an object glass with a < 0.5 mm layer of 2 % water agar. Images were taken with a Zeiss Axioskop 2 plus microscope (Zeiss, Oberkochen, Germany) equipped with a HBO 100 W mercury lamp and a AxioCam MRc (Zeiss, Germany) camera using standard FITC (λ = 450–490 nm, FT510, LP520) filters.

RESULTS

Morphological responses to natamycin during conidial germination

Light microscopy showed that germination of *A. niger* conidia is inhibited in natamycin-treated conidia compared to untreated cells (Fig. 1A). Untreated conidia swell slightly during the first 2 h of germination. The surface area of the cells on the micrographs increased from 17 to 22.6 µm² (Fig. 1B). The conidia enlarged gradually to 46 µm² between 2- and 6 h and their volume further increased up to 8 h. At this stage, the variability in size of the cells was largely due to differences in germ tube emergence and growth. After 6 and 8 h, 10 % and 80 % of the conidia had started to form germ tubes, respectively (Fig. 1C).

Conidia that had been exposed to 3 µM natamycin showed a similar swelling as control cells during the first 2 h. The surface area of the cells on the micrographs increased from 17.7 to 22.1 µm². Between 2- and 6 h, the surface area of the conidia enlarged to 35.6 µm², which had further increased to 40.1 µm² after 8 h of germination. Notably, polarisation and germ tube formation were not observed during the 8 h incubation time (Fig. 1C). The surface area of conidia that had been exposed to 10 µM natamycin for 2 h increased from 17.3 to 21.8 µm² (Fig. 1B). After 3 h the conidia had reached a surface area of 23.4 µm², which remained unchanged up to 8 h of incubation. Polarised cells and germ tubes were not formed throughout culturing (Fig. 1C). All considering, these results show that polarisation and germ tube formation are inhibited at